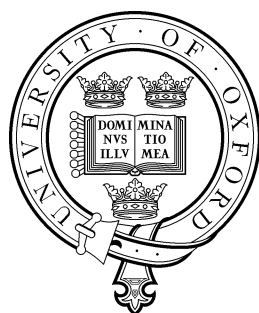


Investigation of Pharmacological and Physiological
Regulation of Pyruvate Dehydrogenase in Diabetes
using Hyperpolarised Magnetic Resonance
Spectroscopy



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Abstract

In type II diabetes, systemic metabolism is perturbed and on a cellular level the balance of fuel use is upset. More specifically, increased fatty acid use is seen alongside decreased glucose metabolism. This altered fuel use is mediated by changes in the activity and expression of multiple enzymes. One such enzyme within the glucose breakdown pathway is pyruvate dehydrogenase, whose activity is known to be reduced in the diabetic state.

The field of real-time metabolic investigation has rapidly expanded over the past few years due to the invention of technology that has enabled the production of ^{13}C labelled hyperpolarised compounds, which can generate high signal levels in magnetic resonance spectroscopy. This has provided the opportunity to measure real-time metabolism of injected hyperpolarised tracers both *ex vivo* and *in vivo*.

This thesis aimed to develop the use of hyperpolarised compounds *in vivo*, to investigate the cardiac and hepatic metabolism of a diabetic rat model. We initially addressed the systemic nature of the disease by establishing a two-slice acquisition for obtaining cardiac and hepatic data during a single injection of hyperpolarised pyruvate. This was tested in the fed and fasted states before being used in the studies described in the subsequent chapters of this thesis.

The value of hyperpolarised compounds in following metabolic modulation by drug treatment was explored in the next chapter. The effect on metabolism of two drugs targeted at pyruvate dehydrogenase, which differed in their isoform specificity, was investigated first in the perfused heart and subsequently *in vivo*, both in control and diabetic animals. Hyperpolarised magnetic resonance spectroscopy was combined with other established techniques to help both our understanding of the systemic changes that had occurred following treatment, and provide links between cardiac metabolism and function.

The final chapter of this thesis explored the use of hyperpolarised ^{13}C pyruvate to understand the effect of hypoxia on pyruvate dehydrogenase, firstly in healthy animals and subsequently in the diabetic, metabolically altered state. Understanding the combination of diabetes and hypoxia was interesting given the existence of several opposing metabolic effects seen in the two states.

Overall this thesis has demonstrated developments in the use of hyperpolarised pyruvate that, when appropriately combined with other techniques, can yield valuable metabolic information, in terms of following disease progression, drug development, and understanding basic metabolism.

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CONTENTS

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Abbreviations

ADP: adenosine diphosphate	EPO: erythropoetin
ALT: alanine aminotransferase	ESV: end systolic volume
AMARES: advanced method for accurate, robust and efficient spectral fitting	FAD: flavin adenine dinucleotide
AMP: adenosine monophosphate	FIH: factor-inhibiting HIF
ANOVA: analysis of variance	FLASH: fast low-angle shot
ATP: adenosine triphosphate	GLUT: glucose transporter
CO: cardiac output	H&E: haematoxylin and eosin
CPT: carnitine palmitoyl transferase	HbA1c: glycated haemoglobin
CVD: cardiovascular disease	HIF: hypoxia-inducible factor
DCA: dichloroacetate	HPLC: high-performance liquid chromatography
DMOG: dimethyloxalyl glycine	HPMC: hydroxypropyl methylcellulose
DMSO: dimethyl sulfoxide	IGF: insulin-like growth factor
DNP: dynamic nuclear polarisation	KH: Krebs-Henseleit
DPP: dipeptidyl peptidase	LDH: lactate dehydrogenase
EC ₅₀ : half maximal effective concentration	LV: left ventricle
ECG: electrocardiogram	LVIDd: LV internal dimension in diastole
EDP: end diastolic pressure	LVIDs: LV internal dimension in systole
EDTA: ethylenediaminetetraacetic acid	MCT: monocarboxylate transporter
EDV: end diastolic volume	MRI: magnetic resonance imaging
EF: ejection fraction	MRS: magnetic resonance spectroscopy
ELISA: enzyme-linked immunosorbent assay	NAD: nicotinamide adenine dinucleotide

NAFLD: non-alcoholic fatty liver disease

ORO: oil-red-O

PBS: phosphate-buffered saline

PC: pyruvate carboxylase

PCr: phosphocreatine

PDC: pyruvate dehydrogenase complex

PDH: pyruvate dehydrogenase

PDK: pyruvate dehydrogenase kinase

PDP: pyruvate dehydrogenase phosphatase

PEPCK: phosphoenolpyruvate carboxykinase

PER: peak ejection rate

PFR: peak filling rate

PPAR: peroxisome proliferator-activated receptor

RF: radiofrequency

RNA: ribonucleic acid

RPP: rate-pressure product

SDS: sodium dodecyl sulphate

SDS-PAGE: SDS-polyacrylamide gel electrophoresis

SGLT: sodium glucose transporter

STZ: streptozotocin

SV: stroke volume

TBS: tris-buffered saline

VEGF: vascular endothelial growth factor

Chapter 1

Thesis overview and aims

1.1 Overview

Worldwide, over 300 million people suffer from type II diabetes, characterised by insulin insensitivity or resistance. With this number only set to rise in the coming years, and with the World Health Organisation predicting a doubling of diabetes-related deaths between 2005 and 2030¹, work towards prevention and cure is vital². Much work is still to be done to fully characterise the underlying metabolic changes that occur during the progression of the disease, so that new treatments can be found.

The risk of developing type II diabetes can be increased by age, socioeconomic status, genetics, and environmental factors such as diet, where increased body fat is associated with an increased risk³. Symptoms of type II diabetes include increased thirst, tiredness, frequent urination and weight loss⁴. Having type II diabetes increases the risk of developing cardiovascular disease (CVD), and in turn CVD is responsible for between 50 and 80% of deaths in people with diabetes¹. Further, the high blood glucose levels that are inherent to the disease cause microvascular problems that can lead to damage of the nerves, kidneys, retina, and feet⁵.

Currently, the initial treatment for type II diabetes is prescription of lifestyle alterations, however this is not always sufficient, and pharmaceutical intervention is commonly required. Even then, after a honeymoon period of response (often requiring combinatory dosing), patients may return to the pre-treatment state⁶. Accurate assessment of the progression of the disease therefore

allows for the most appropriate treatments to be given at each stage. The search for new, improved treatments must also be continued.

The metabolic changes associated with type II diabetes involve a shift in the balance of the fuels that are used for energy production. Generally this involves an increased use of fatty acids, and a decreased use of glucose. There are control points within the metabolic pathways which can be studied to help our understanding of changes in fuel use that occur in disease. Within the glucose pathway, pyruvate dehydrogenase (PDH) is a key enzyme, given its position as one of the gatekeepers to the mitochondrion (figure 1.1).

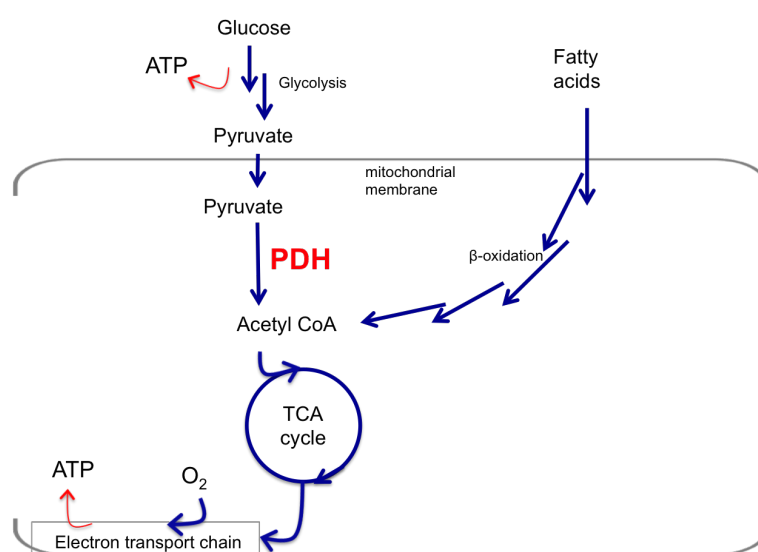


Figure 1.1: Overview of the major cellular metabolic pathways

The activity of PDH is affected by a variety of factors, including pyruvate concentration and insulin. In type II diabetes, PDH activity is decreased⁷, in agreement with the decreased use of glucose mentioned above. This is seen systemically, although given the relevance of cardiovascular health in diabetes already mentioned, it is of high priority to fully understand the response of cardiac PDH to metabolic stressors. In terms of treatment however, targeting cardiac PDH is unlikely to have the systemic effects required to restore fuel balance overall; targeting an organ with more control over whole-body metabolism, such as the liver, may be more successful.

Numerous studies have been carried out investigating cardiac and hepatic metabolism in diabetes, however there is limited *in vivo* data, particularly when looking for information on *in vivo* PDH activity. Recent technical developments in the use of carbon-13 (^{13}C) labelled compounds

provide an opportunity to improve our knowledge in this area; hyperpolarisation of these compounds enables us to assess metabolism *in vivo*⁸. When injected into an anaesthetised animal that has been placed in a magnetic resonance imaging (MRI) system, these hyperpolarised compounds offer the ability to obtain a carbon spectrum in under a second, with an improvement in signal to noise of over 10,000-fold when compared to standard or traditional thermal equilibrium data. If several of these spectra are therefore taken in rapid succession, visualisation of the breakdown, or metabolism, of the injected compound is possible, as the ^{13}C label is transferred to downstream metabolites.

This thesis explores the premise that using this new technique to investigate the metabolic changes in type II diabetes may provide a new perspective on our understanding of the disease, and new insights into its prevention and treatment.

1.2 Aims

Overall, this thesis aims to augment our understanding of type II diabetic metabolism, whilst also assessing the changes that occur to metabolism following physiological and pharmacological interventions. My work has focussed on carbohydrate metabolism, and our ability to assess and understand this using hyperpolarised technology.

As an introduction, in chapter 2 I have discussed the overall effects of diabetes on the body, along with the relevant cardiac and hepatic metabolic pathways central to our understanding. Within this, both healthy and diseased states have been explored, along with current methods of treatment. I have also described the basic principles of the major technique focussed on in this thesis: hyperpolarised magnetic resonance.

In chapter 3, I have detailed techniques used in multiple chapters. Methods specific to individual experiments are detailed in the relevant section.

Previous work using hyperpolarised MR spectroscopy (MRS) has tended to focus on organs in isolation. In order to obtain a more relevant picture of a disease and its treatment, a more systemic view would likely be beneficial. In diabetes, many organs are affected by the hyperglycaemia, and undergo metabolic and functional changes. Further, as discussed in the introduction,

certain organs would be more appropriate than others as a target to enable systemic treatment. Chapter 4 therefore addresses the question of assessing changes in metabolism in multiple organs using hyperpolarised MRS, and aimed to develop a magnetic resonance (MR) acquisition which would provide data on systemic metabolism, followed by validation with regards to a disease model. Previous work in our lab has used a global data acquisition method, with metabolic data assumed to be primarily from the heart due to the location of the radiofrequency coil. Schroeder *et al.* in 2009⁸ stated that this acquisition method was reliable for assessing conversion of cardiac pyruvate to bicarbonate, however they suggested that measurements of lactate and alanine may be contaminated by neighbouring organs such as skeletal muscle and liver. Therefore we believed an improvement in this technique would be to selectively excite nuclei from a particular slice of the animal, focussing on a single organ. This would ensure that the data obtained had no contamination from neighbouring organs. Slice selective hyperpolarised MR has previously been successfully demonstrated in both oncologic⁹ and cardiac¹⁰ studies. My work in chapter 4 developed a related acquisition which obtains spectroscopic data from both the heart and the liver during one injection of hyperpolarised ^{13}C pyruvate. Comparisons of data from global, single slice and two-slice acquisitions provided a proof of this principle, and subsequent validation that the acquisition yields relevant metabolic data was provided by assessment of a range of different metabolic states (fed, fasted, and diabetic).

I then applied this developed acquisition to assess the metabolism and treatment of type II diabetes. The overall aim of the work in chapter 5 was to study the *in vivo* type II diabetic metabolism, and further, pharmacological metabolic modulation as a potential treatment. The chapter documents our experiments into the effects of two metabolic modulators, first on healthy *ex vivo* cardiac metabolism, and subsequently on healthy and diabetic *in vivo* cardiac and hepatic metabolism. Based on previous experimental data, the two compounds assessed were both believed to increase PDH activity^{11,12}, although differing in their specificity. The first, dichloroacetate (DCA) is a non-specific inhibitor of pyruvate dehydrogenase kinase (PDK). PDK inhibits PDH and is partially responsible for maintaining an appropriate fuel-use balance. The three most abundant isoforms of PDK in the heart and liver are 1, 2 and 4, and DCA inhibits all of these. In investigating DCA I also aimed to establish whether observation of a link between cardiac function and metabolism was possible by combining appropriate analytical

techniques. The second drug compound investigated - AZD7545 - is a specific PDK2 inhibitor. The major site of action of both compounds was believed to be the liver, and so a further aim of this chapter was to use the protocol developed in chapter 4 to demonstrate acquisition of data from both heart and liver that contributed to our understanding of the action of this novel compound.

The work described in chapter 6 investigated the effect of hypoxia on PDH flux in the heart and liver. The first aim of this chapter was to establish the effects of hypoxia on PDH *in vivo* in healthy animals, given limited previous work available. Studies were carried out which investigated three different lengths of hypoxic exposure. Secondly, given hypoxia induces metabolic effects that are opposite to those seen in diabetes, it was interesting to look at the effects hypoxia had on the type II diabetic model used in chapters 4 and 5. Finally, as hypoxia is inherently involved in diabetes, for example diabetics having a reduced prognosis following myocardial infarction, and microvascular problems causing reduced peripheral perfusion, understanding the relationship between hypoxia and diabetes more extensively may lead to alternatives in treatment. Previous work by Marfella *et al.*¹³ suggested an impaired hypoxic response in diabetics, and so we hypothesised that the diabetic animals would demonstrate a lesser response to hypoxia. If the hypoxic response could subsequently be enforced, using a drug treatment, it may improve patient outlook following infarction. In the final section of chapter 6, the effect of a small molecule compound (dimethyloxallylglycine, or DMOG) on *in vivo* PDH has been investigated.

Finally, chapter 7 summarises the work of this thesis, discussing any relationships between chapters.

Chapter 2

Background

2.1 Diabetes overview

Diabetes occurs most frequently in two forms; type I and type II. Insulin, a hormone vital in the regulation of carbohydrate and fat metabolism, is central to both diseases. Type I diabetes is typically early-onset and in most cases is believed to be an autoimmune disease. It is characterised by an inability to produce sufficient insulin, and is not currently preventable¹⁴. Type II diabetes usually develops later in life, although it is increasingly being seen in a younger population due to poor diet and a sedentary lifestyle. It involves a combination of compromised insulin secretion and reduced insulin sensitivity, leading to an altered whole body metabolic state. The work in this thesis addresses the metabolic and physiological changes seen in type II diabetes.

There are several well-established indications of type II diabetes, including high fasting blood glucose and abnormal glucose tolerance tests, where, following an oral dose of glucose, *in vivo* plasma glucose levels remain high even after 2 hours¹⁵. Glycated haemoglobin levels (HbA1c) give a good idea of average plasma glucose levels over a longer time period, as the glycation is irreversible in the presence of high blood glucose, and so is maintained until the turnover of red blood cells is complete (approximately four months in humans¹⁶).

Metabolic changes in diabetes involve increased cellular fatty acid uptake and metabolism, and decreased glucose uptake and metabolism, which result in the observed increased plasma glu-

cose. As the disease progresses and the body is subjected to chronically high levels of glucose, basal glucose and insulin levels steadily rise (figure 2.1), until eventually this causes permanent pancreatic damage. In the pancreas there are groups of endocrine cells. These groups are called the islets of Langerhans, and consist of three different types of cells: α -cells, β -cells and δ -cells, which secrete glucagon, insulin and somatostatin respectively¹⁵. The permanent damage includes destruction of the pancreatic β -cells.

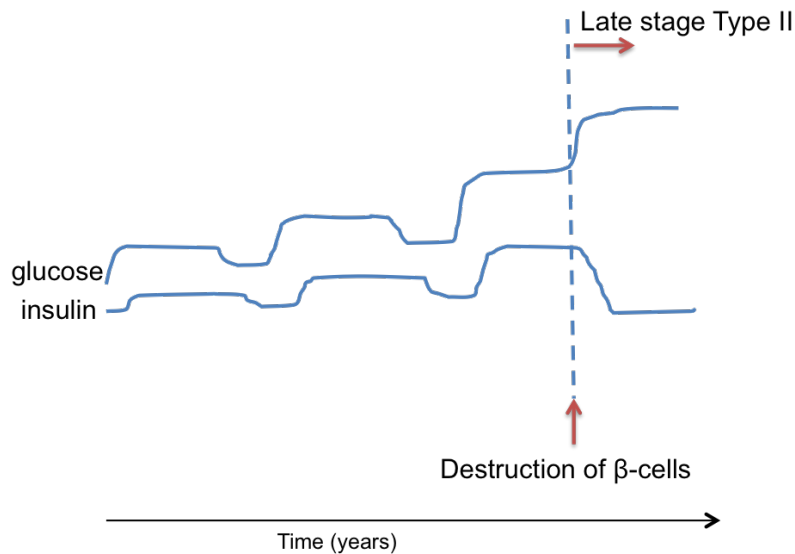


Figure 2.1: Relationship between glucose and insulin as Type II diabetes progresses

2.1.1 Diabetic complications

Cardiovascular complications are the leading cause of death in type II diabetic patients¹. A major component of this is macrovascular complications which are commonly seen in diabetics. Given the alterations in lipid metabolism that are seen, atherosclerosis is common and therefore when this affects the coronary arteries it can lead to fatalities. Further, it is thought that chronic hyperinsulinaemia may cause changes in the arterial wall which contribute to the development of atherosclerosis, or stiffening of the vessels which in turn may cause hypertension¹⁵.

It is generally agreed that hyperglycaemia, the most well-established symptom of diabetes, is damaging if maintained for a long period of time, although there is controversy surrounding the mechanisms by which this damage occurs. Many of the mechanistic steps are not known, but several organs are affected. This includes the kidneys, which usually filter waste products from

the blood, but become overworked on long-term exposure to hyperglycaemia. Glomerulosclerosis occurs, which is a hardening of the glomerulus in the kidney. This damages the kidneys' filtering capacity and useful protein (albumin) begins to leak into the urine. This can develop into end stage renal disease if left untreated. Glucose, and its effect on the vasculature, also has damaging consequences in the retina, feet and nerves. It causes capillary wall damage and therefore has the potential to cause micro-aneurysms, leaks, and blockages, and any new vessels that grow in response are weak-walled. Work has begun to investigate the mechanism for the damage caused by glucose, for example by Lima *et al.* in 2009¹⁷ and Erickson *et al.* in 2013¹⁸ who show post translational modification of proteins as a result of hyperglycaemia, specifically of those important to vascular function.

Although lean type II diabetic patients are seen^{19,20}, obesity is often inherently intertwined with type II diabetes. The two are often addressed together under the umbrella of 'metabolic syndrome' and are associated with an increased risk for cardiovascular disease⁴. Insulin resistance occurs in obesity, and even if initially these patients can maintain normal glucose levels, the amount of insulin produced in response to the high caloric intake is higher. This elevated insulin response can only be maintained for a limited time; the pancreatic islets will start to fail, and diabetes will develop¹⁵. Moreover, diabetes, insulin resistance and obesity are linked with other diseases, such as non-alcoholic fatty liver disease (NAFLD) whose prevalence has been seen to rise in parallel^{21,22}, although this is not unexpected given its association with an altered metabolic lipid profile.

2.2 Physiology and metabolism

2.2.1 Physiology and metabolism in health

The body can use several fuel types, such as carbohydrates, fats and proteins, which are large macromolecules. These are broken down, or metabolised, to smaller molecules such as glucose and fatty acids, and subsequently metabolised further to acetyl Coenzyme A (CoA) in the mitochondria. This substrate can then be used to make adenosine triphosphate (ATP), via the Krebs cycle and oxidative phosphorylation (figure 2.2).

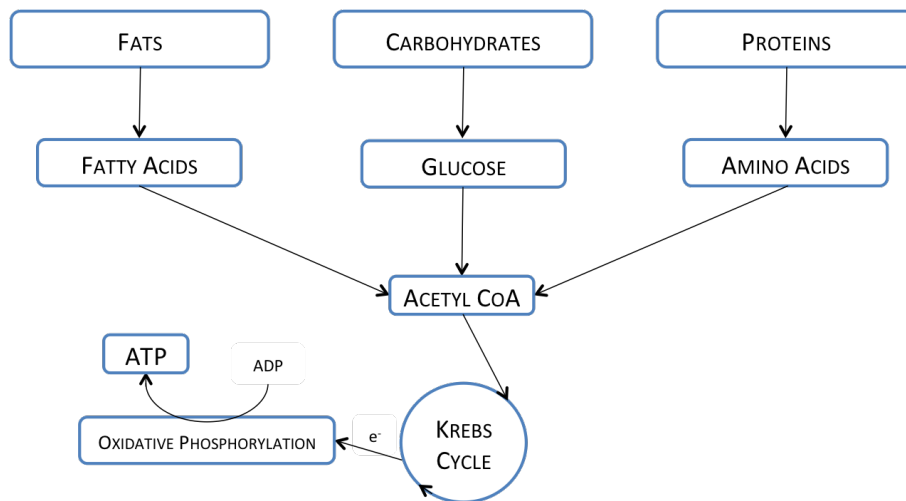


Figure 2.2: Overview of metabolism

2.2.1.1 Role of pyruvate dehydrogenase in healthy metabolism

As mentioned above, the use of glucose (and carbohydrates as a whole) is fundamental for fuelling the body, i.e. producing ATP. Pyruvate dehydrogenase (PDH) is a key enzyme in this process, being a control point within the carbohydrate metabolic pathway. More specifically, when glucose enters the cell, it is broken down into pyruvate by glycolysis. This pyruvate is then decarboxylated and forms acetyl CoA which is used to produce ATP (via the Krebs cycle and oxidative phosphorylation) and it is this reaction which is controlled by PDH (figure 2.3).

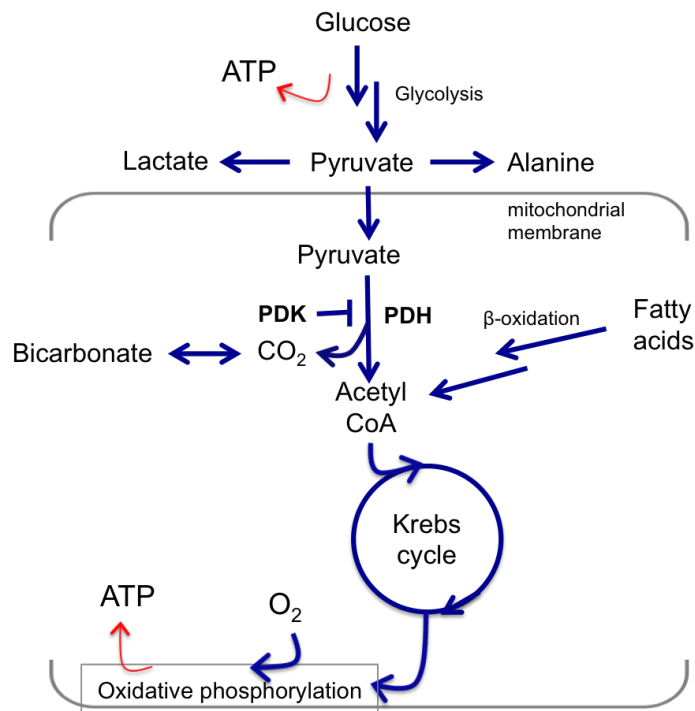


Figure 2.3: Overview of fuel use in the heart. ATP = adenosine triphosphate; PDK = pyruvate dehydrogenase kinase; PDH = pyruvate dehydrogenase

PDH is part of the large pyruvate dehydrogenase complex (PDC), and is the first enzyme within this complex, designated E1. The structure of the PDC and the reactions it controls are visualised in figure 2.4.

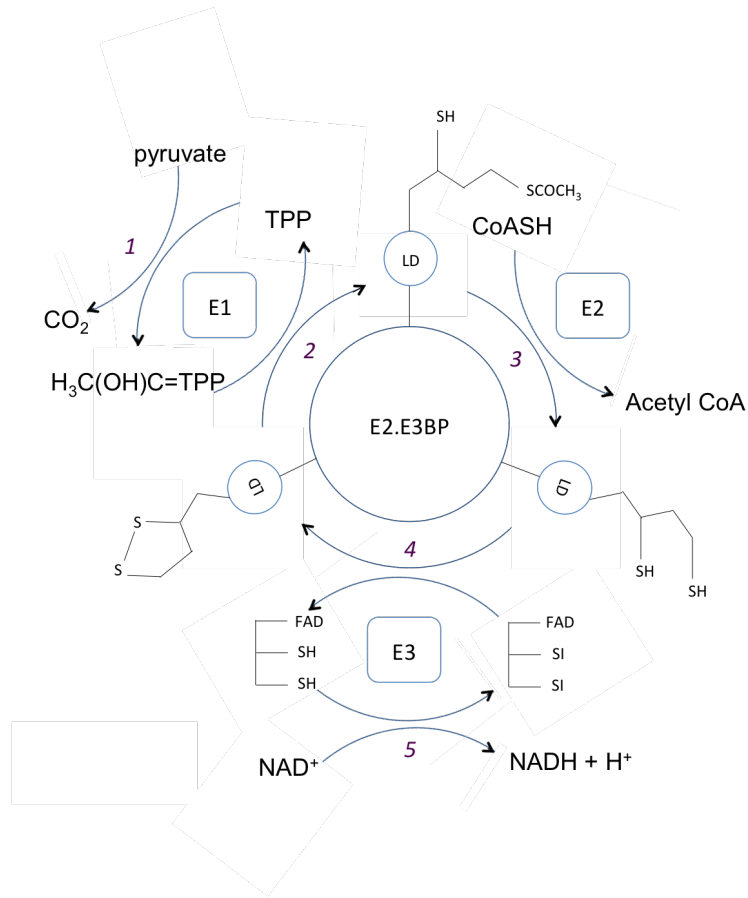


Figure 2.4: Reactions controlled by the pyruvate dehydrogenase complex (PDC). E1 = pyruvate dehydrogenase (PDH). 1 = pyruvate decarboxylation by PDH. 2 = acetylation of lipoyl groups. 3 = transfer of acetyl group to Coenzyme A. 4 = E3 accepts reducing equivalents via thiol flavin adenine dinucleotide (FAD) group. 5 = reducing equivalents transferred to oxidised nicotinamide adenine dinucleotide (NAD^+)

The ratio of active:inactive PDH is controlled by pyruvate dehydrogenase kinase (PDK) and pyruvate dehydrogenase phosphatase (PDP), of which there are four and two known isoforms respectively. The interplay between PDH and the different PDK and PDP isoforms via covalent modification has been excellently reviewed by Sugden and colleagues in 2003, and this is summarised in figure 2.5.

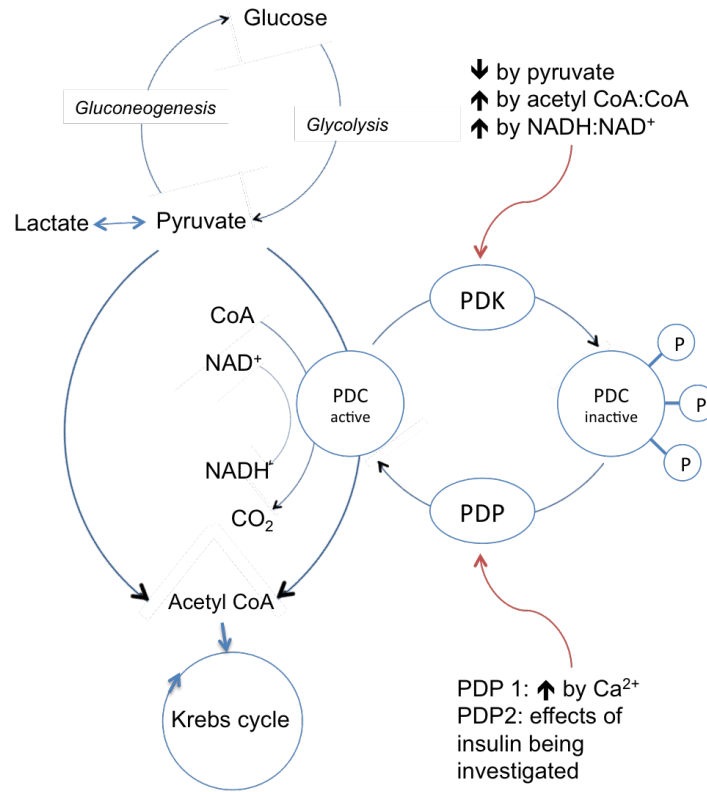


Figure 2.5: Control of PDH and PDK, adapted from Sugden and Holness, 2003²³. PDH = pyruvate dehydrogenase; PDK = pyruvate dehydrogenase kinase; PDC = pyruvate dehydrogenase complex; PDP = pyruvate dehydrogenase phosphatase

There are four known PDKs in rodents and humans, with a high sequence identity between species. Abundance of each isoform varies between tissues, for example PDK1 is highly abundant in the heart compared with other tissues. However, although PDK4 is also highly abundant in cardiac tissue (and skeletal muscle) relative to other tissues, PDK1 and 4 are not equally dominant in the heart. This is partly due to the specific activities (activity of an enzyme per milligram of total protein of the recombinant isoenzymes) varying between isoforms; these are summarised in table 2.1, as found by Bowker-Kinley *et al.*²⁴.

Isoform	Specific activity (nmol/min/mg)
PDK 1	650
PDK 2	50
PDK 3	1250
PDK 4	400

Table 2.1: Variability in specific activities of PDK isoforms

PDK is regulated by products from the PDH-catalysed conversion of pyruvate to acetyl CoA. NADH and acetyl CoA activate PDK²⁴, and this activation can be reversed by high concentrations of NAD⁺ and CoA. Figure 2.6 summarises the factors which can affect the activities of these three isoforms and shows the sites on PDH on which they act.

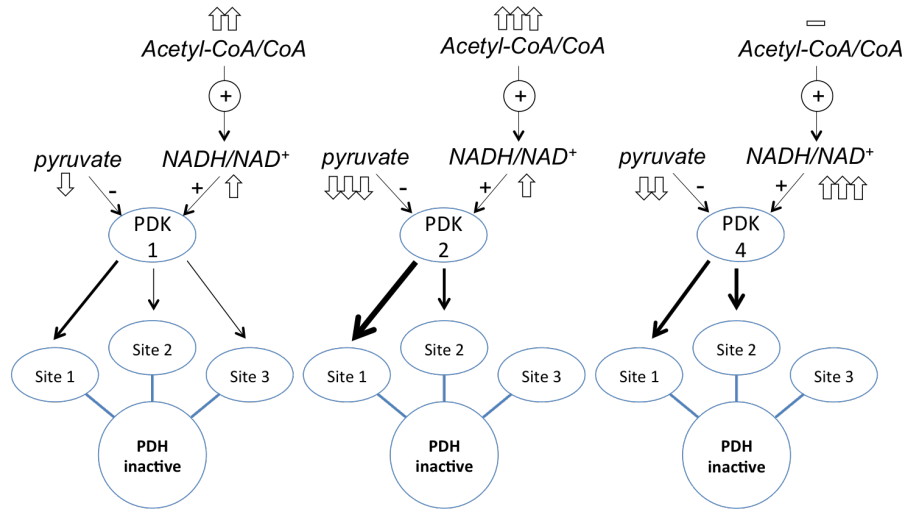


Figure 2.6: Activities of PDK isoforms, adapted from Sugden and Holness, 2003²³.

PDK expression and activity contribute to PDH activity in the long and short term respectively. Transcriptional upregulation of the isoforms can be effected by different factors, for example increased PPAR- α causes increased PDK4, whereas increased HIF can result in increased PDK1 in cells. Although increased PDK expression is not necessarily a result of increased PDK activity, it is possible that the two could be interlinked. For example, increased PDK4 activity would caused decreased PDH activity, and therefore decreased glucose metabolism. This would result

in promotion of fatty acid oxidation (via the Randle cycle), with PPAR- α being a key regulator of this process. As PPAR- α is a transcriptional regulator of PDK4, the longer-term result would be increased PDK4 expression.

PDK activity has been shown to be altered over the timecourse of minutes by Watt *et al.*²⁵, however the work in this thesis focusses on changes in PDK expression. PDK expression is assessed only in the chronic studies, and not in the perfused heart work where transcriptional regulation would not be expected to be affected. Western blot was chosen as the method of assessment due to experience and therefore reliability of the data. PDK activity assays are described in the literature²⁵, however it was not possible to establish and execute them during the time available for this thesis. Future work could address this and it would be particularly interesting to look at the interplay between activity and expression in the longer-term experiments.

PDK1, 2 and 4 were assessed in the heart, and 2 and 4 in the liver due to previous literature describing the tissue-specific presence of these isoforms²⁴.

2.2.1.2 The heart

Cardiac function

The heart consists of four chambers, and is responsible for pumping oxygenated blood around the body and deoxygenated blood back to the lungs. Oxygenated blood arrives from the lungs via the pulmonary veins and enters the left atrium, before moving into the ventricle and out of the heart to the rest of the body, via the aorta. Deoxygenated blood returns to the right side of the heart via the vena cava, before being pumped to the lungs via the pulmonary artery (figure 2.7). Valves between the atria and ventricles, and ventricles and major vasculature prevent backflow of blood. Blood is supplied to the heart muscle itself via a system of coronary arteries which branch from the aorta.

The contraction of the heart consists of three major stages: atrial systole, ventricular systole, and diastole. In systole, the relevant chambers contract, actively forcing blood through the heart and out to the body and lungs. Diastole is relaxation of the heart, and results in passive movement of blood²⁶.

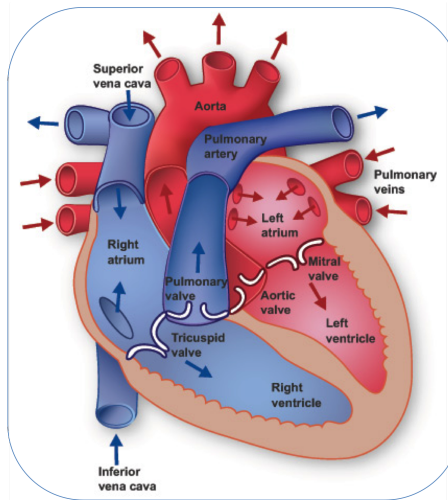


Figure 2.7: Cross-section through the heart, adapted from www.texasheartinstitute.org²⁷

Cardiac metabolism

The heart has a preference for metabolising fatty acids over carbohydrates (approximately 70:30 ratio) and it is this delicate balance which is key to ensuring its continued health²⁸. The metabolism of fatty acids within the cardiomyocyte mitochondria is less oxygen efficient, but produces more ATP per gram than carbohydrate otherwise would. However fatty acids cannot be metabolised if there is not sufficient oxygen present, whereas glucose can be broken down anaerobically (via glycolysis) to produce lactate rather than acetyl CoA. This anaerobic metabolism produces cytosolic ATP during oxygen-independent glycolysis.

The heart can alter its preference for fuel type as required, leading to the description of the heart as ‘metabolically flexible’²⁹. During starvation for example, the heart can reduce its glucose metabolism, ensuring sufficient glucose is available for organs (such as the brain) that cannot use fatty acids as fuel. In temporary fasting conditions the heart uses primarily fatty acids and ketone bodies as fuels. The change is dependent on, and instigated by, the concentrations of both glucose and insulin in the blood³⁰. The balance between the utilisation of glucose and fatty acids was first described by Randle and colleagues in 1963³¹, who suggested that the extent of the oxidation of one fuel directly affects the oxidation of the other. The high levels of fatty acids and their subsequent metabolism cause increased ratios of acetyl CoA/CoA, NADH/NAD⁺, and ATP/ADP which inhibit PDH and therefore lead to a reduction in glucose metabolism.

2.2.1.3 The liver

The liver has a major role in carbohydrate storage and in glucose metabolism although oxidation of amino acids and fatty acids provide the energy for the liver itself¹⁵. The liver's main blood supply is from the portal vein (figure 2.8) which is a meeting point for veins from several parts of the intestine, and as such it sees the postprandial substrates before other organs. The pancreatic veins also join the portal vein; these supply insulin and glucagon. Hepatic veins carry blood away from the liver back towards the heart via the inferior vena cava.

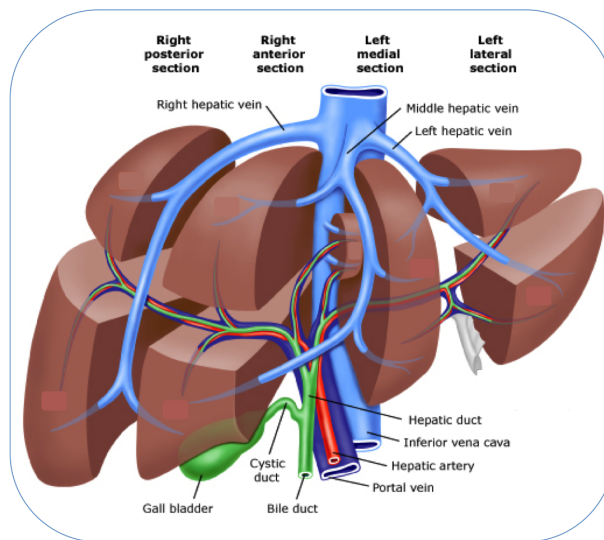


Figure 2.8: Cross-section through the liver, adapted from <http://www.cea1.com>³²

Hepatocytes make up 80% of the liver (by volume) and radiate out from branches of the hepatic veins, creating hexagonal ‘lobules’. The hepatocytes at the edges of these hexagons are therefore next to branches of the portal vein, so are supplied with the substrates from the intestinal tract, and oxygen. As a result, oxidative metabolism is dominant here and it is within these cells that gluconeogenesis occurs. In contrast, the hepatocytes near the hepatic vein in the centre of the lobule become more focussed on glycolysis.

The major glucose transporter (GLUT) in the liver is GLUT2, which is insensitive to insulin. The K_m (a measure of enzyme affinity) of GLUT2 is ~ 15 mM and therefore the concentration of glucose in the hepatocytes is mainly dependent on the concentrations either side of the cell membrane. Glucose, once inside the cells, is phosphorylated to glucose-6-phosphate by

glucokinase, an enzyme found only in the liver and pancreatic β -cells, with a very high K_m for glucose compared to hexokinase found elsewhere in the body. This enables the liver to cope with the high concentrations of glucose that occur during feeding³³. The glucose-6-phosphate can then either be made into glycogen for storage, converted to ribose-5-phosphate via the pentose phosphate pathway, or oxidised to pyruvate (and subsequently lactate, alanine or acetyl CoA) via glycolysis. Insulin has wide-reaching control over these conversions, as shown in figure 2.9. As the insulin is delivered directly from the pancreatic vein to the liver, it can instigate a rapid response to changes in plasma glucose concentrations.

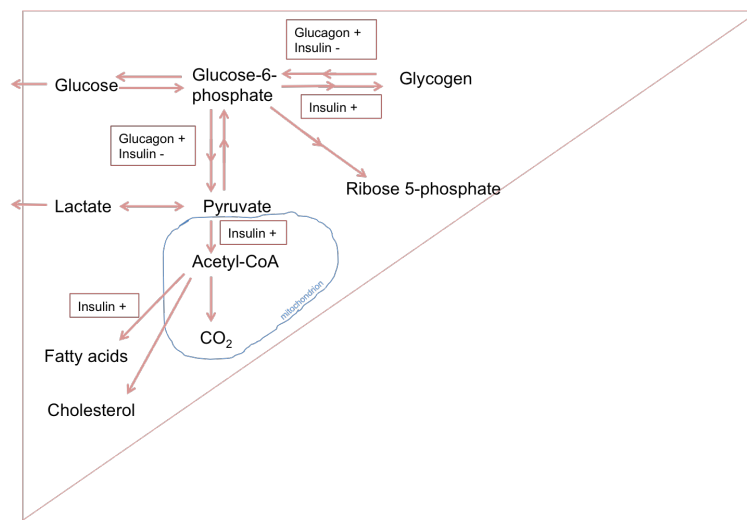


Figure 2.9: Glucose metabolism in the liver. Adapted from Frayn, 3rd Edn.¹⁵

When the body is in the fasted state, the liver synthesises glucose, via two routes stimulated by glucagon. These are glycogenolysis, the breakdown of glycogen, and gluconeogenesis, the formation of glucose from other precursors, including lactate, alanine and glycerol. It also produces ketone bodies such as 3-hydroxybutyrate as an alternative fuel source, via acetyl CoA from fatty acid oxidation³³. Gluconeogenesis causes an increase in NADH, leading to both an inhibition of PDH and isocitrate dehydrogenase, a Krebs cycle intermediate, and an increase in malate production from oxaloacetate, shuttling the carbon skeleton towards glucose production rather than Krebs cycle metabolism. An increase in acetyl CoA is also seen, which inhibits PDH and stimulates pyruvate carboxylase (PC).

2.2.2 Physiology and metabolism in diabetes

2.2.2.1 The diabetic heart

Cardiac function

Patients with diabetes are at an increased risk of developing heart failure³⁴, with increased glycated haemoglobin levels correlating with increased risk (10-15% increase in risk with every unit of HbA1c)³⁵ whilst patients with diabetes who develop heart failure have worse outcomes^{36,37}. Further, it has been demonstrated that an ischaemic insult (such as a myocardial infarction) results in a less successful recovery in a diabetic rodent model in comparison to control animals¹³, and this is supported by patient data which demonstrates a higher mortality rate in diabetics both during a myocardial infarction and in the post-infarction period^{38,39}.

Left ventricular diastolic dysfunction is taken to be an early marker of heart failure⁴⁰, and there is a high incidence of diastolic dysfunction in diabetic patients (type I: Schannwell *et al.*, 2002⁴¹; type II: Patil *et al.*, 2011⁴²), with rates of up to 60% in type II diabetics⁴³. It has been suggested that the diabetic changes in metabolism and left ventricular dysfunction are inherently linked, and this may therefore be a new opportunity for treatment. Von Bibra *et al.*⁴⁴ proposed that insulin resistance leads to diastolic dysfunction and suggested it is mostly due to myocardial energy deficiency, with microvascular problems and the glucose/fat imbalance having less of an impact.

Cardiac metabolism

A substrate utilisation mismatch occurs in the diabetic heart, resulting in an increased preference for fatty acid metabolism. The glucose-fatty acid cycle does not optimise fuel use as the body is not sensitive to insulin and subsequently blood glucose levels are elevated. The lack of insulin sensitivity means that glucose transporter 4 (GLUT4), which is normally translocated to the plasma membrane when more glucose is present, is not relocated, and so less glucose enters the cells. The elevation in fatty acids present also results in high levels of citrate which inhibits phosphofructokinase, reducing the rate of glycolysis. As both fatty acids and carbohydrates are high, increased fatty acid metabolism and resultant acetyl CoA production by β -oxidation has

an inhibitory effect on the enzyme PDH suppressing glucose oxidation. Specifically, PPAR- α is increased, resulting in transcriptional upregulation of PDK4 which suppresses PDH activity. Overall, a reduced uptake and metabolism of glucose in the diabetic heart results in impaired cardiac efficiency⁴⁵.

2.2.2.2 The diabetic liver

In diabetes, the insulin-resistant liver is gluconeogenic, leading to a metabolic state similar to fasting. Insulin, which usually causes the suppression of hormone-sensitive lipase, is not recognised, resulting in fatty acid release from the adipose tissue. These fatty acids are transported to the liver, where they are esterified (to triacylglycerol), before incorporation into very-low-density lipoproteins. Elevated fatty acid metabolism produces high levels of acetyl CoA, inhibiting PDH, and producing ketone bodies. Acetyl CoA also activates PC, leading to production of glucose via phosphoenolpyruvate carboxykinase (PEPCK) - normally suppressed by insulin at the level of DNA transcription, via cyclic AMP³³.

2.2.2.3 Current treatment options

In early diabetes, diet is the recommended treatment, due to the close links between obesity and diabetes, and also due to the fact that the pancreatic islets of Langerhans are still present even if they are not functioning correctly. It has recently been shown by researchers at Newcastle University that the insulin resistance and defective insulin secretion in early diabetic patients can be reversed by an eight week, very low calorie diet⁴⁶, with patients losing an average of 15 kg over eight weeks. Work is ongoing to see whether this effect can be maintained for up to two years.

Subsequent to dietary intervention, several drug options are available, which target different aspects of diabetic metabolism.

The sulphonylureas improve insulin secretion by stimulating the pancreatic β -cells. These cells store and release insulin, and as such are sensitive to the blood glucose levels. As the glucose level rises, GLUT2 transporters in the β -cells take in glucose, and the ATP that is subsequently produced via metabolism of this glucose causes ATP-sensitive potassium channels in the cell

membrane to close. This changes the polarisation of the membrane, and causes calcium channels, also on the membrane, to open. The change in calcium concentration signals for secretion of insulin. Damage to the pancreas can cause the potassium channels to remain open. Sulphonylureas act via modification of the Mg^{2+} -ATP subunit on the ATP-sensitive K^+ channels, forcing them closed and therefore stimulating insulin release. These drugs are fast-acting, but can cause hypoglycaemia and should not be given to patients with later-stage kidney or liver disease⁴⁷.

The biguanide family is another drug option; the most common is metformin. Metformin is widely used, and acts by suppressing excessive hepatic gluconeogenesis. The mechanism however remains elusive, with some research suggesting it acts via AMP-activated protein kinase. It has also been demonstrated to have cardioprotective effects⁴⁸ when compared to sulphonylureas or insulin. However it should not be taken by patients with decreased kidney function, as good kidney function is required to clear the lactate that is not used for gluconeogenesis, which can otherwise cause lactic acidosis.

Further still, there are thiazolidinediones, incretin mimics, dipeptidyl peptidase 4 (DPP-4) inhibitors, and more recently, sodium glucose transporter 2 (SGLT-2) inhibitors, and research continues in order to discover further alternatives, and to overcome the need for combinatory dosing.

Several treatments for diabetes aim to improve glycaemic control, via various therapies including insulin. Reversal of several markers of cardiac dysfunction were seen in rats following insulin treatment⁴⁹. Insulin-like growth factor 1 (IGF-1), the serum levels of which have been shown to be related to glycaemic control in type II diabetes, has been shown to improve myocardial function and suppress myocyte apoptosis⁵⁰.

Improving metabolic control in patients has been shown to improve diastolic function^{51,52} and a study of diabetic animals with lower triglycerides (indicating improved metabolic control) showed a reduced susceptibility to cardiomyopathy⁵³. It has been suggested that the metabolism directly contributes to the diastolic changes⁵⁴. A study investigating genetically diabetic mice with over-expression of GLUT4 showed normal cardiac metabolism and function, further suggesting a causative link between glucose metabolism and cardiomyopathy⁵⁵. There have, however, also

been studies demonstrating uncertainty surrounding the links between diabetic control and functional changes in the heart^{56–58}. The Diabetes Mellitus and Diastolic Dysfunction (DADD) study - a large patient study - concluded that ‘strict glycaemic control induced no improvement in diastolic LV function’⁵².

2.3 Hypoxia

Hypoxia can be defined as the provision of insufficient oxygen to cells or tissues, and is a concern when it comes to the micro- and macro-vascular complications observed in diabetes. The predominant mechanism of the hypoxic response is based on the activation of hypoxia-inducible factor (HIF), a transcription factor comprised of HIF- α and β sub-units. This factor is present in all nucleated cells⁵⁹, with the β sub-unit ubiquitously expressed.

Under normoxic conditions, HIF- α is hydroxylated, resulting in its degradation⁶⁰. This hydroxylation can occur at two conserved sites within HIF- α ; at proline residues, via prolyl hydroxylases, or at asparagine residues, via factor-inhibiting HIF (FIH). Both methods are catalysed by oxygenases that use Fe(II) as a co-factor, and 2-oxoglutarate and oxygen as co-substrates.

Under hypoxic conditions, HIF- α migrates to the nucleus where it dimerises with the β sub-unit. The complex then binds to response elements within the promoter region of genes with the relevant conserved sequence⁶¹ that help the cell cope with the reduced oxygen supply (figure 2.10). The hypoxic response involves both metabolic and physiological changes, and HIF has many downstream targets (figure 2.11). Increases in PDK1 and GLUT1 result in increased anaerobic glycolysis^{62,63}, reducing the dependence on oxidative phosphorylation and attempting to maximise ATP production with low oxygen. Increased vascular endothelial growth factor (VEGF) and erythropoietin (EPO) stimulate angiogenesis and red blood cell production respectively, in an effort to increase oxygen delivery. If normoxia is restored after a period of hypoxia, HIF is rapidly degraded, with a half-life of less than five minutes^{64,65}. Further, long-term exposure to hypoxia does not cause continuous upregulation of HIF; both *in vivo* and *in vitro* it has been shown that following 24hrs of hypoxia, HIF is upregulated, but after one week HIF is not elevated above normoxic levels⁶⁶. These changes in HIF are also organ-specific, with Stroka *et al.*⁶⁷ showing maximal activation in mouse kidney and liver tissue after one hour of housing

in a normobaric hypoxic chamber (fraction of inspired oxygen, or $\text{FIO}_2 = 6\%$), returning to normal levels after four hours, whereas the maximum in brain was at five hours, returning to normal after twelve hours. Some longer term *in vivo* work looking at HIF expression in the peri-infarct region in the hearts of Sprague-Dawley rats shows HIF mRNA remained high up to 4 weeks following the infarct; potentially HIF is still providing a beneficial effect by this time and therefore remained elevated⁶⁸.

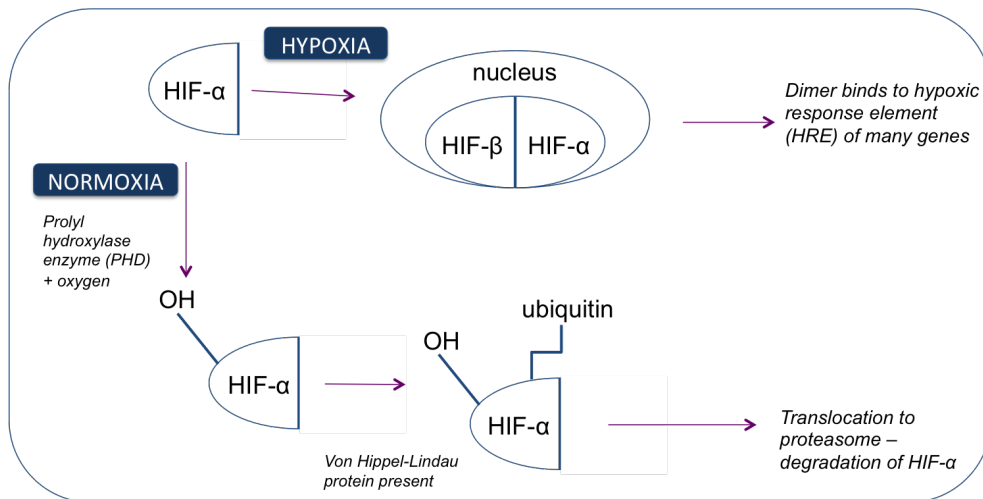


Figure 2.10: Fate of HIF under hypoxia and normoxia

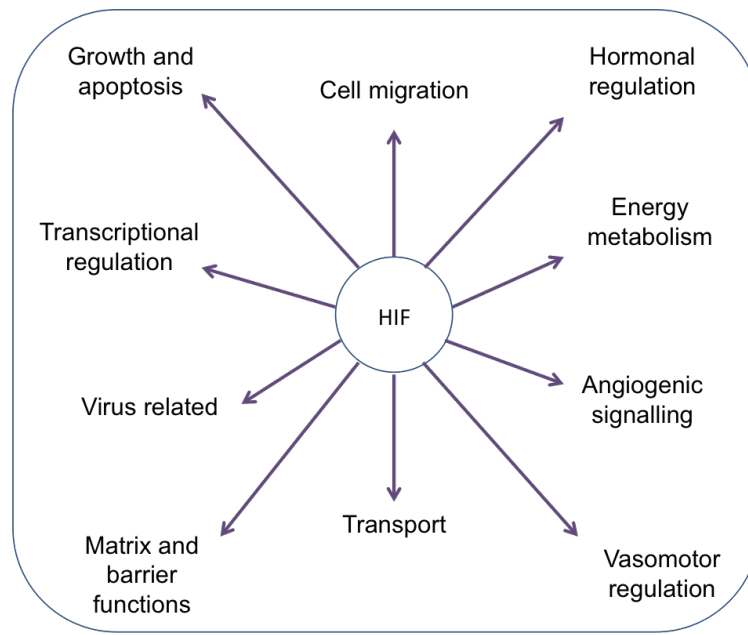


Figure 2.11: The many downstream targets of hypoxia-inducible factor. Adapted from Schofield, C. J., 2004 *et al.*⁶⁹

2.4 Magnetic resonance techniques: thermal and hyperpolarised

There are many methods for investigating the underlying mechanisms behind disease states in animals, from cellular studies to *in vivo* work. Magnetic resonance imaging and spectroscopy provide a means to examine structure, function and metabolism, *in vivo* and non-invasively.

2.4.1 Nuclear magnetic resonance

Magnetic resonance (MR) is a technique that enables us to obtain both images and spectra of metabolites *in vivo* and *ex vivo*. It requires the presence of MR-active nuclei, defined by the property of spin. Spin can be described as the nucleus spinning about an axis, as shown in figure 2.12.

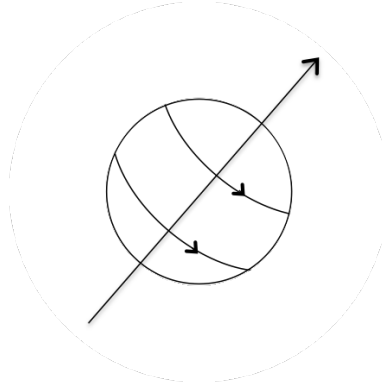


Figure 2.12: Nuclear spin

It is necessary for the spin (or angular momentum) of the nucleus to be non-zero for the nucleus to be MR-active. This occurs in nuclei with odd mass numbers, i.e. those where the number of neutrons and protons are not equal, such as ^1H , ^{13}C or ^{31}P . When placed in a magnetic field, these nuclear spins align either with or against the applied field (B_0). In nuclei with non-zero spin, the number of nuclei aligned with and against B_0 will not be equal. The two orientations that the nuclei fall into are of slightly different energy levels, which can be plotted as a function of B_0 as in figure 2.13.

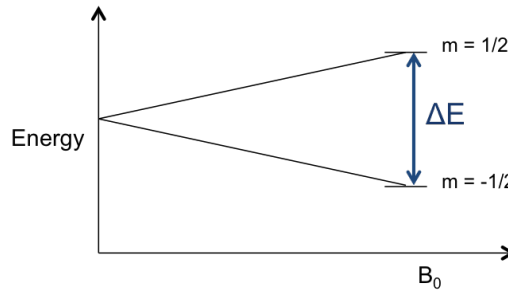


Figure 2.13: Possible energy levels for nuclei in a magnetic field. ΔE = energy difference. B_0 = applied magnetic field

If another magnetic field is applied, perpendicular to B_0 , transitions between these two energy states can be induced. This field, B_1 , is applied at a frequency proportional to ΔE (which is in turn proportional to B_0), and obeys equation 2.1.

$$\Delta E = h\nu_0 \quad (2.1)$$

Given there are an unequal number of spins in each orientation, the overall magnetisation can be depicted as in figure 2.14.

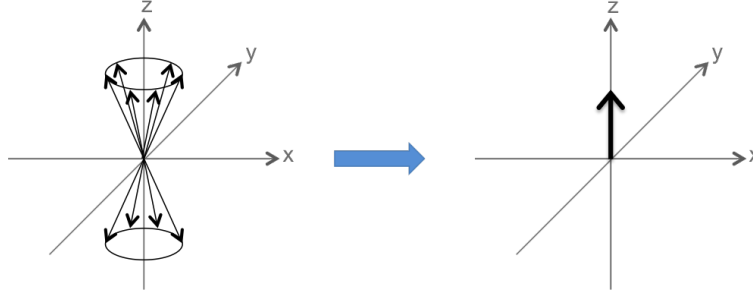


Figure 2.14: Distribution of nuclei in a magnetic field, and depiction of overall magnetisation

The nuclei are precessing around the direction of the main applied field (B_0), along the z axis, at a frequency (ω) depicted by the Larmor equation $\omega = \gamma B_0$, where γ is the gyromagnetic ratio for the relevant nuclei. If a radiofrequency (RF) pulse is applied at the correct frequency, the magnetisation is forced out of this precession, for example moving into the transverse plane, as shown in figure 2.15.

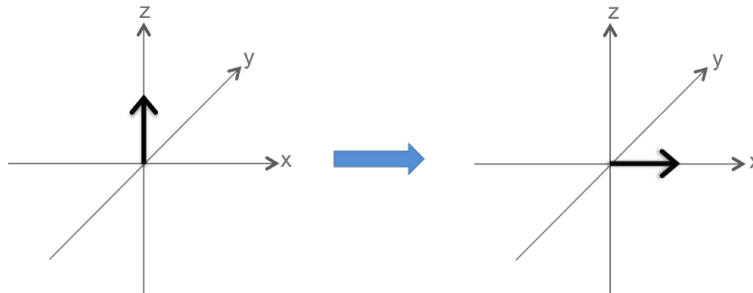


Figure 2.15: Application of a radiofrequency pulse at the Larmor frequency

The way in which these spins then relax to their original state, when the RF pulse ends, provides information on the surroundings of the nuclei, for example their chemical environment.

2.4.2 Imaging

In reality, the difference in the number of nuclei in each energy state is very small, causing the inherent insensitivity of magnetic resonance. For MR imaging (MRI) however, the sheer number of ^1H nuclei present in H_2O around the body is sufficient to overcome this insensitivity. MRI

provides us with *in vivo* images of a particular organ of interest. Cardiac MR for example can produce images such as in figure 2.16.

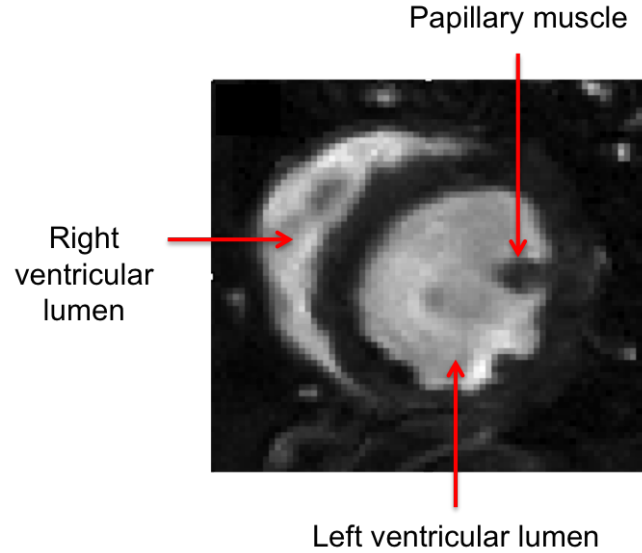


Figure 2.16: Example cine MR image of an *in vivo* rat heart in diastole

When the subject is placed in the magnet, scout images are taken to orientate the user, before several images are taken in slices down the heart (shown in figure 2.17). These images capture the motion of the heart from diastole to systole, with acquisition initiated by the heart beat (measured by electrocardiogram, via leads placed under the skin across the axis of the heart which measure the cardiac electrical impulses) to ensure a whole cardiac cycle is captured. Contrast in these images is due to the different environments of the water, in part due to the constant flow of blood, and the resultant different relaxation times of the nuclear spins. These are characterised by the spin-lattice (T_1) and spin-spin (T_2) relaxation times, which are recovery along B_0 and decay perpendicular to B_0 , respectively.

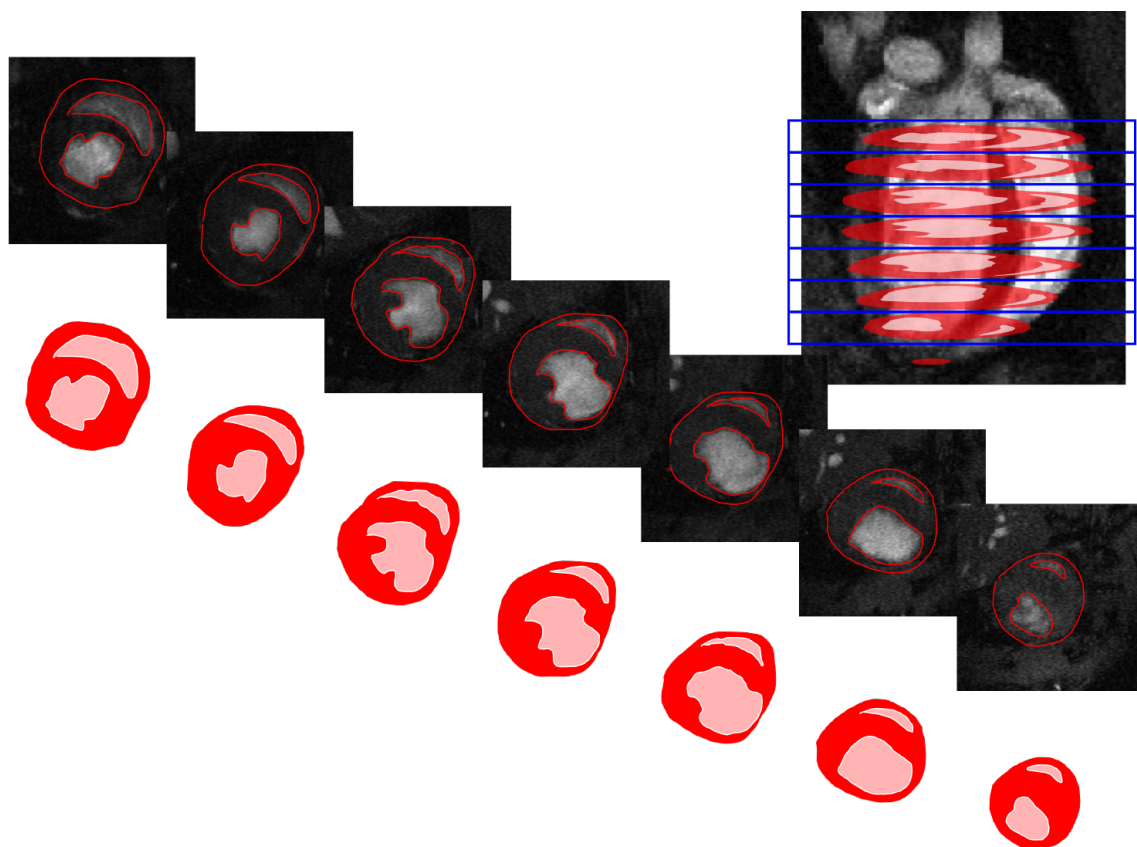


Figure 2.17: Analysis of cine image slices, courtesy of Dr Carolyn Carr

2.4.3 Spectroscopy

2.4.3.1 ^{13}C techniques

^{13}C MR is well-suited to the study of metabolism given that the majority of substrates found in the body are carbon-based. However, the abundance of natural, MR-active ^{13}C is very low ($\sim 1\%$), and so very long scan times are required for data acquisition. The use of hyperpolarised ^{13}C -labelled compounds can overcome these two issues.

2.4.3.2 Hyperpolarisation

Hyperpolarisation is the enhancement of nuclear polarisation (i.e. the population difference between the different energy states), resulting in a vast improvement in MR visibility. Nuclear spins are not maximally polarised when placed in the magnetic field strengths available today. There

is therefore potential for improvement in signal, if this level of polarisation can be enhanced. Dynamic nuclear polarisation (DNP)⁷⁰ is a method which enables an increase in the number of polarised nuclei, i.e. nuclei in the low energy spin state. To achieve this, a sample containing MR-active nuclei is doped with a source of free electrons. This doped sample is then cooled to $\sim 1\text{K}$ in a magnetic field, resulting in non-maximal polarisation of nuclei, but near maximal polarisation of the electrons present. Irradiation of this sample with microwaves at a frequency close to the electron Larmor frequency causes a coupling of the electrons and nuclei, and a subsequent transferral of polarisation by a mechanism known as a flipflop, involving forbidden transitions, resulting in a higher polarisation of nuclei (creation of ' n^+ '; figure 2.18).

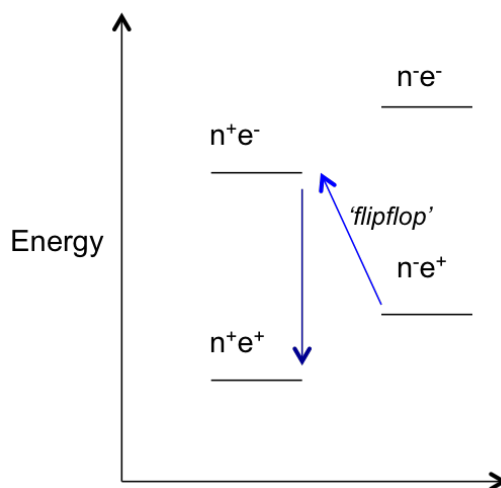


Figure 2.18: Induced energy transitions

There is a second mechanism for transfer of electron polarisation, which instead involves allowed transitions and the electron-electron dipolar interactions which are abundant given the doped sample. The nuclear spin system and the electron system are thermally coupled, so when the electron system is cooled by the microwave irradiation, the nuclei become trapped in the low-energy state, resulting in hyperpolarisation.

Given the low temperatures required for these processes, the hyperpolarised compound is in the solid state. However, a recent development published by Ardenkjaer-Larsen *et al.*⁷¹ enables the rapid dissolution, ejection and neutralisation of these hyperpolarised compounds for injection into model systems (both *in vivo* and *ex vivo*). The MR-active nuclei can then be tracked in real-time via MR spectroscopy. This technique results in an increase in sensitivity of $>10,000$ -

fold and a significant increase in both signal/noise ratio and temporal resolution compared to spectra obtained at thermal equilibrium (figure 2.19).

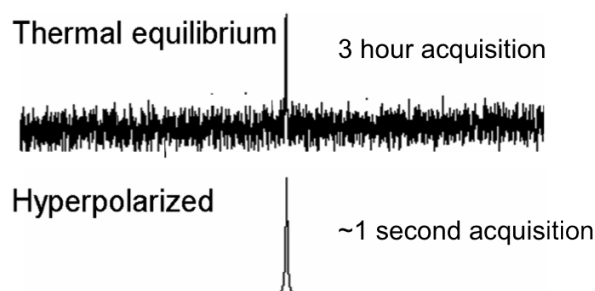


Figure 2.19: Example spectrum obtained from a 3 hour thermal acquisition compared to a hyperpolarised spectrum obtained in ~ 1 second

The most well-established small molecule currently observed in the body using DNP is pyruvate^{8,72}, the breakdown product of glycolysis. Pyruvate can be labelled at either the first⁷³ or second⁷² carbon, or both as demonstrated by Chen *et al.*⁷⁴. These different labels can enable the monitoring of different metabolic pathways, for example labelling at the second carbon results in the label being transferred to acetyl CoA, whose subsequent uptake into the Krebs cycle can then be monitored⁷². All the work in this thesis used $[1-^{13}\text{C}]$ pyruvate.

If hyperpolarised $[1-^{13}\text{C}]$ pyruvate is injected either *in vivo* or into isolated organs, we can see the ^{13}C label transferred to the metabolites downstream of pyruvate (figure 2.20).

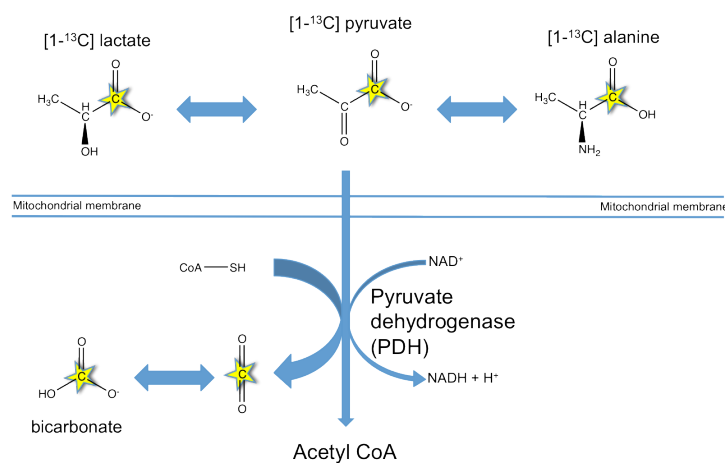


Figure 2.20: Potential fates of the ^{13}C label from $[1-^{13}\text{C}]$ pyruvate (yellow stars represent ^{13}C label)

These include alanine and lactate, whose production is increased under anaerobic conditions, and bicarbonate (in equilibrium with carbon dioxide), increased under aerobic conditions. The signal from the hyperpolarised pyruvate is visible for a short period of time, over which it decays in a predictable manner, and after which the nuclear spins have relaxed back to their thermal equilibrium state. Spectra can be obtained rapidly, for example every second (figure 2.21), allowing the plotting of a timecourse of the arrival of labelled pyruvate, and its metabolism to lactate, alanine and bicarbonate in real-time. Examples of these spectra are shown in figure 2.22. The ratio of metabolite to pyruvate can be used as a measure of flux through the relevant enzyme⁷⁵. Previous work by Atherton *et al.*⁷⁵ in control, DCA-treated and high-fat diet-fed rats demonstrated that hyperpolarised measurements of PDH flux using $[1-^{13}\text{C}]$ pyruvate ‘correlated significantly with *ex vivo* measurements of PDH enzyme activity’, assessed using an established activity assay⁷.

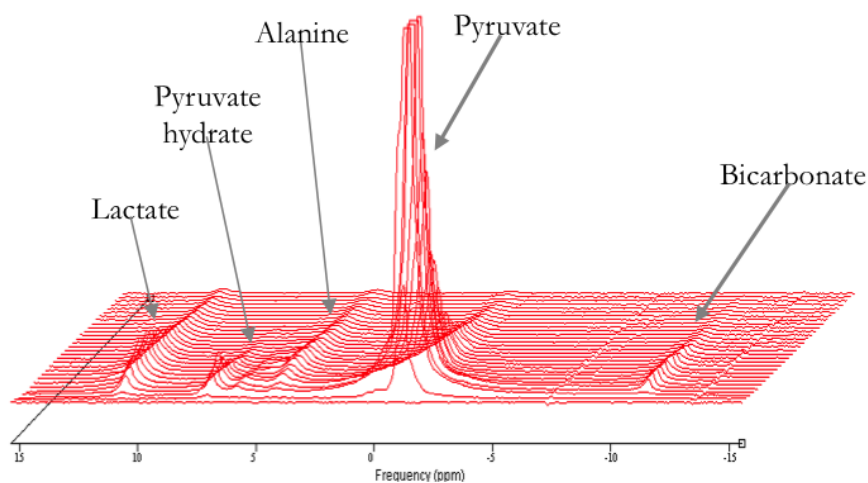


Figure 2.21: Spectra obtained on injection of hyperpolarised $[1-^{13}\text{C}]$ pyruvate *in vivo*

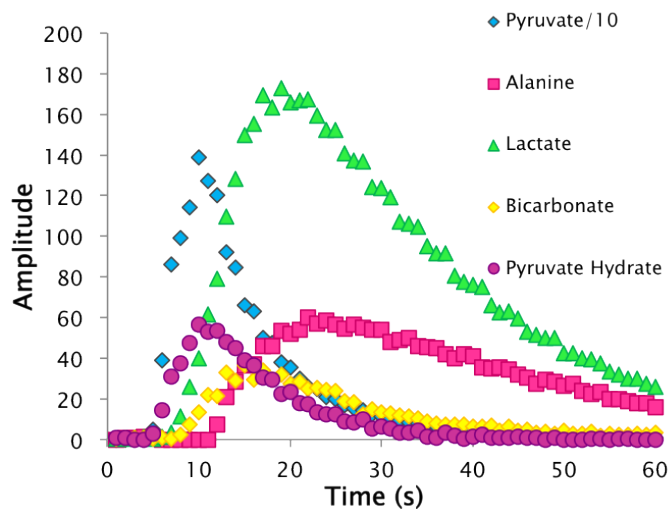


Figure 2.22: Example timecourse of the real-time appearance and metabolism of the ^{13}C -labelled substrates in the rodent heart

Other hyperpolarised probes have been investigated. Ball *et al.*⁷⁶ studied the hyperpolarisation of the short-chain fatty acid butyrate, observing multiple metabolic steps both *in vitro* and *in vivo*. Bastiaansen *et al.*⁷⁷ presented work demonstrating the rate of transformation of acetate into acetylcarnitine using hyperpolarised acetate. Specifically, other probes are being developed for cancer research. Both the metabolic profile of existing tumours⁷⁸, using hyperpolarised lactate and pyruvate, and the response to chemotherapy⁷⁹, using hyperpolarised fumarate, have been studied, and it has been established that metabolic changes in tumours can be seen following treatment and prior to any visible improvements. Work in the field of cancer has been carried out in many animal models, including non-human primates⁸⁰, and the first patients (prostate cancer) have been studied in America⁷³ over the last few years. It is widely agreed that there is much promise for hyperpolarised work in cancer⁸¹, and probes and methods continue to be developed⁸².

2.4.4 Current research focusses using hyperpolarised pyruvate

Many studies have followed the original hyperpolarised work by Ardenkjar-Larsen *et al.* in 2003⁷¹, and hyperpolarised pyruvate in particular has been used to investigate several disease areas.

Work in perfused organs has enabled development of both spectroscopy and metabolic imaging. Use of the perfused heart has allowed direct assessment of metabolic flux without confounding factors such as hormones that would be present in the body. The work by Merritt *et al.*⁸³ demonstrated that when using [1-¹³C]pyruvate, the measurement of bicarbonate is exclusively obtained by flux through pyruvate dehydrogenase. Perfused heart work by Schroeder *et al.* looking at metabolism post-ischaemia showed [2-¹³C]pyruvate could provide information on the Krebs cycle⁷², and hyperpolarised pyruvate has also been demonstrated to be capable of distinguishing between acute and chronic myocardial infarction⁸⁴. Merritt *et al.* demonstrated further use of hyperpolarised pyruvate in assessing perfused liver metabolism, looking at flux through pyruvate carboxylase⁸⁵.

Moving *in vivo*, the early cardiac study by Schroeder *et al.*⁸ established proof of principle by looking at both the differences between the fed and fasted metabolic states and a type I diabetic rat model compared with control. These comparisons demonstrated measurable differences between metabolic states *in vivo*, and in combination with confirmation of physiological relevance by Atherton *et al.*⁷⁵ this supported further work *in vivo* to look at other disease models. Work on a hyperthyroid rat model by Atherton *et al.*⁸⁶ demonstrated the ability to follow treatment response, and Schroeder *et al.* successfully distinguished between early and late heart failure using [2-¹³C]pyruvate in a pig model⁸⁷. Alternative probes are also being developed *in vivo*, as demonstrated by Chen *et al.*⁷⁴ who used pyruvate labelled at both the first and second carbon to look at PDH flux, Krebs cycle flux and pH with one injection of hyperpolarised pyruvate.

Use of hyperpolarised probes in the liver is relevant to several diseases, such as insulin resistance in diabetes; Lee *et al.*⁸⁸ showed increased pyruvate carboxylase flux using [1-¹³C]pyruvate and the exchange of the ¹³C label to malate and aspartate. Josan *et al.* used the conversion of [1-¹³C]pyruvate to lactate to provide information about rat liver metabolism as altered by alcohol⁸⁹. Again with a focus on diabetes, metabolism in the kidneys has been probed with two studies from Laustsen *et al.*^{90,91}, first assessing early changes in diabetes, followed by establishing the effect of hypoxia on the diabetic kidney, relevant given the susceptibility to vascular problems in diabetes.

With sterile systems to produce hyperpolarised compounds being established in several laboratories around the world, clinical applications of hyperpolarised compounds are soon to be

realised. Specific to the work in this thesis, the first clinical polariser to be used for cardiac studies has recently been installed, with the wide clinical implications reviewed by Rider and Tyler in 2013⁹².

Chapter 3

General methods

The methods described in this chapter are used several times throughout this thesis. Further specific methods are detailed in individual chapters.

3.1 Animal handling

All animals were housed in animal facilities at the University of Oxford, on a 12:12 h light/dark cycle. All studies conformed to the Home Office Guidelines on the Operation of the Animal (Scientific Procedures) Act of 1986 and to University of Oxford institutional guidelines. Male Wistar rats were used for all studies, and were between 10 and 11 weeks in age when data were acquired.

All metabolic data was taken in the fed state (i.e. between 6am and 1pm) unless otherwise stated. Fasting to assess fasting metabolism involved removal of food from the animals in the evening (5pm-7pm) until after they were scanned the following morning.

3.2 Induction of a model of type II diabetes

Animals were fed a high fat diet (60% fat, 35% protein, 5% carbohydrate; Special Diet Services, Essex, UK) for 12 days before an overnight fast. The following morning, streptozotocin was dissolved in freshly made citrate buffer (100 ml: 0.655 g citric acid, 0.552 g sodium citrate, pH4)

and administered intraperitoneally (25 mg/kg, unless otherwise stated). Animals continued to be fed the high fat diet for the following week and during any subsequent experimental time.

3.3 Measurements during anaesthesia

3.3.1 Blood glucose

Blood glucose levels were measured (mmol/L) from a tail prick blood sample whilst the animals were under anaesthesia, using an AccuChek monitor (Optium Xceed, Abbot Diabetes Care, UK) and blood glucose test strips (FreeStyle Optium test strips, Abbot Diabetes Care, UK). Twelve hour fasts were carried out for testing fasting blood glucose levels.

3.3.2 Oxygen saturation

Blood oxygen saturation was measured using a red/infrared MouseOx pulse oximeter (Starr Life Sciences, US), placing the sensor on the rat foot. Heart rate and breathing rate were also assessed via this sensor.

3.3.3 Blood sampling

Saphenous vein blood samples were obtained after removal of fur from the thigh with hair clippers, and puncture of the vein with a needle. Blood was taken in heparinised capillary eppendorfs, before being centrifuged at 30,000 rpm for 10 min at 4°C. The plasma was then removed to a fresh eppendorf and frozen in liquid nitrogen before storage at -80°C.

3.4 Animal preparation for *in vivo* magnetic resonance protocol

Animals were anaesthetised with 3.5% isoflurane in oxygen (2 L/min) and maintained via a nose-cone at 2% in a home-built cradle (figure 3.1), with electrocardiographic (ECG) leads placed in the fore-leg which enabled heart rate to be monitored throughout the experiment. The tail vein

was cannulated for administration of hyperpolarised $[1-^{13}\text{C}]$ pyruvate later in the experiment. Animals were maintained at 37°C via air heating, with breathing rate also monitored via a loop of wire placed over the rat abdomen. A home-built ^{13}C butterfly coil was placed under the rat chest to obtain ^{13}C spectra.

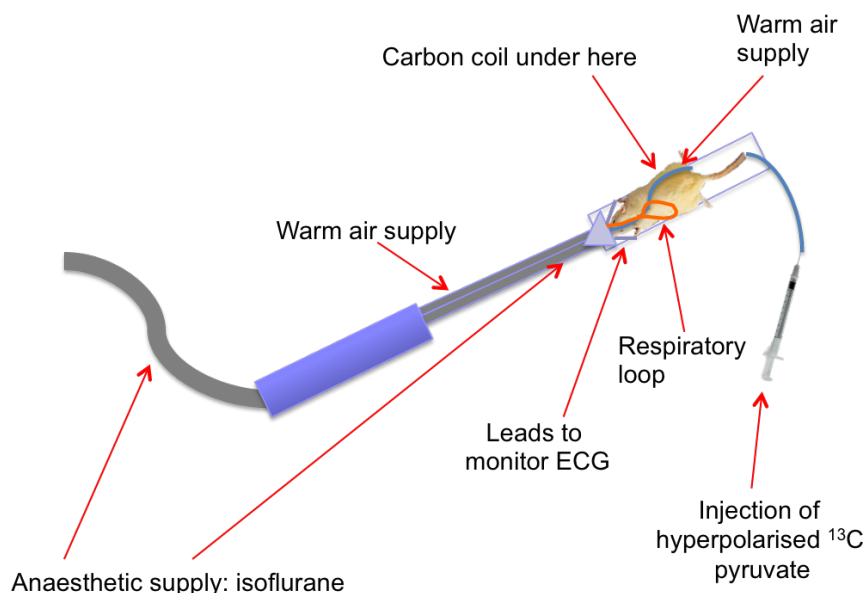


Figure 3.1: Home-built cradle for magnetic resonance imaging and spectroscopy of rodents in 7T magnet system

The cradle was placed inside a horizontal bore 7 Tesla MR system (figure 3.2) interfaced to a Varian console (Varian Inc, Yarnton, UK). Sagittal and axial scout images were taken (FLASH images, TE 1.7 ms, TR 2.33 ms, matrix size 64×64 , FOV 60×60 mm, slice thickness 2.5 mm, excitation flip angle 15°) to ensure the animals were positioned correctly and at the isocentre of the magnet. An ECG-gated shim was used to reduce the proton linewidth to ~ 150 Hz for global scans, and ~ 100 Hz for slice selective scans.

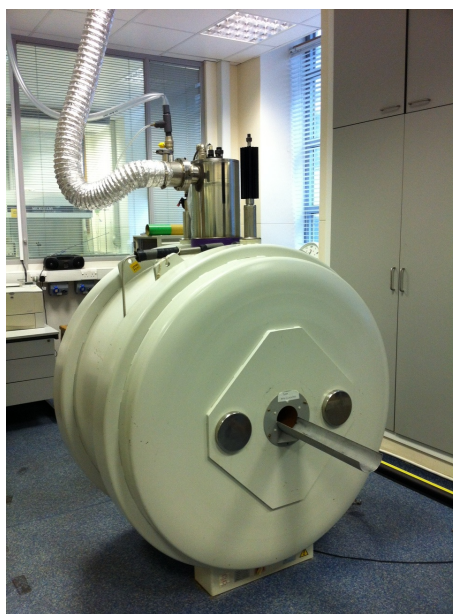


Figure 3.2: 7T magnet system

3.5 Preparation and injection of hyperpolarised pyruvate

30 μl ^{13}C -labelled $[1-^{13}\text{C}]$ pyruvic acid was mixed with 15 mM trityl radical (OXO63, GE Healthcare) and 3 μl 2% Dotarem (Guerbet, Birmingham, UK) and hyperpolarised in a prototype polariser (‘alpha-system’, figure 3.3) for at least 30 minutes at $\sim 1\text{K}$ before being rapidly dissolved in a pressurised, heated alkaline solution (31 mM Tris-base, 60 mM sodium hydroxide, 0.25 mM dipotassium salt of ethylenediaminetetraacetic acid (EDTA))⁷¹. This produced an 80 mM solution of hyperpolarised sodium $[1-^{13}\text{C}]$ pyruvate at physiological temperature and pH, which was immediately injected into the anaesthetised animal in the MR system (1 ml over 10 seconds) whilst acquisition of spectra was initiated. Spectra were obtained from the organ/s of interest over approximately 60 seconds.

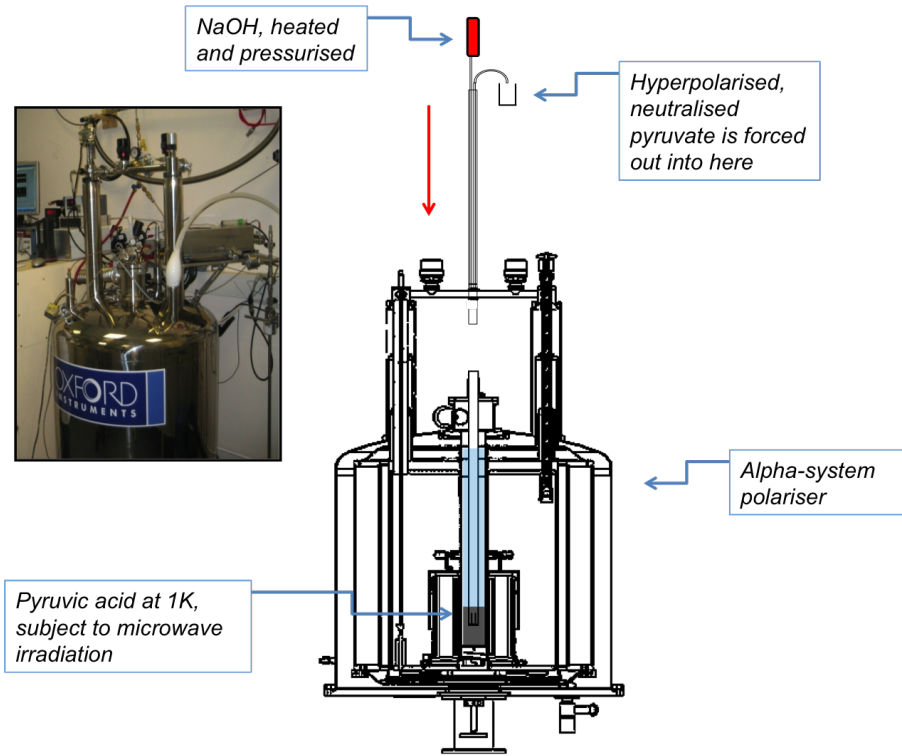


Figure 3.3: Prototype polariser: photograph and schematic

3.6 Spectroscopic data analysis

Unless otherwise stated, data were analysed using the jMRUI software package⁹³ and the inbuilt AMARES algorithm. Spectra were DC offset-corrected based on the last half of the acquired points. The peak areas of the metabolites (pyruvate, bicarbonate, lactate and alanine) at each time point were quantified and used as input data for a kinetic model developed by Atherton *et al.*⁷⁵, based on the original model by Zierhut *et al.*⁹⁴. Firstly the change in labelled pyruvate signal over the acquisition time was fitted to the integrated $[1-^{13}\text{C}]$ pyruvate peak area using equation set 3.1.

$$M_{\text{pyr}}(t) = \begin{cases} 0 & \text{For } t < t_{\text{arrival}} \\ \frac{\text{rate}_{\text{inj}}}{k_{\text{pyr}}} (1 - e^{-k_{\text{pyr}}(t-t_{\text{arrival}})}) & \text{For } t_{\text{end}} > t \geq t_{\text{arrival}} \\ M_{\text{pyr}}(t_{\text{end}}) e^{-k_{\text{pyr}}(t-t_{\text{end}})} & \text{For } t \geq t_{\text{end}} \end{cases} \quad (3.1)$$

In these equations, $M_{pyr}(t)$ represents the $[1-^{13}\text{C}]$ pyruvate peak area as a function of time. The equations fit the parameters k_{pyr} , the rate constant for pyruvate signal decay (s^{-1}), rate_{inj} , the pyruvate arrival rate (a.u. s^{-1}), $t_{arrival}$, the pyruvate arrival time (s) and t_{end} , the time correlating with the end of the injection (s). These parameters were subsequently used in equation set 3.2. In these equations, $t' = t - t_{delay}$, which accounts for delay in the circulation of hyperpolarised pyruvate through the cardiopulmonary system.

$$M_x(t) = \begin{cases} 0 & \text{For } t' < t_{arrival} \\ \frac{k_{pyr \rightarrow x} \text{rate}_{inj}}{k_{pyr} - k_x} \left(\frac{1 - e^{-k_x(t' - t_{arrival})}}{k_x} - \frac{1 - e^{-k_{pyr}(t' - t_{arrival})}}{k_{pyr}} \right) & \text{For } t_{end} > t' \geq t_{arrival} \\ \frac{M_{pyr}(t_{end})k_{pyr \rightarrow x}}{k_{pyr} - k_x} (e^{-k_x(t' - t_{end})} - e^{-k_{pyr}(t' - t_{end})}) + M_x(t_{end})e^{-k_x(t' - t_{end})} & \text{For } t' \geq t_{end} \end{cases} \quad (3.2)$$

3.7 Cine magnetic resonance imaging

Cine-MRI was used to assess cardiac structure and function. A series of both axial and sagittal scout ECG-gated fast low-angle shot (FLASH) images were acquired and used to plan the true short axis orientation required⁹⁵. The coil was tuned and matched, followed by global shimming. Cine-MR images, consisting of 28-35 frames per cardiac cycle were acquired and between ten and twelve slices covered the entire heart⁹⁶. The imaging parameters were: field of view 51.2 x 51.2, matrix size 256 x 256, slice thickness 1.5 mm, TE/TR 1.43/ 4.6 ms, 0.5 ms/17.5° Gaussian RF excitation pulse and 6 averages.

3.8 Cine image analysis

Whole heart images were analysed using the ImageJ software package⁹⁷. Epi- and endo-cardial borders were outlined by freehand selection to obtain lumen size in the end-diastolic and end-systolic frames. These values were then used to quantify the end-systolic volume (ESV) and end-diastolic volume (EDV). These measurements were subsequently used to calculate further parameters - stroke volume ($\text{SV} = \text{end diastolic volume (EDV)} - \text{end systolic volume (ESV)}$),

cardiac output ($CO = SV \times \text{heart rate}$) and ejection fraction ($EF = SV/EDV$)⁹⁶. Left ventricular (LV) mass was calculated using the LV wall volume and the myocardial specific gravity (1.05 g/cm^3).

3.9 Terminal tissue removal

Animals were, unless otherwise stated, terminally anaesthetised with 5% isoflurane in oxygen (2 L/min). Hearts were excised on cessation of both pedal and corneal reflexes. Hearts were washed rapidly with PBS and freeze-clamped in liquid nitrogen using previously cooled tongs. Blood from the cavity was removed into eppendorfs on ice before being centrifuged for ten minutes at 4°C and 30,000 rpm. Plasma was then removed to clean eppendorfs and frozen in liquid nitrogen. Blood samples for haematocrit were also taken at this time in heparinised capillary tubes which were centrifuged for ten minutes before an assessment of the percentage haematocrit (as detailed in section 3.10.6). Liver and skeletal tissue were also excised, briefly washed in PBS, and freeze-clamped in liquid nitrogen. Epididymal fat pad weight was measured, and presented as a ratio of fat:body weight ratio. All tissue samples were then stored at -80°C before being crushed to a fine powder with a pestle and mortar under liquid nitrogen, for use in biochemical analyses.

3.10 Biochemical analyses

All chemicals were obtained from Sigma-Aldrich (Dorset, UK) unless otherwise stated.

3.10.1 Western blotting

3.10.1.1 Buffer preparation

Buffers were prepared for Western blotting as detailed in the tables below. These include those for lysis stock (table 3.1), lysis (table 3.2), resolving gel (table 3.3), stacking gel (table 3.4), sample loading (table 3.5), 10 x electrophoresis (table 3.6), 10 x Tris-buffered saline (TBS) (table 3.7) and sample transfer (table 3.8).

Solution	Weight (g)
1 M Tris-HCl (pH 6.8)	7.88
20% SDS	10
8 mM urea	24

Table 3.1: Components to produce lysis buffer stock solutions. Total volume of chemicals in addition to double-distilled water (ddH₂O) = 50 ml

Solution	Volume of stock (ml)
Tris-HCl (pH 6.8)	3.75
SDS	9.5
Urea	25
Glycerol	10

Table 3.2: Components to produce working solution of lysis buffer. Total volume of chemicals in addition to ddH₂O = 50 ml

Chemical	Weight (g)
1.5 M Tris-base	90.83
0.4 % SDS	2

Table 3.3: Components to produce resolving gel buffer. Total volume of chemicals in addition to ddH₂O = 500 ml. pH to 8.8

Chemical	Weight (g)
0.5 M Tris-base	30.28
0.4% SDS	2

Table 3.4: Components to produce stacking gel buffer. Total volume of chemicals in addition to ddH₂O = 500 ml. pH to 6.8

Chemical	Amount
Tris-base	1.136 g
SDS	3 g
Glycerol	15 ml
Brilliant blue	5 mg

Table 3.5: Components to produce sample loading buffer. Total volume of chemicals in addition to ddH₂O = 50 ml. pH to 6.8

Chemical	Weight (g)
Tris-base	30.3
SDS	10 g
Glycine	144

Table 3.6: Components to produce electrophoresis buffer (10 x working concentration). Total volume of chemicals in addition to ddH₂O = 1 litre. pH to 8.3

Chemical	Weight (g)
NaCl	90
Tris-base	12.19

Table 3.7: Components to produce TBS buffer (10 x working concentration). Total volume of chemicals in addition to ddH₂O = 1 litre. pH to 7.4

Chemical	Weight (g)
Tris-base	4.54
Glycine	21.61
Methanol	300ml

Table 3.8: Components to produce transfer buffer. Total volume of chemicals in addition to ddH₂O = 1.5 litre

3.10.1.2 Sample preparation

Crushed frozen tissue was weighed out (~ 25 mg) into previously-cooled tubes, and the appropriate amount of lysis buffer ($10\ \mu\text{l}/\text{mg}$ sample) added before rapid homogenisation for approximately 30 seconds. Samples were heated at 95°C for 5 minutes and centrifuged at 2000 rpm for 5 minutes at 4°C .

A protein assay was carried out on an aliquot of these samples to establish individual protein concentrations. $5\ \mu\text{l}$ of sample was diluted with $95\ \mu\text{l}$ of ddH₂O, before $25\ \mu\text{l}$ of the diluted sample was pipetted into a 96 well plate along with several standards of known albumin concentration (range 0 - 2 mg/ml). $200\ \mu\text{l}$ of a working solution of protein assay reagents (Reagents A and B, ratio 50:1, Thermo-scientific, Hemel Hempstead, UK) was added to each well before a 30 minute incubation at 37°C . Spectrophotometric absorbance at 550 nm was measured, and absorbances of standards used to establish protein concentrations of unknowns. All samples were run in duplicate.

3.10.1.3 Electrophoresis

Western blotting samples were prepared by addition of β -mercaptoethanol (5%, Bio-rad laboratories), loading buffer and lysis buffer to a volume of sample that resulted in the same concentration of protein for each tissue sample. $30\ \mu\text{l}$ of each sample was loaded on to 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gels along with a rainbow marker (Thermo-scientific); all samples were run in duplicate on different gels. Samples were separated by electrophoresis at 120 V before being transferred to nitrocellulose membrane. Ponceau staining (Fluka analytical) confirmed even protein loading and transfer, and internal standards were used to ensure homogeneity between samples and gels. The membrane was then blocked in 5% milk (dried skimmed milk powder, dissolved in tris-buffered saline Tween (TBS-T)) for one hour, before being incubated with the primary antibody (again in 5% milk solution, with $100\ \mu\text{l}$ sodium azide) overnight. Details of antibodies used are in appendix A, figure 1. The membrane was then washed for 1 hour with TBS-T before incubation with the relevant secondary antibody (Insight Biotechnology, conjugated to horseradish peroxidase) for 1.5 hours. Following a further hour of washing with TBS-T, membranes were covered with ECL

Western blotting detection solution (GE Healthcare, Amersham, UK, 0.75 ml A: 0.75 ml B per membrane) and sandwiched between two sheets of acetate. Photographic film (Fisher Scientific, Loughborough, UK) was then placed on top of the acetate in a dark room before development of the films (Compact X4 automatic X-ray film developer, X-ograph Imaging Systems). Examples of my developed western blot films are shown below in figure 3.4.

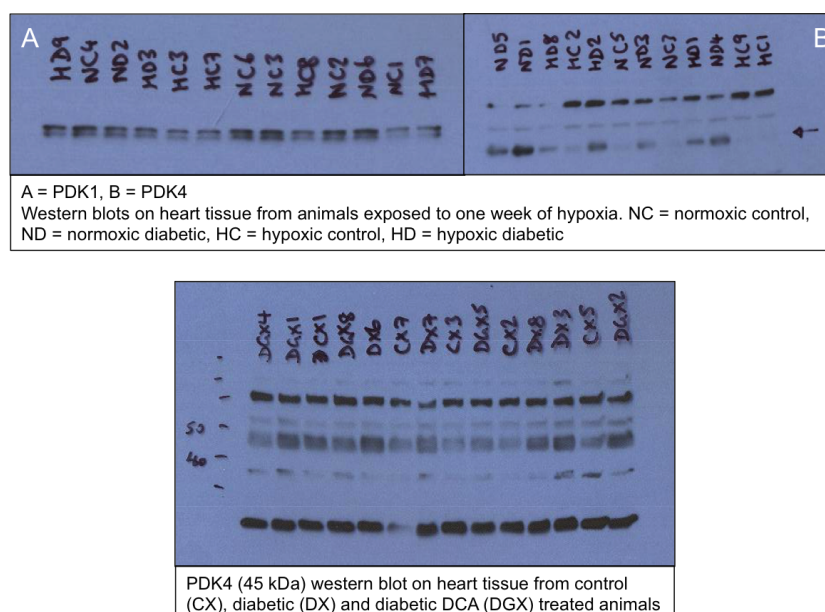


Figure 3.4: Example western blots

3.10.1.4 Data analysis

Intensity of the bands was quantified using the UNSCANit software package (Silk Scientific Inc., USA). Protein expression was standardised using a control sample loaded on all gels, where required.

3.10.2 Triglyceride assay

Frozen tissue was crushed and 25-50 mg weighed (with exact weights noted). 8 ml of Folch solution (chloroform:methanol, 2:1 ratio) was added to all samples, which were rotated for one hour. 2 ml ddH₂O was added and followed by another hour's rotation. Samples were left overnight to allow phase separation. The supernatant and pellets were removed and discarded, and the lower organic phase was dried under air. The sample tubes were then placed on ice, 1

ml of cold ethanol added, and samples mixed. 100 μ l was taken from each sample, placed in an eppendorf and then evaporated to dry overnight. 20 μ l cold ethanol was then used to resuspend each sample for analysis.

The analysis used a triglyceride assay kit (Randox, catalogue number TR 210). 150 μ l of the enzyme reagent was added to each eppendorf. This was incubated for ten minutes before 140 μ l was transferred to a 96 well plate. Absorbance at 500 nm was measured. All samples were run in duplicate.

3.10.3 Plasma insulin assessment

A rat insulin enzyme-linked immunosorbent assay (ELISA) kit (Merckodia, Sweden) was used for assessment of insulin levels in plasma. 10 μ l of each sample (or standard) was added to the assay 96 well plate. 100 μ l of 1 x enzyme conjugate was added to each well before a 2 h incubation at room temperature. The plate was then washed (1 x dilution of wash buffer) five times before 200 μ l 3,3',5,5'-tetramethylbenzidine (substrate TMB) was added to each well. This was incubated for a further 15 minutes at room temperature before 50 μ l stop solution was added. Absorbance was read at 450 nm on a spectrophotometric plate reader.

3.10.4 Plasma metabolite assessment

Blood plasma samples were assessed for a variety of metabolites (including glucose, alanine aminotransferase, lactate and triglyceride) using a clinical chemistry benchtop analyser, ABX Pentra 400 (Horiba Medical). 200 μ l of plasma was required for one set of analyses (in duplicate).

3.10.5 Glycogen assay

This assay involved destroying the endogenous glucose before measuring the glucose as stored as glycogen. 50 mg of tissue was weighed out into eppendorfs. 100 μ l of 30% w/v potassium hydroxide was added and the samples thoroughly mixed. They were then heated at 105°C for one hour before being cooled on ice. 50 μ l 2% Na₂SO₄ was added to each sample before

mixing, and ethanol added to give a final concentration of 75%. Samples were incubated at 4°C overnight. Centrifugation at 16,000 g and 4°C produced pellets; the supernatant was discarded. Samples were then resuspended in 500 μ l of 80% ethanol, before centrifugation and drying at 40°C overnight. 0.375 ml ddH₂O was used to break up the pellet, before addition of 0.125 amyloglucosidase buffer (5 mg amyloglucosidase dissolved in 10 ml of a sodium acetate/acetic acid buffer; 8.2 g of sodium acetate in 100 ml 1 M acetic acid, pH to 5). This solution was incubated at 37°C for one hour before addition of 0.1 ml ddH₂O. Samples were centrifuged for a final time before the supernatant used on the clinical Pentra analyser to measure glucose concentration.

3.10.6 Haematocrit assessment

Plasma was taken in capillary tubes immediately after excision of the heart. Tubes were stoppered with plasticine and centrifuged at 8000 rpm for 10 minutes. Percentage of haematocrit was measured in comparison to the full volume of fluid in the tube.

3.10.7 Statistical analyses and presentation of data

Data were analysed using either an unpaired t-test or a one-way analysis of variance (ANOVA) with a Bonferroni multiple comparison correction, unless otherwise stated. GraphPad Prism software was used for all statistical analyses. Mean values with standard error of the means (SEMs) are plotted for all graphs, unless otherwise stated.

Chapter 4, section 4.5, describes the characterisation of a diabetic rodent model. *In vivo* data from all subsequent studies is summarised in this section, resulting in large data sets for both control and diabetic cardiac (n=41) and hepatic (n=36) data. Thus in chapters 5 and 6, these data are presented for any untreated, normoxic *in vivo* groups. Acquisition of further data sets (for example those treated with a drug, or exposed to hypoxia) are described in the relevant chapter, and the data in graphs are unique to that section.

Chapter 4

Cardiac and hepatic magnetic resonance spectroscopy

4.1 Introduction

The development of type II diabetes is inevitably associated with a systemic alteration of metabolism. Altered hepatic metabolism has a particular dominance due to the liver being a key site of glucose storage and production. Given the prevalence of cardiovascular complications in diabetes¹, it would therefore be interesting to study the interplay between the heart and the liver. Previous work with hyperpolarised compounds has focussed on either the heart or the liver in isolation^{8,98}. However if we were able to study the two organs simultaneously it would provide the maximum information about the current metabolic state. Further it would reduce the number of animals used, and minimise the number of procedures carried out.

Systemic metabolism

Hepatic metabolism is intricately linked to that of the other organs in the body. Two metabolic cycles between the liver and other organs provide an ability to recycle two products of glucose metabolism to produce further fuel for respiration.

The Cori cycle provides a description of the movement of lactate produced in the working mus-

cles, to the liver, where it is converted back to glucose by gluconeogenesis (via pyruvate), which can then be transported back to the muscles. This ensures that lactate levels do not become too high, which can cause lactic acidosis, whilst also maintaining a fuel source for the muscles. A small study in humans by Zawadzki *et al.* using ^{13}C -labelled glucose and blood sampling showed an increased rate of glucose-lactate cycling in patients who were obese, with type II diabetes⁹⁹. This rate did not change when the subjects lost a small amount of weight (approximately 5 kg) however the rate of production of endogenous glucose (a measure of gluconeogenesis) decreased significantly. Boden *et al.* did note that although endogenous glucose production and the rate of gluconeogenesis are important in causing the hyperglycaemia of diabetes, peripheral insulin resistance (i.e. in other organs) is at least as important as these two factors¹⁰⁰. There are many factors key to the control of this cycle, including lactate dehydrogenase, responsible for conversion between pyruvate and lactate, and insulin/glucagon (potentially indicative of fasting or diabetes), responsible for the inhibition/stimulation of gluconeogenesis.

The glucose-alanine cycle, described by Felig *et al.*¹⁰¹ describes the transition of carbon skeletons from proteins to glucose, between the muscle and liver, at a rate approximately 50% of that which occurs in the Cori cycle. It is also a method for moving nitrogen from the peripheral tissues to the liver¹⁰². Glucose metabolism in the muscle produces alanine (along with pyruvate and lactate). This alanine is then transported to the liver where gluconeogenesis can occur via the removal of the amino group from alanine to produce pyruvate. Alanine itself is a prevalent amino acid, important for its role in transporting nitrogen, and alongside production from pyruvate via alanine aminotransferase (also called serum glutamate-pyruvate transaminase), alanine can be formed from protein degradation. In work by Felig and Wahren in 1971¹⁰³, increased exercise intensity resulted in increased alanine production from the legs, which may be a result of increased glucose-alanine cycling, given the importance of alanine as a gluconeogenic precursor. Interference with insulin regulation can also affect glucose-alanine cycling. This has been demonstrated by Layman and Walker¹⁰⁴, who suggested that stimulation of the glucose-alanine cycle by branched-chain amino acids such as leucine could be used for helping patients with the metabolic syndrome lose weight and maintain glycaemic control.

Previous hepatic studies with hyperpolarised compounds

The liver is highly metabolically active, making it suitable for hyperpolarised magnetic resonance studies, as rapid transfer of the ^{13}C label is necessary before the hyperpolarised signal decays. Previous work has successfully followed the metabolism of ^{13}C -labelled pyruvate both in the perfused liver and *in vivo*.

Merritt *et al.*⁸⁵ distinguished between fed and fasted metabolism in perfused mouse livers, in that they showed an increased lactate/alanine ratio in fasted livers. They concluded the technique was sufficiently sensitive to gain a measure of pyruvate carboxylase and PEPCK flux via the observation of incorporation of ^{13}C label into malate and aspartate.

In 2009, Hu *et al.*⁹⁸ showed they were also able to distinguish between fed and fasted animals, but this time *in vivo*. This was achieved by studying ^{13}C label transfer from pyruvate to lactate and alanine in the rat liver, where they saw an increased lactate/alanine ratio in fasted animals compared to fed, driven by a reduction in flux to alanine. They do not report on pyruvate carboxylase, perhaps due to a lack of sensitivity meaning that their ability to measure malate and aspartate was reduced. Further work by Spielman *et al.*¹⁰⁵ began to probe the possibilities for liver hyperpolarised MRS in disease - they demonstrated the use of $[1-^{13}\text{C}]$ pyruvate to investigate the effect of ethanol consumption and look at how the liver reacts - relevant to alcoholic fatty liver disease, hepatitis and cirrhosis. They demonstrated a change in pyruvate to lactate conversion following an injection of ethanol.

Yen *et al.*¹⁰⁶ investigated the use of hyperpolarised magnetic resonance spectroscopy for liver cancer and proposed that the variability in signal from ^{13}C alanine in tumours could distinguish them from healthy tissue. More relevant to the work of this thesis however, is work by Lee *et al.*⁸⁸ which looked at liver metabolism in an insulin resistant mouse model and explored the changes in pyruvate carboxylase flux in diabetes and in treatment. They proposed the use of the rate of conversion of pyruvate to aspartate as a marker for gluconeogenesis and as a measure of pyruvate carboxylase activity.

These studies, and others investigating cardiac metabolism^{8,10,86} used either global acquisitions, localised by the positioning of the radiofrequency coil, or slice selective acquisitions from one organ of interest. Given that the metabolic imbalance that results in diabetes affects the whole

body, it would be of interest to gain a more systemic view of metabolism that could also be used to look at disease and treatment. This chapter discusses the development of an MR acquisition to obtain such data from two organs, selectively acquiring data from the relevant areas, during a single injection of pyruvate (figure 4.1).

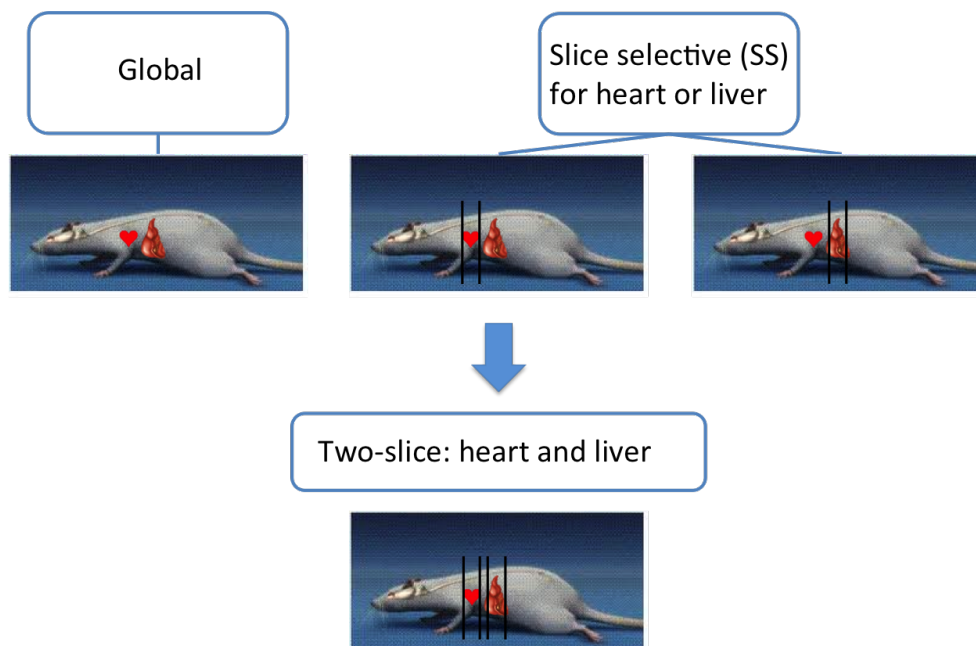


Figure 4.1: Previous acquisition types, and the two-slice acquisition developed in this chapter

4.2 Development of acquisition

Previous work in our laboratory has involved the acquisition of global data. As a result we initially implemented slice selective spectroscopy of individual organs before moving on to the development of the two-slice acquisition. Further, the laboratory had no experience with liver spectroscopy, so it was necessary to investigate the sensitivity that could be achieved with both global and slice selective acquisitions.

4.2.1 Establishing the acquisition protocol

It was first confirmed that we could acquire data from both the heart and the liver by placing the carbon surface coil over the relevant organ. Healthy male Wistar rats ($n=6$) were anaesthetised and placed in the 7T MR system as described in chapter 3, section 3.4, with the carbon coil

placed over the heart or the liver. Shimming achieved linewidths of approximately 150 Hz. Data were acquired globally during and following an injection of hyperpolarised $[1-^{13}\text{C}]$ pyruvate.

This gave spectra such as those seen in figure 4.2. The pyruvate peak was substantially larger in the heart data, in relation to the other metabolites, due to the pools of blood, and therefore hyperpolarised $[^{13}\text{C}]$ pyruvate, in the chambers of the heart. This was not present for the liver, so the pyruvate peak was smaller in relation to lactate, alanine and bicarbonate.

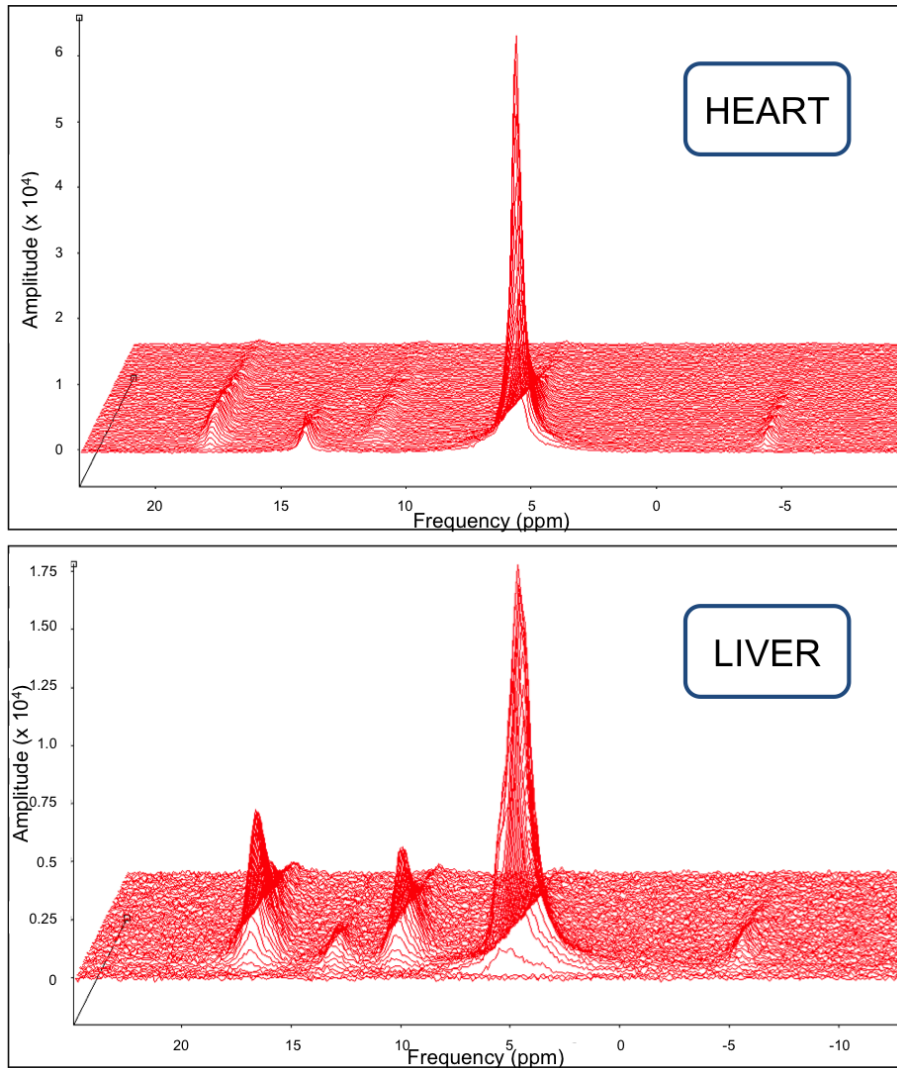


Figure 4.2: Example spectra from global acquisitions of heart and liver metabolism

Following this successful acquisition of both cardiac and hepatic data separately, we next investigated the feasibility of taking data from both organs during one scan. This required the coil to be sensitive over a sufficiently wide area to encompass both organs, which, due to the heart

and liver being in near proximity to each other *in vivo*, was a width of three centimetres.

To assess the sensitivity of our ^{13}C coil, a field map of signal (gradient-echo sequence with a 75 x 75 mm field of view, and an infinite slice to obtain all possible data) was taken across a bottle of $[^{12}\text{C}]\text{urea}$ overnight, with the long scan time necessary due to the low natural abundance of ^{13}C . All phantom data were taken with the help of Mr Jack Miller. We obtained good signal across approximately 4 cm, suggesting that it was possible to obtain data *in vivo* from the rat heart and liver simultaneously.

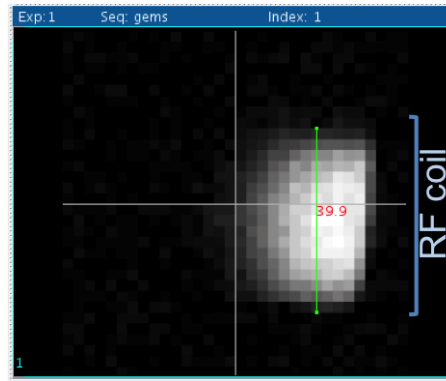


Figure 4.3: Overnight carbon profile across a urea phantom

Next, an assessment of the slice profile was carried out, showing the ability to achieve a good amount of fairly homogeneous signal across a slice width of 1 cm. Once again the urea phantom was used, and figure 4.4 shows the amount of signal across 1 cm, with fairly good homogeneity.

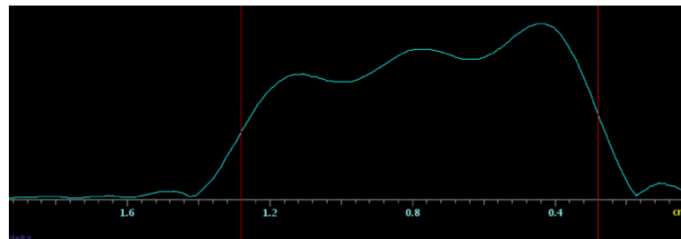


Figure 4.4: Slice profile using proton acquisition on a urea phantom. Distance between the red lines = 1 cm

Finally, the possibility of contamination between slices was ruled out by placing two eppendorfs in close proximity on the coil. One contained $[1-^{13}\text{C}]\text{lactate}$ and the other $[1-^{13}\text{C}]\text{urea}$.

Sagittal and coronal images were taken to confirm the positions of the eppendorfs, along with a global ^{13}C spectrum which showed two spectral peaks, representative of the two different carbon environments present (one in lactate and one in urea, figure 4.5).

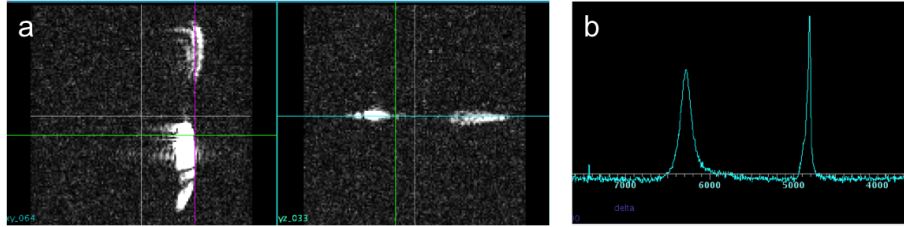


Figure 4.5: a) Location of two eppendorfs, and b) the carbon spectrum peaks arising from the two samples

Then, data were acquired using the two-slice acquisition, with slices placed as they would be *in vivo*, with 1 cm slices placed over the two sources of ^{13}C , 1 cm apart. This resulted in two data sets, each with a single peak, demonstrating no contamination between slices (figure 4.6, a = lactate; b = urea).

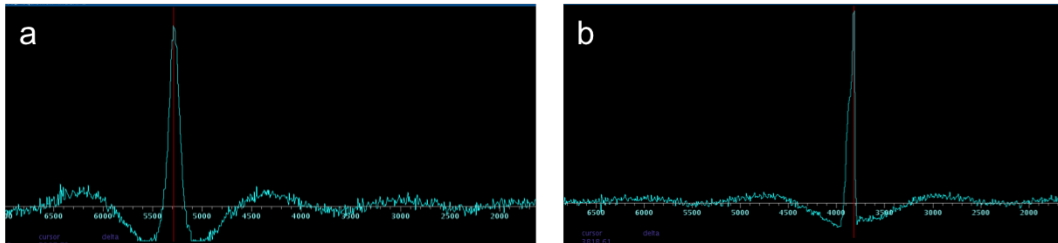


Figure 4.6: Data sets acquired from the two-slice acquisition, showing data from a single ^{13}C environment in each. a = lactate; b = urea

This two-slice acquisition was then tested on an anaesthetised male Wistar rat, prepared for the hyperpolarised pyruvate injection again as described in chapter 3. 1 ml of hyperpolarised $[1-^{13}\text{C}]$ pyruvate was prepared as described in chapter 3, section 3.5 and injected over ten seconds, with data acquired from the start of injection until the hyperpolarised signal had decayed (approximately 60 seconds). Heart and liver data were acquired during this one injection, with the cardiac data acquired every second and triggered by ECG whilst hepatic data were acquired in between cardiac acquisitions. The slices from which data were acquired were 10 mm in thickness, with a 10 mm separation.

This acquisition was successful in that it provided both cardiac and hepatic metabolic data during a single injection of hyperpolarised pyruvate. Optimisation of shims - which led to shimming on individual organs to achieve line widths of approximately 100 Hz - resulted in spectral data such as the example shown in figure 4.7. The baseline roll seen on these spectra was due to first order phase accrual, occurring during the time designated in the sequence for gradient refocussing (necessary because of the slice selective nature of the acquisition).

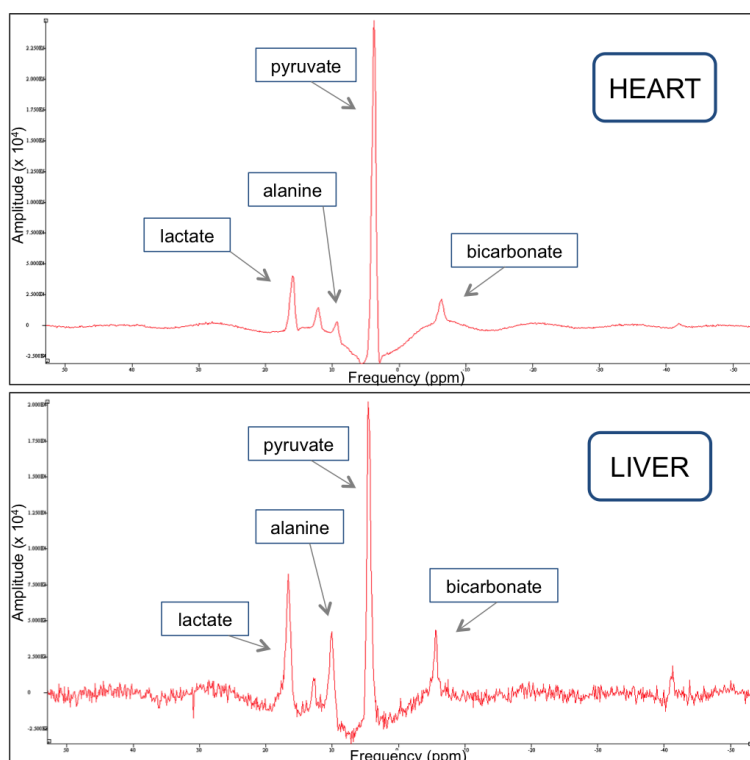


Figure 4.7: Example spectra obtained with the two-slice acquisition

The spectral peaks for lactate and alanine in the heart were again greater in amplitude relative to pyruvate than those seen in the liver, as before due to the pools of blood, and therefore pyruvate, in the heart. The amplitude of bicarbonate was lower in the liver, reflective of a lower PDH activity, and so subsequently, data from several spectra were summed to obtain a measure of PDH activity.

Having established this acquisition protocol, we wished to establish if the choice of scan (global, single slice selective, two-slice selective) would have an effect on the metabolic accuracy or interpretation of the data. As such, the next step was to investigate differences between data

acquired from the different acquisition types across a set of healthy animals.

4.2.2 Comparisons of different scan types

Control male Wistar rats (weight ~ 350 g) were anaesthetised as described in chapter 3, and their tail vein cannulated before they were placed in the horizontal bore 7T MR system. Animals underwent data acquisitions on separate occasions, with at least 48 hours between studies to allow for recovery following anaesthesia. Acquisition of data was begun just prior to injection of hyperpolarised $[1-^{13}\text{C}]$ pyruvate, prepared as described in chapter 3, section 3.5. Data was acquired from the following protocols, on separate days, for all animals:

- Global acquisition (n=6); heart or liver data. The surface carbon coil was placed over the heart or liver, and data was acquired globally.
- Single slice acquisition (n=8); heart or liver data. The surface carbon coil was placed over the organ of interest, and data were selectively acquired from a 10 mm central slice of that organ.
- Two-slice acquisition (n=16); heart and liver data. The surface carbon coil was placed between the heart and the liver, and data were acquired selectively from two slices of 10 mm width, one from the heart and one from the liver. The slices were 10 mm apart.

^{13}C spectra were analysed using the AMARES algorithm in the jMRUI software package⁹³. Spectra were DC offset-corrected based on the last half of acquired points. The peak areas of $[1-^{13}\text{C}]$ pyruvate, $[1-^{13}\text{C}]$ lactate, $[1-^{13}\text{C}]$ alanine and ^{13}C bicarbonate at each time point were quantified and used as input data for a kinetic model based on that developed by Zierhut *et al.*⁹⁴ and Atherton *et al.*⁷⁵. PDH flux was quantified as the rate of ^{13}C label transfer from pyruvate to bicarbonate. The rate of ^{13}C label transfer from pyruvate to lactate and alanine was reflective of lactate dehydrogenase activity and alanine aminotransferase activity respectively.

It was possible to obtain kinetic data for metabolic flux to bicarbonate, lactate and alanine in the heart, and to lactate and alanine in the liver. Flux from pyruvate to bicarbonate was found to be very low in the liver, potentially supported by previous hyperpolarised studies having focussed on lactate and alanine^{98,106}. Therefore for analysis of hepatic data, the first

thirty spectra following the arrival of pyruvate were summed, peaks assigned and amplitudes measured in jMRUI. Following this, the ratio of metabolite:pyruvate was taken as a measure of flux⁷⁵. This allowed us to both measure the hepatic bicarbonate and analyse it in concert with the lactate and alanine.

A one-way analysis of variance (ANOVA) with a Holm-Sidak correction for multiple comparisons was carried out to analyse differences between the types of acquisition.

Results

Cardiac PDH flux was significantly higher in both single slice and two-slice acquisitions compared to the global acquisition. Flux was not significantly different between the single and two-slice sequences. Neither cardiac lactate nor alanine was significantly different between groups (figure 4.8).

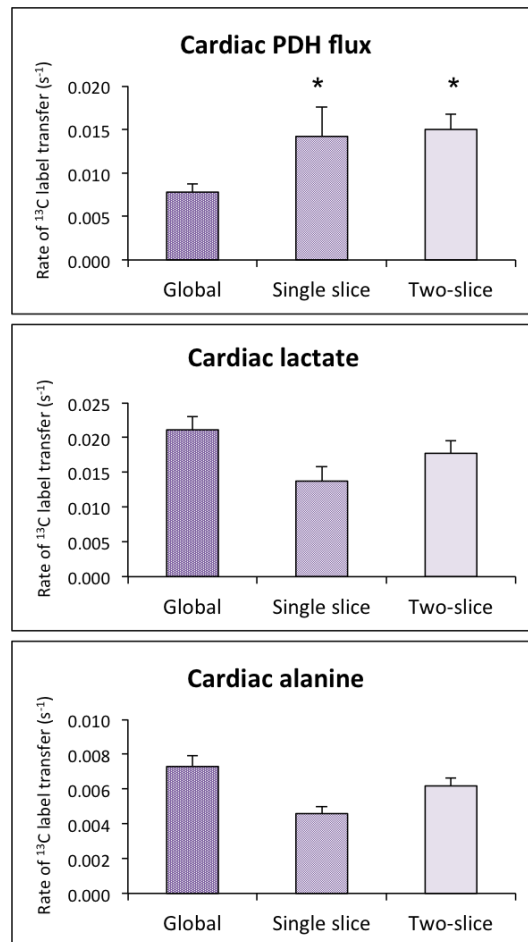


Figure 4.8: Cardiac data from injection of hyperpolarised ^{13}C pyruvate; data acquired globally, slice selectively, and from two slices (placed over the heart and liver) simultaneously. $*p \leq 0.05$ when compared to global data

Hepatic bicarbonate was not significantly different between the acquisition types. Hepatic lactate and alanine showed significant differences between all types of acquisition (figure 4.9).

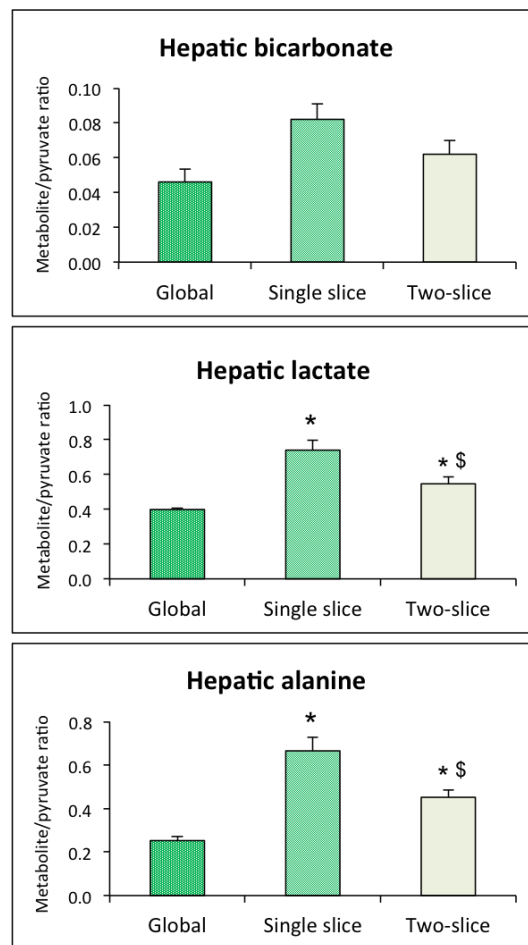


Figure 4.9: Hepatic data from injection of hyperpolarised ^{13}C pyruvate into fed animals; data acquired globally, slice selectively, and from two slices simultaneously. * $p \leq 0.05$ compared to global data; \$ $p \leq 0.05$ compared to single slice data

Absolute pyruvate values were shown to be significantly reduced in both the single and two-slice acquisitions compared to global, in both the cardiac and hepatic data (figure 4.10).

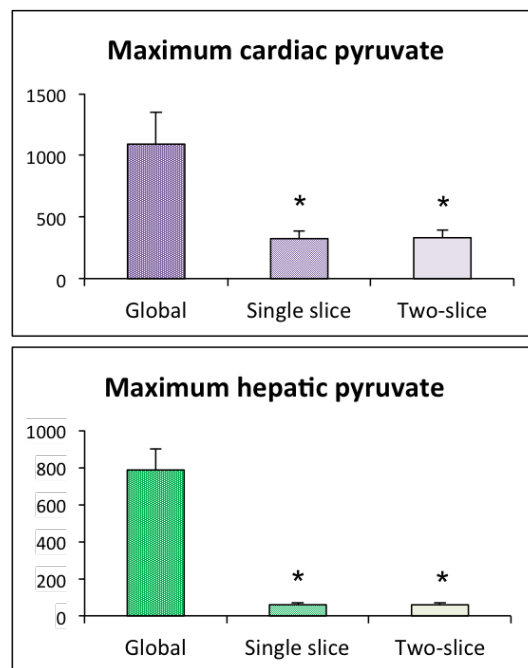


Figure 4.10: Absolute pyruvate data, global, single and two-slice acquisitions. * $p \leq 0.05$ compared to global data

The hepatic metabolic data showed unexpected differences between the single and two-slice acquisitions, in that the two-slice data showed decreased lactate and alanine. There were two hypotheses for this difference: firstly that the organs received twice the number of RF pulses for the two-slice acquisition, which may have caused decay in hyperpolarisation of some pyruvate before it reached the liver. Secondly, the location of the coil between the two organs may have caused a reduced signal in the two-slice acquisitions, since for the single slice acquisitions the coil was placed directly over the organ of interest. To try and establish which of these effects was responsible, the coil was placed between the two organs, but only one slice of data was acquired - the hepatic data. These data, with the surface coil positioned between the heart and the liver ($n=4$), are shown in figure 4.11. Hepatic bicarbonate showed no differences between scan types. Hepatic lactate data obtained from this positioning of the coil showed a trend to be higher than that obtained during a two-slice acquisition ($p=0.08$). Hepatic alanine, however, obtained with the coil in the two-slice position was not significantly different to the two-slice data, and there was in fact a trend towards this data being significantly lower than the single slice data ($p=0.06$).

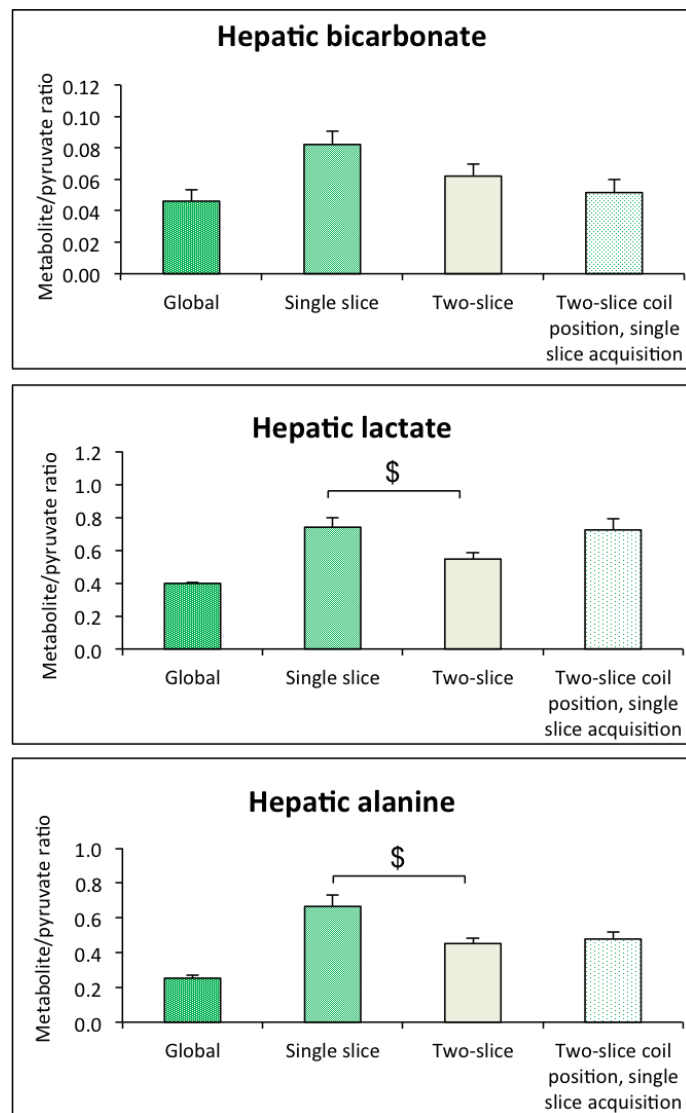


Figure 4.11: Hepatic data comparisons between previously obtained acquisitions and data obtained from a single slice with the coil in the two-slice position. \$ $p \leq 0.05$ compared to single slice data

4.2.3 Discussion

Our comparison between the novel heart/liver dual-slice acquisition sequence and the previously used global acquisition demonstrated a significant difference when measuring cardiac flux from $[^{13}\text{C}]$ -labelled hyperpolarised pyruvate to bicarbonate.

This can be explained by looking at the absolute pyruvate levels, seen to be significantly lower on using a slice-selective scan (data in figure 4.10). This will have a direct effect on the relative

amount of metabolite reported, as metabolite values are effectively normalised to pyruvate by the kinetic model used. Due to the smaller area from which data is acquired in the slice selective acquisition, both the amount of pyruvate seen and the amount of signal from downstream metabolites will be reduced. In terms of cardiac bicarbonate, the levels of bicarbonate outside the heart are low, so narrowing the slice will have little effect on the amount of bicarbonate reported. This, combined with reduced pyruvate will result in the observed increased PDH flux in both single slice and two-slice acquisitions compared to the global acquisition. Conversely, there is some contamination from lactate and alanine outside the slice selective window, most likely from liver metabolism, so on reducing that window, both pyruvate and lactate/alanine signal are reduced, resulting in no net difference between scan types.

Hepatic data showed an apparently higher ^{13}C label transfer from pyruvate to lactate and alanine from the dual-slice acquisition when compared to the global acquisition. This can be explained by the fact that the global acquisition saw the pool of labelled pyruvate (in the blood in the vasculature of the heart) due to proximity. Hepatic data was reported as the ratio of metabolite values relative to pyruvate. Lactate and alanine metabolism was high in the liver, so a reduction in acquisition window would have little effect on the level of these metabolites that is seen. Thus with the slice selective scans, the quantity of pyruvate seen was reduced, and lactate and alanine signal not changed, therefore the relative lactate/alanine values in liver appeared greater.

The differences seen between hepatic single and two-slice acquisitions were unexpected. The subsequent data which investigated whether the lower ^{13}C label transfer to lactate and alanine seen in the two-slice data compared to the single slice data was inconclusive as to whether the position of the coil, or the difference in number of RF pulses had the major effect. We would therefore conclude that it is a combinatory effect of both of these. However, although this difference meant that we were unable to directly compare single slice data and two-slice data in terms of absolute numbers, it did not negate the validity of either acquisition. Indeed, the data demonstrated a significant improvement in organ specificity compared to previously acquired global data. The following experiment was therefore planned to establish whether the two-slice acquisition was able to assess known differences between the fed and fasted conditions.

4.3 Cardiac and hepatic data from fed and fasted animals

Previous studies with hyperpolarised pyruvate have demonstrated the ability to assess the differences between fed and fasted metabolism, both in perfused organs and *in vivo*. Schroeder *et al.* showed a 74% decrease in cardiac bicarbonate production in fasted rats⁸. They did not report the effect of fasting on the conversion of pyruvate to lactate or alanine, due to concern that the global nature of the acquisition may have led to contamination of the data by liver and skeletal muscle metabolism. As discussed earlier, Merritt *et al.*⁸⁵ showed an increased lactate:alanine ratio in fasted, perfused mouse livers, and this was replicated *in vivo* by Hu *et al.*⁹⁸ who saw the same increase in ratio.

Therefore, to test that our developed two-slice sequence provided metabolic data in agreement with that seen previously in the literature, it was initially tested in fed and fasted healthy male Wistar rats.

4.3.1 Methods

Healthy male Wistar rats in the fed state (n=8, body weight ~300 g) were anaesthetised, tail vein cannulated and placed in the 7T MR system as fully described in chapter 3. Cardiac and hepatic metabolic data were obtained via a carbon surface coil placed between the heart and the liver, during a 1 ml injection of hyperpolarised [1-¹³C]pyruvate, prepared as described in chapter 3, section 3.5. Following at least 48 hours given to recover from the procedure, animals were fasted overnight (n=12, approximately 18 hours fasting) and the MR procedure repeated, once again obtaining cardiac and hepatic data. Hepatic summed data is presented, but flux measurements for lactate and alanine were confirmed to mirror the summed data. T-tests were carried out to establish differences between fed and fasted data.

4.3.2 Results

Cardiac PDH flux assessed with the two-slice acquisition was shown to be significantly decreased when animals were fasted compared with the fed state (94% decrease, shown in figure 4.12). ¹³C label transfer to lactate showed a decrease in the fasted state compared to fed. Alanine was

unchanged.

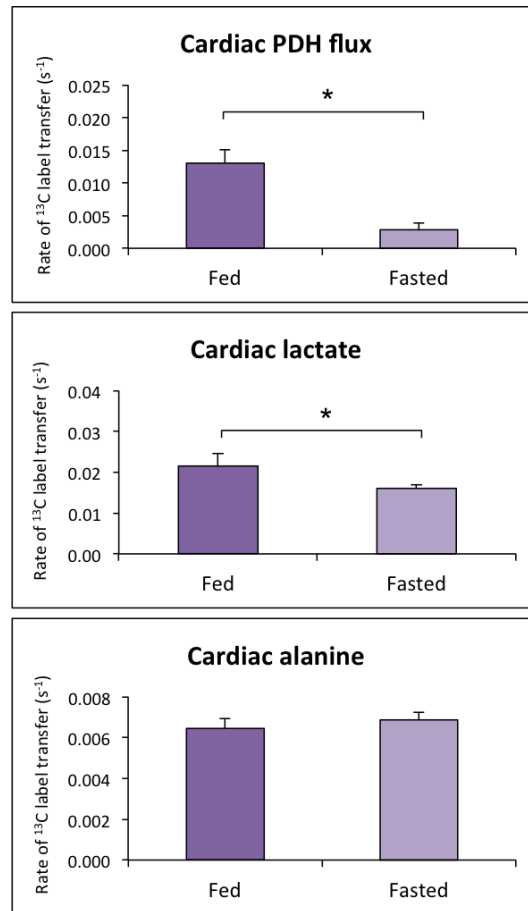


Figure 4.12: Cardiac data from injection of ^{13}C pyruvate into fed and fasted animals, acquired with two-slice acquisition. $*p \leq 0.05$

Hepatic bicarbonate was also significantly decreased in the fasted state compared to the fed, as was hepatic alanine. Hepatic lactate was not significantly different between the two states (figure 4.13).

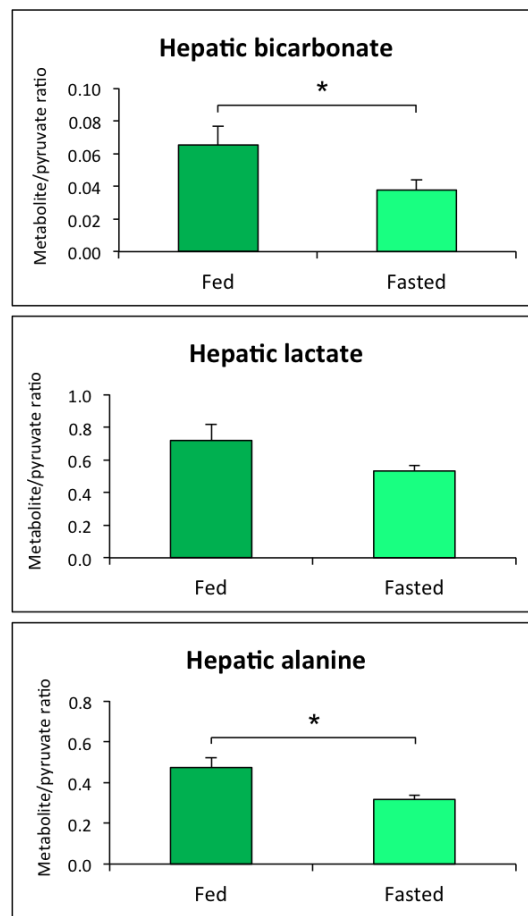


Figure 4.13: Hepatic data from injection of ^{13}C pyruvate into fed and fasted animals, acquired with two-slice acquisition. $*p \leq 0.05$

4.3.3 Discussion

Both cardiac PDH flux and hepatic bicarbonate production were significantly decreased in the fasted state. At this time point after eating, it would be expected that liver glycogen would have been fully broken down, and gluconeogenesis increased (stimulated by an increased glucagon:insulin ratio) to ensure sufficient glucose for cells for which it is essential (such as erythrocytes and the brain). Therefore, decreased PDH flux and hepatic bicarbonate would be expected.

Our study demonstrated a decreased ^{13}C label transfer to cardiac lactate in the fasted state compared to the fed state. Decreased overall glucose metabolism is unlikely to explain this observation, given we administered pyruvate, therefore sidestepping any effect of glucose trans-

porters. However, the monocarboxylate transporters (MCTs), responsible for the movement of pyruvate across the plasma membrane, may be involved. There are several members of the MCT family, with MCT1 particularly prominent in the heart¹⁰⁷. MCT1 has been shown to be upregulated in response to fasting¹⁰⁸ via PPAR- α . If more pyruvate is entering the cells in the fasted state compared to the fed, even though more conversion to lactate will occur, the pool size of pyruvate will be increased relative to the lactate pool. This may result in the apparently lower ^{13}C label transfer to lactate. It may also reflect alterations in the cytosolic NAD^+/NADH ratio, reduced expression or activity of lactate dehydrogenase (LDH), or increased efflux of lactate which would provide a smaller intracellular pool of lactate with which the ^{13}C label could exchange.

Our hepatic data supported that seen *in vivo* by Hu *et al.*, as we also saw a decrease in hepatic alanine, which in combination with no change in hepatic lactate would result in an increased lactate:alanine ratio. This was potentially indicative of the fact that during fasting, muscle protein is broken down into amino acids which can be transaminated to glutamate and alanine, with the latter being transported to the liver for use in gluconeogenesis¹⁰². This would result in the decrease in hepatic conversion of pyruvate to alanine in the liver as shown by our data. The liver would now be using fatty acids and generating ketone bodies rather than metabolising glucose. However, there is a trend to decreased lactate ($p=0.06$). If, on further investigation, decreased hepatic lactate in the fasted state was verified, it may be reflective of lactate being used as a gluconeogenic precursor, resulting in reduced LDH-driven conversion of pyruvate to lactate.

4.4 Assessing metabolism of a disease model with the two-slice acquisition

4.4.1 Aim

The following work aimed to validate the developed two-slice scan in a disease state, to provide support for use of this acquisition to follow progression or treatment of disease. Previous work by Schroeder *et al.* has demonstrated the ability to use hyperpolarised pyruvate to see alterations

in PDH flux in a type I diabetic rodent model⁸. To look at type II diabetes, Srinivasan *et al.* developed a model using a combination of high fat feeding and STZ injection to ‘simulate natural disease progression and metabolic characteristics typical of individuals at increased risk of developing type II diabetes because of insulin resistance and obesity’¹⁰⁹. This high-fat/STZ model has since been further investigated, and the studies in this thesis involving diabetic animals are based on the Wistar model described in Mansor *et al.*¹¹⁰. The following work was carried out in collaboration with Dr. Daniel Ball.

4.4.2 Methods

Male Wistar rats were split into two groups (control, n=6; diabetic n=7). Control animals were fed standard chow. Diabetic animals were fed a high fat diet throughout (60% fat, 35% protein, 5% carbohydrate; Special Diet Services, Essex, UK), with an injection of 35 mg/kg streptozocin after an overnight fast at day 12 (feeding and injection carried out by Dr. Ball). At three weeks, all animals underwent *in vivo* metabolic analysis. Animals were anaesthetised and prepared as described in chapter 3. Whilst anaesthetised in the MR system, animals received an injection of hyperpolarised [1-¹³C]pyruvate, prepared as described in chapter 3, section 3.5. During and following the injection, cardiac and hepatic spectra were acquired. Cine imaging was carried out (as described in chapter 3, section 3.7) to assess cardiac systolic function. Following scanning, animals were given an overdose of pentobarbitone, and the heart removed and perfused free of blood before being snap-frozen. Blood samples were taken and plasma frozen for subsequent analyses of ALT and insulin levels (methods detailed in chapter 3), along with liver tissue samples for Western blot and triglyceride level analysis. T-tests were carried out to establish differences between the control and diabetic groups.

4.4.3 Results

Evaluation of diabetes

Fed plasma glucose levels were significantly elevated in the diabetic group, and insulin levels were decreased, as shown in figure 4.14.

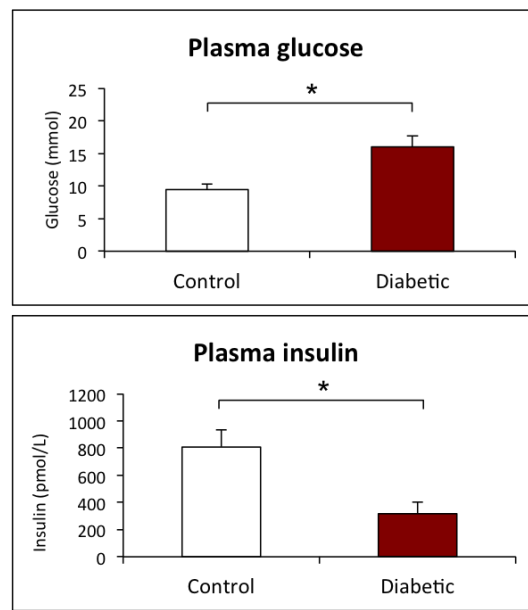


Figure 4.14: Blood glucose and insulin levels in control and diabetic animals. $*p \leq 0.05$

Protein expression of cardiac PDK4 was significantly elevated in the diabetic animals, as assessed by Western blot. Cardiac GLUT4 was significantly decreased (figure 4.15).

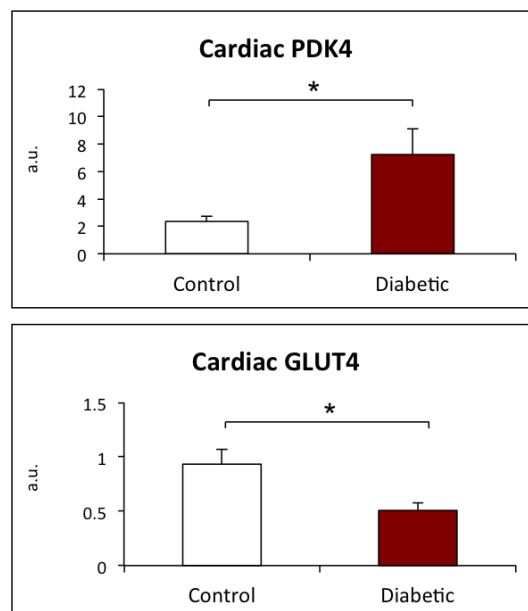


Figure 4.15: Cardiac PDK4 and GLUT4 expression in control and diabetic animals. $*p \leq 0.05$

***In vivo* cardiac and hepatic metabolic assessment**

Hepatic summed data is presented, but flux measurements for lactate and alanine were confirmed to mirror the summed data.

Cardiac PDH flux was significantly decreased in the diabetic animals compared to the control animals. ^{13}C label transfer to lactate and alanine was unchanged (figure 4.16).

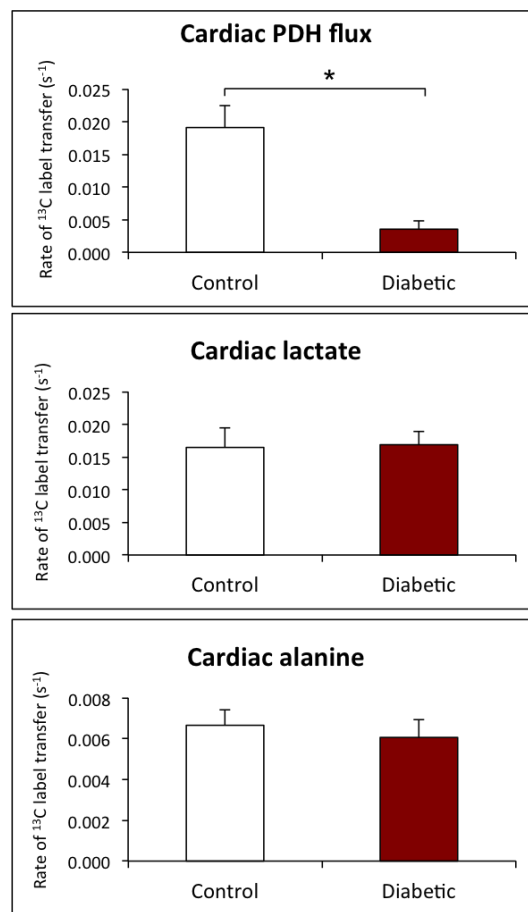


Figure 4.16: *In vivo* cardiac data on injection of ^{13}C pyruvate in control and diabetic animals. $*p \leq 0.05$

No differences in cardiac heart weight to body weight ratio and end diastolic or systolic lumen size were seen between control and diabetic animals. Measurements assessing cardiac function also showed no differences; these included stroke volume, cardiac output, and ejection fraction as shown in figure 4.17.

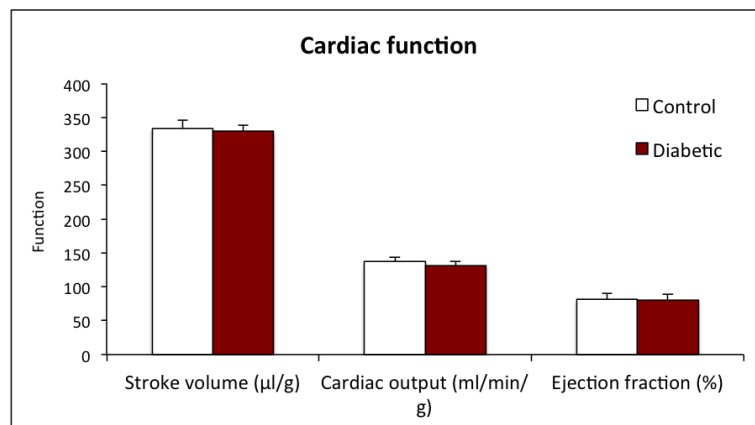


Figure 4.17: *In vivo* cardiac function data from control and diabetic animals

There was a trend for hepatic bicarbonate and lactate to be decreased ($p=0.09$ and 0.08 respectively), and hepatic alanine was significantly decreased in the diabetic animals compared to control (figure 4.18).

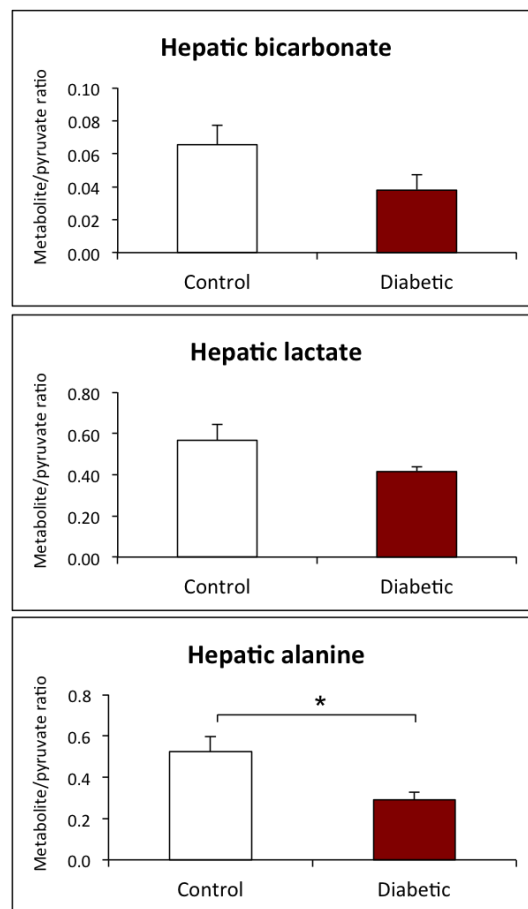


Figure 4.18: *In vivo* hepatic data on injection of ^{13}C pyruvate in control and diabetic animals.
* $p \leq 0.05$

Biochemical analyses

In order to investigate the metabolic changes seen in the liver, protein expression of the hepatic PDK isoforms was measured. There were no significant differences in any of the isoforms between control and diabetic animals (figure 4.19).

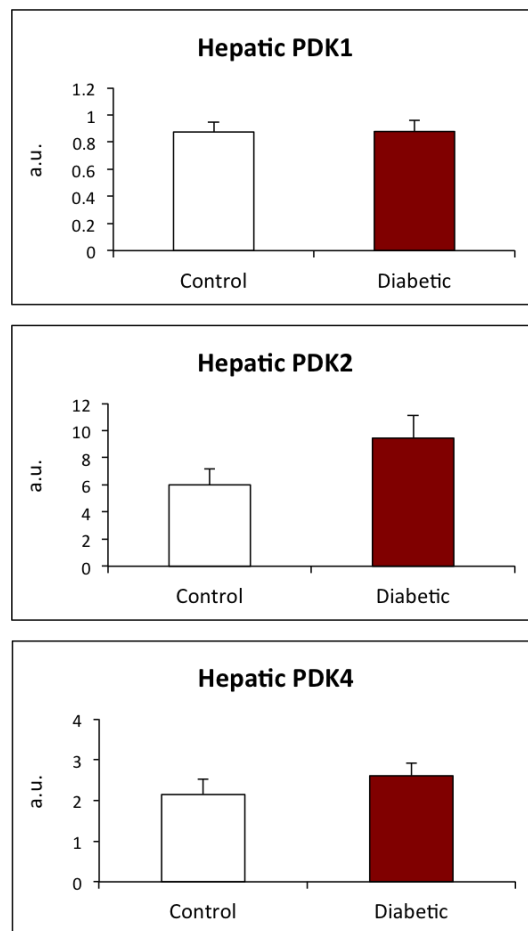


Figure 4.19: Hepatic PDK isoform expression in control and diabetic animals

No significant difference in plasma alanine aminotransferase (ALT) levels was seen ($p=0.16$). Hepatic triglyceride levels were significantly increased in the diabetic liver (figure 4.20).

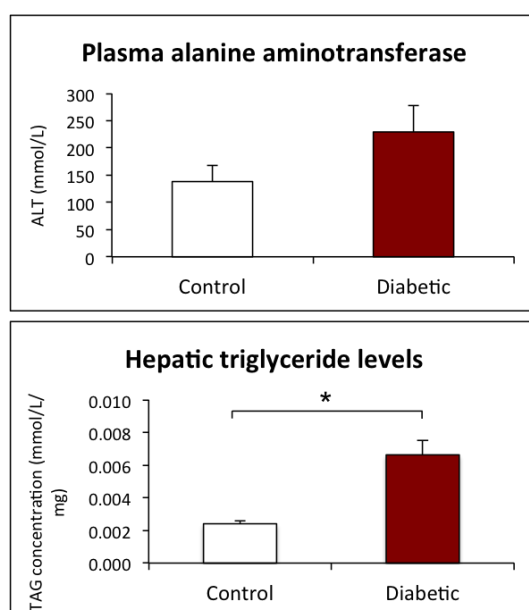


Figure 4.20: Plasma ALT and hepatic triglyceride levels in control and diabetic animals. * $p \leq 0.05$

Finally, neither plasma lactate ($p=0.13$) nor body weight were different between groups (figure 4.21).

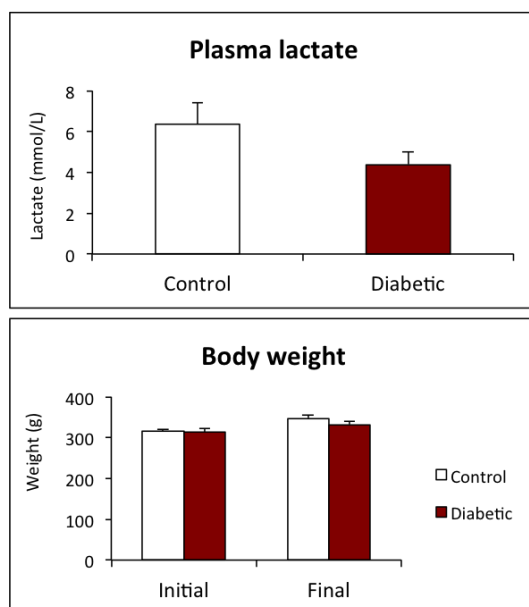


Figure 4.21: Plasma lactate levels and body weights of control and diabetic animals

4.4.4 Discussion

This work has demonstrated our ability to visualise metabolic differences between control animals and a diabetic rodent model induced by high fat feeding and STZ administration.

Our results showed decreased cardiac PDH flux and a trend to decreased hepatic ^{13}C label transfer to bicarbonate in the diabetic rat model, and confirmed our ability to measure *in vivo* bicarbonate production in two organs simultaneously in both health and disease. Decreased PDH activity was expected in diabetes due to the switch to a focus on fatty acid metabolism as discussed in chapter 2; in cardiac tissue this has been demonstrated biochemically *ex vivo* by Seymour and Chatham⁷.

Our model showed an 82% decrease in cardiac flux from pyruvate to bicarbonate, suggesting that the difference was not solely due to the high fat diet, given that similar high-fat feeding for four weeks (55% saturated fat) by Atherton *et al.* only showed a 30% decrease in PDH flux⁷⁵. It was also more extreme than the 65% reduction in cardiac PDH flux seen in the Type I diabetic model in the work by Schroeder *et al.*, induced by an injection of 50 mg/kg STZ, with metabolic assessment five days later⁸.

In addition, we saw an increased overall expression of cardiac PDK4 and decreased expression of GLUT4 in the cardiac tissue from the diabetic animals, indicative of downregulated glucose metabolism, and in support of our *in vivo* cardiac metabolic data. Both have been seen previously in diabetes^{110–112}. This is likely to be due to increased fatty acid metabolism (with acetyl CoA inhibiting PDH) and the resultant effect via the Randle cycle as discussed earlier, along with depressed levels of insulin-stimulated glucose uptake.

Hepatic data showed a trend towards decreased flux through PDH. Potentially if further animals were studied, or diabetes was induced over a longer time period, this may provide a reflection of the diabetic gluconeogenic state, usually prevented by insulin. Hepatic alanine was significantly decreased in the diabetic animals, contrary to the work by Lee *et al.* using an insulin resistant mouse model⁸⁸. However, given the increased fatty acid metabolism in diabetes produces elevated NADH, this may alter the equilibrium of the pyruvate/alanine conversion (see figure 4.22), so reduced ^{13}C label transfer from pyruvate to alanine could be expected.

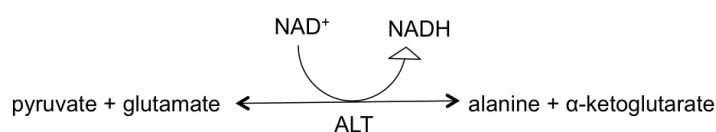


Figure 4.22: Conversion between pyruvate and alanine. ALT = alanine aminotransferase; NADH = reduced nicotinamide adenine dinucleotide; NAD^+ = nicotinamide adenine dinucleotide

The observed increased hepatic triglyceride levels may be an effect of the higher circulating free fatty acids due to the lack of insulin-suppression of hormone-sensitive lipase and resultant increased lipolysis¹¹³.

4.5 Characterisation of a type II diabetic rodent model

The data obtained in the previous section demonstrated that high-fat feeding coupled with administration of a single dose of 35 mg/kg STZ resulted in a fairly severe diabetic model. Subsequent to this study, work by Mansor *et al.* investigated the different phenotypes that resulted from a range of low doses of STZ, and concluded that a dose of 25 mg/kg STZ in combination with the high fat diet better mimicked the metabolic phenotype seen in type II diabetic patients. They established that it induced adiposity, hypercholesterolaemia, and mild hyperglycaemia, without excessively compromising insulin secretion. Further they concluded that it showed the metabolic shift from glucose to fatty acid metabolism seen in diabetes¹¹⁰. The subsequent diabetic studies in this thesis therefore focussed on this diabetic rodent model, in combination with the two-slice acquisition developed in this chapter.

Given that this model was used across several studies in chapters 5 and 6, the figures below summarise the information obtained from all diabetic and control animals studied in this thesis. This is firstly to establish the metabolic differences seen between control and diabetic animals in the model used and to prevent repetitive discussion throughout the thesis, and secondly to increase statistical power for the comparisons. T-tests were used to compare all control and diabetic data, apart from data acquired measuring plasma insulin levels, where a two-way ANOVA with a Holm-Sidak correction for multiple comparisons was used.

4.5.1 Summary of results

The combination of a high-fat diet with a low dose of streptozotocin (25 mg/kg) caused increased fed and fasting plasma glucose concentrations. The statistical analysis on fed and fasting insulin levels in control and diabetic animals showed a significant interaction between diabetes and prandial state. The diabetic animals had decreased fed plasma insulin levels when compared with control animals, as shown in figures 4.23 and 4.24, with samples taken *in vivo*. Post-mortem fasting insulin levels were not different between diabetic and control animals.

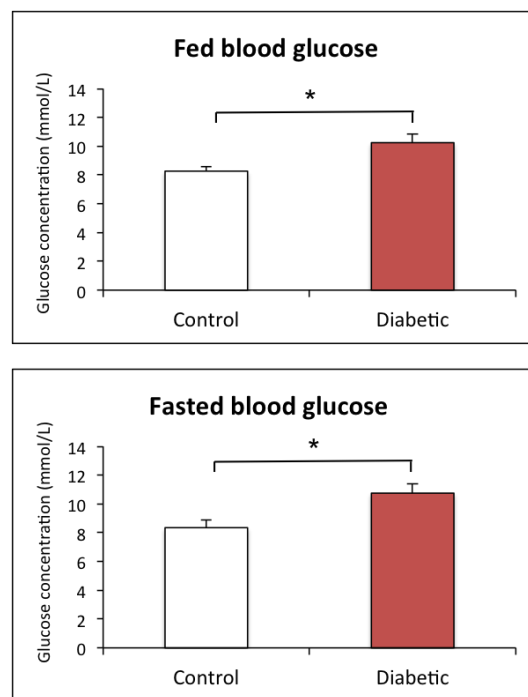


Figure 4.23: Fed (n=28) and fasting (control n=5; diabetic n=6) glucose levels

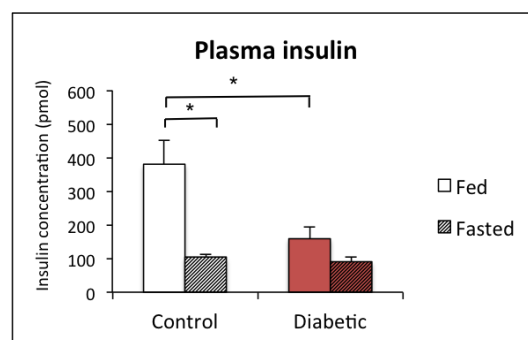


Figure 4.24: Fed (n=28) and fasting (control n=5; diabetic n=6) insulin levels. * $p \leq 0.05$

Cardiac and hepatic metabolic data were also obtained using the two-slice acquisition from many control and diabetic animals, summarised in figures 4.25 and 4.26. The diabetic model demonstrated a significantly decreased cardiac PDH flux, increased ^{13}C label transfer to alanine, and no significant change in the incorporation of the ^{13}C label into lactate.

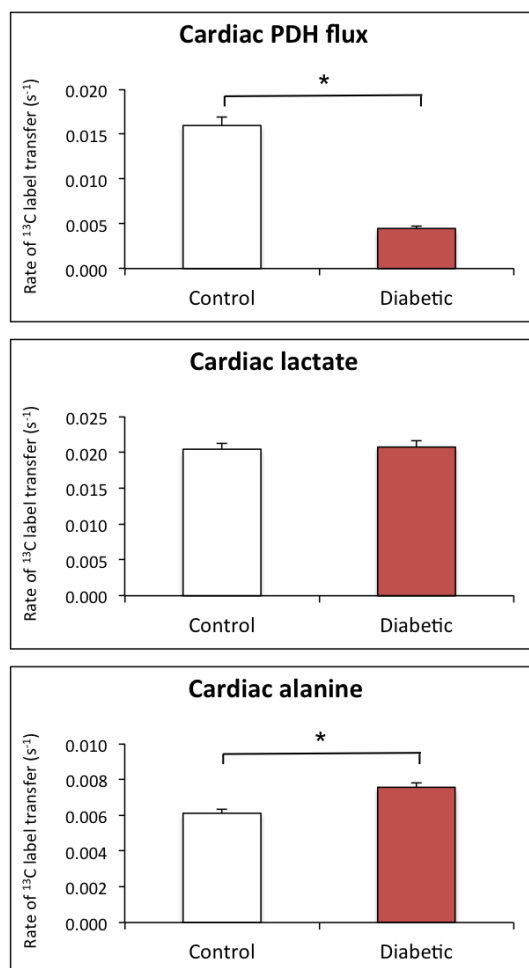


Figure 4.25: Cardiac carbohydrate metabolism data obtained from injection of hyperpolarised $[1-^{13}\text{C}]$ pyruvate ($n=41$)

The hepatic data showed a significant decrease in hepatic bicarbonate in the diabetic animals, but no difference in lactate or alanine.

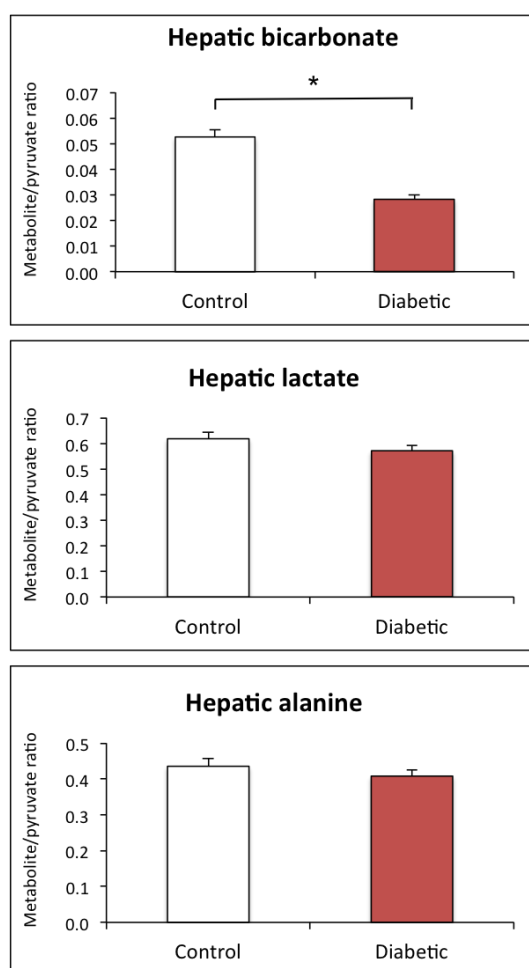


Figure 4.26: Hepatic carbohydrate metabolism data obtained from injection of hyperpolarised $[1-^{13}\text{C}]$ pyruvate ($n=36$)

4.5.2 Discussion

Cardiac PDH flux and hepatic bicarbonate were shown to be significantly decreased in the diabetic model when compared to control animals. This is reflective of the metabolic shift from glucose metabolism to fatty acid metabolism in both organs. Cardiac ^{13}C label transfer to alanine was increased, potentially indicative of the increased supply of gluconeogenic substrates being transported to the liver from other organs.

In comparison to the 35 mg/kg STZ diabetic model, the decrease in cardiac PDH flux is slightly reduced, to 72%. The decrease in hepatic bicarbonate in the lower STZ dose model is significant where it was not in the higher dose model, although this may be an effect of the large group size.

The decreased hepatic alanine seen in the higher dose model is no longer seen with the lower dose of STZ, potentially indicative of a less severe model. The difference in insulin concentration between control and diabetic animals would also support this, with the higher dose causing an 84% decrease in insulin, and a 58% decrease induced in the 25 mg/kg STZ model.

4.6 Overall chapter conclusions

In this chapter the development of a novel two-slice magnetic resonance spectroscopy acquisition was described, for use with hyperpolarised $[1-^{13}\text{C}]$ pyruvate. The initial tests run demonstrated sufficient sensitivity of the coil over the distance required to measure metabolic data from both the heart and the liver, and further showed that no contamination occurred between the two slices. Data from fed and fasted animals using this two-slice acquisition demonstrated the expected differences, supporting the scan's use in a disease model. Further support for the acquisition was provided by assessment of a diabetic model which showed decreased cardiac PDH flux. Potentially the slice selective nature of this acquisition was shown to be more appropriate than previously used global acquisitions, given the certainty of reduced contamination from other organs. Further, the acquisition of two data sets from one experiment reduced anaesthetic time and number of injections for the animals, alongside reducing magnet time and costs for the laboratory, and therefore led to both improved animal welfare and more efficient use of time and resources.

Chapter 5

Investigation of pharmacological PDK inhibition and the effect on PDH: dichloroacetate and AZD7545

5.1 Overview

As pyruvate dehydrogenase (PDH) is a key control point in the glucose metabolism pathway, and flux through PDH is significantly decreased in diabetes as shown in chapter 4 (section 4.5), it is an important enzyme to investigate if we are to fully understand the metabolism of diabetes. It is therefore interesting to specifically modulate PDH in the healthy and diseased state in order to improve our understanding. One method for achieving this is through the use of pharmacological compounds. This chapter investigates two different pharmacological compounds, a general PDK inhibitor, dichloroacetate, and a PDK2-specific inhibitor, AZD7545.

5.2 Chapter aims

This chapter presents work with both the compounds mentioned above. In each case I have first examined the acute effects of the drug on the healthy heart using a Langendorff perfused heart preparation. The perfused heart provides an opportunity to assess the effects of the compounds free from the hormonal and neuronal complications present *in vivo*. Both carbon and phosphorus spectroscopy were used to enable assessment of the metabolic and energetic status of the hearts throughout the experiments. Hyperpolarised [^{13}C]pyruvate experiments enabled assessment of metabolism while phosphorus spectroscopy allowed measurement of the ratio of phosphocreatine (PCr) and adenosine triphosphate (ATP) in the heart. PCr is the major energy reserve in the heart, given the speed at which it can facilitate ATP production, compared to oxidative phosphorylation¹¹⁴. A healthy heart gives a PCr/ATP ratio of approximately two. As the heart transitions into failure, this value falls; PCr is converted to ATP to try to maintain the workload, while PCr cannot be replenished due to a depleted creatine pool^{115,116}.

Following this, *in vivo* studies were carried out to assess the chronic effects of the compounds.

Initial pilot studies provided data on cardiac metabolism and function in control animals. Subsequently, the chronic effect of each drug was assessed *in vivo* in a diabetic rat model. We used the model summarised at the end of chapter 4, based on the work by Mansor *et al.*¹¹⁰. Briefly, this involved the use of a low dose of streptozotocin, in combination with a high fat diet, resulting in insulin resistance enhanced by the high fat diet, emulating the human condition. Further we used the two-slice acquisition developed in chapter 4 to investigate both heart and liver metabolism to gain a more informed understanding of any effects seen.

The methods section below details general methods relevant to studies carried out on both drugs, with any specific methods described in the appropriate compound section. The background, results and discussions for each compound are then detailed separately.

5.3 General methods

5.3.1 Isolated perfused heart studies

Hyperpolarised sample preparation

Approximately 30 mg of $[1-^{13}\text{C}]$ pyruvic acid doped with 15 mM trityl radical (OXO63, GE Healthcare) and a trace amount of Dotarem (Guerbet, France), was hyperpolarised in a Hyper-sense polariser (Oxford Instruments, Abingdon), an automated version of the α -system polariser technique described in chapter 3, with 40 min of microwave irradiation. The polariser automatically dissolved the hyperpolarised pyruvate in a pressurized and heated alkaline solution, containing 100 mg/L EDTA, to yield a solution of, initially, 2.5 mM hyperpolarised sodium $[1-^{13}\text{C}]$ pyruvate when dissolved in 175 ml of KH buffer, maintaining a polarisation of $\sim 30\%$ at physiological temperature and pH. This was later altered to 0.625 mM pyruvate, by instead using 7.5 mg $[1-^{13}\text{C}]$ pyruvic acid.

Perfusion protocol

Healthy male Wistar rats of 200-300 g were used. Perfusions were carried out with the help of Dr. Daniel Ball. Rats were anaesthetised using a 0.5 ml intra-peritoneal injection of pentobarbital sodium (200 mg/ml Euthatal). The hearts were removed and arrested in ice-cold KH buffer, and subsequently cannulated via the ascending aorta and secured to the perfusion apparatus (figure 5.1) where they were perfused in a recirculating retrograde Langendorff mode¹¹⁷.

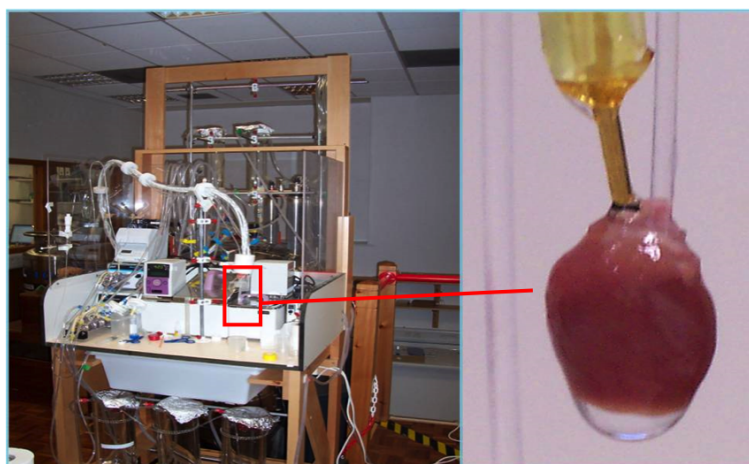


Figure 5.1: Langendorff perfusion rig

The buffer was aerated with a mixture of 95% oxygen to 5% carbon dioxide, which allowed it to remain at a constant working pH of 7.4 at 37°C, and perfused through the heart at a constant pressure of 80 mmHg. Intraventricular pressure development resulting from Thebesian artery drainage was minimised by the insertion of a polytetrafluoroethylene drain through the apex of the heart. A water-filled polyethylene balloon, attached via polyethylene tubing to a pressure transducer was inserted into the left ventricular cavity via the mitral valve and inflated sufficiently to produce an end diastolic pressure (EDP) of ~ 4 mmHg. The left ventricular (LV) pressure and heart rate were recorded using a PowerLab/4SP data acquisition system (ADInstruments Ltd, Oxfordshire, UK). From this, measures of cardiac function could be calculated; the rate pressure product (RPP) was the product of left ventricular developed pressure (DP) and heart rate in beats per minute. The perfused heart was placed in a 20 mm NMR sample tube and positioned inside the bore of a 500 MHz, 11.7T vertical wide bore super-conducting magnet (Bruker) (figure 5.2). The temperature of the heart and perfusate was maintained at 37°C using water-jacketed buffer reservoirs and perfusion lines.

Hearts were initially perfused with control KH buffer, whilst scout images were taken to confirm the location of the heart in the centre of the RF coil. A hyperpolarised sample was prepared and the resulting neutralised solution added to the perfusion buffer, and a ^{13}C scan carried out (as described below). Acquisition of ^{13}C MR spectra began immediately after infusion of the hyperpolarised solution, and infusion continued throughout a two minute acquisition with a spectrum acquired every second. This was followed by a ten minute fully relaxed ^{31}P acquisition

and 35 one minute partially saturated ^{31}P scans. Ten minutes into the latter scans, the control KH buffer was switched for KH buffer that contained the first drug concentration. At the end of the phosphorus scans, a second hyperpolarised dissolution was carried out. The ^{31}P and ^{13}C scans were then repeated with an increased concentration of compound. For an overview, see figure 5.3.



Figure 5.2: 11.7 T vertical bore magnetic resonance system

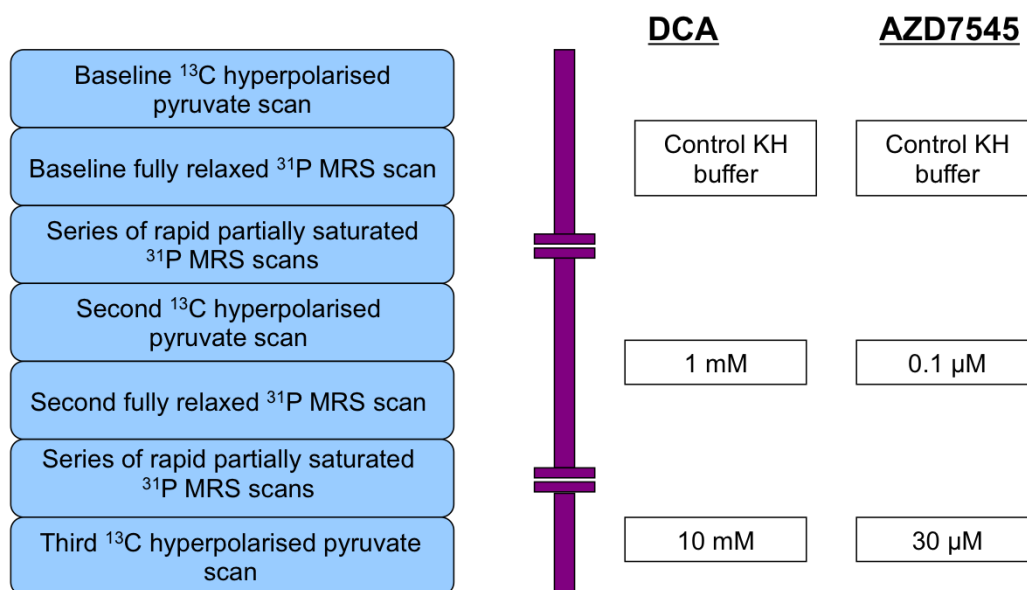


Figure 5.3: Perfusion protocol for administration of DCA or AZD7545 to control rat hearts

Measurement and analysis of ^{13}C spectra

The hyperpolarised solutions of pyruvate were delivered to the hearts over 120 seconds and a series of 120 carbon spectra acquired using a pulse-acquire spectroscopy sequence ($\text{TR}=1\text{s}$, $\text{FA}=30^\circ$, $\text{SW}=180\text{ ppm}$, 4096 pts). Spectra were corrected for DC offset using the last half of the acquired points and referenced to the $[1\text{-}^{13}\text{C}]\text{pyruvate}$ resonance before being fitted using the AMARES algorithm in the jMRUI software package⁹³. The kinetic model described in section 3.6 was not suitable for use with these data as infusion into the perfused heart was continuous rather than as a bolus as in *in vivo* experiments. The metabolite maximum peak areas were therefore used and expressed relative to the maximum pyruvate peak area. A ratio of $(\text{bicarbonate}+\text{CO}_2):\text{pyruvate}$ at the maximum was used as a measure of PDH flux as shown in Atherton *et al.*⁷⁵.

A repeated measures ANOVA was used to assess differences between the two concentrations of compound and the control values, with a Holm-Sidak correction applied for multiple comparisons.

5.3.2 *In vivo* pilot dosing study

Drugs were administered for 28 days. At the end of the dosing period, animals were anaesthetised and prepared for cine imaging as described in chapter 3, section 3.7, however the MR system used was an 11.7T vertical bore scanner (Magnex Scientific, Oxon, UK) interfaced to a Bruker Avance console (Bruker Medical, Ettlingen, Germany). A 60 mm birdcage transmit/receive RF coil was used to obtain MR signals (Rapid Biomedical, Rimpar, Germany). Cardiac images were taken at the end of the 28 day period. Additionally, a further, high temporal resolution acquisition was carried out at the mid-papillary level for assessment of diastolic function (early:active ventricular filling rate, or E/A) as described previously by Stuckey *et al.*¹¹⁸, using the MR images to assess cavity volume change. Whole heart images were analysed using the ImageJ software package⁹⁷ similarly to analysis of systolic cine, where the epicardial and endocardial borders were outlined to obtain lumen size in the end diastolic and end systolic frames. However, analysis of the diastolic scan used automatic thresholding in ImageJ to define the left ventricular lumen and calculate its area in each frame - these measurements were then used to calculate early and late filling rates¹¹⁸.

In vivo cardiac PDH flux, and ^{13}C label transfer to lactate and alanine were measured and assessed after the 28 day dosing period, 48 hours away from the cine measurements, using hyperpolarised $[1-^{13}\text{C}]\text{pyruvate}$ as described in chapter 3, section 3.5. This was achieved using a 7T MR system, and data were acquired with a global sequence focussing solely on cardiac metabolism as the two-slice acquisition detailed in chapter 4 was in development at this time.

Any further procedural details are described in the relevant experimental section.

5.3.3 *In vivo* chronic study: control and diabetic animals

The following protocol was used for the chronic *in vivo* study investigating the effects of the compounds on both control and diabetic animals.

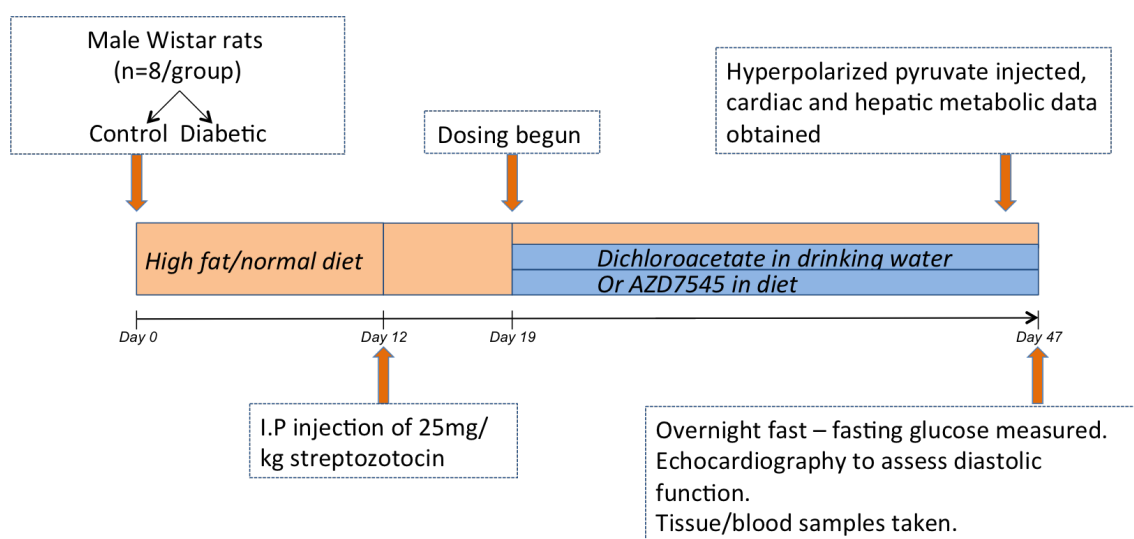


Figure 5.4: Overview of protocol for chronic drug administration to control and diabetic animals

Echocardiography was carried out with the assistance of Dr. Oliver Rider and Dr. Andrew Lewis. Examples of data acquired are shown in figure 5.5. Echocardiographic indices were obtained according to the recommendations of the British Society of Echocardiography. Transthoracic echocardiography was performed in control and diabetic animals with the use of a commercially available Vivid I echocardiography system (GE Healthcare) using an 11.5 Mhz phased array 10S-RS paediatric echo probe. Wall thickness and left ventricular (LV) dimensions were obtained from a short-axis view at the level of the papillary muscles. LV fractional shortening was calculated as $(LVIDd - LVIDs) / LVIDd \times 100$, where LVIDd is the LV internal dimension at the end of diastole and LVIDs is LV internal dimension at the end of systole. 2D guided pulsed-wave Doppler recordings of LV inflow were obtained from the apical 4-chamber view to measure maximal early diastolic peak velocity (E) and late peak velocity (A). In addition, Tissue Doppler Imaging was recorded from the medial mitral valve annulus to record early (E') and active (A') left ventricular diastolic myocardial velocities. This was used to calculate preload independent E/E'.

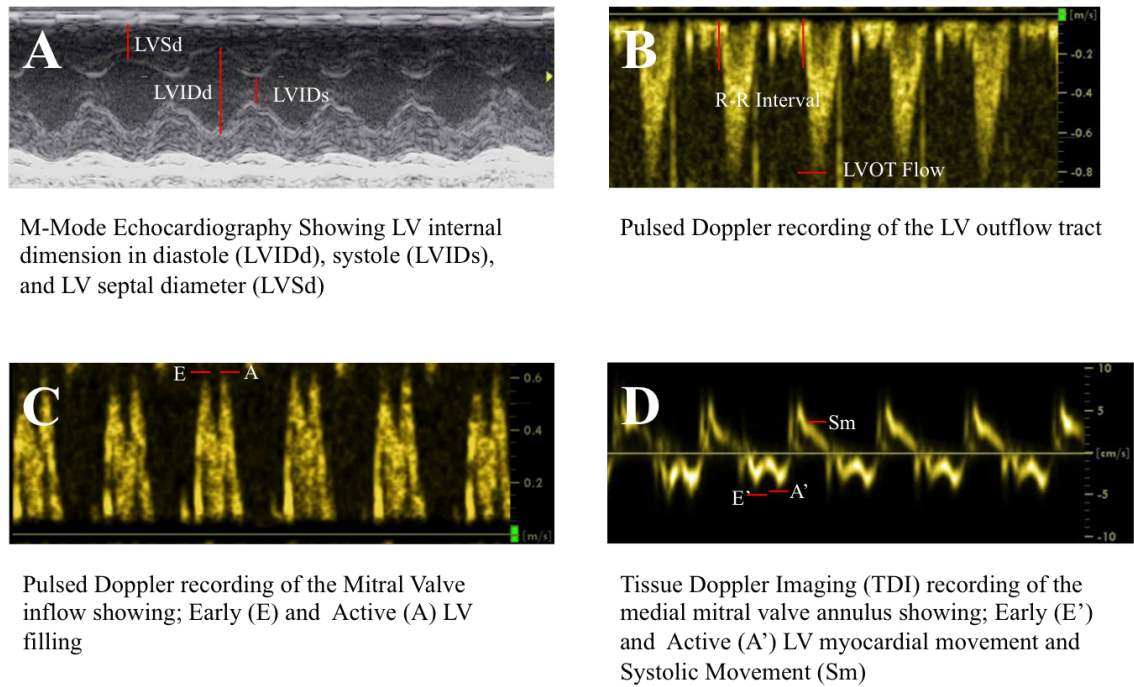


Figure 5.5: Representative images of echocardiographic data acquired

After three weeks of the protocol, and prior to any drug administration, cardiac function of control and diabetic animals was assessed by echocardiography, to establish whether diastolic dysfunction was a characteristic of this model. No differences were seen in fractional shortening or E/A ratio, however a significantly higher E/E' ratio was seen in the diabetic animals at this time point compared with controls animals, indicating that diastolic dysfunction was present in this model prior to any treatment having been given.

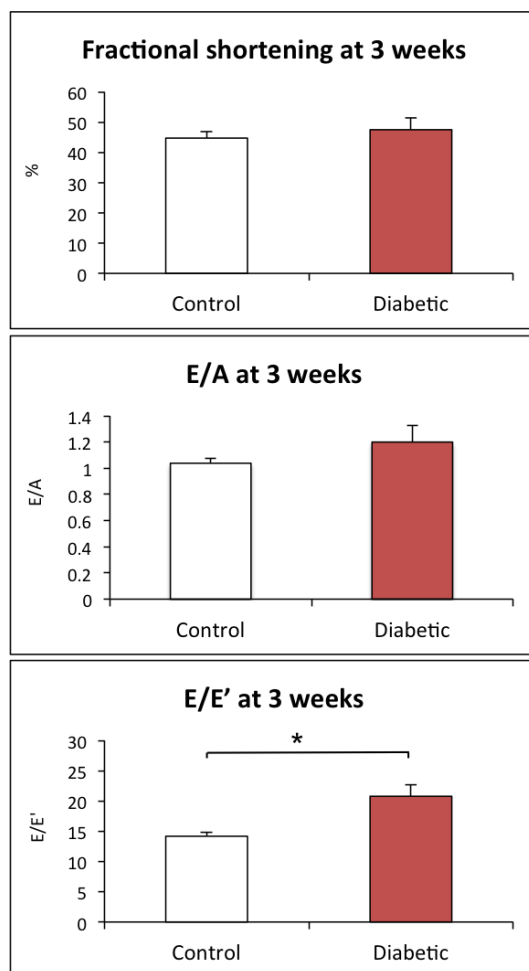


Figure 5.6: Cardiac function at three weeks in untreated control and diabetic animals. * $p \leq 0.05$

5.4 Dichloroacetate

5.4.1 Introduction

The pyruvate mimic dichloroacetate (DCA) is a pharmacological agent that targets PDH, promoting glucose oxidation over glycolysis via inhibition of PDK¹¹⁹ in a dose-dependent manner¹¹. It is a small molecule of 150 Da (figure 5.7) and is therefore able to cross the blood-brain barrier. It also has a resultant high bio-availability; oral dosing can in fact provide 100% bioavailability.

DCA is taken up by monocarboxylate transporters (transporters which enable movement of molecules with one carboxylate group across the plasma membrane)^{120,121}, and subsequently

binds to an allosteric site in the N-terminal domain of all PDK isoforms (unique for pyruvate, and therefore DCA)^{122,123}, causing conformational changes in both the nucleotide- and lipoyl-binding pockets of PDK that prevent it binding to the PDC. Although all of the four rodent PDK isoforms are susceptible to DCA inhibition, the ubiquitous PDK 2 isoform is the most sensitive, with a K_i of 0.2 ± 0.05 mM²⁴. It has in fact been shown that inhibition of PDK2 only (by siRNA) mimics the effect of DCA¹²⁴. PDK3 is the least sensitive, with a K_i of 8.0 ± 1.0 mM (high K_i indicates a high concentration of ligand is required before binding is maximised). PDK 1 and 4 have K_i values of 1.0 ± 0.2 mM and 0.5 ± 0.2 mM respectively. The concentration of DCA shown to be optimal for maximal activation of PDH *in vitro* is 1 mmol/L¹¹.

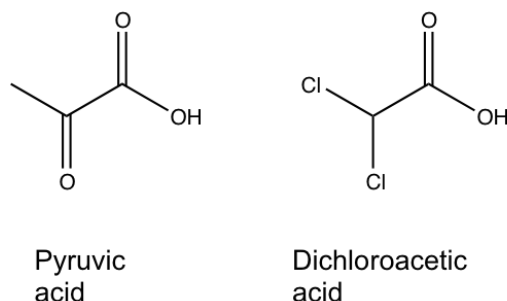


Figure 5.7: Chemical structures of the acids of pyruvate and dichloroacetate

The molecular mechanism of action of DCA is believed to be vitally linked to the presence of ADP as DCA binds to the pyruvate dehydrogenase kinase-ADP complex, increasing the proportion of active, or dephosphorylated, PDH¹²⁵. In fact, it has been shown that the binding of ADP increases the binding of DCA to PDK2, and vice versa¹²⁶.

DCA inhibits hepatic glucose synthesis and stimulates glucose clearance and use by peripheral tissues¹²⁷. It modestly reduces glucose in fasted animals (3 hours after a single dose of 100 mg/kg), and stimulates pyruvate oxidation in isolated hepatocytes in a dose-dependent manner, showing an increase from 124% to 141% of control PDH activity, between 0.5 and 5 mM DCA¹²⁸. In humans the liver is primarily responsible for the metabolism of DCA¹²⁹. Evans showed that rat liver rapidly metabolises DCA to glyoxylate and oxalate; these metabolites do not have a biological effect on PDH¹²⁸.

The clearance of DCA decreases after multiple doses^{130,131}, with the half-life increasing from half an hour to several hours after the first few doses, before reaching a maximum. This has been

shown to be related to DCA-stimulated inactivation of glutathione-transferase $\zeta 1$; this enzyme is responsible for the conversion of DCA to glyoxalate¹³².

In human control studies, DCA has been shown to reduce lactate build up in skeletal muscle during exercise^{133,134}, and activate PDH by 3-6 fold. It has therefore been trialled as a treatment for lactic acidosis¹³⁰.

Given the pivotal role of the liver in the metabolism of DCA, there is potential that patients with reduced liver function (such as diabetics) may have different pharmacokinetics and pharmacodynamics compared with healthy subjects.

In relation to diabetes, DCA was shown to raise the respiratory quotient (i.e. the ratio of carbon dioxide formation to oxygen consumption), indicative of its ability to stimulate glucose oxidation, and lower blood glucose concentration after injection into alloxan-induced diabetic rats by Lorini and Ciman in 1962¹³⁵. In humans, it has been shown to lower plasma glucose in normal fasting subjects¹³⁶ and reduce fasting hyperglycaemia in diabetics¹³⁷, with a concomitant lowering in plasma lactate and alanine - both gluconeogenic precursors - leading to the suggestion of its use as alternative to insulin for improving glucose concentrations.

Given that in diabetes, PDH activity is decreased and pyruvate oxidation impaired^{7,138}, it follows that restoration of this activity, and therefore the fuel balance in the *in vivo* diabetic heart, may restore function. A previous study administering DCA to the perfused working diabetic rat heart by Nicholl *et al.*¹³⁹ has demonstrated improved function as assessed by several parameters, including left ventricular developed pressure and heart rate peak systolic pressure product. Chatham and Forder¹⁴⁰ investigated the effect of DCA on the perfused heart after development of diabetes for one week only, and discovered that although DCA stimulated glucose oxidation as proposed, there was no effect on cardiac function. As yet there have been no direct studies *in vivo* investigating the metabolic effect of DCA on cardiac function in diabetes.

DCA is also being investigated as a cancer therapy. Cancer cells are characteristically glycolytic, exhibiting the Warburg effect¹⁴¹. Initially the cells grow so fast that an hypoxic environment is created as vascularisation does not occur fast enough to provide a suitable concentration of oxygen. Glycolysis is therefore the necessary pathway for ATP production, producing high levels

of lactate, which in itself has been suggested to facilitate tumour growth by, for example, breaking down extra-cellular matrices allowing the tumour to expand¹⁴², and also stimulating angiogenesis (along with hypoxia-inducible factor) to help create a supply network for the tumour¹⁴³. Even after the initial rapid expansion stage, the cancer cells remain glycolytic, suggesting that this metabolic state provides an advantage. As such, should this metabolic state be altered, such as by forcing oxidative phosphorylation by using DCA to stimulate PDH, it may impair the viability of the cancer cells.

One of the most recent studies in humans was carried out by Michelakis *et al.*¹⁴⁴ in five patients with glioblastomas, and demonstrated effective PDH stimulation at a dose level that did not cause hepatic, renal or cardiac toxicity, although did cause a reversible peripheral neuropathy which has been a documented side-effect of DCA for many years^{145,146}. However it has been suggested that this neuropathy is related to thiamine deficiency, and as such may be treatable¹¹.

DCA has also previously been shown to stimulate *in vivo* PDH flux as measured by hyperpolarised pyruvate injection⁷⁵.

5.4.2 Experimental overview: DCA

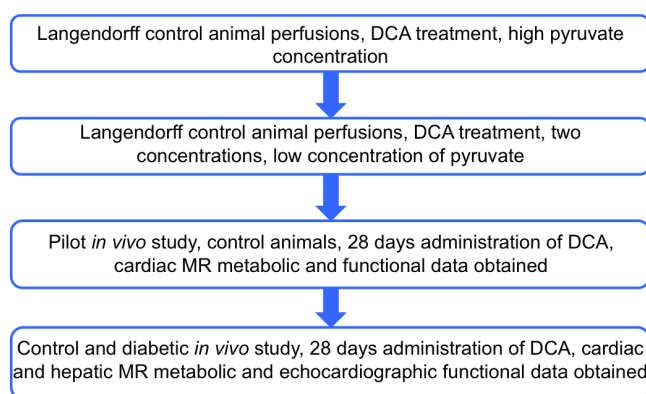


Figure 5.8: Overview of experiments with DCA in this chapter

5.4.3 Acute administration in the isolated perfused heart

We hypothesised that given the previous literature on DCA (discussed above), we should see an increase in PDH flux in the perfused heart. Hearts were perfused as described in section 5.3.1

with KH buffer containing 2.5 mM pyruvate (n=4). The two concentrations of DCA used were 1 mM and 10 mM (neutralised to pH 7.2 with NaOH).

Dose determination

1 mM DCA has been previously shown to increase steady-state glucose oxidation rates in perfused rat hearts¹⁴⁷, and *in vitro* studies demonstrated this concentration to show maximal activation of PDH in the short-term¹¹. This, therefore, was the concentration first used in the perfused heart work. A higher concentration of 10 mM was also assessed in order to establish whether PDH activation could be further activated.

5.4.3.1 Results

No changes were seen in the bicarbonate+CO₂/pyruvate (a measure of PDH flux), or lactate/pyruvate ratios when DCA was added to the perfusion buffer. Decreased ¹³C label transfer to cardiac alanine was shown at both concentrations of DCA (figure 5.9).

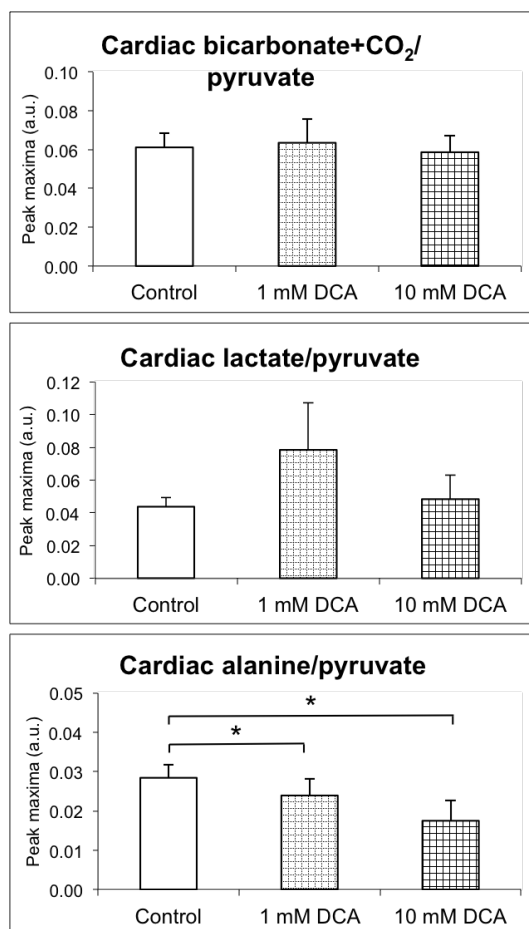


Figure 5.9: Cardiac PDH flux and ^{13}C label transfer to lactate and alanine in the isolated perfused heart on administration of DCA, with 2.5 mM pyruvate in perfusion buffer (n=4).
* $p \leq 0.05$ compared to control

Pyruvate concentration

Our perfusions resulted in no increase in PDH flux when DCA of either concentration was added to the circulating buffer. Given the strong base of evidence supporting the conjecture that an increase in PDH flux is to be expected (as discussed above), we hypothesised that PDH had been maximally activated by the pyruvate in the buffer, via pyruvate inhibition of PDK. Therefore, DCA could have no further effect. A lower concentration of pyruvate (0.625 mM) was therefore used for a subsequent repetition of these experiments (0.625 mM).

Results

Dynamic nuclear polarisation experiments in the isolated perfused heart (n=4 with 0.625 mM pyruvate in KH buffer) showed a significant increase in the rate of conversion of pyruvate to bicarbonate and carbon dioxide by DCA administration (figure 5.10). This is indicative of increased PDH flux due to 1 mM DCA on comparison with control buffer data. This was not further enhanced when a higher concentration of DCA was added - the difference in PDH flux between control and 10 mM DCA administration was not significant (p=0.13). Incorporation of the ^{13}C label into lactate was not significantly altered by either concentration of DCA, but incorporation into alanine was significantly decreased by both 1 and 10 mM DCA.

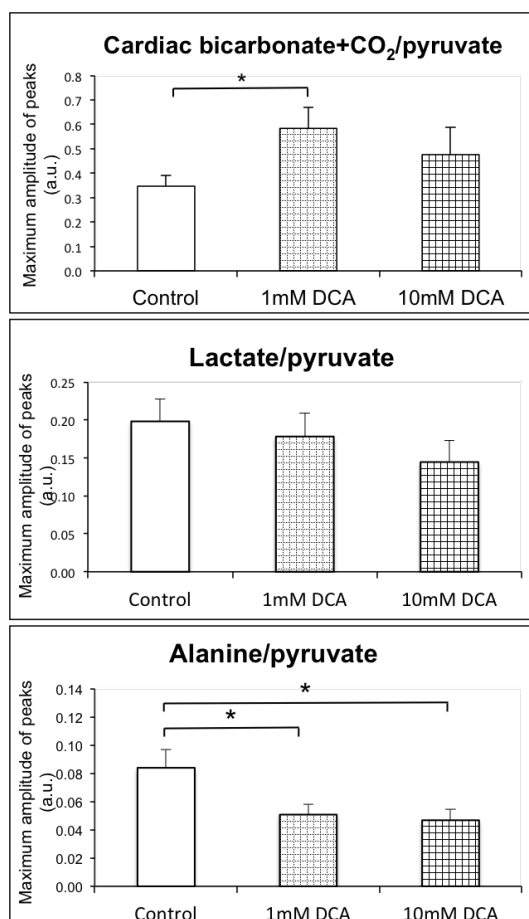


Figure 5.10: Cardiac PDH flux and ^{13}C label transfer to lactate and alanine in the isolated perfused heart on administration of DCA; 0.625 mM pyruvate in buffer (n=6). *p≤0.05

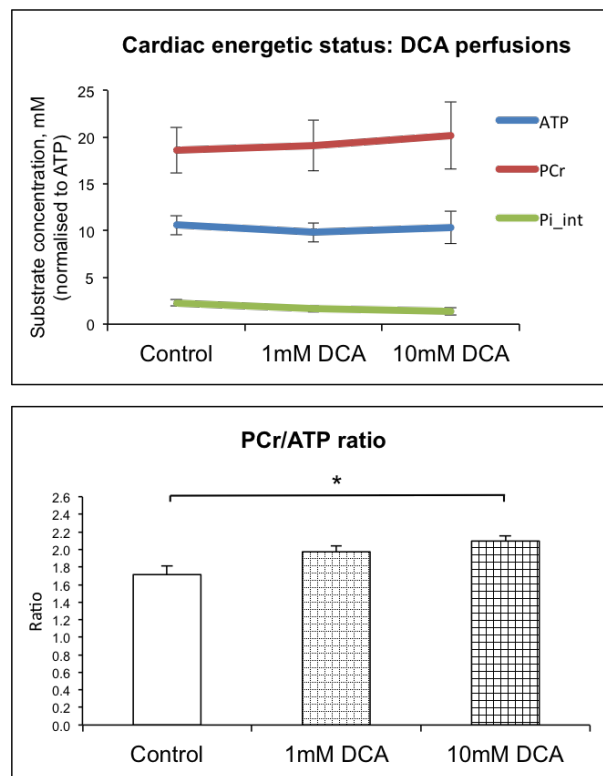


Figure 5.11: Changes in substrate concentrations in the isolated perfused heart on administration of DCA (n=6). * $p \leq 0.05$

There was an increase in phosphocreatine:ATP ratio on addition of DCA that was significant on addition of 10 mM DCA (figure 5.11), with a trend at 1 mM DCA ($p=0.07$). There was no change in rate pressure product (an assessment of heart function), heart rate, or developed pressure (figure 5.12).

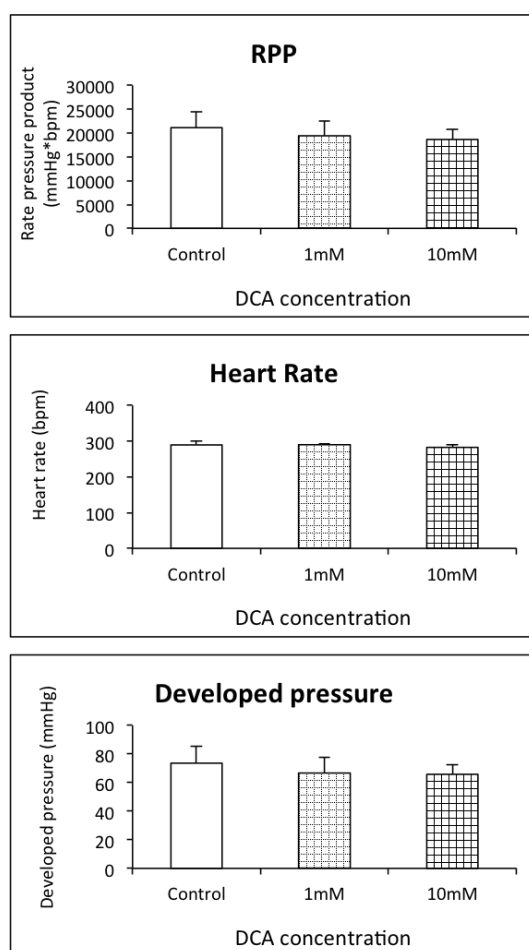


Figure 5.12: Heart function parameters during the administration of DCA (n=5) in the isolated perfused heart.

5.4.3.2 Discussion

This study demonstrated the successful measurement and quantification of an increase in PDH flux in the isolated heart by simple addition of a drug compound to the perfusate. Dichloroacetate was seen to increase PDH flux at 1 mM (although not at 10 mM), measured by increased bicarbonate and carbon dioxide production. No change in function or energetic status of the heart was seen over the course of the experiments, as assessed by the phosphorus NMR data.

5.4.4 *In vivo* pilot chronic dosing study: DCA

Dose determination

Given the successful stimulation of PDH flux in the perfused heart with DCA at 1 mM, but with no further effect seen on increasing the dose to 10 mM, a dose of 1 mM DCA was administered in the pilot chronic dosing study. Previous work by Atherton *et al.*⁸⁶ treated animals with 0.75 g/L DCA (which corresponds to 1 mM) over a 7 day period and saw increased PDH flux *in vivo* via assessment with hyperpolarised pyruvate, further supporting our decision to maintain this concentration for use *in vivo*.

5.4.4.1 Methods

DCA was administered for 28 days to eight control male Wistar rats; cardiac metabolic and functional assessment was then carried out followed by terminal tissue removal. For a protocol summary, see figure 5.13.

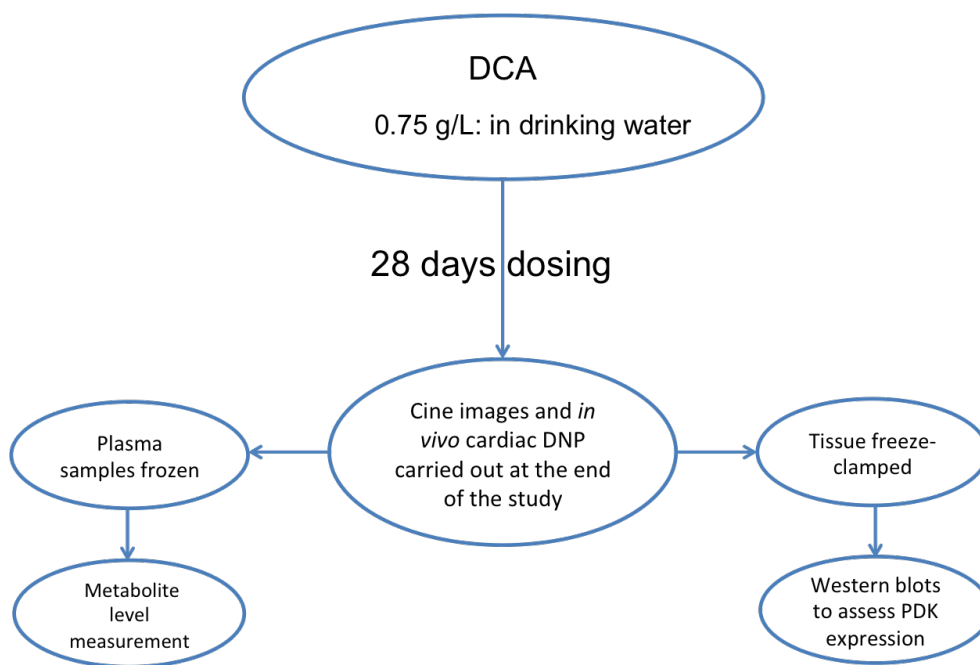


Figure 5.13: Protocol for study assessing the effect of DCA on control Wistar rats

DCA was administered via addition to the drinking water. Water contained DCA (0.75 g/L), neutralised to pH 7.2 with NaOH. This was freshly made every 2/3 days. Body weights were

monitored throughout the experiment to ensure no significant weight loss.

At the end of dosing, hyperpolarised MRS and cine MRI were carried out as described earlier (section 5.3.2). Following the MR protocols the animals were given an overdose of isoflurane and tissue was taken as described in chapter 3, section 3.9.

Lysates were prepared from freeze-clamped cardiac tissue and Western blots run to study PDK1, 2 and 4 expression (detailed in section 3.10 in chapter 3). Blood samples were also taken (similarly detailed in section 3.10) for assessment of metabolite levels.

Cardiac metabolic and functional data was analysed using a one-way ANOVA with a Holm-Sidak correction. All other data sets were analysed using t-tests.

5.4.4.2 Results

The *in vivo* results mirrored those seen in the isolated hearts, in that DCA evoked a significant increase in PDH flux ($p=0.0001$), measured by the rate of conversion of pyruvate to bicarbonate and carbon dioxide. In these DCA treated animals, conversion of pyruvate to alanine was significantly decreased although lactate was not (figure 5.14).

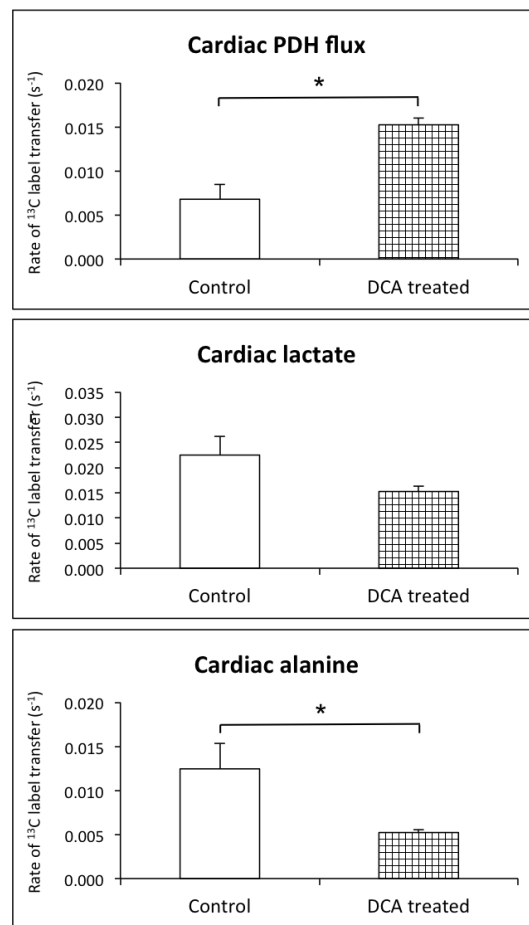


Figure 5.14: Pyruvate metabolism in Wistar rats following 28 days administration of DCA (n=8) compared to control (n=10). * $p \leq 0.05$

The cine images taken enabled structural and functional comparisons between groups. There were no differences in heart weight:body weight ratios or end diastolic or systolic lumen size between any of the groups. Cardiac function was not different between groups (figure 5.15).

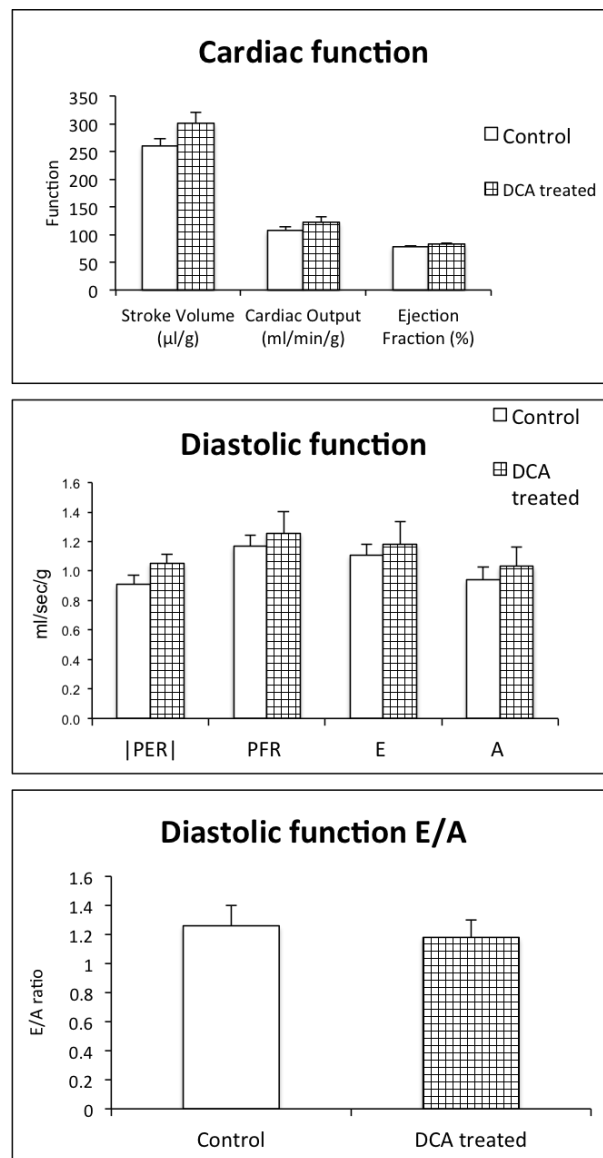


Figure 5.15: Cardiac function following chronic dosing with DCA

Western blots to study pyruvate dehydrogenase kinase expression demonstrated significantly lower PDK2 in both heart and liver tissue from animals treated with DCA. No changes were seen in cardiac PDK1 and 4, or hepatic PDK4 (figure 5.16).

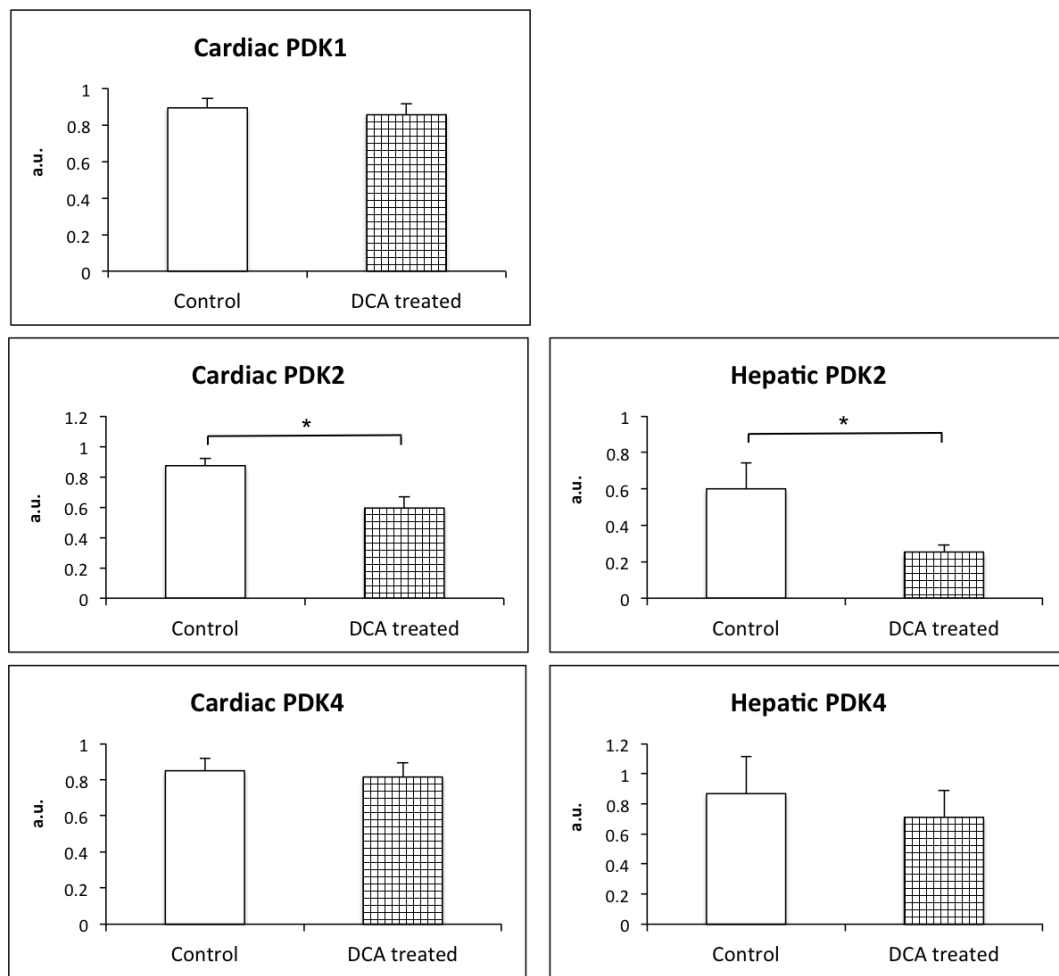


Figure 5.16: Cardiac and hepatic Western blot data from control animals and DCA treated control animals. $*p \leq 0.05$

Unfortunately due to a technical issue the post-mortem plasma samples from the DCA treated group were not analysable, however saphenous samples from these animals were taken before and after DCA treatment. Analysis of these samples showed that DCA elicited no effect on control blood glucose levels, but reduced plasma lactate significantly (figure 5.17).

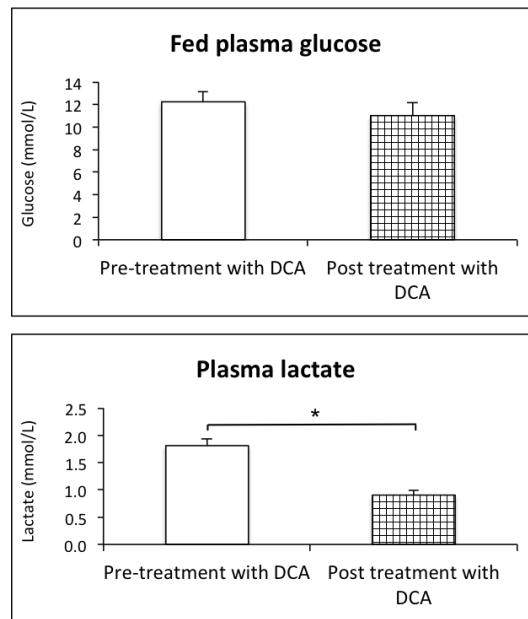


Figure 5.17: Plasma analyses of blood glucose and lactate in control and DCA treated animals.
* $p \leq 0.05$

It is possible that, given the western blotting results, PDK2 is solely responsible for the increased PDH flux seen. However, it is also possible that the activities of PDK1 and 4 have been altered. We cannot conclude either way, but, as discussed in section 2.2.1.1 in chapter 2, this would be an interesting future experiment.

5.4.4.3 Discussion

On moving *in vivo*, an increase in cardiac PDH flux due to administration of 1 mM DCA (to healthy animals) was demonstrated, as with the perfused heart experiments.

No changes were seen in cardiac function in this study, either systolic or diastolic. However, it is possible that the MR assessment of diastolic function that we used was not sufficiently sensitive for determining dysfunction. The E/A measure is pre-load dependent, i.e. it provides a measure that could be affected by similar stiffening of the atria and ventricles. This may have given an inaccurate assessment of function. It has been shown that the E/A ratio does reduce as the heart progresses from health to early failure, however as dysfunction progresses and the left-atrial pressure rises, this ratio can appear 'pseudo normal'. The use of echocardiography

in the place of MR can address this, as it can provide a further measure of diastolic function - E/E' - a pre-load independent measurement.

This pilot study demonstrated the efficacy of DCA, and as such we wished to establish if the visible metabolic alterations that we saw were in any way linked to functional changes in the heart. Given the diabetic heart is prone to diastolic dysfunction (as discussed in chapter 2), it seemed appropriate to probe the effects of this effective metabolic modulator on metabolism and function in our rodent model of type II diabetes.

5.4.5 *In vivo* investigation into treatment of a model of type II diabetes: DCA

This study investigated the effect of DCA on cardiac and hepatic metabolism in control and type II diabetic animals. The dose level was maintained given the successful stimulation of cardiac PDH flux by 1 mM DCA.

5.4.5.1 Methods

An overview of this protocol is given in section 5.3.3. Briefly, diabetes was induced and this group, along with a control group, were administered DCA for 28 days. At the end of the dosing period, DNP data were taken from both the heart and liver using the acquisition developed in chapter 4, to extract more information about the metabolic changes occurring and the effects of the compound. Also, echocardiographic assessment of diastolic function was carried out at the end of the protocol to establish whether a metabolic intervention could have an effect cardiac function. Animals were fasted overnight before echocardiographic data were taken; they were then given an overdose of isoflurane and tissue and blood samples were taken and frozen. Further, epididymal fat pad weights were measured.

Student's t-tests were used to assess differences between the treated groups and their respective untreated groups.

5.4.5.2 Results

All relevant data from this thesis was combined to look at cardiac and hepatic metabolism for control and diabetic untreated animals, as detailed in chapter 4, section 4.5, for control and diabetic animals. In summary, the untreated diabetic animals showed significantly reduced cardiac PDH flux compared to controls; lactate was not changed, and ^{13}C label transfer to alanine was significantly increased. Hepatic bicarbonate was significantly reduced in diabetic animals, and lactate and alanine were unchanged.

Dichloroacetate treatment

Control animals showed no change in cardiac PDH flux when treated with DCA, whereas DCA treatment of diabetic animals resulted in an increase in PDH flux relative to untreated diabetic animals so that it was restored to control levels ($p < 0.0001$; figure 5.18). Lactate was not changed in either controls or diabetics on treatment with DCA, whereas alanine was reduced in both controls and diabetics relative to untreated data ($p < 0.0001$ in both cases; figure 5.18).

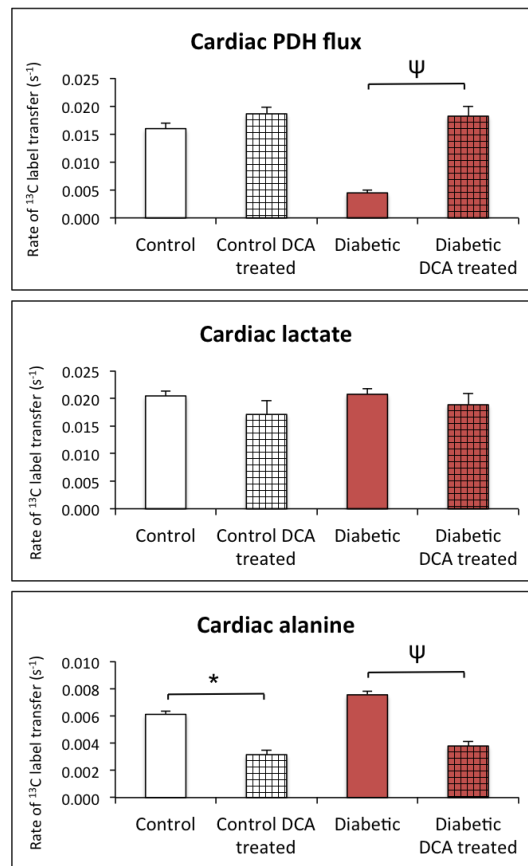


Figure 5.18: *In vivo* cardiac carbohydrate metabolism on injection of hyperpolarised ^{13}C pyruvate in control and diabetic animals, and those treated with DCA. $*p \leq 0.05$ compared to control untreated. $\psi p \leq 0.05$ compared to diabetic untreated

Hepatic bicarbonate was much increased by DCA treatment in both control and diabetic animals ($p < 0.0001$ for both comparisons). Hepatic ^{13}C label incorporation into lactate was seen to be increased in the control animals treated with DCA ($p = 0.0001$), however this effect was not seen in the diabetics. Hepatic ^{13}C label incorporation into alanine was reduced by DCA treatment in diabetics compared with untreated diabetic animals ($p = 0.039$), but there was no difference seen between treated and untreated controls (figure 5.19).

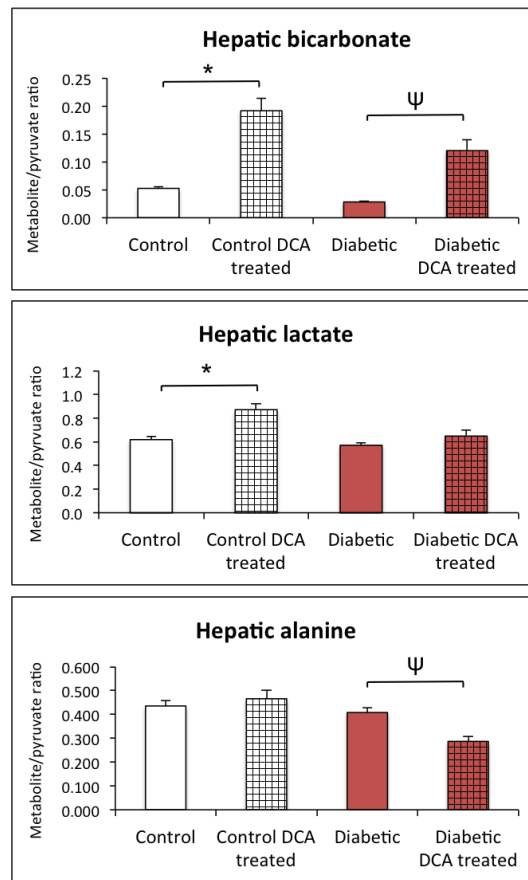


Figure 5.19: *In vivo* hepatic ^{13}C label transfer data on injection of hyperpolarised ^{13}C pyruvate in control and diabetic animals, and those treated with DCA. $*p \leq 0.05$ compared to control untreated. $\psi p \leq 0.05$ compared to diabetic untreated

Ejection fraction and diastolic function as measured by E/A ratio were not different across the groups. However, the E/E' assessment of diastolic function was shown to be significantly higher (indicative of dysfunction) in the diabetic animals at the end of the 7 week treatment period. This dysfunction was not present in the diabetic animals that had been treated with DCA (figure 5.20).

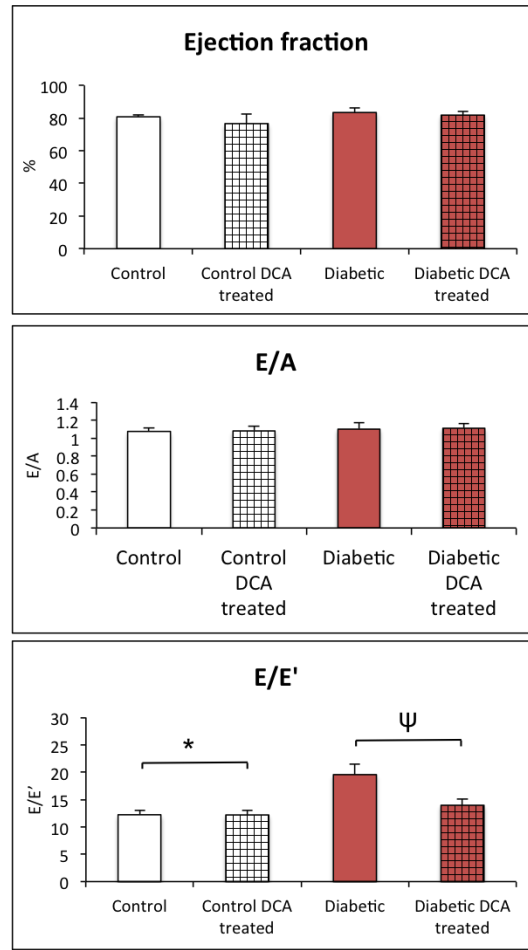


Figure 5.20: Echocardiographic data measuring systolic and diastolic function. Control, diabetic and DCA treated groups. * $p \leq 0.05$ compared to control untreated. $\psi p \leq 0.05$ compared to diabetic untreated

In control animals, treatment with DCA caused a significant decrease in cardiac PDK2 expression, with a trend to decreasing PDK1 ($p=0.07$). PDK4 was not altered. In the diabetic DCA-treated group, levels of cardiac PDK1, 2 and 4 were decreased significantly compared to untreated diabetics (figure 5.21). A separate Western blot with only control, diabetic, and diabetic DCA treated groups was performed, allowing for a larger sample number and reducing error due to spreading samples across multiple blots. This Western blot gave significant differences in cardiac PDK4 between control and diabetic animals ($p=0.026$) and diabetic and diabetic DCA treated animals ($p=0.0014$) (figure 5.22).

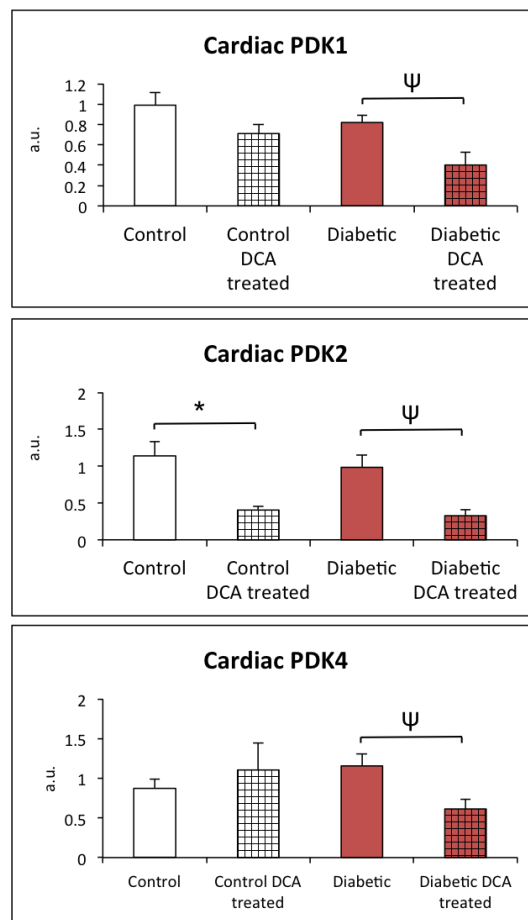


Figure 5.21: Cardiac Western blots for PDK1, 2, and 4 in all groups. $*p \leq 0.05$ compared to control untreated, $\psi p \leq 0.05$ compared to diabetic untreated

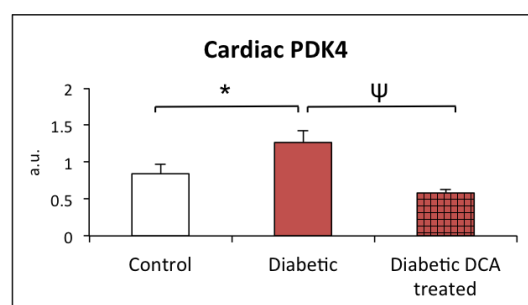


Figure 5.22: Cardiac Western blot for PDK4 in control, diabetic and diabetic DCA treated animals. $*p \leq 0.05$ compared to control untreated, $\psi p \leq 0.05$ compared to diabetic untreated

Hepatic PDK1 was unchanged in both groups. DCA caused a significantly lower PDK2 expression in diabetic animals but not controls. Treatment with DCA resulted in significantly lower hepatic PDK4 in both the control and diabetic groups.

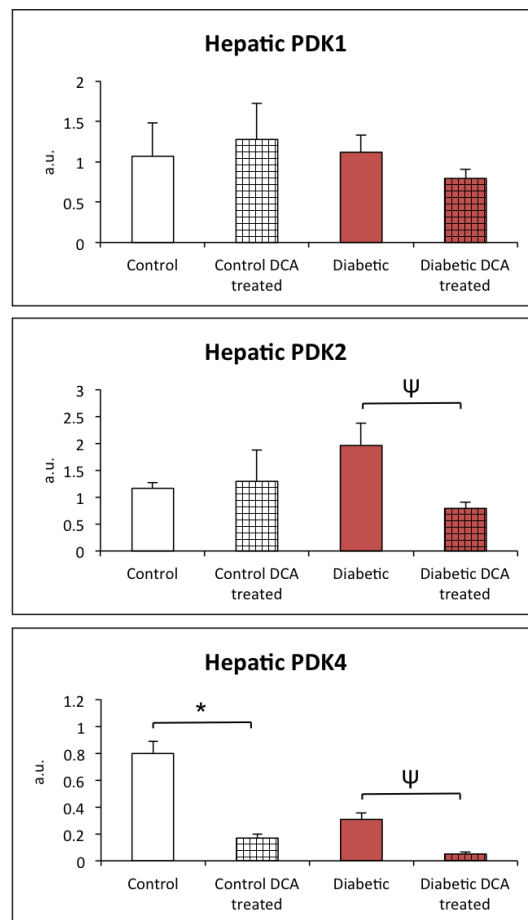


Figure 5.23: Hepatic Western blots for PDK1, 2, and 4 expression in control, diabetic and DCA treated animals. $*p \leq 0.05$ compared to control untreated. $\psi p \leq 0.05$ compared to diabetic untreated

There was a trend towards decreasing fasting blood glucose levels after treatment with DCA in control animals ($p=0.09$), but in the diabetic animals this difference was significant ($p=0.004$; figure 5.24). Epididymal fat was not significantly altered in the control or diabetic DCA-treated groups in comparison to those that were not treated (figure 5.25).

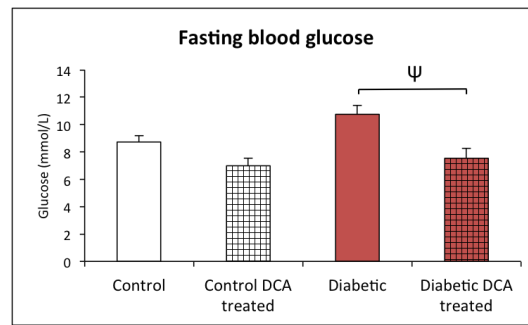


Figure 5.24: Fasting blood glucose levels, measured post-mortem. Statistical analysis carried out comparing treated group and their respective untreated group. $\psi p \leq 0.05$ compared to diabetic untreated

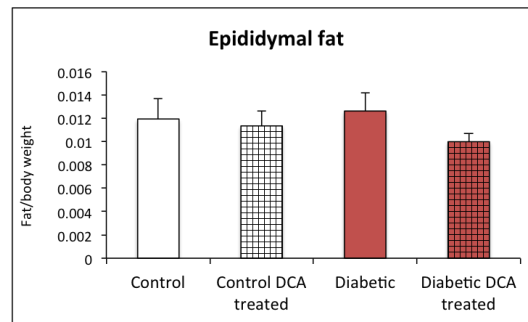


Figure 5.25: Epididymal fat pad weight, untreated and DCA treated control and diabetic animals

When comparing diastolic function (as assessed by E/E') and cardiac PDH flux for all data, a negative correlation is seen ($p=0.002$).

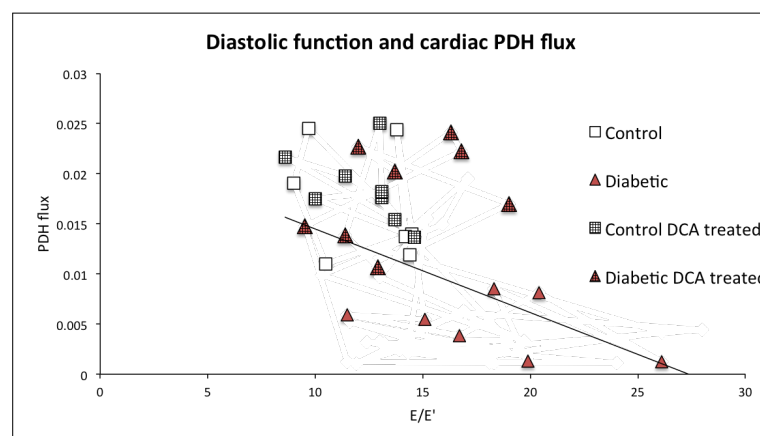


Figure 5.26: Correlation of E/E' ratios with PDH flux. Pearson correlation gives a coefficient of -0.531, with $p=0.002$

5.4.5.3 Discussion

This study involving the treatment of control and diabetic animals with DCA demonstrated our ability to assess metabolism in a disease state non-invasively and *in vivo*. This was coupled with the established technique of echocardiography to provide an assessment of diastolic function that was not possible with MRI, but is relevant due to the high proportion of diabetic patients with diastolic dysfunction.

The use of DCA to treat diabetic animals has demonstrated that by targeting PDH, we have affected cardiac function in diabetes, namely by removing the dysfunction seen (as assessed by E/E'). Modulation of substrate utilisation in the heart is emerging as a candidate mechanism underpinning diabetic cardiomyopathy, and our study has provided support for the idea that PDH may be a target for novel treatments^{148,149}. DCA treatment for 28 days reversed the decreased cardiac PDH flux and inefficient substrate utilisation in the diabetic heart, seemingly with the involvement of cardiac PDK4, and resulted in an improvement in diastolic function and restoration of the fuel use balance seen in healthy hearts. The link between metabolism and function fits alongside previous work on energetic status that has demonstrated that the diabetic heart is less efficient in its production of ATP, and reported decreased PCr/ATP ratios in diabetics^{54,150}. This may be linked to the high energy demand from the cardiac calcium pumps¹⁵¹. Overall, the data in this thesis are the first able to link cardiac metabolism and function directly, *in vivo*.

Cardiac alanine was significantly decreased in both control and diabetic groups treated with DCA, potentially indicative of reduced production of this gluconeogenic precursor for the liver. In diabetic animals only, hepatic ^{13}C label incorporation into alanine was lower in DCA treated animals, potentially in balance with the large increase in hepatic PDH flux seen. However this was not replicated in the control animals, where the large increase in hepatic bicarbonate was not accompanied by a decrease in alanine, but by a significantly increased lactate. Given the different factors which can affect the measure of pyruvate to lactate ^{13}C label transfer, it is possible that the increase seen is indicative of greater LDH activity. This would imply increased lactate consumption, with the liver removing lactate from the plasma, which would support previous literature that has concluded that DCA is a lactate-lowering compound.

In the heart, the PDK isoforms present are 1, 2 and 4, however in diabetes it is PDK4 which is upregulated. We have shown that DCA was successful in downregulating expression of PDK4 in the diabetic heart, and similarly PDK1 and PDK2 were also shown to be decreased. This wider effect only acts to confirm the release of inhibition of cardiac PDH and provides a mechanism for its increased activity. It is interesting to have seen that in this study, in agreement with the pilot study detailed earlier in this chapter, the only cardiac PDK isoform that was affected by treatment of control animals with DCA was PDK2.

Hepatic PDK1 and 2 showed no change between control untreated and DCA treated groups, however the ubiquitous PDK2 was decreased in the hepatic tissue from diabetic treated animals. This supported the increased hepatic bicarbonate seen on DCA treatment of this group. Hepatic PDK4 was high in the control group, most likely due to the overnight fast prior to tissue sampling, meaning data cannot be compared with data from the studies of chapter 6, which showed increased hepatic PDK4 in diabetic animals compared to controls.

The changes seen in cardiac and hepatic metabolism manifested as a significant reduction in fasting blood glucose levels in the diabetic treated group and a trend to a decreased glucose in the control treated group. This demonstrated how metabolic modulation had a whole-body consequence, which was of particular benefit to the diabetic group, where elevated blood glucose levels are at the root of so many of the symptoms and complications as discussed in chapter 2.

5.5 AZD7545

5.5.1 Introduction

The liver has a large role in the control of systemic glucose levels. If we look to target the glucose metabolism of the liver, with an aim to increase glucose oxidation for the treatment of diabetes, a stimulation of PDH activity is required. As discussed in chapter 2, PDK2 is the dominant isoform in the liver, as PDK1 and 4 are abundant in the heart but low in the liver, and PDK3 is only abundant in the testes. This was the logic behind the development of specific PDK2 inhibitors, of which AZD7545, whose structure is shown in figure 5.27, is one^{12, 152}.

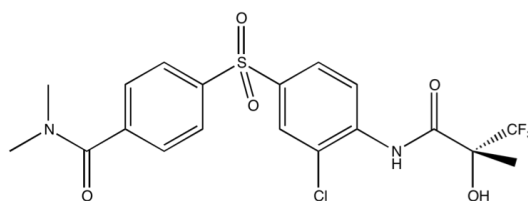


Figure 5.27: Chemical structures of AZD7545

Similar to DCA, AZD7545 prevents PDK-PDC binding, however not by the same mechanism as AZD7545 is not a pyruvate mimic. Instead, it binds to the lipoyl-binding pocket of PDK and prevents kinase-PDC binding (via E2), thus preventing inhibition of PDH¹²².

Mayers *et al.* demonstrated that AZD7545 increased PDH activity, when porcine PDH was in the presence of recombinant human PDK2 and ATP¹². This activity increase was shown to be concentration-dependent, giving an EC_{50} of 5.2 nM (concentration required to give a half-maximal response). In the same assay, they saw DCA give an EC_{50} of 113 μ M. Morrell *et al.* further determined the IC_{50} of AZD7545 for PDK2 to be 6.4 nM (PDK1 was 36.8 nM)¹⁵².

They then moved on to cell work, using the conversion of [¹⁴C]pyruvate to ¹⁴CO₂ as a measure of PDH activity. Pre-incubation of rat hepatocytes with AZD7545 caused a doubling of this conversion rate, and provided an EC_{50} of 105 nM. Again they established that a comparable experiment using DCA showed similar results, but required a concentration of 1mM.

Finally these authors looked at the *in vivo* effects of this compound. A single dose of AZD7545 was given to fed Wistar rats, and an increase in the percentage of active hepatic PDH was seen, with a dose-dependence between 0 and 30 mg/kg (25% to 70% activation). These measurements were taken two hours after the dose was administered orally, and PDH activity was assessed by the assay detailed by Coore *et al.* in 1971. Further, 10 mg/kg AZD7545 was given for seven days to obese Zucker rats, in which the authors state muscle and hepatic PDH activity was seen to be increased compared to lean Zucker or Wistar rats. This treatment protocol resulted in the daily glucose profile of the animals being more stable, removing the increase in blood glucose seen in the untreated animals at around 7pm. Morrell *et al.* concluded that AZD7545 has more of an effect on the liver than skeletal muscle and heart, due to the specificity of its action.

However, work by Aicher *et al.* is in contradiction to this, finding that although similar com-

pounds caused a lactate lowering effect, no effect on the blood glucose level was seen in either Zucker diabetic rats or *ob/ob* mice¹⁵³. This is different to DCA, and the authors suggest it is due to the indirect effects of DCA, such as the effect of PDH on carnitine palmitoyltransferase (CPT). Further, work by Aas *et al.* showed that adding AZD7545 to myotubes in a hyperglycaemic environment did not reverse the induced low glucose oxidation over four days of treatment, even though an acute activation of glucose oxidation was seen (35% and 45% in normo- and hyper-glycaemic environments respectively)¹⁵⁴.

The development of AZD7545 was halted at AstraZeneca due to the observation of lipid droplet build-up in the cardiac tissue and the consequent concerns of cardiac steatosis.

The aims of these studies with AZD7545 were to establish the *in vivo* effects of this novel compound in the heart and the liver, whilst confirming the suitability of techniques used. Further we wished to investigate our ability to visualise the effect of specific isoform inhibition on PDH, and finally assess the use of hyperpolarised techniques in drug development.

5.5.2 Experimental overview: AZD7545

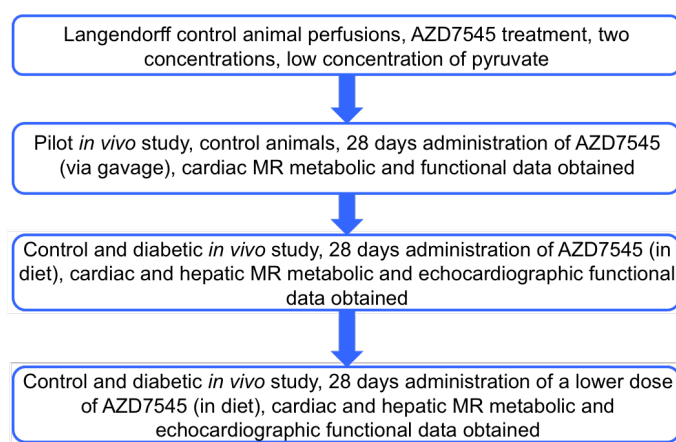


Figure 5.28: Overview of experiments with AZD7545 in this chapter

5.5.3 Acute administration in the isolated perfused heart

We hypothesised that given the previous work in the literature demonstrating the effect of AZD7545 on PDH in skeletal muscle and liver, and of a similar compound in heart tissue¹⁵⁵, a

similar effect would be seen in the *in vivo* heart, although maybe to a lesser extent given the presence of the more dominant PDK1 and 4.

5.5.3.1 Methods

Hearts were perfused as described in section 5.3.1. Given that changes in PDH flux were observed with the low concentration of pyruvate in DCA studies (0.625mM), this concentration was therefore maintained for AZD7545 perfusions.

The concentrations of compound used were 0.1 μ M AZD7545 (dissolved in buffer overnight with continuous stirring) and 30 μ M AZD7545 (dissolved in minimum DMSO initially, then in KH buffer overnight with continuous stirring).

Dose determination

The lower concentration of 0.1 μ M was chosen as work in rat hepatocytes by Mayers *et al.* demonstrated increased PDH activity at this concentration¹². The second dose was chosen to replicate the concentrations used *in vivo* in previous in-house studies at AstraZeneca. This aimed to be sufficiently high to see maximal effects but not cause any sub-acute lethal side effects. Following a one-month oral toxicity study in rats (Report no. 2186-01, AstraZeneca R&D, Sweden), a dose of 300 mg/kg for one month resulted in plasma concentrations of approximately 30 μ M. Therefore this was chosen as the second dose to administer to the perfused heart.

5.5.3.2 Results

Application of AZD7545 showed no change in PDH flux at either concentration of the compound (figure 5.29), disproving our hypothesis. There was also no change in ¹³C label transfer to alanine, however at the higher concentration of AZD7545, a significant increase in lactate was seen.

No significant change in PCr/ATP ratio was seen on addition of either concentration of AZD7545 (figure 5.30). Looking at cardiac function, a significant decrease in rate pressure product, driven

by a significant decrease in heart rate was demonstrated at the higher concentration of 30 μM AZD7545 (figure 5.31), but not at 0.1 μM .

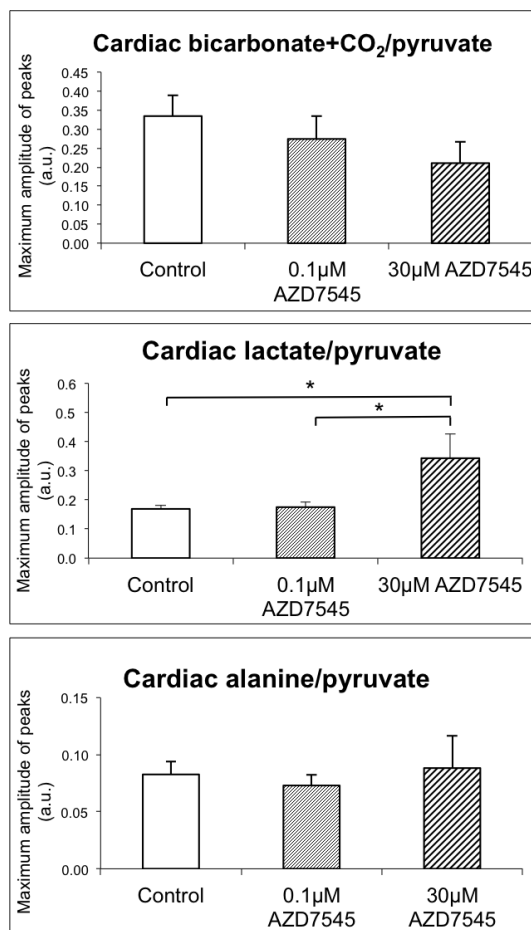


Figure 5.29: Cardiac PDH flux and ^{13}C label transfer to lactate and alanine in the isolated perfused heart on administration of AZD7545 ($n=6$). $p \leq 0.05$

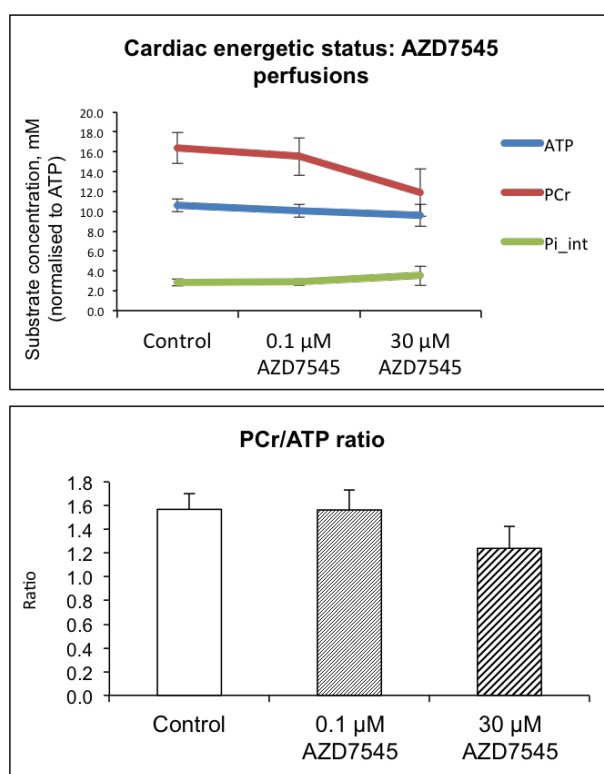


Figure 5.30: Changes in substrate concentrations in the isolated perfused heart on administration of AZD7545 (n=5)

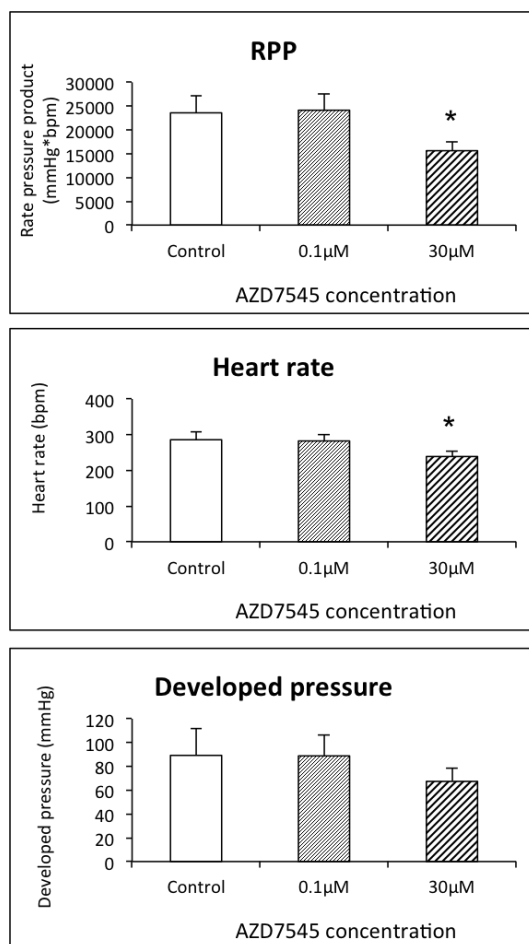


Figure 5.31: Heart function parameters during the administration of AZD7545 ($n=5$) in the isolated perfused heart, * $p < 0.05$ compared to control and 0.1 μ M

5.5.3.3 Discussion

The PDK2 specific inhibitor AZD7545 did not increase PDH flux, and caused a significant decrease in cardiac function at the higher concentration of 30 μ M. This latter effect (RPP significantly decreased at 30 μ M) may be a limitation of the experiment, as decreased heart rate is potentially to be expected over the long timecourse of the protocol (over three hours).

One hypothesis for the inability of AZD7545 to replicate the results seen with DCA (in terms of increased PDH flux) may be its specificity to PDK2. Cardiac PDK activity is a result of a combination of PDK1, PDK2 and PDK4 activity, and potentially changes in the activity of PDK1 and PDK4 counteract the effect of AZD7545 on PDK2, therefore resulting in a different effect to DCA administration. As discussed earlier, assessment of specific activities of purified

PDK isoenzymes by Bowker-Kinley *et al.*²⁴ showed PDK1 and 4 to have higher activities in the heart than PDK2 (650, 400, and 50 nmol/min per mg respectively), giving weight to the possibility that the more active isoenzymes may have helped prevent PDH flux increase in the heart. Given the tight links between metabolic and energetic status, if these changes in activity to maintain PDH flux occurred, they may have contributed to the lack of differences seen in PCr:ATP ratios on drug administration. If the hearts were negatively affected by the drug compounds, a decrease in PCr:ATP ratio would be expected as the stores of phosphocreatine are consumed while more ATP is not produced¹⁵⁶.

Compensation by other isoforms has been seen by Dunford *et al.*¹⁵⁷ in the skeletal muscle of PDK2 knockout mice (albeit with respect to protein expression, not activity). PDK1 was shown to compensate for the loss of activity of PDK2, with PDK1 protein expression becoming almost two-fold higher in the knockouts compared to wild-type. Our short experimental length (relative to the time required for transcription) would be insufficient for protein upregulation so this explanation would require the effect on protein expression to be mirrored in protein activity. AZD7545 has been demonstrated to inhibit PDK1 to a limited extent, and even activate PDK4 at concentrations above 10 nM in cell work; potentially in this study the activity of PDK4 in the heart was increased in response to our inhibition of PDK2¹⁵². The trend to decreased (bicarbonate+CO₂) and increased lactate on administration of 30 μ M AZD7545 would support this, and may provide a reason for the functional decline.

It is also possible that AZD7545 was not administered at a dose sufficient to see an effect on PDK2. In the heart, the ability of PDK2 inhibitors is limited when attempting to maximally activate PDH¹⁵², compared to the liver. It is possible that the limited PDK2 inhibition in the heart resulted in little change, and *in vivo* work with the liver present (with its dominant isoform being PDK2) may give different results. This may then explain the previously observed changes in blood glucose levels¹². Even so, a doubling of PDH activity was expected in the heart¹⁵⁵, which would have been obvious with reference to control animals. Further, the concentrations used for both drugs were calculated to mimic the blood plasma concentrations reached *in vivo*^{12,86}. As discussed earlier, in previous studies at AstraZeneca, 30 μ M AZD7545 corresponded to 30 days dosing with 300 mg/kg/day, which in turn corresponded to the highest tolerated oral dose not to induce sub-acute lethal effects (Dr Johansson, personal communication).

Another possibility is that the AZD7545, having reached the heart, did not enter the cells. However, in the study by Mayers *et al.* in 2003, incubation of rat hepatocytes with the compound demonstrated a doubling of the rate of conversion of [^{14}C]-labelled pyruvate to labelled carbon dioxide. DCA was also demonstrated to have similar effects in their study, at a concentration of 1 mM¹².

5.5.4 *In vivo* pilot chronic dosing study: AZD7545

Dose determination

No effect of AZD7545 was seen on cardiac PDH flux at either concentration in the perfused heart, and therefore the higher concentration (30 μM corresponding to 300 mg/kg/day) was chosen for moving *in vivo*.

5.5.4.1 Methods

AZD7545 was administered for 28 days to ten control male Wistar rats; cardiac metabolic and functional assessment was then carried out followed by terminal tissue removal. For a protocol summary, see figure 5.32.

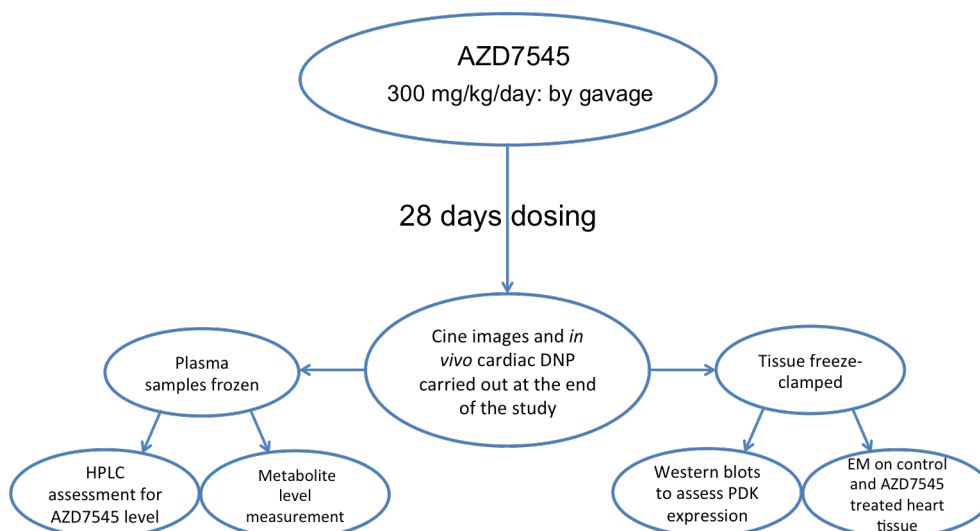


Figure 5.32: Protocol for study assessing the effect of AZD7545 on control Wistar rats

Wistar rats were gavaged daily with a suspension of AZD7545 (300 mg/kg/day) in 0.5% w/v

hydroxypropyl methyl cellulose (HPMC) in 0.1% w/v aqueous polysorbate 80. The volume administered was 1 ml per 100 g body weight. A new suspension was made up every seven days, and the solution stored at 2-8° and protected from light. 10 control male Wistar rats were dosed with the control solution of just HPMC in polysorbate 80. For all groups, body weights were monitored throughout the experiment to ensure no significant weight loss.

At the end of dosing, hyperpolarised MRS and cine MRI were carried out as described earlier (section 5.3.2). Following the MR protocols the animals were given an overdose of isoflurane and, generally, tissue was taken as described in chapter 3, section 3.9. Hearts from control and AZD7545-treated animals were divided in two; half the heart was freeze clamped and the other half prepared for histological staining. They were fixed in 10% neutral buffered formalin for Haematoxylin and Eosin (H&E) and Oil-Red-O (ORO) staining, with samples taken from the left ventricle and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for electron microscopy (EM). This was due to the fact that administration of AZD7545 previously caused cardiac steatosis, as mentioned earlier. As such, this was assessed in this *in vivo* pilot study, to firstly establish whether we could replicate that data, and secondly to investigate the underlying causes. For H&E staining, the middle section of the heart was selected before being processed overnight in formalin, increasing concentrations of alcohol, and xylene before being embedded in wax. These samples were then sectioned (4 μ m) and stained to produce analysable microscope slides of the stained tissue. The tissue not used for H&E staining was fixed in cryogel and slices taken at -14°C for oil-red-O staining (a dye specific to lipids), with several slides being assessed for each animal, and averages taken. These slides were scanned on the Aperio Scanscope XT at x20 objective lens and analysed computationally using an in-house developed programme on the Definiens XD developer version 1.1.2 (AstraZeneca, Alderley Park). Thresholds were established to ensure sole analysis of oil-red-O stained microdroplets, eliminating, for example, red blood cells. The glutaraldehyde EM samples were fixed in resin before sections were cut and stained for analysis.

Lysates were prepared from freeze-clamped cardiac tissue and Western blots run to study PDK1, 2 and 4 expression (detailed in section 3.10 in chapter 3). Blood samples were also taken (similarly detailed in section 3.10) for assessment of metabolite levels and the concentration of AZD7545 achieved in the treated animals (HPLC, in-house method, AstraZeneca, Sweden).

Analysis carried out by Zulma Santisteban-Valencia).

Cardiac metabolic and functional data was analysed using a one-way ANOVA with a Holm-Sidak correction. All other data sets were analysed using t-tests.

5.5.4.2 Results

Treatment with AZD7545 for 28 days caused no change in ^{13}C label transfer to bicarbonate or lactate. A significant decrease in label incorporation into alanine was seen (figure 5.33).

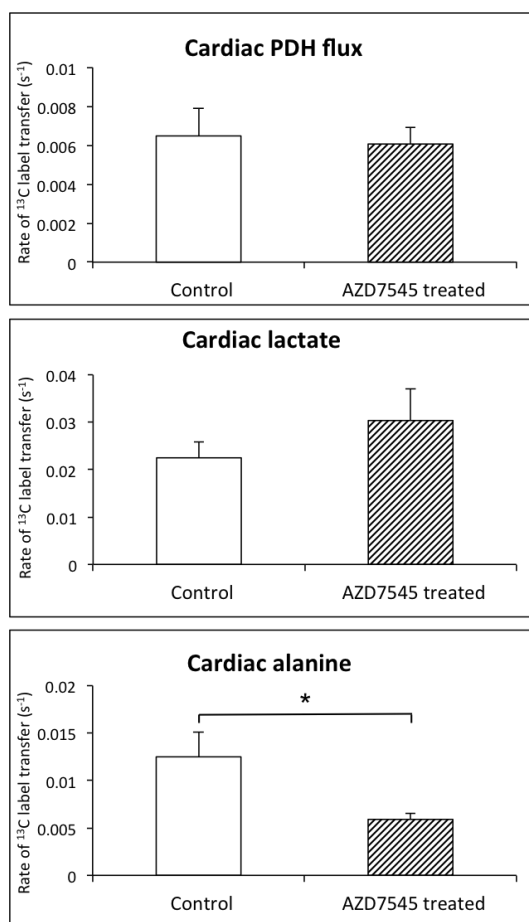


Figure 5.33: Cardiac pyruvate metabolism in Wistar rats following 28 days administration of AZD7545 (n=10) compared to control * $p \leq 0.05$

The cine images taken enabled structural and functional comparisons between groups. There were no differences in heart weight:body weight ratios or end diastolic or systolic lumen size between any of the groups. Cardiac function was not different between groups (figure 5.34).

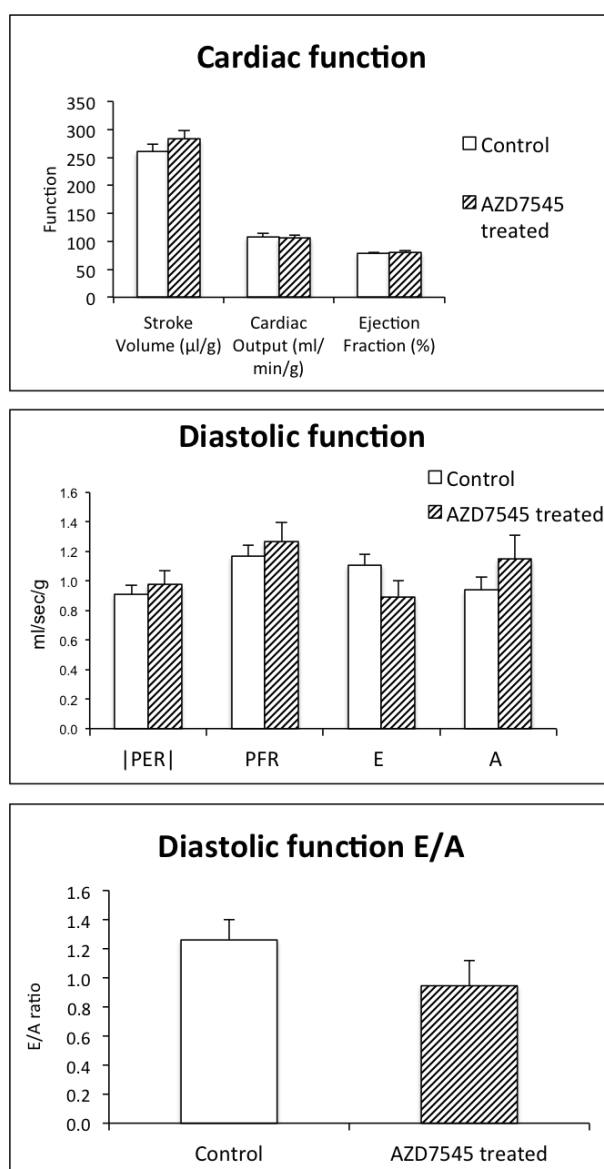


Figure 5.34: Cardiac function following chronic dosing with AZD7545

Haematoxylin and Eosin staining of the cytoplasm and nuclei within tissue from AZD7545 treated animals showed no abnormal morphology in cardiomyocyte structure. There was also no significant increase in oil-red-O staining ($n=4$, $p=0.1$, figure 5.35) in the treated animals compared to controls, although a limited number of treated animals showed increased uptake of the stain (example images in appendix B, figure 2)

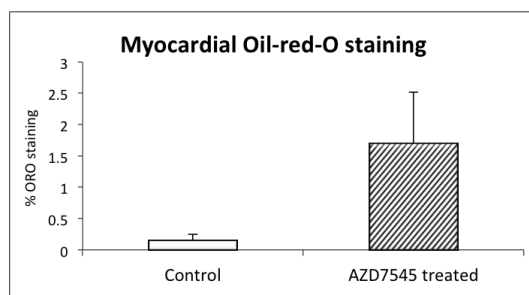


Figure 5.35: % of total area of cardiac tissue stained red with oil-red-O. Samples from control and AZD7545 treated animals

This was then further examined using electron microscopy, where lipid droplets were demonstrated to be present, albeit to a limited degree (appendix B, figure 3).

Western blots to study pyruvate dehydrogenase kinase expression demonstrated no differences in cardiac PDK1 and 4, or hepatic PDK4 (figure 5.16).

No differences in expression of any of the PDK isoforms present in cardiac (1,2 and 4) or hepatic tissue (2 and 4) (figure 5.36).

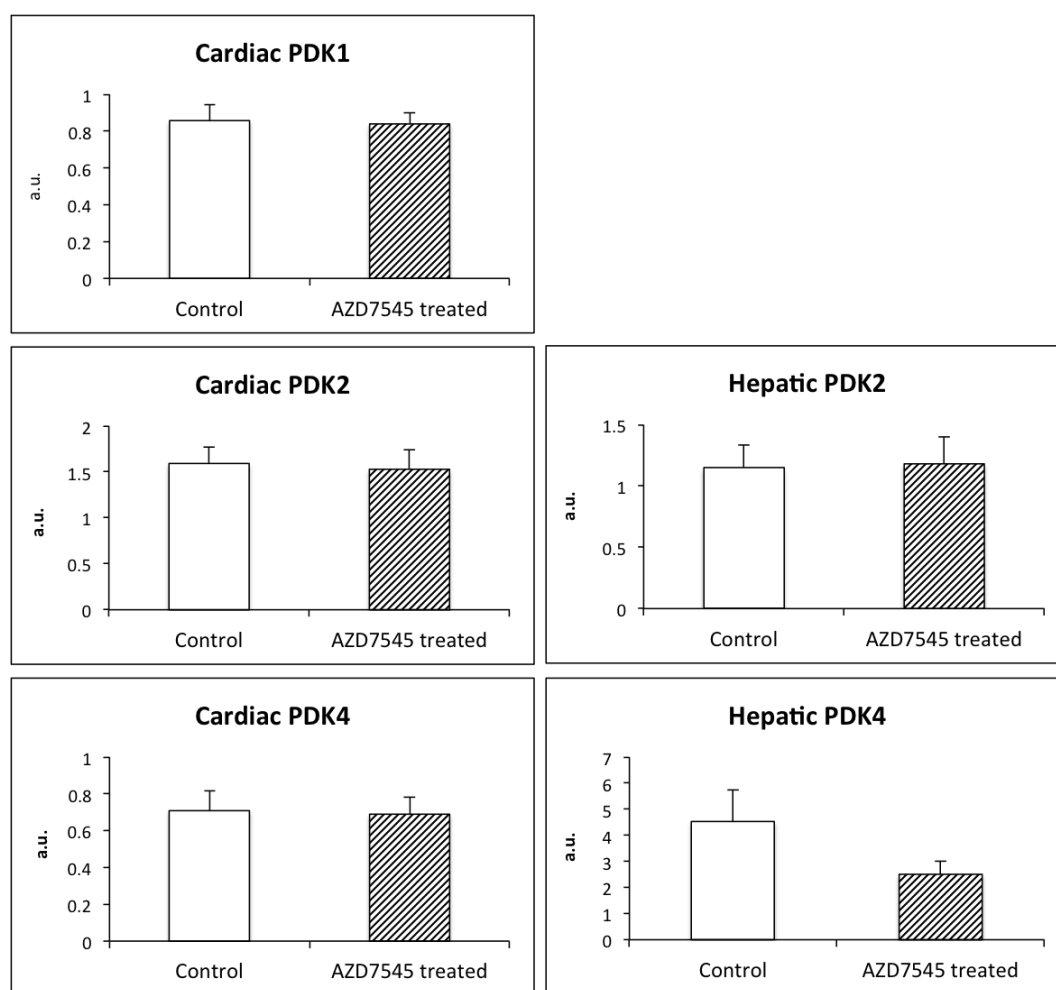


Figure 5.36: Cardiac and hepatic Western blot data from control animals and AZD7545 treated control animals * $p \leq 0.05$

AZD7545 caused no changes in either glucose or lactate (figure 5.37) in plasma samples taken post-mortem.

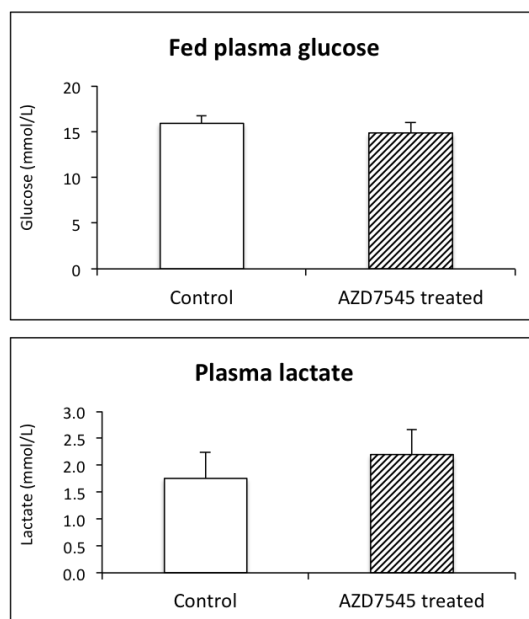


Figure 5.37: Plasma analyses of blood glucose and lactate in control and AZD7545 treated animals. * $p \leq 0.05$

Plasma analysis carried out in-house (AstraZeneca, Mölndal) showed very low concentrations of AZD7545 present. Controls measured $0.012 \mu\text{M} \pm 0.009$, and treated animals gave values of $0.9 \mu\text{M} \pm 0.8$, with the two groups not significantly different in concentration.

5.5.4.3 Discussion

In vivo, $30 \mu\text{M}$ AZD7545 did not cause a change in cardiac PDH flux, and expression of the PDK isoforms was not altered. This may be due to the compound being inactive - there is potential for it having been broken down due to exposure to light (Dr Johansson, personal communication), which may be supported by the low plasma concentrations observed.

AZD7545 was not shown here to have any effect on heart function or metabolism, and as mentioned in the *ex vivo* discussion, this may be due to PDK4 acting to counteract the PDK2 inhibition, although when taking the Western blot data into account, this must be due to changes in activity rather than expression. The majority of the work on this compound previously focussed on liver PDH activity, and given mRNA levels of PDK2 have been shown to be far higher than those of PDK4 in the liver²⁴, we may see a change in PDH activity occurring there

in spite of any compensation being carried out by PDK4.

Previous work by AstraZeneca (in-house report) with the same dosing regime over 28 days documented plasma concentrations of around 30 μM , so we might conclude from this that the method of administration was not successful, so the AZD7545 did not reach the cells, potentially being broken down too quickly - supported by the lack of change seen in expression of the PDK isoforms, or *in vivo* metabolic data.

Potentially the compound may have more of an effect in disease, where PDK levels are increased and there is therefore more scope for an effect. This would be supported by the work mentioned earlier by Mayers *et al.*¹² which showed that a single dose of the compound improved blood glucose control in obese (fa/fa) Zucker rats and, and further studies by Bebernitz *et al.* showing novel PDK inhibitors induced an increase in PDH activity in fasted ZDF rats,¹⁵⁸.

As mentioned previously, development of AZD7545 was stopped due to limited cardiac steatosis being seen in treated animals, with the suggestion that this may be a negative side effect. This study however showed no consistent increase in lipid levels in the heart, no alteration in cardiac function after long-term dosing and no increase in PDH flux in the heart, potentially suggesting that more investigation was required at the time to confirm that the compound was not viable for pursuing. In this study the imaging data have led to further questions surrounding the efficacy of this compound that do not seem to have been an issue in the previous papers by Mayers *et al.* and Morrell *et al.*^{152, 155}.

No changes were seen in cardiac function in this study, either systolic or diastolic. However, similarly to the DCA *in vivo* pilot study, it is possible that the MR assessment of diastolic function that we used was not sufficiently sensitive for determining dysfunction. Once again, echocardiography would provide a more sensitive measure along with the pre-load independent measure of E/E' .

Given the lack of efficacy demonstrated in this study for AZD7545 in healthy animals, we moved on to investigate the effects in a type II diabetic model, mentioned previously¹¹⁰. The same dosing level was maintained, however an alternative delivery route was used, in which the mix was made up more regularly, limiting the time available for breakdown in solution or from exposure to light. In addition, the two-slice acquisition developed in chapter 4 was used to

assess *in vivo* liver metabolism alongside cardiac metabolism, and echocardiography used to assess cardiac diastolic function.

5.5.5 *In vivo* investigation into treatment of a model of type II diabetes: AZD7545

This study investigated the effect of AZD7545 on cardiac and hepatic metabolism in control and type II diabetic animals. The dose level was maintained given the uncertainty surrounding the activity of 30 μ M AZD7545. The justifications for this dose of AZD7545 held from the previous pilot study, but the method of delivery was altered with the aim of reaching 30 μ M plasma concentration.

We hypothesised that metabolic assessment of the liver would show increased PDH flux, whereas this would not be seen in the heart; possibly also a more extreme response would be seen in the diabetic animals compared to controls.

5.5.5.1 Methods

Trial of an alternative drug delivery method

Dietary manipulation was trialled as an alternative to gavaging AZD7545, with an aim to eliminate the stress associated with gavage. Three control animals were given 300 mg/kg/day AZD7545 mixed with sugar-free raspberry jelly and blended rat chow for 7 days. The animals were then given an overdose of isoflurane and blood and tissue samples were taken. The plasma was then analysed for drug concentration (AstraZeneca, Mölndal, in house HPLC method, Table 5.1); this data showed the definite presence of AZD7545, with some values potentially low due to the short dosing period. This method of administration was therefore used for the chronic *in vivo* study.

Rat #	Saphenous (μM)	Post mortem (μM)
1	22	6.16
2	-	19.2
3	7.98	7.58

Table 5.1: AZD7545 plasma concentrations following pilot dietary administration

Chronic study

Diabetes was induced as described in chapter 3, section 3.2 with 25 mg/kg streptozotocin and a high-fat diet throughout the protocol. See figure 5.4 for an overview of the full protocol.

Briefly, DNP data were taken from both the heart and liver using the acquisition developed in chapter 4. Echocardiographic assessment of diastolic function was carried out at the end of the protocol for all groups. As for the DCA study, animals were fasted overnight before echocardiographic data were taken; they were then given an overdose of isoflurane and tissue and blood samples were taken and frozen. Further, epididymal fat pad weights were measured. Blood samples were also assessed for the concentration of AZD7545 present (by HPLC, in-house at AstraZeneca).

Student's t-tests were used to assess differences between the treated groups and their respective untreated groups.

5.5.5.2 Results

All relevant data from this thesis was combined to look at cardiac and hepatic metabolism for control and diabetic animals, as detailed in chapter 4, section 4.5. In summary, these untreated diabetic animals showed significantly reduced cardiac PDH flux compared to controls; lactate was not changed, and ^{13}C label transfer to alanine was significantly increased. Hepatic bicarbonate was significantly reduced in diabetic animals, and lactate and alanine were unchanged.

AZD7545 treatment

Plasma sampled in the fed state from the control and diabetic groups dosed with AZD7545 showed concentrations of $20.2 \pm 0.9 \mu\text{M}$ in control animals, and $18.2 \pm 0.6 \mu\text{M}$ in diabetic animals. These concentrations were in the region of those expected for a dose of 300 mg/kg, given previous literature (AZ, in-house) . Plasma analysed from animals not administered the drug all showed levels below the threshold of analysis ($0.2 \mu\text{M}$).

AZD7545 did not stimulate cardiac PDH flux in either control or diabetic animals. In control animals, treatment resulted in a significantly lower flux ($p < 0.0001$; figure 5.38); in diabetic animals, treatment with AZD7545 did not give a difference in flux. ^{13}C label transfer to lactate was significantly increased in both control and diabetics on treatment with AZD7545 ($p < 0.0001$ for comparisons between both treated groups and their respective untreated group), and alanine was significantly reduced ($p < 0.0001$).

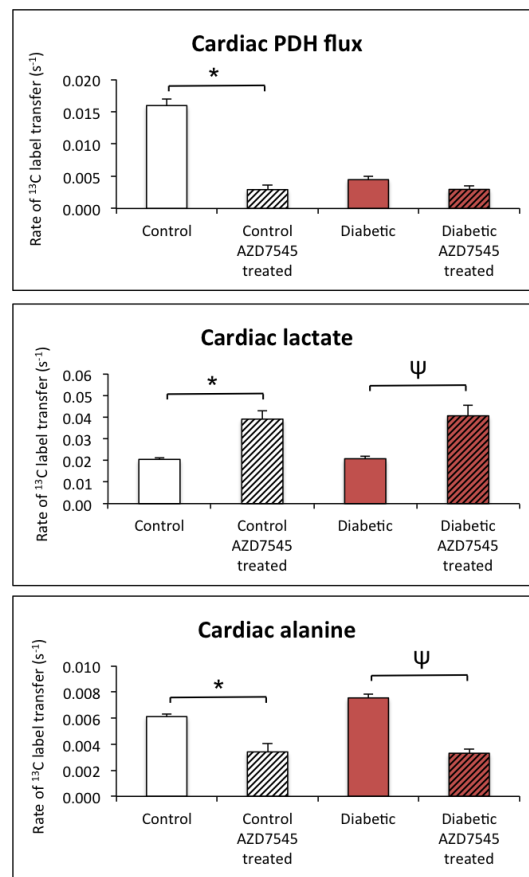


Figure 5.38: *In vivo* cardiac ^{13}C label transfer data to pyruvate, lactate and alanine on injection of ^{13}C pyruvate in control and diabetic animals, and those treated with AZD7545. $*p \leq 0.05$ compared to control untreated. $\psi p \leq 0.05$ compared to diabetic untreated

Hepatic bicarbonate was significantly reduced by AZD7545 treatment in control animals ($p=0.0004$). Diabetic animals showed no further reduction on treatment, having already lower bicarbonate compared to controls. Hepatic lactate was increased in both treated groups compared to untreated ($p < 0.0001$), as was hepatic alanine (controls $p=0.01$, diabetics $p=0.01$; figure 5.39).

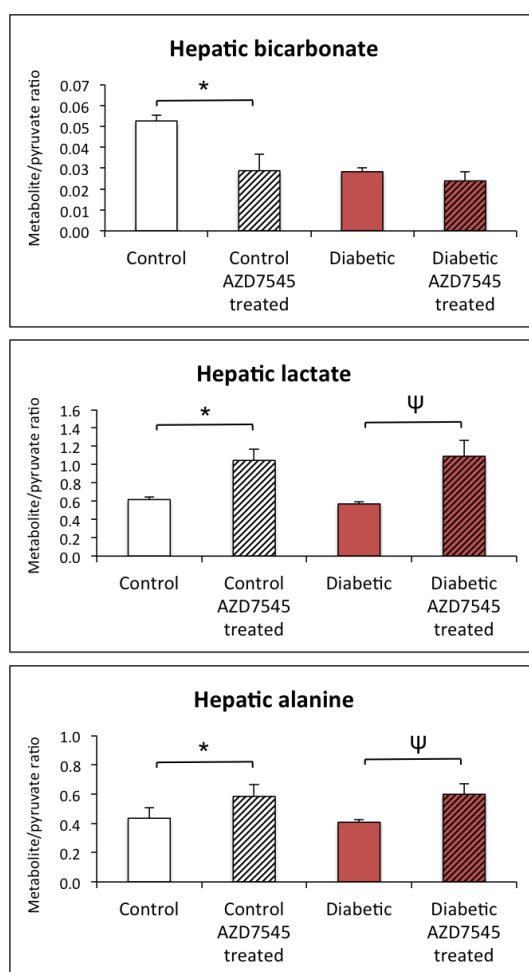


Figure 5.39: *In vivo* hepatic ^{13}C label transfer data on injection of ^{13}C pyruvate in control and diabetic animals, and those treated with AZD7545. $*p \leq 0.05$ compared to control untreated. $\psi p \leq 0.05$ compared to diabetic untreated

Fasting blood glucose levels were not altered by AZD7545 treatment in either control or diabetic animals (figure 5.40).

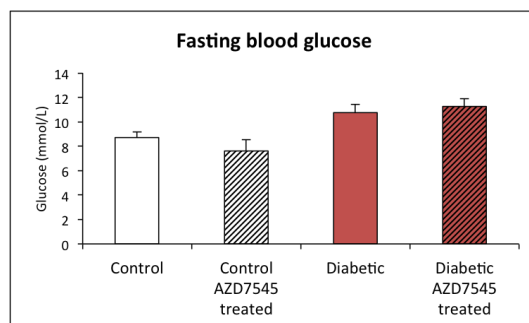


Figure 5.40: Fasting blood glucose levels, measured post-mortem

Epididymal fat was not significantly altered by treatment in the control group (figure 5.41), but diabetic animals treated with AZD7545 had significantly lower epididymal fat than diabetic untreated animals.

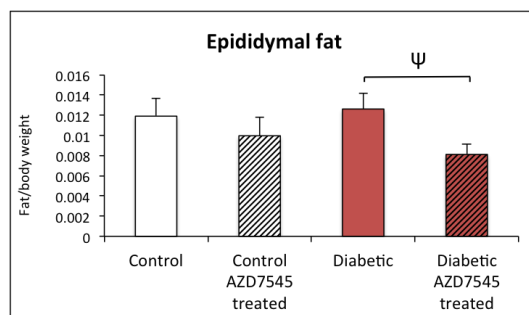


Figure 5.41: Epididymal fat pad weight normalised to body weight; untreated and AZD7545 treated control and diabetic animals. $\psi p \leq 0.05$ compared to diabetic untreated

Cardiac PDK1 was significantly decreased and 4 increased by AZD7545 in control animals (figure 5.42), with no change in PDK2. Diabetic animals showed no change in PDK1 or 2 but 4 was increased.

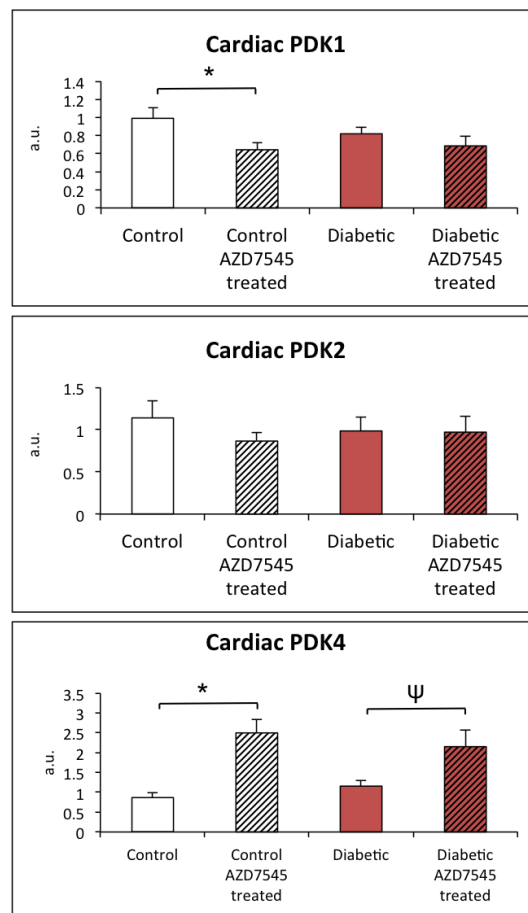


Figure 5.42: Cardiac Western blots for PDK1, 2 and 4 expression in control, diabetic and AZD7545 treated animals. * $p \leq 0.05$ compared to control untreated. $\psi p \leq 0.05$ compared to diabetic untreated

AZD7545 treatment significantly increased both hepatic PDK1 and PDK2 in control animals ($p=0.0008$, $p=0.003$ respectively), whilst decreasing PDK4 ($p=0.0003$). Diabetic animals showed an increase in PDK1 but not in PDK2 or 4 (figure 5.43).

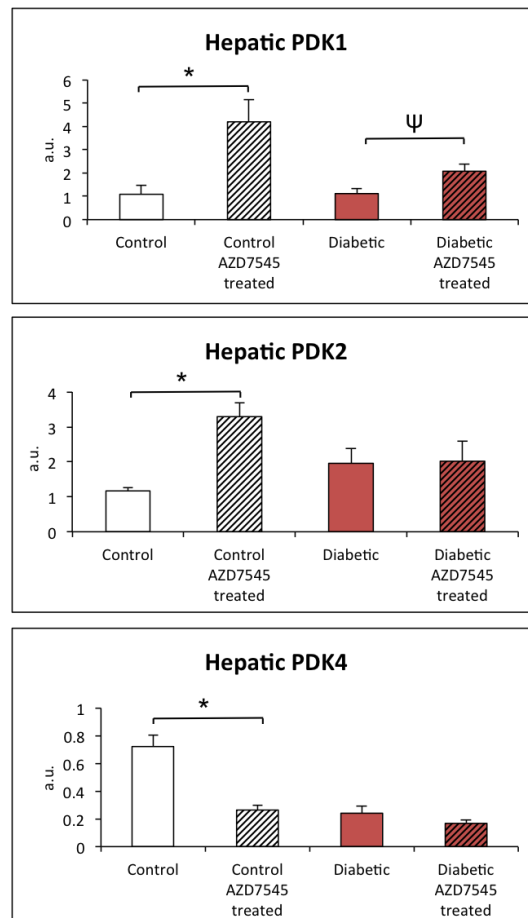


Figure 5.43: Hepatic Western blots for PDK1, 2 and 4 expression in control, diabetic and AZD7545 treated animals. $*p \leq 0.05$ compared to control untreated. $\psi p \leq 0.05$ compared to diabetic untreated

Ejection fraction and E/A ratio were unchanged by AZD7545 treatment in either control or diabetic animals. E/E' following treatment was close to significantly higher in control treated animals compared with untreated ($p=0.056$); this was not seen in the diabetic groups (figure 5.44).

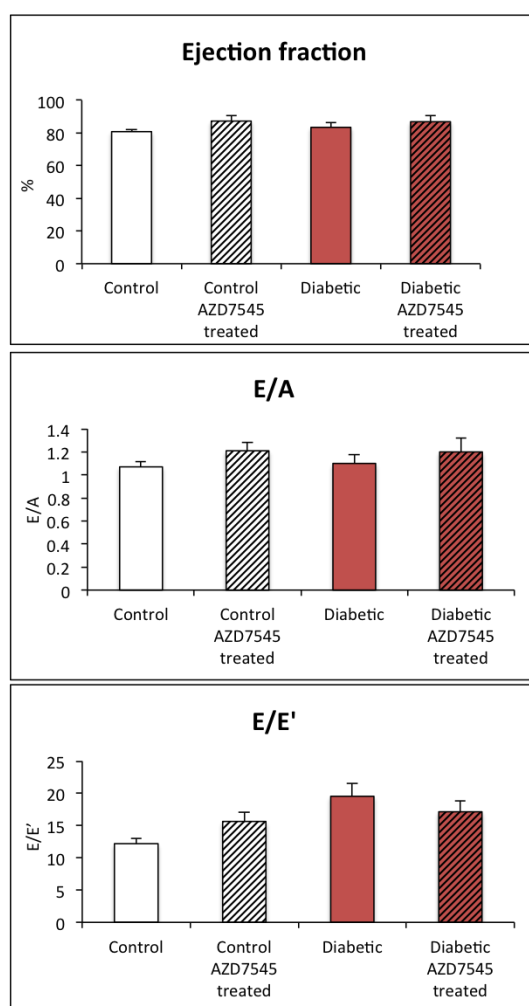


Figure 5.44: Echocardiographic data measuring systolic and diastolic function. Control, diabetic and AZD7545 treated groups.

5.5.5.3 Discussion

Given the plasma concentrations of AZD7545 measured, we can conclude that the method of administration via the diet was successful. The study overall demonstrated a rebuttal of our hypothesis, given that we saw no increase in PDH (via PDK2) in the liver, and in fact saw decreased PDH flux in both the heart and liver. However, the use of hyperpolarised techniques provided interesting data about this novel compound that was not otherwise attainable, and has contributed to a greater understanding of its action.

In contrast with the data from control animals treated with DCA, cardiac PDH flux was shown to be lower in the AZD7545 treated animals, and ^{13}C label transfer to lactate higher. The

reduction in cardiac label transfer to alanine may be a result of the high flux to lactate. Coupled with the protein expression data on cardiac PDK2 and 4 in these animals (figure 5.42) we can conclude that PDK2 was not inhibited, and PDK4 was stimulated. As previously discussed, this upregulation of PDK4 has been seen in cell studies by Mayers *et al.*, although inhibition of PDK2 was maintained. Our study demonstrated that *in vivo*, this dose of AZD7545 appears to have no effect on cardiac PDK2, although there is a reduction in cardiac PDK1 in the control treated animals. In diabetic animals, there was no stimulation of PDH flux, but no further decrease to the low levels seen in the diabetic untreated animals. However, there was an increase in lactate, suggesting that the cardiac metabolism of these animals was further worsened.

There was a similar pattern in the hepatic data, in that treatment with AZD7545 caused a significant decrease in hepatic bicarbonate in control animals and an increase in hepatic lactate, seemingly driven by the increased hepatic PDK1 and 2 (shown by Western blot). Once again, diabetic animals were not further affected in terms of hepatic bicarbonate on treatment with AZD7545, yet saw increased lactate. Potentially the increased lactate alongside increased alanine is indicative of production for gluconeogenesis, indicating that both control and diabetic AZD7545 treated groups had gluconeogenic livers where it was not required.

Given the decreased cardiac PDH flux and hepatic ^{13}C label transfer to bicarbonate seen in animals treated with AZD7545, it is perhaps unsurprising that blood glucose levels were not reduced in the diabetic group as was seen with DCA treatment. Further, there was no restoration of fuel balance in the animals treated with AZD7545, so it follows that no effect on diastolic function was seen in the AZD7545 treated groups.

As this dose of AZD7545 stimulated PDK4 and inhibited PDH, a further small study was carried out with a ten-fold lower dose of AZD7545. The rationale for this dose was based on work by Morrell *et al.* in 2003, where it was shown that 3 hours after an oral dose of AZD7545, doses of both 30 mg/kg and 10 mg/kg showed a significant increase in PDH activity (measured via *ex vivo* assay) in both fed and fasted animals. Further, for another more potent compound in the chemical series of AZD7545, the maximally effective dose was 30 mg/kg. The work by Mayers *et al.* in 2003 demonstrated that a single acute dose of AZD7545 activated PDH in the liver in a dose dependent manner, with an increase from 24.7 to 70.3% at 30 mg/kg¹². This was therefore the dose used in the following study, where we supposed that a lower dose would not stimulate

PDK4 and may allow for effective PDK2 inhibition.

5.5.6 *In vivo* investigation into treatment of a model of type II diabetes: low dose of AZD7545

5.5.6.1 Methods

Control (n=8) and diabetic (n=7) animals began drug administration at 3 weeks, as in the previous study. 30 mg/kg AZD7545 was administered via the diet; this was continued for 4 weeks. Following this, cardiac and hepatic ^{13}C spectroscopy was carried out, with blood samples taken for analysis of plasma concentration of the AZD7545. The animals were then fasted overnight and fasting blood glucose measurements taken. Echocardiography was also carried out for diastolic function analysis, and followed by tissue removal for subsequent biochemical analyses.

Statistical analyses involved one-way ANOVAs with a Holm-Sidak correction, with comparisons made between the lower dose of AZD7545 (control and diabetic) and their respective control and high dose groups.

5.5.6.2 Results

Plasma concentrations of animals dosed with 30 mg/kg were analysed as previously described (section 5.5.5.2). Control animals showed a plasma concentration of $4.6 \pm 0.3 \mu\text{M}$; diabetic animals measured $4.5 \pm 0.5 \mu\text{M}$.

Control animals treated with 30 mg/kg AZD7545

The low dose of AZD7545 in control animals caused a cardiac PDH flux that was significantly different to both untreated and the high dose of AZD7545 (figure 5.45), in that the low dose caused PDH flux to be lower than in untreated animals but higher than that seen in the high dose group. ^{13}C label transfer to lactate was still increased compared to control levels in the low dose control animals. There was no effect on alanine.

Hepatic metabolic data from control low dose AZD7545 animals showed bicarbonate levels not significantly different to control untreated animals - an improvement on the reduced bicarbonate seen in the high dose animals (figure 5.46). There were no differences in hepatic lactate or alanine between the low dose groups and untreated or high dose groups (figure 5.46).

Diabetic animals treated with 30 mg/kg AZD7545

Diabetic animals showed no differences between doses when looking at cardiac PDH flux. Diabetic animals treated with the lower dose of AZD7545 showed cardiac lactate levels not different to either untreated or the high dose group - i.e. the dose caused an intermediate effect. This low dose of AZD7545 caused diabetic animals to demonstrate ^{13}C label transfer to alanine lower than in untreated diabetic animals (figure 5.45), but significantly higher than the previously seen cardiac alanine level in the diabetic group treated with 300 mg/kg AZD7545.

Hepatic data showed that the low dose of AZD7545 in diabetic animals caused a decreased ^{13}C label transfer to bicarbonate, similar to that seen in the high dose group. No significant differences were seen between the diabetic untreated, or either treated group in lactate or alanine (figure 5.46).

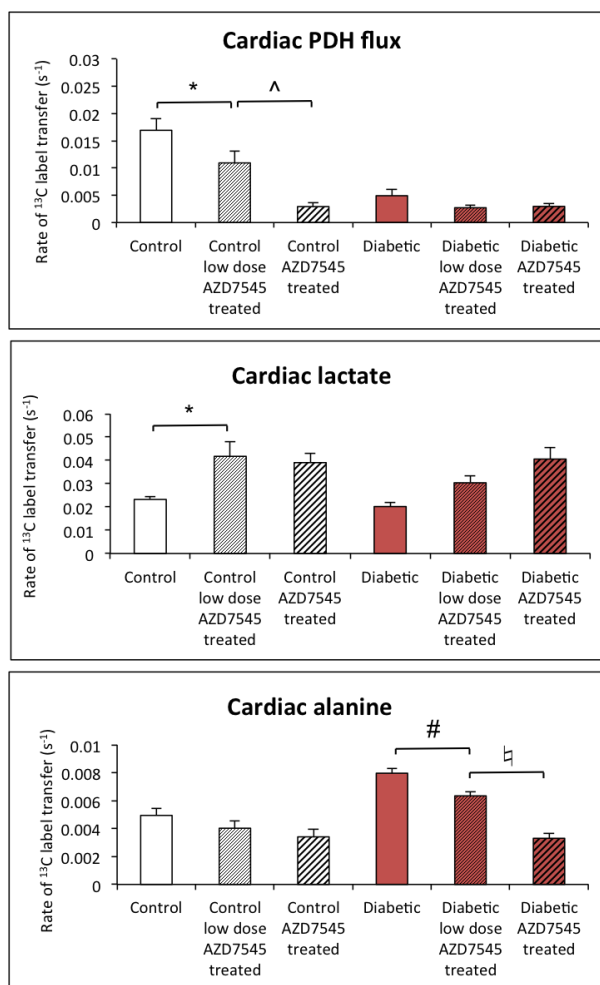


Figure 5.45: Low dose AZD7545 treatment of control and diabetic animals, as compared with untreated and high dose AZD7545-treated animals. * $p \leq 0.05$ compared to control untreated, $^{\wedge}p \leq 0.05$ compared to control, low-dose treated AZD7545 animals, # $p \leq 0.05$ compared to diabetic untreated animals, $^{\natural}p \leq 0.05$ compared to diabetic, low-dose treated AZD7545 animals

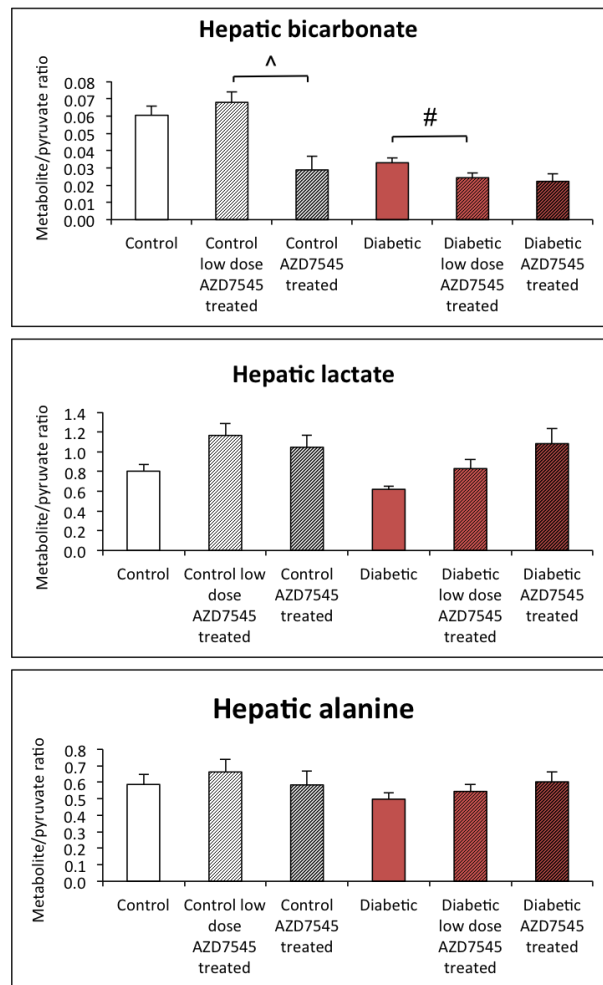


Figure 5.46: Low dose AZD7545 treatment of control and diabetic animals, as compared with untreated and high dose AZD7545-treated animals. [^] $p \leq 0.05$ compared to control, low-dose treated AZD7545 animals, [#] $p \leq 0.05$ compared to diabetic untreated animals

E/A ratios were unaffected by the low dose of AZD7545. The treated groups gave E/E' ratios that were not significantly different to their respective untreated groups (figure 5.47).

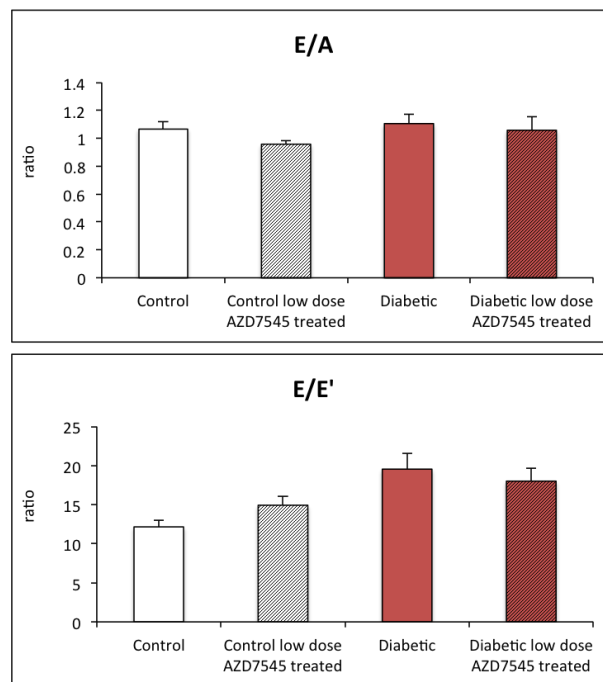


Figure 5.47: Effect of a lower dose of AZD7545 on control and diabetic animals. $*p \leq 0.05$

The low dose (30 mg/kg) of AZD7545 resulted in a significantly increased hepatic PDK2 expression in control animals, and a similar trend in diabetic animals ($p=0.054$) (figure 5.48).

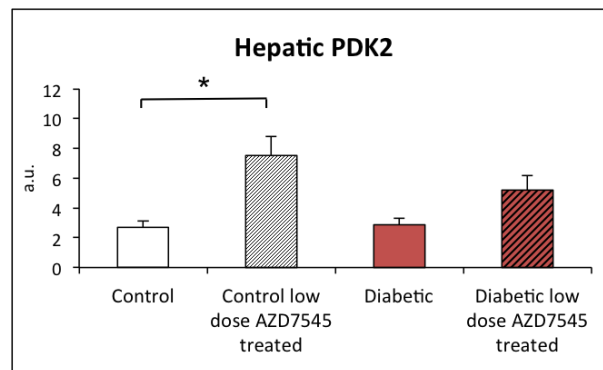


Figure 5.48: Effect of a lower dose of AZD7545 on hepatic PDK2 expression in control and diabetic animals. $*p \leq 0.05$

5.5.6.3 Discussion

The low dose of 30 mg/kg AZD7545 had a different effect on the cardiac and hepatic metabolism of both control and diabetic animals when compared to the previous, higher dose of 300

mg/kg.

In control animals treated with the higher dose of 300 mg/kg AZD7545, we saw a significant reduction in cardiac PDH flux due to upregulation of PDK4. With the lower dose, this reduction was less, although still significantly lower than the control untreated animals. Cardiac lactate remained elevated in the control low dose treated group, and alanine remained unaffected. This would suggest that although the lower dose has a lesser effect on carbohydrate metabolism, it remains a negative change, given the aim of stimulating PDH flux.

Hepatic bicarbonate in control animals treated with the lower dose of AZD7545 did not cause the reduction in bicarbonate seen in the higher dose experiment; values seen were instead not different to those seen in untreated animals. In combination with the protein expression data for PDK2, we can conclude that we have not seen the inhibition of hepatic PDK2 (figure 5.48) and concurrent stimulation of PDH flux that was our target with this lower dose of AZD7545.

The diabetic animals demonstrated a similar response to the control animals, when treated with 30 mg/kg of AZD7545, in that the effect of the compound was not as extreme, but there were no beneficial effects such as stimulation of PDH flux. This suggests that PDK4 was still upregulated with this dose of AZD7545, and PDK2 was not inhibited. It then follows that the E/E' measure of diastolic dysfunction showed the AZD7545 treated animals being no different from their untreated group, indicating in fact that dysfunction remained in the diabetic animals treated with the low dose of AZD7545.

5.6 Overall chapter conclusions

5.6.1 Dichloroacetate

We demonstrated in this chapter that dichloroacetate allowed us to modulate carbohydrate metabolism systemically in both the control and diabetic rat. Previous work by Wall and Lopaschuk showed increased glucose oxidation improving function in perfused diabetic rodent hearts via inhibition of carnitine palmitoyltransferase-1 and therefore fatty acid oxidation¹⁵⁹. We have shown we are able to link cardiac metabolism and function for the first time *in vivo*, by using a combination of hyperpolarised MRS and echocardiography, and provided evidence

for modulating PDH as a therapeutic target for diabetic cardiomyopathy. This has wide clinical implications, given that the incidence of diabetes is continually increasing, with over 600 million people worldwide expected to have type II diabetes by 2030¹⁶⁰. Further, subclinical diastolic dysfunction has now been shown to be linked to an increased risk of heart failure¹⁶¹. Therefore a therapeutic target that improves both general glycaemic control and reverses cardiac dysfunction is likely to be of great clinical importance. DCA has previously been the subject of clinical trials as a diabetic treatment¹³⁷. However, despite the benefits in glycaemic control seen, it has not been widely used as a diabetic treatment due to its side effect profile which includes peripheral neuropathy¹⁴⁵. Although other agents are now available that increase PDH activity, including phenylbutyrate¹⁶², they have so far generally been limited to the treatment of rare conditions of inborn errors in metabolism, including congenital PDH deficiency¹⁶³. Despite this, the findings in this study support further work into the development of pyruvate dehydrogenase kinase and pyruvate dehydrogenase modulators as targets for the treatment of type II diabetes. Identifying reversible changes in cardiac substrate selection could help improve our treatment of cardiac dysfunction in diabetes, with imminent translation to the clinic, given the advent of clinical hyperpolarisation studies⁹².

5.6.2 AZD7545

When looking at the assessment of AZD7545, it did not provide the stimulation in PDH flux observed in the previous literature, therefore unfortunately we cannot make comparisons between single isoform/multi-isoform PDK inhibition, or discuss our ability to visualise single isoform effects on PDH. In fact stimulation of cardiac PDK4 by AZD7545 overwhelmed any potential inhibition of PDK2. This was apparent in both the heart and the liver. It is possible that the lower dose of 30 mg/kg was still not sufficiently low to allow PDK2 inhibition alone to occur. Given the statement in Mayers *et al.*¹² that stimulation of PDK4 occurred above concentrations of 10 nM in cell work, it may well be valuable to do further pilot studies to assess the exact oral dosing concentration that is required to achieve a cellular concentration below this threshold. If we refer back to the work by Morrell *et al.*¹⁵² which showed increased PDH activity after one dose of 30 mg/kg we could propose that for future studies, either a shorter study is carried out to confirm PDK2 inhibition, or the dose lowered further to prevent overexpression of PDK4 in

the long term.

The work detailed in this chapter has however demonstrated that it may be valuable to use hyperpolarised MRS techniques during the drug discovery process to directly study the effect of novel therapies, rather than relying on *ex vivo* cell and tissue experiments.

Chapter 6

Physiological alteration of pyruvate dehydrogenase regulation

6.1 Introduction

Understanding the metabolic changes that occur following exposure to low oxygen is relevant in many diseases, such as cancer, myocardial infarction, and type II diabetes. However, these are multi-factorial diseases, so to fully understand the occurrences in disease we must first establish the effect of hypoxia in isolation.

As discussed in chapter 2, hypoxia-inducible factor instigates transcriptional responses when cells are exposed to hypoxia, with involvement of specific genes having been established previously. Studies have been carried out in a variety of different models and at varying concentrations of oxygen for different lengths of hypoxia, such as work by Adroque *et al.*¹⁶⁴ who exposed Wistar rats to 11% oxygen for 4, 10 and 12 weeks, Ebert *et al.*¹⁶⁵, whose work involved exposure of cells to 1% oxygen for 14-16 hours, and Fuller *et al.*¹⁶⁶ who demonstrated the method of modelling ischaemia-reperfusion using isolated perfused rat hearts and 15 minutes of hypoxia at a partial oxygen pressure of <150 mmHg.

Carbohydrate metabolism is affected during hypoxia, in that the decrease in oxygen provision means that oxidative metabolism in the mitochondrion is reduced. Glycolysis is increased, with an aim to maintain cellular ATP production, with GLUT1 (a cell membrane glucose transporter)

and PDK1 having been shown to be downstream targets of HIF^{167,168}. It may be hypothesised that increasing PDK1 will cause a decrease in PDH activity via inhibitory phosphorylation. However, *in vivo* studies in mice have demonstrated a decrease in PDK4 (Dr Amira Abd-Jamil, personal communication), which one might conclude would have the opposite effect on PDH and result in its increased activity. The decrease in PDK4 may be due to the reduced oxygen levels decreasing fatty acid oxidation, or suppression of PPAR- α , which has been demonstrated to be involved in PDK4 regulation¹⁶⁹. There is also evidence of hypoxia causing altered mitochondrial structure, function and dynamics due to oxidative stress, potentially involving nitric oxide¹⁷⁰; this could cause a multitude of changes including an effect on PDH. It is therefore key to understand the effect of hypoxia on PDH *in vivo*, and this may lead to novel therapeutic approaches for diseases in which hypoxia plays a part, including type II diabetes.

As previously discussed, type II diabetes causes changes in PDH activity, and there is a preference for fatty acid metabolism. A down-regulation of both glycolysis and glucose oxidation contributes to a diabetic phenotype that involves elevated plasma glucose levels and increased susceptibility to cardiovascular disease, fatty liver disease and other complications. There are also several other metabolic alterations such as decreased GLUT1, increased expression of PPAR- α target genes, and increased reactive oxygen species which are associated with energetic inefficiency⁴⁵.

The metabolic changes caused by hypoxia are therefore the reverse of those seen in diabetes (i.e. increased glycolysis, decreased fatty acid oxidation). An inability to adapt to hypoxia due to the metabolic inflexibility of the diabetic heart may result in a reduced ability to cope with hypoxia in disease⁴⁵. Hypoxia is an inherent part of diabetes^{171–174}, and may play a role in the increased risk of cardiovascular disease and the worsened prognosis following myocardial infarction¹⁷⁵. Taken together, this means that understanding the hypoxic response in type II diabetes is key to understanding the diabetic metabolic phenotype as a whole.

In this chapter we have firstly studied the effects of acute hypoxia, and subsequently undertaken chronic hypoxic studies *in vivo*. One of the most common methods for replicating long-term hypoxia in the laboratory is with the use of a normobaric, or hypobaric hypoxic chamber for housing the animals. These are commonly investigated with an oxygen concentration of 11% - examples include the previous studies by Calmettes *et al.*^{176,177} - one of which showed a

decreased PCr/ATP ratio in isolated perfused rodent hearts following 3 weeks of housing at 11% oxygen. This level of oxygen simulates 5500m of altitude; however previous studies place the animals immediately in this low concentration^{164,178–181}, with the potential for a severe hypoxic insult to be caused by the sudden change in oxygen concentration. Our study therefore included an adaptation week, with oxygen concentration in the chamber being reduced steadily over one week, before two weeks of 11% oxygen. Details of this protocol are described in section 6.4.1 in this chapter.

Several investigators have explored the links between diabetes and hypoxia. Marfella *et al.* demonstrated that myocardial infarct size was statistically greater in a hyperglycaemic diabetic rat model than in normoglycaemic or non-diabetic animals¹³. Size of infarct was assessed by isolating and perfusing the hearts. They also showed an impairment in HIF-1 α mRNA expression associated with infarct size in the hyperglycaemic diabetic rats¹³. This link between HIF-1 α and diabetes was then explored by further work in patients¹⁷⁵; ventricular biopsies from diabetic and control subjects showed decreased HIF-1 α RNA and VEGF in diabetic patients, along with a correlation between glycated haemoglobin and HIF-1 α . Work with diabetic mice and human fibroblasts by Thangarajah *et al.*¹⁸² also demonstrated a reduced upregulation of VEGF in response to hypoxia. Catrina *et al.* used dermal fibroblasts and endothelial cells to show the effect of glucose level on HIF stabilisation - namely that increasing levels of glucose resulted in inhibited stabilisation¹⁸³. In support of this, Thangarajah *et al.* demonstrated that the glycolytic metabolite methylglyoxal has the capacity to covalently modify proteins and disrupt their interaction with HIF-1 α ¹⁸². It therefore seems that the hypoxic response is reduced in diabetes and may offer some explanation for the poor cardiovascular prognosis for diabetic patients.

Given that the metabolic changes seen in hypoxia are opposite to those seen in diabetes, stimulating the hypoxic response in the presence of normal oxygen levels may improve the diabetic phenotype. Xue *et al.*¹⁸⁴ activated HIF using metallothionein, and demonstrated that it increased hexokinase expression in control cell conditions and attenuated the diabetes-induced suppression of hexokinase. This work built on their previous demonstration that overexpression of HIF-1 α (in a cardiac-specific manner) prevented both the metabolic change of decreased glycolysis and the associated cardiac remodelling in a diabetic mouse model¹⁸⁵. Mice over-

expressing HIF-1 α were given an STZ injection to induce diabetes, and after assessing their metabolism the authors concluded that the constitutive cardiac upregulation of HIF relieved the diabetic suppression of GLUT1 and hexokinase II (the predominant form of hexokinase in the heart), which led to restored ATP production and VEGF expression. If VEGF can be manipulated in diabetics in this manner it may help with peripheral perfusion and the associated diabetic retinopathy and nephropathy. Small-molecule compounds allow us to investigate the effects of altering a specific set of pathways, and the final study described in this chapter used dimethyloxalylglycine (DMOG). DMOG is the cell permeable ester of the active compound N-oxalyl glycine (NOG), which is a di-acid, 2-oxoglutarate analogue competitive inhibitor of the prolyl hydroxylases. It has been shown to prevent the degradation of HIF and initiate the HIF hypoxic response¹⁸⁶. DMOG also inhibits FIH, and so this combination allows stabilisation of HIF, and therefore mimicry of the hypoxic response, whilst in the presence of normal oxygen levels.

Previous *ex vivo* and *in vivo* work has been carried out using DMOG in several animal models, to investigate a range of biological states. VEGF-dependent gastrointestinal repair processes were stimulated by Marchbank *et al.*¹⁸⁷ in Sprague-Dawley rats, and similar use to improve vascularisation was demonstrated in the study by Kelly *et al.*¹⁸⁸. The latter authors delivered DMOG on a stent next to an occluded porcine coronary artery *in vivo*, and showed local angiogenic effects, although this failed to translate to physiological parameters. A mouse skeletal muscle study from Milkiewicz *et al.*¹⁸⁹ investigated ischaemic insults, and concluded that DMOG may have potential as a therapeutic following an ischaemic injury, due to the significant increase in HIF and the resultant capillary growth following the ischaemic insult combined with DMOG treatment. Two recent neurological studies^{190,191} looked at cerebral ischaemia; Ogle *et al.* reported a reduced loss of local blood flow near the infarct and reduced behavioural deficits, and Nagel *et al.* demonstrated a reduced infarct volume on treatment with DMOG, whilst also showing that the lower of two dose options had more beneficial effects. One further cardiac study in 2005¹⁹² demonstrated a reduction in cardiac infarct size in rabbits treated with DMOG for 24 hours prior to ischaemia-reperfusion.

From the current available evidence, the effect of hypoxia on PDH activity and regulation *in vivo* is unclear and published data is conflicting. Therefore the work in this chapter investigated the

effect of a range of lengths of hypoxic exposure, in both the control and diabetic rat, looking at both cardiac and hepatic metabolism. Further, we investigated the ability of a pharmacological agent, DMOG, to mimic the effects of hypoxia in normal oxygen levels.

These studies were all carried out with the help of Miss Victoria Noden, Dr Oliver Rider and Dr Andrew Lewis. The PDK Western blots on chronically hypoxic cardiac tissue were performed by Victoria.

6.1.1 Chapter aims

The initial aim of this chapter was to characterise the *in vivo* effect of hypoxia on PDH activity and regulation using hyperpolarised ^{13}C pyruvate. Secondly, we looked to investigate the effects of hypoxia on PDH activity and regulation in diabetes, interesting given both the opposite metabolic changes observed in the two states, and also the suggested compromised HIF signalling pathway in diabetes.

To understand any changes and adaptations of carbohydrate metabolism to hypoxia, a protocol for hypoxic anaesthesia for magnetic resonance was first developed, allowing the animals to be anaesthetised at the same level of oxygen experienced during long-term hypoxic housing. Subsequently, animals were studied following an acute hypoxic insult (30 minutes) and a return to normoxia, 1 week of hypoxic housing (adaptation period only) and 3 weeks of hypoxic housing (adaptation period followed by a further 2 weeks housed at 11% oxygen). Finally, further investigation of the specific HIF response was carried out using the hypoxia mimetic, DMOG. All studies were carried out on control and diabetic animals.

6.2 Development of hypoxic anaesthesia

6.2.1 Understanding hypoxia and anaesthesia

Anaesthetic agents have a range of effects on the body, including on the cardiovascular, respiratory and thermoregulatory system, with respiratory depression under anaesthesia particularly concerning for patients with sleep apnea, or who are obese or older¹⁹³. Most relevant to our pre-clinical studies is the observation that isoflurane causes dose-dependent reductions in heart rate

and respiratory rate^{194,195}, although it has less of an effect on cardiac function than injectable anaesthetics¹⁹⁶. Our laboratory set-up ensured that the body temperature of the animals was maintained under anaesthesia. The cardiovascular and respiratory changes were not addressed per se, but were monitored throughout the experiments.

Exposure to hypoxia causes an initial response involving increased breathing rate and tachycardia¹⁹⁷. This is via activation of the chemoreceptors of the carotid body¹⁹⁸ - a group of cells near the fork in the carotid artery in the neck. The carotid body is very sensitive to carbon dioxide and oxygen levels in the blood, and stimulates increased ventilatory rate via the brain¹⁹⁹. This carotid body response is considered the fast (peripheral) response, and is then followed by a further (central) response to stabilise breathing if hypoxia continues^{200,201}. If exposure to hypoxia continues for more than approximately 5 minutes, breathing rate slows. This is termed an hypoxic ventilatory decline^{202,203}.

We wished to look at the effects of an acute hypoxic insult in both control and diabetic animals, following stabilisation of these initial physiological changes, and subsequently to investigate the metabolic changes that occur following longer periods of hypoxia. For this, we aimed to develop a protocol to maintain anaesthetised animals at a level of hypoxia equivalent to that experienced during chronic housing at 11% oxygen, whilst keeping in mind that delivery of 11% oxygen may present a more severe hypoxic challenge to an anaesthetised animal than an awake animal, given the ventilatory decline mentioned above. In addition, the use of hypoxic anaesthesia aimed to remove any potential effects of a brief return to normoxia after chronic hypoxic housing. It has been established that re-oxygenation following hypoxia in cells causes injury, including mitochondrial dysfunction, mediated by reactive oxygen and nitrogen species^{204,205}. Studies on relocation of people living at high altitude to sea-level have discussed the length of time required to acclimatise and the physiological differences such as lower mitochondrial number and higher haemoglobin levels^{206,207}, but make little mention of metabolic changes. Hence, we believed our experiments would provide a more accurate representation of the hypoxic environment if metabolic data were acquired at the same level of hypoxia, removing the possibility of unknown metabolic changes caused by a return to normoxia for assessment.

In conclusion, our protocol was developed to allow us to:

1. Assess the short-term, acute effects of hypoxia, following stabilisation of the physiological hypoxic response.
2. Assess the metabolic effects which occurred due to chronic hypoxia, without removing the animals from their hypoxic environment to give us data not confounded by a return to normoxia.

6.2.2 Protocol development

Six male Wistar rats were housed inside a normobaric hypoxic chamber (see figure 6.1), and six in room air outside the chamber. Following one week of adaptation to low oxygen, where the oxygen concentration of the chamber was lowered by one or two percent per day by replacement of air with nitrogen, the hypoxic animals were housed at 11% for two weeks (figure 6.2).



Figure 6.1: Normobaric hypoxia chamber, with nitrogen cylinders

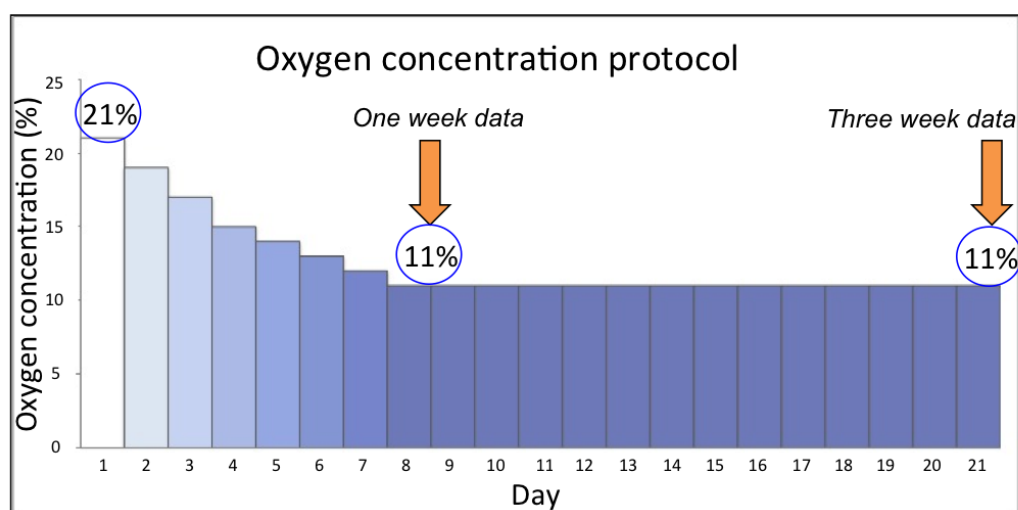


Figure 6.2: Hypoxic protocol showing decrease in oxygen concentration in the hypoxia chamber and experimental time points

As mentioned above, hypoxic anaesthesia was developed with two types of experiment in mind. Firstly we wished to assess metabolism in anaesthetised animals exposed to acute hypoxia, by lowering of the percentage of oxygen delivered after anaesthetic induction at 100% oxygen. Secondly we wished to be able to maintain the hypoxic environment for animals which had been chronically housed in hypoxia, so that when metabolic measurements were being taken, these would not be affected by short-term normoxic exposure. It was logical therefore that an hypoxic anaesthetic protocol should result in the same blood oxygen saturation as was achieved by those living in hypoxia for longer time periods.

Therefore, during the housing of these animals at 11% for two weeks, oxygen saturation measurements were taken from the feet of both the hypoxic and normoxic animals (whilst awake), using a pulse oximeter (MouseOx, Starr Life Sciences). Hypoxic animals gave blood oxygen saturation values of approximately 75% compared to normoxic measurements of approximately 94% (data shown in figure 6.3).

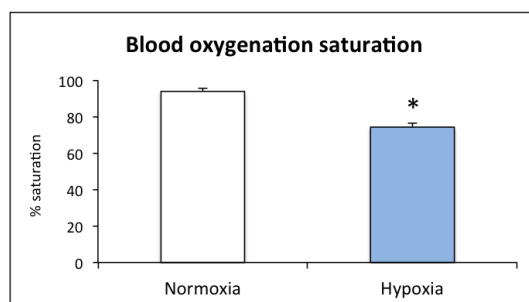


Figure 6.3: Blood oxygen saturation of awake control animals housed in 11% oxygen. $*p \leq 0.05$

A group of control animals ($n=6$) were subsequently anaesthetised at 100% oxygen as described in section 3.4, and then oxygen delivery altered whilst pulse oximeter readings were continuously monitored. When oxygen delivery was altered by replacement of the oxygen with nitrogen, blood oxygen saturation was seen to rapidly respond. It was established that an oxygen percentage delivery of 16% resulted in a blood oxygen saturation of approximately 75%. This was then used on all further hypoxically-anaesthetised animals.

The values of oxygen supplied were higher in the anaesthetised animals compared to awake (16% oxygen compared to 11% in the chamber) due to the depressed breathing rate under anaesthesia. We also concluded that animals should be placed prone in the cradle, as placing them supine caused breathing difficulty. We further observed changes in heart rate and breathing rate over the first fifteen minutes of hypoxia, as expected given the literature summarised above, with both tending to stabilise by 30 minutes.

This hypoxic anaesthesia then enabled us to study both the acute and chronic effects of hypoxia on metabolism *in vivo*. The two-slice scan developed in chapter 4 was used in all hypoxic studies, enabling us to assess both heart and liver metabolism *in vivo*.

6.3 Acute hypoxia

We hypothesised that exposure of control animals to acute hypoxia would cause pyruvate metabolism to lactate to increase, as the anaerobic respiration known to occur in hypoxia promotes glycolysis over glucose oxidation. We could potentially extrapolate and say that PDH flux is expected to decrease, however this relationship is not always seen. For example in this thesis,

the control and diabetic cardiac data in figure 4.25 show a decreased PDH flux in the diabetic animals compared to controls, but no difference in lactate is present. We believed it was possible that following re-oxygenation for 30 minutes, levels of metabolites would return to pre-hypoxic levels, given the observed rapid dissipation of the effects of hypoxia in humans²⁰⁸.

Although there is evidence to suggest diabetics have a reduced hypoxic response, this involves the reaction of HIF and upregulation of related genes. Over an acute time period, this is unlikely to have an effect, and therefore we expected the diabetic group to also increase pyruvate to lactate conversion. However we had previously seen that the diabetic PDH flux was severely reduced, and so considered it unlikely that any further reduction would occur on exposure to acute hypoxia.

6.3.1 Methods

Male Wistar rats (control n=7, diabetic n=8) were placed in the 7T horizontal bore scanner as described in section 3.4 with a nose cone for administration and scavenging of anaesthetic (2% isoflurane in oxygen or an appropriate oxygen/nitrogen mix). All animals were anaesthetised at 100% oxygen. Oxygen delivery was then reduced to 16% as discussed above (section 6.2.2). Pulse oximetry was used to confirm blood oxygen saturations. Control and diabetic animals were assessed with the same protocol, with diabetes being induced as described in chapter 3.

Pyruvate metabolism was assessed at three timepoints:

1. After 30 minutes of normoxia
2. After 30 minutes of hypoxia
3. After 30 minutes of re-oxygenation (return to 100% oxygen anaesthesia following 30 minutes of hypoxia)

To minimise the length of anaesthesia, animals underwent either procedures 1 and 2, or 2 and 3, in any one scanning session. Animals were given at least 48 hours to recover before further anaesthesia. The two hypoxic data sets were averaged for each animal. A one way ANOVA was used to assess differences between the three states of oxygenation, with all groups compared to each other and a Holm-Sidak post-hoc correction applied. $P \leq 0.05$ was considered statistically

significant. On presentation of diabetic data, control values are displayed alongside as a reminder of the control response, but are not included in statistical analyses.

6.3.2 Results

Control animals

Control animals: *in vivo* cardiac data

In control animals, thirty minutes of hypoxic anaesthesia resulted in a significant decrease in cardiac PDH flux, along with a significant increase in ^{13}C label transfer to lactate, as hypothesised. No change in label transfer to alanine was seen (figure 6.4).

Following hypoxia, thirty minutes of normoxia resulted in values of PDH flux and label transfer to lactate that were not significantly different to those initially seen in normoxia. Lactate was significantly lower following 30 minutes of re-oxygenation than the measurement following hypoxia.

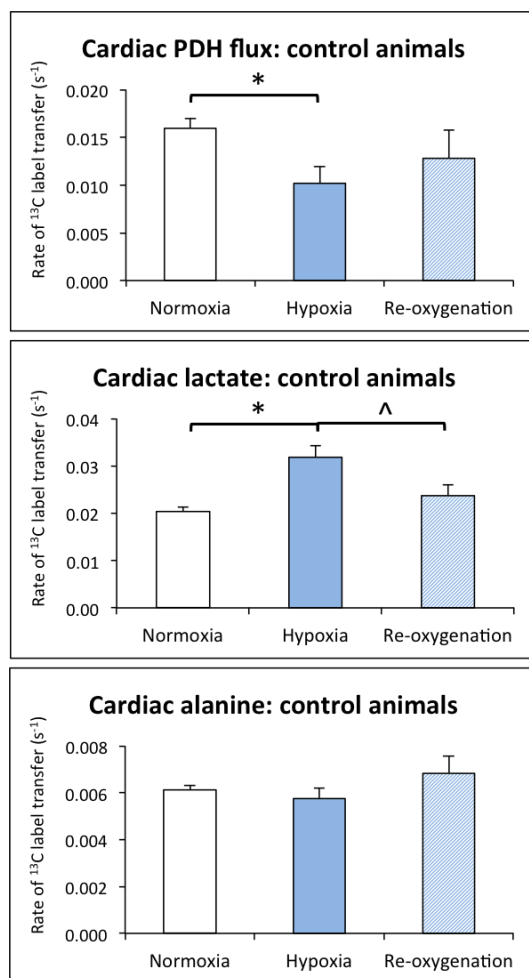


Figure 6.4: *In vivo* cardiac PDH flux on injection of ^{13}C pyruvate in control animals, before, during and after acute hypoxia. $*p \leq 0.05$ compared to normoxia, $^{\wedge}p \leq 0.05$ compared to hypoxia

Control animals: *in vivo* hepatic data

Hepatic bicarbonate and alanine were unchanged by hypoxia (figure 6.5). However, hepatic lactate was significantly increased after thirty minutes of hypoxia. Lactate was then seen to return to normoxic levels after re-oxygenation. Hepatic bicarbonate was significantly lower than normoxic levels following re-oxygenation, and hepatic alanine was not affected.

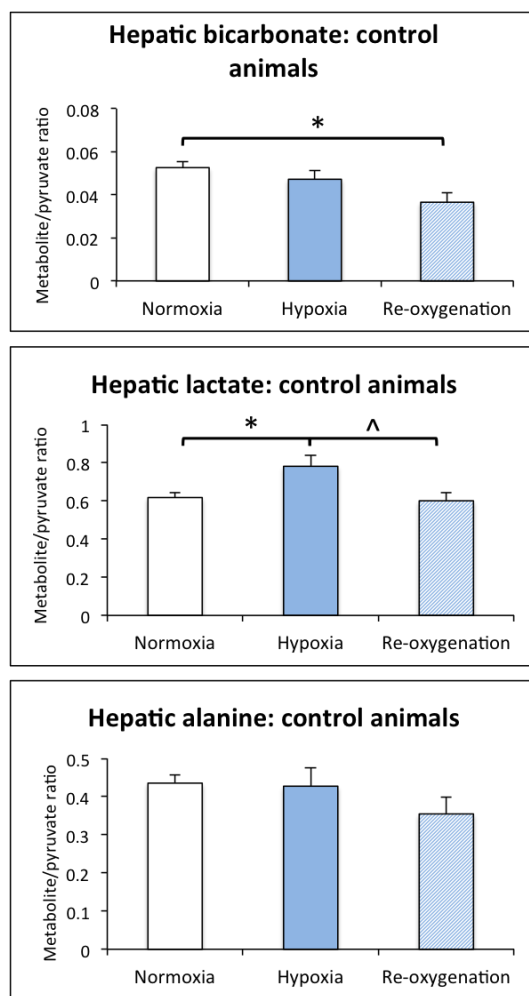


Figure 6.5: *In vivo* hepatic pyruvate/metabolite ratio on injection of ^{13}C pyruvate in control animals before, during and after acute hypoxia. * $p \leq 0.05$ compared to normoxia, ^ $p \leq 0.05$ compared to hypoxia

Diabetic animals

To recap, the results presented at the end of chapter 4 showed that the metabolic differences between control and diabetic animals included significantly decreased cardiac PDH flux and hepatic bicarbonate in the diabetic animals, and an increased ^{13}C label transfer to alanine in the heart.

Diabetic animals: *in vivo* cardiac data

In contrast to the control animals, but as conjectured, diabetic animals demonstrated no significant changes in cardiac PDH flux following thirty minutes of hypoxia. ^{13}C label transfer to lactate showed a significant increase following 30 minutes of hypoxia (figure 6.6). No change was seen in alanine measurements. Re-oxygenation caused no changes in PDH flux or label transfer to alanine; the rate of label transfer to cardiac lactate was not significantly different to either normoxia or hypoxia at this point.

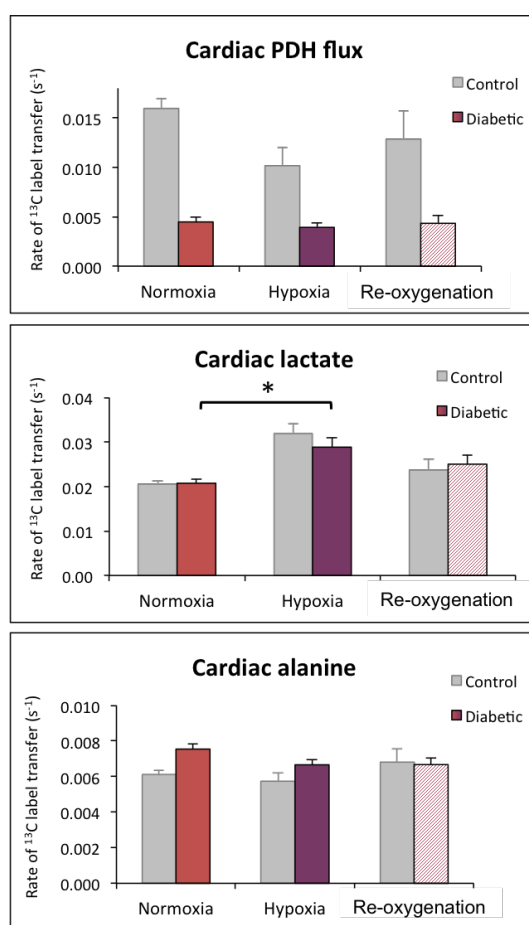


Figure 6.6: *In vivo* cardiac ^{13}C label transfer on injection of ^{13}C pyruvate in diabetic animals before, during and after acute hypoxia. * $p \leq 0.05$

Diabetic animals: *in vivo* hepatic data

The diabetic animals demonstrated unchanged hepatic bicarbonate after acute hypoxia, however lactate was significantly increased, and alanine showed a trend to increase ($p=0.058$). Following

a return to normoxia the hepatic lactate was returned to a normoxic level (figure 6.7), with a similar trend in alanine ($p=0.056$).

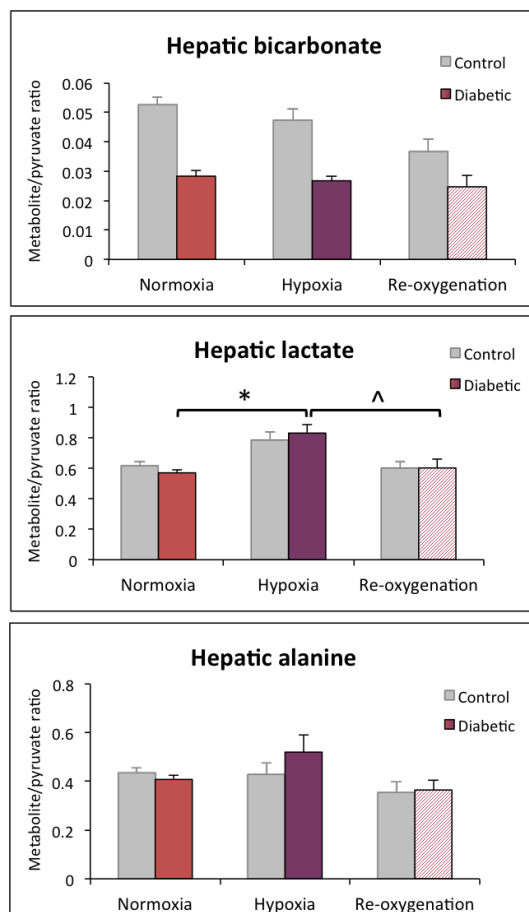


Figure 6.7: *In vivo* hepatic pyruvate/metabolite ratio on injection of ^{13}C pyruvate in diabetic animals before, during and after acute hypoxia. $*p \leq 0.05$ compared to normoxia, $^{\wedge}p \leq 0.05$ compared to hypoxia

Assessment of heart rate

Heart rate was variable over the 30 minutes of hypoxia, although the initial and end rates were not significantly different in either the control animals or the diabetics (figure 6.8). Data was not collected at all timepoints for all animals due to poor ECG signal. However on looking at the individual data, the control animals appeared to present a more variable profile over time; the diabetics being more steady in their response to hypoxia.

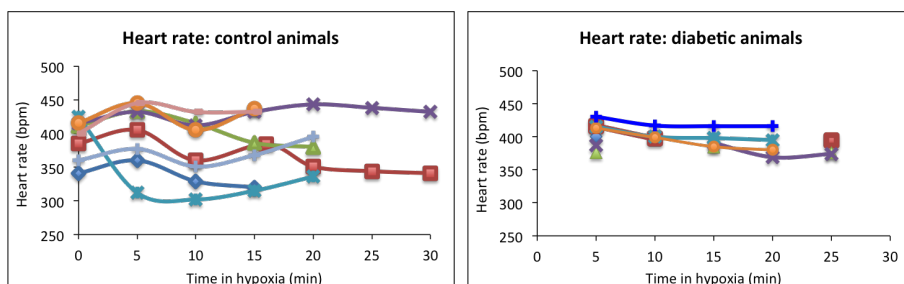


Figure 6.8: Average heart rate during hypoxia for individual control and diabetic animals

6.3.3 Discussion

Response of cardiac and hepatic carbohydrate metabolism to acute hypoxia in control animals

Control animals demonstrated a decreased cardiac PDH flux following 30 minutes of hypoxia, and a concomitant increase in ^{13}C label transfer to lactate. It could therefore be suggested that this is indicative of increased glycolysis, and decreased glucose oxidation, focussing metabolism on anaerobic ATP production rather than oxidative phosphorylation (the Pasteur effect), given the reduction in oxygen available. Although glucose is known as a more oxygen efficient fuel source i.e. requires less oxygen per ATP molecule produced than fatty acids, glycolysis alone is not sufficient to maintain ATP production if long periods of hypoxia are experienced, as most of the ATP from glucose is made during oxidation³³. Both PDH flux and lactate returned to levels not significantly different to normoxia, after 30 minutes of re-oxygenation.

Given the short time frame, and ability to return to a pre-hypoxic state with 30 minutes of re-oxygenation, we would suggest that this response was due to changes in the redox state of the cells, i.e. the NAD^+/NADH ratio, to which lactate dehydrogenase (LDH) is very sensitive, rather than to transcriptional changes due to HIF. LDH regenerates the NAD^+ required for glycolysis to continue. We may need to take into account the induction of ROS by hypoxia, demonstrated by previous cell work to occur in a time frame of minutes following exposure²⁰⁹, however due to the limited *in vivo* work carried out over such a short time frame, it cannot be confirmed to translate from cells to *in vivo*. There has been work with hyperpolarised pyruvate which demonstrated an assessment of pH using $^{13}\text{CO}_2$ and ^{13}C bicarbonate ratios²¹⁰,

however unfortunately in our studies the signal:noise ratios were not sufficient to be confident in measuring $^{13}\text{CO}_2$ peaks and therefore the changes in pH.

Previously, the effect of hypoxia on hepatic metabolism has been interesting from the perspective of maintenance of livers used for transplantation. Ischaemia-reperfusion studies have been carried out on the perfused liver, as injury due to ischaemia-reperfusion results in increased morbidity and resection for patients^{211,212}, in spite of the liver's regenerative capacity. The liver depends heavily on oxygen for its metabolism, being a very aerobic organ, although differences exist between hepatic cell types as discussed in chapter 2. However in the event of hypoxic exposure, coping mechanisms are present, such as the use of glycogen (alongside already present glucose) as a substrate for anaerobic glycolysis. Hepatocytes from fed rats have been shown to be more resistant to anoxic injury than those from fasted rats²¹³.

Hypoxia-inducible factor is likely to have dimerised and moved to the nucleus in this period of hypoxia, given its rapid response to hypoxia. Kallio *et al.* in 1999²¹⁴ demonstrated an increase after 4h treatment of HeLa cells with CoCl_2 , a hypoxia mimetic, and further in 2001, Jewell *et al.*⁶⁵ demonstrated the presence of HIF-1 α in the nucleus of cells following 2 minutes of hypoxia, where there had been none in normoxia. However, following thirty minutes, the downstream upregulation of factors due to HIF are unlikely to have had any effect. Reoxygenation causes HIF to be broken down, with a half-life of less than 5 minutes.

Hepatic ^{13}C label transfer to bicarbonate was significantly decreased on re-oxygenation following hypoxia. The dominant metabolism of hepatocytes (on glycolysis or oxidative phosphorylation) is dependent on their location relative to the portal and hepatic veins. During hypoxia, the glycolytic hepatocytes will be able to continue functioning as normal, but those more focussed on oxidative phosphorylation will not. Potentially, the decrease in bicarbonate seen on re-oxygenation was due to a slow response to the replenished oxygen supply by the latter hepatocytes. However, it may be expected that thirty minutes of replenished oxygen supply would be sufficient for a return to normal function, in which case the decrease may instead be due to damage, similar to that seen in ischaemia-reperfusion experiments. HIF-1 α accumulates during hypoxia, upregulating transferrin if hypoxia is maintained, which has been suggested to contribute to iron-dependent reactive species formation²¹⁵. These in turn can contribute to liver injury, although once again, it would be surprising if the lengths of hypoxia/re-oxygenation

were sufficiently long to see these effects. Previously, two hours of ischaemia has been shown to cause hypoxic lesions²¹⁶. Further, the level of hypoxia should not be so low as to cause hepatic damage. Phosphorus NMR could be used to assess ATP levels in these livers following hypoxia, as intracellular ATP has been previously used to indicate the presence of hepatic liver damage following transplant^{217,218}. However overall the mechanism behind this result remains unclear.

The hepatic data from the control animals demonstrated an increase in lactate similar to the cardiac data. As above, this may be indicative of increased glycolysis, although this is not seen alongside a decrease in hepatic bicarbonate, and the liver is not often regarded as glycolytic. Potentially instead it shows increased lactate uptake, given production of lactate elsewhere in the body will be increased, and the Cori cycle will result in the liver responding in an attempt to maintain homeostasis. This is then reversed on re-oxygenation. The trend to an increase in ¹³C label transfer to alanine (a gluconeogenic precursor) during hypoxia suggests we could hypothesise that this short period of hypoxia induces the liver to increase production of glucose to supply the rest of the body for the increased glycolysis that is occurring, although this would require an increase in ATP which may not be available. The increased glucose supply for the rest of the body may instead come from the hepatic glycogen stores.

Response of cardiac and hepatic carbohydrate metabolism to acute hypoxia in diabetic animals

Cardiac PDH flux in the diabetic animals did not change on exposure to hypoxia. The level of normoxic PDH flux in the diabetic heart was shown in chapter 4 to be substantially depleted compared to controls, and the lack of reaction to hypoxia seen in these experiments is potentially indicative of cardiac metabolic inflexibility⁴⁵ due to this prior depletion. This variation from the control response, and an inability to respond as successfully to hypoxia, may contribute to an explanation for the increased morbidity and mortality following myocardial infarction in diabetics.

Lactate however was significantly increased in the diabetic heart following 30 minutes of hypoxia. In terms of the interpretation of hyperpolarised data, it is not possible to determine the exact

contribution from NAD^+/NADH ratio change, LDH activity, and lactate pool size in the heart. However overall this points to increased glycolysis as in the control animals. Alanine once again was not seen to change.

Hepatic diabetic data showed, similarly to control data, a significant increase in lactate on exposure to hypoxia, which is no longer present after 30 minutes of re-oxygenation. Mansor *et al.*¹¹⁰ showed no reduction in hepatic glycogen in this diabetic model, so it would not be expected that diabetic livers be more susceptible to hypoxic injury than controls.

Future developments

For future studies pertaining to acute hypoxia/re-oxygenation measurements, it would be optimal to continuously measure the animals' blood oxygen saturation, using a magnet-safe pulse oximeter. Given the initial changes in respiratory and cardiac rate, it is difficult to establish certainty of a stable saturation. On measuring their saturation following the experimental procedure, most animals did maintain a constant saturation, however continuous monitoring would be preferable to confirm that this was the case throughout the procedure. Alternatively the protocol could be repeated without metabolic data taken, outside the magnet, to confirm steady oxygen saturation.

With regards to anaesthesia and its effect on the hypoxic response, we have observed some effects, which allows us to conclude that the response is not entirely ablated. Work by Pandit *et al.*²¹⁹ has suggested that isoflurane has the largest effect of the commonly used gaseous anaesthetics on suppressing hypoxic response so if these studies were repeated with an alternative volatile anaesthetic, the differences seen here may be more obvious.

6.4 One week of hypoxia

The acute hypoxic data showed both the control and diabetic animals could respond to hypoxia to some extent, and therefore we concluded that the redox state was altered in both groups. In terms of a longer period of hypoxia however, it was difficult to predict whether the redox state would still be affected or whether downstream targets of HIF (which would now have likely been

upregulated) would change the outcome that would be observed. This was further complicated by the inconclusiveness of evidence surrounding the effects of hypoxia on the expression of the different PDK isoforms. In any case we believed the control animals would demonstrate a metabolic response to the hypoxic exposure, in order to maintain sufficient ATP production in the reduced oxygen environment.

6.4.1 Methods

Control and diabetic (n=10 per group) animals were housed either in normoxia or in a normobaric hypoxic chamber (figure 6.1) for one week, with diabetes induced as described in chapter 3, section 3.2. Oxygen levels in the chamber were reduced by one or two percent per day from 21% to 11%, with animals living at 11% oxygen for the final day prior to scanning (figure 6.2). Animals were then removed from the chamber, anaesthetised at the relevant oxygen concentration (see section 6.2.2) and blood glucose measured before being placed in the magnet (chapter 3, section 3.4). Cardiac and hepatic metabolic data were obtained during one injection of hyperpolarised ^{13}C pyruvate. Animals were then given an overdose of isoflurane. Blood samples were analysed for haematocrit levels and tissue and blood was taken and snap frozen for later analyses, as described in chapter 3. Statistically, the responses of control and diabetic animals to hypoxia were treated separately, therefore t-tests were used to assess differences. On presentation of diabetic data, control values are displayed alongside as a reminder of the control response, but are not included in statistical analyses.

6.4.2 Results

Control animals: *in vivo* cardiac data

Following one week of hypoxia, control animals demonstrated some response to hypoxia in that they showed increased cardiac ^{13}C label transfer to lactate (figure 6.9). Cardiac PDH flux and label transfer to alanine were, however, unchanged.

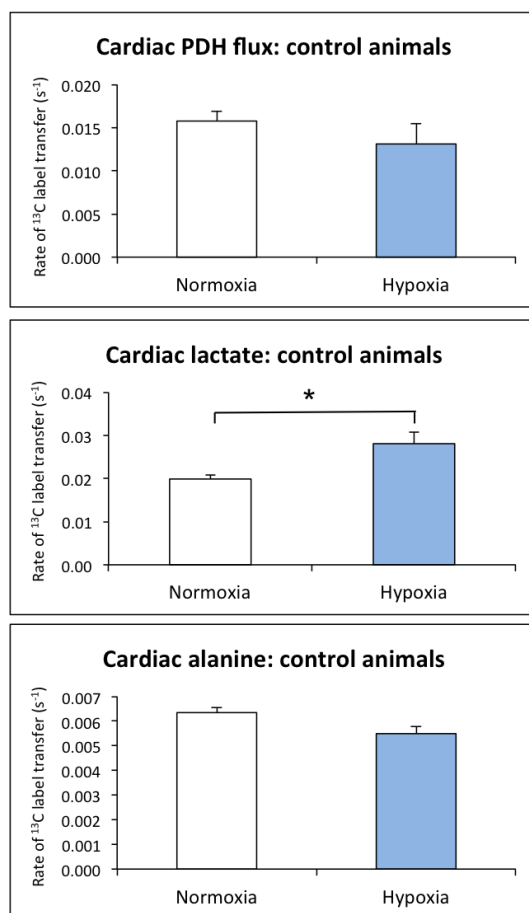


Figure 6.9: Metabolic effects of one week of hypoxia on the cardiac glucose metabolism pathway in control animals. $*p \leq 0.05$

Control animals: *in vivo* hepatic data

After one week of hypoxic housing, control hepatic metabolism demonstrated increased ^{13}C label transfer to lactate. Bicarbonate and alanine were unchanged (figure 6.10).

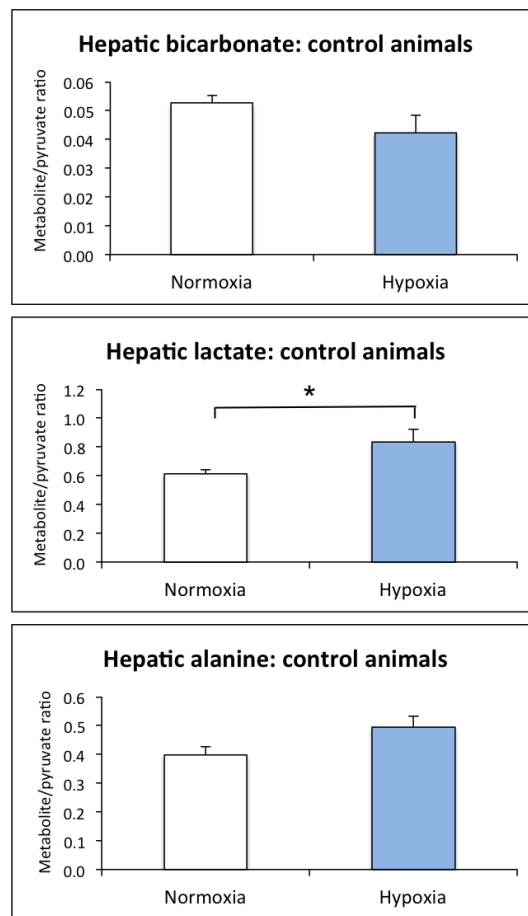


Figure 6.10: Metabolic effects of one week of hypoxia on hepatic glucose metabolism in control animals. * $p \leq 0.05$

Control animals: cardiac Western blot data

Western blots demonstrated no changes in the major pyruvate dehydrogenase kinase isoforms found in the heart (figure 6.11), although there was a trend to decreased PDK2 in hypoxic animals ($p=0.07$).

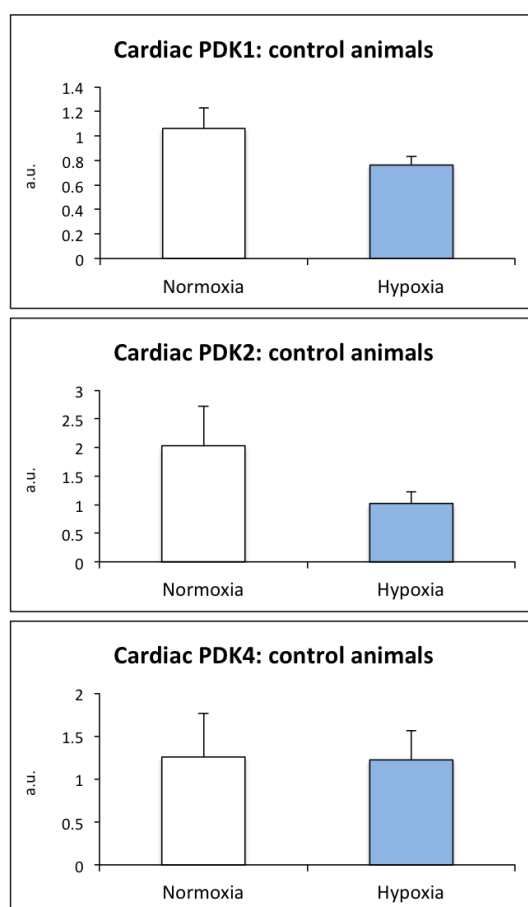


Figure 6.11: Effect of one week of hypoxia on the pyruvate dehydrogenase kinase isoforms in the control heart. $*p \leq 0.05$

Control animals: hepatic Western blot data

Similar to cardiac tissue, these control animals showed no PDK expression changes in liver tissue (figure 6.12).

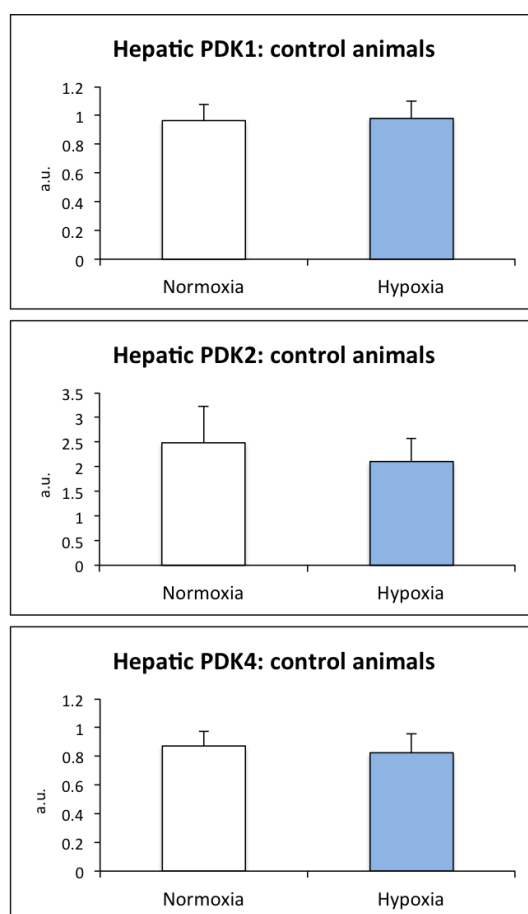


Figure 6.12: Effect of one week of hypoxia on the pyruvate dehydrogenase kinase isoforms in the control liver. $*p \leq 0.05$

Control animals: further biochemical assessments

Control animals demonstrated a substantial decrease in insulin following one week of hypoxia compared to normoxic animals, but no change in plasma glucose. Body weights were significantly decreased by 9% (figure 6.13). Haematocrit was shown to be significantly increased (figure 6.14).

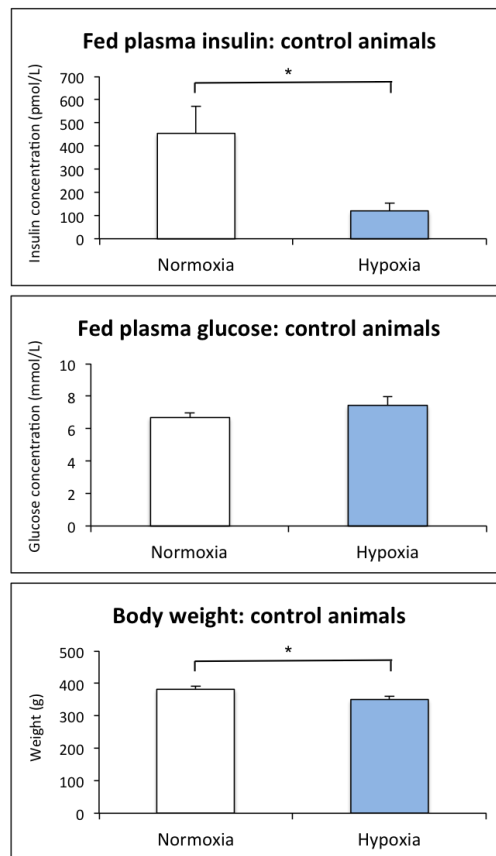


Figure 6.13: Effect of one week of hypoxia on plasma glucose and insulin levels, and on body weight. $*p \leq 0.05$

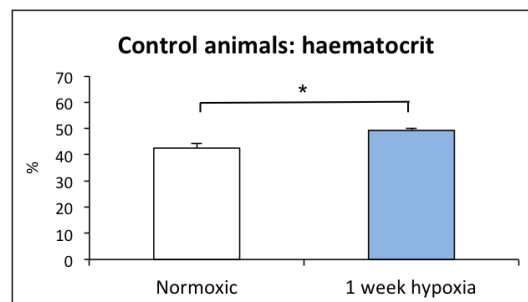


Figure 6.14: Effect of one week of hypoxia on haematocrit in control animals. $*p \leq 0.05$

Diabetic animals: *in vivo* cardiac data

Diabetic animals demonstrated a significant decrease in cardiac PDH flux and ^{13}C label transfer to alanine, in contrast to control animals which were not affected in this manner. However in terms of ^{13}C label transfer to lactate, diabetic animals showed an increase, similar to control

animals (figure 6.15).

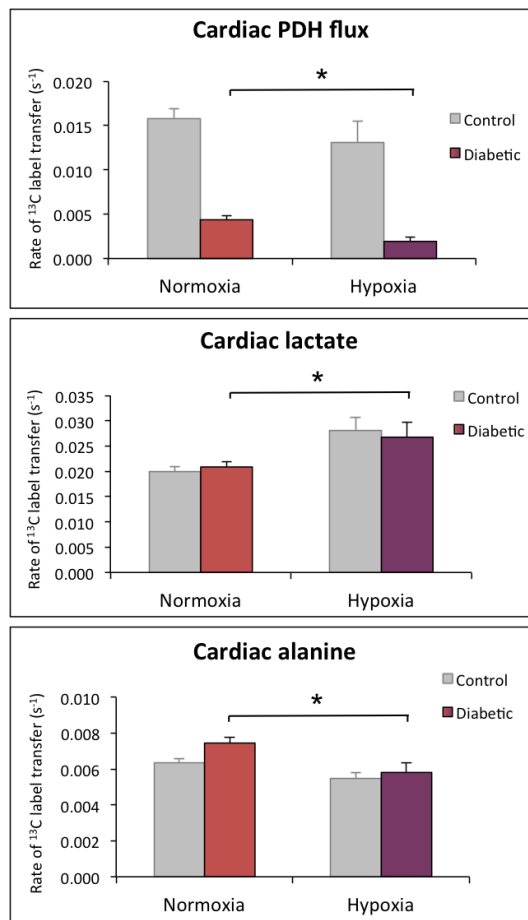


Figure 6.15: Metabolic effects of one week of hypoxia on the cardiac glucose metabolism pathway in diabetic animals. * $p \leq 0.05$

Diabetic animals: *in vivo* hepatic data

Hepatic data from diabetic animals showed a significant decrease in bicarbonate not seen in control animals; lactate was shown to be increased following one week of hypoxia (figure 6.16). Alanine was unchanged.

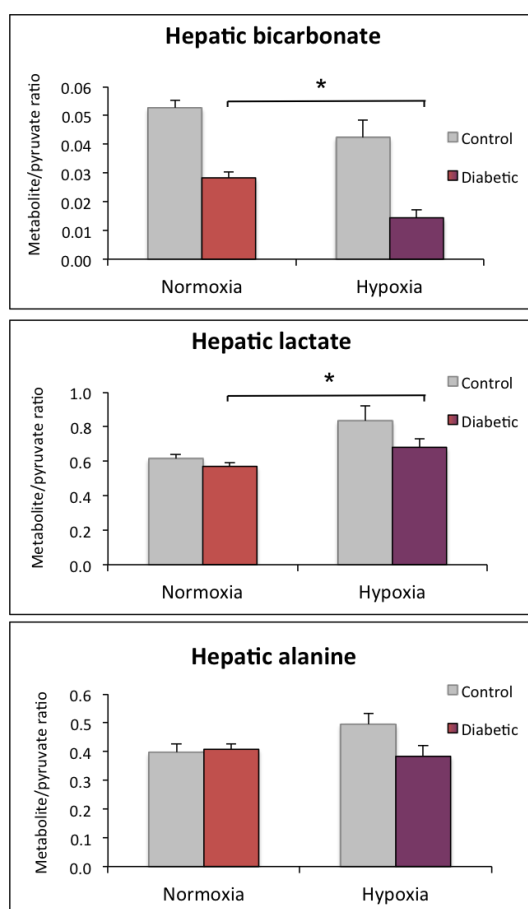


Figure 6.16: Metabolic effects of one week of hypoxia on the hepatic glucose metabolism pathway in diabetic animals. $*p \leq 0.05$

Diabetic animals: cardiac Western blot data

Diabetic animals showed decreased cardiac PDK1 expression in the hypoxic group compared with the normoxic group. There was no significant change in either cardiac PDK2 or PDK4 (figure 6.17).

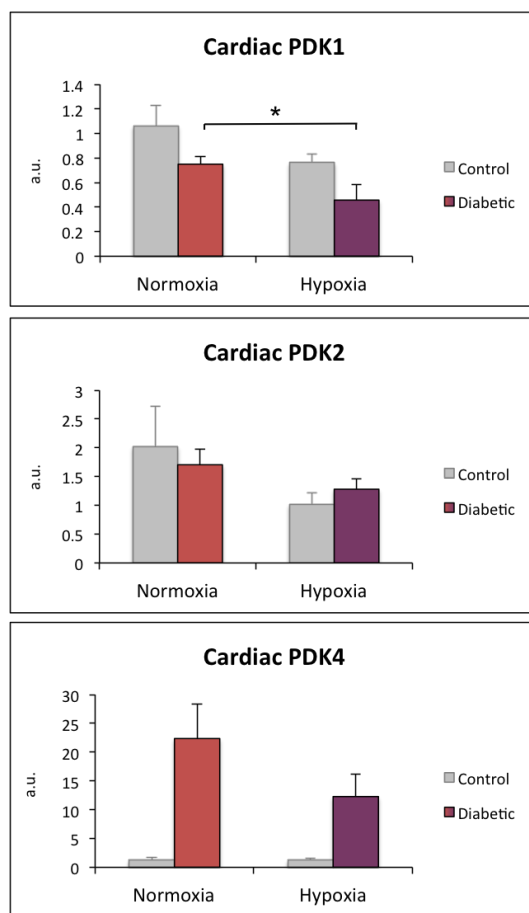


Figure 6.17: Effect of one week of hypoxia on the pyruvate dehydrogenase kinase isoforms in the heart $*p \leq 0.05$

Diabetic animals: hepatic Western blot data

Diabetic liver tissue varied from control tissue in that increased PDK1 and PDK4 protein levels were demonstrated in the animals housed in hypoxia for one week, compared to those housed in normoxia. Any differences in PDK2 failed to reach statistical significance (figure 6.18).

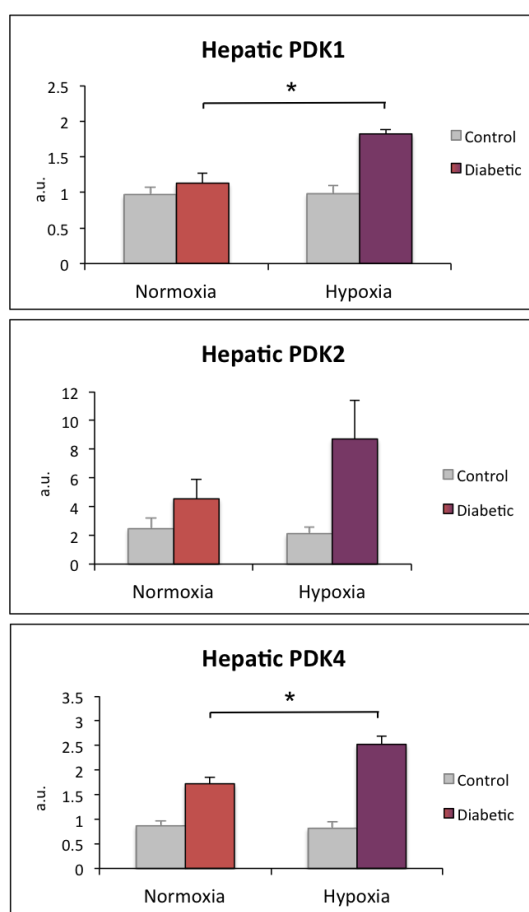


Figure 6.18: Effect of one week of hypoxia on the pyruvate dehydrogenase kinase isoforms in the heart $*p \leq 0.05$

Diabetic animals: further biochemical assessments

Plasma insulin and glucose were not seen to be altered by one week of hypoxic exposure in the diabetic groups, but body weight was shown to be lower (by 13%) in the hypoxic group (figure 6.19). Haematocrit was significantly increased following one week of hypoxia (figure 6.20).

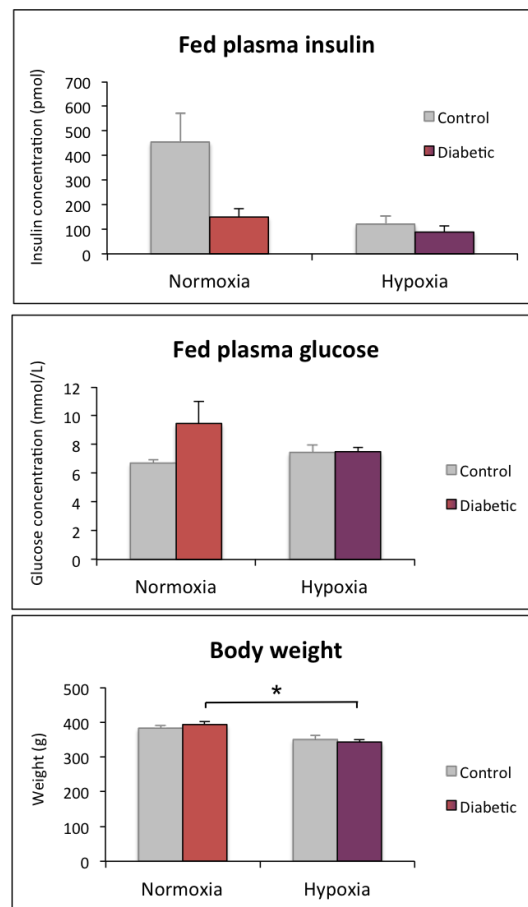


Figure 6.19: Effect of one week of hypoxia on plasma glucose and insulin levels, and on body weight. * $p \leq 0.05$

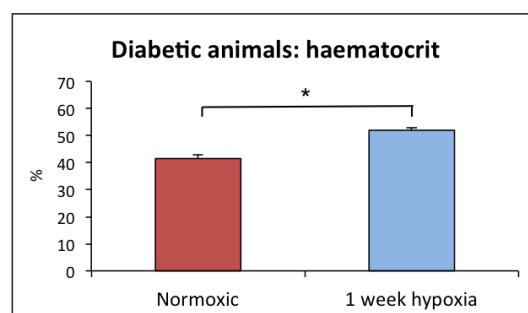


Figure 6.20: Effect of one week of hypoxia on haematocrit in diabetic animals. * $p \leq 0.05$

6.4.3 Discussion

Control response of cardiac and hepatic carbohydrate metabolism to one week of hypoxia

After one week of hypoxic exposure, it could be concluded from the *in vivo* data that cardiac and hepatic metabolism in the control animal retained a focus on glycolysis, away from oxidative phosphorylation. There are many metabolic changes that occur due to hypoxia, and one of the major regulators of this is hypoxia-inducible factor, as explained in chapter 2. However, it is experimentally difficult to measure HIF levels with *ex vivo* tissue analyses, given the rapid degradation time once tissues are exposed to normoxia. It is not found to be such a problem when using cells for hypoxic studies. For *in vivo* work however, given the inevitable variations in response to isoflurane overdose and slight variations in time taken to snap freeze tissue, HIF levels subsequently measured by Western blot are likely to be unreliable. Measuring mRNA levels of HIF-1 α was also discounted, as work by Wenger *et al.* suggested increased DNA binding activity of HIF-1 α in hypoxia was not due to transcriptional changes and therefore mRNA levels would not be a useful measurement²²⁰. We therefore looked at the response of downstream targets of HIF as markers of its up- or down-regulation.

The week of gradual adaptation to hypoxia should limit any severe hypoxic insult whilst providing sufficient time for activation of genes downstream of HIF to take effect, such as increased expression of lactate dehydrogenase A⁶², which would support our observed increase in ¹³C label transfer to lactate. Erythropoietin (EPO) is also a HIF target, and induces red blood cell production by the kidney and liver to increase the oxygen-carrying capacity of the blood. Further, HIF has control over not only the EPO gene²²¹ and its receptor²²², but also several genes involved in uptake, transport and utilisation of iron - necessary for synthesising haemoglobin. After one week of hypoxia, we observed that haematocrit was shown to be significantly increased from control levels, however potentially it was not increased to a great enough extent to restore the normoxic glycolysis/oxidative phosphorylation balance.

Another major HIF target, PDK1^{62,223}, was shown to be not changed in the heart after one week of hypoxia. Further, there was no change in cardiac PDH flux. In support of this, a study by Le Moine *et al.*⁶⁶ also demonstrated no change in skeletal muscle PDK1 expression after a

week of (12%) hypoxic housing. Work by Kim *et al.*⁶² only looked as far as 72 hours of hypoxia, so cannot confirm the expression of PDK1 beyond this. PDK1 was not altered in the hepatic tissue either, although the dominant hepatic isoforms are PDK2 and 4. Expression of PDK2 and 4 were not affected in the liver or heart when comparing hypoxic and normoxic animals. Previous data from our laboratory (Dr Amira Abd-Jamil, personal communication) showed a decrease in PDK4 in healthy mouse cardiac tissue following 3 weeks of hypoxia; potentially the response is time-dependent as for PDK1, or species specific.

As cardiac lactate was increased in control animals, similar to the acute data, it may be hypothesised that a redox effect still remained at this timepoint, in that the increased NAD^+/NADH ratio caused the pyruvate/lactate equilibrium to shift to a greater production of lactate. Also, HIF-stimulated increased LDH expression may have also played a role. A similarly increased ^{13}C label transfer to lactate is seen in the hepatic data, suggestive of a similar sensitivity to the cellular redox state.

Our data shows a significant reduction in insulin in the hypoxic control animals. Hypoxia has previously been demonstrated to reduce insulin production, by having a direct effect on the pancreatic β -cells. Further, an increase in GLUT1, a HIF target gene, would reduce the need for insulin in sensitising the cells to increase glucose uptake. Insulin has also been shown previously to stimulate pyruvate dehydrogenase phosphatase (PDP) in adipose and muscle³³, which causes dephosphorylation and resultant activation of PDH. If, as seen here, animals' insulin levels were reduced, we might hypothesise that this effect on PDP is also relevant in other tissues, such as the heart and liver, although no other literature is available to support this. Both isoforms of PDP (1 and 2) are present in rat heart²²⁴.

Body weight was significantly lower in the hypoxic animals compared to normoxic, i.e. growth was slower. This may be due to reduced physical activity of these animals as a response to the lack of oxygen²²⁵, resulting in a reduction in food intake. Alternatively, leptin (a protein that affects appetite) in adipose tissue has been shown to be a HIF target protein²²⁶. However in studies looking at the response of leptin to altitude, the direction of change remains controversial²²⁷, and is even suggested to be dependent on the initial weight of the subject.

Diabetic response of cardiac and hepatic carbohydrate metabolism to one week of hypoxia

In the diabetic animals, cardiac PDH flux and ^{13}C label transfer to alanine were significantly decreased in animals exposed to decreasing concentrations of oxygen over a week. ^{13}C label transfer to cardiac lactate was significantly increased. This is potentially indicative of a switch to glycolysis, unlike the metabolic inflexibility demonstrated following acute hypoxia. If the HIF-response is blunted as suggested by previous literature¹⁷⁵, the diabetic animals would not have a mechanism for coping with non-acute hypoxia. However, their increase in haematocrit levels would suggest a preserved HIF response.

Insulin levels in the normoxic diabetic animals were already significantly lower than control, and so potentially a lack of any further significant decrease due to hypoxia is not surprising. Induction of diabetes involves an injection of streptozotocin, which after being taken up via GLUT2²²⁸ causes a low level of necrosis of the pancreatic β -cells by DNA alkylation, specifically by O-glycosylation²²⁹. There would therefore be a reduced volume of healthy β -cells to be susceptible to the hypoxic environment. Further, the links between hypoxia and insulin are an active area of research, with Taniguchi *et al.* suggesting that specific inhibition of Phd3 could treat type II diabetes by improving insulin sensitivity²³⁰. In addition, work by Treins *et al.* has shown that insulin plays a part in regulating HIF-1 action²³¹, and so the lack of insulin in the diabetic animals may contribute to their inability to respond to HIF. Under normoxia, HIF-1 α was shown to be stabilised in the presence of insulin²³², although the mechanism of the eventual stabilisation of HIF is not yet known²³³. Insulin also induces genes coding for GLUT1²³⁴, similar to hypoxia, and also glycolytic enzymes, EPO²³⁵, and VEGF²³⁶ although again, the signaling pathways of these inductions are unknown.

Diabetic cardiac tissue showed a significant decrease in levels of PDK1 protein, but no significant change in PDK4. Despite this transcriptional change there was a decrease in cardiac PDH flux following exposure to hypoxia. We may conclude that because PDK4 is the major controller of PDH activity in diabetes, the alterations in PDK1 expression were not sufficient to counteract the substantially elevated cardiac PDK4 levels seen due to diabetes. Potentially, given the low levels of cardiac PDP2 seen in diabetes²²⁴, even if PDK levels were lowered, there would not be

sufficient PDP to activate PDH. The decrease in PDK1 that we observed in response to hypoxia was the opposite of that previously seen by Kim *et al.* in 2006⁶², although their observations were in isolated mouse embryo fibroblasts. Previous work by Le Moine *et al.*⁶⁶ that studied the temporal changes in glucose metabolism in muscle showed that following one week of hypoxia, HIF, PDK1 and PDH activity returned to pre-normoxic levels (after seeing changes at 24h), and although this was in healthy exercising mice, demonstrated the variability in HIF expression and its downstream effects over a short period of time.

It is perhaps surprising that cardiac PDK4 levels were unchanged in control animals in response to hypoxia, and once again in our diabetic model. PDK4 is regulated by PPAR- α , which is in turn HIF-responsive²³⁷. Work by Razeghi *et al.*⁶³ in rat heart demonstrated that hypoxia decreases PPAR- α , so should decrease PDK4 expression.

Diabetic hepatic data, on the other hand, demonstrated decreased bicarbonate and increased lactate, supported by an increase in both PDK1 and 4 expression. Thus, in this tissue, PDK4 may not be solely under the control of PPAR- α , given in hypoxia PPAR- α is decreased⁶³ which would suggest lower levels PDK4 could have been expected.

6.5 Chronic hypoxia: three weeks

Based on the available literature concerning the effect of hypoxia on the different PDK isoforms discussed above, we theorised that the as experiment by Dr. Abd-Jamil involving a decrease in cardiac PDK4 in mice housed in hypoxia for three weeks mimicked our experiments more closely that *in vitro* cell studies, we might expect to see an increase in cardiac PDH flux in the control animals. Given the increased cardiac PDK4 in our diabetic model, if hypoxia were to decrease PDK4, this could ameliorate the diabetic state and improve the hyperglycaemia of the disease.

6.5.1 Methods

Thirty-two male Wistar rats were split into four groups of eight and designated to normoxic control, normoxic diabetic, hypoxic control and hypoxic diabetic. Type II diabetes was induced

as described in chapter 3. Control groups were fed normal chow. Hypoxic animals were housed in the normobaric hypoxic chamber as shown previously (figure 6.1) and experienced one week of adaptation to low oxygen levels, where the oxygen concentration was decreased over the course of a week to reach 11%, and a further two weeks of housing at 11% (figure 6.2). This time period was termed ‘chronic’. Animals were weighed daily, during which time they were exposed to brief normoxia. Scanning was carried out after this time using the appropriate anaesthetic oxygen level, with preparation for MRI as described in section 3.4. Cardiac and hepatic metabolic data were taken during an injection of hyperpolarised ^{13}C pyruvate. Achieving a good ECG in the hypoxic animals proved difficult, so a brief assessment of cardiac function was carried out - two long-axis cine images (6-8 averages) were taken, and a short axis image at the mid-point of the heart. Ejection fraction was subsequently calculated from these data²³⁸. Animals were subsequently given an overdose of isoflurane, and tissue samples were snap frozen. Blood samples were analysed for haematocrit levels; further blood plasma was frozen for later metabolite analyses (details in chapter 3, section 3.10). Biochemical analyses were carried out on the tissue (protocols are described in chapter 3); measurement of glycogen levels was carried out with the help of Dr. Lisa Heather. Statistically, the responses of control and diabetic animals to hypoxia were treated separately, therefore t-tests were used to assess differences. On presentation of diabetic data, control values are displayed alongside as a reminder of the control response, but are not included in statistical analyses.

6.5.2 Results

Control animals: *in vivo* cardiac data

Perhaps surprisingly, when considering previous experiments, there were no differences observed between control animals housed in normoxia or hypoxia, with respect to their cardiac carbohydrate metabolism (figure 6.21).

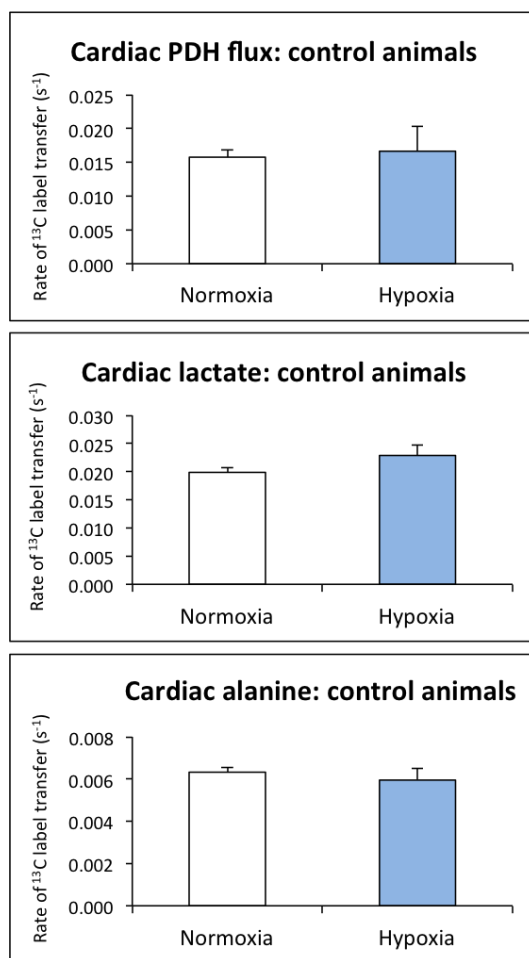


Figure 6.21: Effect of three weeks of hypoxia on control cardiac carbohydrate metabolism

Control animals: cardiac function

There was no effect of chronic hypoxic exposure on the ejection fraction of control animals (figure 6.22).

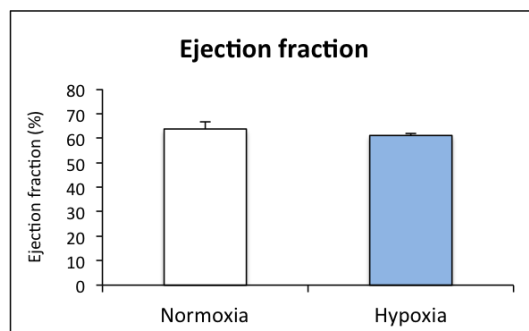


Figure 6.22: Effect of chronic hypoxia on ejection fraction in control animals

Control animals: *in vivo* hepatic data

Hepatic bicarbonate was decreased in the hypoxic control animals in comparison with normoxic controls. There was a trend for hepatic lactate to be decreased in the hypoxic group ($p=0.06$, figure 6.23). Alanine was unchanged.

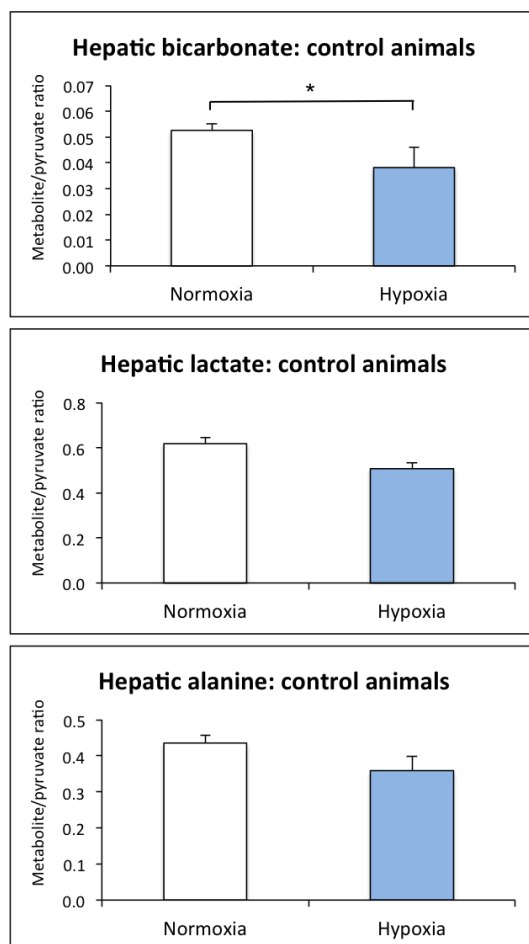


Figure 6.23: Effect of chronic hypoxia on control hepatic carbohydrate metabolism. $*p \leq 0.05$

Control animals: cardiac Western blot data

In control animals, three weeks of hypoxic exposure did not have any significant effects on PDK1, 2 or 4 in the heart (figure 6.24).

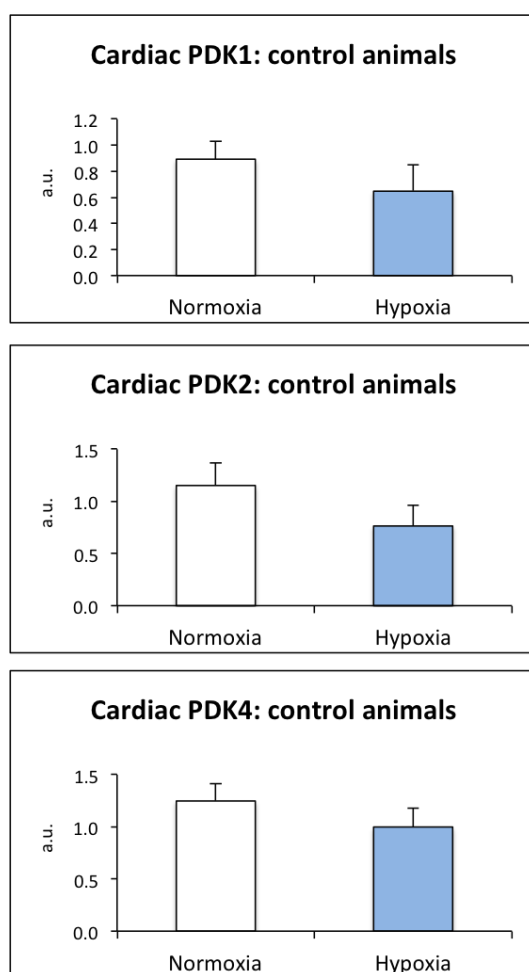


Figure 6.24: Effect of chronic hypoxia on control cardiac PDK expression

Control animals: hepatic Western blot data

No significant differences in PDK isoform expression were seen between control normoxic and hypoxic hepatic tissue (figure 6.25).

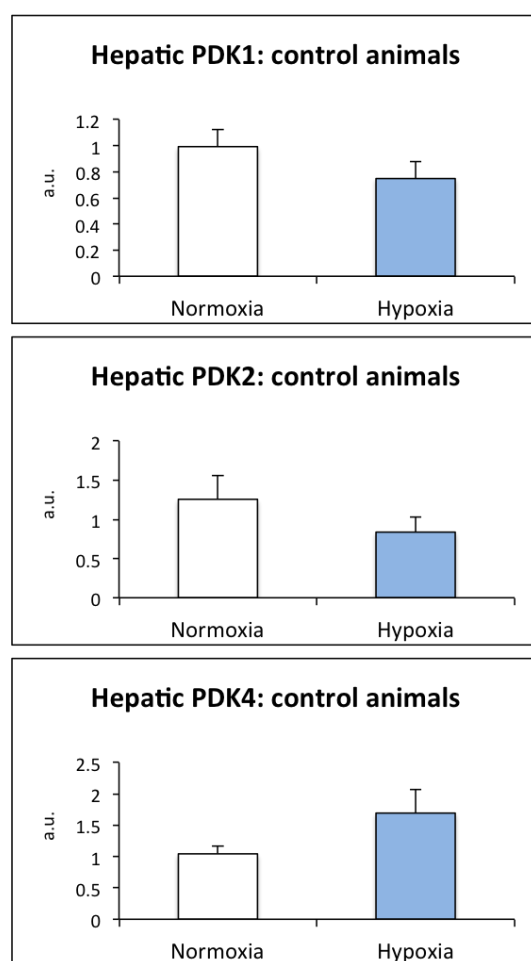


Figure 6.25: Effect of chronic hypoxia on control hepatic PDK expression

Control animals: plasma analyses

Fed plasma insulin and lactate levels were not seen to be different in the hypoxic control animals compared to normoxia. However plasma glucose levels were significantly decreased in the hypoxic animals (figure 6.26).

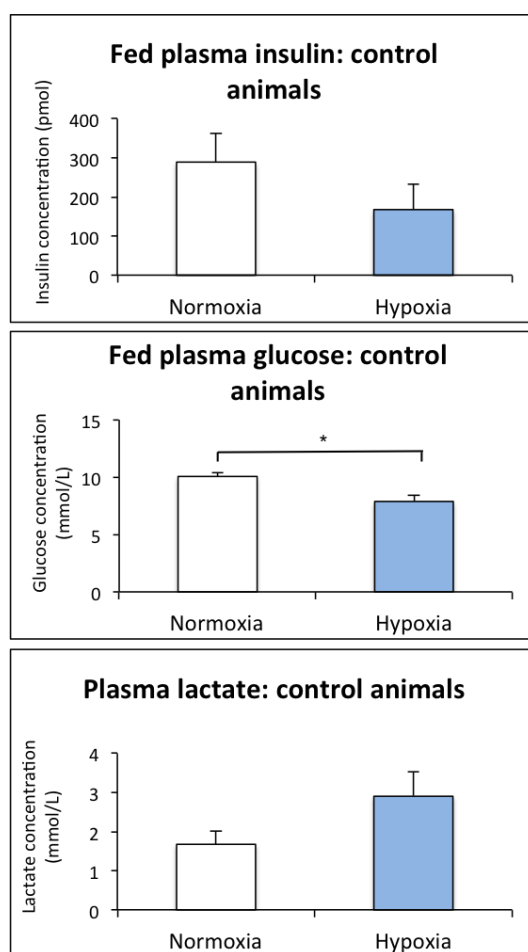


Figure 6.26: Effect of chronic hypoxia on control fed insulin, glucose and lactate plasma levels.

* $p \leq 0.05$

Control animals: further biochemical analyses

Cardiac glycogen and body weight were not different in the hypoxic control group compared with the normoxic group. Haematocrit was significantly increased in control animals housed in hypoxia for three weeks (figure 6.27).

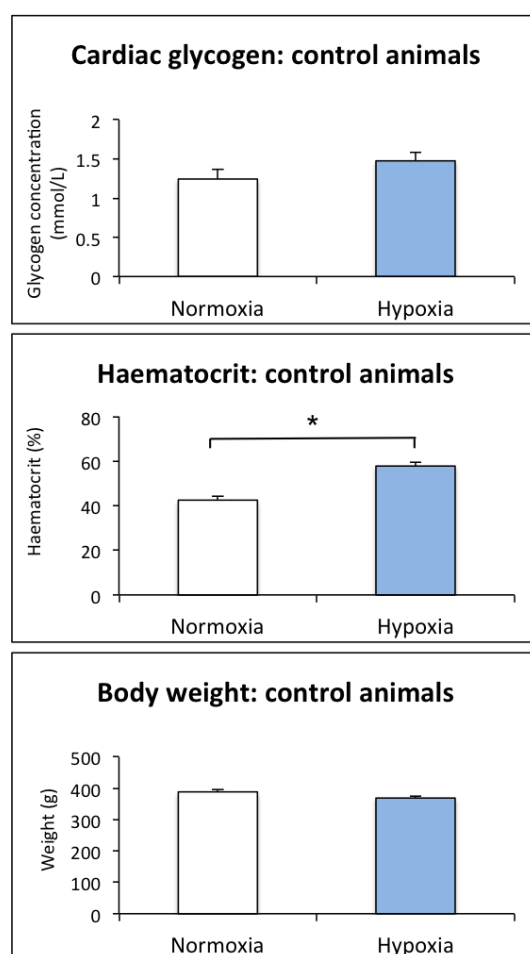


Figure 6.27: Effect of chronic hypoxia on control cardiac glycogen, haematocrit, and body weight. * $p \leq 0.05$

Diabetic animals: *in vivo* cardiac data

Similar to the control animals, cardiac PDH flux and ^{13}C label transfer to lactate in diabetic animals were not seen to be different between normoxia and hypoxia. However, cardiac alanine was shown to be lower in the hypoxic group (figure 6.28), when comparing the diabetic groups.

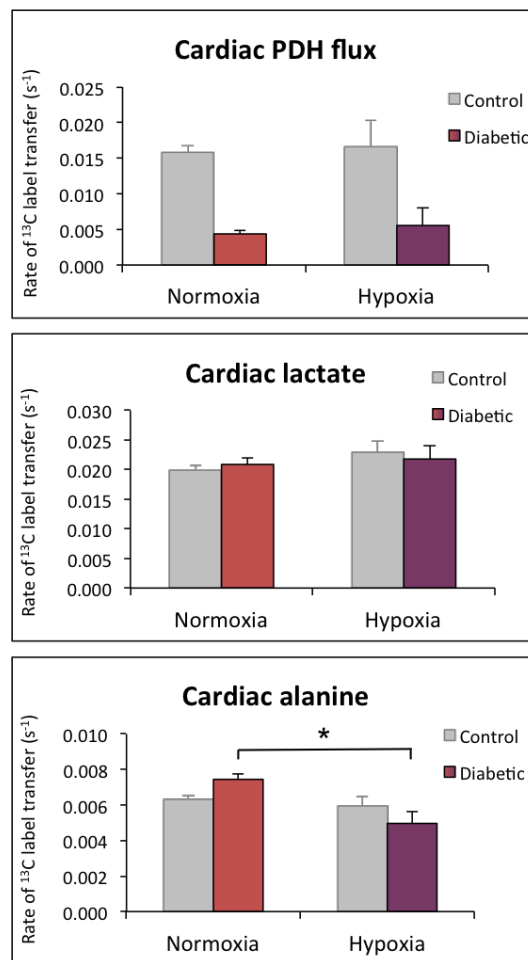


Figure 6.28: Effect of chronic hypoxia on diabetic cardiac carbohydrate metabolism. *p ≤ 0.05

Diabetic animals: cardiac function

No effect of three weeks of hypoxia was seen on ejection fraction of diabetic animals (figure 6.29).

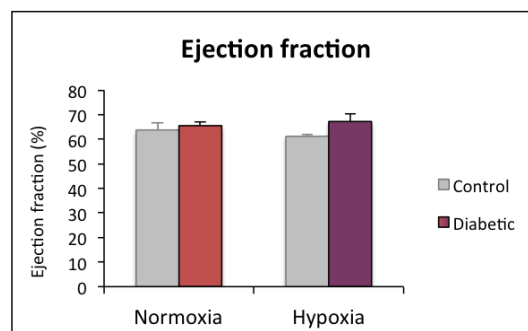


Figure 6.29: Effect of chronic hypoxia on ejection fraction in diabetic animals

Diabetic animals: *in vivo* hepatic data

No differences were seen in hepatic carbohydrate metabolism between diabetic animals housed in normoxia and hypoxia (figure 6.30).

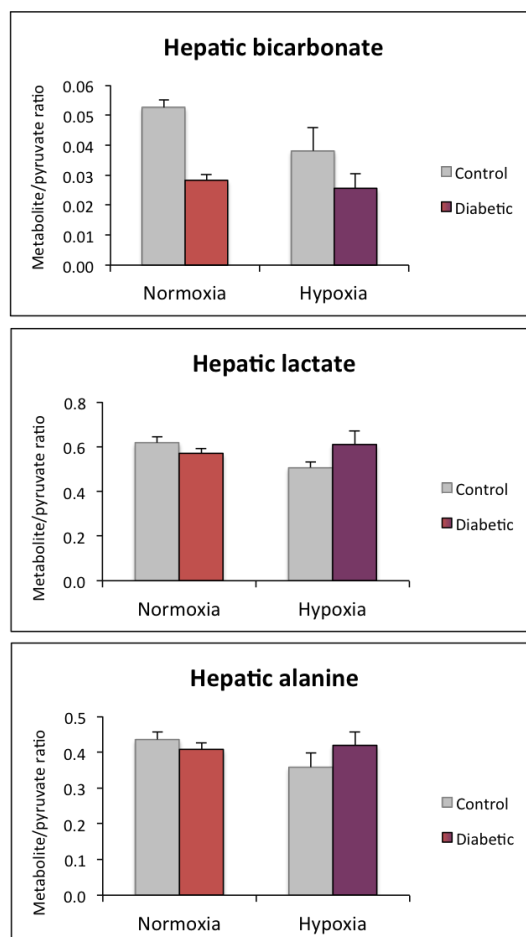


Figure 6.30: Effect of chronic hypoxia on diabetic hepatic carbohydrate metabolism. * $p \leq 0.05$

Diabetic animals: cardiac Western blot data

Diabetic animals showed no changes in cardiac PDK expression as a result of hypoxic exposure, although there was a trend for PDK1 to be increased ($p=0.08$, figure 6.31).

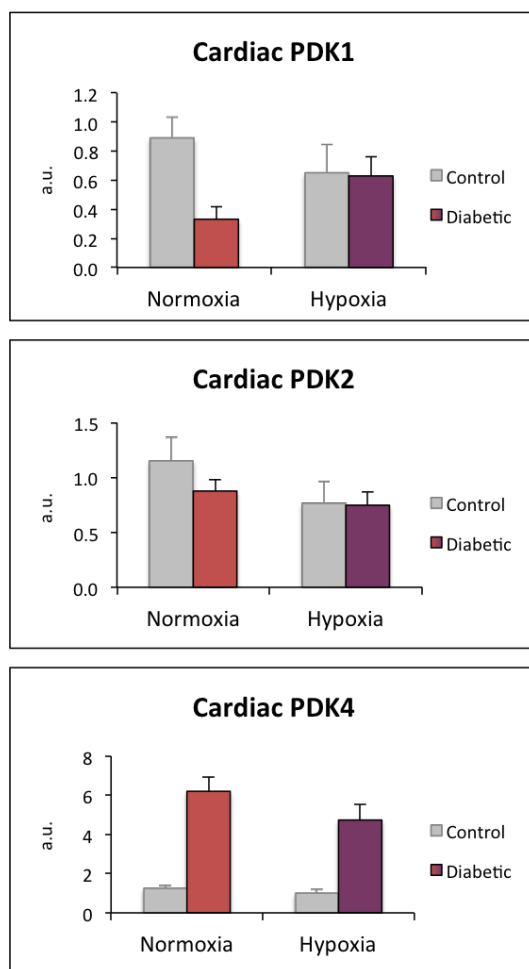


Figure 6.31: Effect of chronic hypoxia on diabetic cardiac PDK expression

Diabetic animals: hepatic Western blot data

No effect of hypoxia was seen on hepatic PDK expression (figure 6.32).

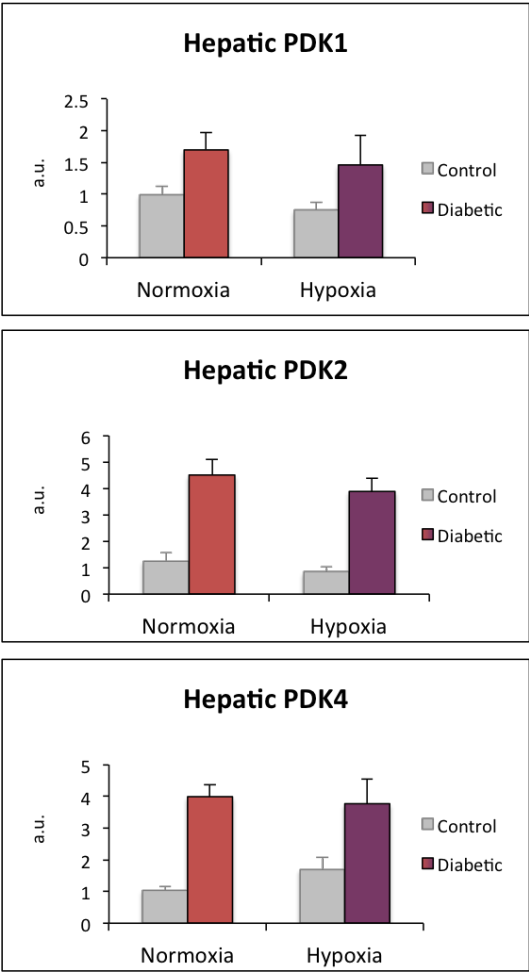


Figure 6.32: Effect of chronic hypoxia on diabetic hepatic PDK expression

Diabetic animals: plasma analyses

Similar to control animals, plasma insulin and lactate were not seen to be significantly different between normoxia and hypoxia, and plasma glucose was significantly decreased in the hypoxic group (figure 6.33).

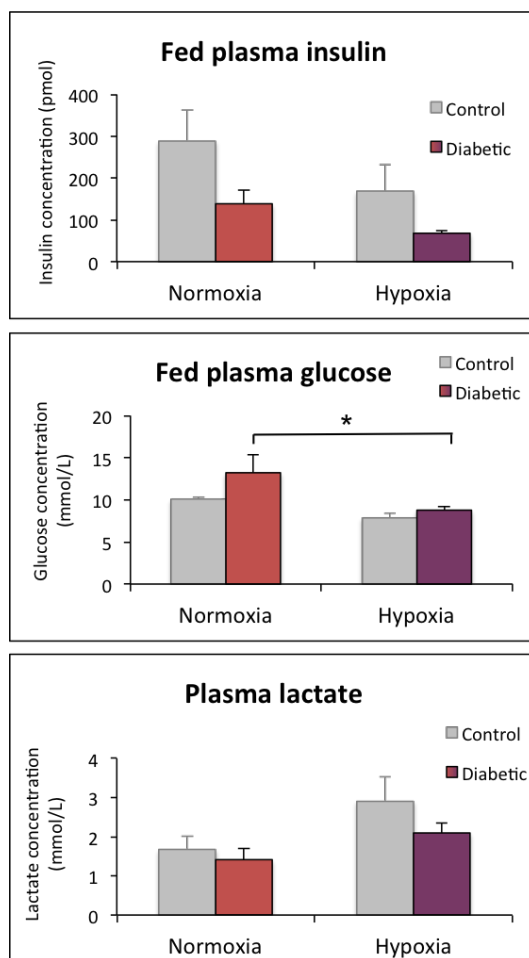


Figure 6.33: Effect of chronic hypoxia on diabetic fed insulin, glucose and lactate plasma levels.

* $p \leq 0.05$

Diabetic animals: further biochemical analyses

Cardiac glycogen levels were not different between control and diabetic normoxic tissue (comparison not shown). Increased glycogen was seen in diabetic hypoxic cardiac tissue compared to diabetic normoxic tissue ($p=0.053$). Haematocrit was significantly increased in diabetic hypoxic animals compared to diabetic animals housed in normal air. Body weight was not affected by hypoxia (figure 6.34).

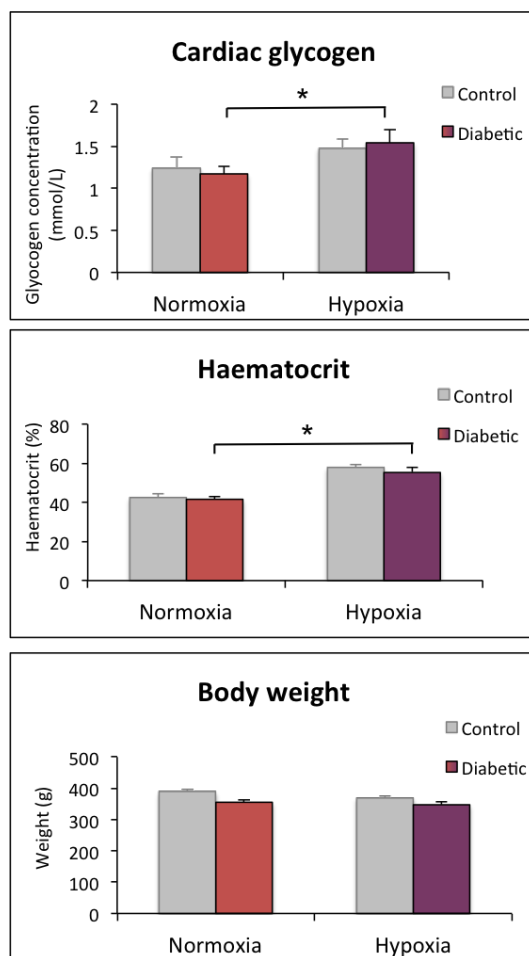


Figure 6.34: Effect of chronic hypoxia on diabetic cardiac glycogen, haematocrit, and body weight. $*p \leq 0.05$

6.5.3 Discussion

Overall, carbohydrate metabolism was largely unaffected by hypoxia at the three week timepoint, both in the heart and liver *in vivo*. This is potentially unexpected, however given the chronic nature of this experiment, we could have expected that physiological adaptations, including the increased haematocrit (which was also increased when compared to values at one week of hypoxia) have compensated for the lack of oxygen. Indeed Sharma *et al.*¹⁸⁰ have suggested that the switch to favour glucose as a fuel reverts after 14 days in hypoxia.

Both control and diabetic animals in hypoxia did have a reduced systemic blood glucose level, although this did not appear to be due to altered hepatic PDH activity. In the diabetic animals,

but not in control animals, cardiac glycogen was significantly increased in the hypoxic group, offering a potential endpoint for the glucose removed from the blood. This was supported by the similar result seen by Pescador *et al.* in 2010, who conclude that glycogen synthase is a HIF target gene¹⁶⁸. The glycogen produced can be used as an on-site anaerobic fuel source, should the hypoxia become more severe.

The control group did not show the same increase in cardiac glycogen levels, although did reduce its blood glucose concentration. Plasma lactate levels in both control and diabetic groups remained the same between normoxia and hypoxia. When looking at both control and diabetic data, there is a pattern of decreased insulin levels in hypoxic groups, which is in accord with previous discussion about the effect of hypoxia on the pancreatic β -cells.

6.6 Conclusions and future directions from studies of hypoxia

6.6.1 Conclusions

Our data has demonstrated that the cardiac redox response (reduced PDH flux and increased lactate) seen in the control animals exposed to acute hypoxia was not present in the diabetic group, suggesting the response is blunted. The response of both groups was different following one week of hypoxic exposure; the control group showed fewer metabolic changes, suggesting that changes have occurred (potentially due to a response to HIF) that enable these animals to overcome the redox pressure, whereas the diabetic animals demonstrated a metabolic state similar to the redox-responsive acute hypoxic exposure of the control animals, potentially suggesting that they are less responsive to HIF. By three weeks, however, differences between groups no longer existed; we can conclude that both control and diabetic animals have responded to the low oxygen conditions and established a mechanism to provide sufficient oxygen for cellular metabolism, albeit via different routes.

Overall, we demonstrated the different responses that occurred in response to different lengths of hypoxia, both in control and diabetic animals, and in the heart and the liver. These are summarised in figures 6.35 and 6.36.

<i>Control cardiac metabolism</i>	Acute hypoxia	1 week hypoxia	3 weeks hypoxia
PDH flux	↓	↔	↔
¹³ C label transfer to lactate	↑	↑	↔
¹³ C label transfer to alanine	↔	↔	↔

<i>Control hepatic metabolism</i>	Acute hypoxia	1 week hypoxia	3 weeks hypoxia
Bicarbonate	↔	↔	↓
Lactate	↑	↑	↔
Alanine	↔	↔	↔

Figure 6.35: Summary of changes seen in cardiac and hepatic metabolism on exposure of control animals to different lengths of hypoxia

<i>Diabetic cardiac metabolism</i>	Acute hypoxia	1 week hypoxia	3 weeks hypoxia
PDH flux	↔	↓	↔
¹³ C label transfer to lactate	↑	↑	↔
¹³ C label transfer to alanine	↔	↓	↓

<i>Diabetic hepatic metabolism</i>	Acute hypoxia	1 week hypoxia	3 weeks hypoxia
Bicarbonate	↔	↓	↔
Lactate	↑	↑	↔
Alanine	↔	↔	↔

Figure 6.36: Summary of changes seen in cardiac and hepatic metabolism on exposure of diabetic animals to different lengths of hypoxia

6.6.2 Future directions

Re-oxygenation following hypoxia

Further studies could include studying metabolic changes seen both immediately following, and sometime after, a return to normoxia following hypoxic exposure, as a response to some interesting data that arose during these studies. Previous work studying humans who trekked to Everest base camp (low oxygen conditions at 5300 m) demonstrated that changes in diastolic function and cardiac PCr/ATP ratios were maintained four days after a return to normal altitude²³⁹. During the execution of our hypoxia experiments, a practical issue beyond our control resulted in a group of control animals (n=6) experiencing 18 days of hypoxia, including the one week adaptation period, followed by two days of normoxia. These animals underwent metabolic assessment (as detailed in chapter 3, sections 3.4 and 3.5) at normal oxygen anaesthesia to assess cardiac and hepatic metabolism. The data from these animals is below, compared to the normoxic control group data from all other studies (figures 6.37 and 6.38). It was shown that cardiac PDH flux was significantly elevated following the return to normoxia, and cardiac alanine was significantly decreased. Cardiac lactate remained unchanged. Hepatic metabolism demonstrated no changes.

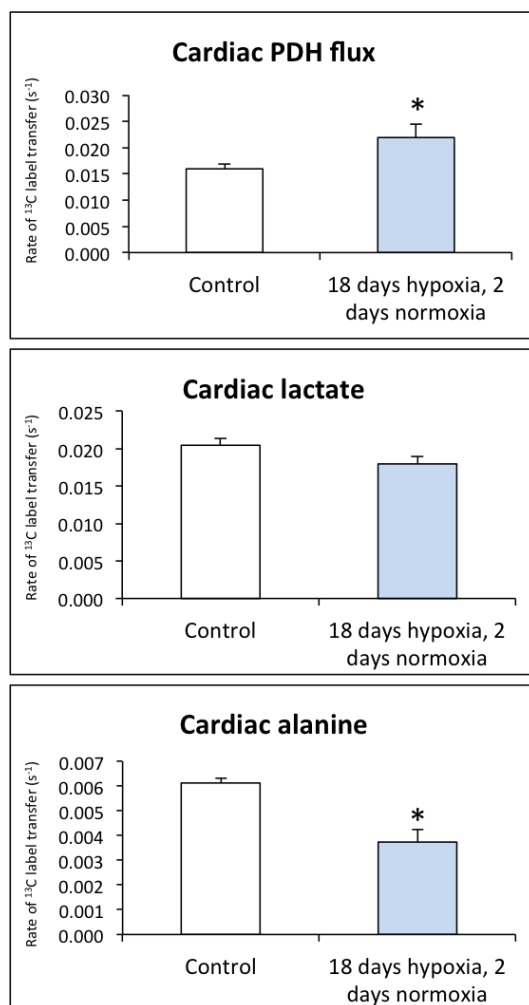


Figure 6.37: Cardiac metabolism of animals returned to normoxia following 18 days of hypoxia.

* $p \leq 0.05$

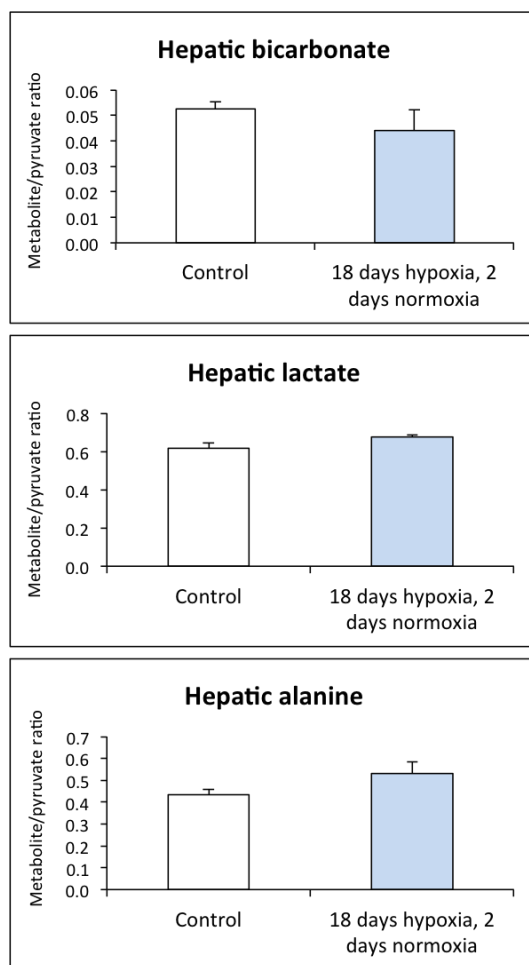


Figure 6.38: Hepatic metabolism of animals returned to normoxia following 18 days of hypoxia

It is interesting that although three weeks, or 21 days hypoxic exposure caused no differences between hypoxic and normoxic animals, a return to normoxia for 2 days could elicit differences. It would be interesting to investigate the underlying mechanisms further. It may be necessary to take into consideration the changes in body weight seen - over the 18 days of hypoxia the animals put on an average of 10 ± 3 g, and following the 2 days of normoxia put on a further 15 ± 3 g.

Choice of anaesthetic agent

Inhalational anaesthetics have been shown to reduce the hypoxic response^{193,240}, affecting both the peripheral and central responses. A meta-analysis by Pandit *et al.* in 2014²⁴¹ concluded that anaesthetic in general depressed the hypoxic response of the carotid body by 24%, but

the mechanism remains elusive. It has not even been fully established how the carotid bodies sense oxygen levels, although it has been suggested to be linked to the sensitivity of their potassium channels. These are usually opened by hypoxia, causing calcium influx and release of neurotransmitters, and it may be that they are sensitive to reactive oxygen species caused by hypoxia²⁴². One study in 2002 demonstrated an alleviation of the depression of hypoxic response by halothane using anti-oxidant treatment in humans, although this was at sub-anaesthetic levels¹⁹³. Further, although halothane is in the same family as isoflurane, different anaesthetic agents have been found to have variable effects²¹⁹, so the relevant literature is limited. Work could be carried out to repeat our experiments with an alternative anaesthetic agent, potentially paving the way for more stark variations to be seen.

Oxidative stress caused by diabetes

Previous literature has discussed the idea that diabetes and hypoxia are inherently interlinked, suggesting that hyperglycaemia causes a pseudo-hypoxic state, i.e. there are similarities to hypoxia but without the reduced oxygen saturation. There is still uncertainty surrounding this, such as is demonstrated by the work by Hodson *et al.* which found no evidence for adipose hypoxia in obesity causing metabolic dysfunction²⁴³. However, if the hyperglycaemia can simulate hypoxia, this may provide an explanation as to why hypoxia did not directly counteract the metabolic changes seen in diabetes. In 1993, Williamson *et al.* compared the vascular changes that occur in hypoxia due to the redox imbalance (vasodilation and increased blood flow in response to acute hypoxia) to the similar response in hyperglycaemic tissue, also due to changes in NAD^+/NADH ratio¹⁷¹. It is suggested by these authors that hyperglycaemia causes this change in ratio by increasing the metabolism of glucose via the sorbitol pathway. Chung *et al.*²⁴⁴ looked specifically at the two enzymes involved in this pathway - aldose reductase and sorbitol dehydrogenase - to try and understand the oxidative stress caused by diabetes. In lenses and nerves, the two enzymes were shown to have different contributions to this oxidative stress. Specifically relevant to our work, Ochiai *et al.* demonstrated an increase in HIF-1 α in mouse liver following a high-fat/high-sugar diet²⁴⁵. This therefore complicates the conclusions from our studies, as if diabetes was exacerbated by hypoxia, a more severe diabetic state in normoxia is required to enable a comparison between hypoxic and normoxic diabetic groups.

However, given the cyclical nature of the diabetes/hypoxia relationship, we could suggest a mechanism for our chronic hypoxic data. As discussed above, it is plausible that hyperglycaemia is least partially responsible for blunting the HIF response in the diabetic group^{183,246}. In our data, hypoxia is shown to reduce blood glucose levels (in both our control and diabetic groups). This therefore may play a part in alleviating HIF suppression, allowing the diabetic animals to respond to hypoxia in the long-term, so we do not see any differences in metabolism between groups following chronic hypoxic exposure.

6.7 Pharmacological manipulation of the hypoxic pathway in diabetes

It is possible that, as the metabolic changes in hypoxia are opposite to those seen in diabetes, stimulating the hypoxic response in diabetics in the presence of normal oxygen levels may improve the diabetic phenotype. In support of this, we saw in section 6.5 that three weeks of hypoxia caused a decreased blood glucose when compared to normoxic animals. The use of dimethyloxallylglycine (DMOG) in this section allows us to be certain of targeting HIF, and in terms of a potential treatment, a small molecule drug is obviously of greater benefit than a change of environment such as hypoxia. We hypothesised that stimulating HIF pharmacologically would enable increased glycolysis, and therefore promote glucose metabolism in place of fatty acid oxidation. In the diabetic model, this would be beneficial, and further, given the presence of oxygen, would not hinder glucose oxidation also occurring. The metabolic balance may possibly be restored. This may be made more likely when considering the lack of increased PDK 1 seen in diabetic heart tissue following one week of hypoxic exposure compared to that previously described in cell work.

Previous *in vivo* work administering DMOG by injection has involved a range of doses to treat a variety of conditions, and as such, specific pharmacodynamic and pharmacokinetic data has not been established for male Wistar rats. Limited studies have investigated the effects of varying doses, for example Sears *et al.*²⁴⁷ administered increasing doses of DMOG to retinal cells and saw the maximal effect at 1 mM, with lower expression of HIF seen both below and above this dose. This was replicated by Ayrapetov *et al.*²⁴⁸, who saw this maximal activation at 1 mM in

mouse embryonic fibroblasts. However Sears *et al.* also gave varying injections of DMOG *in vivo* (125, 250 and 500 mg/kg) and showed similar ocular EPO expression (a downstream gene of HIF, and therefore a marker of the effect of DMOG) for all doses. As an aside, the liver was shown to upregulate VEGF by Sears *et al.* on DMOG treatment (200 mg/kg), so the use of our two-slice acquisition is particularly relevant here.

Doses used in previous literature include 40 mg/kg and 200 mg/kg in rats, approximately 260 mg/kg in mice, and 20 mg/kg in rabbits. Most of these doses are, however, given only once, and so potentially for a longer-term study a lower dose is required to prevent over-dosing and potential toxic effects. Song *et al.* saw restoration of VEGF and GLUT1 levels in rats subject to a nephrectomy, following mini-pump infusion of 15 mg/kg/day DMOG for 4 weeks²⁴⁹. However, our supplier of DMOG, Cayman Chemicals, recommended not storing an aqueous solution of DMOG for more than 24 hours, and therefore injection was the administration route of choice for our experiment. Literature reporting multiple injections of DMOG utilises alternate days for administration^{189,250}; this protocol was therefore used in our study.

6.7.1 Methods

Control (n=12) and diabetic male Wistar animals (n=9) were treated with DMOG (Cayman Chemicals, USA) over a one week period. Type II diabetes was induced as described in chapter 3, section 3.2. Just prior to the 6 week endpoint, animals (both diabetic and control) were scanned under normoxic anaesthesia (as in section 3.4), with an injection of hyperpolarised pyruvate and cine imaging, before recovery. Subsequently, four injections of dimethyloxallylglycine (15 mg/kg, dissolved directly in phosphate-buffered saline) were administered intraperitoneally, on alternate days, between 9am-12pm. Animals were scanned on day 2, day 4, and day 8; all scanning sessions were on days when injections were not administered (see figure 6.39 for clarification). After the final scanning session animals were given an overdose of isoflurane and heart and liver tissue snap frozen. Blood haematocrit levels were measured and further blood samples were taken and frozen for later metabolite analysis.

A two-way ANOVA was used in all cases to assess differences between groups and timepoints. Repeated measures analyses were not used however due to only six full data sets of all timepoints

in each group. Baseline data includes all control and diabetic animal data obtained in this thesis.

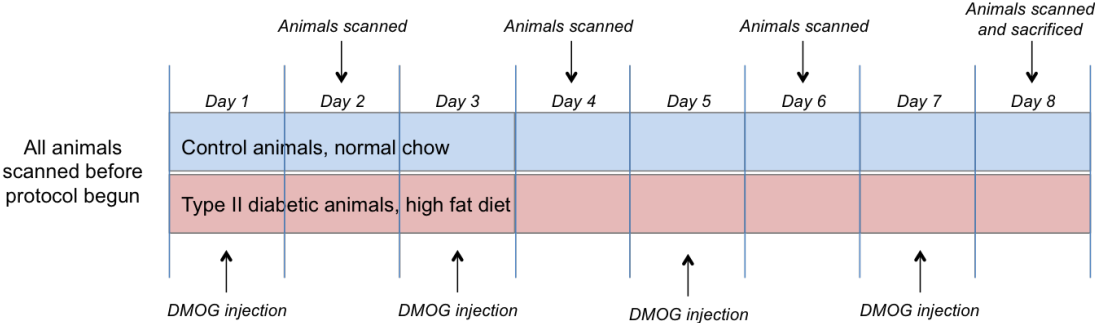


Figure 6.39: Protocol for DMOG administration to control and diabetic animals over one week.

6.7.2 Results

Cardiac PDH flux was significantly lower in the diabetic group compared with the control group at all timepoints except day 8, following all four injections of DMOG. There was a significant statistical interaction between diabetes and time point, shown by the fact that at day 8, cardiac PDH flux in the control group was decreased compared to that seen prior to dosing whereas the diabetic group did not respond and by that point the control and diabetic groups showed similar cardiac PDH flux (figure 6.40). Cardiac ^{13}C label transfer from pyruvate to lactate showed no differences either between groups or following DMOG treatment. Label incorporation into alanine was only significantly different at baseline between control and diabetic animals - no differences were seen at the other individual points during the protocol, although overall the ANOVA was significant for diabetes.

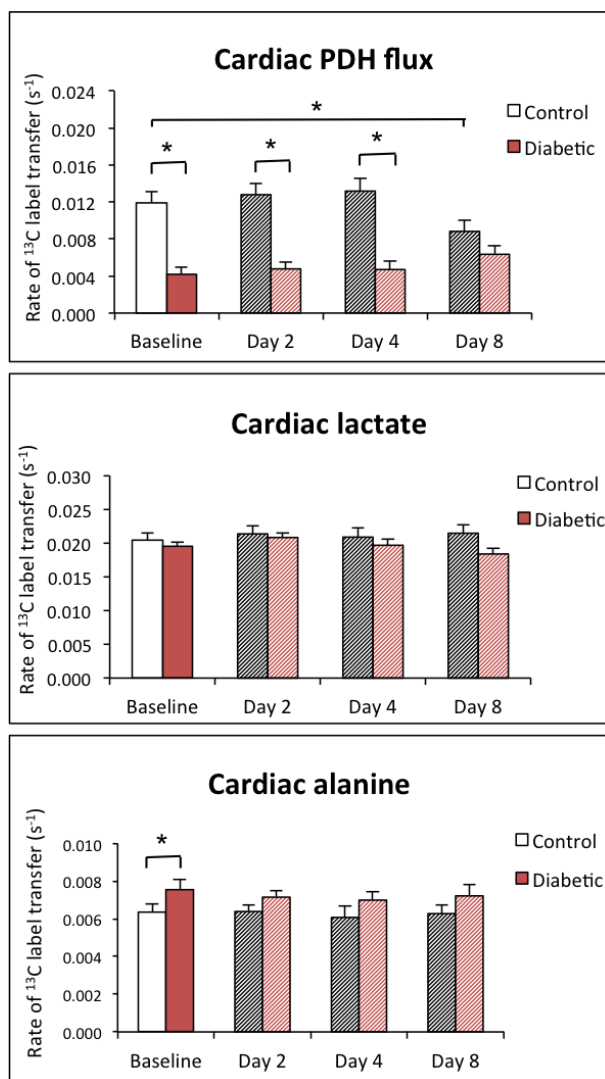


Figure 6.40: Effect of one week DMOG administration on cardiac PDH flux, and ^{13}C label transfer to lactate and alanine, control and diabetic groups. $*p \leq 0.05$

The diabetic animals showed a significantly reduced level of hepatic bicarbonate compared to control animals, until after the fourth injection of DMOG. At this point, neither control nor diabetic animals showed a significant difference in hepatic bicarbonate to that shown prior to dosing, however, they were not significantly different to each other either (figure 6.41). Hepatic lactate, similar to in the heart, showed no differences between groups or across the protocol of injections. Hepatic alanine demonstrated no variations either.

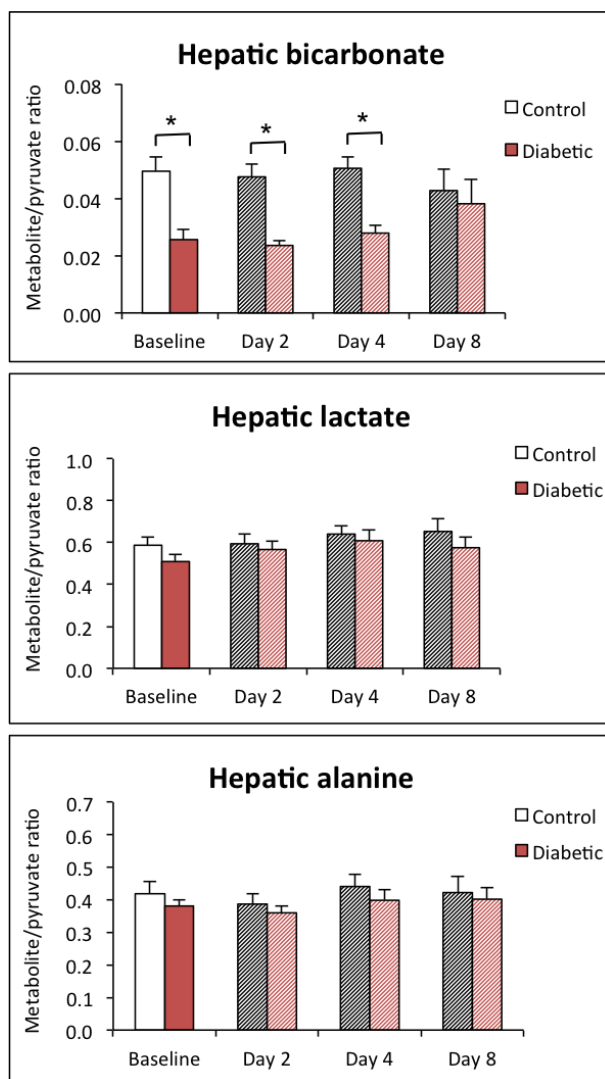


Figure 6.41: Effect of one week DMOG administration on hepatic ^{13}C label transfer to bicarbonate, lactate and alanine, control and diabetic groups. $*p \leq 0.05$

There was a significantly elevated blood glucose level in the diabetic animals prior to dosing, however this was no longer present after DMOG injections (figure 6.42). Individual t-tests between baseline and day 8 data show that this was driven by a significant lowering of blood glucose level in the diabetics. Haematocrit was not altered by DMOG treatment. The diabetic animals were significantly heavier prior to dosing; this difference was no longer present by the end of the week of treatment.

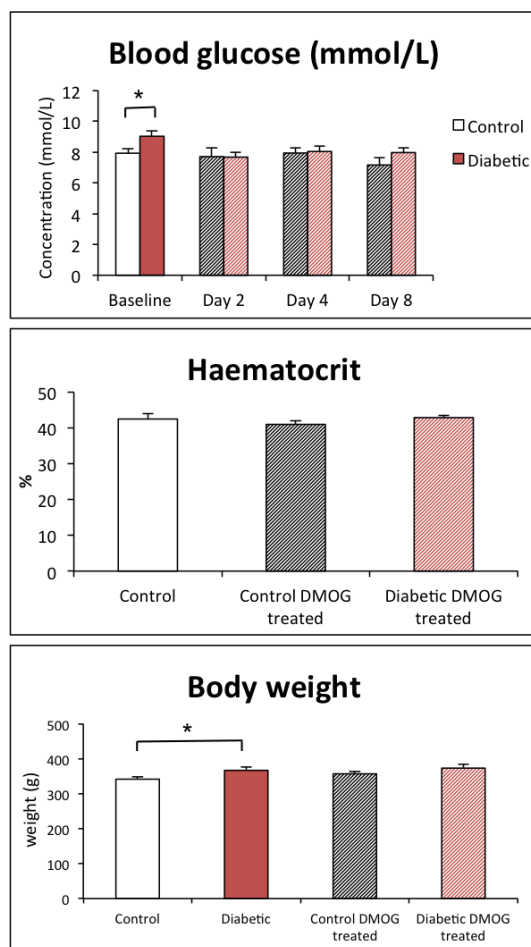


Figure 6.42: Effect of one week DMOG administration on body weight and blood glucose concentration, control and diabetic groups. Statistics assessed by repeated measures 2-way ANOVA. $*p \leq 0.05$

All Western blot data assessed samples taken at the end of DMOG treatment, compared with untreated control and diabetic tissue from animals of the same age from a separate study. DMOG treatment resulted in cardiac PDK1 expression being significantly different between control DMOG treated and diabetic DMOG treated, i.e. the interaction between disease and treatment was significant. Diabetes had a significant effect on cardiac PDK2, but there was no effect of treatment. Diabetes was shown to have an effect on cardiac PDK4, although DMOG treatment did not, in both the control and diabetic group (figure 6.43).

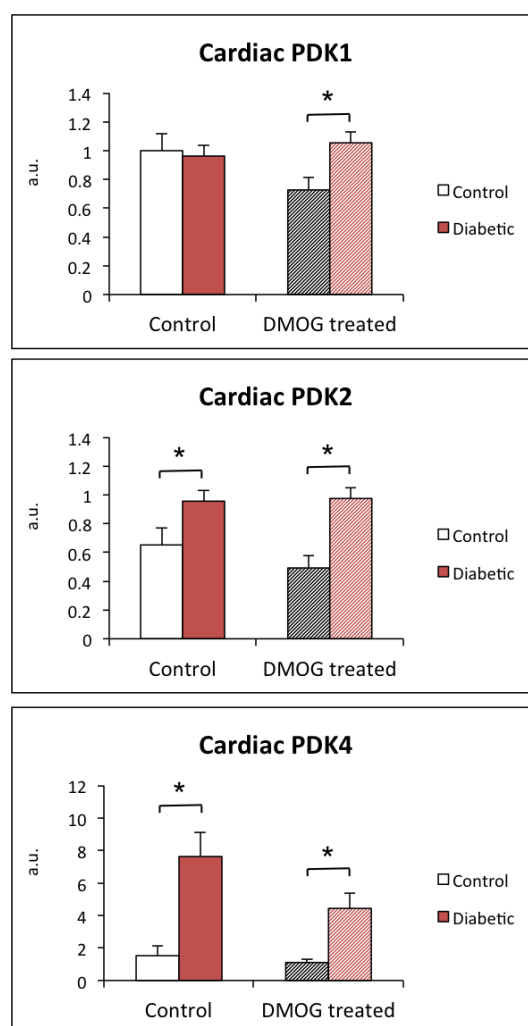


Figure 6.43: Effect of one week DMOG administration on cardiac pyruvate dehydrogenase kinase expression. A two-way ANOVA was used to assess the differences between groups. $*p \leq 0.05$

No significant changes in hepatic PDK1 and PDK4 expression were measured. On assessment by two-way ANOVA, hepatic PDK2 was shown to be affected by diabetes ($p < 0.0001$) and DMOG treatment ($p = 0.017$), but no interaction was seen, i.e. both control and diabetic group were affected similarly by DMOG treatment (figure 6.44), although when looking at individual comparisons, a trend to increased hepatic PDK2 was seen in the diabetic treated animals compared to untreated ($p = 0.1$).

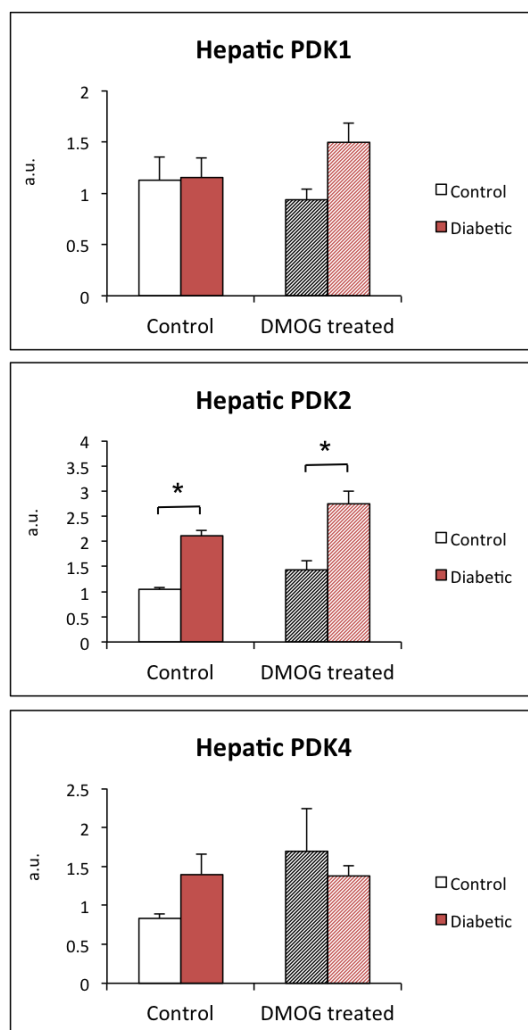


Figure 6.44: Effect of one week DMOG administration on hepatic pyruvate dehydrogenase kinase expression. A two-way ANOVA was used to assess the differences between groups. * $p \leq 0.05$

6.7.3 Discussion

Treatment of control and diabetic animals with DMOG allowed us to specifically study the effects of increased expression of hypoxia-inducible factor (HIF) and its downstream targets.

We can conclude from the *in vivo* data that, in the control group at least, DMOG has caused downstream effects which have affected PDH activity. Following the four DMOG injections, cardiac PDH flux was significantly decreased in comparison to baseline data. This would be expected to be a consequence of altered PDK expression, specifically PDK1, based on past literature⁶². However, it has yet to be established whether the other PDK isoforms are regulated

by HIF²⁵¹. Given we saw no effect of DMOG on cardiac PDK1, 2 or 4, it is not clear what is driving the decreased PDH flux.

There was no effect of DMOG seen in the rate of incorporation of ¹³C label into lactate or alanine in the control group. It is difficult to establish why these are not altered in the light of the altered PDH flux, as if DMOG was stimulating the HIF pathway, GLUT1 should be increased; it may be expected that this extra glucose entering the cells would be converted to lactate or alanine. However, it is possible pyruvate has been diverted via pyruvate carboxylase (stimulated by acetyl CoA) to oxaloacetate and into the Krebs cycle - further experiments would be required to establish whether this is the case. [2-¹³C]pyruvate could be used *in vivo* to measure Krebs cycle intermediates prior to and following treatment⁷² in combination with protein expression analysis.

The hepatic data from control animals showed no significant change in bicarbonate between baseline data and the end of the dosing protocol. There were no effects either on hepatic lactate or alanine. This was supported by a lack of PDK protein expression changes in the liver as assessed by Western blot.

The diabetic group responded in a different way to control animals to DMOG administration. Cardiac PDH flux remained significantly lower than that seen in control animals until the final metabolic assessment on day 8. However this was primarily driven by the reduction in PDH flux in the control animals, rather than a significant increase in the diabetic animals. Coupled with the lack of changes in cardiac lactate and alanine, and supported by the Western blot data, this indicated that any effect of DMOG on HIF has not elicited any response in the diabetic cardiac metabolism. This may be due to the hypothesised reduced HIF response in the diabetic model¹³ as discussed earlier. Given the reduced cardiac PDH flux seen in control animals, this may not actually be a positive effect even if it were seen in the diabetic animals. However, there is some evidence that diabetic complications are to some extent a result of accumulation of partially metabolised lipid derivatives, leading to insulin resistance²⁵². In this situation, a decrease in PDH flux may cause a further increase in fatty acid oxidation, reduced insulin resistance, and reduced blood glucose levels. Before dosing was begun, the blood glucose level measured was significantly higher in the diabetic group compared to the control group, and by the end of the protocol this difference no longer existed, driven by a reduced glucose in the

diabetic animals. Potentially this was due in part to a slight increase in hepatic PDH flux, which may be indicated by the *in vivo* hepatic bicarbonate data. These data showed that the diabetic animals had significantly lower hepatic bicarbonate at all stages except day 8. By that point, they were no longer significantly lower, however unlike in the cardiac data where it is clear that this was driven by a reduction in PDH flux in the control group, the hepatic control data was not significantly lower at day 8 than at baseline. It is difficult to say with certainty that the diabetic animals had altered their metabolism, and the difference in hepatic PDK2 between control and diabetic animals did remain following DMOG treatment; a larger study and/or an increased dose of DMOG would provide more certainty either way. In any case, administration of DMOG in this study normalised the hyperglycaemia seen in the diabetic group prior to treatment.

6.7.4 Conclusions

Administration of DMOG for one week can potentially provide some insight into the mechanisms of change in the animals exposed to one week of hypoxia, although it needs to be confirmed that the dose of DMOG given is an accurate representation of the level of hypoxic exposure. I have assumed this is the case for this discussion. Looking at the *in vivo* assessment of cardiac metabolism in the control groups, animals exposed to one week of hypoxia showed no alteration in PDH flux, whereas DMOG (solely activating the HIF pathway, in the presence of oxygen) caused a significant reduction in PDH flux. This potentially indicates that, in terms of cardiac metabolism at least, HIF was not (or no longer was) the major driving force behind the changes seen after one week of hypoxia, and with the lack of oxygen present, the redox effect was the greater contributor. This was further supported by the fact that ^{13}C label transfer to lactate was also not comparable between these two experiments; where DMOG treatment caused no change over time, one week of hypoxia resulted in a significant increase in ^{13}C label transfer to lactate. The differences between one week hypoxia and DMOG treatment in the heart were maintained in the liver, leading to a conclusion that the redox effect was systemic in its control over the metabolic balance, rather than the effects of HIF, in the control animals, at this time point.

The cardiac data from the diabetic animals treated with DMOG generally showed little response to the HIF-stimulator, and this was in stark contrast to the data from diabetic animals exposed

to one week of hypoxia, where cardiac PDH flux and alanine were both decreased, and lactate was increased. As in control groups, this led us to conclude that the redox alterations caused by hypoxia produced these latter effects, and it is interesting to note that this redox effect seems more extreme. If in fact, the cardiac HIF response was inhibited (as suggested by the DMOG study data), the lack of ability to respond using HIF and its downstream targets may result in this more extreme metabolic response in the face of an altered redox state.

Chapter 7

Thesis conclusions and future directions

Since the original work by Ardenkjaer-Larsen *et al.*⁷¹ in 2003 demonstrated the ability to produce solutions of hyperpolarised ^{13}C -labelled compounds which could be used for *in vivo* metabolic measurements, the field has rapidly expanded into several disease areas. Cancer^{253,254} and cardiovascular disease^{8,83} are two such areas. The first clinical studies have recently been carried out in prostate cancer patients and reported by Nelson *et al.* in 2013⁷³, and studies in patients with cardiovascular disease are imminent⁹². The technique continues to be developed for diagnosis, assessment of treatment response, and for improving our understanding of any underlying metabolic changes that occur in disease. The studies described in this thesis have addressed the use of hyperpolarised ^{13}C pyruvate for these purposes in relation to type II diabetes, and have produced data that will hopefully be relevant and informative for upcoming clinical work.

Chapter 4 developed a data acquisition method to increase our wider understanding of disease metabolism, through assessing both cardiac and hepatic carbohydrate metabolism during one injection of hyperpolarised pyruvate. Further, the slice selective nature of the acquisition removed contamination from neighbouring organs, and thereby provided a more accurate reflection of metabolism. The two-slice acquisition, although not quantitatively comparable to global or single-slice data, provided metabolically relevant data, shown through measurement of fed, fasted, and diabetic metabolism in rodents. The final section of this chapter summarised

the data achieved over several studies in this thesis, from both control animals and a slightly less severe, and therefore hopefully more clinically relevant, diabetic rodent model, as developed by Mansor *et al.*¹¹⁰. This diabetic model was found to have a significantly lower cardiac and hepatic PDH flux compared with control animals, and a significantly higher cardiac alanine. Compilation of these data confirmed the metabolic alterations caused by this model of diabetes, and provided increased statistical power.

Having presented the successful use of the two-slice scan for assessment of metabolic alterations *in vivo*, chapter 5 demonstrated its use in the investigation of PDH modulation by pharmacological means, namely DCA and AZD7545. These compounds differed in their PDK specificity. Prior to *in vivo* studies and whilst the two-slice scan was in development, initial studies were carried out which assessed the effect of these drugs on metabolism in the control perfused heart, which provided data independent of circulating hormones. DCA increased PDH flux in the perfused heart, in agreement with previous literature, however AZD7545 caused no such change. A pilot *in vivo* chronic dosing study of control animals was therefore implemented to provide further information on this. The data from this *in vivo* study, again looking at both DCA and AZD7545, showed trends comparable to the perfused work. A significant increase in PDH flux was seen following 28 days of DCA administration, but no change was seen in PDH flux in the AZD7545-treated animals, supported by no changes in cardiac or hepatic PDK expression. To try and further explain the lack of effect of AZD7545 on PDH flux in these control animals, plasma levels of the compound were measured, and found to be comparable to control levels. As a result, the next study, which involved assessment of the effect of these drug compounds on cardiac and hepatic metabolism in both control and diabetic animals, employed an alternative AZD7545 administration method. DCA was, as in the pilot study, administered via the drinking water.

This penultimate *in vivo* study combined the use of the two-slice acquisition of hyperpolarised data with echocardiographic data to assess diastolic dysfunction. The diabetic data in particular provided an interesting insight into the link between cardiac function and metabolism. Diastolic dysfunction, which was present in the diabetic animals prior to treatment, was no longer present following treatment with DCA. This was observed alongside restoration of cardiac and hepatic PDH flux, and cardiac alanine to control levels.

AZD7545 was confirmed to be present in the plasma of the treated animals at levels comparable with previous studies at this dose. In control animals, this dose caused a significant decrease in both cardiac and hepatic PDH flux, increased ^{13}C label incorporation into lactate, and decreased incorporation into alanine, indicating that it was not acting as expected. Concomitantly, PDK4 was shown to be upregulated. A subsequent study with a lower dose of AZD7545 was therefore carried out, however once again PDH flux was not increased in either the heart or the liver. Levels of hepatic PDK2 protein were not decreased, and so it could be hypothesised that PDK4 remained activated as in the previous study at a higher dose of AZD7545. In previous work at AstraZeneca, doses comparable to the low dose demonstrated an inhibition of hepatic PDK2, without stimulating PDK4 to a level at which it prevents the desired increase in PDH flux. Our studies did not support this observation.

Overall, chapter 5 demonstrated the value of hyperpolarised pyruvate as a tool for understanding metabolism and also for following treatment response, particularly in combination with other established techniques. In addition, the work with AZD7545 supported the use of hyperpolarised compounds during the drug development process. The data acquired was not in support of previous literature, and as such provided a new perspective, along with further questions, on the *in vivo* efficacy and mechanism of action of the drug.

Finally, chapter 6 provided further foundation for the use of hyperpolarised compounds in understanding metabolism. Following development of hypoxic anaesthesia for the laboratory, initial studies demonstrated that the real-time aspect of hyperpolarised MRS could be exploited to assess the effect of acute hypoxia (30 minutes) and subsequent re-oxygenation on carbohydrate metabolism. Metabolism was also assessed following one week and three weeks of hypoxia; all studies were carried out in control and diabetic animals at each time point. The data showed different responses from control and diabetic animals, and as a result has opened many avenues for further investigation. Overall, we hypothesised that we have shown the gradual change in balance between redox effects and the HIF response, and resultant changes in protein expression, although obviously further work is needed to confirm this. Considering the results from chapter 5, it is possible that administering DCA to (and therefore lowering blood glucose of) diabetic animals in hypoxia, or prior to an acute hypoxic insult, may enable them to initiate a successful hypoxic response. The last section of chapter 6 explored the effect on carbohydrate metabolism

of upregulating HIF in the presence of oxygen, with a particular interest in the response of the diabetic animals. Interestingly, although no alterations were seen in carbohydrate metabolism in the diabetic group, measured using hyperpolarised pyruvate, the elevated blood glucose levels were normalised by the DMOG treatment. Future studies with higher concentrations, more frequent administrations, or a longer period of administration of DMOG may cause a more distinct response, and either enable the diabetic animals to improve their PDH flux, or allow sufficient time for the observed decreased blood glucose level to have a positive effect on their fuel balance.

The aim of this thesis was to address the use of hyperpolarised MRS in a type II diabetic rodent model, and it has done so partially through investigation of metabolic modulation by drug compounds and an hypoxic environment. Much of this work could be further understood using alternative hyperpolarised probes to investigate metabolic changes elsewhere in the cell. Many such probes have been developed recently, such as hyperpolarised butyrate⁷⁶ for studying fatty acid metabolism; hopefully these will become more widely used in the near future to provide a fuller picture of the *in vivo* metabolism. In addition to this, we believe the data presented in chapter 5 provides a strong argument for combining hyperpolarised probes with other established analytical techniques. In terms of clinical relevance, an injection of hyperpolarised pyruvate would only add a few minutes to a routine MR protocol, and this would be particularly valuable in light of the conclusion of chapter 4 that data is attainable from two organs during a single hyperpolarised injection.

We have shown that *in vivo*, real-time data can be acquired from multiple organs in both healthy and diseased animals using hyperpolarised ¹³C pyruvate, and that this technique can then be used to both follow treatment effects and inform our basic understanding of diabetes, via pharmacological or physiological means.

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Appendices

Appendix A

Primary antibody	Primary antibody supplier	Dilution	Secondary antibody	Dilution
PDK1	Cell Signalling Technology	1:3000	Goat anti-rabbit	1:3500
PDK2	Abgent	1:3000	Goat anti-rabbit	1:3500
PDK4	Gift from Prof. Mary Sugden, QMUL, UK	1:10000	Goat anti-rabbit	1:3500
GLUT4	Gift from Dr. Geoffrey Holman, University of Bath, UK	1:4000	Goat anti-rabbit	1:3500

Figure 1: Details of western blotting antibodies used

Appendix B

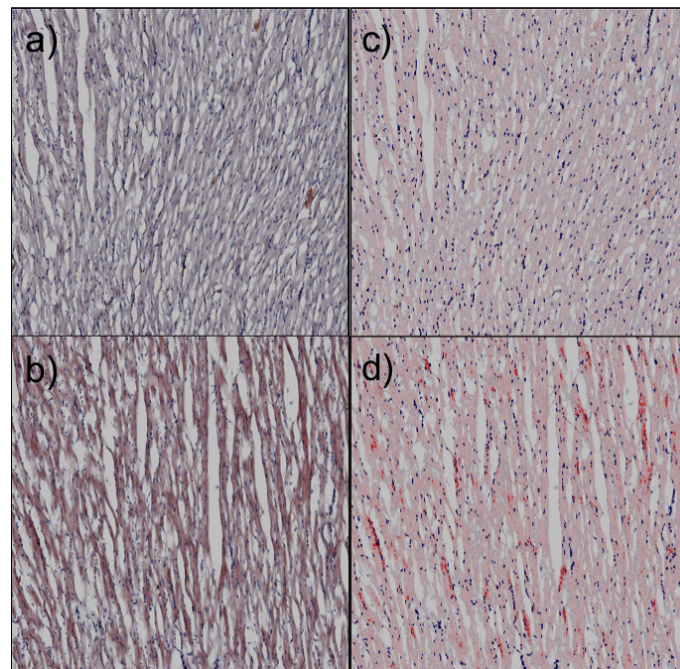


Figure 2: Example section of cardiac tissue stained for lipids (10 x magnification), from a) control, b) AZD7545 treated, and subsequently with a computational threshold applied, c) control, and d) AZD7545 treated

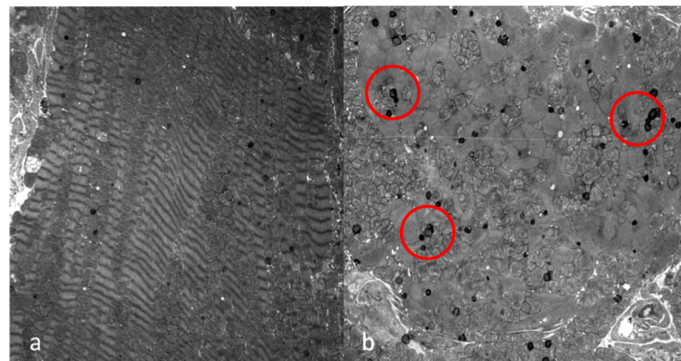


Figure 3: Example section of cardiac tissue from a) control and b) an AZD7545 treated animal, viewed with an electron microscope (15000 x magnification). Lipid droplets are circled in red

Appendix C

List of publications

L. S. Mansor, K. Mehta, D. Aksentijevic, C. A. Carr, T. Lund, M. A. Cole, **L. M. Le Page**, M. L. S. Fialho, M. J. Shattock, E. Aasum, K. Clarke, D. J. Tyler, L. C. Heather, 'Increased oxidative metabolism following hypoxia in the type 2 diabetic heart, despite normal hypoxia signalling and metabolic adaptation', *Journal of Physiology*, 2016

L. M. Le Page, O. J. Rider, A. J. Lewis, V. Ball, K. Clarke, E. Johansson, C. A. Carr, L. C. Heather, D. J. Tyler, 'Increasing pyruvate dehydrogenase flux as a treatment for diabetic cardiomyopathy: a combined ^{13}C hyperpolarised magnetic resonance and echocardiography study', *Diabetes*, 2015

D. R. Ball, B. Rowlands, M. S. Dodd, **L. M. Le Page**, V. Ball, C. Carr, K. Clarke, D. J. Tyler, 'Hyperpolarized butyrate: a metabolic probe of short chain fatty acid metabolism in the heart', *Magnetic Resonance in Medicine*, 2013

M. S. Dodd, D. R. Ball, M. A. Schroeder, **L. M. Le Page**, H. J. Atherton, L. C. Heather, A-M. Seymour, H. Ashrafian, H. Watkins, K. Clarke, D. J. Tyler, 'In vivo alterations in cardiac metabolism and function in the spontaneously hypertensive rat heart', *Cardiovascular Research*, 2012