

Pharmacological manipulation of hypoxic signalling as a therapeutic target to improve metabolism and function in the type 2 diabetic heart



Maria da Luz Sousa Fialho

Wadham College

University of Oxford

A thesis submitted for the degree of

Doctor of Philosophy

Trinity 2018

Acknowledgements

I would like to thank the British Heart Foundation (BHF) for the Studentship, which allowed me to take on this DPhil project over the past four years. It has been a pleasure to be part of the great work funded by the BHF, and to attend symposia, funding events, and lab visits where I could meet the incredible people behind this valuable organisation. I would also like to thank the Department of Physiology, Anatomy and Genetics (DPAG) for the support and environment provided during my studies. I am eternally grateful to all members of the Cardiac Metabolism Research Group (CMRG) and its subgroup leaders. I would like to express my gratitude to Prof. Kieran Clarke, Prof. Carolyn Carr, Dr Rhys Evans, and my wonderful co-supervisor Prof. Damian Tyler, for always being available to support and advise me, and for providing constructive feedback throughout my DPhil.

I owe my deepest gratitude to my supervisor Prof. Lisa Heather, without whom this work would not have been possible. Without her guidance, support, advice, and encouragement, this work would hardly have been completed. I am also extremely grateful to Jon, Evie, and Poppy Shaw for allowing Prof. Heather time to review my thesis chapters.

I would like to thank our collaborators Prof. Jan Glatz, Dr Joost Luiken, and Mr Will Coumans (Maastricht University, The Netherlands) for their help and collaboration on the FAT/CD36 inhibition study in Chapter 3; and Prof. Julian Griffin and Dr James West (University of Cambridge) for their assistance and collaboration in Metabolomics for Chapters 3 and 4.

I am grateful to all members of CMRG, past and present, for their continuous assistance and support. Never have I met such a welcoming, positive, supportive laboratory group. I am truly pleased to have been a part of it, and hope to have contributed accordingly. I would like to thank Mrs Vicky Ball for her invaluable help with animal work. I would not have been able to carry out my initial experiments without her generous assistance. I would also like to thank her for her optimism, encouragement, and entertainment near the end of my DPhil, which helped me to complete this thesis. I would also like to thank Dr Latt Mansor for the training and advice which he provided, and for the work he contributed to Chapter 3. Additionally, I must thank Miss Elizabeth Eggleston, for helping me settle in at the start, Dr Lucia Giles, for her support and company in the lab and at Wadham

College; Dr Arne Bruyneel, for his help and entertainment during the start of my DPhil; Dr Jack Miller for his singing, and for teaching me more than I would ever have known about the World (and actually, not just Physics!); Mrs Nicola Dixon, for bringing us back down to Earth when we got too "sciencey"; Mrs Yvonne Green, for some of the loveliest Christmas dinners I have ever attended; Dr Cher-Rin Chong, for always making me feel positive about the present; Dr Kertin Timm, for bringing an extra European feel to the group; and Dr Michael Dodd, for being a great conference buddy, and a great postdoc to work with.

I would also like to thank the project students that helped complete the work in this thesis. Ms Helen Thompson (Chapter 4), Mr George Stannard (Chapter 4), and Ms Noelia Diaz (Chapter 5) provided invaluable help with the work carried out, and supervising them was a great experience for me. Ms Claudia Montes also provided precious help during her Master's project in our group, and I would like to thank her for her assistance, and for her friendship, which has been a fundamental source of support throughout the last part of this DPhil. I also thank Miss Megan Mary Thomas for her professional help in preparing Figure 2.4 of this thesis.

Special thanks go to my fellow CMRG DPhil students, who have made this journey so very special, and who helped me to persevere when I was carrying the weight of the World on my shoulders. I thank Mr Azrul Danial Haikal for his motivation. I thank Dr Sophia Malandraki-Miller, for being one of the most understanding people I have met, and for her priceless advice; soon-to-be Dr Dragana Savic, for always listening, and for knowing what to say even before I started speaking; soon-to-be Dr Colleen Lopez, for bringing extra fun into my life, and for being an amazing housemate; Mr Matthew Kerr, for being one of the kindest men I have ever encountered, for always being there, and for reminding me not to take some things too seriously. I could not have wished for more generous, admirable friends. I thank Ms Marta Madureira for her warm, supportive encouragement and friendship. I would also like to thank my friends all over the World, who have always supported me.

This thesis would not have been completed without the help and support from my dearest partner, Dr Alexander Vervuurt. My deepest thanks go to him for his kindness, patience, love, and friendship, and for challenging me to think in new and different ways.

Finally, I would like to express my gratitude and appreciation to my family for their encouragement and unconditional support. I would like to thank my mother for giving me the example of patience and kindness, and for being the best friend a daughter could ask for. I am grateful to my father, for teaching me that the passing of time is a friend, not a foe. And I am eternally grateful to my brother, João Maria, for reminding me to always consider what matters most.

Abstract

The work presented in this thesis investigates the metabolic and functional benefits of metabolic modulatory treatment strategies in a rodent model of type 2 diabetes. The first strategy, using a fatty acid translocase (FAT/CD36) inhibitor was investigated, in an attempt to reduce fatty acid oxidation and force glucose uptake and glycolysis. A second strategy, using prolyl hydroxylase domain (PHD) inhibitors, was investigated, to activate hypoxia inducible factor 1 alpha (HIF-1 α) to stimulate glycolysis and inhibit fatty acid oxidation. Type 2 diabetes was induced using high fat feeding and low dose streptozotocin in male Wistar rats. Hearts were excised for Langendorff perfusion, to assess substrate metabolism and cardiac function in the intact contracting organ. The use of *ex vivo* treatment with fatty acid uptake inhibitor sulfo-*N*-succinimidyl oleate (SSO) was investigated using a protocol of acute hypoxia and reoxygenation. The use of *in vivo* treatment with the prolyl hydroxylase domain inhibitors, dimethyloxallylglycine (DMOG) and Molidustat, (BAY85-3934) was investigated using a protocol of acute low-flow ischaemia and reperfusion. Diabetic hearts showed a significant impairment in functional recovery following both hypoxia-reoxygenation and ischaemia-reperfusion, and this was associated with lower glycolytic rates, and higher fatty acid oxidation rates, compared with controls. Metabolomics analysis revealed non-uniform changes to the Krebs cycle intermediates in diabetes, with a depletion of intermediates in the first span of the cycle, and accumulation of intermediates within the second span. When perfused hearts were given SSO just four minutes prior to the onset of hypoxia, a significant improvement in functional recovery was seen in diabetic hearts, which displayed similar recovery levels to control hearts. This improvement was associated with decreased fatty acid oxidation rates, increased glycolytic rates, and decreased triacylglyceride accumulation following hypoxia.

Diabetic hearts from rats given three doses of DMOG (40 mg/kg) *in vivo* showed significantly improved functional recovery following ischaemia-reperfusion. However, this was not accompanied by the predicted improvement in substrate metabolism. No significant changes were seen in glycolytic rate, instead fatty acid oxidation was found to be significantly increased in DMOG-treated diabetics. Changes in several metabolites found through metabolomics suggested the metabolic effect seen with DMOG treatment was likely due to off-target effects on other α -ketoglutarate-dependent enzymes. No evidence was found for significant upregulation of HIF-1

signalling with DMOG treatment, other than increased VEGFA mRNA. Additionally, hearts from control rats that received DMOG recovered to a much lesser extent than untreated control hearts following ischaemia-reperfusion.

Diabetic hearts from rats treated with five daily oral doses of Molidustat (5 mg/kg) *in vivo* also showed significantly improved functional recovery following ischaemia-reperfusion. This was paired with increased baseline glycolytic rates, and decreased fatty acid oxidation rates. Molidustat treatment resulted in significantly increased blood haemoglobin levels in both control and diabetic rats, suggesting stimulation of HIF-1 signalling, although no increases in HIF-1 α protein or downstream target glucose transporter 1 (GLUT1, SLC2A1) were found in cardiac tissue from treated rats.

This work has validated the potential of metabolic modulation as a therapeutic avenue for the treatment of the diabetic heart, harnessing substrate metabolism as a driving force for functional improvement, and to improve function under stress conditions. We have shown that metabolic modulation improves the diabetic heart's ability to recover in acute hypoxia, and that hypoxic signalling upregulation improves recovery in ischaemia. This provides a new opportunity for the investigation and development of drugs which can selectively target metabolism in the heart to relieve the long-term maladaptation caused by diabetes, allowing it to regain its inherent metabolic flexibility, which is critical for survival under stress.

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List of Abbreviations

ACADM		Medium-chain acyl-coA dehydrogenase
ADP		Adenosine diphosphate
AMP		Adenosine monophosphate
AMPK		AMP-activated protein kinase
ATP		Adenosine triphosphate
CAT		Carnitine acyltranslocase
CBP		CREB binding protein
CPT		Carnitine palmitoyl transferase
DCM		Diabetic cardiomyopathy
DMOG		Dimethyloxalylglycine
EPO		Erythropoietin
FABP_{pm}		Fatty acid binding protein (plasma membrane)
FAD⁺		Flavin adenine dinucleotide
FADH		Reduced flavin adenine dinucleotide
FAT/CD36		Fatty acid translocase
FATP (SLC27)		Fatty acid transporter protein
FIH		Factor inhibiting HIF
F-2,6-BP		Fructose-2,6-bisphosphate
GLUT (SLC2A)		Glucose transporter
G-6-P		Glucose-6-phosphate
HBS		HIF binding sites
HIF		Hypoxia-inducible factor
HMOX1		Haem oxygenase 1
HRE		HIF response elements
IFM		Interfibrillar mitochondria

KO	Knock-out
LCFA	Long chain fatty acid
LDH	Lactate dehydrogenase
MCT	Monocarboxylate transporter
MI	Myocardial infarction
mRNA	Messenger ribonucleic acid
NAD⁺	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NEFA	Non-esterified fatty acid
PCR	Polymerase chain reaction
PDH	Pyruvate dehydrogenase
PDK	Pyruvate dehydrogenase kinase
PFK	Phosphofructokinase
PGK	Phosphoglycerate kinase
PHD	Prolyl hydroxylase domain
PPAR	Peroxisome proliferator-activated receptor
pVHL	von Hippel-Lindau tumour suppressor
ROS	Reactive oxygen species
RPP	Rate-pressure product
SSM	Subsarcolemmal mitochondria
SSO	Sulfo-N-succinimidyl oleate
STZ	Streptozotocin
T2DM	Type 2 diabetes mellitus
UCP	Uncoupling protein
VEGF	Vascular endothelial growth factor

*Go to your bosom; Knock there, and ask your heart
what it doth know.*

— William Shakespeare

1

General Introduction

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1.1 The burden of type 2 diabetes

The 2016 WHO global report on diabetes found that 422 million adults were living with diabetes mellitus in the year 2014, equating to a doubling of the global prevalence of diabetes since 1980 [158]. Type 2 diabetes mellitus (T2DM) accounts for over 90% of all cases of diabetes, and in the past was almost exclusively seen in adults, but is now becoming prevalent in children too [158]. Additionally, there is a worrying economic burden associated with diabetes, which according to the International Federation of Diabetes (IDF) was estimated to have tripled over the period 2003 to 2013 as a result of increased incidence of diabetes worldwide [61]. Patients with T2DM are likely to develop a number of microvascular complications (diabetic nephropathy, neuropathy, and retinopathy) and macrovascular complications (coronary artery disease, peripheral arterial disease, and stroke) [71]. Though all diabetic complications cause some degree of morbidity, cardiovascular disease is the major cause of mortality in these patients. T2DM is an independent risk factor for cardiac events, and not only is it associated with reduced survival rate following a myocardial infarction (MI), but the baseline risk of MI in diabetic patients is similar to that of nondiabetics who have already had a previous MI [85, 77]. The lack of oxygen and metabolic substrate supply to the myocardium, resulting from the ischaemic period during an MI, is likely to play a key role in the effects observed in diabetic patients. From a therapeutic perspective, it is therefore invaluable to better understand the mechanisms underlying ischaemia-reperfusion injury and how these may differ in the diabetic heart.

Insulin resistance is one of the hallmarks of this disease, which highlights the key role of metabolic dysregulation in the pathophysiology of T2DM [199]. Insulin, a hormone secreted by the β -cells of the pancreas, is one of the key regulators of systemic metabolism, as it acts as the signal for upregulation of glucose metabolism following feeding [73]. The current guidelines for diagnosis of T2DM are: a random

venous plasma glucose concentration ≥ 11.1 mmol/l, or a fasting plasma glucose concentration ≥ 7.0 mmol/l, or plasma glucose concentration ≥ 11.1 mmol/l two hours after 75g anhydrous glucose in an oral glucose tolerance test (OGTT) [157]. It is important to note that T2DM is a progressive disease and, for this reason, a single high blood glucose measurement alone is not enough for firm diagnosis. Late stage T2DM is characterised by systemic hyperglycaemia, hypoinsulinaemia, dyslipidaemia and peripheral insulin resistance. However, a patient may develop insulin resistance, and early-stage T2DM, before developing β -cell impairment [175]. Unfortunately, as there are virtually no symptoms associated with the initial stages of the pathophysiology, the disease may go unnoticed for many years, but this slow progression at least offers some opportunity for early intervention when the disease or its cardiac complications could still be prevented.

1.2 Myocardial metabolism in the healthy heart

The normal healthy heart relies on a constant supply of oxygen and metabolic substrates to ensure its continuous contraction, requiring approximately 6kg of adenosine triphosphate (ATP) per day [156]. It therefore comes as no surprise that this is one of the most tightly metabolically regulated organs in the body, as well as the most metabolically flexible. The heart can be viewed as a metabolic omnivore, able to metabolise a range of different substrates, including fatty acids, glucose, pyruvate, ketone bodies, and lactate [193, 208]. This allows for a degree of metabolic flexibility, switching between substrates in response to changing physiological conditions, including substrate availability, hormone levels, oxygen supply, coronary blood flow, and workload [194, 52]. Under normal physiological conditions, the healthy heart uses 60-70% fatty acids for energy generation [156, 130]. Pancreatic hormones, insulin and glucagon, are responsible for maintaining tight control of systemic metabolism, by regulating the uptake and use of glucose in several organ systems. Insulin stimulates glucose uptake in muscle (including the heart) and fat, and inhibits hepatic glucose production following feeding [176]. Another level of regulation is achieved within the heart muscle cells themselves to maintain energy homeostasis in extreme conditions, when adenosine monophosphate (AMP) levels start to rise, creating a shift in the AMP:ATP ratio. This shift is sensed by the protein complex AMP-activated protein kinase (AMPK), which acts to stimulate glycolysis, inhibit glycogen synthesis, inhibit fatty acid synthesis and stimulate fatty acid uptake and β -oxidation [106].

1.2.1 Fatty acid metabolism

Circulating fatty acids are either transported as esterified triacylglycerol (TAG) in lipoproteins such as chylomicrons, or as non-esterified fatty acids (NEFA) bound to albumin. NEFAs are primarily taken up by transport mechanisms involving fatty acid translocase (FAT/CD36), plasma membrane fatty acid binding protein

(FABPpm) and fatty acid transporter proteins (SLC27A) 1 and 6. Once in the cytoplasm, fatty acids can be esterified by acyl-CoA synthetase, and either enter the intracellular TAG pool or enter fatty acid oxidation (Fig. 1.1). This allows entry through the outer mitochondrial membrane, and carnitine palmitoyltransferase-1 (CPT1) in the intermembrane space then substitutes the CoA group on the long chain acyl-CoA with carnitine, which allows the newly formed acyl-carnitine to be shuttled across the inner mitochondrial membrane by carnitine acyltransferase (CAT) in exchange for free carnitine [52, 127] (Fig. 1.1). The regenerated long chain fatty acyl-CoA can then undergo β -oxidation in the mitochondrial matrix, leading to the production of acetyl-CoA and reduced cofactors NADH and FADH₂, which can enter the electron transport chain to produce ATP [127]. CPT1 serves as a key regulatory step in mitochondrial fatty acid oxidation, through its inhibition by malonyl-CoA (synthesised by acetyl CoA carboxylase in the fatty acid synthetic pathway) [52]. In the same way that long chain acyl-CoAs must be transported across the mitochondrial membrane through CPT with carnitine, acetyl CoA is transferred from the mitochondria to the cytoplasm as citrate, which can freely cross the mitochondrial membrane, and is then cleaved by ATP-citrate lyase to form acetyl CoA in the cytoplasm [37, 12].

1.2.2 Glucose metabolism

In the myocardium, glucose is taken up by glucose transporters, either SLC2A1 or SLC2A4 and enters glycogenesis, the pentose phosphate pathway, or the glycolytic pathway. An important distinction exists between the isoforms of cardiac glucose transporters, whereby SLC2A1 is responsible for basal glucose uptake, while SLC2A4 is known as the insulin-sensitive glucose transporter, cycling between the sarcolemma and intracellular vesicles in response to insulin stimulation and AMPK activation, and therefore serving as the primary regulator of glucose uptake [90]. The phosphorylation of glucose to glucose-6-phosphate (G-6-P) by hexokinase is the first step of glucose metabolism, and G-6-P acts as a negative feedback inhibitor of hexokinase when it begins to accumulate if the downstream

steps of glycolysis are slowed down (Fig. 1.2). In the heart, glycolysis is the major pathway for glucose utilisation, generating pyruvate, NADH, and a small amount of ATP via substrate-level phosphorylation. This is invaluable to cardiac energy metabolism, as it provides an oxygen-sparing alternative to oxidation under anaerobic conditions. Phosphofructokinase-1 (PFK-1) presents a key regulatory step in the glycolytic pathway, which is modulated by the cellular energy status and metabolite concentrations [168, 169]. The end product of glycolysis, pyruvate, can either be reduced to lactate in the cytosol or enter the mitochondria for oxidation. Under anaerobic conditions, lactate dehydrogenase reduces pyruvate to lactate for export, utilising NADH derived from glycolysis. Under aerobic conditions, pyruvate enters the mitochondria and is converted to acetyl CoA prior to entry into the Krebs cycle for glucose oxidation (Fig. 1.2). The oxidative decarboxylation of pyruvate is catalysed by pyruvate dehydrogenase (PDH), a key regulated enzyme complex in glucose metabolism [93] (Fig. 1.4). PDH is phosphorylated and inhibited by pyruvate dehydrogenase kinase (PDK) enzymes 1-4, which are activated by NADH and acetyl-CoA. Additionally, PDH can be dephosphorylated and activated by insulin signalling and calcium via PDH phosphatase [132].

Anaplerotic role of pyruvate

Levels of oxaloacetate can be replenished by the carboxylation of pyruvate directly into oxaloacetate, via the action of pyruvate carboxylase [103]. This allows the Krebs cycle to be rapidly replenished if intermediates are drawn for biosynthetic pathways. Pyruvate carboxylase is positively regulated allosterically by acetyl CoA, as oxaloacetate will be consumed when acetyl CoA is available [159]. The anaplerotic role of pyruvate via its carboxylation is therefore crucial to Krebs cycle functioning and maintenance, which in turns is essential to normal myocardial functioning. This mechanism has been implicated in cardiac function and disease [174].

Alternative glucose metabolic pathways

Glucose metabolism can also branch into pathways other than glycolysis, including the pentose phosphate pathway and the hexosamine biosynthetic pathway.

The pentose phosphate pathway is one such branch of glycolysis, producing pentoses and NADPH, as well as ribose-5-phosphate, which is a precursor for generation of nucleotides, and erythrose 4-phosphate, a precursor for aromatic amino acids [95, 96].

The hexosamine pathway accounts for only a small proportion of glucose utilisation, and produces UDP-N-acetylglucosamine (UDP-GlcNAc) as its end product [141]. Diabetes has been linked to increased hexosamine biosynthesis, with increased N-acetylglucosamine in O-linkage in proteins, possibly leading to changes in the development and progression of insulin resistance [50, 30, 141].

1.2.3 The Randle cycle

As mentioned previously, the healthy heart exhibits a degree of metabolic flexibility. This is primarily achieved through a phenomenon known as the Randle cycle (or glucose-fatty acid cycle). This phenomenon was first described by Philip Randle in his 1963 publication illustrating the competition between glucose and fatty acids, and the metabolic inhibition of one by the other in the absence of any hormonal input [168]. This mechanism prevents fuel waste by ensuring that two substrates are not simultaneously broken down. When the heart oxidises fatty acids, it has been shown that key steps in glucose utilisation, including glucose uptake, hexokinase, PFK and PDH, are inhibited [168, 169]. We now know that when β -oxidation is increased, this causes an increase in acetyl-CoA and NADH, and these in turn are able to inhibit PDH by activating PDKs [191]. An increase in acetyl-CoA also increases citrate concentrations via the Krebs cycle, and the citrate efflux inhibits PFK1 [34]. This inhibition of PFK1 leads to an increase in G-6-P, which in turn inhibits hexokinase [42]. If the increase in fatty acid metabolism is prolonged, the fatty acids present in the cell can bind to the transcription factor peroxisome proliferator-activated receptor α (PPAR α), stimulating the transcription and expression of several genes involved in fatty acid uptake as well as mitochondrial and peroxisomal fatty acid β -oxidation, including FAT/CD36, medium chain acyl-CoA dehydrogenase (ACADM) and uncoupling protein 3 (UCP3) [181, 48, 217]

(Fig. 1.4). Additionally, PPAR α also upregulates PDK4 mRNA and protein levels in the heart, which leads to the inhibition of glucose metabolism [93, 92].

On the other hand, an increase in glucose metabolism leads to the inhibition of fatty acid oxidation [168]. An increase in glycolysis will produce increasing amounts of acetyl-CoA, which can be carboxylated by acetyl-CoA carboxylase (ACC) to produce malonyl-CoA, a potent inhibitor of CPT1, which will prevent entry of fatty acids into the mitochondria for oxidation [128]. ACC is an important point of Randle cycle regulation, as both citrate and glutamate are allosteric activators of ACC, whereas long and medium chain fatty acids are negative feedback inhibitors of ACC [142, 60]. Hormonal regulation is, of course, another important player in cardiac metabolic flexibility, as it allows tissues to shift their metabolism in response to changing systemic conditions. Insulin, for instance, plays a role in ACC regulation by stimulating its activation via dephosphorylation, as well as stimulating glucose uptake and glycolytic enzymes [176].

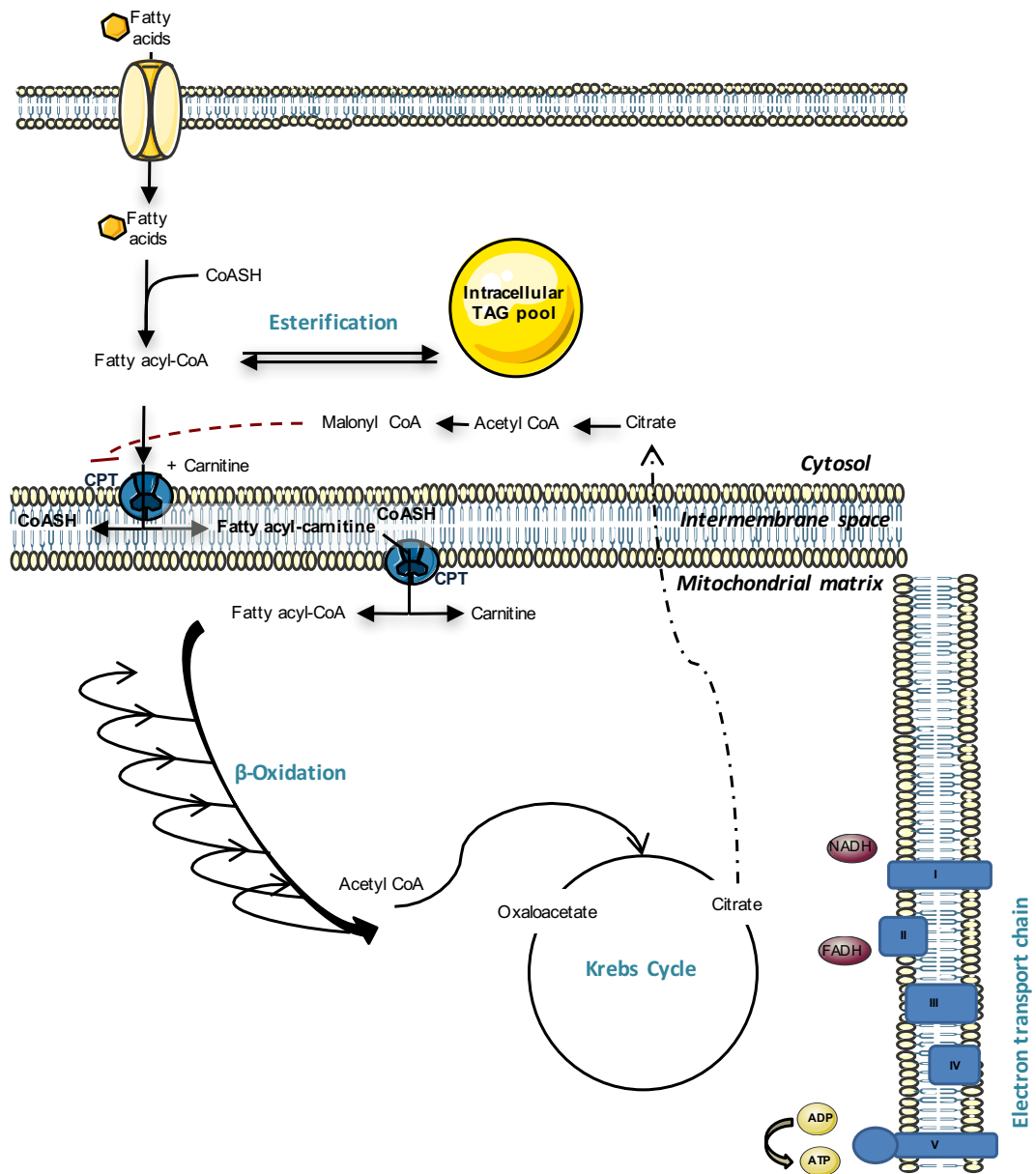


Figure 1.1: Fatty acid uptake and utilisation in the heart. Non-esterified fatty acids (NEFAs) enter the cardiomyocyte either through passive mechanisms or through transporters such as FAT/CD36, FABPpm and SLC27A1. Once inside the cell, NEFAs can then undergo esterification to fatty acyl-CoA, and enter the cellular triacylglyceride pool, or enter the mitochondria following conversion to fatty acyl-CoA. Transportation into the mitochondria occurs through CPT1, using carnitine. This can be allosterically inhibited by malonyl-CoA, as a feedback inhibition mechanism. Once in the mitochondrial matrix, fatty acyl-CoA can undergo β -oxidation, producing acetyl CoA to be used in the Krebs cycle, and reduced cofactors NADH and FADH₂, which enter the electron transport chain to produce ATP. Acetyl CoA can be transported to the cytoplasm through the movement of citrate, which is able to cross the mitochondrial membrane when the mitochondrial concentration is elevated, and can be cleaved by ATP-lyase once in the cytoplasm, forming acetyl CoA.

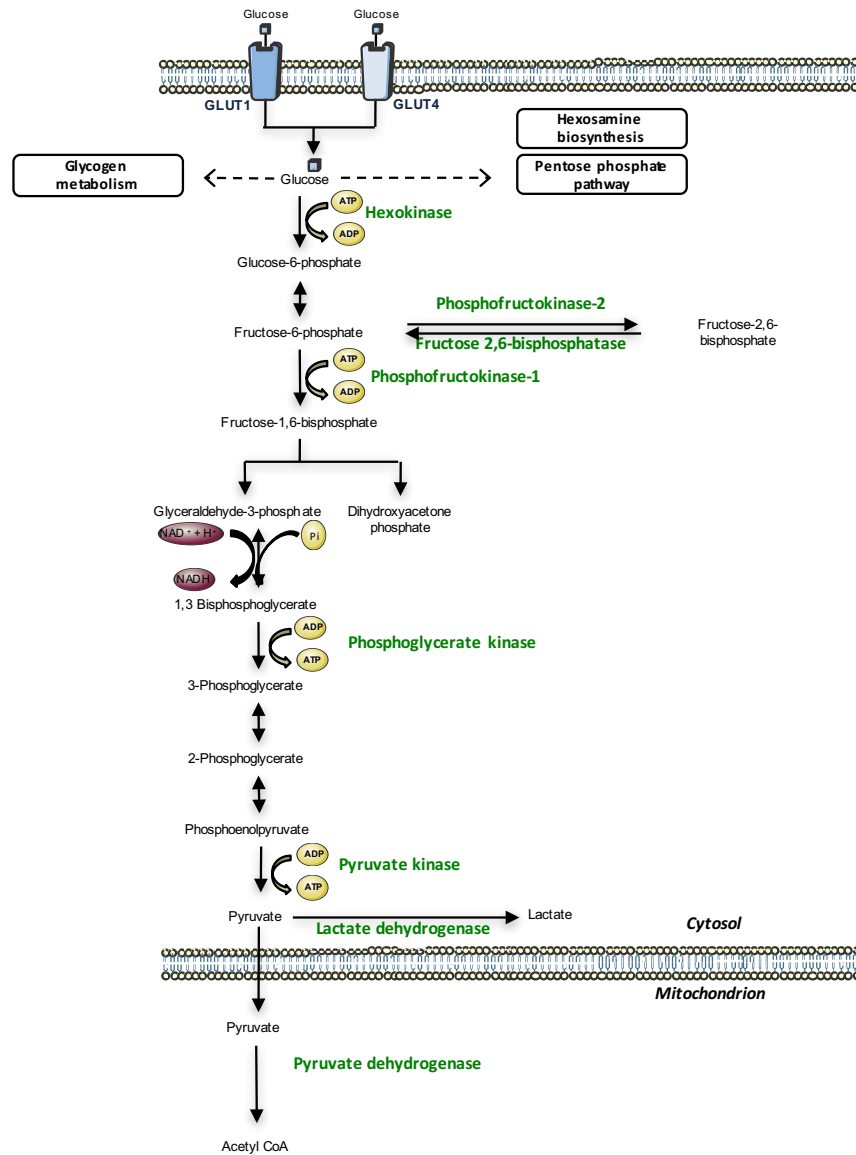


Figure 1.2: Glucose uptake and utilisation in the heart. Glucose enters the cardiomyocyte through glucose transporters (GLUT) 1 and 4 (SLC2A1 and SLC2A4). It is then rapidly phosphorylated by the enzyme hexokinase to glucose-6-phosphate, which subsequently enters many metabolic pathways including glycolysis, glycogenesis, the pentose phosphate and hexosamine biosynthetic pathways, though glycolysis is the main pathway for glucose use in the heart. Glycolysis produces a small quantity of ATP anaerobically, as well as generating pyruvate, which can enter the mitochondria for oxidation. Mitochondrial pyruvate dehydrogenase (PDH) catalyses the oxidative decarboxylation of pyruvate, and is a key regulated enzyme complex in glucose metabolism.

1.3 Cardiac metabolic dysfunction in type 2 diabetes

In the T2DM heart, energy metabolism is greatly remodelled, due to systemic dysregulation of glucose and fatty acid metabolism. Interestingly, the T2DM heart is exposed to both increased glucose and increased NEFA concentrations, but changes its metabolism in favour of fatty acids. Cardiac metabolic flexibility is consequently impaired, as the presence of insulin can no longer stimulate SLC2A4 translocation to the sarcolemma, and glycolysis is not upregulated when required. In T2DM, we see increased cardiac fatty acid uptake, increased fatty acid oxidation, and increased cardiac triacylglyceride (TAG) accumulation (Fig. 1.3) [201, 59, 23]. In parallel, glucose uptake is decreased, as are glycolysis and glucose oxidation [17, 196, 86].

The T2DM heart is therefore at risk, as it is predisposed to take up fatty acids to a much greater extent, and has decreased ability to take up glucose. Though the pathophysiology of diabetes and insulin resistance is not yet fully understood, many of the mechanisms at play in this disease state have been studied and elucidated, and our understanding of these at the molecular level has unravelled over the past few decades. Perhaps noteworthy is the role of fatty acids in the development of insulin resistance. The increase in NEFA concentration in the blood might also lead to increased triglyceride deposition within the liver, skeletal muscle, and cardiac tissue, as well as other lipid intermediates that may have a role in lipotoxicity, such as diacylglycerol, fatty acyl-CoA and ceramides [185].

There is an increasing body of evidence suggesting that increased circulating NEFA levels can stimulate increased hepatic glucose output, leading to increased circulating glucose levels, further inhibiting glucose uptake and utilisation in peripheral tissue such as heart and skeletal muscle via a Randle cycle effect [17]. The decrease in glucose utilisation means that insufficient glycolysis-derived acetyl-CoA is produced for ACC to generate malonyl-CoA, which could inhibit CPT1 in order

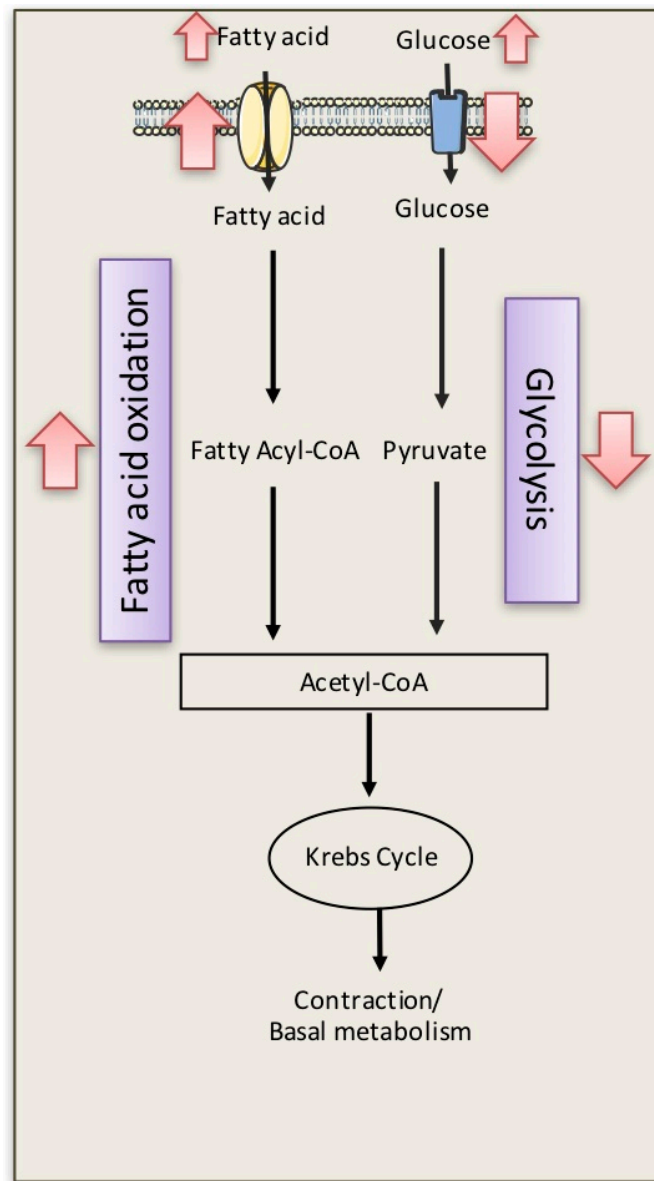


Figure 1.3: Effects of type 2 diabetes on metabolic processes. Circulating levels of non-esterified fatty acids and glucose are increased. Fatty acid uptake is increased, while glucose uptake is decreased. Fatty acid oxidation is increased, while glycolysis is decreased.

to limit fatty acid oxidation. This sustained increase in fatty acid metabolism in the T2DM heart allows for the stimulation of PPAR α activity, increasing the expression of a number of its metabolic target genes, which further aggravates the metabolic imbalance [65]. For instance, increased PDK4 expression leads to the greater inhibition of PDH flux, a further inhibition of glucose utilisation and oxidation, allowing for a continued increase in fatty acid β -oxidation (Fig. 1.4).

Another PPAR α target, ACADM, is responsible for the oxidation of medium chain (6 to 12 carbon) fatty acids in β -oxidation, yet another stimulus for increased fatty acid utilisation. Furthermore, UCP3 expression is upregulated, which uncouples mitochondrial respiration from ATP synthesis [24].

Together, these chronic adaptations in diabetes result in cardiac metabolic inflexibility. The heart is unable to change substrate metabolism in response to insulin, leading to a self-perpetuating disease state. Additionally, the heart becomes metabolically inflexible in response to other stimuli, such as AMPK activation. Cardiac flexibility is particularly important in the event of a myocardial infarction, when the heart needs to sustain function in low oxygen and substrate conditions. However, the insulin resistant state and the sustained increase in fatty acid metabolism seen in the T2DM heart do not allow for this metabolic flexibility and put the heart at greater risk under an ischaemic insult. Additionally, the increase in lipid uptake and metabolism leads to increased peroxidation by reactive oxygen species, and therefore potential damage to the mitochondria which may decrease mitochondrial oxidative capacity. This is particularly dangerous in a heart that is unable to upregulate anaerobic metabolism when required.

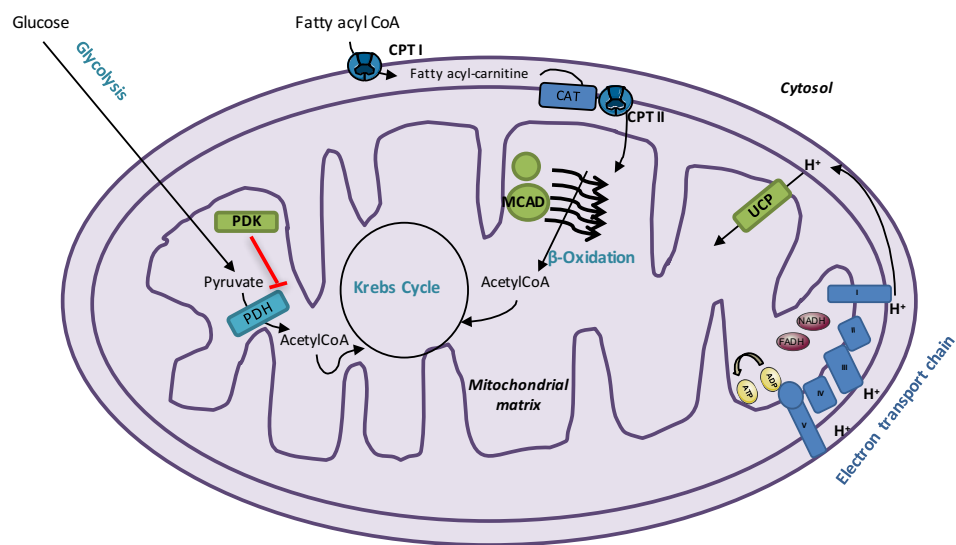


Figure 1.4: Mitochondrial metabolism and PPAR α target genes. A number of PPAR α target genes act to regulate mitochondrial metabolism, stimulating fatty acid oxidation and inhibiting glucose, by inhibiting incorporation of acetyl-CoA from pyruvate into the Krebs cycle. PDH: pyruvate dehydrogenase; PDK: pyruvate dehydrogenase kinase; CPT: carnitine palmitoyl transferase; CAT: carnitine acyltransferase; MCAD: medium-chain acyl-CoA dehydrogenase; UCP: uncoupling protein.

1.4 Hypoxic response and HIF signalling

1.4.1 Regulation of HIF

Hypoxia, a lack of oxygen availability, is likely to be encountered in diabetes, either due to ischaemia in the event of a myocardial infarction, or due to co-morbidities often seen in T2DM, such as sleep apnoea, lung disease, or anaemia [122]. The human body, like many organisms ranging from Cnidarian sea anemones to mammals, are well adapted to coping with a hypoxic insult, via highly conserved mechanisms. These mechanisms involve the activation of the hypoxia-inducible factors (HIFs), a family of obligate heterodimeric transcription factors whose target genes are key in mediating the cellular response to hypoxia [186]. The heterodimers are formed of an unstable α -subunit, and a constitutively-expressed aryl hydrocarbon receptor nuclear translocator (ARNT), which forms the β -subunit [102, 202]. Stabilisation of the α -subunit is tightly and acutely controlled by cellular oxygen concentrations (see Fig. 1.5). Under normal oxygen tension, the HIF- α subunits have a very short half-life (<5 min) and are continually transcribed, translated and degraded. This occurs because specific prolyl hydroxylase domain (PHD) enzymes are able to hydroxylate certain highly-conserved HIF- α proline residues [76] (Fig. 1.5). The Von Hippel-Lindau tumour suppressor protein (pVHL) is then able to bind the hydroxylated residues, forming the substrate recognition component of the E3 ubiquitin ligase complex [155]. This activates the polyubiquitination of specific lysine residues in HIF- α , tagging it for proteasomal degradation [155]. In hypoxia, however, the lack of oxygen availability results in the inability of PHD enzymes to hydroxylate HIF- α subunits, therefore allowing it to be stabilised and to translocate to the nucleus, where it can dimerise with its β -subunit [104]. The HIF- α/β complex recruits transcriptional co-activators p300 and CREB-binding protein (CBP) [170], allowing it to bind to specific hypoxia response elements (HRE) promoter regions of target genes to initiate their transcription [104]. The recruitment of these

transcriptional co-activators is also oxygen-dependent and provides another point of regulation, as it is inhibited by factor inhibiting HIF (FIH) [135]. FIH is an asparaginyl hydroxylase, which catalyses the hydroxylation of conserved asparagine residues in the HIF COOH-terminal transactivation domain (CAD) (N803 in HIF-1, N847 in HIF-2) under normoxic conditions, resulting in a steric clash with the coactivator protein p300, thereby preventing transcriptional activation of target genes [144]. Though other pathways have been identified that interact with HIF, the PHD-VHL-HIF axis is widely accepted as the primary regulatory pathway of the cellular hypoxic response.

1.4.2 HIF and its target genes

HIF- α/β complexes can bind the HREs at specific binding sites known as HIF binding sites (HBS), which include the consensus sequence RCGTG (where R is A or G). The ability of HIF complexes to bind to the HBS is also affected by epigenetic mechanisms such as CpG methylation or DNA damage due to the formation of reactive oxygen species (ROS) [206, 171].

In humans, there are three isoforms of HIF- α : HIF-1 α , HIF-2 α , and HIF-3 α . Although there is good evidence for the responsiveness of all three isoforms to low oxygen tension, resulting in their binding to HRE core sequences, the effects of HIF-3 α activation have not yet been well characterised. Several splice variants of HIF-3 α have been found, with high cardiac expression in humans [145]. In the mouse, one splice variant of HIF-3 α has been identified as the inhibitory Per/Arnt/Sim domain (IPAS) [136], a protein previously identified as an inhibitory regulator of the hypoxic response [136, 49]. This variant lacks the carboxy-terminal region of the protein, and therefore lacks the CAD transactivation domain [136]. It is now known that activation of HIF-1 α leads to the transcription of HIF-3 α , which may have a regulatory role in decreasing the HIF signalling response following the acute hypoxic insult, in preparation for the effects of reoxygenation [81, 46, 47].

HIF-1 α and HIF-2 α have been studied more extensively, and although the two share similar protein sequences and structures, the two isoforms have been

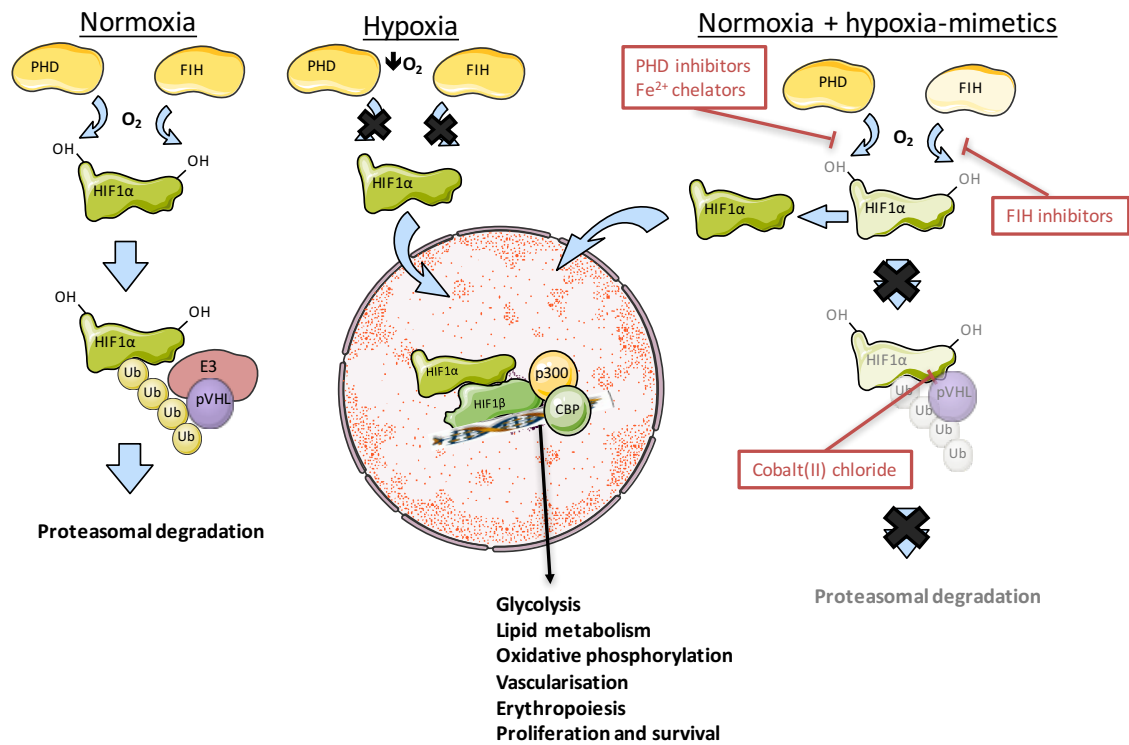


Figure 1.5: HIF-1α regulation and signalling. Under normoxic conditions, HIF-1α is constantly synthesised, hydroxylated and degraded. This hydroxylation occurs through the action of Prolyl Hydroxylase Domain (PHD, EGLN) and Factor Inhibiting HIF (FIH) enzymes, which are oxygen dependent. Once hydroxylated, HIF-1α is then poly-ubiquitinated by pVHL-E3 ligase complex and undergoes subsequent proteasomal degradation. In hypoxia, the PHDs and FIH are inhibited by lack of their substrate oxygen, so cannot hydroxylate HIF-1α, which is therefore stabilised and able to translocate to the nucleus, where it binds to HIF-1β. This complex recruits co-activators of transcription p300 and CBP, which allows the initiation of transcription of target genes. Compounds developed as hypoxia-mimetics target either the PHDs or FIH, preventing hydroxylation of HIF-1α and allowing for stabilisation and transcription of target genes under normoxic conditions.

shown to mediate different physiological responses, with distinct target genes [99]. *In vitro* work has shown that HIF-1α drives the acute hypoxic response (within 24h), and HIF-2α mediates the response during a chronic hypoxic challenge (>24h) [91, 111]. This shows that the cellular hypoxic response is a complex mechanism made up of different isoforms of HIF, each with specific temporal and functional roles. Broadly, HIFs regulate the transcription of hundreds of target genes, which allow the cell to adapt to the new lower oxygen environment, downregulating processes

that consume oxygen, upregulating anaerobic processes, and contributing to the restoration of normal oxygen delivery. Metabolism is one of the key targets of the hypoxic adaptation regulated by HIF, since oxygen is the terminal electron acceptor in oxidative phosphorylation. Cellular metabolism shifts away from oxygen-using processes and favours oxygen-sparing anaerobic glycolysis [39]. This is crucial in allowing the cell to generate ATP under low oxygen tension. Hypoxia-responsive genes may respond exclusively to HIF-1 α , or to HIF-2 α , or to both. Additionally, HIF-1 α and HIF-2 α do share certain target genes [98]. HIF-1 α promotes the expression of genes encoding proteins and enzymes involved in the regulation of the glycolytic pathway, and for this reason is of particular interest to our work. These include glucose transporter 1 (GLUT1, SLC2A1), phosphoglycerate kinase (PGK), and lactate dehydrogenase A (LDHA). Both HIF-1 α and HIF-2 α regulate the expression of SLC2A1). HIF-1 signalling activation therefore leads to increased glycolysis, and increased glucose uptake, as well as decreased expression of peroxisome proliferator-activated receptor alpha (PPAR α) targets, such as PDK4, ACADM, and UCP3, leading to decreased fatty acid oxidation rates. The Randle effect produced by stimulating glycolysis via HIF also acts to intracellularly inhibit fatty acid oxidation. HIF-1 also regulates cytochrome c oxidase (COX; complex IV) to decrease oxidative phosphorylation and optimise oxygen efficiency [182, 74]. HIF also regulates the expression of genes involved in oxygen delivery, including erythropoietin (EPO) and vascular endothelial growth factor (VEGF), which help accelerate the process of restoring a normoxic environment in response to hypoxia [203, 69]. The effects of hypoxia will vary across different tissues, which can be explained by the fact that the expression patterns of different isoforms of both the HIF transcription factors and the regulatory HIF hydroxylases are tissue-specific, and that different tissues will naturally be exposed to varying degrees of hypoxia depending on the hypoxic insult in question.

1.4.3 Regulation of HIF hydroxylases

The prolyl hydroxylases are part of the non-haem Fe(II) and α -ketoglutarate (α -KG)-dependent dioxygenase superfamily of proteins. Each oxygen bound is split, with one atom of oxygen used to catalyse the decarboxylation of α -KG into succinate and CO_2 , and the other incorporated into the hydroxylated proline residue of HIF- α [88]. The unusually slow reaction of the hydroxylase with oxygen is thought to be a key factor in facilitating the enzyme's role as a hypoxia sensor [67]. Three different isoforms of the PHD enzymes exists, PHD1-3 (EGLN1-3), all of which are near-ubiquitously expressed, but differentially distributed across different organ tissues [209]. Intracellular compartmentalisation is also different between isoforms, with PHD1 found exclusively in the nucleus, PHD2 primarily in the cytoplasm, and PHD3 found in both [111]. PHD2 and PHD3 are both more highly expressed in the heart than in other tissues [207]. In hypoxia, mRNA levels of both PHD2 and PHD3 are increased, as these are transcriptional targets of HIF-1, forming a feedback loop when hypoxia is resolved. There may be some selectivity of specific isoforms of PHD enzymes for specific isoforms of HIF, evidenced by a greater impact of PHD2 silencing on HIF-1 α levels, and PHD3 silencing on HIF-2 α [7].

FIH affects the transcriptional activity of HIF rather than its protein stability. Also a Fe(II) and α -KG-dependent dioxygenase, FIH catalyses the hydroxylation of asparaginyl residues in HIF- α in normoxia, causing a steric clash with co-activator p300, whose recruitment is crucial to the initiation of transcription of target genes [135]. FIH may provide a 'fine-tuning' role in the regulation of HIF, as it is inhibited by hypoxia to a lesser extent than PHDs [40, 112]. Both PHDs and FIH are likely to be controlled by factors other than oxygen tension, allowing greater flexibility within the hypoxic response. Changes in Fe(II) levels have been shown to modulate HIF stabilisation, as these are Fe(II)-dependent dioxygenases [101]. Additionally, levels of certain Krebs cycle intermediates, and in particular succinate, which is the product of the hydroxylation reaction with α -KG, have modulatory effects on these hydroxylases [44]. This has been evidenced by the effects of mutations in Krebs cycle enzymes in cancer, where genetic defects in succinate dehydrogenase (SDH)

lead to elevated succinate, upregulating HIF- α in normoxia [134]. Mutations in fumarate hydratase also increase HIF- α in tumors [163, 154].

1.5 The therapeutic potential of HIF signalling

1.5.1 HIF signalling in cardiovascular disease

An MI exposes the myocardium to an episode of ischaemia and reperfusion, triggering an important hypoxic response in the myocardium. Following myocardial infarction or ischaemia, HIF signalling is stimulated in the affected tissue and neighbouring region [120]. The hypoxic response involving metabolic and angiogenic adaptations coordinated by HIF is key in enabling the tissue to cope following the ischaemic insult and alleviate long-term effects of ischaemia-reperfusion. Studies performed on ventricular biopsies from patients undergoing coronary bypass surgery showed that HIF-1 α activity was increased in this tissue [120]. Increased expression of HIF-1 α protein, as well as HIF-1 α target gene mRNA, was also found in rat tissue from a coronary ligation model, in the first week after infarction [207]. In a HIF-1 α conditional KO mouse model, Wei *et al* [205] found that KO mice developed cardiac hypertrophy and quicker decompensation when subjected to pressure overload, concluding that HIF-1 α signalling plays a key role in limiting the development of cardiac hypertrophy. Work in a transgenic mouse model of constitutively active HIF-1 α showed reduced infarct size and improved cardiac function just weeks following coronary ligation [109]. Studies using gene silencing to prevent PHD activity led to attenuated ischaemia-reperfusion injury in the mouse heart [151]. Similarly, PHD4 silencing in endothelial cells showed cardioprotection in a left anterior descending (LAD) coronary artery ligation mouse model of cardiac ischaemia [107].

1.5.2 Manipulation of HIF signalling

Endogenous activation of HIF has been shown to have therapeutic potential in the context of ischaemia-reperfusion through the use of ischaemic pre-conditioning. Short bursts of ischaemia given prior to an ischaemic insult have led to improved tissue survival, and have been shown to relate to upregulation of the HIF signalling pathway [54]. HIF1A locus deletion studies in mouse models, where mice underwent

pre-conditioning, showed that infarct size was reduced in control mice with pre-conditioning, but this was not observed in mice with the HIF1A deletion [108]. This forms a hopeful framework for the development of strategies that target HIF in the context of acute cardiac disease, or chronic cardiac risk factors. Though HIF stability and activity are naturally regulated through oxygen tension, it is also possible to achieve this genetically or pharmacologically.

HIF-activating compounds fall into one of several classes, all of which target HIF-1 α regulatory enzymes. Examples of these are listed in Table 1.1. The first class includes α -KG mimetic tool compounds, which have been used in pre-clinical work. Pre-treatment with dimethyloxallylglycine (DMOG) in a rabbit model of myocardial ischaemia-reperfusion led to attenuated post-ischaemic injury, as measured by a significant decrease in infarct size [153]. In line with this, hearts from rats pre-treated with DMOG showed improved functional recovery following ischaemia-reperfusion in an *ex vivo* perfused heart setup [164]. These compounds have been criticised for their poor selectivity and risk of off-target effects due to their inhibition of other α -KG dependent enzymes [72, 101]. However, they provide an extremely valuable tool to inexpensively test hypotheses relating to the inhibition of HIF hydroxylation, and are easily accessible.

A second group of HIF-activating compounds are iron (Fe(II)) chelators, such as desferrioxamine and hydralazine, which have been shown to have HIF stabilising properties through PHD inhibition, but provide little selectivity or potency. Desferrioxamine treatment showed improved brainstem blood flow and reduced vasospasm in a rat model of aneurysmal subarachnoid haemorrhage [89].

Cobalt chloride (CoCl₂) was found to have hypoxia-mimetic effects, and it is now known to act by directly inhibiting the interaction between HIF-1 α and pVHL, bypassing the regulation by hydroxylases [219]. Pre-treatment with CoCl₂ has been shown to produce a protective preconditioning effect in both rat and mouse models of cardiac ischaemia-reperfusion via HIF-1 α activation [15, 210].

However, a new generation of PHD inhibitors has since been developed, several of which are now undergoing clinical trials for use in conditions such as anaemia

associated with renal disease, inflammatory diseases, and brain haemorrhage. Some of these new compounds have even shown potential in cardiovascular disease. For instance, in a rat model of established heart failure, treatment with orally-active PHD inhibitor GSK360A resulted in increased expression of HIF targets, accompanied by improved recovery post-MI, showing long term improvements in remodelling and left ventricular function [11]. Additionally, the clinical PHD inhibitor Daprodustat (GSK1278863) has now completed Phase I clinical trials for the treatment of diabetic foot ulcer (NCT01831804), as well as perioperative ischaemia, suggesting that repurposing of these compounds for use in cardiovascular disease may not be far. Roxadustat (FG-4592) has completed Phase II trials for use in anaemia, and showed good tolerability, improved circulating haemoglobin, as well as reduced levels of total cholesterol independent of the use of lipid-lowering agents [166, 165, 18]. Vadadustat (AK-6548) has gone through Phase II clinical trials, showing enhanced iron mobilisation and increased hemoglobin levels in patients with renal disease, while showing no significant changes in blood pressure, VEGF or total cholesterol levels [143]. JTZ-951, a recently developed PHD inhibitor, completed Phase II clinical trials showing improved haemoglobin levels in patients with end-stage renal disease [3]. Molidustat, another orally-available PHD inhibitor, has now completed Phase II clinical trials for treatment of anaemia associated with renal disease (DIALOGUE trial, NCT02021370) and is about to enter Phase III clinical trials (NCT03351166).

1.5.3 Diabetes, HIF signalling, and metabolic modulation

As mentioned above, cardiovascular disease is the main cause of death in patients with T2DM [114]. This highlights the critical deleterious effects of diabetes pathology on the heart, including contractile dysfunction and energetic defects [26]. The metabolic remodelling of the diabetic heart is evidently a key player in this maladaptation, with a dramatic dysregulation of substrate metabolism, and impaired ability to respond to stimuli [152]. The diabetic heart has increased fatty acid uptake and utilisation, paired with decreased glucose uptake and utilisation.

Table 1.1: HIF stabilising compounds.

Class	Compound	Current use
α -KG mimetics	Dimethyloxallylglycine	Tool compound - PHD inhibitor
	<i>N</i> -oxalyl-d-phenylalanine	Tool compound - FIH inhibitor
Hypoxia-mimetic metals	Cobalt(II) chloride	Binds HIF-1 α preventing pVHL binding and consequent proteasomal degradation. Shown to produce beneficial hypoxic preconditioning in mouse and rat models of cardiac ischaemia-reperfusion
Iron (Fe(II)) chelators	Desferrioxamine	Phase II clinical trials for use in brain haemorrhage
	Hydralazine	Licensed as an antihypertensive, recently found to also have a HIF mechanism of action
	AKB-4924	Phase I clinical trials for the treatment of inflammatory bowel diseases
PHD active-site blockers	GSK360A	Pre-clinical studies showed improved left-ventricular function and remodelling in rat model of established heart failure
	Daprodustat (GSK1278863)	Phase III clinical trials for use in anaemia associated with chronic kidney disease. Also in Phase II for the treatment of perioperative ischaemia and diabetic foot ulcers
	Roxadustat (FG-4592)	NDA filed in China for treatment in anaemia associated with chronic kidney disease. Also in Phase III clinical trials for use in anaemia associated with chronic kidney disease
	Vadadustat (AKB-6548)	Phase III clinical trials for use in anaemia associated with chronic kidney disease
	JTZ-951	Phase III clinical trials for use in anaemia associated with chronic kidney disease
	Molidustat (BAY85-3934)	Phase II clinical trials for use in anaemia associated with chronic kidney disease

This metabolic adaptation is essentially opposite to the metabolic changes driven by HIF signalling, where fatty acid utilisation is inhibited, while glucose uptake and glycolysis are upregulated. Whether HIF signalling is impaired in diabetes is not fully understood, but there has been some evidence for defects in HIF signalling in diabetes, including inability to upregulate downstream targets of HIF-1 following ischaemia or MI [25, 195, 20]. This connection suggests that, if HIF signalling is impaired in diabetes, the ability to respond to an ischaemic insult or to hypoxia would pose an even greater risk to the diabetic heart, as the defect in HIF could contribute to the metabolic dysregulation already present in diabetes. However, this also highlights a potential novel therapeutic target, as pharmacological HIF activation could provide a mechanism to correct abnormal metabolism in diabetes in hypoxia. Additionally, the reverse may also be of interest, as metabolic modulation, such as fatty acid uptake inhibition, could improve the diabetic heart's hypoxic response.

1.6 Thesis aims and hypothesis

The work presented in this thesis aimed to investigate the relationship between hypoxia and metabolism in diabetes, and the potential of hypoxia signalling manipulation in the treatment of the type 2 diabetic heart.

Investigating the effect of acute hypoxia on the diabetic heart and the therapeutic potential of fatty acid uptake inhibition. The relationship between hypoxia and diabetic metabolic remodelling was initially assessed in control and diabetic hearts by investigating the effect of acute hypoxic stress in the isolated perfused heart. In this instance, a fatty acid uptake inhibitor was used to assess the therapeutic benefit of a Randle cycle shift in metabolism, away from fatty acid oxidation and towards anaerobic glycolysis in the diabetic heart (Chapter 3). Metabolomics were performed on heart samples following perfusion to assess the changes observed in a wide range of metabolites, and to begin to elucidate the mechanisms involved in tissue damage and recovery. Subcellular fractionation was used to investigate the compartmentalisation of both glucose and fatty acid uptake proteins and assess the involvement of these in the processes studied.

Investigating the effect of ischaemia-reperfusion on the diabetic heart and the therapeutic potential of prolyl hydroxylase inhibition. The hypothesis of this work was that hypoxia mimetics may alter substrate metabolism, towards anaerobic glycolysis and away from fatty acid oxidation, and that this would drive improved recovery in ischaemia-reperfusion, by allowing the diabetic heart to move into a more oxygen-sparing state during the ischaemic challenge (Chapter 4). This was firstly done using dimethyloxallylglycine (DMOG), a tool compound widely available, which is relatively inexpensive, which was given to rats as a chronic pre-treatment. Metabolism and function were assessed in a model of cardiac ischaemia-reperfusion, and metabolic markers were investigated, to elucidate the

mechanisms by which the observed changes may be regulated. The metabolic effects of this compound were unexpected despite an improvement in functional recovery.

Investigating the therapeutic potential of prolyl hydroxylase inhibition by a clinically-trialled compound. The hypothesis of prolyl hydroxylase inhibition was taken further, using Molidustat, a clinical compound which is available commercially (Chapter 5). This allowed us to better test the hypothesis with a more potent and selective compound, with no off-target effects, and allowed for better clinical translatability. Similarly to the previous study, this work involved chronic pre-treatment prior to isolated heart experiments using ischaemia-reperfusion.

Working Hypothesis The working hypothesis of this thesis is that, as T2DM is a metabolic disorder directly and indirectly affecting the heart, a metabolic intervention will be of benefit to the diabetic heart's health and ability to respond under stress. Specifically, we hypothesise that the diabetic heart has impaired functional recovery in response to acute hypoxia or ischaemia, and that pharmacologically manipulating substrate metabolism to increase glycolysis and decrease fatty acid oxidation will result in improved functional recovery in the diabetic heart following either a hypoxic or ischaemic insult. We hypothesise that this can be achieved by inhibiting fatty acid uptake directly, using a FAT/CD36 blocker, infused into the circulating perfusion buffer; and that this can also be achieved indirectly, using prolyl hydroxylase inhibitor pre-treatment to stimulate HIF-1 signalling *in vivo*.

By three methods we may learn wisdom: First, by reflection, which is noblest; Second, by imitation, which is easiest; and third by experience, which is the bitterest.

— Confucius

2

General Methods

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2.1 Animal work

All animal work was conducted in accordance with the Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act of 1986 and University of Oxford Institutional Guidelines, under the Project Licence PPL 30/3322. Rodents were housed in a dedicated facility (Biomedical Sciences Services, University of Oxford, UK) under a 12 h light-dark cycle and maintained on standard laboratory chow (Harlan Laboratories, Kent, UK) and standard laboratory water, given ad libitum unless otherwise stated.

2.2 Rat model of type 2 diabetes

The high fat fed, low dose streptozotocin rat model of type 2 diabetes was used for all studies [137]. Male Wistar rats (Harlan Laboratories, Kent, UK) were given either normal chow consisting of 4% kcal from fat, 16% kcal from protein, and 48% kcal from carbohydrate (Envigo, see Appendix A), or a high fat diet consisting of 60% kcal from lipid, 35% kcal from protein, and 5% kcal from carbohydrate (Special Diet Services, see Appendix A) for five weeks. On day 14, high fat fed rats were given an intraperitoneal injection of the β -cell toxin streptozotocin (STZ, 25 mg/kg body weight, in 0.3 ml citrate buffer pH 4). STZ is an alkylating agent, which, given its selectivity for GLUT2 transporters, is particularly toxic to pancreatic β -cells, inducing DNA damage and cytotoxicity [192]. High fat diet was continued for a further 20 days (Fig. 2.1). Fasting blood samples were collected on day 33 after overnight fast, and rats were terminated on day 35 in the fed state (Fig. 2.1). Hearts were excised under terminal anaesthesia in the fed state, and cannulated immediately for perfusion (see section 2.5). In parallel, blood samples were taken to measure fed state blood glucose, and centrifuged to obtain plasma samples, which were snap frozen in liquid nitrogen and stored at -80°C for subsequent analysis. The liver was harvested, snap frozen in liquid nitrogen, and stored at -80°C for subsequent metabolic profiling. Epididymal fat pads were collected and weighed to assess adiposity. Time of tissue collection (morning/afternoon) was alternated between groups to ensure an even distribution across all groups.

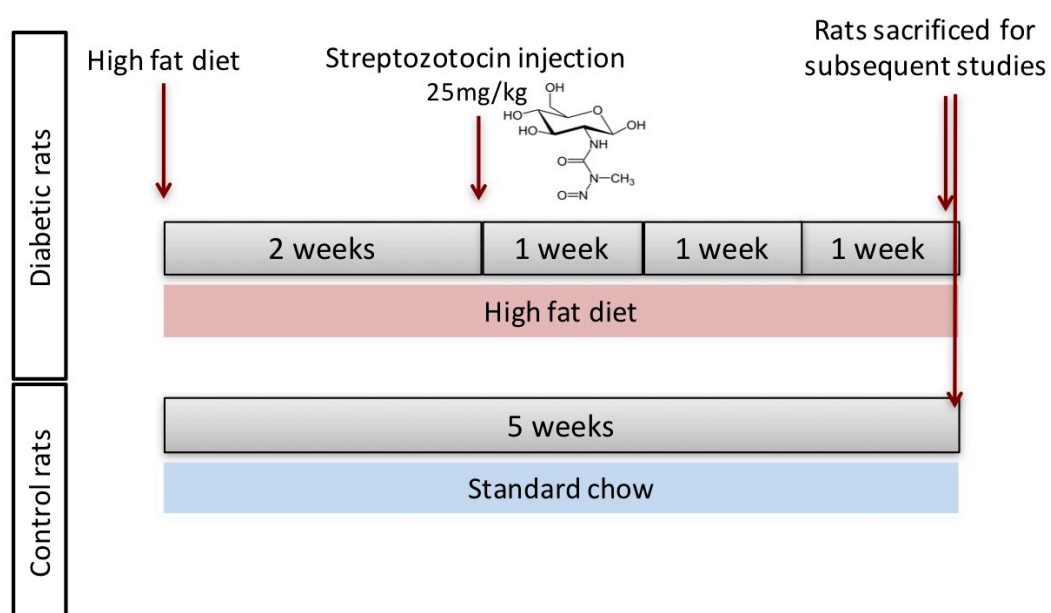


Figure 2.1: Rat model of type 2 diabetes and timeline.

2.3 Dimethyloxallylglycine treatment

Prolyl hydroxylase domain (PHD) inhibitor dimethyloxallylglycine (DMOG; Cayman Chemical, UK) was given by intraperitoneal injection (40 mg/kg) on three alternate days. DMOG was prepared in dimethyl sulfoxide (DMSO, 5% final concentration, Sigma Aldrich, Dorset, UK) before diluting in fresh sterile saline (0.9%) to inject a final dose of 40 mg/kg in 0.3 ml saline. DMOG treatment was prepared freshly each day immediately prior to injection. This dose was chosen as previous work within our at a dose of only 15 mg/kg had shown no evidence of HIF signalling upregulation. Most work shown in the literature has used alternate day dosing (48 h intervals) over three to five days of treatment course [218, 198], therefore to achieve a chronic dose effect we chose to inject intraperitoneally on alternate days as shown by other groups to induce HIF signalling (Fig. 2.2).

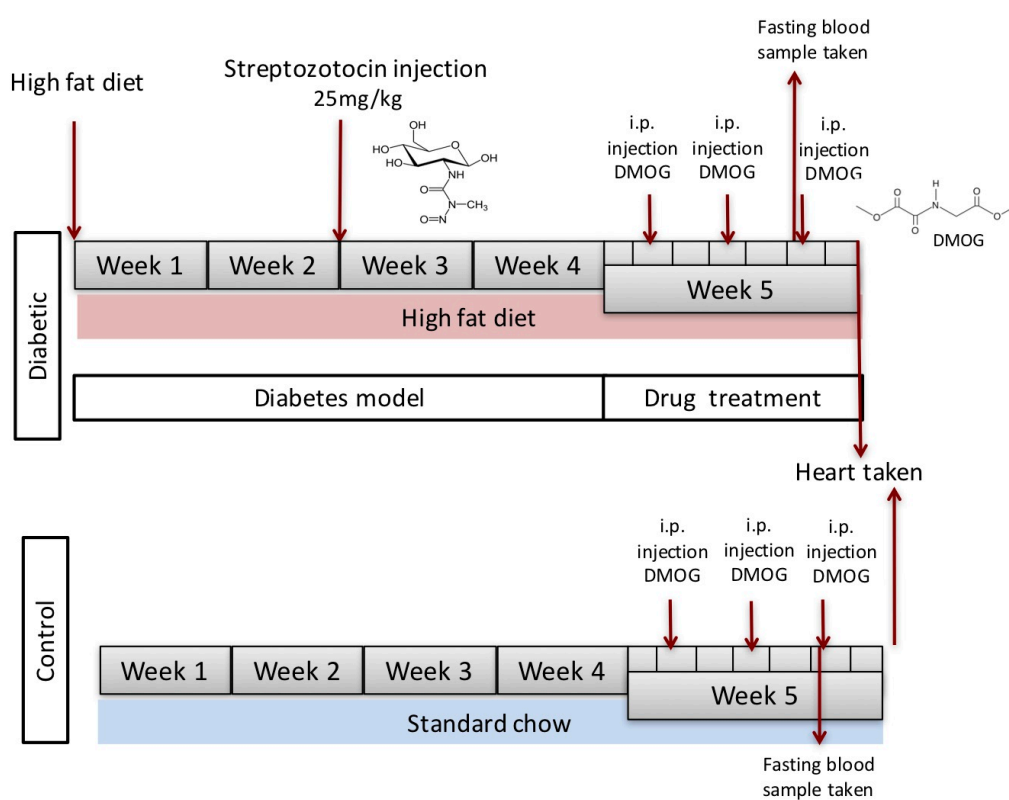


Figure 2.2: Diabetes model induction and DMOG treatment regimen (Chapter 4).

2.4 Molidustat treatment

Prolyl hydroxylase domain inhibitor BAY85-3934, aka Molidustat (ADV465750852, Sigma Aldrich, UK) was given orally (5 mg/kg) in 1 ml sucrose-free jelly, once-daily over five days (Fig. 2.3). Prepared Molidustat jelly was given to rats in solitary housing during the week of treatment. Control rats were given the same jelly prepared without drug. This dose of drug was chosen as it has been shown to increase in blood markers of HIF signalling such as circulating haemoglobin, packed cell volume and erythropoietin (EPO) by the pharmaceutical group that developed the drug [66].

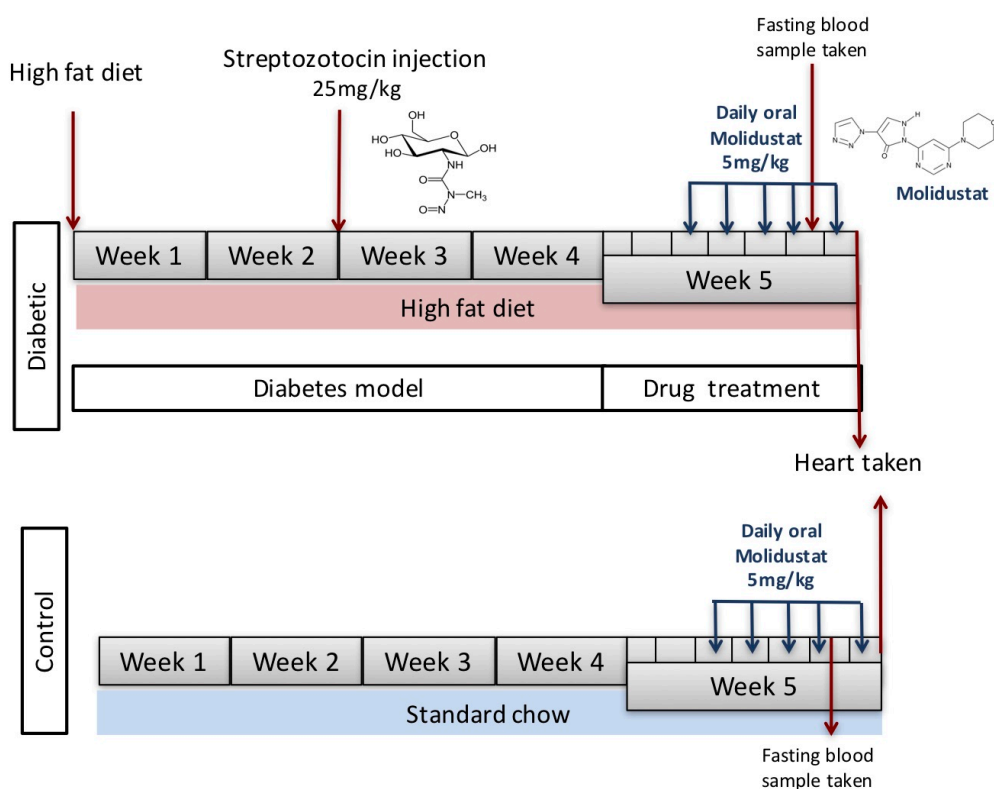


Figure 2.3: Diabetes model induction and Molidustat treatment regimen (Chapter 5).

2.5 Radiolabelled isolated heart perfusion

2.5.1 Preparation of Krebs-Henseleit buffer

Perfusion studies were performed in re-circulating modified Krebs-Henseleit buffer containing 11 mM glucose and 1.5% (w/v) bovine serum albumin bound to 0.4 mM palmitate. This was prepared using 118 mM NaCl, 4.7 mM KCl, 12 mM MgSO₄, 1.3 mM CaCl₂, 0.5 mM EDTA, 25 mM NaHCO₃, 1.2 mM KH₂PO₄ and 11 mM glucose. This was prepared using deionised water (dH₂O) and gassed with 95% O₂ and 5% CO₂. The buffer was filtered prior to addition of bovine serum albumin (BSA, 1.5 % final concentration; Lampire, USA). The BSA was allowed to dissolve completely before heating the buffer to 30 °C, at which point palmitate was added to the solution to a final concentration of 0.4 mM, using a heated pipette tip to ensure binding of the palmitate to BSA. This KH-palmitate-BSA buffer was placed in dialysis tubing and submerged in the standard KH buffer overnight, to allow equilibration of free calcium. The free calcium concentration was checked immediately prior to use using a radiometer blood gas analyser and adjusted to a final concentration of 1.35 mM.

2.5.2 Palmitate stock preparation

Palmitate stock (100 mM) was prepared in advance, to be used in the modified KH buffer. This was prepared by dissolving 2.56 g of palmitic acid (Sigma Aldrich, Dorset, UK) in 2 ml of ethanol (Sigma Aldrich, Dorset, UK), in a water bath warmed to 70 °C. K₂CO₃ was dissolved in a minimum volume of water, and added slowly to the palmitic acid solution. The precipitate was allowed to dry fully, and the salt was subsequently dissolved in 100 ml of saline (0.9%). This palmitate stock was warmed to 70 °C within a water bath, prior to addition to the KH-albumin buffer, to ensure binding of palmitate to albumin.

2.5.3 Langendorff heart perfusion

Rats were terminally anaesthetised in the fed state using an intraperitoneal injection of sodium pentobarbital (400 mg/kg; Euthatal, Merial, Woking, UK). Hearts were excised immediately following the cessation of the pedal and corneal reflexes and placed in ice-cold Krebs-Henseleit (KH) buffer. The aorta was then cannulated on the perfusion apparatus, and perfused with standard KH buffer supplemented with glucose only. The pulmonary artery was cut, a drain was inserted into the left ventricle and pushed through the apex to allow drainage of the ventricle, and a PVC balloon was inserted, also into the left ventricle (Fig. 2.4). At this stage, the buffer was switched, to be perfused in re-circulating KH-palmitate-BSA buffer containing glucose and albumin-palmitate, as described above. Hearts were perfused in Langendorff mode at a constant pressure of 100 mmHg and end-diastolic pressure of 4-8 mmHg, gassed with 95% O₂ and 5% CO₂ at 37 °C, as previously described [129, 87]. The fluid-filled PVC balloon, which was attached to a bridge amplifier and PowerLab data acquisition system (ADInstruments, Oxfordshire, UK), was used to record functional data throughout the protocol (Fig. 2.4). Left ventricular developed pressure (LVDP) was determined as peak-systolic minus end-diastolic pressure, and rate pressure product (RPP), used as the measure of cardiac function, was the multiple of developed pressure and heart rate (see Fig. 2.5).

2.5.4 Perfusion protocols

Acute hypoxia

In the acute hypoxia perfusions (Chapter 3), hearts were first perfused under normoxic conditions for 20 min, before induction of acute hypoxia, by replacing the gas with 95% N₂ and 5% CO₂ to decrease the pO₂ from 400 ± 10 mmHg to 90 ± 10 mmHg for 32 minutes. The perfusate pO₂ was measured by taking a 0.5 ml sample and immediately running it through a blood gas analyser. Hearts were then subsequently reoxygenated with 95% O₂ and 5% CO₂ for 20 minutes. Hearts were freeze-clamped either at the end of normoxia ($t = 20$ min), hypoxia ($t = 52$ min), or reoxygenation ($t = 72$ min).

Ischaemia-reperfusion

In ischaemia-reperfusion perfusions (Chapters 4 and 5), 3 U/l insulin (NovoRapid Penfill, Novo Nordisk, Denmark) was added to the circulating buffer at the start of the perfusion protocol. Hearts were initially perfused under normal flow for 20 min, before induction of 30 min of low-flow ischaemia at a continuous flow rate of 0.3 ml/min/gww using a calibrated peristaltic pump. The hearts were then reperfused at full flow for the remainder of the protocol, for a further 30 min. Hearts were freeze-clamped at the end of reperfusion.

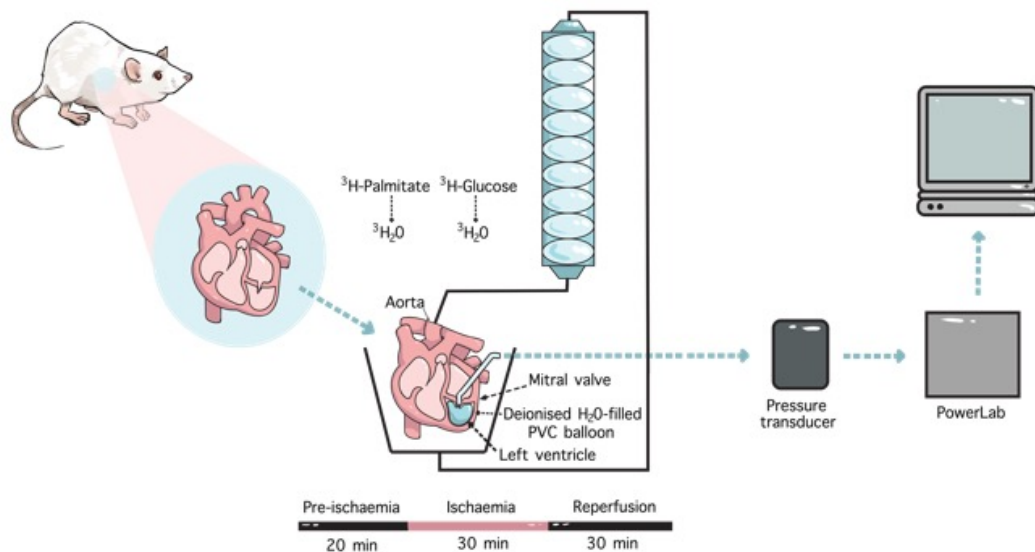


Figure 2.4: Langendorff perfusion apparatus and protocol

2.5.5 Metabolic rates

For the measurement of glycolytic flux and palmitate oxidation rates, the perfusate was supplemented with 0.2 $\mu\text{Ci/ml}$ of either $[5\text{-}^3\text{H}]\text{-glucose}$ or $[9,10\text{-}^3\text{H}]\text{ palmitate}$. A sample volume of 1 ml of perfusate was taken every four minutes during the perfusion, for the assessment of glycolytic flux and 1.5 ml taken every four minutes during the perfusion, for the assessment of fatty acid oxidation rate.

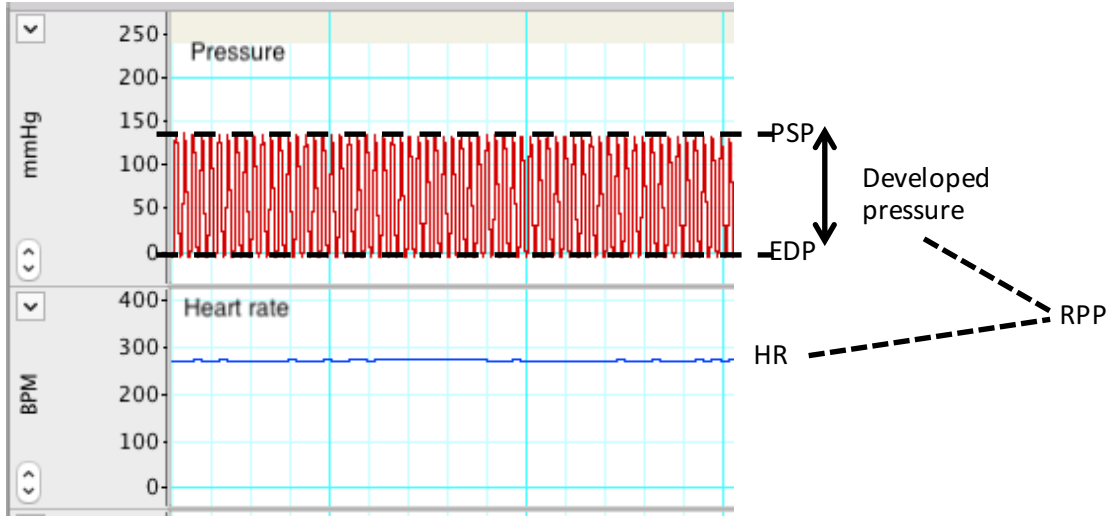


Figure 2.5: Measurement of cardiac function in the Langendorff perfused heart using PowerLab data acquisition system. The software measures the pressure on the balloon in the left ventricle, and calculates heart rate. Left ventricular developed pressure was determined as peak systolic (PSP) minus end-diastolic pressure (EDP), and rate pressure product (RPP) is the multiple of developed pressure and heart rate (HR).

Glycolytic rates

Glycolytic rate was determined based on $^3\text{H}_2\text{O}$ generated by the enolase reaction of glycolysis following separation through a Dowex resin anion-exchange column, as described by Lopaschuk and colleagues [126] (Fig. 2.6). The Dowex resin was prepared in 1.35 M NaOH and 1.62 M boric acid before washing repeatedly to achieve pH 8. For each collected perfusate sample, a Dowex column was prepared using a short nose glass Pasteur pipette plugged with glass wool and filled with Dowex. A volume of 200 μl perfusate sample was added to the top of the column, incubated for 15 min to bind to the resin, before the column was washed with 2 ml of dH_2O (Fig. 2.7). The efflux was collected in scintillation vials, to which 10 ml of Ecolite scintillation cocktail (BP Medical, USA) was added prior to reading for tritium on a scintillation counter. The specific activity was measured directly from 200 μl perfusate sample taken at $t = 0$ min.

The cumulative glucose consumption within each sample was then calculated as follows:

$$\frac{(\text{CPM}_{t=x} - \text{CPM}_{t=0}) \times \text{Vol}_{\text{circ}}}{(\text{SA} - \text{CPM}_{t=0}) \times [\text{glucose}]} \quad (2.1)$$

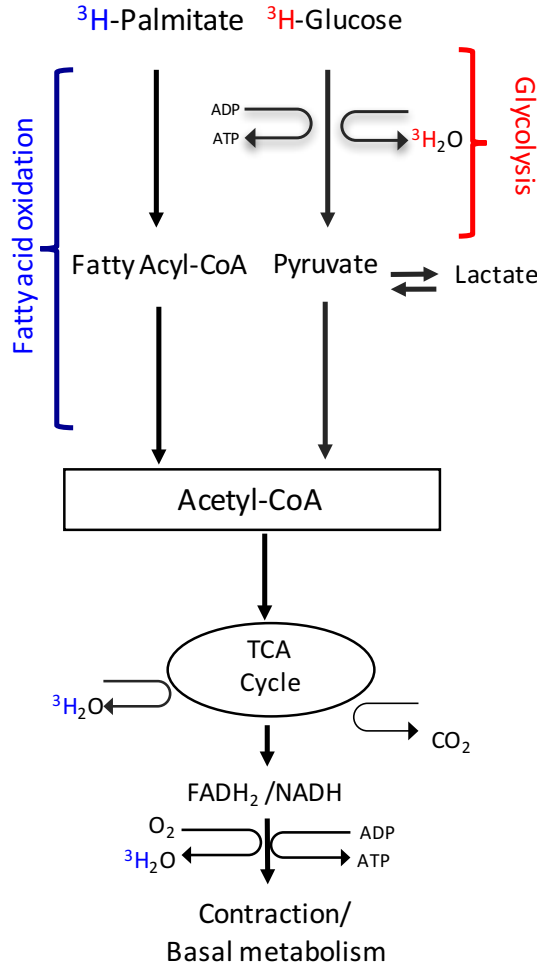


Figure 2.6: Measurement of glycolytic and fatty acid oxidation rates in the Langendorff perfused heart. The perfusate is supplemented with either tritiated glucose or palmitate, and the tritium label converted to $^3\text{H}_2\text{O}$ when the substrate is metabolised by the heart. Samples are taken at regular intervals and analysed for $^3\text{H}_2\text{O}$ concentration, which allows for the calculation of metabolic rates over time.

where $\text{CPM}_{t=x}$ is the scintillation count per minute at time $t = x$, $\text{CPM}_{t=0}$ is the scintillation count per minute at time $t = 0$, Vol_{circ} is the total remaining volume of circulating perfusate, SA is the specific activity at $t = 0$, and $[\text{glucose}] = 11 \text{ mM}$.

The glucose consumption for each sample was plotted as a function of time, and the rate of glycolysis ($\mu\text{mol}/\text{min}$) was taken from this plot and then normalised to heart weight given as gram of wet weight tissue (gww).

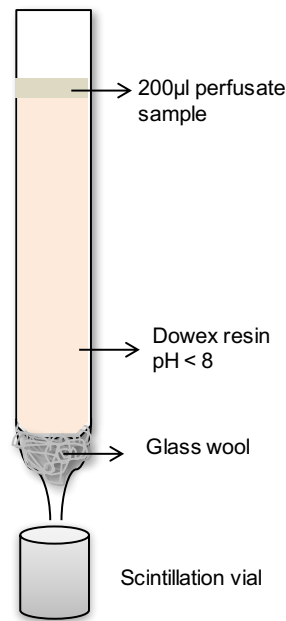


Figure 2.7: Dowex resin anion-exchange column for the measurement of glycolytic rate.

Fatty acid oxidation rates

The KH buffer was supplemented with [9,10- ^3H] palmitate at the time of binding the non-labelled palmitate to albumin, when preparing the perfusion buffer (as described in Section 2.5.2). Palmitate oxidation rates were determined from $^3\text{H}_2\text{O}$ generated by cytochrome c of the electron transport chain and the Krebs cycle, following Folch extraction, as described by Lopaschuk and colleagues [126] (Fig. 2.6). Using a Folch method extraction, $^3\text{H}_2\text{O}$ was separated from [9,10- ^3H] palmitate, as follows: 0.5 ml of perfusate sample was added to 1.88 ml Folch solution (1:2 chloroform:methanol), vortexed for 10 sec, and mixed for 10 min. An additional 0.625 ml chloroform, as well as 0.625 ml 2 M KCl prepared in 0.4 M hydrochloric acid (KCl-HCl), was added to the sample-solution mixture and mixed on a rotator for 15 min, then left to stand for 1 hour to allow separation of the aqueous and organic phases. The aqueous phase was removed and placed in a fresh tube, to which a further 1 ml chloroform followed by 1 ml methanol and 0.9 ml of KCl-HCl were added. The solution mixture was rotated as before for 15 min and again

allowed to stand for 1 hour to allow for a second separation. The aqueous phase was again carefully removed and retained. A volume of 0.5 ml of this aqueous phase was mixed with 10 ml Ecolite scintillation cocktail (BP Medical, USA) and counted for tritium on a scintillation counter. The specific activity was measured directly from 0.5 ml perfusate sample taken at $t = 0$ min.

The cumulative palmitate consumption within each sample was then calculated as follows:

$$\frac{(\text{CPM}_{t=x} - \text{CPM}_{t=0}) \times 8.26 \times 2 \times \text{Vol}_{\text{circ}}}{(\text{SA} - \text{CPM}_{t=0}) \times 2 / [\text{palmitate}]} \quad (2.2)$$

where $\text{CPM}_{t=x}$ is the scintillation count per minute at time $t = x$, $\text{CPM}_{t=0}$ is the scintillation count per minute at time $t = 0$, 8.26×2 is the dilution factor in the Folch extraction, Vol_{circ} is the total remaining volume of circulating perfusate, SA is the specific activity at $t = 0$, and $[\text{palmitate}] = 0.4$ mM.

The palmitate consumption for each sample was plotted as a function of time, and the rate of palmitate oxidation ($\mu\text{mol}/\text{min}$) was taken from this plot and then normalised to heart weight given as gram of wet weight tissue (gww).

2.6 Blood analysis

Fasting metabolites were measured from saphenous vein blood samples following an overnight fast. Following terminal anaesthesia, fed state blood was collected from the chest cavity immediately after the heart was excised, and plasma was separated by centrifugation on a microcentrifuge at 36,000 rpm for 10 minutes. Whole blood glucose concentration was measured using an Accu-check Aviva glucose meter. Hemoglobin concentrations were measured using a HemoCue 201+ System spectrophotometric device (HemoCue, Sweden). Hematocrit was measured by drawing blood from the chest cavity into capillary tubes, which were sealed and centrifuged at 15000g for 5 min, and calculating the ratio of the column of packed erythrocytes to the total length of the sample in the capillary tube, measured with a graphic reading device. Plasma glucose, lactate, and β -hydroxybutyrate were measured on an ABX Pentra 400 clinical analyser (Horiba ABX Diagnostics, Irvine, CA, USA), non-esterified fatty acids (NEFA) using a NEFA colorimetric benchtop assay kit (Randox, UK), triacylglycerides using a Randox TRIGS colorimetric benchtop assay kit (Randox, UK). Insulin levels were measured using a rat insulin ELISA kit (Mercodia, Sweden).

2.7 Tissue analysis

Heart tissue was frozen immediately at the end of the perfusion protocol, or immediately after excision for hearts that did not undergo perfusion. Frozen tissue was ground to a fine powder using a pestle and mortar under liquid nitrogen. Livers were collected and frozen at -80°C immediately following heart excision, and epididymal fat pads were weighed to assess adiposity.

2.7.1 Measurement of cardiac pyruvate dehydrogenase activity

Pyruvate dehydrogenase activity was measured following the protocol established by Seymour & Chatham [183]. Approximately 50 mg tissue was weighed out and homogenised using a Polytron (Brinkman Instruments) for 30 sec at $5,680 \times g$ in an extraction buffer containing 25 mM HEPES (pH 7), 25 mM KF, 1 mM dichloroacetate, 3 mM EDTA, 1 mM ADP, 1 mM dithiotreitol, 0.05 mM leupeptin, and 1% Triton. Samples were freeze-thawed 3 times and then centrifuged at $4,930 \times g$ for 5 min at 4°C to remove debris. The assay buffer for the PDH activity assay, containing 50 mM HEPES (pH 7.2), 1 mM MgCl_2 , 80 μM EGTA, 1 mM dithiotreitol, 4 μM rotenone, 1.67 mM NAD, 0.1 mM co-enzyme A, 0.2 mM thiamine pyrophosphate, 16.7 mM lactate, and 2 U/ml LDH, was incubated at 4°C prior to the addition of each sample. Following addition of 25 μl of sample to 975 μl of assay buffer, the reaction was followed spectrophotometrically at 340 nm over a 2 minute period. The initial rate of NADH production was determined by the gradient over the first 60 seconds, from which PDH activity was then calculated. The 5 min incubation period was to allow production of pyruvate from lactate, and then the addition of sample lysate started the conversion of pyruvate to acetyl-CoA by the action of PDH.

2.7.2 Measurement of myocardial and hepatic glycogen

Glycogen content was measured by the conversion of glycogen to glycosyl units following digestion with amyloglucosidase, following a protocol adapted from Chan & Exton [33]. A volume of 100 μl of 30% potassium hydroxide (KOH) solution was added to approximately 50 mg of frozen powdered tissue and heated for 1 hour to 105°C . A volume of 50 μl of 2% sodium sulphate (NaSO_3) solution and 600 μl ethanol (for a final concentration of 75%) were then added and the solution mixture was incubated overnight at 4°C . Following this incubation, samples were centrifuged for 5 min at $16,000\times g$ in a refrigerated centrifuge (Eppendorff Centrifuge 5415R, Eppendorff, Hamburg, Germany) and the supernatant was discarded before resuspension of the pellet in 500 μl of 80% ethanol using sonication. The ethanol was left to evaporate overnight, and the remaining pellet was resuspended in 375 μl of dH_2O before the addition of 125 μl of amyloglucosidase-containing buffer (5 mg amyloglucosidase, (Sigma Aldrich, Dorset, UK) in 10 ml of 1 M sodium acetate). The reaction mixture was incubated for 1 hour at 37°C prior to the addition of a further 100 μl of dH_2O and a final centrifugation for 5 min (as before). The resulting supernatant was analysed for glucose using an ABX Pentra 400 clinical analyser (Horiba ABX Diagnostics, California, USA). Total glycogen content was extrapolated from the concentration of glucose in the final sample suspension, and normalised to the exact weight of tissue used for the assay, given as gram of wet weight tissue (gww).

2.7.3 Measurement of myocardial and hepatic triacylglycerides

Triglyceride content was assessed following Folch extraction, using a triglyceride benchtop assay kit (Randox, UK). A volume of 5 ml of Folch solution (2:1 chloroform:methanol) was added to approximately 25 mg of frozen powdered tissue and the mixture was homogenised using a Polytron (Brinkman Instruments) tissue homogeniser. A further 3 ml Folch solution was added to the mixture, before mixing on a rotator for 1 hour. A volume of 2 ml dH_2O was added into the tube, and this

was again mixed on a rotator for a further hour. The tube was allowed to stand overnight to allow separation of the aqueous and organic phases. The aqueous phase was discarded and the organic phase evaporated to dryness under an air blower (kept below 55°C). The sample was then placed on ice, and 1 ml of cold 100% ethanol was added to resuspend the pellet. Two samples of 100 µl were taken from this suspension, and evaporated to dryness. These final pellets were resuspended in 20 µl cold ethanol and this was assayed for triacylglyceride concentration using the Randox TRIGS colorimetric benchtop assay kit (Randox, UK) following manufacturer's instructions. Total triglyceride content was extrapolated from the triglyceride concentration obtained according to the dilution from the extraction protocol, and was normalised to the exact weight of tissue used for the assay, given as gram of wet weight tissue (gww).

2.7.4 Metabolomics

Metabolomics was carried out in the laboratory of Prof J Griffin (MRC, Cambridge), under the supervision of Dr J West. Metabolites were extracted from approximately 50 mg of frozen powdered heart tissue using the methanol:chloroform method described by Le Belle and colleagues [118] followed by homogenisation using a tissue lyser (Qiagen, Hilden, Germany). After centrifugation the aqueous and organic fractions were separated. Chromatographic separation was performed using a Vanquish LC system (Thermo Scientific), with an ACE C18-PFP 2 µm column (2.1 x 150 mm) (Advanced Chromatography Technologies Ltd) coupled to a TSQ Quantiva Triple Quadrupole Mass Spectrometer (Thermo Scientific). The mobile phase gradient was run at 0.5 mL/min using water with 0.1% formic acid (mobile phase A) and acetonitrile with 0.1% formic acid (mobile phase B). The gradient started at 100% A, and increased to 60% B from 1.6 to 4.5 minutes, followed by re-equilibration for 2 minutes. The injection volume was 2.5 µL. Data were acquired in both positive and negative ionisation modes using a capillary spray voltages of 3.5 kV and 2.5 kV respectively. The ion transfer tube was set to operate at 350°C whilst the vaporizer temperature was set to 400°C. Compound dependent parameters (RF

lens and collision energy) where determined on infusion of standards at 100 nM prepared in 4:1 acetonitrile (water with 0.1% acetic acid). Samples from the C18 PFP analysis were dried and reconstituted in 100 μ L 7:3 acetonitrile: water followed by filtration in 96 well 0.66 μ m filter plates. Samples were filtered using a positive pressure 96 processor collected into a 300 μ l polypropylene 96 well plate and sealed using silicone pre-slit sealing mats. Chromatographic separation was performed using a Vanquish LC system with a bridged ethylene hybrid amide column (100 $\times$ 2.1 mm, 1.7 μ m; Waters Ltd.). The mobile phase gradient was run at a flow rate of 0.6 mL/min using 100 mM ammonium carbonate as mobile phase A and mobile phase B was acetonitrile. The following linear gradient was used: 30% A was held for 1.5 minutes followed by a linear gradient to 60% A at 3.5 minutes with further re-equilibration for 2 min. The injection volume was 5.0 μ L. Data were acquired in both positive and negative ionisation modes using a capillary spray voltages of 3.5 kV and 2.5 kV respectively. Compound dependent parameters (RF lens and collision energy) where determined on infusion of standards at 100 nM prepared in 4:1 acetonitrile: water with 0.1% acetic acid. Data were processed using the QuanBrowserTM application within the Xcalibur software, both from Thermo Scientific where the chromatographic trace of each compound was integrated and normalised to that of the internal standard that had the closest retention time.

2.7.5 Protein expression: Western blotting

Approximately 50 mg of frozen tissue were crushed to a fine powder and lysis buffer containing 75 mM Tris-HCl (pH 6.8), 3.8% SDS, 4 M urea, 20% glycerol, and 0.1% Triton-X-100, was added before homogenising using a Polytron (Brinkman Instruments) tissue homogeniser. Complete Mini EDTA-free Protease Inhibitor Cocktail (Sigma Aldrich, Dorset, UK) was used in the lysis buffer. A Pierce BCA protein assay (Thermo Fisher Scientific, USA) was used to quantify total protein before addition of 5% β -mercaptoethanol. Protein samples were loaded onto SDS-PAGE gels and separated by electrophoresis, at a constant voltage of 200 V. Protein bands were transferred onto nitrocellulose membranes using wet transfer method

at a constant current of 180 mA for 60 min. Membranes were blocked in TBS-T containing 5% milk for 1 h at room temperature before incubating in primary antibody at 4 °C overnight. Membranes were exposed using C-DiGit blot scanner system (Li-Cor Biotechnology, USA). Band intensities were quantified using Li-Cor ImageStudio software. Equal loading was confirmed using Ponceau staining after transfer, and by using cyclophilin B and β -actin as housekeeping controls. To avoid the metabolically-sensitive instability of GAPDH as a housekeeping gene, cyclophilin B and β -actin were initially selected as loading controls for Western blots. However, both of these genes have been shown to be altered by hypoxia [6, 167, 221, 68]. The use of several such housekeeping genes as loading controls has been put into question [63, 172, 70] and therefore reversible Ponceau staining was additionally used as a loading control, which was checked for every gel run, before progression to primary antibody incubation. A list of the primary antibodies used can be found in Appendix B.1.

2.7.6 Subcellular fractionation

For the measurement of sarcolemmal and microsomal expression of FAT/CD36 and SLC2A4, separation of the sarcolemmal membrane fraction from intracellular endosomes was carried out according to the established method of Luiken and colleagues [22]. Approximately 500 mg tissue was added to 10 ml of a high salt solution containing 20 mM HEPES (pH 7.4), 2 M NaCl, and 5 mM NaN₃. The tissue was then cut up using scissors for a duration of 4 min, whilst kept cold on ice. The mixture was incubated on ice for 15 min with stirring, before centrifuging at $1,000 \times g$ for 5 min at 4 °C. The high salt supernatant was discarded, and the pellet was resuspended in 6 ml of a buffer containing 20 mM HEPES (pH7.4), 2 mM EDTA(K⁺), 1 mM MgCl₂, 250 mM sucrose, and 5 mM NaN₃. This suspension was homogenised 10 times using a glass handheld homogeniser, on ice, before centrifuging at $1,000 \times g$ for 5 min at 4 °C. The supernatant was kept, and the pellet was resuspended in 3 ml of the same buffer, then homogenised another 10 times, before adding the supernatant back into the same suspension. The mixture

was centrifuged at $100 \times g$ for 10 min, and the pellet was resuspended in 300 μ l Western blotting lysis buffer, forming the first subcellular fraction. The supernatant was centrifuged at $5,000 \times g$ for 10 mins. The pellet was resuspended in 300 μ l Western blotting lysis buffer, forming the second subcellular fraction. The supernatant was centrifuged at $20,000 \times g$ for 30 min. The pellet was resuspended in 300 μ l Western blotting lysis buffer, forming the third subcellular fraction. The supernatant was centrifuged at $50,000 \times g$ for 30 min. The pellet was resuspended in 300 μ l Western blotting lysis buffer, forming the fourth subcellular fraction. The supernatant was centrifuged at $190,000 \times g$ for 30 min. The pellet was resuspended in 300 μ l Western blotting lysis buffer, forming the fifth and final subcellular fraction. The fractions were stored at -80°C and, upon thawing, were used for SDS-polyacrylamide gel electrophoresis followed by Western blotting.

2.7.7 Gene expression: RNA extraction, cDNA conversion, and qPCR

For the extraction of RNA from tissue, a Qiagen RNeasy Mini Kit was used (Qiagen, Venlo, Netherlands). Approximately 30 mg of powdered frozen tissue was added to 300 μ L RLT buffer, and this was homogenised using syringe-and-needle method on ice. Then, 590 μ L of RNase-free water and 10 μ L of proteinase K solution (Qiagen, Venlo, Netherlands) was added to the lysate and mixed. This mixture was incubated at 55°C for 10 min before centrifugation at room temperature at $10,000 \times g$ (Eppendorff Centrifuge 5415R, Eppendorff, Hamburg, Germany). The supernatant was transferred to a fresh tube and to this 450 μ L of 100% ethanol was added before mixing thoroughly. Approximately half of this lysate was transferred to the RNeasy spin column (placed in a 2 ml collection tube) and this was centrifuged for 15 sec at $10,000 \times g$. The flow-through was discarded. This step was repeated with the remainder of the lysate using the same column, and the flow-through was discarded once again. A volume of 350 μ L of RW1 buffer was added to the column and this was centrifuged for 15 sec at $10,000 \times g$. The flow-through was discarded. A volume of 500 μ L of RPE buffer was added to the column and this was centrifuged for 15 sec at $10,000 \times g$. This step was repeated with a further 500

μ L of RPE buffer, with a longer centrifugation step of 2 min to wash the membrane. The spin column was placed onto a new collection tube and dried with a further centrifugation step, before eluting the RNA using 30 μ L RNase-free water into a fresh RNase-free collection tube. RNA yields were measured using a Nanodrop Microvolume Spectrophotometer (Thermo Fisher Scientific, USA) and this was used to calculate the volume used for cDNA conversion.

RNA was converted into complementary DNA (cDNA) using a high capacity cDNA reverse transcriptase kit (Applied Biosystems) following manufacturer's instructions. Briefly, 10 μ g of RNA, 2 μ l RT random primers, 2 μ l RT buffer, 1 μ l MultiScribe Reverse Transcriptase enzyme, 0.8 μ l deoxynucleotide triphosphate (dNTP) mix, and 4.2 μ l of water was used to a total volume of 20 μ l per reaction. The samples were then incubated in a SensoQuest Basic Thermal Labcycler (SensoQuest, GeneFlow): 10 min at 25 °C, 120 min at 37 °C, and 5 min at 85 °C. The cDNA samples were stored at -20°C for further use.

The resulting cDNA solutions were used for quantitative polymerase chain reaction (qPCR) using SYBR Green technology (SYBR, Thermo Fisher Scientific, USA). A 20 μ l reaction volume was used per reaction, containing 12.5 ng cDNA (in 5 μ L) + 10 μ L SYBRGreen + 2 μ L primer mix + 3 μ L water per well. A list of primers used for each target can be found in Appendix [B.2](#). The samples were loaded in a 96-well format qPCR plate (MicroAmp Fast, Applied Biosystems) and a standard SYBR cycle was started, using a StepOnePlus Real-Time PCR system (Applied Biosystems). The denaturation was set to 95 °C for 5 min on the initial cycle, and 30 s on the rest. The annealing temperature was set to 60 °C for 30 s, and the extension temperature was set to 72 °C. There were 30 cycles.

Quantification of PCR data was carried out using the traditional double delta Ct relative quantification method [\[178\]](#). Expression levels were measured by the cycle threshold (Ct), defined by the number of cycles required for one sample's fluorescent signal to reach the threshold of background signal. The Ct of each target gene was normalised firstly to the average Ct value for the housekeeping gene (SDHA), and then a second time to the experimental reference sample.

2.8 Statistics

SPSS (IBM) and Prism 7 (GraphPad) softwares were used for the statistical analyses. Results, presented as means $\pm$ SD, were considered significant at $p < 0.05$. All data sets were tested for Gaussian distribution using D'Agostino & Pearson normality test and Shapiro-Wilk normality test. Where data passed the normality tests, one-way ANOVA, two-way ANOVA, and unpaired t-tests were used, depending on the chapter. Sidak post-hoc corrections were made for multiple comparisons, and Welch's correction was made for t-tests. Specific statistics are given in each respective results chapter. Where data did not pass normality tests, the statistical analysis was performed using Kruskal-Wallis test instead (non-parametric).

SPSS (IBM) and Prism 7 (GraphPad) softwares were used for the statistical analyses.

*Money isn't the most important thing in life, but
it's reasonably close to oxygen on the 'gotta have it'
scale.*

— Zig Ziglar

3

Effects of fatty acid uptake inhibition on the response to acute hypoxia in the diabetic heart

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3.1 Chapter abstract

Type 2 diabetes is a metabolic disorder, which causes abnormal substrate metabolism in the heart, and has been associated with impaired recovery following myocardial infarction (MI). A key factor of MI is sudden acute hypoxia, resulting in immediate metabolic stress to the myocardium. The diabetic heart is metabolically over-reliant on fatty acids for energy generation, and unable to switch to anaerobic glucose metabolism under situations of stress. In this chapter we investigated the effects of acute hypoxia-reoxygenation on the diabetic heart, and the therapeutic potential of a metabolic treatment strategy, using fatty acid uptake inhibitor sulfo-*N*-succinimidyl oleate (SSO). We hypothesised that blocking fatty acid translocase (FAT/CD36) using SSO would result in rapidly improved substrate metabolism in the diabetic heart, consisting of decreased fatty acid oxidation and increased glycolysis, and, consequently, improved functional recovery following hypoxia-reoxygenation.

Hearts from control and type 2 diabetic rats were perfused in normoxia, acute hypoxia and reoxygenation, with the FAT/CD36 inhibitor SSO infused *ex vivo* 4 min prior to hypoxia. Diabetic hearts showed significantly higher baseline fatty acid oxidation rates, lower baseline and hypoxic glycolytic rates, and poorer functional recovery following hypoxia-reoxygenation, compared with control hearts. SSO infusion into diabetic hearts significantly decreased the fatty acid oxidation rate and myocardial triacylglyceride concentration compared with untreated diabetic hearts, restoring fatty acid metabolism to control levels following hypoxia-reoxygenation. SSO infusion concomitantly increased the glycolytic rate in diabetic hearts during hypoxia, and increased pyruvate dehydrogenase activity during reoxygenation. In addition, SSO treatment of diabetic hearts increased intermediates within the second span of the Krebs cycle, namely fumarate, oxaloacetate, and the FAD total pool. The cardiac dysfunction in diabetic hearts at reoxygenation was prevented by SSO-infusion prior to the hypoxic stress.

In conclusion, pharmacological inhibition of the sarcolemmal fatty acid transporter, FAT/CD36, provides a rapid mechanism to suppress fatty acid oxidation and promote glycolysis, as well as preventing cardiotoxic lipid accumulation, by targeting the primary step in the fatty acid metabolism pathway. This provides a proof-of-concept for metabolic strategies in the type 2 diabetic heart.

3.2 Introduction

Acute hypoxia is an consequent factor of myocardial ischaemia and MI, which has a number of important metabolic consequences. The ability to sense and respond immediately to the acute changes in oxygen levels is essential for the heart to survive the MI and to limit long term damage to the myocardium [113]. A major aspect of the response to hypoxia is a metabolic shift towards anaerobic glycolysis, and away from oxygen-expensive fatty acid oxidation. It is now well established that diabetic patients are less likely to survive a MI, and though the mechanisms for this are not yet fully elucidated, the reasons may be of metabolic origin. The metabolic inflexibility associated with diabetes is known to have functional consequences. In acute hypoxia, the heart must quickly adapt substrate metabolism to adjust to the change in physiological conditions, upregulating glucose use and downregulating fatty acid uptake and oxidation. This is partly achieved by AMP-activated protein kinase (AMPK) activation, which acts as the energy stress sensor in the cell [13]. AMPK triggers a number of immediate changes in energy metabolism, including translocation of glucose transporter 4 (SLC2A4) to the sarcolemma, allowing for a fast upregulation of glucose uptake and utilisation, as well as translocation of fatty acid transporter FAT/CD36 away from the sarcolemma, decreasing fatty acid uptake and oxidation [87, 22]. Failure to quickly mount an appropriate response to acute hypoxia may result in functional deficit, and contractile dysfunction may persist following reoxygenation [1].

Contractile dysfunction has been shown in a number of studies using rat models of type 1 and type 2 diabetes [216, 56, 55, 62]. These studies also suggest a metabolic basis for the dysfunction observed in diabetic hearts. Metabolic modulation may therefore provide a viable therapeutic avenue for the diabetic heart, and has already received some attention in the field of diabetes. Compounds targeting mitochondrial fatty acid oxidation have already been developed and

studied, primarily in the context of heart failure [179, 119, 2]. However, targeting mitochondrial fatty acid oxidation does not prevent the entry of fatty acids into the cell, or the consequent accumulation of lipids in the cytoplasm, which was one concern reported with etomoxir treatment (CPT inhibition) in diabetic rats [180]. For this reason, in diabetes, it may prove more beneficial to target fatty acid metabolism at its origin, by blocking fatty acid uptake at the level of the sarcolemma. This strategy has already been supported by genetic studies using PPAR α -overexpressing mice, prone to lipotoxic cardiomyopathy, which showed great improvement in cardiac contractile function and decreased lipid accumulation when crossed with FAT/CD36-deficient mice [213].

Therefore, understanding whether the diabetic heart shows abnormal metabolic flexibility in acute hypoxia, and if it can be reinstated by pharmacological inhibition of fatty acid uptake, would be of great interest. In this Chapter, we investigate the acute hypoxic response in the type 2 diabetic rat heart, by perfusing hearts through a protocol of acute hypoxia and reoxygenation, and measuring metabolic rates as well as contractile function throughout. Sulfo-*N*-succinimidyl oleate (SSO) was chosen as a metabolic modulator, to inhibit fatty acid uptake by blocking FAT/CD36. SSO is a competitive FAT/CD36 inhibitor which binds irreversibly to lysine residue 164 in the fatty acid binding site of FAT/CD36 [116], and has been shown to reduce fatty acid uptake in isolated cells, including rat adipocytes [82] and cardiomyocytes [131].

Although this compound had been extensively used in isolated cell studies, it was unknown whether it would have an effect on the intact heart. As the compound had not been studied *in vivo*, and we were interested in its effects acutely, we chose to administer it directly in the circulating perfusion buffer prior to hypoxia-reoxygenation. This allowed us to investigate whether SSO pre-treatment would result in decreased fatty acid oxidation rates, decreased TAG accumulation, and increased glycolytic rates. Additionally, we investigated whether this was paired with an improvement in contractile function in hypoxia-reoxygenation. The aims of this chapter were:

- Firstly, to identify any metabolic differences between our type 2 diabetic rat hearts and healthy controls
- Secondly, to assess the effects of acute hypoxia-reoxygenation on control and diabetic hearts, and
- Finally, to assess the effects of acute treatment with sulfo-*N*-succinimidyl oleate (SSO) in hearts exposed to acute hypoxia-reoxygenation

3.3 Methods

3.3.1 Animal work

Control rats were fed for 42 days on a standard chow diet (Envigo). Type 2 diabetes was induced in rats by feeding a high-fat diet (60% calories from fat, Special Diet Services) for 42 days and, on day 14, a single low dose intraperitoneal injection of streptozotocin (STZ, 25 mg/kg) was given (Fig. 3.1). On day 40, control and diabetic rats had fasting blood collected from the saphenous vein, under recovery isoflurane anaesthesia, for measurement of glucose and insulin (Fig. 3.1). On day 42, rats were terminally anaesthetized using an intraperitoneal injection of sodium pentobarbital (150 mg/kg bodyweight, Euthatal) (Fig. 3.1). Hearts were excised for perfusion and blood was collected for analysis of plasma NEFA and β -hydroxybutyrate.

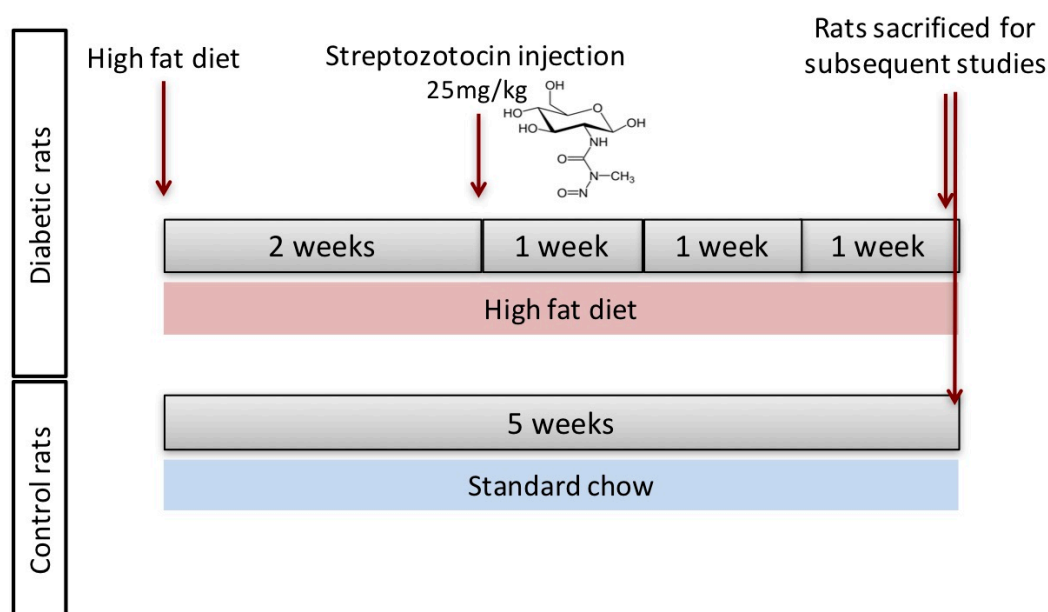


Figure 3.1: Timeline of animal work for Chapter 3 - Control rats were fed normal chow, and diabetics were fed high fat diet, for four weeks prior to the start of treatment. Low-dose (25 mg/kg) STZ was given to diabetic rats two weeks into high fat feeding.

3.3.2 Sulfo-*N*-succinimidyl oleate

Sulfo-*N*-succinimidyl oleate (SSO) was obtained from collaborators Dr J Luiken & Dr W Coumans (Maastricht, Netherlands). A *N*-hydroxysulfosuccinimide sodium salt was synthesised according to the protocol by Staros [190]. *N*-hydroxysulfosuccinimide sodium salt (2 mM), dicyclohexylcarbodiimide (2.2 mM), and oleic acid (4 mM) were dissolved in dimethylformamide overnight at room temperature, followed by stirring for 3 h at 4 °C. The product was filtered, washed three times with dimethylformamide, and sulfo-*N*-succinimidyl oleate (SSO) was precipitated from the solution using 20 volumes of ethyl acetate, filtered, and stored under vacuum in a desiccator. Following optimization of dose and time to effect, a final concentration of 0.5 mM SSO (dissolved in 200 µL DMSO) was added to the recirculating perfusion buffer. SSO was added 4 min prior to the induction of hypoxia and recirculated throughout the hypoxia-reoxygenation period. Hearts perfused with SSO were freeze-clamped at the end of reoxygenation.

3.3.3 Acute hypoxia-reoxygenation isolated heart perfusion

Heart perfusions and metabolic rate analysis for this chapter only were carried out by Dr Latt Mansor. Hearts were perfused at a constant pressure of 100 mmHg and an end-diastolic pressure of 4–8 mmHg. Hearts were perfused with Krebs-Henseleit buffer containing 11 mM glucose and 1.5% (w/v) fatty acid-free bovine serum albumin bound to 0.4 mM palmitate at 37 °C. Hearts were initially perfused in normoxia for 20 min by gassing the buffer with 95% O₂ and 5% CO₂ to give an oxygen partial pressure (PO₂) of 413 ± 15 mmHg (Fig. 3.2). Following normoxia, hearts were made acutely hypoxic by replacing the gas with 95% N₂ and 5% CO₂ to decrease the PO₂ to 90 ± 10 mmHg for 32 min, followed by reoxygenating the buffer with 95% O₂ and 5% CO₂ for a further 20 min (Fig. 3.2). Cardiac function was measured using a fluid-filled PVC balloon inserted into the left ventricle, attached to a bridge amplifier and PowerLab data acquisition system (ADInstruments). Left ventricular developed pressure was determined as peak systolic minus end-diastolic pressure, and rate pressure product (RPP) was the multiple of developed pressure

and heart rate. Functional data were averaged over the last 5 min at the end of normoxia, hypoxia, and reoxygenation. Coronary flow rates were determined by measuring the volume of perfusate flow from the heart during one minute.

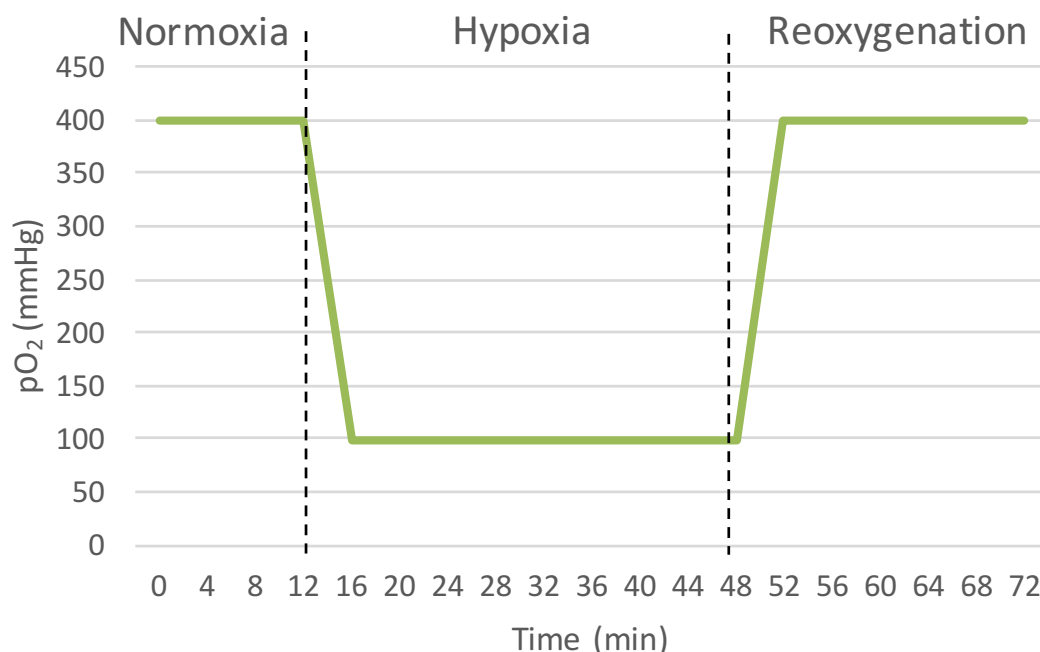


Figure 3.2: Oxygen manipulation in the hypoxia-reoxygenation isolated heart perfusion. Hearts were initially perfused in normoxia for 20min by gassing the buffer with 95% O₂ and 5% CO₂ to give an oxygen partial pressure (PO₂) of 413±15mmHg. Following normoxia, hearts were made acutely hypoxic by replacing the gas with 95% N₂ and 5% CO₂ to decrease the PO₂ to 90±10mmHg for 32min, followed by reoxygenating the buffer with 95% O₂ and 5% CO₂ for a further 20min.

3.3.4 Metabolic rates and tissue analysis

For measurement of glycolytic rates, buffer was supplemented with 0.2 µCi/ml [5-³H]-glucose. For measurement of palmitate oxidation rates, buffer was supplemented with 0.2 µCi/ml [9,10-³H] palmitate. Metabolic rates were calculated during normoxia, hypoxia and reoxygenation, from perfusate samples collected at 4 min intervals for a minimum of five linear data points. In three separate groups, hearts were freeze-clamped on the cannula at the end of normoxia, following normoxia-hypoxia, or following normoxia-hypoxia-reoxygenation. All tissue analysis was carried out by Maria da Luz Sousa Fialho. Glycogen content was determined by the conversion of

glycogen to glycosyl units, which were assayed using amyloglucosidase. Triglyceride content was measured following Folch extraction. Pyruvate dehydrogenase (PDH) activity was measured in the active form according to the protocol of Seymour and Chatham [183]. PDH activity was measured as a surrogate marker for glucose oxidation, as this pathway could not be measured with the [^3H]-glucose label.

3.3.5 Metabolomics

Extraction for Metabolomics and subsequent analysis was carried out as detailed in section 2.7.4. Data are expressed in arbitrary units as the area ratio of metabolite peak relative to the internal standard, normalized to the protein content. In total, 76 metabolites were identified and quantified per heart.

3.3.6 Statistical analysis

Data are expressed as means $\pm$ standard deviation of the mean (SD), and $p < 0.05$ was taken as the level of statistical significance. All data sets were tested for Gaussian distribution using D'Agostino & Pearson normality test and Shapiro-Wilk normality test. Physical and blood characteristics assessed between controls and diabetics were analysed using unpaired, two-tailed Student's t tests.

Glycolytic rates, fatty acid oxidation rates, and cardiac function were measured on the same heart in normoxia, hypoxia and reoxygenation. Therefore, these were analysed using repeated-measures two-way ANOVA with post-hoc Sidak correction, to assess effects of diabetes and oxygenation. The effect of SSO was analysed separately, using two-way ANOVA within each oxygenation state, where diabetes and drug were the two factors analysed. All other data that were from separate hearts collected at the end of each oxygenation stage, were analysed using two-way ANOVA in the same way. Where data did not pass normality tests, the statistical analysis was performed using Kruskal-Wallis test instead (non-parametric).

Graphpad Prism 7 software was used for all statistical analysis.

3.4 Results

3.4.1 Metabolic profiling of the type 2 diabetic rat model

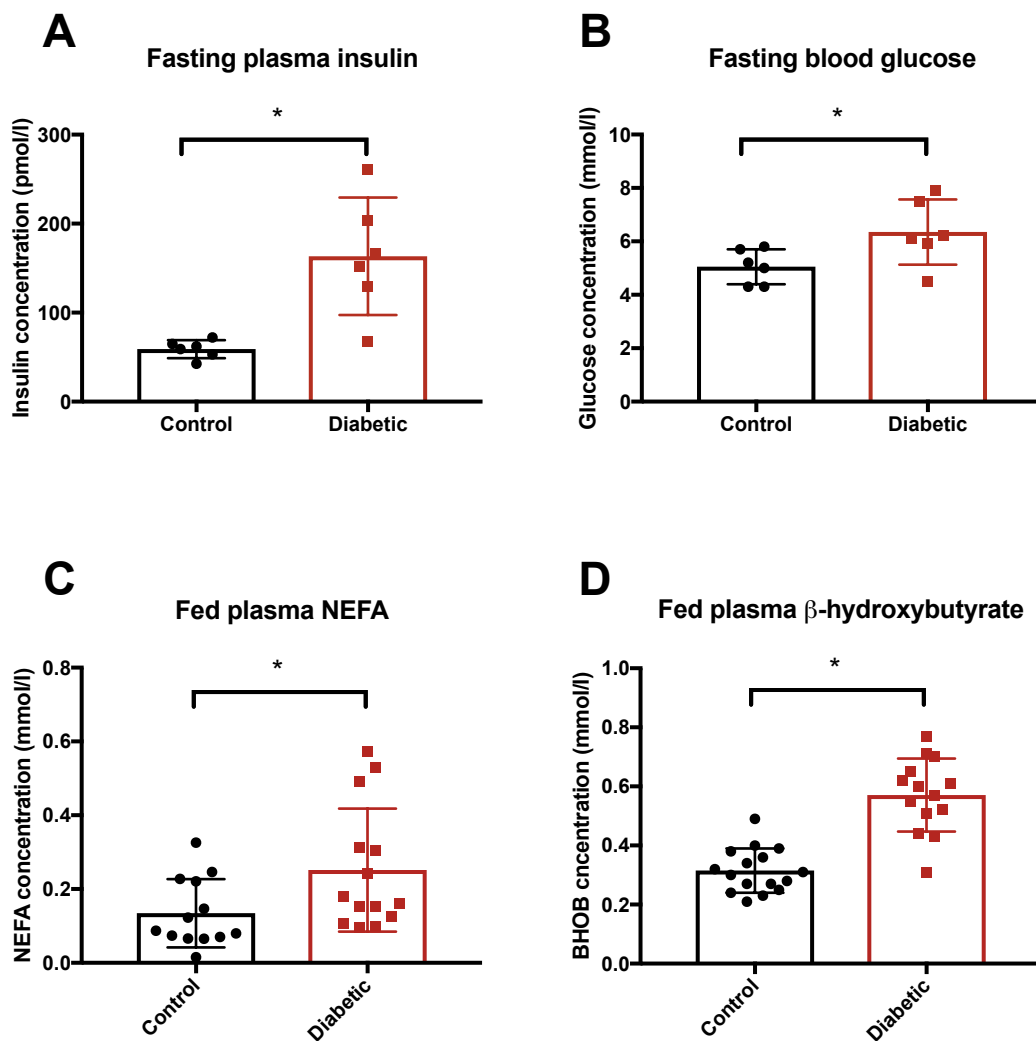


Figure 3.3: Concentrations of fasting state plasma insulin (A), fasting state blood glucose (B), fed state plasma non-esterified fatty acids (NEFA, C), and fed state plasma β -hydroxybutyrate (D) from control and diabetic rats. The control measurements of NEFA in graph C are repeated in figure 4.2 * $p < 0.05$ vs control.

In diabetic rats, fasting plasma insulin showed a 2.7-fold increase, and fasting blood glucose was increased by 26%, compared with controls (Fig. 3.3A,B). The concentration of circulating non-esterified fatty acids (NEFA) in the fed state

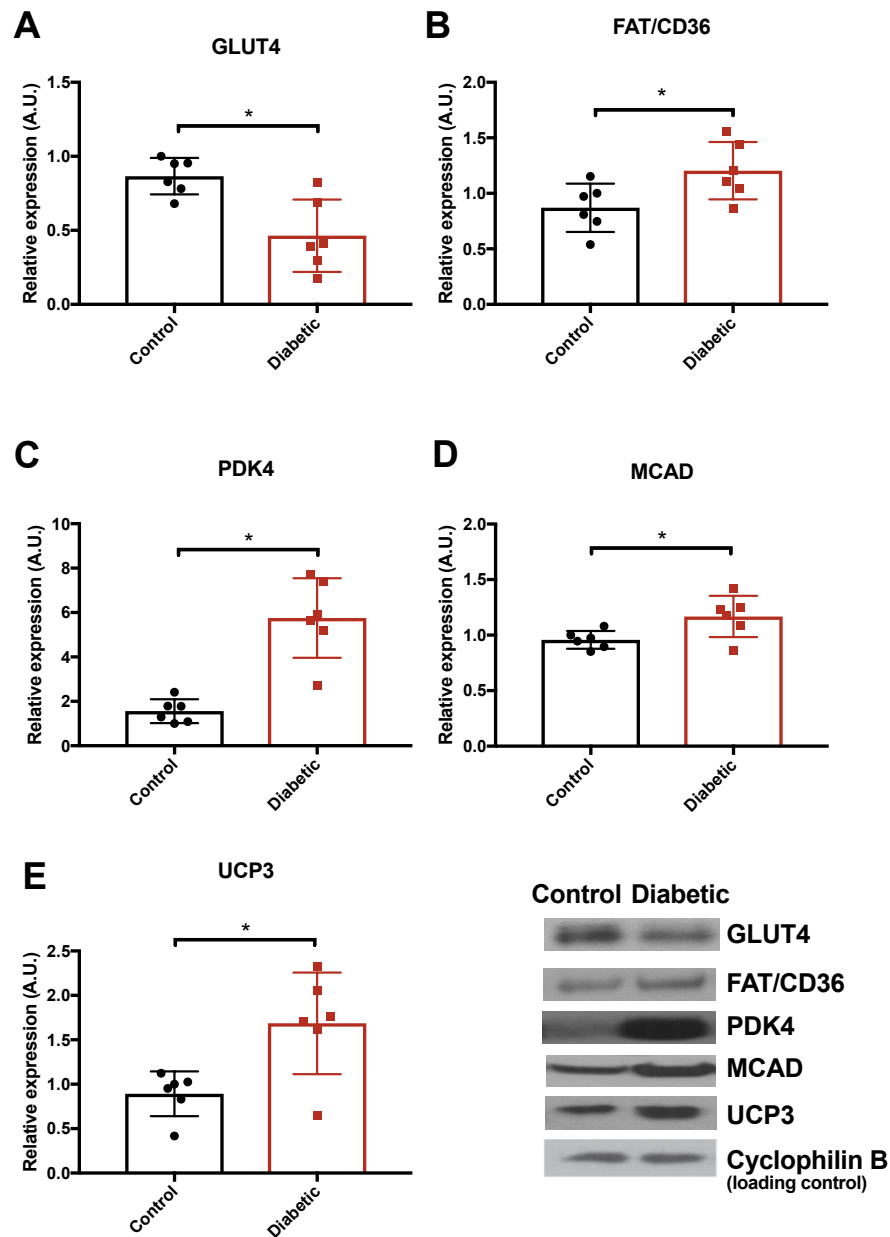


Figure 3.4: Protein levels of glucose transporter 4 (GLUT4, SLC2A4), fatty acid translocase (FAT/CD36), pyruvate dehydrogenase kinase 4 (PDK4), medium chain acyl co-enzyme A dehydrogenase (ACADM), and uncoupling protein 3 (UCP3) in control and diabetic hearts. Normalised to cyclophilin B loading control, as shown in representative blot images. * $p < 0.05$ vs control.

were 83% higher in diabetic rats compared with controls, and ketone body β -hydroxybutyrate levels showed a nearly 2-fold increase (Fig. 3.3C,D). Tissue analysis revealed that the total amount of glucose transporter 4 (SLC2A4) in diabetic hearts was 46% lower than that seen in controls (Fig. 3.4A). Protein expression of

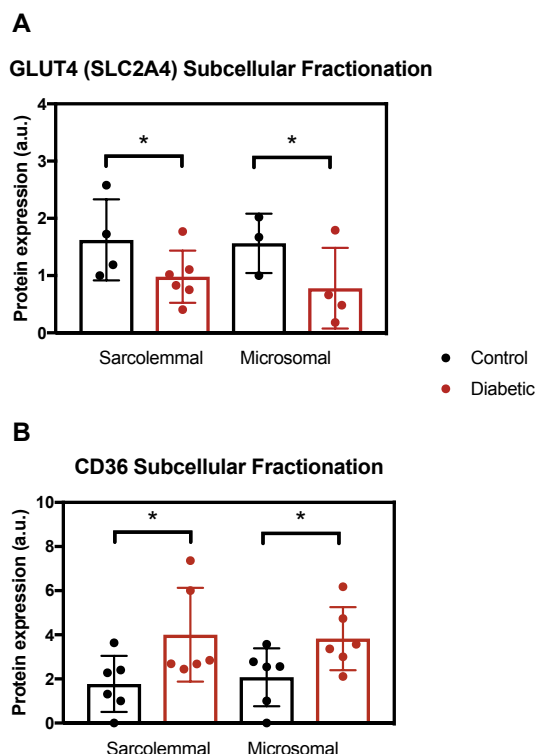


Figure 3.5: Levels of sarcolemmal and microsomal glucose transporter 4 (SLC2A4, A) and fatty acid translocase (FAT/CD36) in isolated perfused control and diabetic rat hearts. * $p < 0.05$ vs control.

PPAR α targets fatty acid translocase (FAT/CD36), pyruvate dehydrogenase kinase 4 (PDK4), medium chain acyl-CoA dehydrogenase (ACADM), and uncoupling protein 3 (UCP3) were all significantly increased (Fig. 3.4C,D,E,F).

Subcellular fractionation analysis of the glucose transporter GLUT 4 (SLC2A4) and fatty acid translocase FAT/CD36 demonstrated that sarcolemmal content of GLUT 4 (SLC2A4) was decreased by 72% and sarcolemmal content of FAT/CD36 was increased by 26% in diabetic hearts compared with controls (Fig. 3.5). In line with this, glycolytic rates in perfused hearts were 53% lower and myocardial glycogen concentration was decreased by 32% in diabetic hearts compared with controls (Fig. 3.6A,B). Fatty acid oxidation rates were 37% higher in diabetic hearts, but myocardial triacylglyceride (TAG) content remained unchanged (Fig. 3.6C,D). Pyruvate dehydrogenase (PDH) activity was significantly impaired in diabetic hearts in normoxia, compared with controls (Fig. 3.7A). Additionally, levels of free carnitine were 44% lower in diabetic hearts compared with controls

(Fig. 3.7B), while levels of acetyl CoA were 60% higher in diabetics (Fig. 3.7C). No significant differences were found in contractile function in type 2 diabetic hearts compared with control hearts during baseline normoxic perfusion (Fig. 3.8).

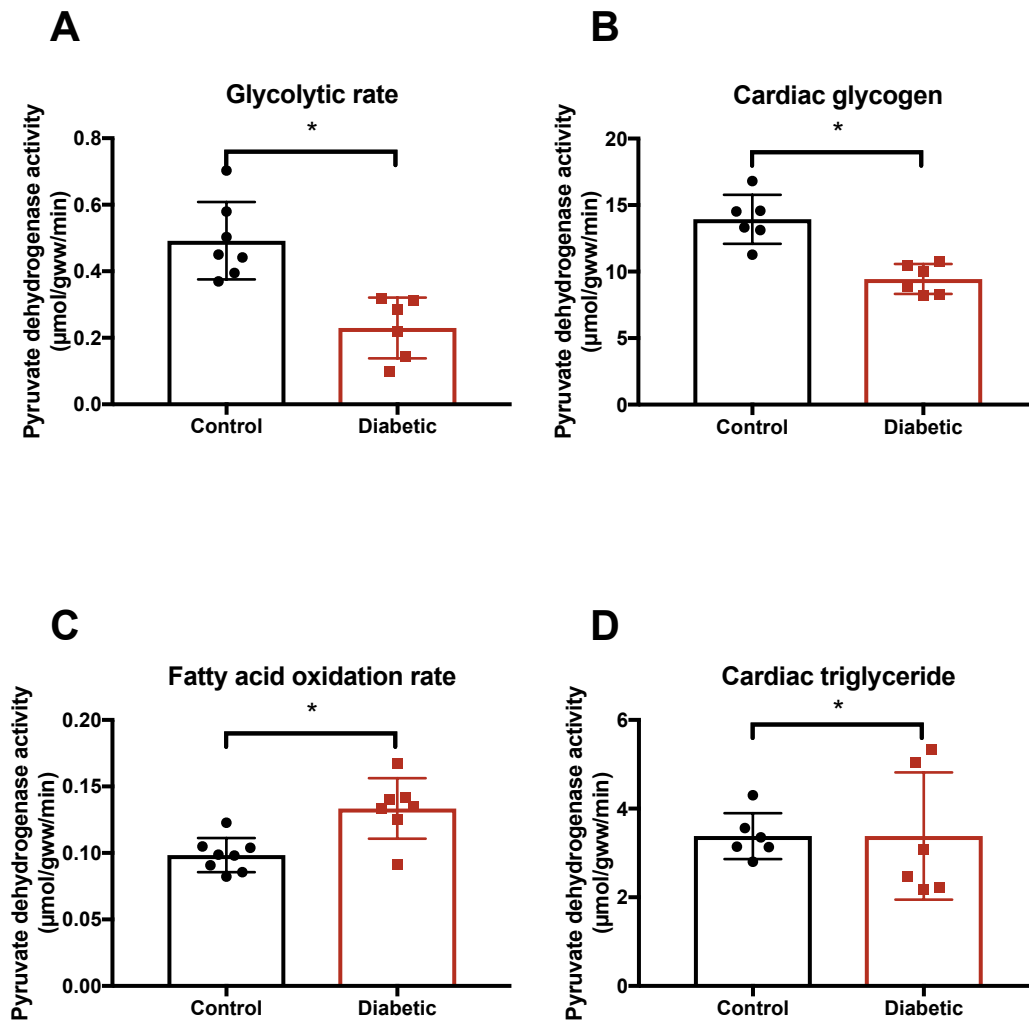


Figure 3.6: Glycolytic rates (A), cardiac glycogen stores (B), fatty acid oxidation rates (C), and cardiac triacylglyceride content (D) in control and diabetic rat hearts perfused under normoxic conditions. These data are repeated in figure 3.9. * $p < 0.05$ vs control.

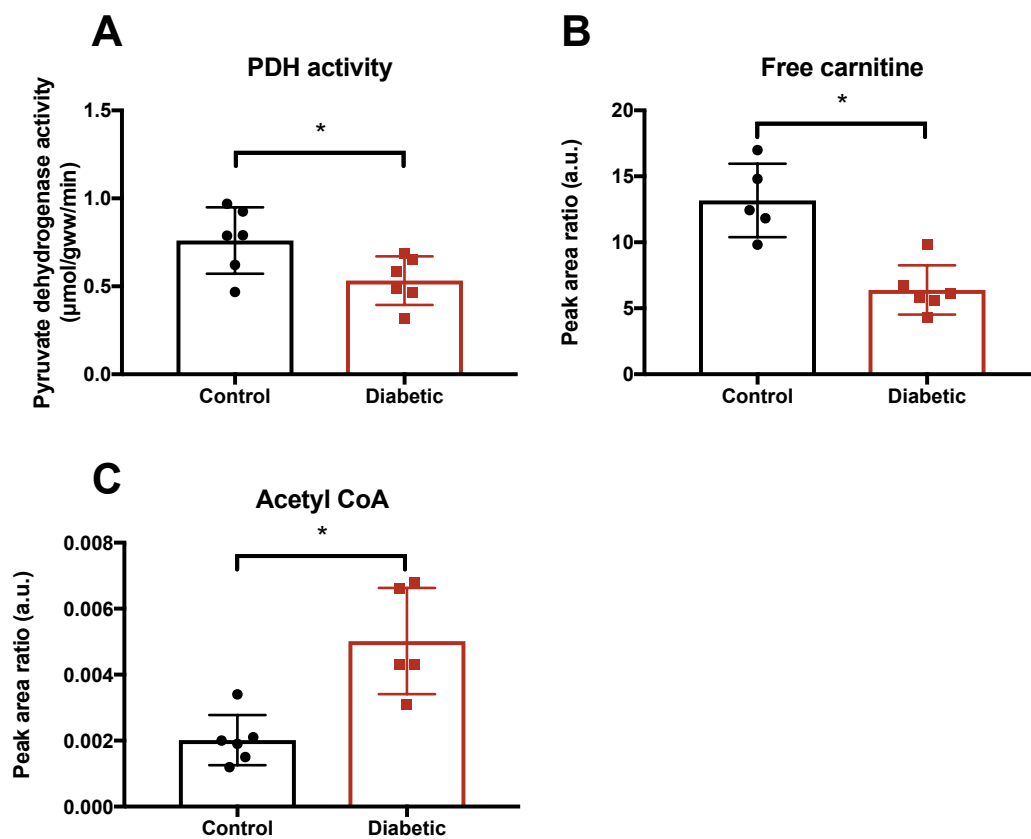


Figure 3.7: Pyruvate dehydrogenase enzyme activity (A), levels of free carnitine (B), and levels of acetyl CoA (C) in control and diabetic rat hearts perfused in normoxic conditions. * $p < 0.05$ vs control.

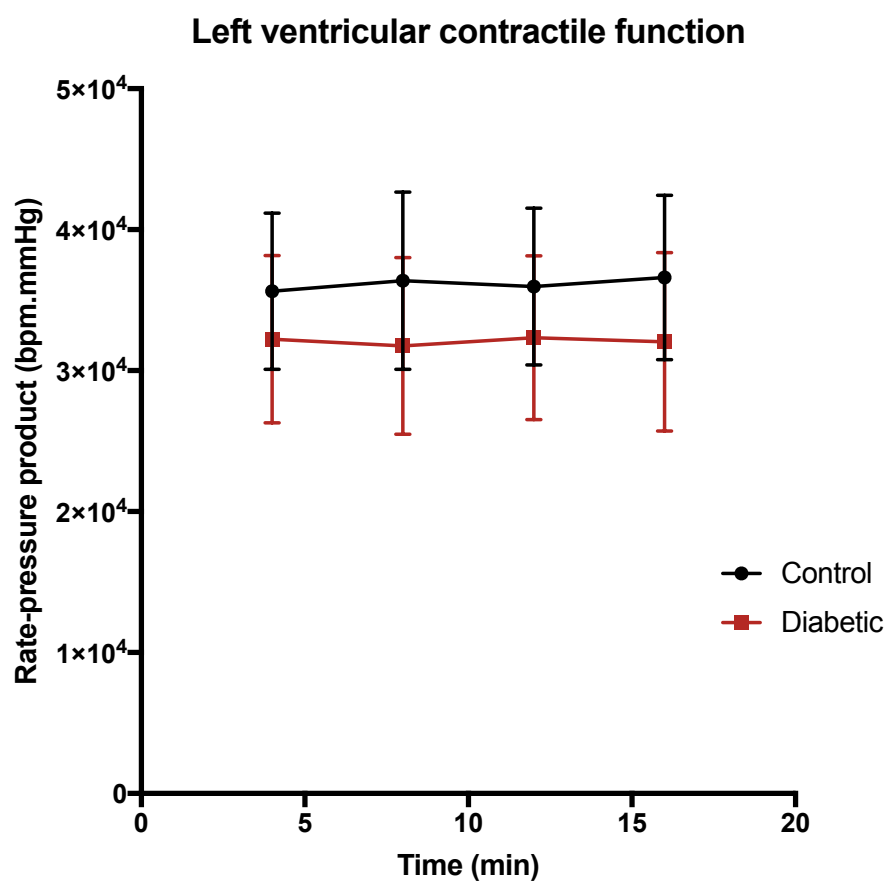


Figure 3.8: Rate-pressure product (RPP) in isolated perfused hearts from control and diabetic rats, during normoxia. These data are repeated in figure [3.16](#)

3.4.2 Effects of acute hypoxia-reoxygenation on control and type 2 diabetic rat hearts

When exposed to acute hypoxia, isolated perfused control and diabetic hearts all displayed significantly increased glycolytic rates, suggesting activation of HIF signalling in both groups (Fig. 3.9A). However, in diabetic hearts absolute glycolytic rates remained 55% lower than in control hearts during hypoxia (Fig. 3.9A). In parallel, hypoxia resulted in a significant decrease in fatty acid oxidation rates in all hearts (Fig. 3.9B). At reoxygenation, glycolytic rate and fatty acid oxidation rate returned to normal in control hearts. Diabetic hearts continued to show significantly lower glycolytic rate and significantly higher fatty acid oxidation rate compared with controls (Fig. 3.9A,B). A pyruvate dehydrogenase (PDH) activity assay performed on hearts freeze-clamped at the end of each oxygenation stage of perfusion showed that PDH activity was significantly increased in control hearts during hypoxia, and this was maintained throughout reoxygenation (Fig. 3.9C). However, diabetic hearts did not show any significant increase in PDH activity during hypoxia, remaining lower than in controls, but increased following reoxygenation (Fig. 3.9C).

Total AMP-activated protein kinase (AMPK) was unchanged between control and diabetic hearts in normoxia, as was the phosphorylated (active) form of the protein, P-AMPK (Fig. 3.10). Both control and diabetic rat hearts upregulated P-AMPK in response to hypoxia (Fig. 3.10). P-AMPK activation results in phosphorylation, and inhibition, of its downstream target, acetyl CoA carboxylase (ACC). P-ACC levels were significantly upregulated in both control and diabetic hearts at reoxygenation (Fig. 3.10). Together, these findings suggest this pathway was not affected in our diabetic model. Protein expression of HIF-1 α was assessed via Western blot, but this was undetectable in samples from hearts subjected to acute hypoxia, indicating that HIF-1 α stabilisation was not significantly increased by this degree of hypoxia (Fig. 3.11).

In normoxia, there were no significant differences in any Krebs cycle intermediates between control and diabetic hearts (Fig. 3.12). In response to hypoxia-reoxygenation, intermediates within the first span of the Krebs cycle, namely citrate,

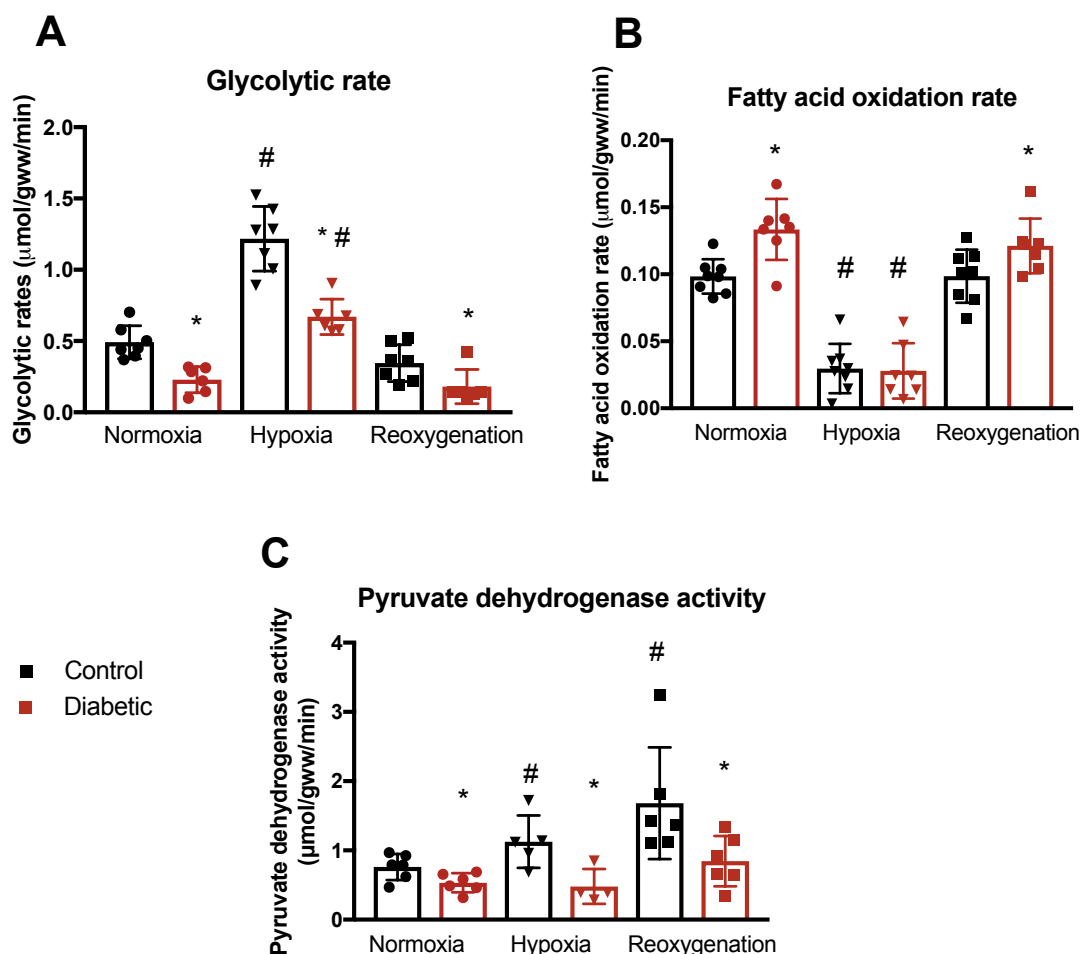


Figure 3.9: Glycolytic (A) and fatty acid oxidation (B) rates, and pyruvate dehydrogenase (PDH) enzyme activity (C), in isolated perfused hearts from control and diabetic rats, during normoxia, hypoxia, and reoxygenation. The reperfusion data are repeated in subsequent figures [3.13](#) and [3.14](#). *p<0.05 vs control, # p<0.05 vs normoxia.

aconitate, and isocitrate, were decreased in control and diabetic hearts. Levels of citrate were significantly lower in diabetic hearts at reoxygenation, and levels of isocitrate significantly lower in diabetic hearts in hypoxia. Sequential intermediates in the second span of the Krebs cycle, namely succinate, fumarate, and malate, were increased in control hearts, by between two- and five-fold, following hypoxia (Fig. [3.12](#)). In diabetic hearts, levels of succinate, fumarate, and malate were 28–54% lower following hypoxia compared with controls.

Several amino acids were modulated in diabetic hearts exposed to hypoxia-reoxygenation. Lysine, aspartate, and asparagine were increased in diabetic hearts

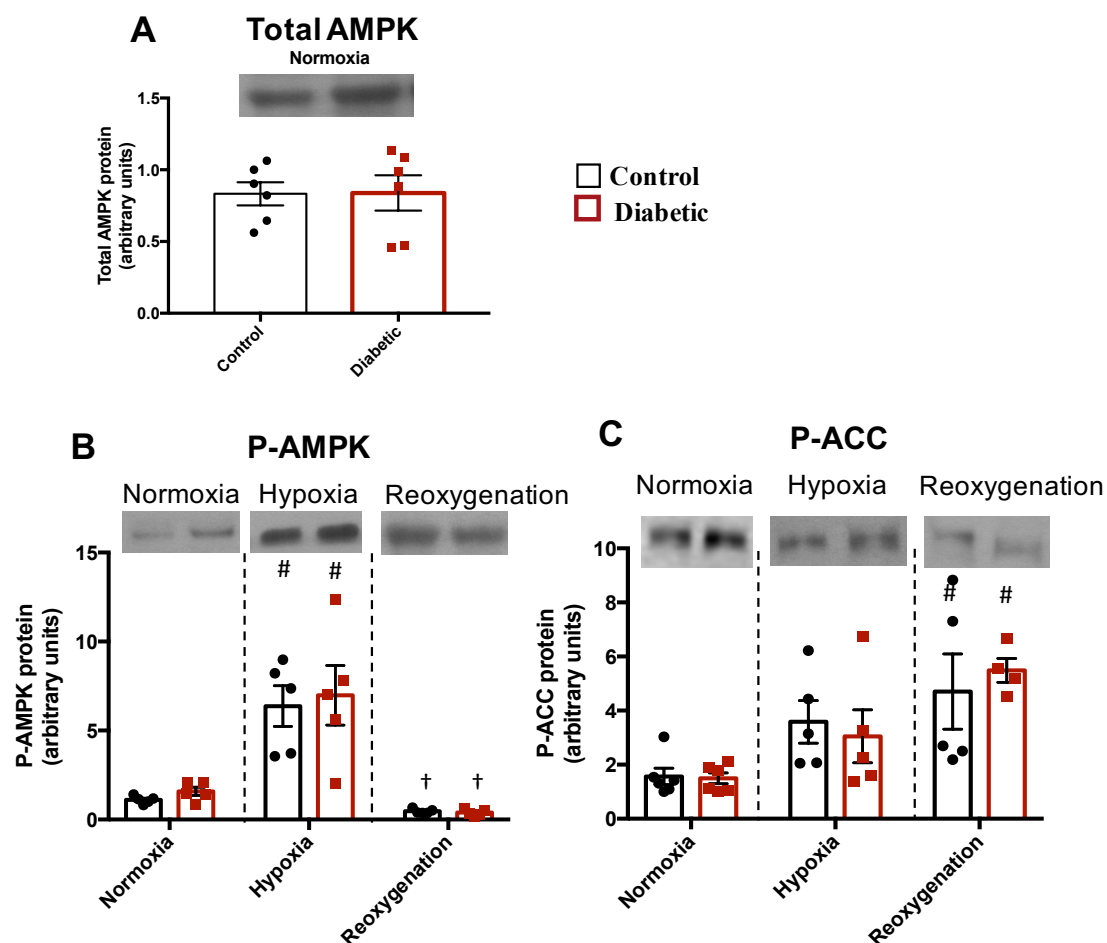


Figure 3.10: Protein levels of total cellular AMP-activated protein kinase (AMPK) (A), phosphorylated AMPK (B), and phosphorylated acetyl-CoA carboxylase (ACC) (C) in control and diabetic hearts, during normoxia, hypoxia, and reoxygenation. Normalised to cyclophilin B. # $p < 0.05$ vs. normoxia within same disease state, † $p < 0.05$ vs. hypoxia within same disease state.

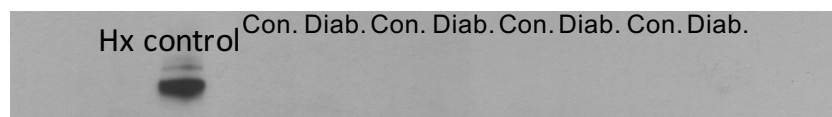


Figure 3.11: Western blot analysis of HIF-1 α protein in control and diabetic hearts subjected to acute hypoxia. Hx control = hypoxic control, cell lysates harvested following 8h hypoxia.

in normoxia (Table 3.1). Leucine, isoleucine, and phenylalanine were increased following hypoxia, and histidine and phenylalanine were decreased following reoxygenation in diabetic hearts compared with controls (Table 3.1).

The total FAD pool (FAD and FADH₂), and NAD pool were selectively depleted

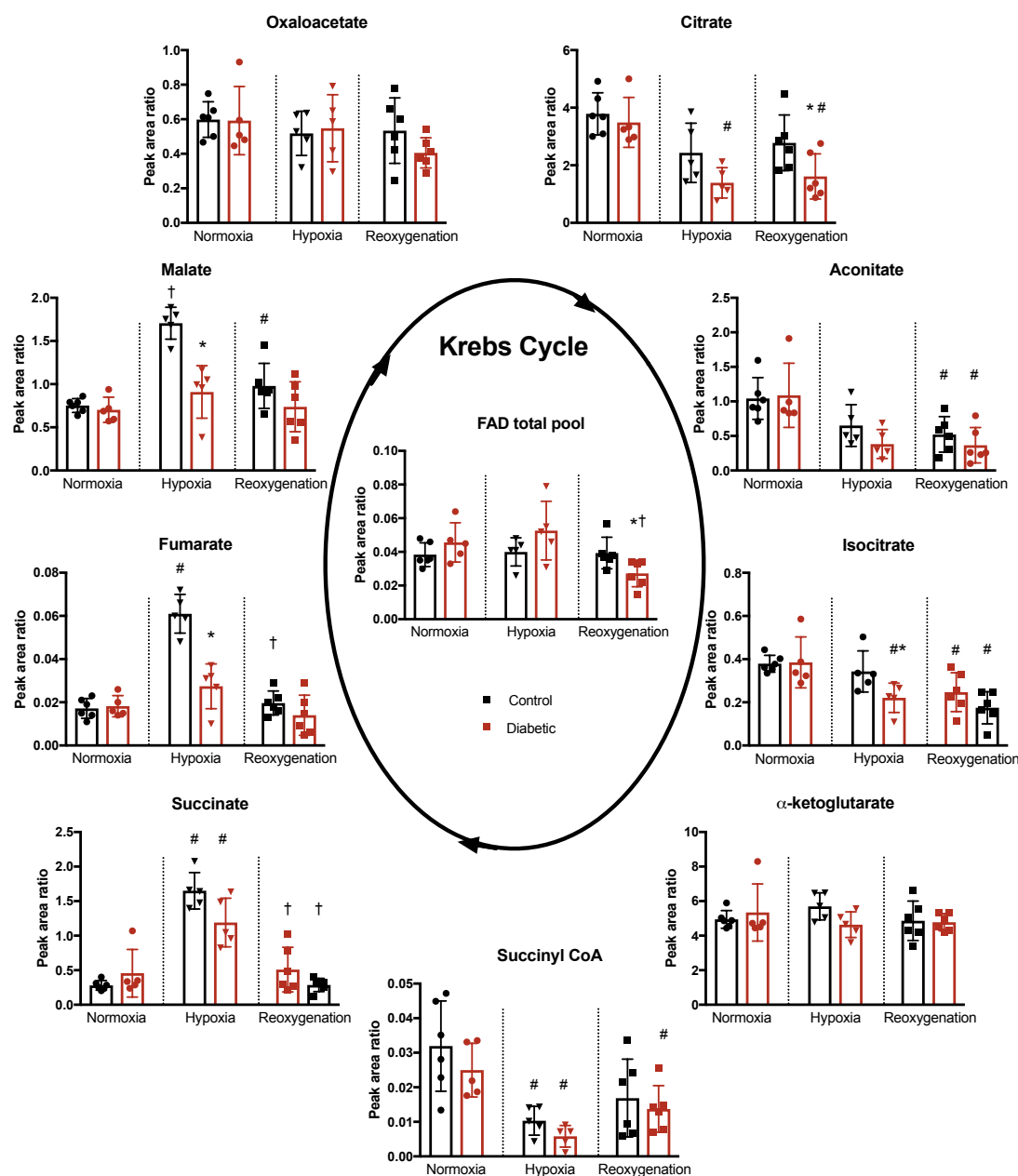


Figure 3.12: Metabolomics analysis of Krebs cycle intermediates in control and diabetic hearts, during normoxia, hypoxia, and reoxygenation. * $p < 0.05$ vs control, # $p < 0.05$ vs normoxia, within same disease state, † $p < 0.05$ vs hypoxia within same disease state.

by 31% in diabetic hearts following reoxygenation, which resulted in these being lower compared with controls at reoxygenation. Despite this decrease in total FAD pool, no significant differences were found in phosphocreatine, or ATP ((Table 3.2)). One-carbon metabolism intermediates choline, betaine, methionine, and S-adenosylmethionine were all depleted in diabetic hearts at reoxygenation, compared

Table 3.1: Table of aminoacids in control and diabetic cardiac tissue in normoxia, acute hypoxia, and reoxygenation.

Amino acids	Normoxia		Hypoxia		Reoxygenation	
	Control	Diabetic	Control	Diabetic	Control	Diabetic
Leucine	0.072 ± 0.011	0.097 ± 0.027	0.188 ± 0.024 #	0.146 ± 0.027 * #	0.108 ± 0.031 †	0.079 ± 0.019 †
Isoleucine	0.06 ± 0.012	0.083 ± 0.024	0.17 ± 0.015 #	0.135 ± 0.028 * #	0.088 ± 0.034 †	0.06 ± 0.016 †
Valine	0.036 ± 0.007	0.05 ± 0.013	0.095 ± 0.010 #	0.082 ± 0.016 #	0.061 ± 0.021 # †	0.042 ± 0.010 †
Phenylalanine	0.052 ± 0.010	0.055 ± 0.014	0.08 ± 0.022	0.053 ± 0.010 *	0.076 ± 0.016	0.054 ± 0.015 *
Lysine	0.16 ± 0.015	0.32 ± 0.09 *	0.17 ± 0.04	0.25 ± 0.07	0.22 ± 0.05	0.22 ± 0.05
Aspartate	0.11 ± 0.02	0.16 ± 0.03 *	0.09 ± 0.02	0.14 ± 0.06	0.09 ± 0.03	0.09 ± 0.02 #
Asparagine	0.015 ± 0.003	0.027 ± 0.004 *	0.017 ± 0.002	0.018 ± 0.012	0.014 ± 0.005	0.015 ± 0.005
Glutamate	17.6 ± 3.23	19.3 ± 6.54	10.2 ± 1.66 #	12.2 ± 2.02 #	9.6 ± 3.41 #	7.7 ± 1.94 #
Glutamine	6.2 ± 0.8	6.6 ± 1.9	4.0 ± 0.8 #	4.8 ± 0.2	3.8 ± 0.9 #	2.6 ± 0.5 # †
Histidine	0.28 ± 0.03	0.25 ± 0.02	0.25 ± 0.07	0.2 ± 0.03 #	0.2 ± 0.05 #	0.13 ± 0.03 * # †
Alanine	0.19 ± 0.04	0.22 ± 0.13	0.42 ± 0.07 #	0.37 ± 0.14	0.16 ± 0.08 †	0.11 ± 0.05 †

* $p < 0.05$ vs control at same oxygenation, # $p < 0.05$ vs. normoxia within same disease state, † $p < 0.05$ vs. hypoxia within same disease state.

with control hearts (Table 3.2). Levels of adenine were significantly lower in diabetic hearts compared with controls in hypoxia and in reoxygenation (Table 3.2).

Table 3.2: Table of aqueous metabolites relating to energetics and one carbon metabolism, in control and diabetic cardiac tissue in normoxia, acute hypoxia, and reoxygenation.

	Normoxia		Hypoxia		Reoxygenation	
	Control	Diabetic	Control	Diabetic	Control	Diabetic
Energetics						
FAD total pool	0.38 ± 0.007	0.46 ± 0.012	0.40 ± 0.009	0.53 ± 0.017	0.39 ± 0.009	0.27 ± 0.008 #
NAD total pool	0.93 ± 0.12	0.96 ± 0.252	0.95 ± 0.11	0.89 ± 0.15	0.82 ± 0.29	0.6 ± 0.13 #
Phosphocreatine	36.2 ± 6.23	32.4 ± 10.52	16.3 ± 6.64 #	13.3 ± 7.77 #	38.9 ± 12.75 †	28.5 ± 7.8 †
ATP	8 ± 0.9	9.2 ± 2.1	5.4 ± 2.1	4.1 ± 1.5 #	4.7 ± 2.81 #	5.5 ± 1.28 #
One carbon metabolism and signalling molecules						
Choline	0.16 ± 0.03	0.17 ± 0.06	0.26 ± 0.07 #	0.19 ± 0.04	0.25 ± 0.05 #	0.15 ± 0.04 *
Betaine	0.39 ± 0.13	0.12 ± 0.04 *	0.39 ± 0.18	0.09 ± 0.02 *	0.31 ± 0.09	0.09 ± 0.09 *
Methionine	0.017 ± 0.001	0.014 ± 0.002	0.023 ± 0.002	0.012 ± 0.002 *	0.023 ± 0.006	0.015 ± 0.004 *
S-adenosyl methionine	0.016 ± 0.003	0.015 ± 0.002	0.017 ± 0.003	0.016 ± 0.002	0.015 ± 0.003	0.01 ± 0.003 * # †
Methylhistidine	0.02 ± 0.004	0.02 ± 0.004	0.014 ± 0.003	0.017 ± 0.005	0.014 ± 0.004 #	0.008 ± 0.002 # †
UDP-glcNAC	4.4 ± 1.26	4.4 ± 1.65	4.1 ± 0.90	3.4 ± 1.20	3.7 ± 1.19	2.3 ± 0.72 #
cAMP	0.018 ± 0.003	0.021 ± 0.007	0.035 ± 0.020	0.024 ± 0.008	0.018 ± 0.005	0.011 ± 0.003 †
Adenine	0.013 ± 0.002	0.013 ± 0.004	0.016 ± 0.001	0.013 ± 0.002 *	0.016 ± 0.003	0.011 ± 0.002 *

* $p < 0.05$ vs control at same oxygenation, # $p < 0.05$ vs. normoxia within same disease state, † $p < 0.05$ vs. hypoxia within same disease state.

3.4.3 Effects of sulfo-*N*-succinimidyl oleate (SSO) on control and diabetic hearts in acute hypoxia-reoxygenation

Metabolic rates and fuel stores

Sulfo-*N*-succinimidyl oleate (SSO) was added to the perfusate 4 min prior to the onset of hypoxia. In diabetic hearts, treatment with SSO was able to increase glycolytic rate by 46% compared with untreated diabetic hearts during hypoxia, resulting in a glycolytic rate no longer significantly different to that of control hearts (Fig. 3.13A). However, SSO had no significant effect on glycolytic rate at reoxygenation (Fig. 3.13A). Following reoxygenation, SSO treatment of diabetic hearts normalized the fatty acid oxidation rate back to that found in control hearts, decreasing fatty acid oxidation by 29% compared with untreated diabetic hearts (Fig.

3.13B). SSO treatment had no significant effect on myocardial glycogen concentration following hypoxia-reoxygenation (Fig. 3.14A). However, SSO treatment resulted in an improvement in myocardial TAG in diabetics. While in untreated diabetic hearts myocardial triglyceride concentration was 73% greater than in control hearts at reoxygenation, this was restored to control levels by SSO infusion (Fig. 3.14B). SSO also led to increased PDH activity in diabetic hearts by 53%, so that it was no longer significantly different to controls (Fig. 3.14C).

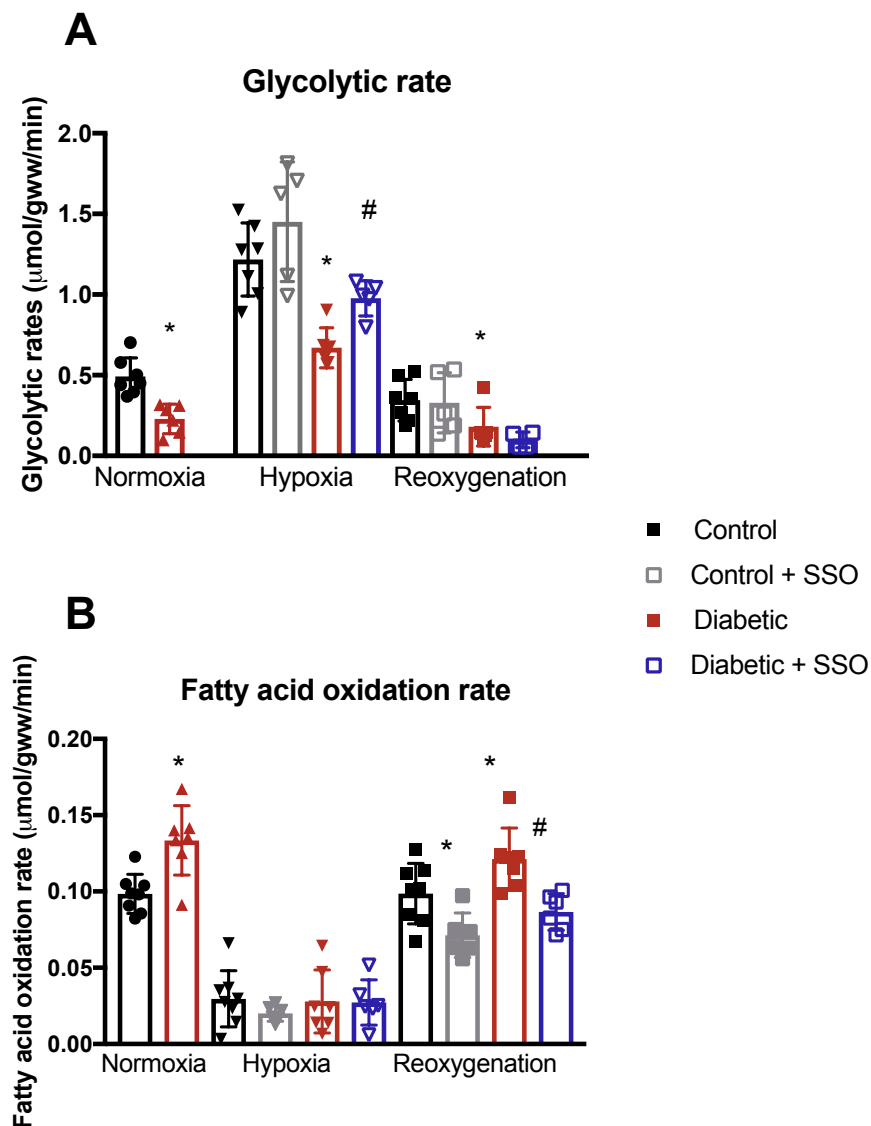


Figure 3.13: Glycolytic rates (A) and fatty acid oxidation rates (B) in isolated perfused control and diabetic hearts following hypoxia-reoxygenation, with and without SSO treatment. * $p < 0.05$ vs control untreated, # $p < 0.05$ vs. diabetic untreated.

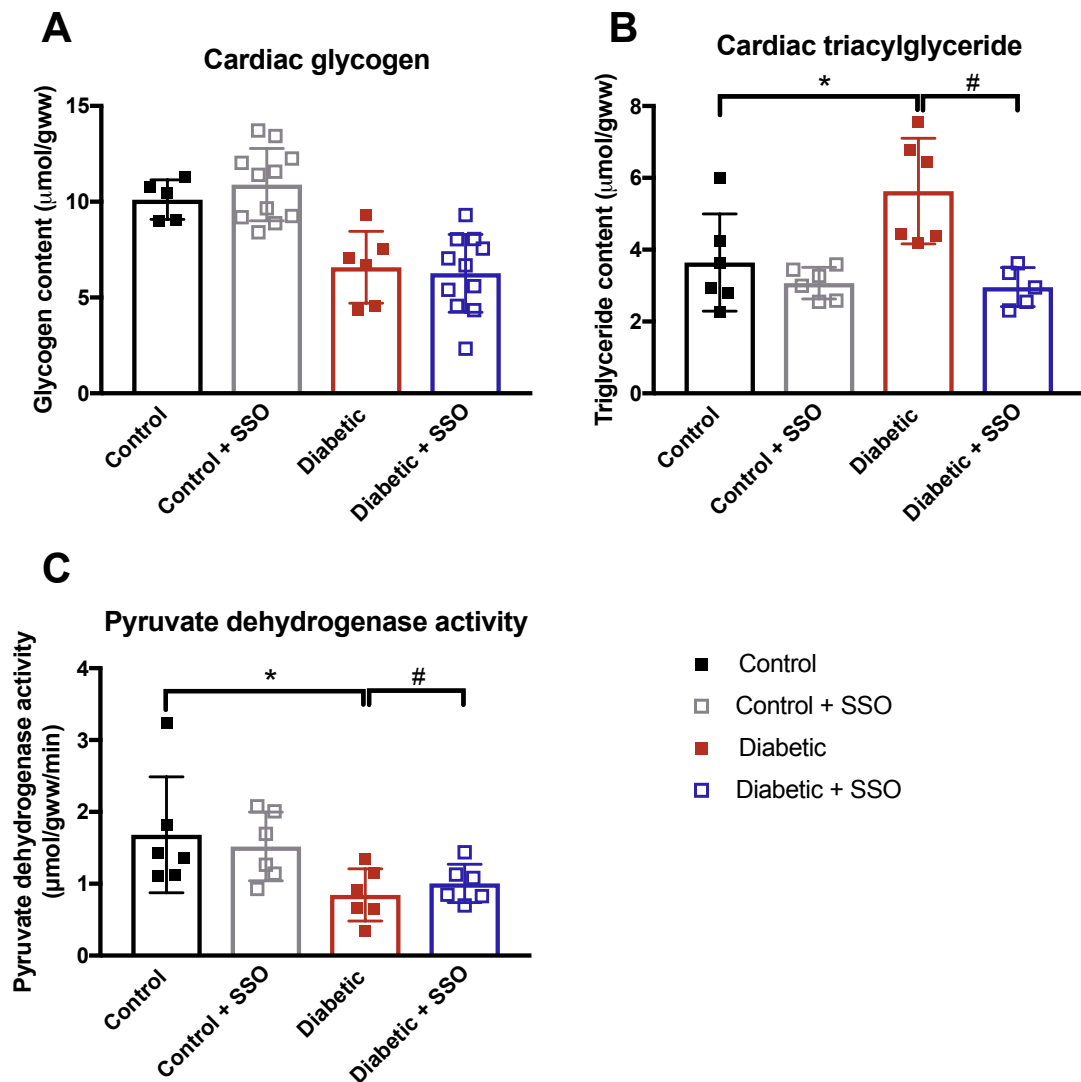


Figure 3.14: Glycogen stores (A), triacylglyceride stores (B), and pyruvate dehydrogenase activity (C) in control and diabetic hearts following hypoxia-reoxygenation, with and without SSO treatment. *p<0.05 vs control, # p<0.05 vs. diabetic untreated.

Cardiac function

SSO had a beneficial effect on RPP in diabetic hearts both during hypoxia and after reoxygenation. Control hearts treated with SSO recovered to a similar extent as untreated controls. Diabetic hearts treated with SSO recovered to the same extent as controls at the end of reoxygenation, showing a 28% improvement in function compared with untreated diabetics (Fig. 3.15). SSO treatment significantly increased heart rate in diabetic hearts during hypoxia, and following reoxygenation restored

heart rate in diabetic hearts to control levels (Fig. 3.16A). Following SSO treatment, end-diastolic pressure was normalized in diabetic hearts during hypoxia, and was no longer significantly different to control hearts (Fig. 3.16B). SSO had no significant effect on developed pressure or coronary flow rates in any group (Fig. 3.16D).

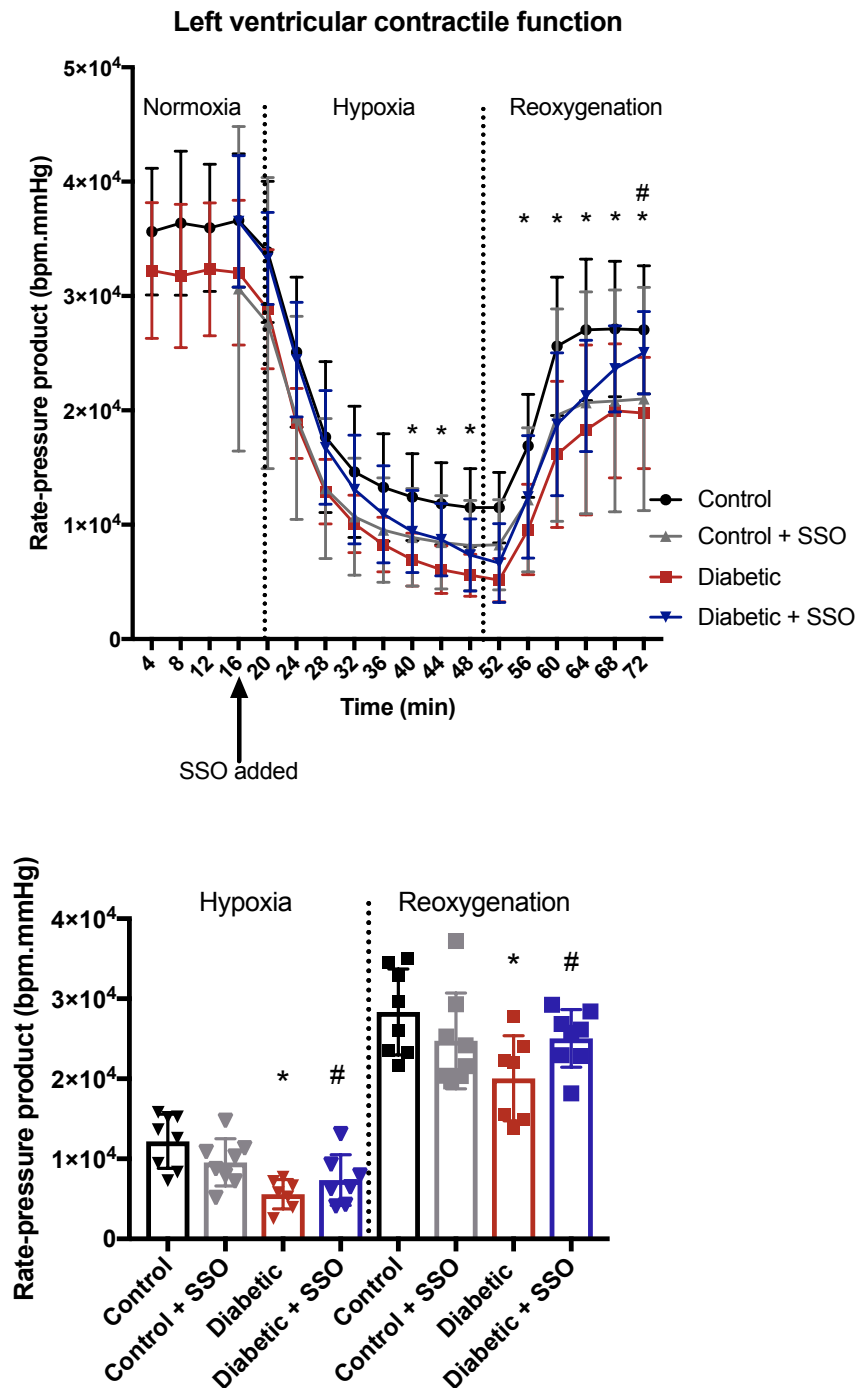


Figure 3.15: Rate-pressure product in isolated perfused control and diabetic hearts exposed to hypoxia-reoxygenation, with and without SSO treatment. *p<0.05 vs control, # p<0.05 vs. diabetic untreated.

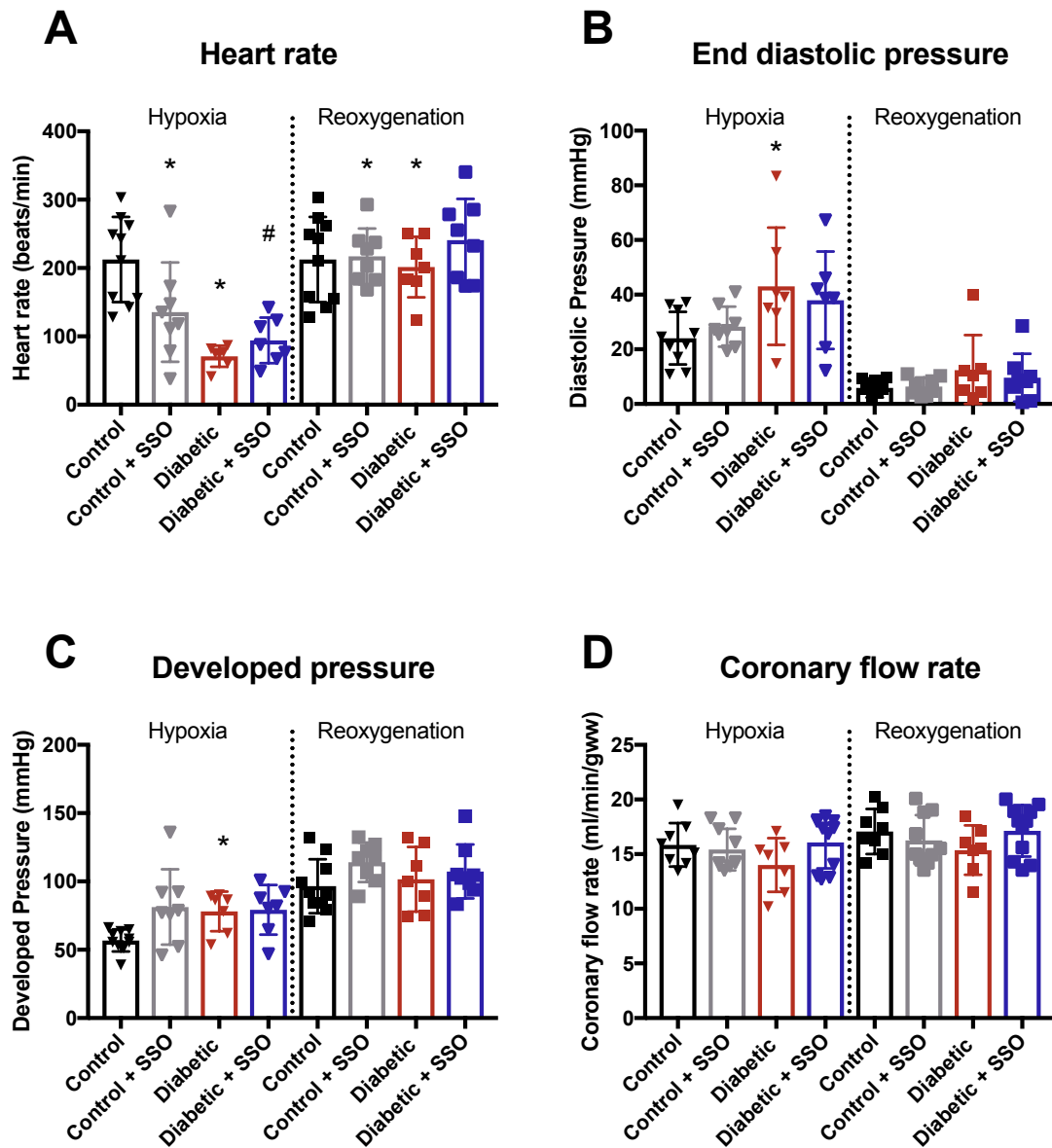


Figure 3.16: Heart rate (A), end diastolic pressure (B), developed pressure (C), and coronary flow rate (D) in isolated perfused control and diabetic hearts following hypoxia-reoxygenation, with and without SSO treatment. * $p < 0.05$ vs control, # $p < 0.05$ vs. diabetic untreated.

Metabolic intermediates

SSO treatment of diabetic hearts had an anaplerotic effect on selective steps within the Krebs cycle (Fig. 3.17). SSO treatment of diabetic hearts resulted in increased levels of Krebs cycle intermediates in the second span of the cycle, doubling the amount of fumarate, and increasing oxaloacetate by 61% compared with untreated diabetic hearts. The total FAD pool, which was depleted at reoxygenation in diabetic hearts, was significantly increased by 67% by SSO treatment of diabetic hearts (Fig. 3.17).

SSO treatment resulted in significantly increased levels of glutamate in diabetic treated hearts compared to treated controls, following hypoxia-reoxygenation (Table 3.3). Additionally, one-carbon metabolism intermediates choline, betaine and methionine, were significantly higher in SSO-treated diabetics at reoxygenation compared with treated controls (Table 3.3).

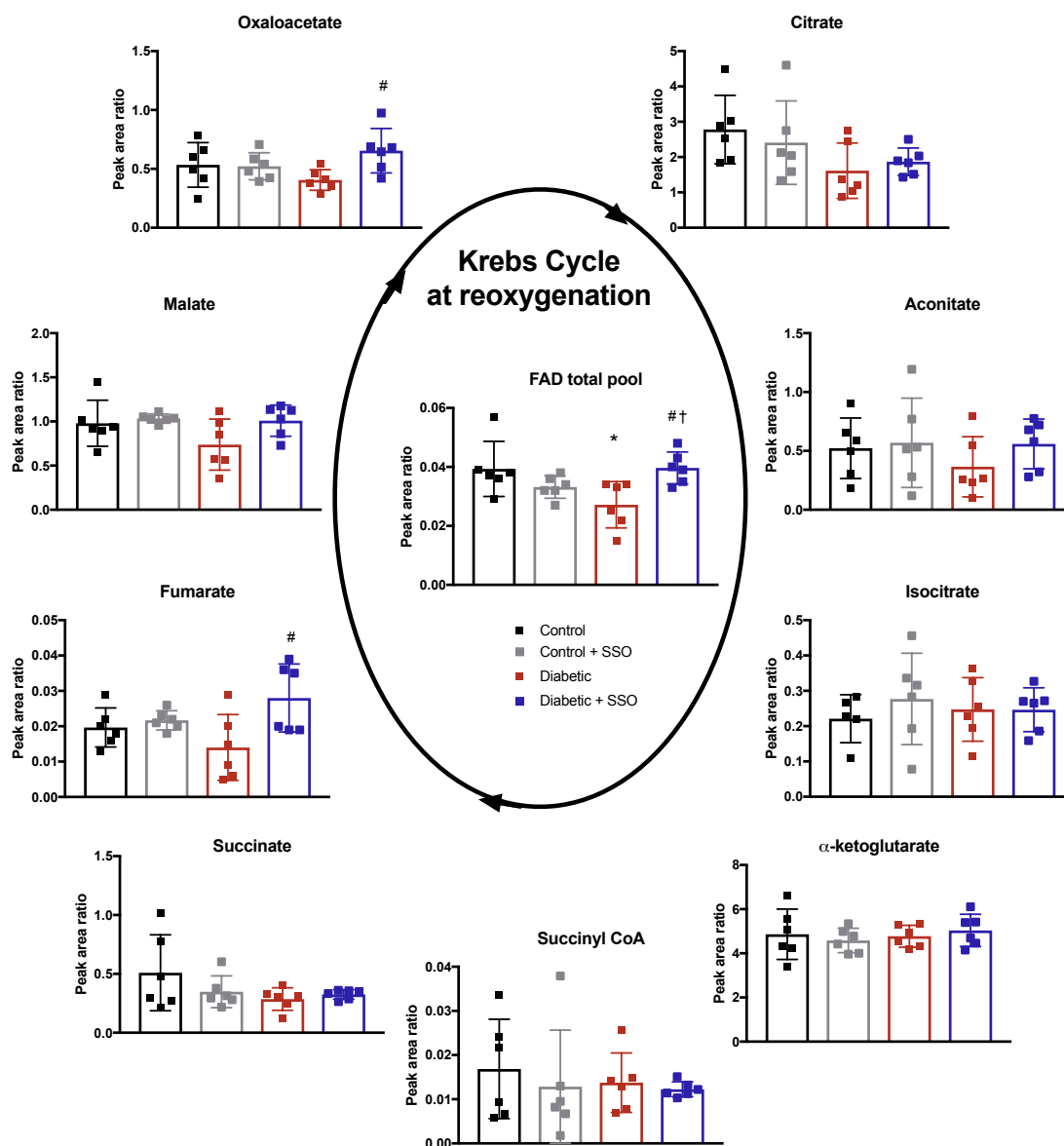


Figure 3.17: Metabolomics analysis of Krebs cycle intermediates in control and diabetic hearts following hypoxia-reoxygenation, with and without SSO treatment. * $p < 0.05$ vs control, # $p < 0.05$ vs. diabetic untreated, † $p < 0.05$ vs. control + SSO.

Levels of short-chain acylcarnitines were not significantly different between control and diabetic hearts, and remained unchanged with SSO treatment (Fig. 3.18). However, levels of dodecanoylcarnitine (C12) were significantly higher in diabetics following reoxygenation, compared with controls, and SSO treatment normalised this to control levels (Fig. 3.19). Levels of tetradecanoylcarnitine (C14) were also significantly higher in diabetics following reoxygenation, and this was significantly decreased to control levels by SSO treatment (Fig. 3.19). SSO treatment

Table 3.3: Metabolomics analysis of aminoacids and one-carbon metabolism intermediates, in control and diabetic hearts following hypoxia-reoxygenation with and without SSO treatment.

Reoxygenation	Control	Control + SSO	Diabetic	Diabetic + SSO
Leucine	0.108 ± 0.031	0.082 ± 0.019	0.079 ± 0.017	0.080 ± 0.014
Isoleucine	0.088 ± 0.034	0.063 4 ± 0.016	0.060 ± 0.010	0.065 ± 0.013
Valine	0.061 ± 0.021	0.043 ± 0.010	0.042 ± 0.007	0.047 ± 0.010
Phenylalanine	0.076 ± 0.016	0.058 ± 0.015	0.054 ± 0.011 *	0.054 ± 0.008
Lysine	0.22 ± 0.05	0.19 ± 0.05	0.22 ± 0.02	0.25 ± 0.036
Aspartate	0.09 ± 0.03	0.08 ± 0.0	0.09 ± 0.01	0.15 ± 0.07
Asparagine	0.014 ± 0.005	0.016 ± 0.005	0.015 ± 0.003	0.015 ± 0.004
Glutamate	9.6 ± 3.4	7.6 ± 1.9	7.7 ± 0.9	9.7 ± 1.8 †
Glutamine	3.8 ± 0.9	3.1 ± 0.6	2.6 ± 1.1	3.1 ± 1.0
Histidine	0.20 ± 0.04	0.17 ± 0.03	0.13 ± 0.03 *	0.16 ± 0.03
Alanine	0.16 ± 0.08	0.13 ± 0.05	0.11 ± 0.05	0.13 ± 0.04
NAD total pool	0.82 ± 0.29	0.75 ± 0.13	0.60 ± 0.24	0.76 ± 0.17
Phosphocreatine	38.9 ± 12.7	33.3 ± 7.8	28.5 ± 14.1	41.4 ± 11.7
Choline	0.25 ± 0.05	0.19 ± 0.04 *	0.15 ± 0.03 *	0.16 ± 0.01 †
Betaine	0.31 ± 0.09	0.29 ± 0.08	0.09 ± 0.05 *	0.08 ± 0.02 †
Methionine	0.023 ± 0.006	0.018 ± 0.004	0.015 ± 0.002 *	0.015 ± 0.002 †
Adenine	0.016 ± 0.003	0.014 ± 0.002	0.011 ± 0.001 *	0.013 ± 0.001

* $p < 0.05$ vs control, † $p < 0.05$ vs. control + SSO, n = 5 - 6 per group.

also resulted in depletion of pentadecanoylcarnitine (C15), and a significant increase in 3-OH-octadecenoylcarnitine (C18:1-OH), in both control and diabetic hearts following reoxygenation (Fig. 3.19). Odd-chain fatty acids, particularly C15, have been associated with risk of type 2 diabetes [177], however this can not explain why our data shows no difference between control and diabetic hearts, but shows a consistent change only in SSO-treated hearts compared with their untreated counterparts. The increase in C18:1-OH is curious, given that SSO itself is derived from oleic acid, and could indicate a contamination of the fatty acid in the SSO solution from the synthesis.

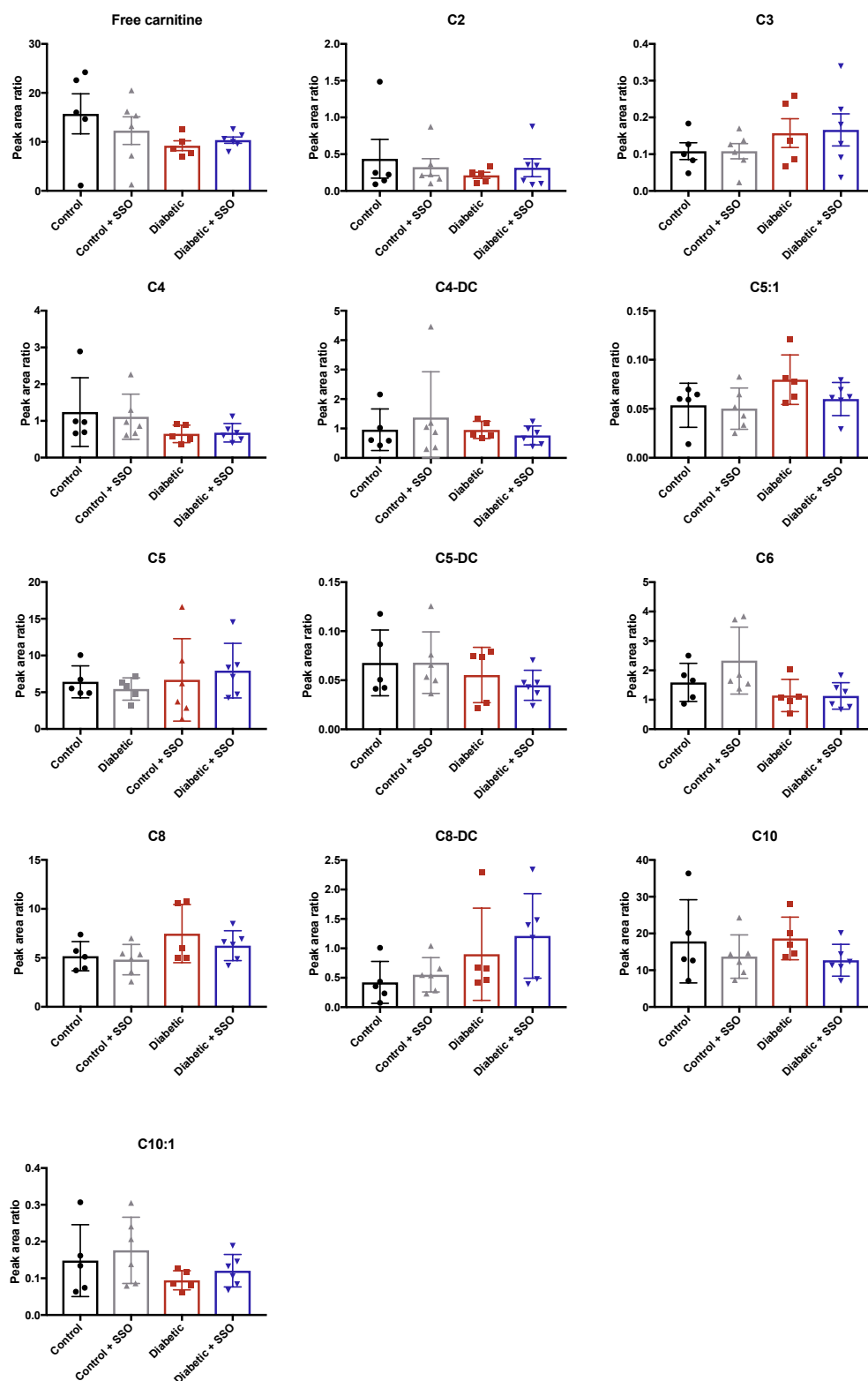


Figure 3.18: Metabolomics analysis of short- and medium-chain acylcarnitines in control and diabetic hearts following hypoxia-reoxygenation, with and without SSO treatment, ordered by acyl chain length. OH = monocarboxylic, DC = dicarboxylic.

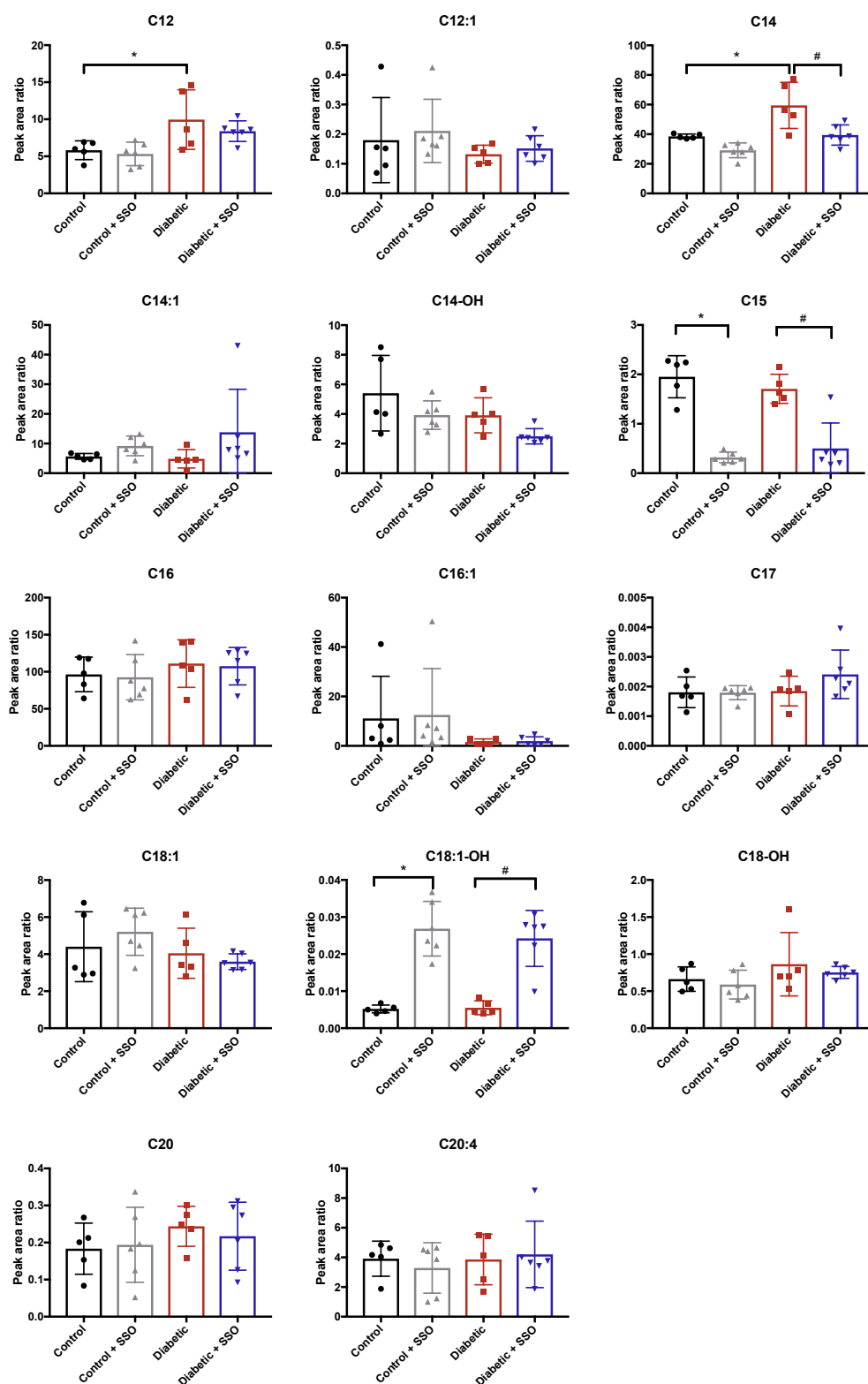


Figure 3.19: Metabolomics analysis of medium- to long-chain acylcarnitines in control and diabetic hearts following hypoxia-reoxygenation, with and without SSO treatment, ordered by acyl chain length. *p<0.05 vs control, # p<0.05 vs. untreated diabetic.

3.5 Discussion

Type 2 diabetes is a metabolic disorder affecting a number of different organs, with a pathological systemic overdependence on fatty acid utilisation, and an inability to respond appropriately to insulin, therefore failing to upregulate glucose utilisation [199]. We have demonstrated that our type 2 diabetic rat model exhibits the hallmarks of the disease, with increased fasting insulin and glucose levels, and increased circulating fatty acids. This rat model of type 2 diabetes was developed in our group relatively recently [137], and it was therefore important to characterise it fully, to ensure that the observed changes resembled other models and human disease. Additionally, we showed that this metabolic dysregulation extended to the cardiac tissue, with increased expression of several markers of fatty acid oxidation, suggesting a sustained upregulation of PPAR α activity, after just five weeks of disease model generation.

3.5.1 Diabetic hearts retained a degree of metabolic flexibility

Work from other groups has previously suggested an absolute lack of metabolic flexibility and responsiveness to stimuli in diabetes, suggesting glycolysis would not have been upregulated in these conditions [152, 147]. In response to hypoxia, we found that diabetic hearts, were able to upregulate glycolysis to some extent, showing a similar fold increase in glycolytic rates to that seen in control hearts. However, the rates reached by diabetic hearts remained significantly lower than those achieved by controls, demonstrating indeed some impaired metabolic flexibility in the diabetic heart. Having additionally found that diabetic hearts were not able to stimulate translocation of SLC2A4 to the sarcolemma to the same extent as control hearts, and that this was accompanied by a lack of increase in pyruvate dehydrogenase activity, we questioned whether this was due to impaired energy status signalling. Under acute stress, the low energy levels within the cell generate

AMP, which is sensed by AMPK [106]. Interestingly, our diabetic hearts did not exhibit impaired stimulation of AMPK, as we found that the active, phosphorylated form of the protein, P-AMPK, was increased to the same levels in both control and diabetic hearts in response to hypoxia. In agreement with this, other groups have previously found intact AMPK signalling despite insulin resistance [115]. We therefore showed that the diabetic heart retained a degree of inherent metabolic flexibility, but that this required the activation of multiple pathways, as intrinsic stimulation of AMPK signalling alone was not sufficient to reach the maximal glycolytic response. This abnormal glycolytic increase, despite normal AMPK activation, indicated an alternative mechanism for the suppression of anaerobic metabolism in hypoxia. We hypothesised that this was a Randle cycle-mediated effect, due to the increase in fatty acid metabolism [168]. By inhibiting fatty acid uptake, we exploited the Randle cycle in the opposite direction, inhibiting fatty acid oxidation, leading to increased glucose uptake, and even restoring PDH activity.

3.5.2 Treatment with SSO improved post-hypoxic function in diabetic hearts

We investigated the effects of infusing the FAT/CD36 inhibitor sulfo-*N*-succinimidyl oleate (SSO) added into the perfusion buffer just 4 min prior to hypoxia. Our results suggest that the abnormalities leading to functional deficit in the diabetic heart are of metabolic nature, and could be treated via a metabolic therapeutic strategy. SSO has previously been shown to reduce fatty acid uptake in isolated rat cardiomyocytes but had never been tested in an intact beating heart [131]. We found that this short window of pre-treatment was sufficient to significantly improve substrate metabolism, reverting the diabetic heart to a profile comparable to healthy controls, and resulted in functional rescue of the diabetic hearts following hypoxia-reoxygenation. We found that diabetic hearts had impaired functional recovery following reoxygenation. Diabetic hearts displayed lower heart rates in hypoxia and reoxygenation compared with controls, driving the post-hypoxic functional impairment. Additionally, end diastolic pressure was significantly higher in diabetic hearts during hypoxia, which is in line with the clinical observation

that diabetic patients develop diastolic dysfunction [27, 5]. SSO treatment in diabetic hearts improved both of these parameters, preventing the significant increase in end diastolic pressure. SSO therefore prevented the cardiac dysfunction in diabetics, in agreement with previous studies showing diastolic dysfunction in diabetic rats, associated with decreased PDH flux, which was reversed when PDH flux was increased. Additionally, SSO had the added benefit of preventing the accumulation of intracellular triglycerides, a benefit that does not occur with compounds that target mitochondrial fatty acid oxidation directly, where fatty acid uptake and esterification may continue unaffected, resulting in lipotoxic effects, as has been reported for CPT1 inhibitor etomoxir [43]. Lipotoxicity resulting from intramyocardial lipid accumulation, together with sustained PPAR α upregulation, have been implicated in the development of cardiac hypertrophy and heart failure in diabetes [222, 64, 184]. Genetic studies have previously shown that FAT/CD36 ablation can prevent cardiac lipotoxicity linked to PPAR α upregulation, which provided hope for metabolic strategies in the context of obesity and diabetes [213]. We have demonstrated that this is translatable into an *ex vivo* setting, where treatment just minutes preceding a hypoxic insult was sufficient to restore the metabolic phenotype and rescue contractile function in the diabetic heart.

3.5.3 Acute hypoxia resulted in differential depletion of metabolic intermediates in controls and diabetics

Our metabolomics analysis allowed us to use a non-hypothesis approach to the investigation of the effects of hypoxia-reoxygenation and SSO treatment. This led to the identification of non-uniform changes to the Krebs cycle intermediates, with a depletion of intermediates in the first span of the cycle, and accumulation of intermediates within the second span. This pattern was formed around enzymatic steps involving NAD-dependent dehydrogenases or substrate level phosphorylation steps, suggesting that this profile may be due to redox-dependent mitochondrial regulation. Consistent with these findings, Banke *et al.* found abnormal Krebs cycle flux in diabetic hearts only when stressed by ischaemia [10]. Interestingly, our data show that diabetic hearts had lower levels of succinate, as well as fumarate,

during hypoxia compared with controls. Succinate accumulation during ischaemia has been implicated in the generation of mitochondrial reactive oxygen species and reperfusion injury, via reverse electron transport by complex I [36]. This might suggest that in diabetes, the succinate-mediated reperfusion injury would be reduced, however, we cannot assess this in our reoxygenated tissue (due to the acute nature of the hypoxia). In our study, control hearts exhibit increased succinate levels in hypoxia, which go hand in hand with improved functional recovery upon reoxygenation compared with diabetics. This suggests a differential effect of hypoxia and ischaemia, or a longer time frame needed to see the consequent effects. Branched chain aminoacids, leucine and isoleucine, were depleted during hypoxia in diabetic hearts compared with controls. This could be explained by these aminoacids feeding into the Krebs cycle via acetyl CoA under hypoxic stress. Few studies have focused on aminoacid metabolism in the heart, but branched-chain aminoacids have previously been reported to be protective in the heart in ischaemia [200, 123]. Finally, our data showing increases in oxaloacetate, and the total FAD pool in diabetic hearts with SSO treatment, suggest a further anaplerotic benefit from metabolic therapy in diabetes, replenishing metabolic intermediates and preventing energy stress [41].

In this chapter, we demonstrated the potential of metabolic therapy in a setting of acute hypoxia, using an acute treatment strategy. Therefore, the effects observed here are changes induced immediately, exploiting the available existing proteins within the tissue. Chronic SSO studies could reveal whether additional beneficial effects could also occur, via transcriptional and translational regulation from decreased fatty acid metabolism. This would be of interest, to investigate the effects of metabolic therapy longer term, particularly on cardiac function and metabolism.

Four simple chambers.
A thousand complicated doors.
One of them is yours.

— Jill Alexander Essbaum

4

Effects of dimethyloxallylglycine on cardiac ischaemia-reperfusion in diabetes

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4.1 Chapter Abstract

Whether the diabetic heart has an impaired HIF signalling response to hypoxia is unclear in the literature. In Chapter 3, we demonstrated that in our rat model of type 2 diabetes, the heart did not adapt to an acute hypoxic insult to the same extent as a healthy control in absolute terms, but showed a similar fold increase in glycolytic rate. In this chapter, we aimed to investigate the therapeutic potential of hypoxia mimetics in a clinically relevant setting of ischaemia-reperfusion. Our hypothesis was that HIF-1 α stabilisation was abnormal, and that stimulating HIF signalling pharmacologically using hypoxia mimetic dimethyloxallylglycine (DMOG) would improve substrate metabolism and function in the diabetic heart. Control and diabetic rats were treated with DMOG (40 mg/kg) intraperitoneally on three alternate days over the period of a week following establishment of the diabetic model. Hearts from these rats were perfused in Langendorff mode, using a protocol of low-flow ischaemia followed by reperfusion. Substrate metabolism was measured throughout using radiolabelled substrates (glucose and palmitate), and function was measured using a pressure transducer connected to a balloon in the left ventricle. Hearts were frozen following perfusion for subsequent studies, including fuel store assays, protein quantification, and metabolomics analysis. We found that diabetic hearts recovered to a lesser extent following ischaemia-reperfusion, compared with control hearts. DMOG-treated diabetic rat hearts showed significantly improved functional recovery compared with untreated diabetic hearts. Surprisingly, DMOG-treated rat hearts did not show any significant differences in glycolytic rates, and instead showed increased fatty acid oxidation rates at baseline. Subsequent analysis, including blood haemoglobin levels, HIF-1 α protein, and HIF-1 target gene mRNA and protein levels, revealed no evidence of HIF-1 signalling upregulation with DMOG treatment, suggesting the observed effects may be due to an off-target mechanism of DMOG.

4.2 Introduction

Hypoxia inducible factor (HIF) signalling is crucial in the heart, as it forms a central part of the response to hypoxia. This is clinically very relevant, as a myocardial infarction will inevitably cause localised hypoxia in the affected region of the myocardium, and an appropriate HIF response is required to protect the tissue from ischaemic injury, and adapt to the new environment. HIF signalling has a number of effects including improved oxygen delivery via angiogenesis and increased hematocrit [203, 69]. However, the metabolic effects of HIF stimulation are particularly important to the heart's ability to maintain function following MI. HIF signalling orchestrates the metabolic transition away from oxygen-expensive fatty acid oxidation and oxidative metabolism, towards oxygen-sparing anaerobic glycolysis, allowing the heart to produce sufficient ATP anaerobically to fuel contraction [53, 86].

Whether the diabetic heart has an impaired HIF signalling response to hypoxia is unclear in the literature. Studies have found blunted HIF-1 α stabilisation in STZ-diabetic rats following MI [139], and lower levels of HIF-1 α protein and target gene expression in diabetic patients compared with nondiabetics [140].

There has additionally been some evidence for impaired hypoxia signalling in diabetic models, though these have mostly been studies in contexts not directly relating to the heart [25, 125]. As hypoxia and diabetes have fundamentally opposing effects on substrate metabolism, hypoxia mimetics may pose an interesting avenue of treatment for the metabolic abnormalities observed in the diabetic heart (as discussed in Chapter 1). Hypoxia stimulates increases in glycolytic rates, and decreases in fatty acid oxidation and oxidative metabolism. In contrast, diabetes leads to decreased glycolysis and increased fatty acid uptake and oxidation.

There is a strong link between HIF signalling and PPAR α regulation, which is relevant to the chronic metabolic adaptations observed in type 2 diabetes. It has

been shown that PPAR α upregulation by high fat feeding leads to impaired hypoxic signalling and cardiac dysfunction in chronic hypoxia [38]. In the diabetic heart, studies have shown evidence for insulin responsiveness and therefore a degree of metabolic flexibility [79], while other studies support cardiac metabolic inflexibility in diabetes [152]. Work from our group showed that in our model of type 2 diabetes, the heart retained the ability to adapt its metabolism in response to chronic hypoxia [138]. However, this study also showed that the diabetic heart's response was superimposed on the abnormal metabolic disease state, which resulted in lower absolute rates of glycolysis and levels of glycogen stores, and higher absolute rates of fatty acid oxidation and mitochondrial oxygen consumption [138]. Therefore, while it appears that the diabetic heart maintains some flexibility and ability to respond to stimuli, the fact that the baseline metabolic state is so dramatically altered, affects the level to which anaerobic metabolism can be upregulated. Thus, additional pharmacological stimuli, to encourage further metabolic flexibility in diabetes may be beneficial.

The development of pharmacological compounds upregulating hypoxia signalling has focused on HIF-1 α stabilisation. One of the first classes of HIF-1 α stabilising drugs were α -ketoglutarate (α -KG) mimetics. Dimethyloxalylglycine (DMOG) is a commercially-available prodrug, which is metabolised to *N*-oxalylglycine, a cell-permeable α -KG mimetic, that binds to and inhibits prolyl hydroxylase domain (PHD) enzymes, preventing the hydroxylation of HIF-1 α even under normoxic conditions [173, 110]. Treatment with DMOG has been shown to increase levels of HIF-1 α in cultured cells as well as *in vivo* [58, 9]. Additionally, some pre-clinical work has been done using DMOG in models of cardiac disease. In a rabbit model of myocardial ischaemia-reperfusion, pre-treatment with DMOG led to a significant decrease in infarct size [153]. Further to this, a study using a perfused heart setup showed that pre-treatment of rats *in vivo* resulted in improved functional recovery following ischaemia-reperfusion *ex vivo* [164].

In this Chapter, we investigated the effects of chronic *in vivo* pre-treatment with DMOG on diabetic rat hearts in an *ex vivo* model of low-flow ischaemia-

reperfusion. We hypothesised that pre-treatment with DMOG would result in increased baseline glycolytic rates in the diabetic heart, allowing the heart to better cope with ischaemia, and consequently improving post-ischaemic cardiac function. While in Chapter 3 our focus was on investigating the response of the diabetic heart to acute hypoxia, in this chapter we wanted to assess the therapeutic effect of hypoxia mimetics for the diabetic heart in a clinically relevant situation of cardiac stress. For this reason, we chose to use a model of low-flow ischaemia-reperfusion, as it provided better clinical translation to a setting of myocardial infarction. We therefore set out to clarify, firstly, whether HIF is abnormal in the diabetic heart, and, secondly, whether DMOG treatment would benefit metabolism and post-ischaemic cardiac function in diabetes.

The aims of this chapter were:

- Firstly, to assess the effects of low-flow ischaemia on the diabetic heart, and
- Secondly, to assess the effects of *in vivo* DMOG pre-treatment on the diabetic heart in ischaemia-reperfusion.

4.3 Methods

4.3.1 Animal work and *in vivo* DMOG treatment

Diabetic rats were fed high fat diet (HFD) for four weeks prior to the start of the treatment (Fig. 4.1), and given an i.p. injection of streptozotocin at the end of the second week of high fat feeding, following our diabetic model protocol. Control rats were weight-matched to the diabetics at the start of the study and continued on normal chow throughout the five weeks of study. Rats were treated *in vivo* with DMOG during the final week prior to heart perfusion, to allow time for changes in gene transcription and protein translation induced by HIF upregulation. To have a chronic effect, which would be more clinically relevant, we chose to treat on three alternate days in the period of a week, taking the heart 24h after the last dose. Rats were given 40 mg/kg DMOG via intraperitoneal (i.p.) injection on three alternate days, as described in Chapter 2. Fasting blood samples were taken two days before perfusion, under recovery isoflurane anaesthesia (2.5 ml/min). Fed blood samples were taken at the time of heart excision, together with the liver, and blood haemoglobin levels were measured immediately, as a measure of systemic HIF activation. In a subset of rats, hearts were frozen immediately at the time of isolation and not taken for perfusions. This allowed us to assess effects of DMOG treatment independent of the effect of ischaemia-reperfusion or perfusion of the heart.

4.3.2 Ischaemia-reperfusion heart perfusion

In total, four groups were studied: control, diabetic, control + DMOG, and diabetic + DMOG. Hearts from all groups were isolated and perfused in Langendorff mode with Krebs-Henseleit (KH) buffer containing 11 mM glucose, 0.4 mM palmitate, and 3 U/l insulin, as described in section 2.5.4. Pre-ischaemic function and metabolism were assessed for a period of 20 minutes, following which the hearts were subjected to low-flow ischaemia at a flow rate of 0.3 ml/min/gww for 30 minutes. This ischaemic flow rate was chosen following preliminary experiments to optimise the functional

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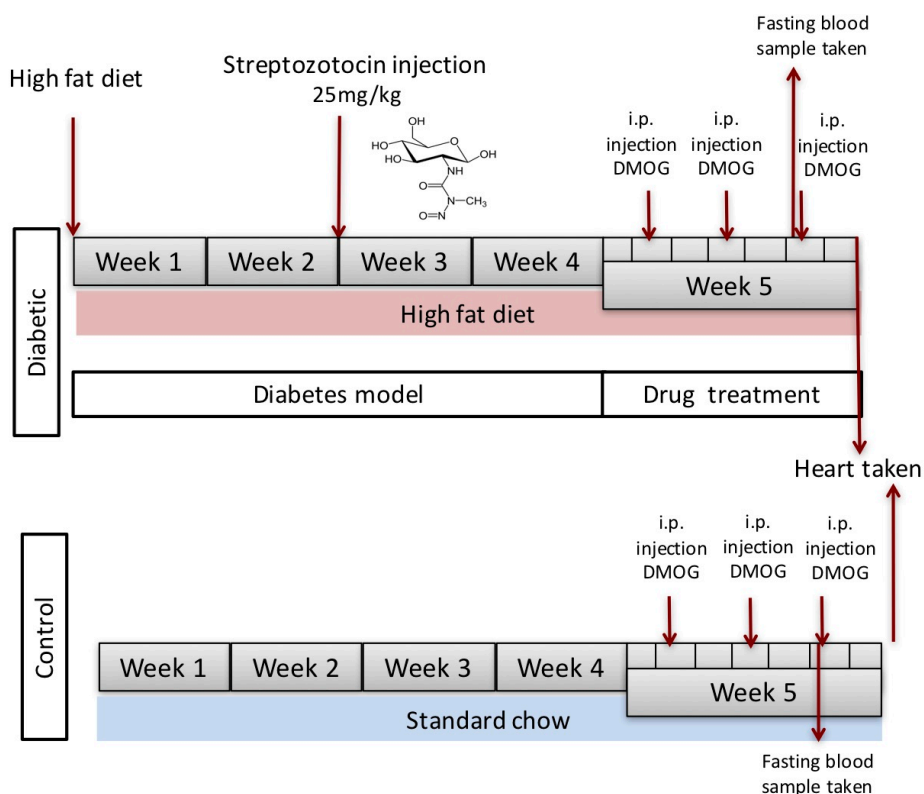


Figure 4.1: Timeline of animal work for Chapter 4 - Control rats were fed normal chow, and diabetics were fed high fat diet, for four weeks prior to the start of treatment. Low-dose (25 mg/kg) STZ was given to diabetic rats two weeks into high fat feeding. Two weeks after STZ dosing, rats receiving DMOG treatment were given 40 mg/kg DMOG intraperitoneally on three alternate days over the final week. Fasting bloods were taken two days prior to sacrifice. Hearts were excised 24h-48h following the final dose of DMOG.

recovery percentage. Normal flow was resumed for a further 20 minutes. Hearts were rapidly freeze-clamped on the cannula at the end of the reperfusion period, and stored at -80°C for subsequent analysis. The perfusion buffer was supplemented with $0.2 \mu\text{Ci/ml}$ of either $[5-^3\text{H}]$ -glucose for the measurement of glycolytic rates, or $[9,10-^3\text{H}]$ palmitate for the measurement of fatty acid oxidation rates. A sample of perfusate (1 ml in volume) was taken every 4 minutes over the perfusion period and used to calculate myocardial glycolytic flux as previously described section [2.5.5](#) and fatty acid oxidation rates as described in section [2.5.5](#). A subset of untreated control and diabetic hearts were frozen at the end of ischaemia to initially study the effects of acute ischaemia on the diabetic heart.

4.3.3 Statistical Analysis

Data are expressed as means $\pm$ standard deviation from the mean (SD), and $p < 0.05$ was taken as the level of statistical significance.

Analyses made between controls and diabetics alone were carried out using an unpaired, two-tailed Student's *t* test. All data shown as the four groups (controls and diabetics with and without drug treatment) were analysed using a two-way ANOVA, where the two factors were diabetes and drug, using Sidak correction for multiple comparisons. Cardiac function and metabolism with drug treatment were compared within each perfusion state separately. Where data did not pass normality tests, the statistical analysis was performed using Kruskal-Wallis test instead (non-parametric).

The correlation between levels of HIF-1 α in acute ischaemia and *in vivo* levels of plasma NEFA was analysed using a linear regression of HIF-1 α protein levels versus NEFA levels to check linear relationship, and then using Pearson correlation coefficient analysis.

GraphPad Prism 7 Software Package (GraphPad Softwares, California, USA) was used for all statistical analyses.

4.4 Results

4.4.1 Effects of acute ischaemia on cardiac metabolism and function in diabetes

The effects of acute ischaemia were investigated in control and diabetic rats. In this subset of rats, no differences in fed state blood glucose were seen between controls and diabetics (Fig. 4.2A), but fasting blood glucose concentration was significantly increased in diabetic rats, as were the levels of circulating NEFAs (Fig. 4.2B, C).

Hearts perfused in normoxic conditions showed that HIF-1 α protein levels were not detectable either in controls or diabetics (Fig. 4.2D). Hearts frozen at the end of 30min low-flow ischaemia showed that HIF-1 α protein levels were significantly lower in diabetic hearts compared with controls, demonstrating impaired HIF-1 α stabilisation in diabetes in response to ischaemia (Fig. 4.2E). However, levels of HIF-1 β were not significantly different between the two groups (Fig. 4.2F). Interestingly, levels of HIF-1 α protein correlated negatively with circulating plasma NEFA concentration (Fig. 4.2G).

Cardiac function was measured throughout the isolated heart perfusion. No significant differences were found in rate-pressure product between control and diabetic hearts pre-ischaemia or during ischaemia (Fig. 4.3A). Developed pressure, end diastolic pressure and heart rate all remained unchanged between control and diabetic hearts throughout the perfusion (Fig. 4.3B, C, D).

4.4.2 Effects of DMOG treatment on physical parameters

Body mass was increased in diabetic rats compared with controls (Fig. 4.4A). There were no significant differences in heart mass and liver mass between controls and diabetics (Fig. 4.4B,C). However, epididymal fat pad to body mass ratio, a marker of adiposity, was significantly higher in diabetic rats compared with controls (Fig. 4.4D). DMOG treatment resulted in no significant changes in body mass, heart mass, liver

mass or fat pad to body mass ratio, in either control or diabetic rats compared with their respective untreated groups (Fig. 4.4).

Circulating levels of NEFA were significantly higher in the fed state in diabetic rats compared with controls (Fig. 4.5A). There were no significant differences in fasting state plasma NEFA levels or fed state plasma triacylglyceride (TAG) levels between controls and diabetics. DMOG treatment did not lead to any changes in circulating NEFA in the fed or fasted state, or plasma TAG levels (Fig. 4.5).

Blood glucose concentration was significantly higher in diabetic rats compared with controls, in both the fed (Fig. 4.6A) and fasted states (Fig. 4.6B). Plasma insulin levels were unchanged in the fed state (Fig. 4.6C), but there was a significant increase in fasting plasma insulin concentration in diabetic rats compared with controls (Fig. 4.6D). DMOG treatment resulted in no changes in fed state (Fig. 4.6A) or fasted state (Fig. 4.6B) blood glucose concentrations in either control or diabetic rats. Fed state insulin levels were also unchanged with DMOG treatment in both control and diabetic rats (Fig. 4.6C). However, DMOG treatment resulted in increased fasting plasma insulin concentrations in diabetic rats only (Fig. 4.6D).

4.4.3 Effects of DMOG treatment on cardiac function in ischaemia-reperfusion

No significant differences in rate-pressure product (RPP) were observed prior to ischaemia between any of the four groups (Fig. 4.7). Although all groups showed a drop in RPP to virtually zero during ischaemia, control hearts showed no significant impairment in RPP recovery following reperfusion (Fig. 4.7C). However, post-ischaemic RPP recovery was 20% lower in diabetic hearts compared with controls (Fig. 4.7A,C). DMOG treatment resulted in a 36% improvement in recovery of RPP at reperfusion in diabetic hearts compared with untreated diabetics (Fig. 4.7C). RPP in treated diabetic hearts was therefore no longer significantly different to untreated control hearts. However, in control hearts, DMOG treatment led to a significant decrease of in RPP recovery following ischaemia-reperfusion compared with untreated controls. DMOG-treated control hearts

therefore exhibited a functional profile similar to untreated diabetic hearts following ischaemia-reperfusion (Fig. 4.7).

The individual functional parameters; developed pressure, end-diastolic pressure and heart rate, were not significantly different between control and diabetic hearts throughout ischaemia and reperfusion (Fig. 4.8). However, DMOG treatment in diabetic rats resulted in increased developed pressure at reperfusion compared with untreated diabetics (Fig. 4.8A), with no significant changes in either end-diastolic pressure or heart rate (Fig. 4.8). End diastolic pressure was significantly increased with DMOG treatment in control hearts, suggesting diastolic dysfunction in these hearts (Fig. 4.8).

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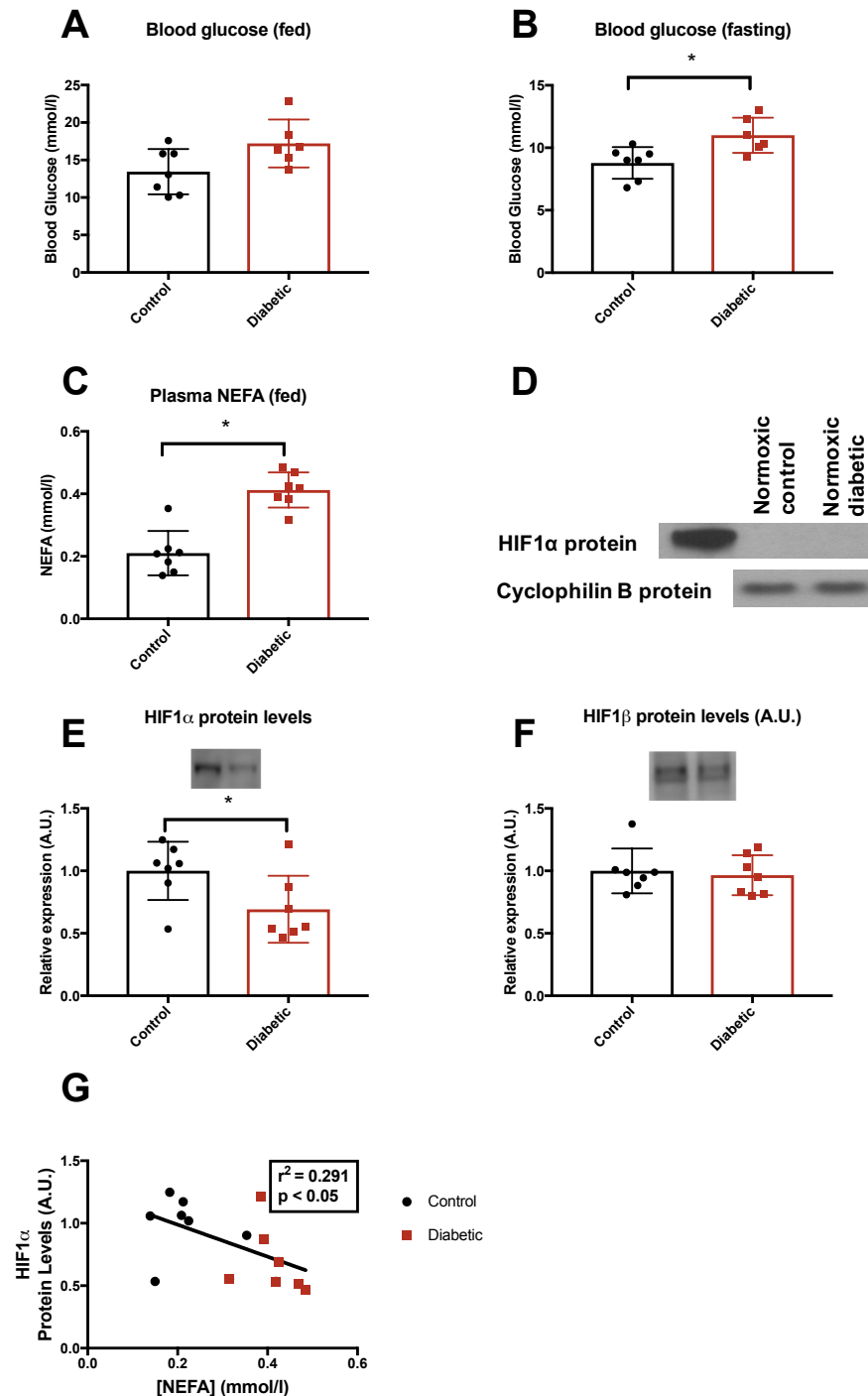


Figure 4.2: Type 2 diabetes and acute ischaemia. Concentrations of fed state blood glucose (A), fasting blood glucose (B) and fed state NEFA (C) in control and diabetic rats. Protein expression of HIF-1 α in normoxic control and diabetic isolated perfused rat hearts (D), protein expression of HIF-1 α (E) and HIF-1 β (F) in control and diabetic isolated perfused rat hearts following 30min low-flow ischaemia. (G) Correlation between levels of HIF-1 α in acute ischaemia and *in vivo* levels of plasma NEFA. * $p < 0.01$ vs untreated control.

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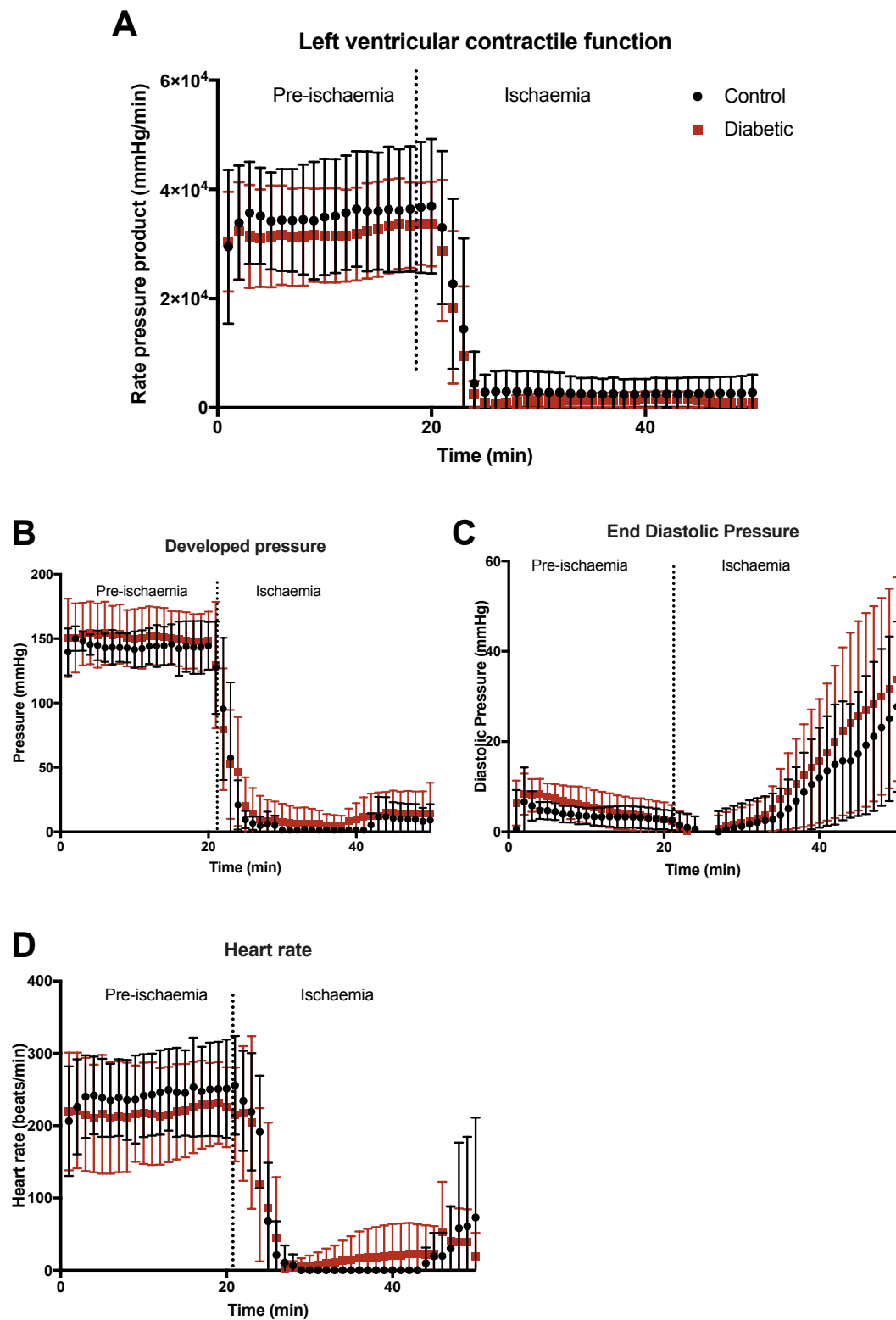


Figure 4.3: Type 2 diabetes and cardiac function in acute ischaemia. Rate-pressure product (A), developed pressure (B), end diastolic pressure (C) and heart rate (D) in control and diabetic isolated perfused rat hearts following 30min low-flow ischaemia.

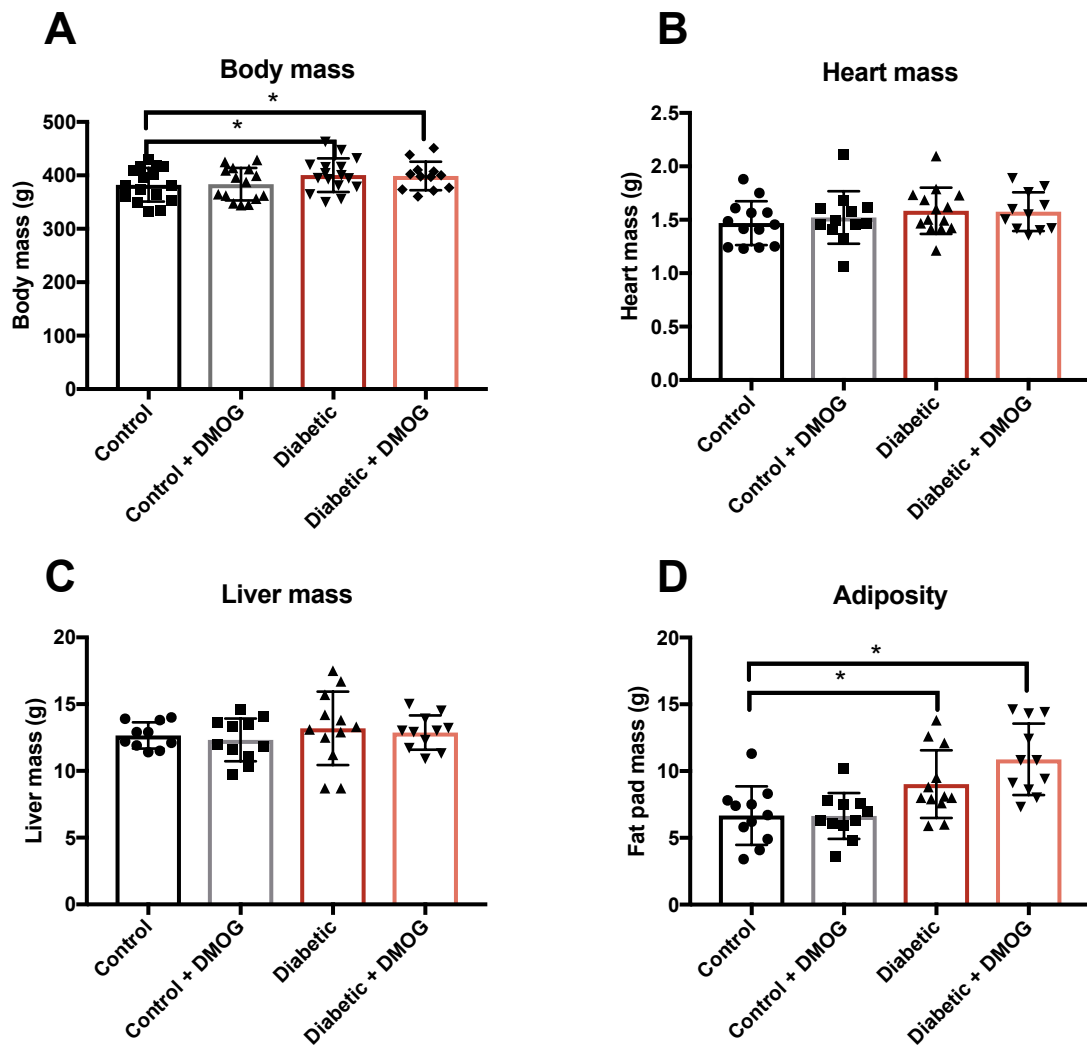


Figure 4.4: Body mass (A), heart mass (B), liver mass (C), and adiposity (D) in control and diabetic rats, with or without DMOG treatment (40 mg/kg). * $p < 0.05$ vs untreated control.

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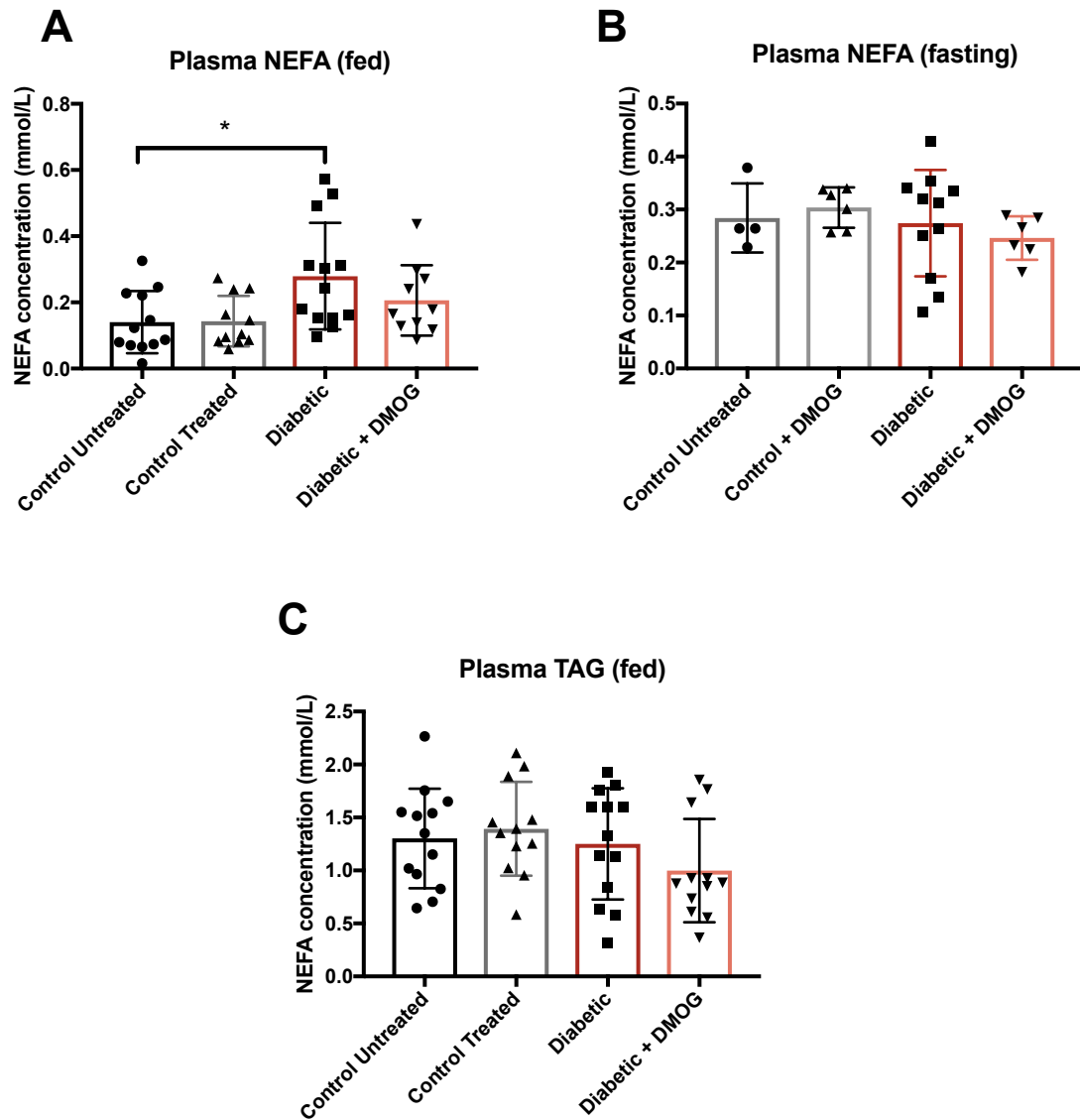


Figure 4.5: Plasma levels of circulating non-esterified fatty acids (NEFA) in the fed (A) and fasted state (B), and triacylglycerides in the fed state (C). *p<0.01 vs untreated control.

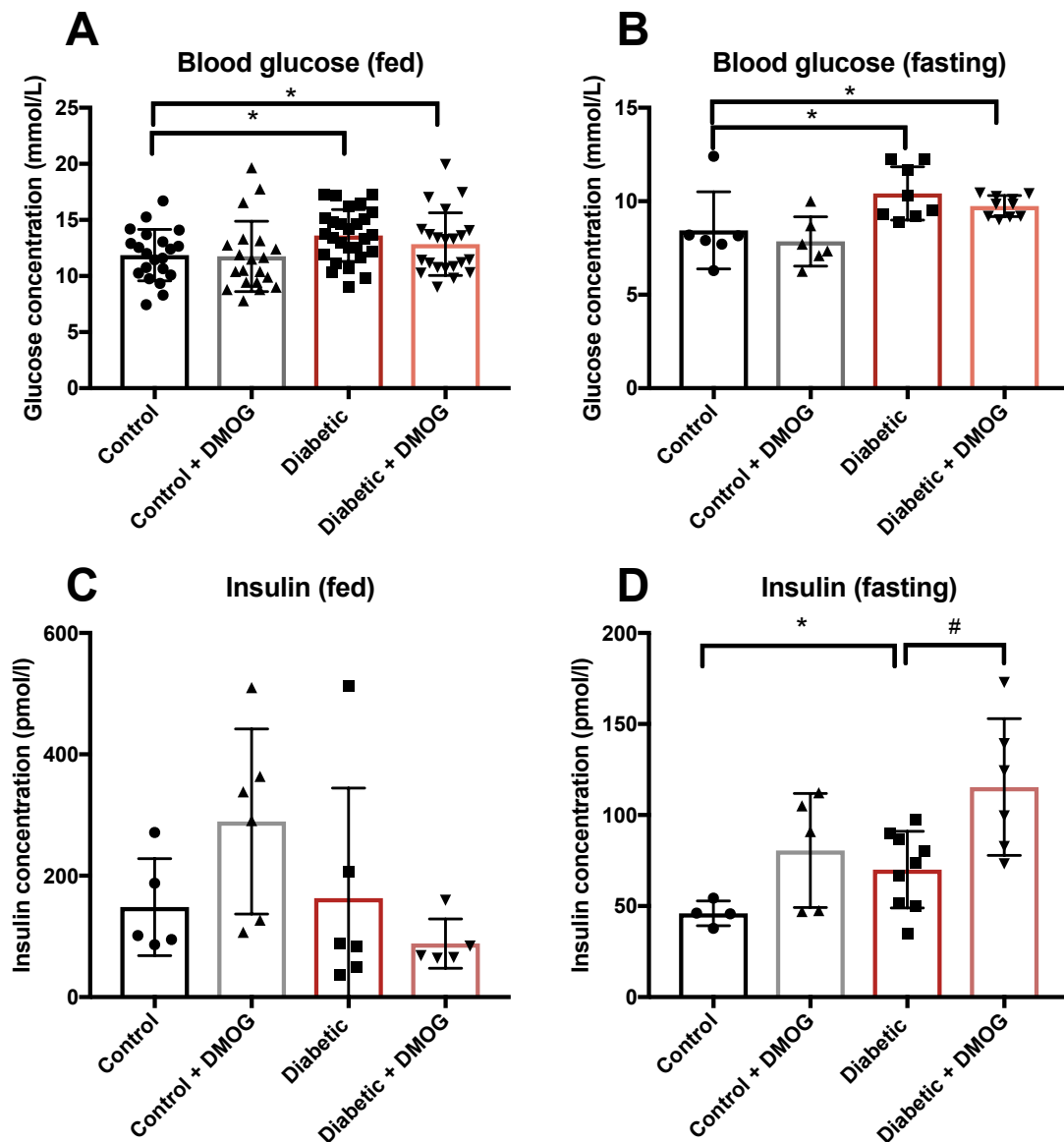


Figure 4.6: Plasma levels of circulating glucose in the fed state (A) and the fasted state (B), plasma levels of insulin in the fed state (C) and fasted state (D) in control and diabetic rats with or without DMOG treatment. * $p < 0.01$ vs untreated control; # $p < 0.05$ vs untreated diabetic.

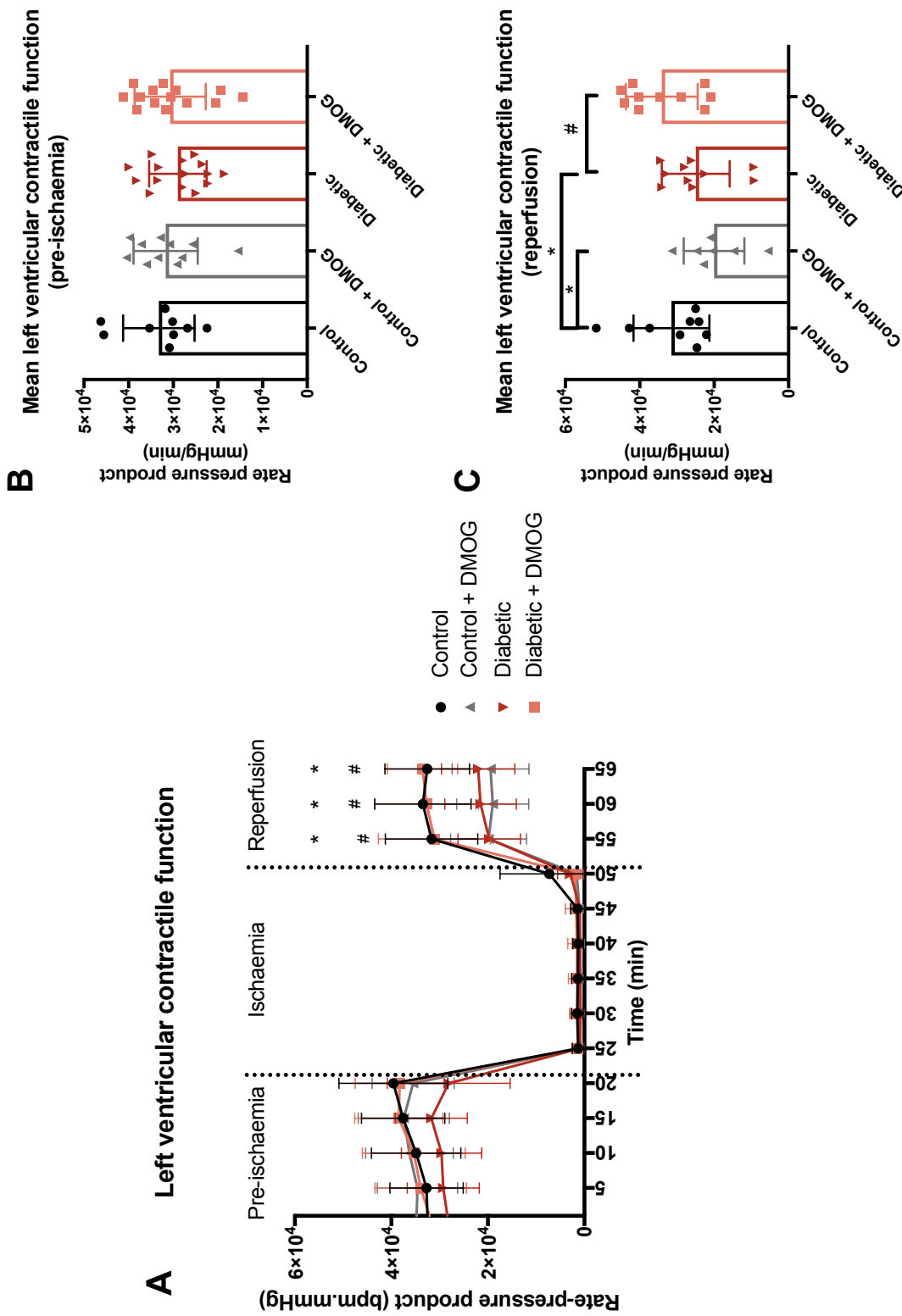


Figure 4.7: Left ventricular contractile function, as measured by rate-pressure product in the perfused heart, from control and diabetic rats untreated or given pre-treatment with DMOG. Rate-pressure product trace throughout the perfusion protocol (A), mean rate-pressure product in the pre-ischaemic period (B), and mean rate-pressure product in the reperfusion period (C). * $p < 0.01$ vs untreated control; # $p < 0.05$ vs untreated diabetic.

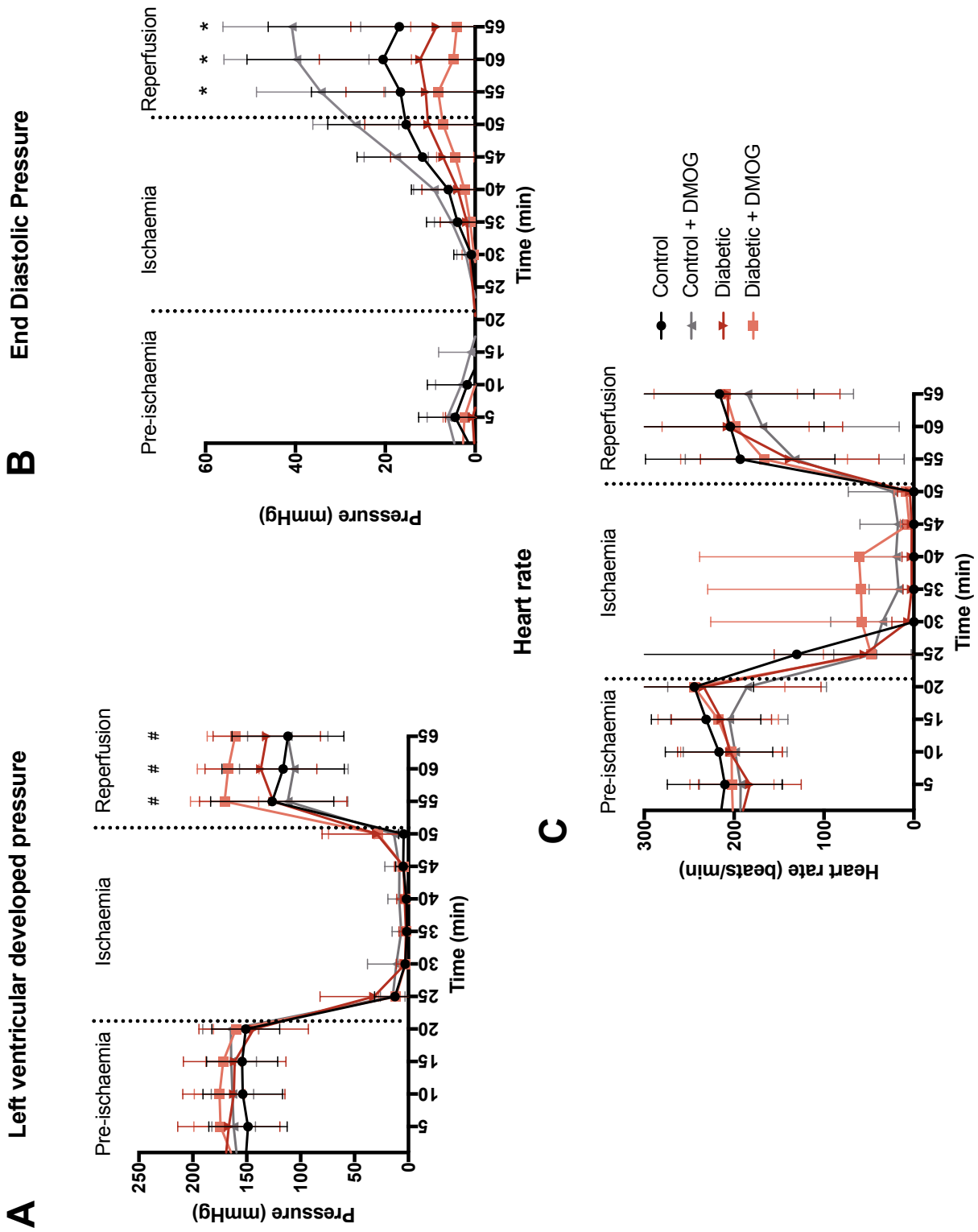


Figure 4.8: Parameters of left ventricular contractile function in the perfused heart, from control and diabetic rats untreated or given pre-treatment with DMOG. Developed pressure (A), end-diastolic pressure (B), and heart rate (C) throughout the perfusion protocol. * $p < 0.05$ vs control, # $p < 0.05$ vs untreated diabetic.

4.4.4 Effects of DMOG treatment on cardiac metabolic rates

During pre-ischaemia, rates of glycolysis were significantly lower in diabetic hearts compared with controls, and this difference was maintained following reperfusion, despite an increase in glycolysis during ischaemia in both control and diabetic hearts (Fig. 4.9A). Treatment with DMOG resulted in no significant increase in glycolytic rates in either control or diabetic hearts during pre-ischaemia, ischaemia, and reperfusion (Fig. 4.9A). Instead, during reperfusion, glycolytic rates were significantly lower in DMOG-treated control hearts compared with untreated controls (Fig. 4.9A). Diabetic glycolytic rates remained unchanged with treatment (Fig. 4.9A). Additionally, cardiac glycogen stores following reperfusion were decreased in diabetic hearts compared with controls (Fig. 4.9B). DMOG treatment resulted in decreased glycogen stores following reperfusion in control hearts compared with untreated controls (Fig. 4.9B). However, this effect of DMOG on glycogen was not present in diabetic hearts (Fig. 4.9B). An interaction term was found between drug and disease in glycogen stores with DMOG treatment. Livers from diabetic rats also showed significantly lower glycogen stores compared with controls, despite untreated diabetics not reaching a statistically significant difference (Fig. 4.9C). Hepatic glycogen levels remained unchanged with treatment in control rats (Fig. 4.9C).

Fatty acid oxidation rates did not reach significance between control and diabetic hearts during pre-ischaemia, ischaemia or reperfusion (Fig. 4.10A). However, in pre-ischaemia, a trend for increased fatty acid oxidation rate was observed in diabetic hearts compared with controls ($p=0.09$). Unexpectedly, DMOG treatment resulted in significantly increased fatty acid oxidation rates in both control and diabetic hearts during pre-ischaemia (Fig. 4.10A). Ischaemic fatty acid oxidation rates dropped to virtually zero (Fig. 4.10A). Following reperfusion, fatty acid oxidation rates were significantly increased in both control and diabetic hearts compared with their respective untreated group (Fig. 4.10A). Cardiac triacylglyceride (TAG) stores following reperfusion were not significantly different between control and diabetic hearts (Fig. 4.10B). However, DMOG treatment resulted in a significant

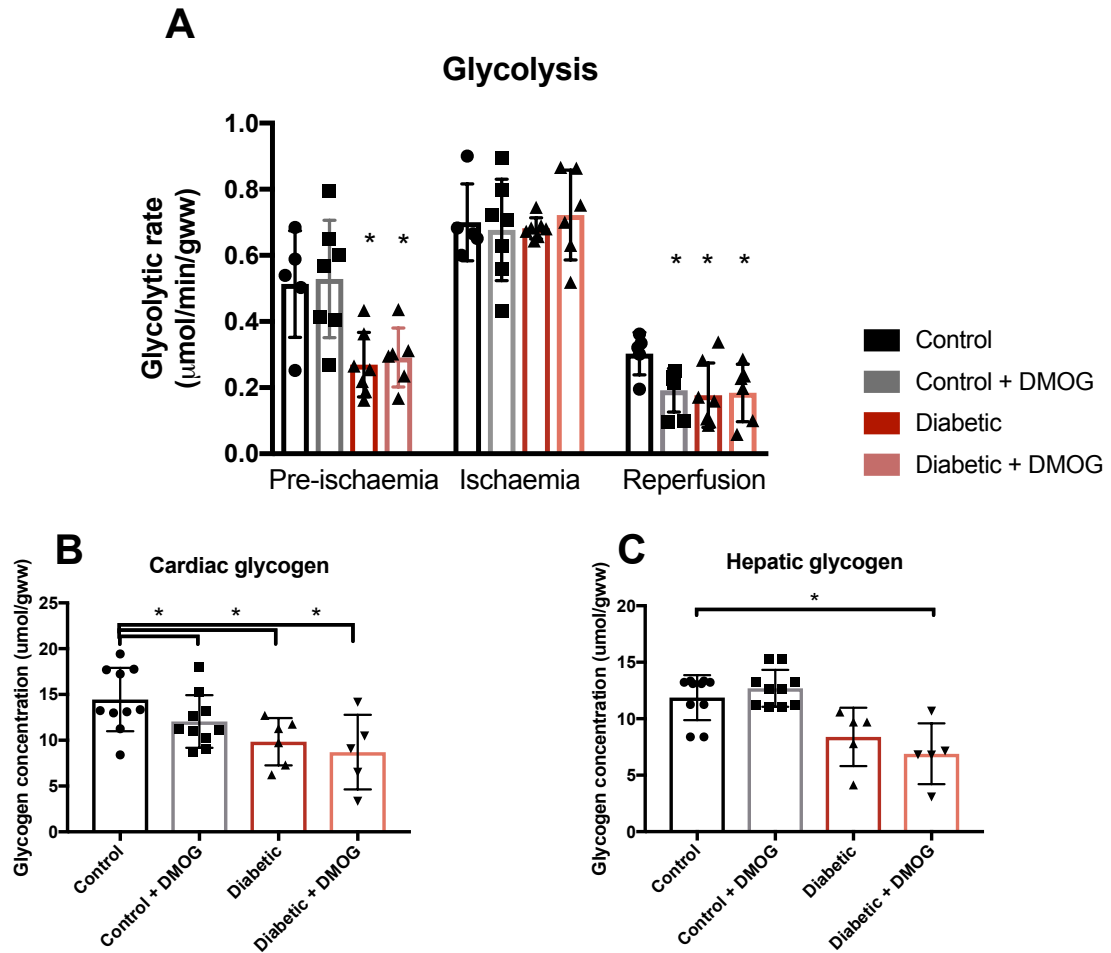


Figure 4.9: Glycolysis and glycogen handling in control and diabetic rats with and without pre-treatment with DMOG. Glycolytic rates of isolated hearts in pre-ischaemia, ischaemia and reperfusion (A), cardiac glycogen stores following reperfusion in the perfused heart (B) and hepatic glycogen stores in the intact liver (C). * $p < 0.05$ vs untreated control. $n = 6-8$ per group

increase in post-reperfusion cardiac TAG stores in diabetic hearts, but had no significant effect on TAG stores in control hearts (Fig. 4.10B). Livers from diabetic rats showed significantly increased TAG stores compared with controls (Fig. 4.10B). DMOG treatment had no effect on hepatic TAG stores in either control or diabetic rats (Fig. 4.10C).

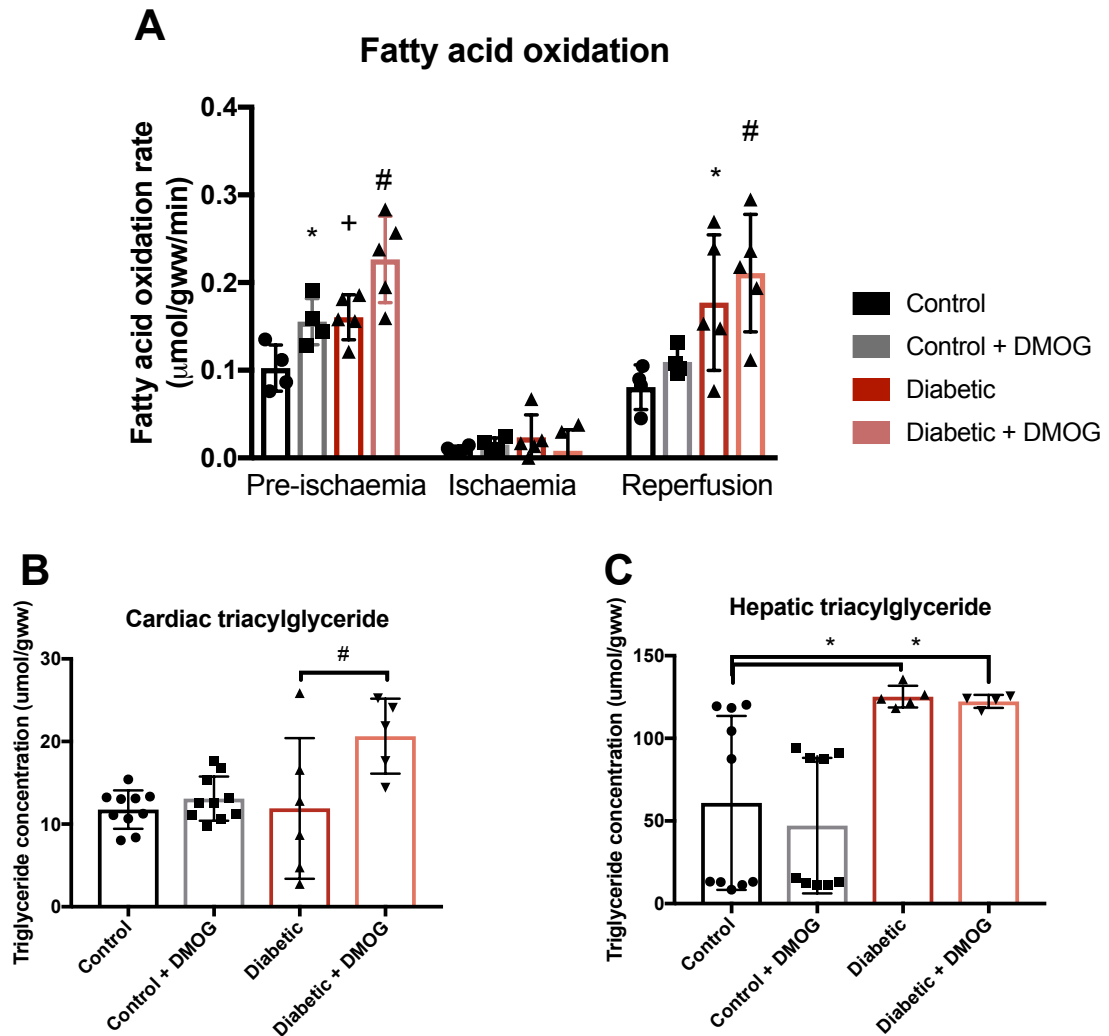


Figure 4.10: Fatty acid oxidation and triacylglycerides (TAG) in control and diabetic rats with and without pre-treatment with DMOG. Palmitate oxidation rates of isolated hearts in pre-ischaemia, ischaemia and reperfusion (A), cardiac TAG stores following reperfusion (B) and hepatic TAG stores in the intact liver (C). * $p < 0.05$ vs untreated control; # $p < 0.05$ vs untreated diabetic, + $p = 0.09$ vs untreated control. $n = 6-8$ per group

4.4.5 Effects of DMOG treatment on cardiac metabolic markers

Following the results of increased fatty acid oxidation shown above (Fig. 4.10A), the expression of PPAR α targets was investigated as a possible cause for the observed increase. The quantification was performed in tissue from the same hearts used for the perfusions in which fatty acid oxidation rates were measured. Protein expression of PDK4 was significantly increased in diabetic hearts compared with

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controls (Fig. 4.11A). Levels of FAT/CD36, ACADM, and UCP3, were unchanged in diabetic rat hearts compared with controls (Fig. 4.11B,C,D). DMOG treatment resulted in no significant changes in levels of any of these proteins in diabetic hearts. In control hearts, ACADM protein expression was significantly reduced with DMOG treatment (Fig. 4.11C).

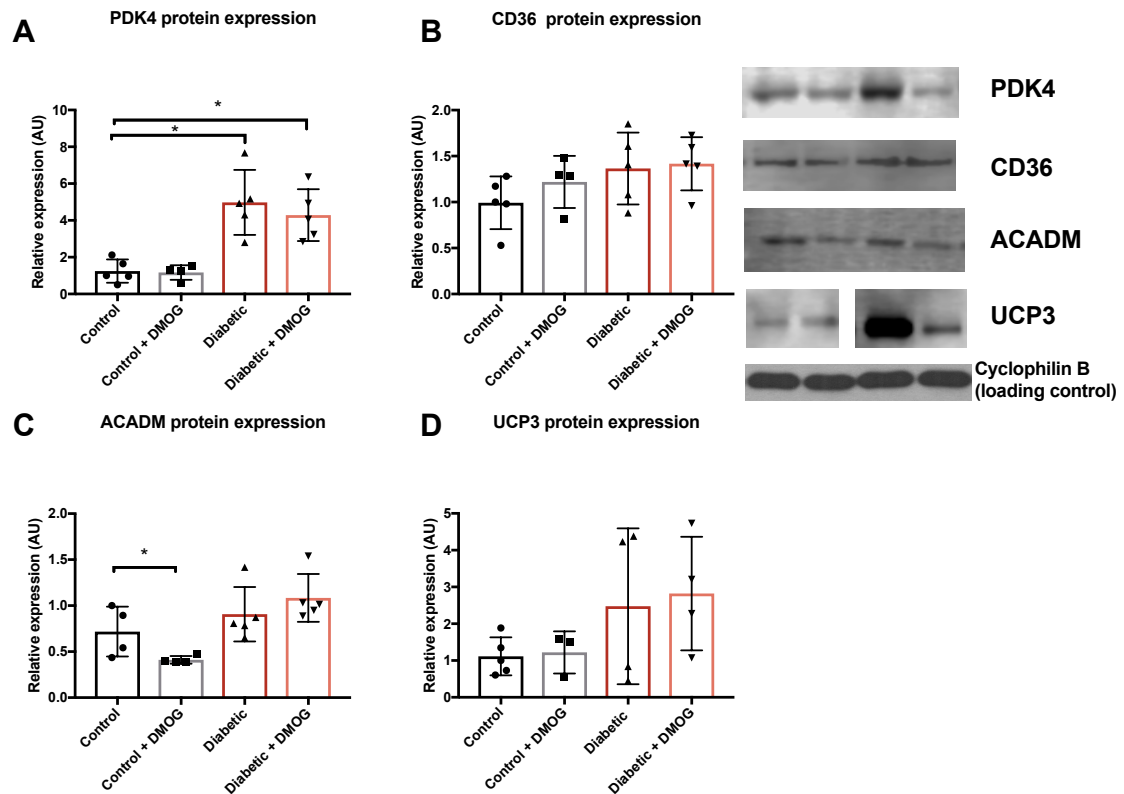


Figure 4.11: PPAR α targets protein expression in control and diabetic rats with or without DMOG treatment, following ischaemia-reperfusion. Relative expression of pyruvate dehydrogenase kinase 4 (PDK4, A), fatty acid translocase (CD36, B), medium-chain acylCoA dehydrogenase (ACADM, C) and uncoupling protein 3 (UCP3, D). * $p < 0.05$ vs untreated control.

From the metabolomics analysis performed on cardiac tissue frozen at the end of reperfusion, we found that several metabolites relating to the energy status of the myocardium, namely PCr and ATP, were significantly lower in control hearts with DMOG treatment, compared with untreated controls (Fig. 4.12A,B). In contrast, these were not changed between untreated diabetics and DMOG-treated diabetics. This resulted in them being significantly higher in DMOG-treated diabetics compared with DMOG-treated controls. The FAD and NAD pools

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were unchanged between untreated control and diabetic hearts, but significantly higher in DMOG-treated diabetics compared with DMOG-treated controls (Fig. 4.12G,H). NAD and NADP levels were significantly lower in DMOG-treated controls, compared with their untreated counterparts (Fig. 4.12H,I).

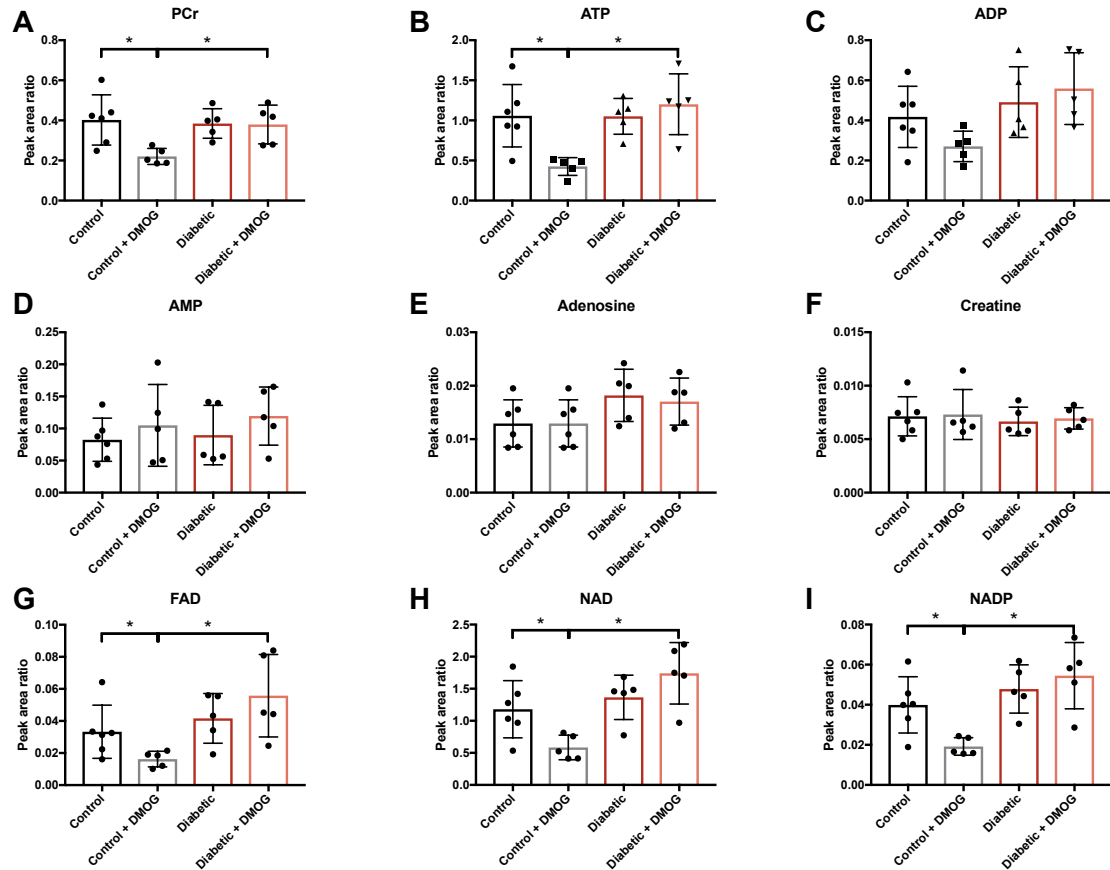


Figure 4.12: Levels of metabolic intermediates relating to cardiac energetics, in control and diabetic hearts following ischaemia-reperfusion, with and without DMOG pre-treatment. *p<0.05

No significant differences were seen between untreated control and diabetic hearts in Krebs cycle intermediates (Fig. 4.13). DMOG-treated control hearts had significantly lower levels of isocitrate compared with untreated controls (Fig. 4.13). In DMOG-treated diabetic hearts, levels of α -ketoglutarate were significantly lower than in untreated diabetics (Fig. 4.13). Additionally, DMOG-treated control hearts had significantly lower levels of citrate and isocitrate compared with DMOG-treated diabetics (Fig. 4.13).

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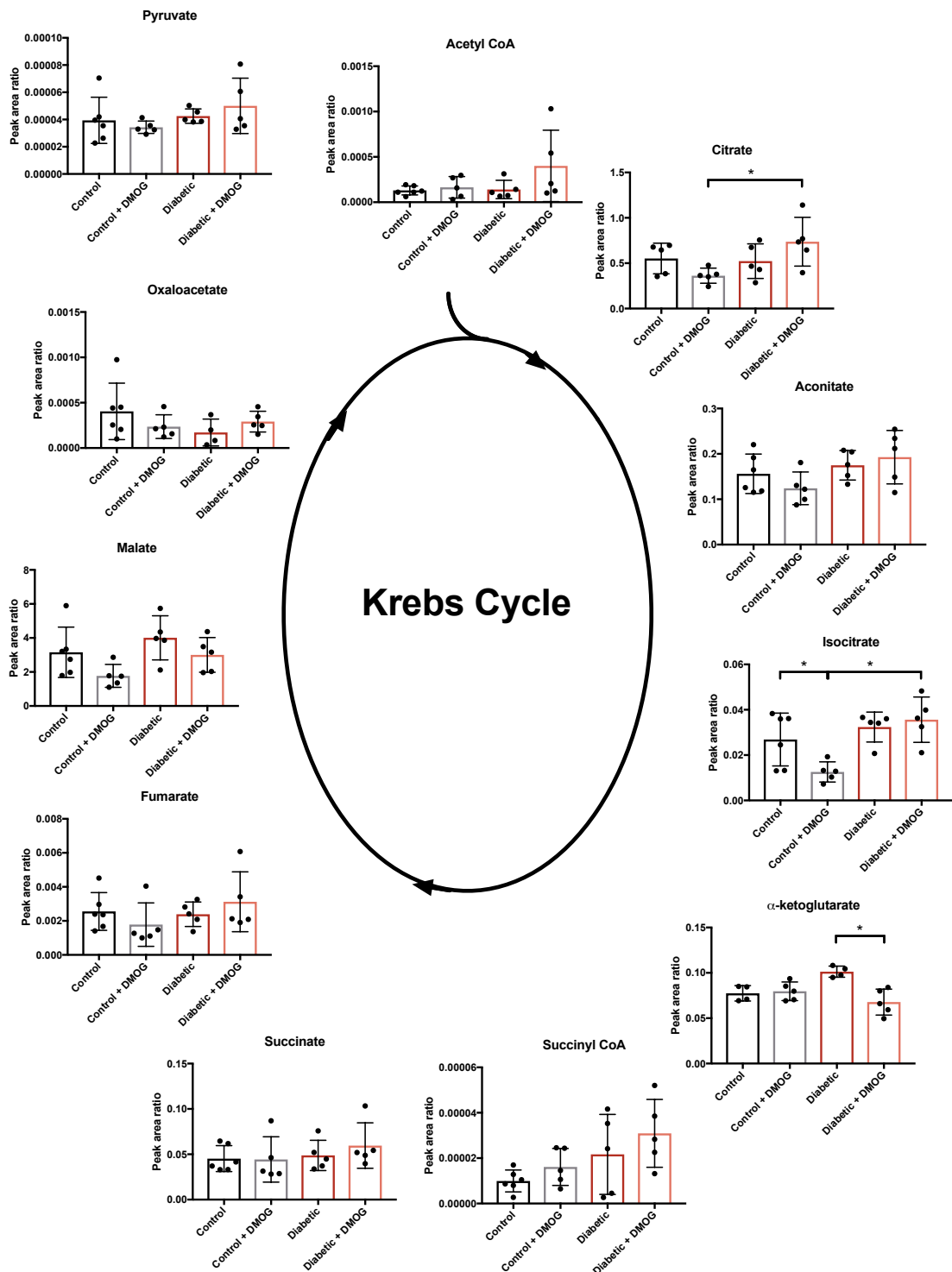


Figure 4.13: Levels of Krebs cycle intermediates in control and diabetic hearts following ischaemia-reperfusion, with and without DMOG pre-treatment. * p < 0.05 .

DMOG-treated control hearts had significantly decreased levels of fructose-6-phosphate/glucose-6-phosphate (F6P-G6P) compared with untreated controls

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(Table 4.1). This was not the case in diabetic hearts. No differences in any other glycolytic intermediates were found between any of the groups (Table 4.1).

DMOG-treated diabetic hearts showed significantly higher levels of lysine, asparagine, aspartate, and glutamate, compared with DMOG-treated controls (Table 4.2). DMOG treatment resulted in a significant decrease in levels of glutamate in controls (Table 4.2). In diabetics, DMOG treatment resulted in a significant decrease in levels of ornithine (Table 4.2). No other significant differences in aminoacid levels were found between any groups.

Levels of cytidine triphosphate (CTP) were also significantly decreased in DMOG-treated control hearts compared with untreated controls (Table 4.3). CTP levels were also significantly higher in DMOG-treated diabetic hearts compared with DMOG-treated controls. Levels of UDP, UDPglc, UDP-N-acetylglucosamine, and UTP, were all significantly decreased in DMOG-treated controls (Table 4.3). Additionally, UDP, UDPglc, and UTP were significantly higher in DMOG-treated diabetics compared with DMOG-treated controls. Levels of glutathione (GSSH) were significantly decreased in DMOG-treated control hearts compared with untreated controls (Table 4.3). Additionally, GSSH levels were significantly higher in DMOG-treated diabetic hearts compared with DMOG-treated controls. Levels of GDP and GTP were also significantly higher in DMOG-treated diabetic hearts compared with DMOG-treated controls, as were the levels of S-adenosylmethionine, and malonyl-CoA. Many other metabolites were measured, but no significant differences were found (Table 4.3). Overall, many metabolites were decreased by DMOG treatment of control hearts, but none were changed by DMOG treatment of diabetics.

In our measurements of acylcarnitines, we found no significant differences in free carnitine between control and diabetic hearts or with treatment. The levels of dodecenoyl carnitine (C12:1) and 3-hydroxy-octadecenoyl carnitine (C18:1) were significantly higher in untreated diabetic hearts compared with controls (Table 4.4). Additionally, the levels of acetyl-carnitine (C2), butyryl-carnitine (C4), and tiglyl carnitine (C5:1) were significantly lower in DMOG-treated diabetic hearts compared with DMOG-treated controls (Table 4.4). An interaction term was also found

between diabetes and DMOG treatment on the levels isovaleryl-carnitine (C5) of C5:1 (Table 4.4). DMOG-treated control hearts showed significantly increased levels of pentadecanoylcarnitine (C15), eicosadienoyl carnitine (C20:2), and arachidonoyl carnitine (C20:4) compared with untreated controls (Table 4.4). DMOG-treated diabetic hearts showed significantly lower levels of these compared to DMOG-treated controls.

Table 4.1: Table of glycolytic intermediates in control and diabetic cardiac tissue following ischaemia-reperfusion.

	Control	Control + DMOG	Diabetic	Diabetic + DMOG
F6P/G6P	0.398 ± 0.014	0.197 * ± 0.049	0.410 ± 0.084	0.429 ± 0.11
F16BP	0.096 ± 0.055	0.061 ± 0.040	0.090 ± 0.087	0.091 ± 0.057
G3P	0.00082 ± 0.00031	0.00082 ± 0.00033	0.00113 ± 0.00075	0.00110 ± 0.00069
DHAP	0.0009 ± 0.0004	0.0007 ± 0.0004	0.0013 ± 0.0011	0.0011 ± 0.0007
2PG/3PG	0.012 ± 0.003	0.009 ± 0.002	0.008 ± 0.002	0.011 ± 0.002
PEP	0.014 ± 0.004	0.008 ± 0.001	0.011 ± 0.002	0.011 ± 0.002

* $p < 0.05$ vs control.

Table 4.2: Table of amino acids and their derivatives in control and diabetic cardiac tissue following ischaemia-reperfusion, with and without DMOG treatment.

	Control	Control + DMOG	Diabetic	Diabetic + DMOG
Arginine	0.113 ± 0.033	0.079 ± 0.018	0.104 ± 0.025	0.116 ± 0.026
Histidine	0.029 ± 0.006	0.029 ± 0.004	0.031 ± 0.009	0.027 ± 0.005
Lysine	0.051 ± 0.021	0.033 ± 0.012	0.062 ± 0.014	0.089 \$ ± 0.043
Proline	0.018 ± 0.009	0.022 ± 0.008	0.029 ± 0.02	0.026 ± 0.02
Phenylalanine	0.007 ± 0.003	0.007 ± 0.004	0.016 ± 0.019	0.009 ± 0.007
Tryptophan	0.0008 ± 0.0003	0.0009 ± 0.0005	0.0019 ± 0.0023	0.0010 ± 0.0008
Methionine	0.0006 ± 0.00020	0.0005 ± 0.00015	0.0009 ± 0.00072	0.0006 ± 0.0002
Leucine	0.009 ± 0.005	0.012 ± 0.007	0.025 ± 0.034	0.014 ± 0.013
Isoleucine	0.006 ± 0.003	0.008 ± 0.004	0.015 ± 0.019	0.009 ± 0.007
Valine	0.006 ± 0.002	0.007 ± 0.003	0.014 ± 0.017	0.008 ± 0.008
Alanine	0.019 ± 0.006	0.015 ± 0.003	0.025 ± 0.008	0.022 ± 0.009
Serine	0.005 ± 0.001	0.004 ± 0.001	0.004 ± 0.003	0.005 ± 0.002
Glutamine	0.740 ± 0.298	0.597 ± 0.132	0.805 ± 0.145	0.746 ± 0.121
Glycine	0.00011 ± 0.00003	0.00008 ± 0.00002	0.0001 ± 0.00004	0.0001 ± 0.00007
Asparagine	0.006 ± 0.001	0.005 ± 0.001	0.006 ± 0.001	0.007 \$ ± 0.002
Threonine	0.016 ± 0.003	0.012 ± 0.003	0.019 ± 0.007	0.017 ± 0.004
Tyrosine	0.003 ± 0.001	0.003 ± 0.001	0.007 ± 0.007	0.004 ± 0.002
Aspartate	0.012 ± 0.003	0.008 ± 0.003	0.022 * ± 0.003	0.020 \$ ± 0.009
Glutamate	2.166 ± 0.604	1.421 * ± 0.438	2.370 ± 0.267	2.425 \$ ± 0.293
Citrulline	0.136 ± 0.050	0.143 ± 0.069	0.099 ± 0.016	0.104 ± 0.026
Ornithine	0.0008 ± 0.00018	0.00103 ± 0.0004	0.00121 ± 0.0003	0.00075 # ± 0.00013

* $p < 0.05$ vs control, # $p < 0.05$ vs untreated diabetic, \$ $p < 0.05$ vs control + DMOG.

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Table 4.3: Table of additional aqueous metabolic intermediates in control and diabetic cardiac tissue following ischaemia-reperfusion.

	Control	Control + DMOG	Diabetic	Diabetic + DMOG
alpha-Aminobutyrate	0.00014 ± 0.00006	0.00009 ± 0.00003	0.00012 ± 0.00005	0.00011 ± 0.00004
beta-Hydroxybutyrate	0.0003 ± 0.0002	0.0004 ± 0.0001	0.0002 ± 0.0002	0.0003 ± 0.0002
Betaine	0.061 ± 0.032	0.067 ± 0.023	0.024 ± 0.006	0.042 ± 0.043
cAMP	0.010 ± 0.005	0.006 ± 0.003	0.009 ± 0.003	0.011 ± 0.004
Carnosine	0.027 ± 0.013	0.019 ± 0.006	0.038 ± 0.014	0.046 \$ ± 0.019
CDP	0.0013 ± 0.001	0.0008 ± 0.0003	0.0015 ± 0.0009	0.0019 * ± 0.0008
CDP-choline	0.044 ± 0.018	0.044 ± 0.015	0.063 ± 0.031	0.080 ± 0.051
Choline	0.02 ± 0.003	0.024 ± 0.005	0.025 ± 0.019	0.023 ± 0.013
Creatinine	0.0046 ± 0.001	0.0045 ± 0.001	0.0046 ± 0.001	0.0040 ± 0.002
CTP	0.0006 ± 0.0003	0.0002 * ± 0.00008	0.0005 ± 0.00009	0.0006 \$ ± 0.0003
Cytidine	0.0006 ± 0.001	0.0010 ± 0.001	0.0017 ± 0.003	0.0009 ± 0.001
Cytidine monophosphate	0.0011 ± 0.0005	0.0008 ± 0.0004	0.0011 ± 0.0007	0.0015 ± 0.0006
Cytosine	0.0006 ± 0.0004	0.0008 ± 0.0005	0.0015 ± 0.0023	0.0009 ± 0.0008
GDP	0.0007 ± 0.0003	0.0004 ± 0.0002	0.0009 ± 0.0005	0.0012 ± 0.0007
GMP	0.007 ± 0.005	0.006 ± 0.003	0.011 ± 0.007	0.015 ± 0.008
GSSH	0.039 ± 0.0013	0.023 * ± 0.08	0.044 ± 0.009	0.045 \$ ± 0.006
GTP	0.003 ± 0.0012	0.001 ± 0.0004	0.002 ± 0.0007	0.004 \$ ± 0.0021
Inosine	0.0004 ± 0.0001	0.0005 ± 0.0001	0.0006 ± 0.0001	0.0006 ± 0.0001
Malonyl CoA	0.00012 ± 0.00005	0.00005 ± 0.00002	0.00014 ± 0.00004	0.00020 \$ ± 0.00011
Ribose-5-phosphate	0.0005 ± 0.0003	0.0009 ± 0.0005	0.0009 ± 0.0004	0.0009 ± 0.0005
S-Adenosylmethionine	0.0006 ± 0.0002	0.0005 ± 0.0002	0.0008 ± 0.0002	0.0009 ± 0.0002
Taurine	0.022 ± 0.004	0.019 ± 0.005	0.020 ± 0.002	0.021 ± 0.004
UDP	0.0011 ± 0.0004	0.0005 * ± 0.0001	0.0012 ± 0.0003	0.0010 \$ ± 0.0003
UDPglc	0.261 ± 0.081	0.113 * ± 0.031	0.224 ± 0.037	0.290 \$ ± 0.065
UDPNacetylglucosamine	0.450 ± 0.236	0.169 * ± 0.049	0.488 ± 0.174	0.369 ± 0.178
UMP	0.0005 ± 0.0003	0.0003 ± 0.0001	0.0004 ± 0.0002	0.0005 ± 0.0002
UTP	0.024 ± 0.011	0.008 * ± 0.002	0.020 ± 0.005	0.025 \$ ± 0.011

* $p < 0.05$ vs control, \$ $p < 0.05$ vs control + DMOG.

Table 4.4: Table of acylcarnitines in control and diabetic cardiac tissue following ischaemia-reperfusion, with or without DMOG treatment.

	Control	Control + DMOG	Diabetic	Diabetic + DMOG
C0	2.710 ± 0.918	2.188 ± 0.819	1.603 ± 0.452	7.381 ± 0.343
C2	1.633 ± 0.527	2.079 ± 0.231	1.233 ± 0.322	1.428 ± 0.481
C3	0.076 ± 0.021	0.116 ± 0.036	0.088 ± 0.013	0.076 ± 0.035
C4	0.090 ± 0.021	0.119 ± 0.029	0.076 ± 0.009	0.078 ± 0.006
C5	0.004 ± 0.003	0.009 ± 0.002	0.008 ± 0.007	0.004 ± 0.002
C5:1	0.003 ± 0.0011	0.005 ± 0.002	0.003 ± 0.0005	0.002 \$ ± 0.002
C6	0.032 ± 0.005	0.045 ± 0.020	0.028 ± 0.006	0.031 ± 0.008
C8	0.013 ± 0.003	0.018 ± 0.009	0.012 ± 0.004	0.014 ± 0.009
C8:1	0.00014 ± 0.00006	0.00012 ± 0.00006	0.00008 ± 0.00005	0.00008 ± 0.00004
C10	0.018 ± 0.005	0.027 ± 0.014	0.019 ± 0.005	0.022 ± 0.011
C12	0.054 ± 0.018	0.074 ± 0.032	0.062 ± 0.014	0.067 ± 0.029
C12:1	0.0012 ± 0.0004	0.0014 ± 0.0006	0.0007 * ± 0.0001	0.0008 ± 0.0004
C14	0.337 ± 0.144	0.419 ± 0.214	0.317 ± 0.109	0.392 ± 0.211
C14:1	0.002 ± 0.001	0.003 ± 0.001	0.005 ± 0.003	0.003 ± 0.001
C14:2	0.0004 ± 0.0004	0.0010 ± 0.0006	0.0006 ± 0.0003	0.0005 ± 0.0002
C15	0.00007 ± 0.00004	0.00022 * ± 0.00007	0.00010 ± 0.00003	0.00007 \$ ± 0.00001
C16	1.199 ± 0.489	1.350 ± 0.586	0.878 ± 0.257	1.041 ± 0.482
C16:1	0.005 ± 0.004	0.007 ± 0.002	0.008 ± 0.005	0.007 ± 0.003
C16:2	0.002 ± 0.001	0.003 ± 0.002	0.002 ± 0.002	0.002 ± 0.001
C16-OH	0.028 ± 0.015	0.037 ± 0.022	0.018 ± 0.005	0.024 ± 0.017
C18	0.099 ± 0.080	0.120 ± 0.039	0.066 ± 0.033	0.097 ± 0.043
C18:1-OH	0.001 ± 0.0007	0.0018 ± 0.0008	0.0016 ± 0.0005	0.0016 ± 0.0007
C18:2	0.019 ± 0.010	0.032 ± 0.018	0.014 ± 0.003	0.019 ± 0.007
C18:1	0.021 ± 0.012	0.042 ± 0.019	0.047 * ± 0.017	0.049 ± 0.016
C20	0.015 ± 0.020	0.023 ± 0.012	0.009 ± 0.007	0.011 ± 0.006
C20:1	0.002 ± 0.002	0.004 ± 0.001	0.003 ± 0.002	0.004 ± 0.002
C20:2	0.002 ± 0.001	0.003 ± 0.001	0.001 ± 0.001	0.002 ± 0.001
C20:4	0.005 ± 0.002	0.018 * ± 0.006	0.006 ± 0.003	0.007 \$ ± 0.002

* $p < 0.05$ vs control, \$ $p < 0.05$ vs control + DMOG.

4.4.6 Effects of DMOG on markers of HIF-1 signalling

Evidence of HIF-1 signalling changes was assessed by measuring blood haemoglobin levels immediately at the time of heart isolation, as well as subsequently measuring levels of HIF-1 target mRNA and protein. Neither blood hematocrit nor concentration of blood haemoglobin were changed between control and diabetic rats (Fig. 4.14). Additionally, with DMOG treatment, readings remained unchanged in controls and diabetics.

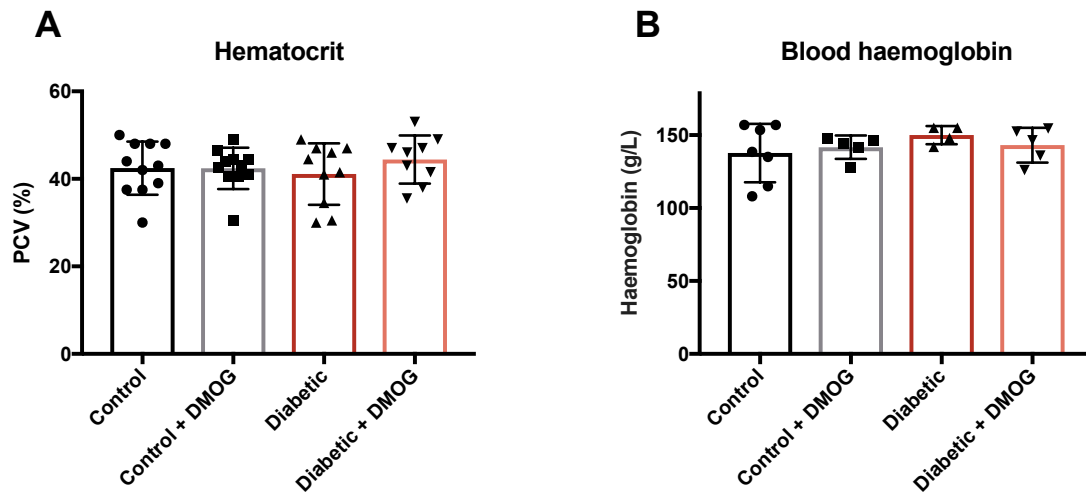


Figure 4.14: Blood hemoglobin levels in control and diabetic rats with or without DMOG treatment. Readings of packed cell volume (hematocrit) (A) and concentrations of haemoglobin (B) from terminal fed state blood samples.

Relative quantification of mRNA was performed on heart samples following ischaemia-reperfusion. Levels of PHD3 and haem oxygenase 1 (HMOX1) mRNA were unchanged between control and diabetic hearts (Fig. 4.15). However, mRNA levels of VEGF were significantly reduced in diabetic hearts compared with controls (Fig. 4.15C). With DMOG treatment, mRNA levels of HMOX1 were significantly reduced in controls, and unchanged in diabetic hearts (Fig. 4.15B). VEGF mRNA, however, was significantly increased with DMOG treatment in diabetics, though still significantly lower than those in control hearts (Fig. 4.15C).

Protein expression of HIF-1 α was assessed in samples from perfused hearts following ischaemia-reperfusion. However, this resulted in extremely difficult quantification, as no HIF-1 α bands were found in our samples by Western blot (Fig.

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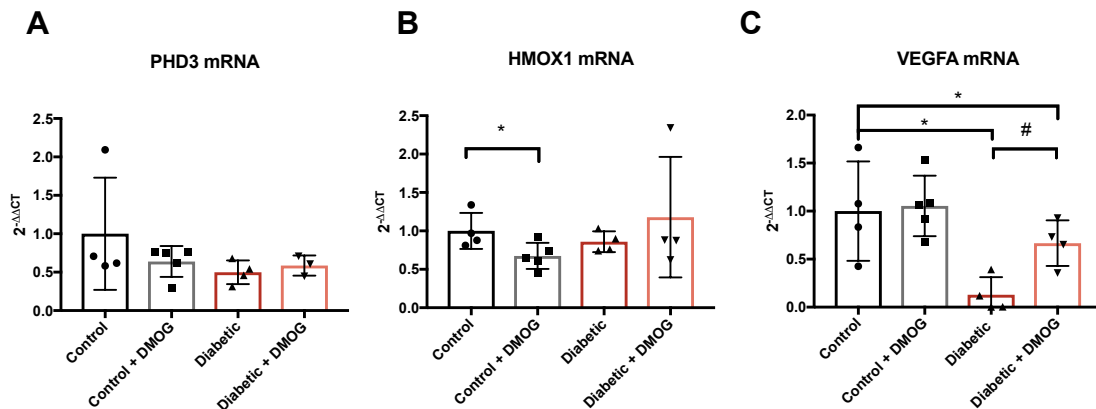


Figure 4.15: Levels of HIF targets mRNA in control and diabetic rat hearts with or without DMOG treatment, following ischaemia-reperfusion. mRNA relative expression of PHD3 (A), HMOX1 (B), and VEGF (C). * $p < 0.01$ vs untreated control; # $p < 0.05$ vs untreated diabetic.

4.16). Subsequently, Western blots were performed on hearts frozen immediately after isolation (not perfused), but this, again, was extremely difficult to quantify via Western blot, and no significant difference was seen with DMOG treatment in these hearts (Fig. 4.17). HIF-1 target glucose transporter 1 (GLUT1, SLC2A1) was then chosen as a surrogate marker for HIF-1 α upregulation and measured in samples from non-perfused hearts. No significant difference was seen in levels of GLUT1 (SLC2A1) protein expression between control and diabetic hearts (Fig. 4.18). Furthermore, DMOG treatment resulted in no significant changes in GLUT1 (SLC2A1) protein expression, in either control or diabetic hearts (Fig. 4.18), consistent with the lack of change in glycolytic rate presented in Fig. 4.9A.

The expression of the phosphorylated form of acetyl-CoA carboxylase (P-ACACA) was measured, to investigate whether the increase in fatty acid oxidation rates observed in Fig. 4.10A could be due an increase in the phosphorylation of ACACA caused by DMOG treatment, which has previously been reported in the literature [220]. Protein expression of P-ACACA was not significantly different between control and diabetic hearts (Fig. 4.19). We found no effect of DMOG treatment on P-ACACA expression in either control or diabetic hearts (Fig. 4.19).



Figure 4.16: Western blot to assess HIF-1 α protein expression in control and diabetic hearts following ischaemia-reperfusion, with or without DMOG treatment.

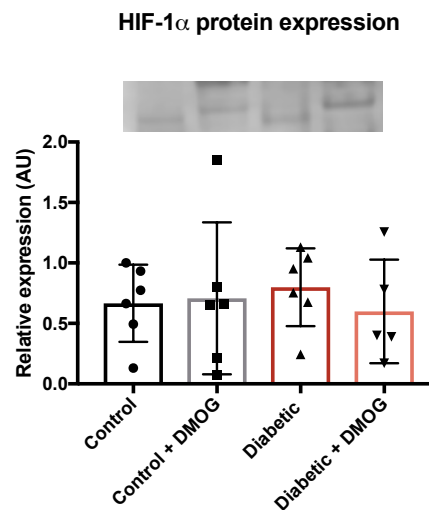


Figure 4.17: HIF-1 α protein expression in control and diabetic hearts with or without DMOG treatment.

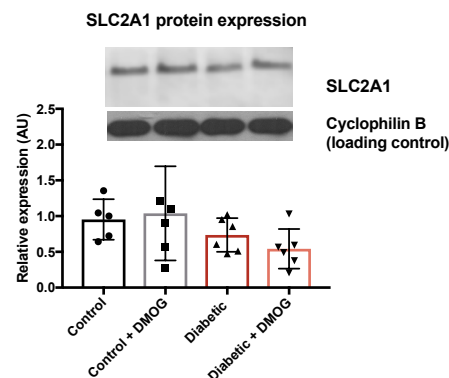


Figure 4.18: Glucose transporter 1 (SLC2A1) protein expression in control and diabetic rats with or without DMOG treatment.

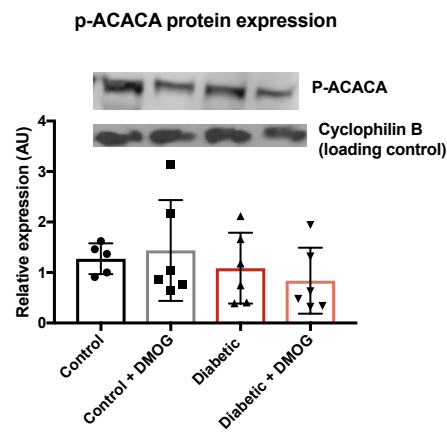


Figure 4.19: Phospho-acetylCoA carboxylase (P-ACACA) protein expression in control and diabetic rats with or without DMOG treatment.

4.5 Discussion

In this Chapter we investigated the effects of acute ischaemia-reperfusion on the type 2 diabetic heart, and the therapeutic potential of DMOG treatment to improve substrate metabolism and function. We aimed to modulate substrate metabolism by causing an increase in glycolysis, to force the diabetic heart away from its dependency on fatty acid oxidation and allow it to switch to oxygen-sparing anaerobic glycolysis to improve recovery following ischaemia-reperfusion. Diabetic hearts had significantly decreased stabilisation of HIF-1 α during ischaemia, demonstrating that HIF activation was impaired by diabetes. However, the results obtained following DMOG treatment were somewhat surprising. We found an improvement in functional recovery in DMOG-treated diabetic hearts following ischaemia-reperfusion, which was not supported by the expected changes in substrate metabolism and metabolic markers. Additionally, we found that control hearts showed poorer functional recovery post-reperfusion with DMOG treatment.

Interestingly, the levels of fasting blood glucose and plasma NEFA were higher in this cohort compared with those found in the previous chapter. This could be due to a number of reasons, including the time of year of treatment and sacrifice, which may have resulted in differences in the light/dark cycle of the housing facilities due to poor light isolation. Additionally, it must be noted that the animal handling and sample collection of chapter 3 was performed by a different individual, Latt Mansor, than those in the present chapter. This could impact on the levels of stress experienced by the rats and lead to changes in plasma metabolites.

4.5.1 Diabetic hearts had impaired HIF-1 α following ischaemia-reperfusion

Similarly to the observations in acute hypoxia in the previous chapter, acute ischaemia-reperfusion resulted in a drastic reduction in heart rate during the acute insult, followed by functional recovery during reperfusion. In diabetic hearts, no

differences in functional parameters were found, either prior to or during ischaemia. However, we found that diabetic hearts recovered to a lesser extent following reperfusion compared with healthy controls, which is consistent with the widely observed reduced survival in diabetics following myocardial infarction [77]. Here we found that diabetic hearts also had decreased levels of HIF-1 α during ischaemia, demonstrating that indeed the ability to upregulate HIF-1 signalling was impaired in diabetes. We found that, in ischaemia, diabetic hearts were able to upregulate glycolysis to the same extent as controls. This may be explained by the fact that all hearts in this Chapter were perfused with 3 U/l insulin, which was not done in the previous Chapter. It may be that this level of insulin is enough to stimulate glucose uptake and utilization to a certain extent in our diabetic rat hearts under stress, despite significantly lower glycolytic rates at baseline.

DMOG treatment had no significant effects on physical or systemic parameters such as adiposity, plasma NEFAs or blood glucose, but did lead to an increase in fasting plasma insulin in diabetic rats. A regulatory relationship between HIF-1 and insulin has been known for some time, and HIF-1 β has previously been found to be important in maintaining glucose-stimulated insulin release from pancreatic β -cells [162]. HIF-1 α has been demonstrated to be important for the expression of HIF-1 β [35], which could explain the observation that DMOG treatment led to increased insulin in diabetic rats. This is supported by studies showing that HIF-1 β ablation leads to abnormal insulin secretion and hepatic gluconeogenesis, and that this was regulated by the presence of HIF-1 α , highlighting HIF-1 as a potential therapeutic target for the treatment of pancreatic β -cell dysfunction in type 2 diabetes [204, 35].

4.5.2 DMOG treatment resulted in improved functional recovery post-reperfusion in diabetic hearts only

In ischaemia-reperfusion isolated heart perfusions, DMOG-treated diabetic hearts showed a significant improvement in functional recovery in RPP post-reperfusion, rendering them no longer different to healthy controls. When we examine the individual parameters of cardiac function, it becomes apparent that this is driven by a significant increase in developed pressure, while there was no change in either

end-diastolic pressure or heart rate. This is beneficial, as these hearts therefore have an increased systolic pressure throughout reperfusion, allowing them to pump more efficiently than untreated diabetics. DMOG-treated control hearts, however, showed a surprising change in functional recovery post-reperfusion. These hearts recovered to a much lesser extent than their untreated counterparts, with a recovery RPP similar to that of the untreated diabetic hearts. This was driven by a stark increase in end-diastolic pressure during ischaemia-reperfusion, with a sustained increase throughout reperfusion, suggesting these hearts entered a state of contracture. It is worth noting that in the untreated diabetic hearts, the individual parameters of developed pressure, end-diastolic pressure and heart rate were not significantly different to untreated controls throughout ischaemia and reperfusion, suggesting the effect seen on the RPP between these two groups was not driven by a single parameter, but rather by the additive effect of small changes within each of these (Fig. 4.8). Therefore, although the recovery seen in untreated diabetics and treated controls was similar in RPP measures, this was not driven by the same mechanism.

4.5.3 DMOG treatment resulted in no changes to glycolytic rates and increased fatty acid oxidation

We were surprised to find that DMOG treatment had no effect on the baseline glycolytic rates of either controls or diabetics. Instead, we found that fatty acid oxidation was increased at baseline, in both treated controls and treated diabetics, and this profile was maintained following ischaemia-reperfusion. DMOG treatment therefore affected substrate metabolism in a similar fashion in control and diabetic hearts, despite disparate changes in function following ischaemia-reperfusion. It appears that the increase in fatty acid oxidation caused by DMOG treatment was tolerated by the diabetic hearts, which were adapted to the utilisation of fatty acids, but not by the controls, which could not cope with this increase and may have suffered increased mitochondrial stress.

Furthermore, treated diabetic hearts not only had increased fatty acid oxidation rates, but also showed significantly higher levels of cardiac TAG following ischaemia-reperfusion. Together, these results suggest an increase in total lipid utilisation

in diabetic hearts following DMOG treatment. In DMOG-treated controls, end-diastolic pressure at reperfusion was significantly higher than in any of the other groups, suggesting that DMOG treatment resulted in diastolic dysfunction in controls. This was paired with lower glycolytic rates than untreated controls, and depleted glycogen stores, which is in line with the belief that ischaemic contracture results from a lack of glycolytic ATP production [28].

These were surprising findings, which do not support the hypothesis of increased HIF-1 signalling with DMOG treatment, where substrate metabolism would be expected to shift in favour of glycolysis, and away from fatty acid oxidation, as seen in previous studies reporting increases in glycolysis [4, 197, 189, 188]. The data described in this Chapter, showing improved functional recovery in diabetics despite increased lipid utilisation, resemble the widely reported paradoxical observation in diabetic patients that clinical outcomes are often better in patients with higher adiposity, obesity, or BMI [80, 45, 117]. The mechanisms for this are poorly understood, but may relate to the fact that the diabetic heart is well adapted to handling high levels of fatty acids and triacylglycerides.

4.5.4 DMOG treatment resulted in no increased HIF-1 signalling

Circulating levels of haemoglobin would be expected to be higher when HIF-1 signalling is activated, which is why we chose this as our first measurement to quickly determine whether *in vivo* DMOG treatment had any effect on HIF-1 signalling. Having assessed levels of hematocrit in one group of animals and found no effect of DMOG on packed cell volume percentage, we questioned whether our method was powerful enough to detect a difference, and whether it took too long to take the measurement, possibly affecting any subtle differences we might have seen. We therefore moved to a faster, more automated method using a HemoCue machine, in our subsequent group of animals. However, this too showed no difference with DMOG treatment. Increased hematocrit with *in vivo* DMOG treatment has only been shown in one study, which used DMOG in conjunction with chronic hypoxia, using a high dose (320 mg/kg) in mice, and expressed hematocrit as a percentage

of day 0 in normoxia [187]. Therefore, it appears that hematocrit is not necessarily increased by DMOG treatment, and would unlikely be significantly increased in our study, where rats were housed in normoxia. The batch of DMOG (Cayman Chemicals) used for these experiments was sent for mass spectrometric analysis to confirm purity and rule out any contaminants which would have interfered with the compound's action.

Metabolomic analysis revealed some of the discrepancies between control and diabetic hearts following DMOG treatment that could explain the divergent functional outcomes. DMOG-treated control hearts appeared to be in an energetic crisis, displaying depleted pools of ATP, PCr, and NAD. Additionally, intermediates in the first span of the Krebs cycle, such as citrate, aconitate, and isocitrate, were depleted in DMOG-treated controls. This may contribute to the energy defect, and consequently impair contractile function. In diabetic hearts, DMOG treatment resulted in a decrease in α -ketoglutarate, which was not the case in controls. In contrast, levels of glutamate were depleted in DMOG-treated controls but not diabetics, suggesting that in control hearts α -ketoglutarate levels may have been replenished by the action of transaminases, which would also have contributed to the depletion of the NAD pool. This could be the result of DMOG acting on α -ketoglutarate dehydrogenase, due to its poor specificity and its similarity to α -ketoglutarate, which, together with DMOG's active metabolite NOG, is known to affect a number of different Fe(II)/ α -ketoglutarate-dependent hydroxylases [105, 84].

We found no significant differences in the expression of any PPAR α target proteins, suggesting this was not the mechanism by which fatty acid oxidation rates had been increased. We postulated that an off-target mechanism of DMOG might be involved and, having found that other groups had reported an effect of DMOG on acetyl-CoA carboxylase (ACC) phosphorylation or upstream AMPK activation [220, 212], we measured the levels of phosphorylated ACC in hearts from DMOG-treated animals that had not been taken for perfusion. However, no differences were found in these either. Nevertheless, we believe this is an avenue worth pursuing, and it may be that our current antibody is not best suited for this

measurement. If the mechanism for increased ACC phosphorylation by DMOG is indeed via AMPK activation, our metabolomics data support this hypothesis, as DMOG-treated control hearts are energy stressed, showing depleted PCr, ATP, and ADP. Our data show an interaction term between diabetes and DMOG on malonyl-CoA levels, which were depleted in DMOG-treated control hearts and increased in DMOG-treated diabetic hearts. This is in agreement with our findings that several acyl-carnitines were increased in DMOG-treated control hearts, but not diabetics, where levels of malonyl-CoA were presumably sufficient to limit entry of fatty acids into the mitochondria, via inhibition of carnitine acyltransferase [146]. We would therefore expect levels of phosphorylated ACC to inversely correlate with the levels of malonyl-CoA, as this is the product of ACC activity.

With regards to our hypothesis for the therapeutic potential of HIF-1 signalling upregulation, at this stage it is not possible to accept or reject the hypothesis, as we have found a functional benefit, but no signalling evidence to support this hypothesis. Nevertheless, our finding that HIF-1 α protein was lower in ischaemic diabetic hearts compared with controls may in fact suggest that therapeutic targeting of HIF-1 α would be particularly beneficial in diabetes. In conclusion, DMOG treatment showed potential in diabetic hearts, with improved post-ischaemic functional recovery. However, off-target effects have complicated the assessment of this hypothesis of beneficial effects of HIF-activating treatment in the diabetic heart. A more potent and selective compound is required to further test our hypothesis. For this reason, we proceeded to assess this using a different compound, within the same disease model and perfusion protocol.

*So many of our dreams at first seem impossible, then
they seem improbable, and then, when we summon
the will, they soon become inevitable.*

— Christopher Reeve

5

Effects of Molidustat on the diabetic heart in ischaemia-reperfusion

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5.1 Chapter abstract

Clinical compounds that activate HIF via prolyl hydroxylase domain (PHD) inhibition have recently been developed for use in the treatment of anaemia. This has provided an array of drugs which could be repurposed for other diseases settings, such as brain or cardiac ischaemia. We hypothesise that treating diabetic rats with a clinical PHD inhibitor, Molidustat, can help re-balance cardiac substrate metabolism, which is abnormal in diabetes. Our hypothesis is that, by improving substrate metabolism, with increased glycolysis and decreased fatty acid oxidation, Molidustat treatment will also improve cardiac functional recovery following ischaemia-reperfusion. Molidustat treatment was first validated in healthy control rats, using a single dose and a five-day chronic daily dose of 5mg/kg. Subsequently, control and diabetic rats were given Molidustat orally once daily for five consecutive days, before excising the heart for isolated heart perfusion. Hearts were subjected to low-flow ischaemia-reperfusion and freeze-clamped for subsequent studies. We found that 5mg/kg Molidustat resulted in sustained upregulation of circulating haemoglobin, indicating systemic HIF signalling activation. Baseline glycolytic rates were increased with Molidustat treatment, and fatty acid oxidation rates were decreased with treatment. Untreated diabetic hearts displayed impaired functional recovery compared with controls, whis was fully rescued by Molidustat treatment.

In this study, we demonstrate for the first time that treatment with a clinically-relevant HIF-stabilising compound leads to improved recovery post-ischaemia in diabetic rat hearts, paired with improved substrate metabolism.

5.2 Introduction

The effects of hypoxia inducible factor (HIF) activation are multi-factorial, and include stimulation of angiogenesis and secretion of erythropoietin (EPO), which has been of particular interest for research in renal anaemia. Investigations into HIF-activating strategies for the treatment of renal anaemia prompted the development of a new class of clinical small molecule inhibitors, the prolyl hydroxylase domain (PHD) inhibitors, several of which have proceeded into clinical trials [148]. This has provided a framework for research into the repurposing of these compounds for additional unmet clinical needs. One such case may be ischaemic disease, as HIF has been a point of focus in studies investigating the benefits of ischaemic pre-conditioning, including models of brain and cardiac ischaemia [151, 54, 108, 150]. In the heart, HIF signalling is crucial, as it provides most of the indispensable cellular responses to changes in oxygen availability. This becomes particularly important in a situation of ischaemia, such as a myocardial infarction (MI). The benefits of HIF signalling activation may be of most interest in the context of diabetes, where the maladaptations to the disease are essentially opposing the normal adaptation to hypoxia. As previously discussed, diabetes shifts the metabolic profile towards fatty acid oxidation and away from glucose use, while HIF signalling downregulates fatty acid oxidation and PPAR α targets, and upregulates glycolysis, providing energy at a lower oxygen cost. The diabetic heart appears to have lost its natural metabolic flexibility, which is particularly important in the event of an MI [152]. A growing body of evidence suggests that impaired HIF signalling may be an underlying factor in diabetic complications, including microangiopathy and macrovascular disease, due to reduced angiogenesis, increased reactive oxygen species formation, and impaired metabolic remodelling [16, 139].

To date, the cardioprotective effects of HIF signalling upregulation have predominantly been studied using either pre-conditioning frameworks or genetic models

[54, 151, 108]. While this provides valuable evidence for the benefit of this treatment approach, pharmacological intervention is required to validate this treatment strategy and take it forward into the clinic. In the small number of studies which have used a pharmacological approach, the compounds used were not developed for clinical use, and therefore do not have suitable potency, selectivity, or specificity. Therefore, new proof-of-concept studies are needed to validate the use of potent and selective PHD inhibitors in cardiac ischaemic disease. The orally-available PHD inhibitor, Molidustat (BAY85-3934), was one of the novel compounds developed for use in anaemia associated with chronic kidney disease [66], and has already completed Phase II clinical trials (DIALOGUE trial, NCT02021370), showing good safety and tolerability in pre-dialysis patients, and increased EPO and haemoglobin [133]. Molidustat was characterised *in vivo* in male Wistar rats, and resulted in increased EPO release and increased reticulocyte counts after a single dose as low as 1.25 mg/kg, as well as increased packed cell volume after just one week of daily dosing [66], suggesting sustained upregulation of downstream HIF signalling. Molidustat treatment also showed increased HIF1- α protein levels in three different cell lines [66]. However, cardiac tissue was not studied by this group in any of the cell or animal models used in the characterisation of Molidustat.

A comparative study of the potencies and selectivities of several clinical PHD inhibitors revealed that Molidustat was more potent than FG-4592, Vadadustat, and GSK1278863 in an antibody-based assay, similarly potent in a cell-based assay, and selective for the PHD enzymes against other α -ketoglutarate dioxygenases [214]. Molidustat is a triazole-based PHD inhibitor, which confers additional stability upon binding, and improved selectivity for PHD enzymes [14]. DMOG is cleaved to form NOG, an α -ketoglutarate analogue, with a simple chemical structure, and little ability to stabilise binding, as well as poor selectivity, predicted to inhibit several α -ketoglutarate-dependent dioxygenases [57]. Molidustat therefore exhibits a much more desirable selectivity profile, as it is unlikely to exhibit off-target effects when administered *in vivo*, as observed with DMOG in the previous Chapter. Molidustat was therefore chosen for this Chapter, and administered to control and

diabetic rats as a daily pre-treatment before *ex vivo* heart perfusion with low-flow ischaemia-reperfusion, to assess the potential of this drug in preventing damage from cardiac ischaemia in type 2 diabetes.

Therefore, the aims of this chapter were:

- Firstly, to assess the effects of Molidustat treatment on systemic markers of HIF signalling in healthy control rats
- Secondly, to assess the effects of Molidustat pre-treatment on metabolism and function in control and diabetic rat hearts subjected to ischaemia-reperfusion.

5.3 Methods

5.3.1 Animal work

Pilot study

To validate the ability of Molidustat to upregulate HIF signalling *in vivo* in male Wistar rats, and in order to test our chosen treatment regimen, healthy control rats were given either a single dose, or five consecutive daily doses of 5 mg/kg Molidustat orally, to assess the effects of acute and chronic Molidustat treatment (Fig. 5.1). This dose was selected based on the findings by Flamme *et al.* that this was the lowest dose to result in increased hematocrit within one week of treatment [66]. Rats were given Molidustat orally, by dissolving the dose of drug in a jelly fed to each rat, as described in Chapter 2. Animals treated acutely were sacrificed either 24h or 72h following treatment, to detect any delay in downstream HIF signalling upregulation due to the requirement for gene transcription and translation. Animals treated with the chronic five-day dosing were sacrificed 24h following the final dose.

Main study

Diabetic rats were fed high fat diet (HFD) for four weeks prior to the start of the treatment (Fig. 5.2), and given an i.p. injection of streptozotocin at the end of the second week of high fat feeding, following our diabetic model protocol. Control rats were weight-matched to the diabetics at the start of the study and continued on normal chow throughout the five weeks of study. Rats were treated *in vivo* with Molidustat during the final week prior to heart perfusion, to allow time for changes in gene transcription and protein translation induced by HIF upregulation. Rats were treated daily for the final five days, taking the heart 24h after the last dose, as in the chronic pilot study. Fasting blood samples were taken from a subset of animals on the fourth day of treatment, under recovery isoflurane anaesthesia (2.5 ml/min). Fed blood samples were taken at the time of heart excision, together with the liver, and blood haemoglobin levels were measured

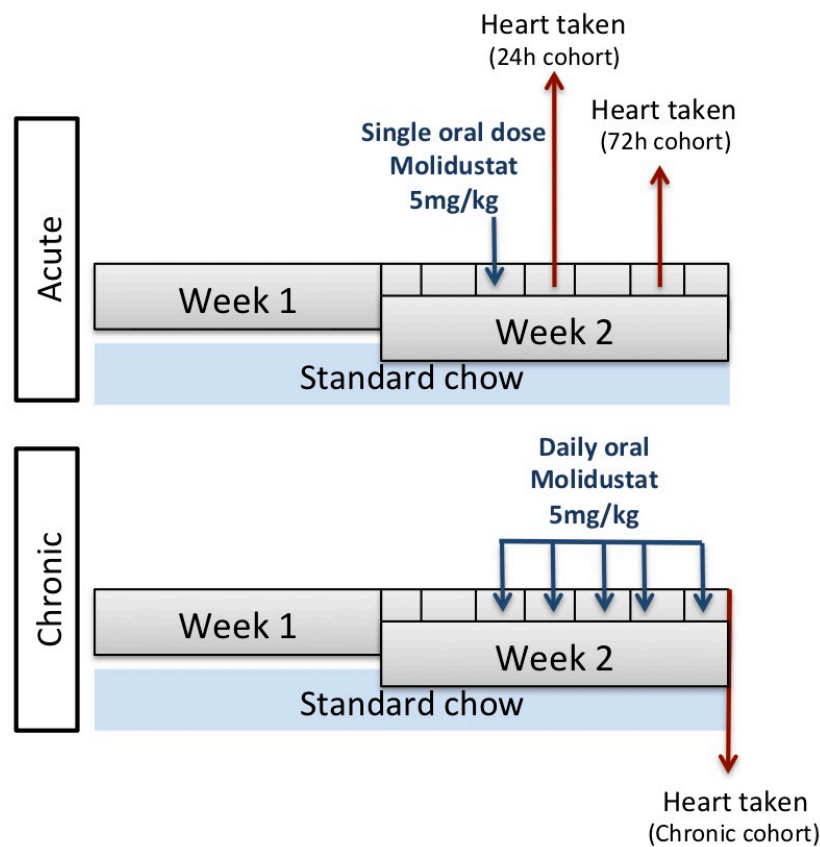


Figure 5.1: Timeline of animal work for pilot Molidustat study. Control rats were given 5mg/kg Molidustat either acutely as a single oral dose, or chronically as five consecutive daily oral doses. Hearts were excised 24h or 72h following the single dose, and 24h following the final dose in the chronic cohort.

immediately, as a marker of systemic HIF activation. In a subset of rats, hearts were frozen immediately at the time of isolation and not taken for perfusions. This allowed us to assess effects of Molidustat treatment independent of the effect of ischaemia-reperfusion or perfusion of the heart.

5.3.2 Ischaemia-reperfusion heart perfusion

In total, four groups were studied: control, diabetic, control + Molidustat, and diabetic + Molidustat. Hearts from all groups were isolated and perfused in Langendorff mode with Krebs-Henseleit (KH) buffer containing 11 mM glucose, 0.4 mM palmitate, and 3 U/l insulin, as described in section [2.5.4](#). Pre-ischaemic function and metabolism were assessed for a period of 20 minutes, following which the hearts were subjected to low-flow ischaemia at a flow rate of 0.3 ml/min/gww

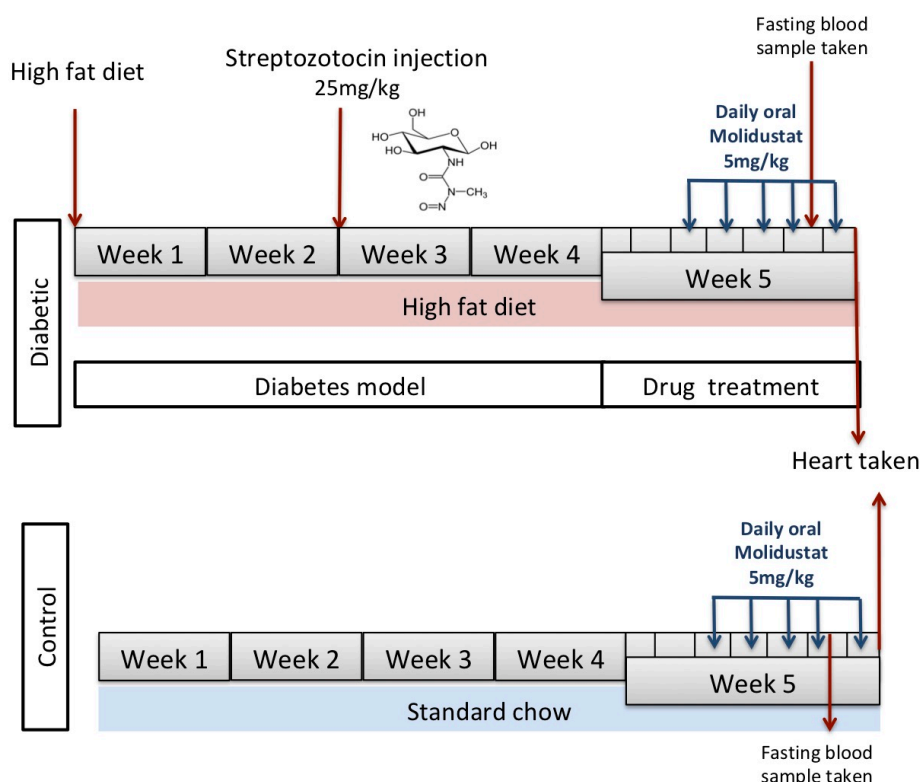


Figure 5.2: Timeline of animal work for the main Molidustat study. Control rats were fed normal chow, and diabetic rats were fed high fat diet (HFD) for four weeks prior to the start of the treatment. Rats receiving treatment were given five daily oral doses of 5mg/kg Molidustat. Fasting blood samples were taken two days prior to sacrifice. Hearts were excised 24h following the final dose of Molidustat.

for 30 minutes. Normal flow was resumed for a further 20 minutes. Hearts were rapidly freeze-clamped on the cannula at the end of the reperfusion period, and stored at -80°C for subsequent analysis. The perfusion buffer was supplemented with $0.2 \mu\text{Ci/ml}$ of either $[5-^3\text{H}]$ -glucose for the measurement of glycolytic rates, or $[9,10-^3\text{H}]$ palmitate for the measurement of fatty acid oxidation rates. A sample of perfusate (1 ml in volume) was taken every 4 minutes over the perfusion period and used to calculate myocardial glycolytic flux and fatty acid oxidation rates as described in section [2.5.5](#).

5.3.3 Statistical analysis

Data are expressed as means $\pm$ standard deviation of the mean (SD), and $p < 0.05$ was taken as the level of statistical significance. Results from the pilot study were

analysed using one-way ANOVA, as these experiments were all conducted in control rats only. All data shown as the four groups (controls and diabetics with and without drug treatment) were analysed using a two-way ANOVA, where the two factors were diabetes and drug, using Sidak correction for multiple comparisons. Cardiac function and metabolism with drug treatment were compared within each perfusion state separately. GraphPad Prism 7 Software Package (GraphPad Softwares, California, USA) was used for all statistical analyses.

5.4 Results

5.4.1 Pilot study

In control rats, Molidustat treatment, either acute or chronic, had no significant effect on either body mass or adiposity, measured by the ratio of epididymal fat pad mass to body mass (Fig. 5.3).

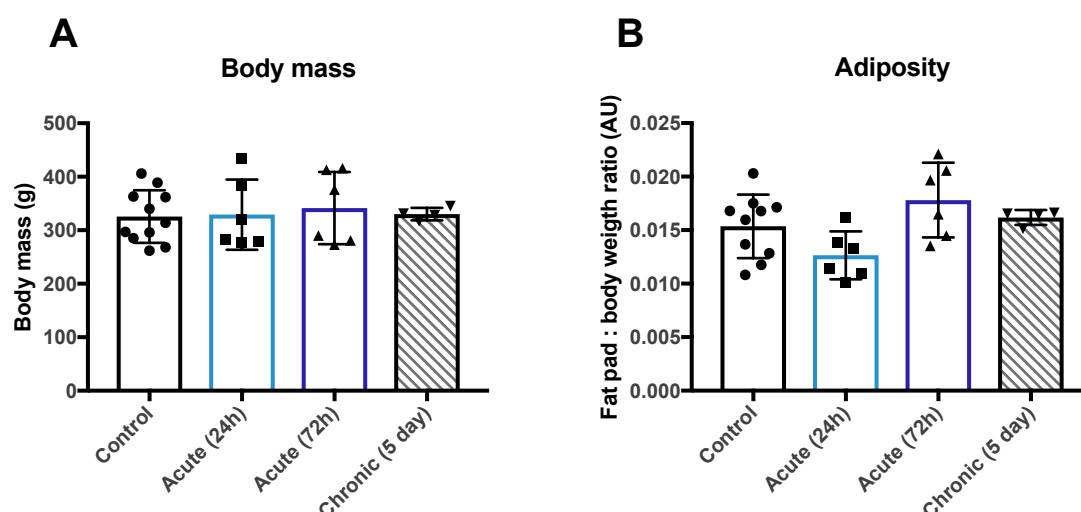


Figure 5.3: Body mass (A) and adiposity (B) of control rats 24h or 72h following a single oral dose of 5mg/kg Molidustat, and 24h following five daily chronic doses of 5mg/kg Molidustat.

No significant difference was found in circulating haemoglobin in rats treated acutely, either 24h or 72h following treatment (Fig. 5.4A). However, levels of circulating haemoglobin were significantly increased in rats given five consecutive daily doses, indicating stimulation of the HIF signalling pathway (Fig. 5.4A). No significant differences were found in blood glucose in animals treated with a single dose, or those given chronic five day treatment (Fig. 5.4B). Similarly, mRNA levels of vascular endothelial growth factor (VEGFA) were not significantly upregulated in heart tissue of rats given a single dose of Molidustat, whether measured 24h or 72h following dosing, but were significantly increased in hearts from rats treated with five daily doses (Fig. 5.5A). In contrast, mRNA levels of haem oxygenase 1 (HMOX1)

were significantly upregulated in heart tissue from rats just 24h following a single dose of Molidustat, but this was not seen 72h after dosing (Fig. 5.5). Additionally, no significant change in mRNA levels of HMOX1 were found in hearts from rats following five daily doses of Molidustat. With the exception of HMOX1 mRNA levels, these results validated that chronic treatment over five days was sufficient to cause systemic upregulation of HIF-1 signalling, and this treatment regimen was chosen for the subsequent main study in diabetic rats.

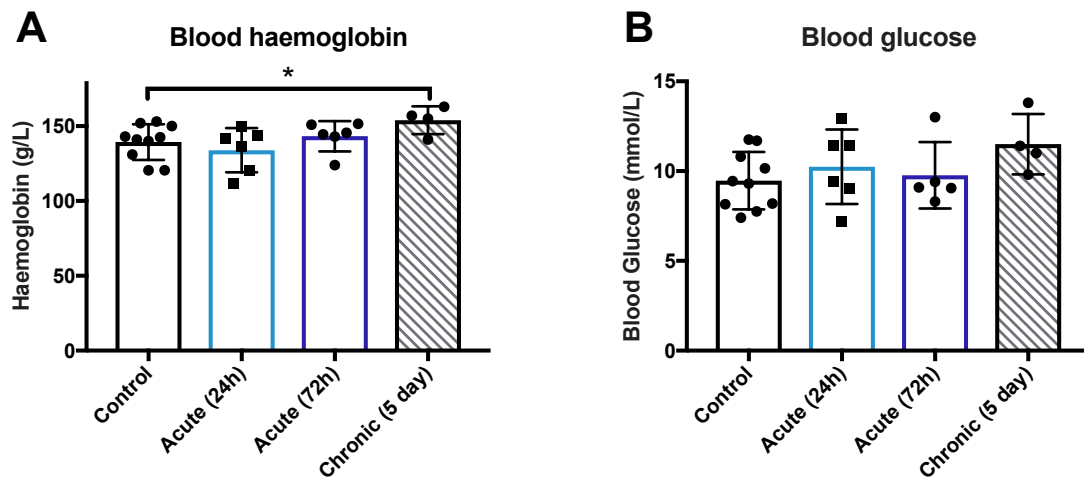


Figure 5.4: Fed state blood haemoglobin (A) and blood glucose (B) levels in control rats 24h or 72h following a single oral dose of 5mg/kg Molidustat, and 24h following five daily chronic dosing of 5mg/kg Molidustat. * $p < 0.05$ vs control untreated.

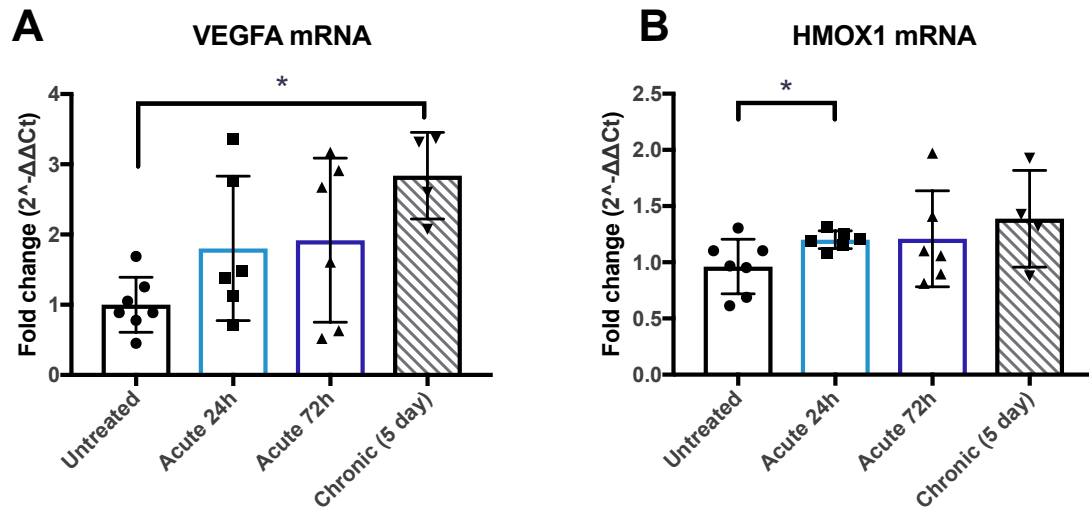


Figure 5.5: Cardiac levels of vascular endothelial growth factor A mRNA (A) and haem oxygenase 1 mRNA (B) in control rats 24h or 72h following a single oral dose of 5mg/kg Molidustat, and 24h following five daily chronic dosing of 5mg/kg Molidustat. * $p < 0.05$ vs control untreated.

5.4.2 Main study

Effects of diabetes and Molidustat treatment on physical parameters

Diabetic rats, whether treated with Molidustat chronically or untreated, had significantly higher body mass compared with control rats (Fig. 5.6A). Molidustat treatment had no effect on body mass in either control or diabetic rats (Fig. 5.6A). Relative heart mass remained unchanged by diabetes or Molidustat treatment, with all groups exhibiting comparable heart mass to body mass ratios (Fig. 5.6B). Adiposity was increased in diabetic rats, who showed increased epididymal fat pad mass to body mass ratio compared with controls (Fig. 5.6C). Molidustat treatment had no effect on adiposity, in either control or diabetic rats. However, both control and diabetic rats treated with Molidustat exhibited significantly elevated levels of circulating haemoglobin compared with their untreated counterparts (Fig. 5.6D), indicating systemic stimulation of the HIF signalling pathway.

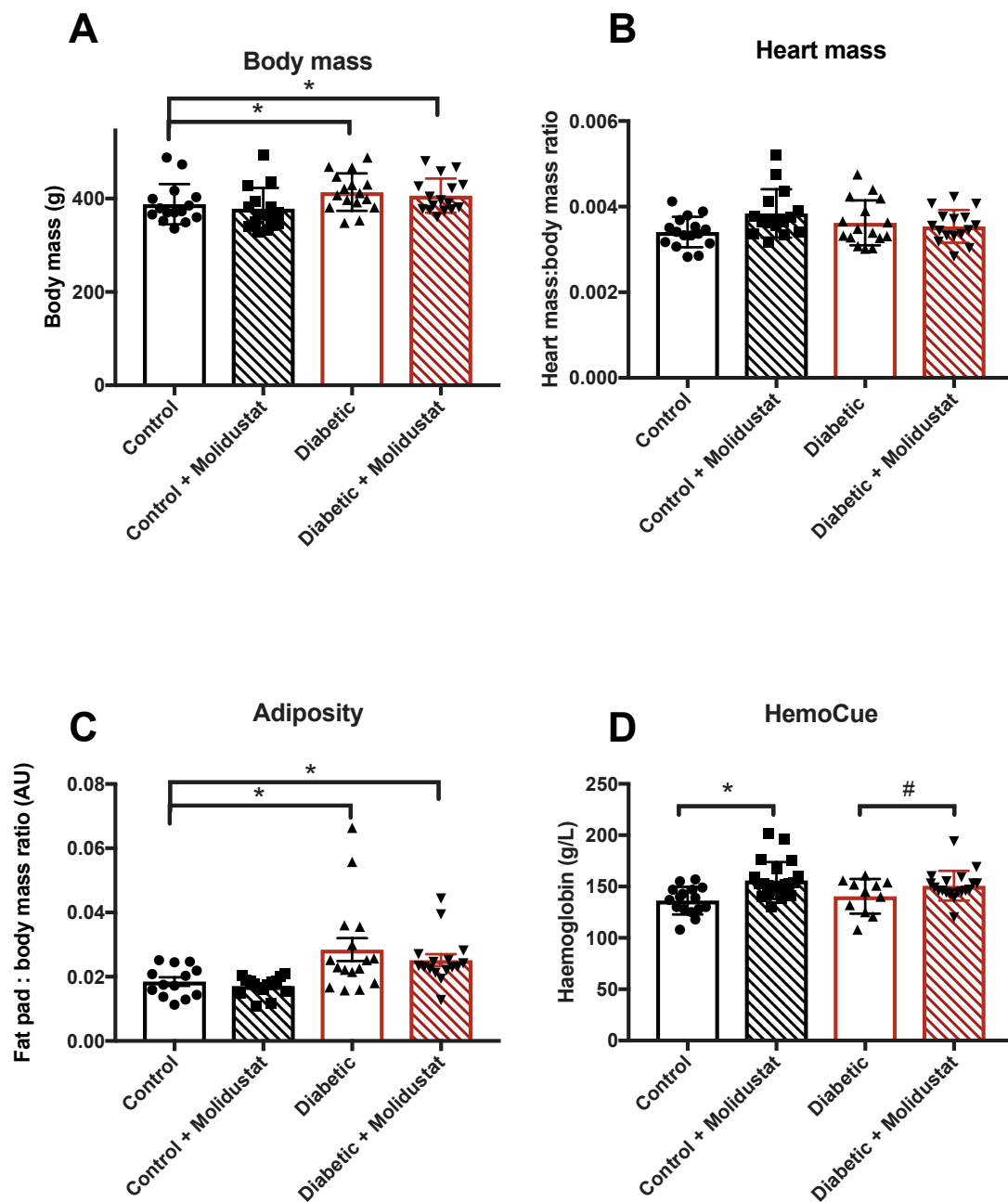


Figure 5.6: Body mass (A), heart mass (B), adiposity (C), and circulating haemoglobin (D) in control and diabetic rats with and without Molidustat treatment. * $p < 0.05$ vs control untreated, # $p < 0.05$ vs diabetic untreated.

Effects of diabetes and Molidustat treatment on systemic metabolic markers

Diabetic rats had elevated fed state blood glucose levels compared with controls, and this was unchanged by Molidustat treatment, with treated diabetics showing similar

glucose concentrations to untreated diabetics (Fig. 5.7A). Similarly, fasting blood glucose concentration was higher in diabetic rats, and unchanged with Molidustat treatment (Fig. 5.7B). Molidustat treatment had no significant effect on either fed or fasted state blood glucose in control rats (Fig. 5.7A,B). Fed state plasma NEFA concentrations were not significantly different between diabetics and controls, and no significant differences were found with Molidustat treatment, in either group (Fig. 5.7D). No significant differences were found in fasted state plasma NEFA concentrations between any of the four groups on this occasion (Fig. 5.7D). Plasma insulin concentration, which was significantly increased in diabetic rats compared with controls, was significantly decreased in Molidustat-treated diabetics (Fig. 5.7E).

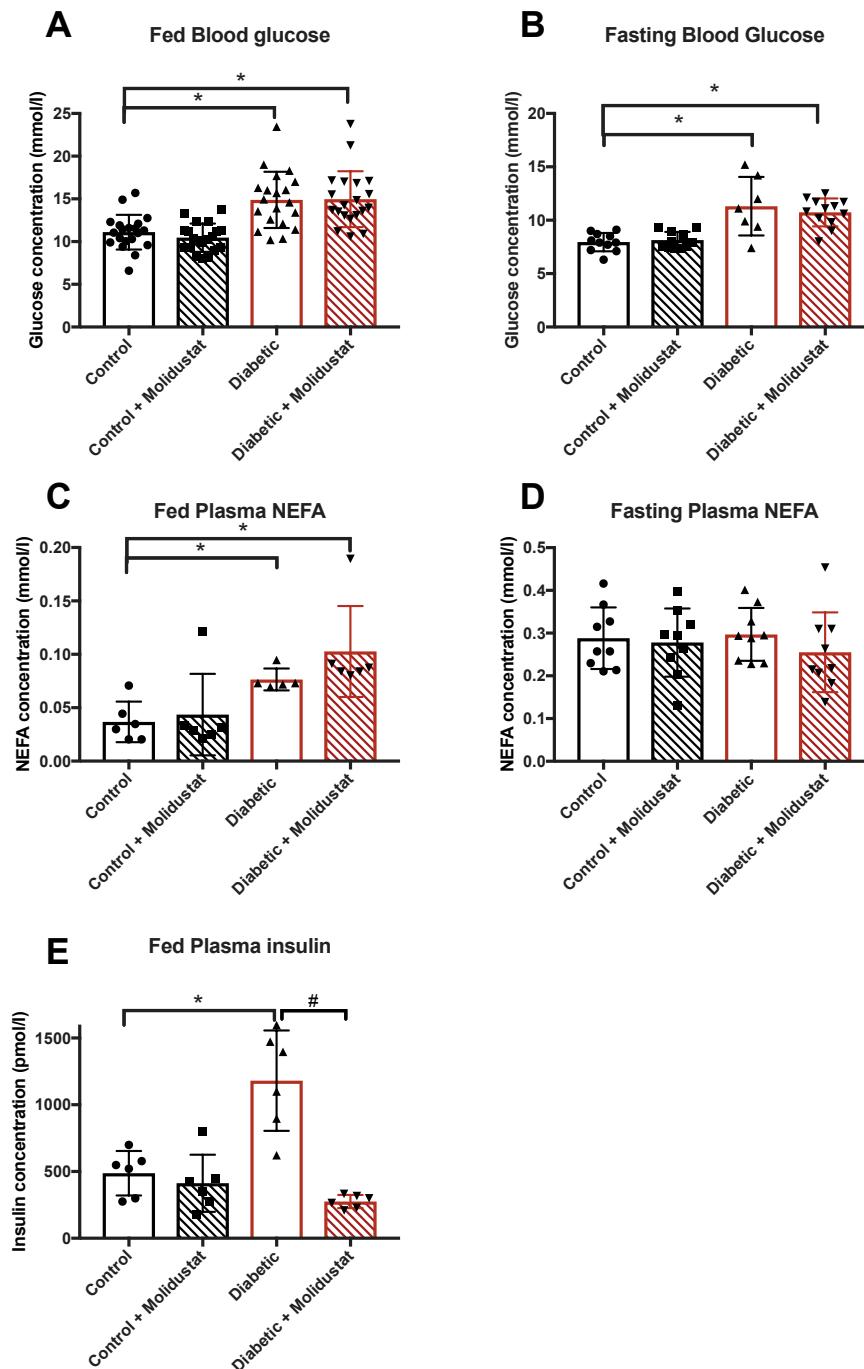


Figure 5.7: Concentrations of fed state (A) and fasting state (B) plasma glucose, fed state (C) and fasting state (D) plasma non-esterified fatty acids (NEFA), and fed state plasma insulin concentrations (E) in control and diabetic rats with and without Molidustat treatment. * $p < 0.05$ vs control untreated, # $p < 0.05$ vs diabetic untreated.

Effects of Molidustat treatment on metabolism in the isolated heart

In the isolated perfused heart, pre-ischaemic glycolytic rates were significantly lower in diabetic hearts compared with controls (Fig. 5.8A). With Molidustat

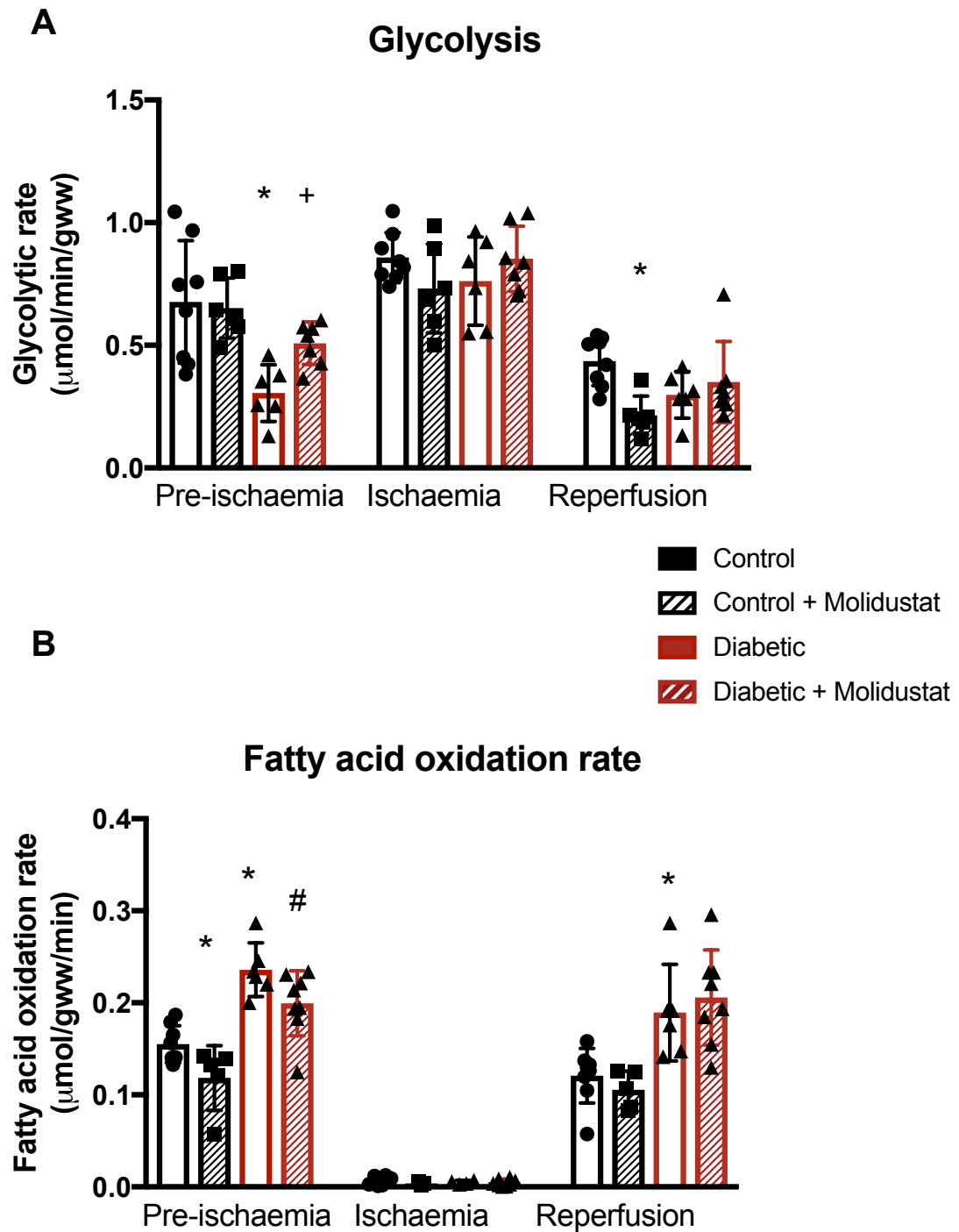


Figure 5.8: Glycolytic rates (A) and fatty acid oxidation rates (B) in isolated perfused hearts from control and diabetic rats with and without Molidustat treatment, during pre-ischaemia, ischaemia and reperfusion. * $p < 0.05$ vs control untreated, + $p = 0.07$ vs control, # $p < 0.05$ vs diabetic untreated. $n = 6-8$ per group.

treatment, glycolytic rate was increased in diabetic hearts, to a level no longer significantly different to controls, showing a trend for increased rates compared with untreated diabetics but which was not statistically significant ($p=0.07$, Fig. 5.8A). Molidustat treatment did not result in changes to pre-ischaemic glycolytic rates in control hearts (Fig. 5.8A). During low-flow ischaemia, glycolytic rates were increased in all groups, and not significantly different between any of the four groups. Glycolytic rates in Molidustat-treated diabetic hearts following reperfusion were not significantly different to either control or untreated diabetic hearts. Conversely, Molidustat-treated control hearts did show significantly decreased glycolytic rates at reperfusion compared with controls (Fig. 5.8A). Pre-ischaemic fatty acid oxidation rates were significantly higher in diabetic hearts compared with controls (Fig. 5.8B). Molidustat treatment resulted in a significant decrease in fatty acid oxidation rates in both control and diabetic hearts, compared with their untreated counterparts (Fig. 5.8B). Fatty acid oxidation rates during low-flow ischaemia were minimal in all groups. At reperfusion, diabetic hearts once again showed significantly higher fatty acid oxidation rates than control hearts (Fig. 5.8B). Molidustat treatment had no significant effect on fatty acid oxidation rates during reperfusion (Fig. 5.8B).

Effects of Molidustat treatment on cardiac function in the isolated heart

Left ventricular contractile function, measured by rate-pressure product (RPP), was unchanged by diabetes or Molidustat treatment during pre-ischaemic perfusion (Fig. 5.9A,B). During low-flow ischaemia, RPP dropped to negligible levels in all hearts (Fig. 5.9A). At reperfusion, diabetic hearts displayed impaired contractile function, with significantly lower RPP compared with controls (Fig. 5.9A,C). Molidustat-treated diabetic hearts displayed significantly improved functional recovery compared with untreated diabetics, with RPP levels no longer significantly different to control hearts (Fig. 5.9A,C). In control hearts, Molidustat treatment had no significant effect on contractile function (Fig. 5.9). An interaction term was found between drug and disease for RPP during reperfusion, indicating a divergent effect of the drug in diabetes.

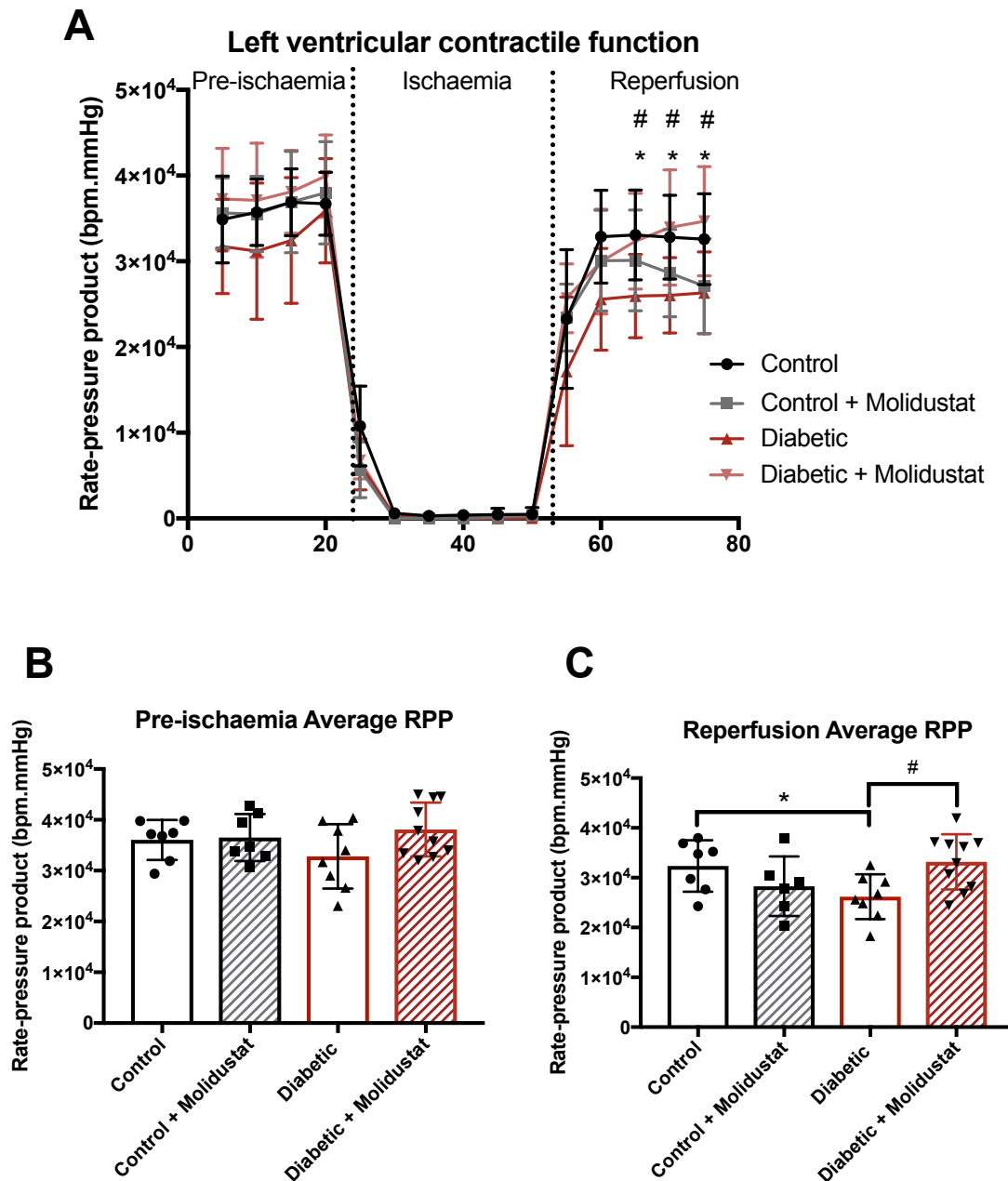


Figure 5.9: Left ventricular contractile function in hearts from control and diabetic rats with and without Molidustat treatment, during pre-ischæmia, ischæmia and reperfusion (A). Average rate-pressure product at baseline (B) and average rate-pressure product during reperfusion (C). * $p < 0.05$ vs control untreated, # $p < 0.05$ diabetic untreated vs diabetic + Molidustat.

The different parameters of contractile function were analysed, including heart rate, developed pressure, end-diastolic pressure, and systolic pressure, and none of these were significantly changed in any of the four groups during pre-ischæmic

measurements (Fig 5.10). During low-flow ischaemia, end diastolic pressure increased in all groups to varying degrees, though no significant differences were found between any groups (Fig 5.10C). At reperfusion, heart rate was found to be significantly lower in untreated diabetic hearts compared with controls (Fig 5.10A). Molidustat treatment resulted in increased heart rate at the end of reperfusion in diabetic hearts, compared to untreated diabetics, restoring this to control levels (Fig 5.10A). Developed pressure was significantly increased by Molidustat treatment in diabetic hearts at the beginning of reperfusion, which was no longer seen in later stages of reperfusion (Fig 5.10B). No significant differences in end-diastolic pressure or systolic pressure were found between groups (Fig 5.10C,D).

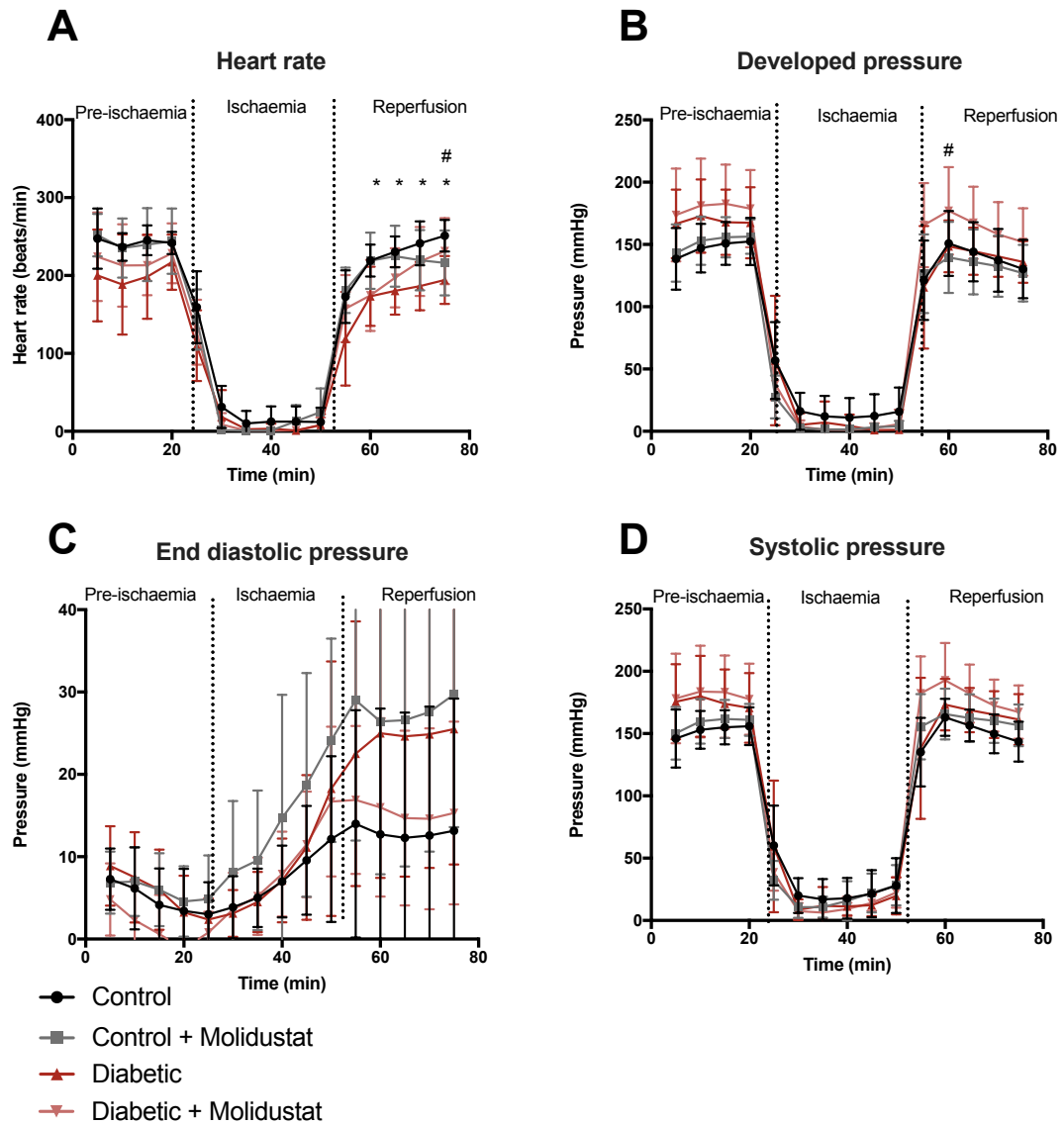


Figure 5.10: Functional parameters in hearts from control and diabetic rats with and without Molidustat treatment, during pre-ischaemia, ischaemia and reperfusion: heart rate (A), developed pressure (B), end-diastolic pressure (C), and systolic pressure (D). * $p < 0.05$ control untreated vs diabetic untreated, # $p < 0.05$ diabetic untreated vs diabetic + Molidustat.

Effects of Molidustat treatment on cardiac tissue metabolic markers

Cardiac tissue from hearts perfused through ischaemia-reperfusion was analysed for glycogen and triacylglyceride (TAG) content. No significant differences were found in glycogen (Fig. 5.11A) or TAG (Fig. 5.11B) stores between control and diabetic hearts following ischaemia-reperfusion. Molidustat treatment resulted in no significant changes in glycogen stores or TAG stores in either control or diabetic rat hearts (Fig. 5.11).

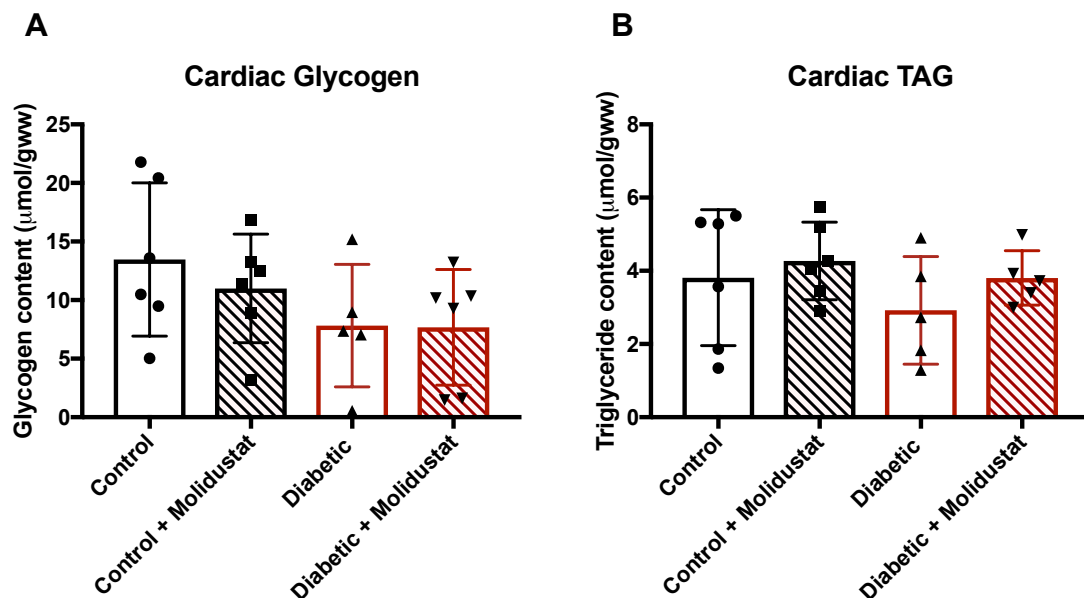


Figure 5.11: Glycogen (A) and triacylglyceride (B) content in hearts from control and diabetic rats with and without Molidustat treatment, following isolated heart perfusion with ischaemia-reperfusion.

Evidence of cardiac HIF-1 α upregulation with Molidustat treatment was investigated by measuring protein expression levels of glucose transporter 1 (GLUT1, SLC2A1) and HIF-1 α in hearts frozen immediately at the time of excision. No significant differences were found in GLUT1 (SLC2A1) or HIF-1 α protein expression levels between control and diabetic rats (Fig. 5.12A,B). Molidustat treatment resulted in no significant upregulation of either GLUT1 (SLC2A1) or HIF-1 α protein expression levels (Fig. 5.12A,B).

PPAR α target proteins were assessed in hearts frozen immediately following excision. No significant differences were found in protein expression levels of fatty

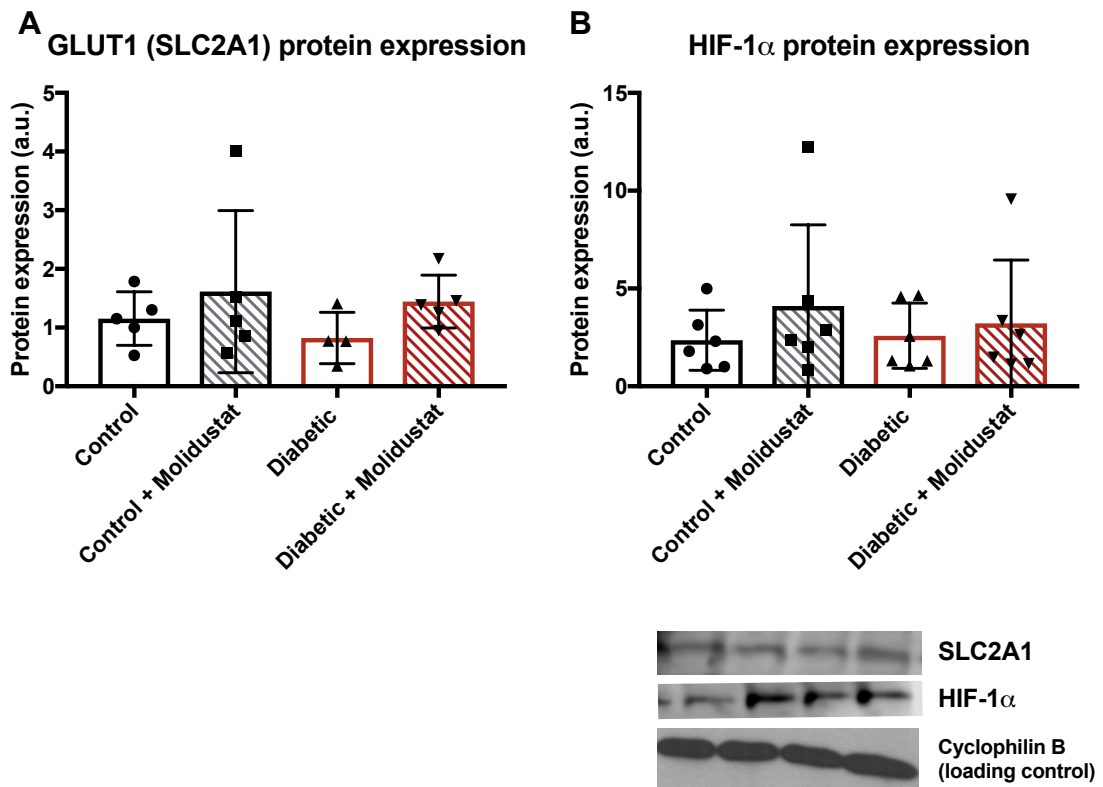


Figure 5.12: Protein expression levels of glucose transporter 1 (GLUT1) (A) and hypoxia-inducible factor 1 alpha (HIF-1 α) (B) in hearts from control and diabetic rats with and without Molidustat treatment.

acid translocase (FAT/CD36), medium-chain acyl-CoA dehydrogenase (MCAD), or pyruvate dehydrogenase kinase 4 (PDK4) with either diabetes or Molidustat treatment on this occasion (Fig. 5.13).

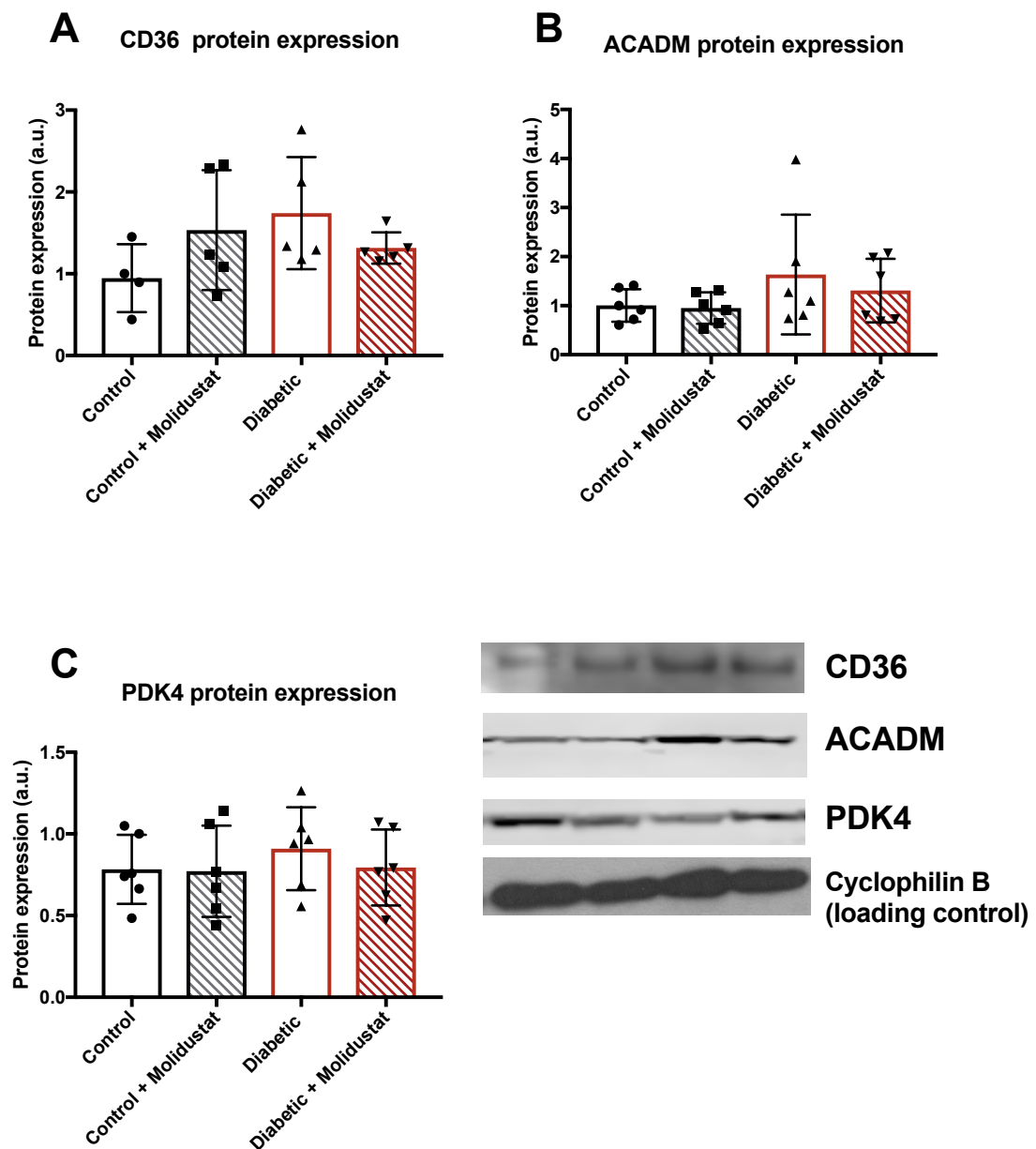


Figure 5.13: Protein expression levels of fatty acid translocase (FAT/CD36, A), medium-chain acyl-CoA dehydrogenase (MCAD, B), and pyruvate dehydrogenase kinase 4 (PDK4, C) in hearts from control and diabetic rats with and without Molidustat treatment.

5.5 Discussion

The diabetic heart is at increased risk in the event of myocardial ischaemia, a hallmark of acute myocardial infarction (MI) [85, 77]. This is due, in great part, to the metabolic maladaptations caused by diabetes, which deprive the heart of its metabolic flexibility and, consequently, of its natural ability to switch substrate metabolism to a more favourable glycolytic state to save oxygen and prevent oxidative damage [152]. In this Chapter, our hypothesis that treatment with Molidustat, a compound known to upregulate the activation of HIF, can improve functional recovery in the diabetic heart following an ischaemic insult, was validated. By measuring real time metabolic rates, we have shown that the improvement in functional recovery was related to increased glycolytic rate, and decreased fatty acid oxidation rates in diabetic hearts following Molidustat treatment.

5.5.1 Molidustat treatment showed promising results in the diabetic heart

HIF-1 α activation via genetic overexpression or prolyl hydroxylase domain (PHD) inhibition has become of interest in the past decade, though only recently in the field of ischaemia [8, 211, 11]. Genetic studies and ischaemic pre-conditioning studies have highlighted the important role and therapeutic potential of HIF-1 α signalling in the context of ischaemia [94, 54, 149, 151]. However, in the context of cardiac ischaemia, very few studies have examined this pharmacologically. Improved cardiac recovery has been shown in only two studies using clinically-developed HIF-activating compounds in rat models of MI. Philipp *et al.* showed that rats treated with PHD inhibitor FG2216 prior to induction of MI had improved left ventricular contractility and ejection fraction [161]. Bao *et al.* showed that rats treated with PHD inhibitor GSK360A had improved ejection fraction and ventricular dilation up to three months following MI [11]. Here, we investigated this treatment strategy in the type 2 diabetic heart, where we believe HIF activation would be of

particular interest and benefit, as its cardiac metabolic effects essentially oppose those of diabetes. Our results are in agreement with the literature, having now shown that Molidustat treatment in diabetic rats resulted in improved functional recovery following ischaemia-reperfusion. The fact that we do not see a significant difference with treatment in controls may be due to the fact that our model of low-flow ischaemia is not as severe as the *in vivo* MI models used in these studies, as our control hearts recover fully following reperfusion. However, we show for the first time that this therapeutic strategy has potential in the type 2 diabetic heart. Until now, no studies have investigated the use of HIF-activating compounds in diabetes.

5.5.2 Molidustat treatment improved post-ischaemic cardiac functional parameters

In examining the parameters used here to measure left ventricular function, we found that heart rate was the main driver of functional improvement in Molidustat-treated diabetic hearts. A myocyte-specific HIF-1 α deletion study showed no significant differences in heart rate, while our results show increased heart rate with Molidustat treatment following ischaemia-reperfusion [100]. We also found that developed pressure rose transiently at early reperfusion in Molidustat-treated diabetic hearts, contributing to the improvement in rate-pressure product (RPP), which is supported by Poynter *et al.* who found that pre-treatment with DMOG resulted in greater recovery of left ventricular developed pressure following ischaemia-reperfusion in isolated rat hearts [164]. This is also in line with Huang *et al.*'s myocyte-specific HIF-1 α deletion study, where developed pressures were significantly lower in the cardiac HIF-1 α KO mice [100]. We found great variability in end-diastolic pressures (EDP) across all groups, however it appears that diabetic hearts tended to have higher EDP after ischaemia, whereas Molidustat-treated diabetic hearts were closer to controls. Despite this not reaching significance, this general trend may be of interest in the context of diabetes, if one of the main problematic aspects of diabetic cardiomyopathy is diastolic dysfunction [21, 5]. Further studies are warranted to investigate whether HIF stabilising treatment could also rescue diastolic function *in vivo* in the diabetic heart under ischaemic stress.

5.5.3 Molidustat treatment improved substrate metabolism in the diabetic heart

It is worth noting that the dose of drug used in this Chapter was relatively low compared with doses used in previous *in vivo* studies for similarly potent compounds, often up to 30 mg/kg [161, 11]. This difference could explain the trends for differences that did not show statistical significance in targets such as glycolytic rate, GLUT1 (SLC2A1) protein and HIF-1 α protein. Nevertheless, increased circulating haemoglobin, and increased glycolytic rates suggest that the observed functional effects are related to HIF signalling activation. These data are in line with the significant upregulation of hematocrit found by Flamme *et al.* in their characterisation of the compound [66]. Glycolytic rate or flux have not previously been assessed with Molidustat treatment, though glycolytic flux has been shown to be upregulated by PHD1 knockout (KO) in skeletal muscle, in an attempt to reprogramme basal metabolism [8]. Here we show, for the first time, that treatment with the PHD inhibitor, Molidustat, resulted in significantly improved substrate metabolism in the diabetic rat heart, showing increased glycolytic rates, as well as decreased fatty acid oxidation rates. These results corroborate our hypothesis, that PHD inhibition led to HIF-1 activation, resulting in increased stimulation of glycolysis, as well as inhibition of fatty acid utilisation, presumably via Randle cycle inhibition and inhibition of PPAR α targets.

5.5.4 Molidustat treatment resulted in differential modulation of different HIF targets

Interestingly, in the pilot study of this Chapter we found that HIF targets mRNA transcription varied between different targets, with significant increases in VEGFA mRNA only with chronic dosing, while increases in HMOX1 mRNA were seen within 24h and were not sustained. This is in line with a recent report showing that PHD inhibitors resulted in varying degrees of target gene upregulation, and that Molidustat treatment resulted in lower VEGFA mRNA upregulation compared with similarly potent compounds, in a cell-based assay [214].

The data shown in this Chapter have validated and corroborated the hypothesis of HIF-activating treatment in the diabetic heart, showing re-balanced substrate metabolism, increased glycolysis and decreased fatty acid oxidation, paired with significantly improved post-ischaemic functional recovery. This was supported by significant increases in systemic markers of HIF activation, further validating this therapeutic strategy. Improvement in ischaemic survival and recovery has been previously reported with HIF-activating treatment, but this had not been shown in diabetes, where we argue it would be most beneficial.

Freedom is the oxygen without which science cannot breathe.

— David Sarnoff

6

General Discussion

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6.1 Summary of findings

The work shown in this thesis has demonstrated hope for novel therapeutic avenues to treat the diabetic heart and improve chances of survival and recovery following myocardial infarction (MI). The relationships between acute hypoxic stress, hypoxia signalling, substrate metabolism, and contractile function were investigated in a rat model of type 2 diabetes, to identify the ability of metabolic modulators to improve contractile function. Initially, the effects of acute hypoxia on the diabetic heart were investigated, using isolated heart perfusion, and by subjecting hearts to acute hypoxia-reoxygenation (Chapter 3). This work revealed that the metabolic maladaptation resulting from the diabetic pathology drives the loss of contractile function following acute cardiac hypoxia, despite intact AMPK activation. This highlighted the potential for metabolic therapy in rescuing functional post-hypoxic recovery. Indeed, treatment with FAT/CD36 inhibitor SSO to block fatty acid uptake, just minutes prior to the induction of hypoxia, was sufficient to completely rescue functional recovery, as well as preventing the accumulation of TAG in the diabetic hearts (Fig. 6.1). This was paired with increased glycolytic rate during hypoxia and decreased fatty acid oxidation rate at reoxygenation, supporting the hypothesis that re-balancing substrate metabolism could prevent damage to the diabetic heart in acute hypoxia. This hypothesis was taken further, to investigate whether chronic pre-treatment with a hypoxia mimetic, prolyl hydroxylase domain (PHD) inhibitor, DMOG, could re-balance baseline substrate metabolism in the heart, in order to prevent functional impairment in a clinically-relevant model of ischaemia-reperfusion (Chapter 4). Importantly, impaired HIF-1 α stabilisation was found in diabetic hearts in low-flow ischaemia. This demonstrated that HIF activation could be a viable therapeutic strategy. DMOG treatment did lead to improved functional recovery following ischaemia-reperfusion, but this was not associated with re-balanced substrate metabolism, as glycolysis remained unchanged,

and fatty acid oxidation was increased following DMOG treatment (Fig. 6.2). Due to DMOG's widely acknowledged lack of potency and selectivity, this hypothesis was tested once more, using the same model of ischaemia-reperfusion, using a clinically-developed compound, Molidustat (Chapter 5). Molidustat treatment did lead to increased glycolysis, as well as decreased fatty acid oxidation, associated with improved functional recovery following ischaemia-reperfusion, corroborating the hypothesis that pre-treatment with a HIF-1 α -stabilising compound could protect the diabetic heart from damage and functional impairment in ischaemic stress (Fig. 6.2).

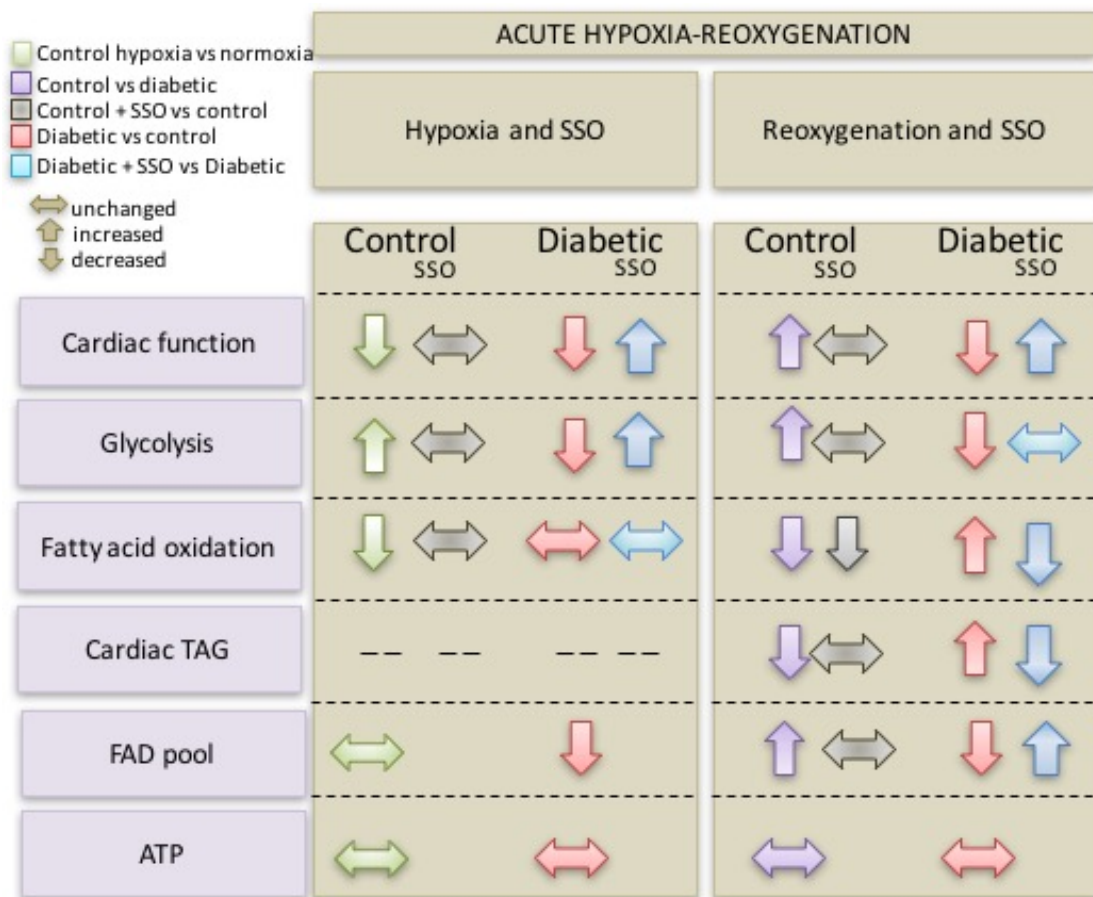


Figure 6.1: Summary of findings with fatty acid uptake inhibitor SSO in acute hypoxia and reoxygenation. Effects of SSO during acute hypoxia and following reoxygenation in control and diabetic rat hearts.

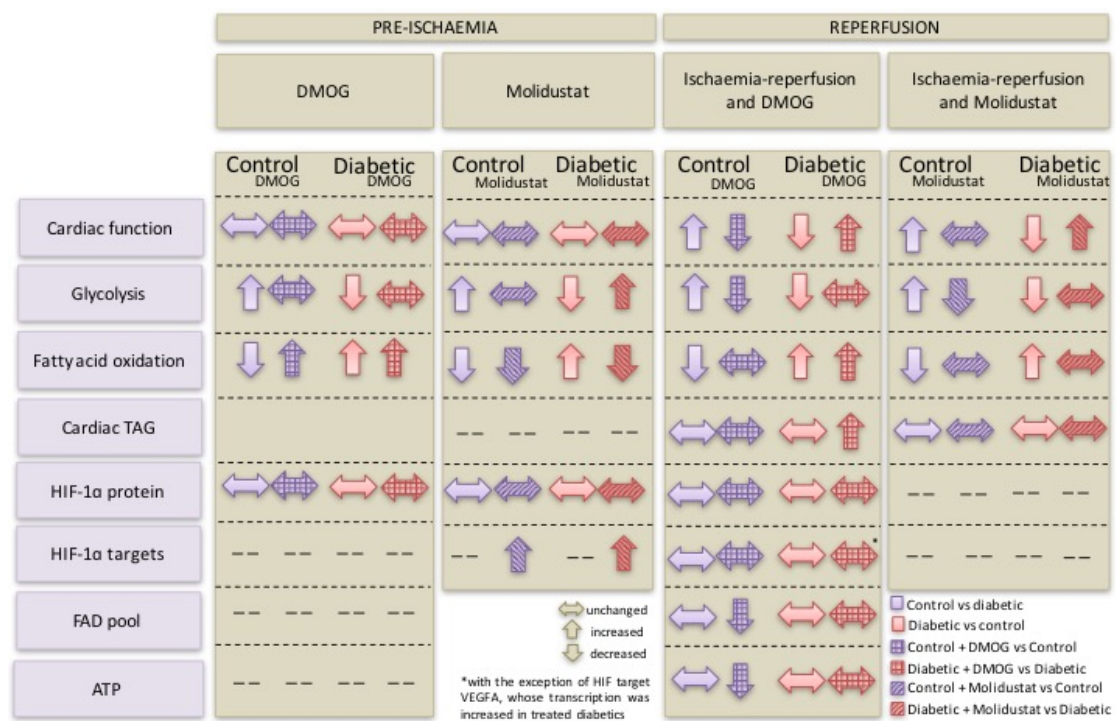


Figure 6.2: Summary of findings with chronic PHD inhibitor treatments. Effects of PHD inhibitors DMOG and Molidustat at baseline (pre-ischaemia) and following ischaemia-reperfusion in control and diabetic rat hearts.

6.2 Study limitations

This work provides a valuable contribution to the study of the type 2 diabetic heart, but it is important to note the limitations of the study designs, which influence how these data can be interpreted.

It must be noted, for instance, that the majority of results shown are derived from *ex vivo* experiments, using Langendorff isolated heart perfusion. This implies three key limitations. Firstly, the biological processes (namely, metabolism and cardiac function) studied are not as physiologically relevant as would be those observed in an *in vivo* setting, such as an MI model, which could be performed in the same animal model. This may have implications on the clinical translatability of the studies, though it still provides valuable insight into the response of the heart to an acute stress, and provides real-time information of both cardiac function and metabolism, at a lower financial cost and higher consistency between cohorts. Secondly, the Langendorff perfusion itself is a retrograde perfusion, which does not require the heart to perform the amount of hydraulic work that a working heart perfusion would. Therefore, it is not as physiologically translatable, as the metabolic load is not as comparable to an *in vivo* setting. Nevertheless, the hearts were perfused at constant pressure, and the balloon inserted into the left ventricle was inflated to provide a diastolic pressure of 4 – 8 mmHg, to provide workload conditions as translatable as possible. Finally, the perfusion apparatus was arranged to re-circulate the perfusate throughout the protocol. This allowed for the measurement of metabolic rates, but also provided a risk of accumulation of lactate in the circulating buffer, which may have implications for the heart's substrate use.

Two additional limitations relating to the perfusion conditions should be considered. One is the substrate composition of the re-circulating buffer, which included glucose and palmitate, but did not include additional substrates, and therefore limited the availability of physiologically-relevant substrates. The second limitation

is the use of [5- ^3H]-glucose and [9,10- ^3H] palmitate as radioactive tracers for the measurement of glycolytic rate and palmitate oxidation rate. The fact that both tracers are ^3H -labelled requires perfusions to be done separately for each substrate, which prevents the ability to get parallel measures of both metabolic pathways in the same heart. This could be achieved by using a ^{14}C -labelled substrate and a ^3H -labelled substrate, however this would require complete restructuring of the perfusion apparatus, and additional safety clearance due to the nature of the isotope. Additionally, these tracers are associated with a risk of overestimating the flux through these pathways, as the hydrogen labels can be converted to $^3\text{H}_2\text{O}$ at non-specific steps, and, in the case of glucose, in the pentose phosphate pathway and hexosamine biosynthetic pathway [75, 121]. This may be particularly problematic in the context of diabetes, where there is evidence for increased activity of the hexosamine pathway [50]. Nevertheless, these tracers do provide comparable measures of metabolic rates, and have been consistent over several studies, giving us confidence in the observed changes.

A further limitation of the presented work is that hearts from Chapter 4 were all freeze-clamped at the end of reperfusion, which did not allow assessment of the effects of DMOG treatment in tissue during ischaemia without the confounding factor of reperfusion. This also prevented the comparison of metabolomics analysis between hypoxic tissue and ischaemic tissue. It would be of interest to perform metabolomics analysis in ischaemic tissue following DMOG treatment.

Finally, the rat model of type 2 diabetes has its limitations, and it is apparent that the severity of diabetes was not consistent across studies. For instance, the levels of plasma NEFA were not significantly raised in diabetics in Chapter 5, while they had shown increased concentrations in previous chapters. The high fat-fed, low-dose streptozotocin model was chosen as it provides insight into earlier stages of disease progression compared with other, more severe models in which metabolic intervention could be more difficult to assess. However, the consistency of the model is important, and in future it would be beneficial to validate each cohort as early as possible in the study, to exclude any animals that do not match

the expected diabetic phenotype. In the case of plasma NEFA levels, however, it appears that these were higher in controls compared with previous cohorts, which could also be due to a different limitation of the study design, where this cohort was used during summer months only, and therefore may have experienced a different light/dark cycle to previous cohorts due to the housing facilities on site not having adequate light isolation.

6.3 Discussion of findings

Increased plasma NEFA was found in diabetics in both Chapters 3 and 4, and increased fatty acid oxidation rate was found in diabetics across all Chapters. This was associated with impaired functional recovery in both hypoxia-reoxygenation, and ischaemia-reperfusion (Fig. 6.1 and 6.2). Previous studies support that increased lipid metabolism is associated with decreased cardiac efficiency and post-ischaemic functional impairment [29, 78, 97]. However, the question of whether the diabetic heart retains any metabolic flexibility is still controversial. In the work presented here, decreased baseline glycolytic rates were found in all diabetic hearts. However, in Chapter 3, diabetic hearts showed increased glycolysis in response to hypoxia, but were unable to upregulate glycolysis to the same level as control hearts. This clarified that the lower baseline rates were not the maximal rate available to diabetics, as they were still able to upregulate this significantly in response to hypoxia. In fact, the fold increase in glycolytic rate was similar between controls and diabetics, though the absolute rate was lower in diabetic hearts. In Chapter 4, diabetic hearts displayed similarly low glycolytic rates at baseline, but were able to upregulate glycolysis to the same level as control hearts in response to ischaemia. This may be due to the presence of insulin in the perfusate used in Chapter 4, which was not used in acute hypoxic perfusions in Chapter 3. Together, these results suggest that the diabetic heart retains some responsiveness to insulin, which is in agreement with other studies [79], and to hypoxia as well, which contradicts the generally-accepted notion that the diabetic heart is metabolically inflexible. This also highlights that the diabetic model used in this thesis is representative of early-stage type 2 diabetes, showing hyperglycaemia and increased adiposity, without compromising insulin secretion [137].

What is also apparent is that control hearts in Chapter 3 showed a greater upregulation of glycolysis in response to hypoxia, compared to control hearts in

Chapter 4, in response to ischaemia. This does not appear to be due to the presence of insulin, as the baseline rates are comparable across the two groups, but may in fact be a result of the differing characteristics of hypoxia and ischaemia, whereby substrate continues to be freely available in hypoxia, while ischaemia restricts both oxygen and substrate availability, limiting glucose delivery and preventing a greater increase in glucose uptake and utilisation. For this reason, ischaemia was chosen for all future studies, as it provides a more clinically-relevant model of MI.

Having found that there was an accumulation of TAG in the diabetic heart following hypoxia-reoxygenation, but not following ischaemia-reperfusion, and that SSO prevented this accumulation while DMOG resulted in increased accumulation in diabetics, the question of whether TAG accumulation is deleterious or beneficial must be raised. The question of lipotoxicity has caused much debate among the field. There has been much concern over the risk of increased TAG accumulation in the diabetic heart and its functional consequences [222, 64, 184]. However, it could be argued that TAG themselves are not detrimental, and that an increased TAG pool in the diabetic heart, provides a useful energy source when substrate availability is low. However, though the TAG themselves are likely to be harmless to the myocardium, it is the generation of other lipid types is likely to be detrimental. In fact, it has been shown that the accumulation of TAG under increased oleate concentration was well tolerated, while excess palmitate led to increased apoptosis, with no increase in TAG pool [124]. It appears that it is not TAG themselves that cause lipotoxicity, but other lipid forms that can cause severe cytotoxicity, and the risk is then that the diabetic heart may more easily convert TAG into toxic lipid types. Ceramide, in particular, is believed to be a cytotoxic lipid, as it can induce apoptotic signals via iNOS upregulation [19]. Therefore, the present study would benefit from investigating the levels of lipid types other than TAG in the diabetic heart, as well as following therapeutic intervention, to give a better overview of the benefits of metabolic therapies on lipotoxicity.

Pre-treatment with 40 mg/kg DMOG on three alternate days over the course of a week was found to result in increased fatty acid oxidation rates in both control and

diabetic hearts, with no significant effect on glycolytic rate (Chapter 4). Surprisingly, this was associated with improved functional recovery in treated diabetic hearts, and impaired recovery in treated control hearts. This unexpected finding brings to mind the poorly understood phenomenon known as the "obesity paradox", whereby in a number of studies, patients who are overweight and obese show better survival rates than lean or very lean participants [80]. Specifically in diabetes, a study found that within participants diagnosed with T2DM and cardiovascular co-morbidity, those that were overweight and obese had lower mortality compared with patients with normal weight [45]. Furthermore, in that study, weight gain rather than weight loss, was associated with decreased mortality and morbidity [45]. Though the obesity paradox has been classically described in human studies at the whole-body level, it may still be valid at the tissue level, and could help explain the differing effects observed in control and diabetic hearts following DMOG treatment. This could be explained, in part, by the fact that the diabetic heart has undergone long-term metabolic adaptations, resulting in highly upregulated machinery to process fatty acids, including enzymes involved in fatty acid uptake and oxidation [65]. Control hearts were simply not used to handling increased levels of fatty acids.

The work described in Chapter 4 and Chapter 5 highlights the importance of good drug development for both appropriate hypothesis testing, and prevention of undesirable off-target effects. DMOG treatment has been previously shown to lead to HIF-1 α stabilisation, but few studies have used the compound *in vivo*. Additionally, due to its close similarity with α -ketoglutarate, it has been criticised for its poor selectivity, and risk of off-target effects. This is mainly due to the fact that DMOG is a small-molecule α -ketoglutarate analogue, which does not have any additional chemical groups to stabilise the interaction specifically with PHD enzymes, rendering it poorly selective and likely to target other α -ketoglutarate-dependent dioxygenases. Conversely, triazole-based inhibitors such as IOX4, have been shown to be far more potent, compared with DMOG, and display significantly higher selectivity for prolyl hydroxylase domain-containing proteins (PHDs) [32]. Molidustat is a triazole-based PHD inhibitor, closely related to IOX4, and has been

shown to have similar potency to IOX4 in antibody-based, as well as cell-based assays [214], and has been well characterised *in vivo*, showing significant upregulation of HIF-1 signalling [66]. Together, these factors may explain the differing effects observed in both control and diabetic rats following treatment with these two compounds (Fig. 6.2). These divergent observations have highlighted the need for potent compounds to be developed in order to investigate the potential of metabolic modulation in the diabetic heart. Fatty acid oxidation rates were differentially modulated by DMOG treatment and Molidustat treatment (Chapters 4 and 5). This is likely to be explained by DMOG exhibiting off-target effects, and Molidustat having a potent HIF-activating effect. While Molidustat treatment appears to have led to significant systemic upregulation of downstream HIF targets, and resulted in the expected decrease in fatty acid oxidation pre-ischaemia in diabetic hearts, DMOG treatment did not show evidence of HIF activation, and resulted in increased fatty acid oxidation rates pre-ischaemia. As discussed in Chapter 4, this may be partly explained by an effect on AMPK activation, and ACACA phosphorylation. If, as we suspect, ACACA phosphorylation has been increased by DMOG treatment, this would inhibit the enzyme and result in increased fatty acid oxidation, which would explain the metabolic rates found in Chapter 4.

Molidustat treatment allowed us to demonstrate the benefit of HIF-stabilising treatment on cardiac outcomes following ischaemia-reperfusion (Chapter 5). This was clearly associated with metabolic benefits, including a significant decrease in fatty acid oxidation, and increased glycolysis. These metabolic changes are precisely what was expected with PHD inhibition, and support a HIF-activating mechanism of action of Molidustat treatment.

There is some concern as to whether HIF-activating treatment may cause detrimental effects long term [83], and treatment with the clinically-trialed PHD inhibitor Vadastutat led to several systemic side effects, as well as three deaths, in the treatment arm of a recent clinical trial [160]. However, it is worth noting that these observations have been reported in studies not related to diabetes, and it could be argued that HIF-activating strategies may be most beneficial in the context of

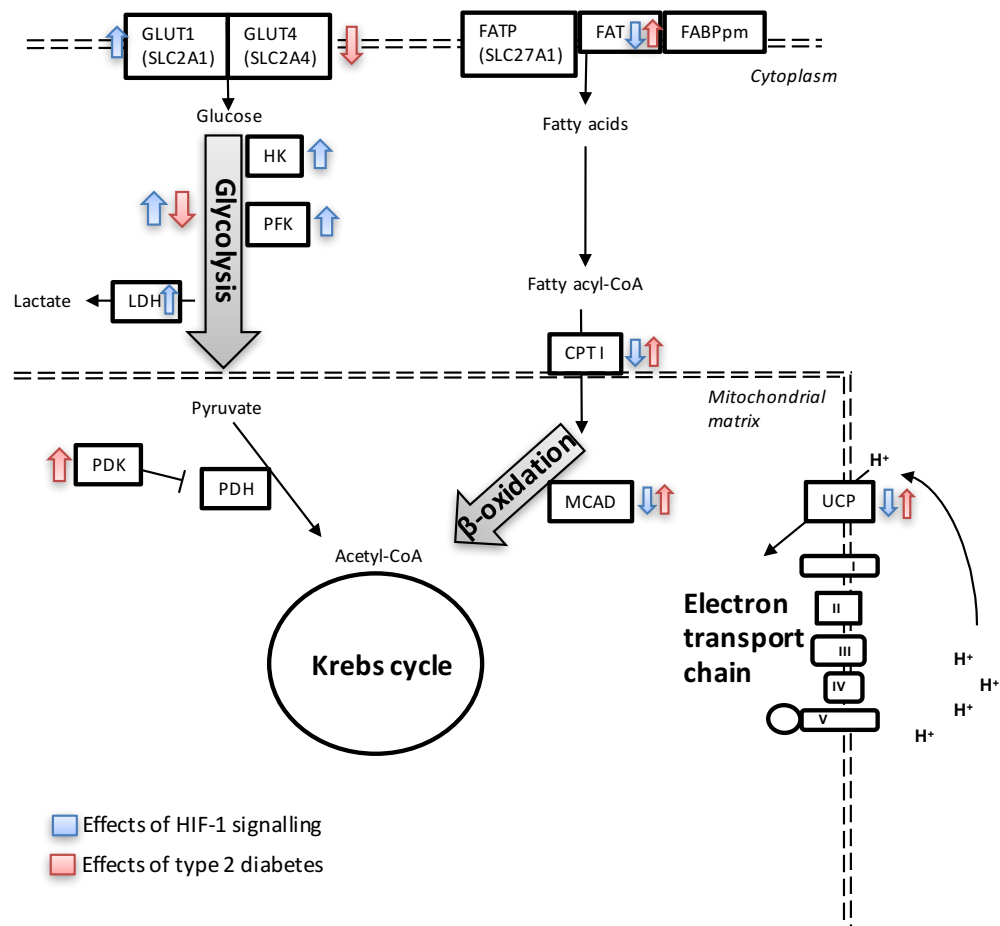


Figure 6.3: The opposing effects of type 2 diabetes (red) and HIF-1 signalling (blue) on cellular metabolism.

diabetes, where there is a significant upregulation of machinery opposing the effect of HIF signalling (Fig. 6.3). Whereas in the setting of heart failure, for instance, the heart is already primed to upregulate glycolysis and downregulate fatty acid oxidation, and HIF-activating treatment may not be of benefit in such a disease setting. Hypoxia mimetics developed for use in the treatment of anaemia have received interest in the context of ischaemia, but have thus far not been investigated in diabetes, where we argue they would be of most benefit. Nevertheless, it is important that investigation into the repurposing of available inhibitors into cardiovascular diseases and diabetes is done on a compound-specific basis, with careful assessment of individual compounds, as there may be a risk with chronic treatment. In the present study, we have shown beneficial effects within this short-term chronic treatment, however longer-term treatment regimens may show

different effects. Prolongued activation of hypoxic signalling will have a number of effects on the myocardium, including the possibility of decreased cytochrome oxidase activity, potentially leading to a change in redox state and the risk of increased production of reactive oxygen species [51].

Interestingly, the PHD inhibitor Daprodustat has recently entered Phase II for the treatment of perioperative ischaemia and diabetic foot ulcers (Clinical Trial NCT03153007), suggesting that the potential of HIF-activating treatment in diabetes has begun to receive attention. Biopsies of foot ulcers from diabetic patients have previously shown decreased HIF-1 α levels compared with venous ulcers not exposed to hyperglycaemia, but which share a similarly hypoxic environment [31]. However, it is important to acknowledge that diabetic complications include cases of uncontrolled small-vessel proliferation, such as ocular neovascularization, where HIF-1 α appears to play a role [215], and it is vital that HIF-activating treatment be carefully targeted to prevent further complications to these co-morbidities.

In this thesis, the use of novel metabolic modulating approaches in the diabetic heart has been investigated, identifying the use of fatty acid uptake inhibition and PHD inhibition as potential therapeutic avenues. Nevertheless, it would be important to ensure cardiac selectivity of any compounds that are investigated further, as well as appropriate potency and enzyme selectivity, to prevent undesirable off-target effects. Following promising results using fatty acid uptake inhibitor SSO, it would be valuable to perform further studies *in vivo*, and to assess cardiac function using cine MRI to better characterise the effects of this compound on the heart. Similarly, these studies would be particularly interesting using Molidustat, to obtain further information on the functional and structural changes that may be underlying the improvement in function observed, as well as differences between control and diabetic hearts following treatment. The work presented here provides a relevant basis for the development and characterisation of metabolic therapies for the diabetic heart, and further high throughput screens should be performed to identify potential drugs that may exploit the treatment strategies discussed in this thesis.

Appendices

*The heart of animals is the foundation of their life,
the sovereign of everything within them, the sun of
their microcosm, that upon which all growth depends,
from which all power proceeds.*

— William Harvey, 1628



Content specifications of the rodent diets

A.1 Content specifications of the standard chow

2016

+++
ENVIGO

Teklad Global 16% Protein Rodent Diet

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

Ingredients (in descending order of inclusion)- Ground wheat, ground corn, wheat middlings, corn gluten meal, calcium carbonate, dicalcium phosphate, soybean oil, brewers dried yeast, iodized salt, L-lysine, DL-methionine, choline chloride, magnesium oxide, vitamin E acetate, menadione sodium bisulfite complex (source of vitamin K activity), manganous oxide, ferrous sulfate, zinc oxide, niacin, calcium pantothenate, copper sulfate, pyridoxine hydrochloride, riboflavin, thiamin mononitrate, vitamin A acetate, calcium iodate, vitamin B₁₂ supplement, folic acid, biotin, vitamin D₃ supplement, cobalt carbonate.

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

Teklad Diets are designed and manufactured for research purposes only.



© 2015 Envigo

Standard Product Form: Pellet

Vitamins		
Vitamin A ^{e, f}	IU/g	15.0
Vitamin D ₃ ^{e, g}	IU/g	1.5
Vitamin E	IU/kg	110
Vitamin K ₃ (menadione)	mg/kg	50
Vitamin B ₁ (thiamin)	mg/kg	17
Vitamin B ₂ (riboflavin)	mg/kg	15
Niacin (nicotinic acid)	mg/kg	75
Vitamin B ₆ (pyridoxine)	mg/kg	18
Pantothenic Acid	mg/kg	33
Vitamin B ₁₂ (cyanocobalamin)	mg/kg	0.08
Biotin	mg/kg	0.40
Folate	mg/kg	4
Choline	mg/kg	1030
Fatty Acids		
C16:0 Palmitic	%	0.5
C18:0 Stearic	%	0.1
C18:1ω9 Oleic	%	0.7
C18:2ω6 Linoleic	%	2.0
C18:3ω3 Linolenic	%	0.1
Total Saturated	%	0.6
Total Monounsaturated	%	0.7
Total Polyunsaturated	%	2.1
Other		
Cholesterol	mg/kg	—

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

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

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

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

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

^f 1 IU vitamin A = 0.3 µg retinol

^g 1 IU vitamin D = 25 ng cholecalciferol

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

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

0915

Teklad Diets + Madison WI + envigo.com + tekkladinfo@envigo.com + (800) 483-5523

A.2 Content specifications of the high fat diet



DIET FORMULATION AND SPECIFICATION DATA

BASIC DIET INFORMATION:

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

CALCULATED ANALYSIS:

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

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

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

[illegible]

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

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

B

Reagent specifications

Table B.1: Primary antibodies used for Western blotting. *MCAD and UCP3 antibodies used for samples from non-perfused hearts due to the previously used antibodies being discontinued by the provider.

Target protein	Source	Dilution
AMPK (total)	Cell Signaling (# 2603)	1:1000
FAT/CD36	private donation	1:50000
GLUT1 (SLC2A1)	Abcam (ab652)	1:1000
GLUT4 (SLC2A4)	private donation	1:12500
FABPpm	private donation	1:1000
HIF-1 α	Cayman chemicals (10006421)	1:500
MCAD (ACADM)	Santa Cruz (sc-49047)	1:1000
NaKATPase	private donation	1:200
Phospho-ACC (ACACA)	Cell Signaling (# 3661)	1:1000
Phospho-AMPK	Cell Signaling (# 2535)	1:1000
PDK4	Abgent (AP7041b)	1:2000
rab4	BD Biosciences (610888)	1:2500
UCP3	Abcam (ab3477)	1:2500
MCAD (ACADM)*	Abcam (ab92461)	1:5000
UCP3*	Abcam (ab180643)	1:1000

Table B.2: Primer sequences used for qPCR experiments.

Target	Primer sequence
HMOX1	Fp: GTC-AGG-TGT-CCA-GGG-AAG-G
HMOX1	Rp: CTC-TTC-CAG-GGC-CGT-ATA-GA
VEGFA	Fp: CAA-GCC-AAG-GCG-GTG-AGC-CA
VEGFA	TCT-GCC-GGA-GTC-TCG-CCC-TC
PPAR α	Fp: ACG-ATG-CTG-TCC-TCC-TTG-ATG
PPAR α	Rp: ACG-ATG-CTG-TCC-TCC-TTG-ATG
PHD3	Fp: CTA-TGT-CAA-GGA-GCG-GTC-CAA
PHD3	Rp: GTC-CAC-ATG-GCG-AAC-ATA-ACC

C

Publications

Inhibition of sarcolemmal FAT/CD36 by sulfo-*N*-succinimidyl oleate rapidly corrects metabolism and restores function in the diabetic heart following hypoxia/reoxygenation

Latt S. Mansor^{1†}, Maria da Luz Sousa Fialho^{1†}, Georgina Yea¹, Will A. Coumans², James A. West³, Matthew Kerr¹, Carolyn A. Carr¹, Joost J.F.P. Luiken², Jan F.C. Glatz², Rhys D. Evans¹, Julian L. Griffin³, Damian J. Tyler¹, Kieran Clarke¹, and Lisa C. Heather^{1*}

¹Department of Physiology, Anatomy and Genetics, University of Oxford, Parks Road, Oxford, OX1 3PT, UK; ²Department of Molecular Genetics, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University, Maastricht, The Netherlands; ³Department of Biochemistry, University of Cambridge, and MRC Human Nutrition Research, Cambridge, UK

Received 21 October 2016; revised 16 January 2017; editorial decision 22 February 2017; accepted 23 March 2017

Time for primary review: 62 days

Aims

The type 2 diabetic heart oxidizes more fat and less glucose, which can impair metabolic flexibility and function. Increased sarcolemmal fatty acid translocase (FAT/CD36) imports more fatty acid into the diabetic myocardium, feeding increased fatty acid oxidation and elevated lipid deposition. Unlike other metabolic modulators that target mitochondrial fatty acid oxidation, we proposed that pharmacologically inhibiting fatty acid uptake, as the primary step in the pathway, would provide an alternative mechanism to rebalance metabolism and prevent lipid accumulation following hypoxic stress.

Methods and results

Hearts from type 2 diabetic and control male Wistar rats were perfused in normoxia, hypoxia and reoxygenation, with the FAT/CD36 inhibitor sulfo-*N*-succinimidyl oleate (SSO) infused 4 min before hypoxia. SSO infusion into diabetic hearts decreased the fatty acid oxidation rate by 29% and myocardial triglyceride concentration by 48% compared with untreated diabetic hearts, restoring fatty acid metabolism to control levels following hypoxia-reoxygenation. SSO infusion increased the glycolytic rate by 46% in diabetic hearts during hypoxia, increased pyruvate dehydrogenase activity by 53% and decreased lactate efflux rate by 56% compared with untreated diabetic hearts during reoxygenation. In addition, SSO treatment of diabetic hearts increased intermediates within the second span of the Krebs cycle, namely fumarate, oxaloacetate, and the FAD total pool. The cardiac dysfunction in diabetic hearts following decreased oxygen availability was prevented by SSO-infusion prior to the hypoxic stress. Infusing SSO into diabetic hearts increased rate pressure product by 60% during hypoxia and by 32% following reoxygenation, restoring function to control levels.

Conclusions

Diabetic hearts have limited metabolic flexibility and cardiac dysfunction when stressed, which can be rapidly rectified by reducing fatty acid uptake with the FAT/CD36 inhibitor, SSO. This novel therapeutic approach not only reduces fat oxidation but also lipotoxicity, by targeting the primary step in the fatty acid metabolism pathway.

Keywords

Metabolism • Energy • Fatty acid • Hypoxia • Glucose

* Corresponding author. Tel: +44 1865 282048; fax: +44 1865 282272, E-mail: lisa.heather@dpag.ox.ac.uk

† The first two authors contributed equally to the study.

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1. Introduction

To power contraction the heart requires a constant supply of ATP, which can be obtained from a range of metabolic fuels. Metabolism of fatty acids produces more ATP per carbon atom but requires more oxygen per ATP generated, when compared with glucose. In type 2 diabetes, the heart becomes metabolically abnormal, oxidizing more fatty acids, and less glucose to meet its energy requirements, and this is associated with higher myocardial oxygen consumption.^{1–7} The primary step in myocardial fatty acid metabolism is fatty acid import into the cardiomyocyte, which is regulated by a family of sarcolemmal fatty acid transporters. Fatty acid translocase (FAT/CD36), the predominant fatty acid transporter, is increased in diabetes,⁸ consequently more lipid is imported into the diabetic heart fuelling increased fatty acid oxidation, lipid storage and, via the Randle effect, suppressing glucose metabolism.^{9,10}

Strategies that modify fatty acid metabolism are of great interest for the diabetic heart. Compounds such as ranolazine, etomoxir, perhexiline, and trimetazidine, which target mitochondrial fatty acid oxidation, have been investigated predominantly in heart failure and angina.^{11–15} However, one concern with these mitochondrial fatty acid oxidation inhibitors is that entry of fat into the cardiomyocyte can still continue, and additional fat may accumulate as lipid within the cytosol, as reported in diabetic rats following chronic etomoxir treatment.¹⁶ Thus, a better approach would be to block fatty acid metabolism at its primary point, at the level of the sarcolemmal fatty acid uptake, by inhibiting FAT/CD36. Genetic studies with FAT/CD36-deficient mice showed particular benefit when crossed with PPAR α -overexpressing mice prone to lipotoxic cardiomyopathy, as loss of FAT/CD36 reduced lipid accumulation and reversed contractile dysfunction.¹⁷ However, to exploit this genetic knowledge, therapeutic strategies that pharmacologically inhibit FAT/CD36 are needed.

Metabolic dysfunction in diabetes is regulated by complex mechanisms relating to increased fatty acid availability and insulin resistance. Metabolic changes in the diabetic heart become of greater consequence under conditions of cardiac stress, e.g. when oxygen is restricted. Published data suggest that the diabetic heart has limited metabolic flexibility to change metabolism in response to stimuli,^{18,19} and decreased mitochondrial energetics in response to anoxia.^{20,21} To acutely adapt to low oxygen availability the heart must downregulate fatty acid metabolism and increase anaerobic glycolysis, mediated in part by AMP-activated protein kinase (AMPK) activation.²² Interestingly, patients with type 2 diabetes have a number of hypoxia-associated complications, resulting in the heart being exposed, locally or systemically, to reduced oxygen supply.^{23–25} Therefore, understanding whether metabolic flexibility in diabetes in response to hypoxic stress is abnormal, and if it can be reinstated by pharmacological agents that suppress fatty acid uptake, is of interest.

Given the accessible location of FAT/CD36 on the extracellular surface of the cardiomyocyte, and its elevated sarcolemmal localization in the diabetic heart, FAT/CD36 may be an attractive target for metabolic intervention, as it may simultaneously reduce both fatty acid oxidation and lipid deposition. In this study, we investigate the FAT/CD36 inhibitor, sulfo-*N*-succinimidyl oleate (SSO) as a metabolic modulator, to study its effects in the intact heart of the type 2 diabetic rat. We questioned whether inhibition of FAT/CD36 using SSO may provide a novel pharmacological way to improve metabolism and function of the diabetic heart challenged by hypoxic stress.

2. Methods

2.1 *In vivo* rat model of type 2 diabetes

Male Wistar rats ($n = 71$, starting body weight ~ 250 g) were obtained from a commercial breeder (Harlan, UK). This investigation conformed to the United Kingdom Home Office guidelines under The Animal (Scientific Procedures) Act, 1986, and was approved by the University of Oxford local ethics committee.

Control rats were fed for 42 days on a standard chow diet (Harlan Laboratories). Type 2 diabetes was induced in rats by feeding a high-fat diet (60% calories from fat, Special Diet Services) for 42 days and, on day 14, a single low dose intraperitoneal injection of streptozotocin (STZ, 25 mg/kg bodyweight, in citrate buffer, pH 4)^{7,26–28} was given. We have previously demonstrated that it is the combination of high-fat diet and low dose STZ that induces type 2 diabetes,²⁷ as low dose STZ alone does not impair insulin secretion, which is in contrast to type 1 diabetes models where a high dose of STZ is used to destroy the β -cells. This model of type 2 diabetes was chosen over others available as it induces hyperinsulinaemia, mild hyperglycaemia, adiposity and diastolic dysfunction,^{7,26,27} thereby mirroring the human disease, yet avoiding extreme hyperglycaemia and genetic manipulation present in other models.²⁹ On day 40, control and diabetic rats had fasting blood collected from the saphenous vein to confirm hyperinsulinaemia and hyperglycaemia (blood glucose measured by Accu-check Aviva blood glucose testing system and plasma insulin concentrations measured by ELISA, R&D systems). On day 42, rats were terminally anaesthetized using an intraperitoneal injection of sodium pentobarbital (150 mg/kg bodyweight, Euthatal). Hearts were excised for perfusion and blood was collected for analysis of plasma non-esterified fatty acids (NEFA).

2.2 Isolated heart perfusion

Hearts were perfused at a constant pressure of 100 mmHg and an end-diastolic pressure of 4–8 mmHg. Hearts were perfused with Krebs-Henseleit buffer containing 11 mM glucose and 1.5% (w/v) fatty acid-free bovine serum albumin bound to 0.4 mM palmitate at 37 °C. Hearts were initially perfused in normoxia for 20 min by gassing the buffer with 95% O₂ and 5% CO₂ to give an oxygen partial pressure (P_{O_2}) of 413 ± 15 mmHg. Following normoxia, hearts were made acutely hypoxic by replacing the gas with 95% N₂ and 5% CO₂ to decrease the P_{O_2} to 90 ± 10 mmHg for 32 min, followed by reoxygenating the buffer with 95% O₂ and 5% CO₂ for a further 20 min. Cardiac function was measured using a fluid-filled PVC balloon inserted into the left ventricle, attached to a bridge amplifier and PowerLab data acquisition system (ADInstruments). Left ventricular developed pressure was determined as peak systolic minus end-diastolic pressure, and rate pressure product (RPP) was the multiple of developed pressure and heart rate. Functional data were averaged over the last 5 min at the end of normoxia, hypoxia, and reoxygenation.

2.3 Sulfo-*N*-succinimidyl oleate

N-hydroxysulfosuccinimide sodium salt was synthesized according to the protocol of Staros.³⁰ *N*-hydroxysulfosuccinimide sodium salt (2 mM), dicyclohexylcarbodiimide (2.2 mM), and oleic acid (4 mM) were dissolved in dimethylformamide overnight at room temperature, followed by stirring for 3 h at 4 °C. The product was filtered, washed three times with dimethylformamide, and sulfo-*N*-succinimidyl oleate (SSO) was precipitated from the solution using ~ 20 volumes of ethyl acetate, filtered, and stored under vacuum in a desiccator. Following optimization

of dose and time to effect, a final concentration of 0.5 mM SSO (dissolved in 200 μ L DMSO) was added to the perfusion buffer. SSO was added 4 min prior to the induction of hypoxia and recirculated throughout the hypoxia-reoxygenation period. Hearts perfused with SSO were freeze-clamped at the end of reoxygenation.

2.4 Metabolic rates and tissue analysis

For measurement of glycolytic rates, buffer was supplemented with 0.2 μ Ci.mL⁻¹ [5-³H]-glucose.³¹ For measurement of palmitate oxidation rates, buffer was supplemented with 0.2 μ Ci.mL⁻¹ [9,10-³H] palmitate.³¹ Lactate efflux rates were measured in timed aliquots using lactate dehydrogenase. Metabolic rates were calculated during normoxia, hypoxia and reoxygenation, from perfusate samples collected at 4-min intervals for a minimum of five linear data points. In three separate groups, hearts were freeze-clamped on the cannula at the end of normoxia, following normoxia-hypoxia, or following normoxia-hypoxia-reoxygenation. Glycogen content was determined by the conversion of glycogen to glycosyl units, which were assayed using amyloglucosidase. Triglyceride content was measured following Folch extraction.³² Pyruvate dehydrogenase (PDH) activity was measured in the active form according to the protocol of Seymour and Chatham.³³ PDH activity was measured as a surrogate marker for glucose oxidation, as this pathway could not be measured with the ³H-glucose label.

2.5 Metabolomics

In brief, metabolites were extracted using methanol/chloroform from ~50 mg wet weight of powdered, frozen heart tissue.³⁴ The organic and aqueous fractions were separated by the addition of water and chloroform. The aqueous layer was analysed subsequently for metabolomics. Mass spectrometry was performed on an AB Sciex 5500 (Warrington, UK) coupled to an Acquity ultra performance liquid chromatography (UPLC) system from Waters Ltd (Atlas Park, Manchester, UK). Part of the aqueous extract was dissolved in 70:30 acetonitrile: water containing 20 μ M deoxy-glucose 6 phosphate and 20 μ M [U-¹³C,¹⁵N] glutamate as internal standards. For chromatography of nucleotides the strong mobile phase was 100mM ammonium acetate, the weak mobile phase was acetonitrile and the LC column used was the ZIC-HILIC column from Sequant (100 $\times$ 2.1 mm, 5 μ m). Metabolites were quantified using pre-defined mass transitions on the mass spectrometer in positive ion mode. For the analysis of Krebs cycle intermediates the sample from the above analysis was recovered and analysed in a second UPLC chromatography assay with the mass spectrometer in negative ion mode. The strong mobile phase was 10 mM ammonium acetate with 0.05% ammonium hydroxide and the weak mobile phase was acetonitrile, and the LC column used was a BEH amide HILIC column (100 $\times$ 2.1 mm, 1.7 μ m; Waters Ltd). Compound specific parameters such as cone voltage, collision energy, and mass transitions were used for quantification of the metabolites. For the analysis of amino acids, the remaining aqueous sample was dried under nitrogen and derivatized with 3M HCl in BuOH for 15 min at 65°C. After further drying, the sample was reconstituted in 9:1 0.1% formic acid in water/acetonitrile. For chromatography, the strong mobile phase used for analysis was acetonitrile and the weak mobile phase was 0.1% formic acid in water, and a HSS T3 column (100 $\times$ 2.1 mm, 1.7 μ m) from Waters Ltd was used. Again, cone voltage, collision energy and mass transitions were optimized for each metabolite for quantification. Data are expressed in arbitrary units as the area ratio of metabolite peak relative to the internal standard, normalized to the protein content. In total, 76 metabolites were identified and quantified per heart.

2.6 Western blotting

For western blotting, protein lysates were prepared from frozen tissue, protein was loaded onto SDS-PAGE gels and separated by electrophoresis.³⁵ Even protein loading and transfer were confirmed by normalizing to loading controls, either cyclophilin B, or ponceau staining. For measurement of sarcolemmal transporters, separation of the sarcolemmal membrane fraction from intracellular endosomes was carried out according to the established method of Luiken *et al.*³⁶ Differential centrifugation was used to separate the membrane fractions, and the different fractions were analysed by western blotting for FAT/CD36 and GLUT4. As described previously, sarcolemmal fractions had three-fold enrichment of Na⁺K⁺-ATPase protein levels, whereas microsomal fractions had nine-fold enrichment of Rab4 protein levels.³⁷ Bands were quantified using UN-SCAN-IT gel software (Silk Scientific, USA), and all samples were run in duplicate on separate gels to confirm results.

2.7 Statistics

Results, presented as means $\pm$ SEM, were considered significant at $P < 0.05$ (SPSS Statistics 18 and GraphPad Prism). Data are presented as scatter graphs, with n numbers displayed on graphs. Baseline function and metabolism were compared between control and diabetic hearts using an unpaired t-test, and characterization of the SSO effect in control hearts were compared by paired t-test. Metabolomic data were compared using a two-way ANOVA (two factors being diabetic status and oxygen level). Cardiac function and metabolism with SSO treatment were compared within the same oxygenation status, using a two-way ANOVA (two factors being diabetes and drug). *Post-hoc* comparisons were made using Tukey test with corrections made for multiple comparisons.

3. Results

3.1 Characterization of type 2 diabetic rats

There were no significant differences in body weights (377 $\pm$ 7 g control vs. 393 $\pm$ 9 g diabetic rats) or in heart weights (1.40 $\pm$ 0.03 g control vs. 1.46 $\pm$ 0.03 g diabetic rats) between groups. Epididymal fat pad weight, a marker of adiposity, was increased by 64% in diabetic rats compared with controls (5.3 $\pm$ 0.3 g vs. 8.7 $\pm$ 0.5 g in control and diabetic rats, respectively, $P < 0.05$). Fasting blood glucose concentrations were increased by 26% from 5.1 $\pm$ 0.3 mmol/L in control rats to 6.4 $\pm$ 0.6 mmol/L in diabetic rats ($P < 0.05$), and fasting plasma insulin concentrations were increased 2.7-fold from 59 $\pm$ 5 pmol/L in control rats to 163 $\pm$ 29 pmol/L in diabetic rats ($P < 0.05$). Plasma NEFA concentrations in the fed state were increased by 83% from 0.06 $\pm$ 0.01 mmol/L in control rats to 0.11 $\pm$ 0.01 mmol/L in diabetic rats ($P < 0.05$).

There were no differences in contractile function in type 2 diabetic hearts compared with control hearts during baseline normoxic perfusion (Figures 1 and 6). Glycolytic rate was 53% lower and myocardial glycogen concentration was decreased by 32% in diabetic hearts compared with controls (Figure 1). In contrast, fatty acid oxidation rate was increased by 37%, acetyl CoA level was 2.5-fold higher, and free carnitine was 50% lower in diabetic hearts compared with controls, but with no significant difference in myocardial triglyceride concentration. Metabolic changes in the diabetic heart are associated with increased PPAR α transcriptional activity,^{38,39} as PPAR α targets PDH kinase 4 (PDK4), medium chain acyl co-

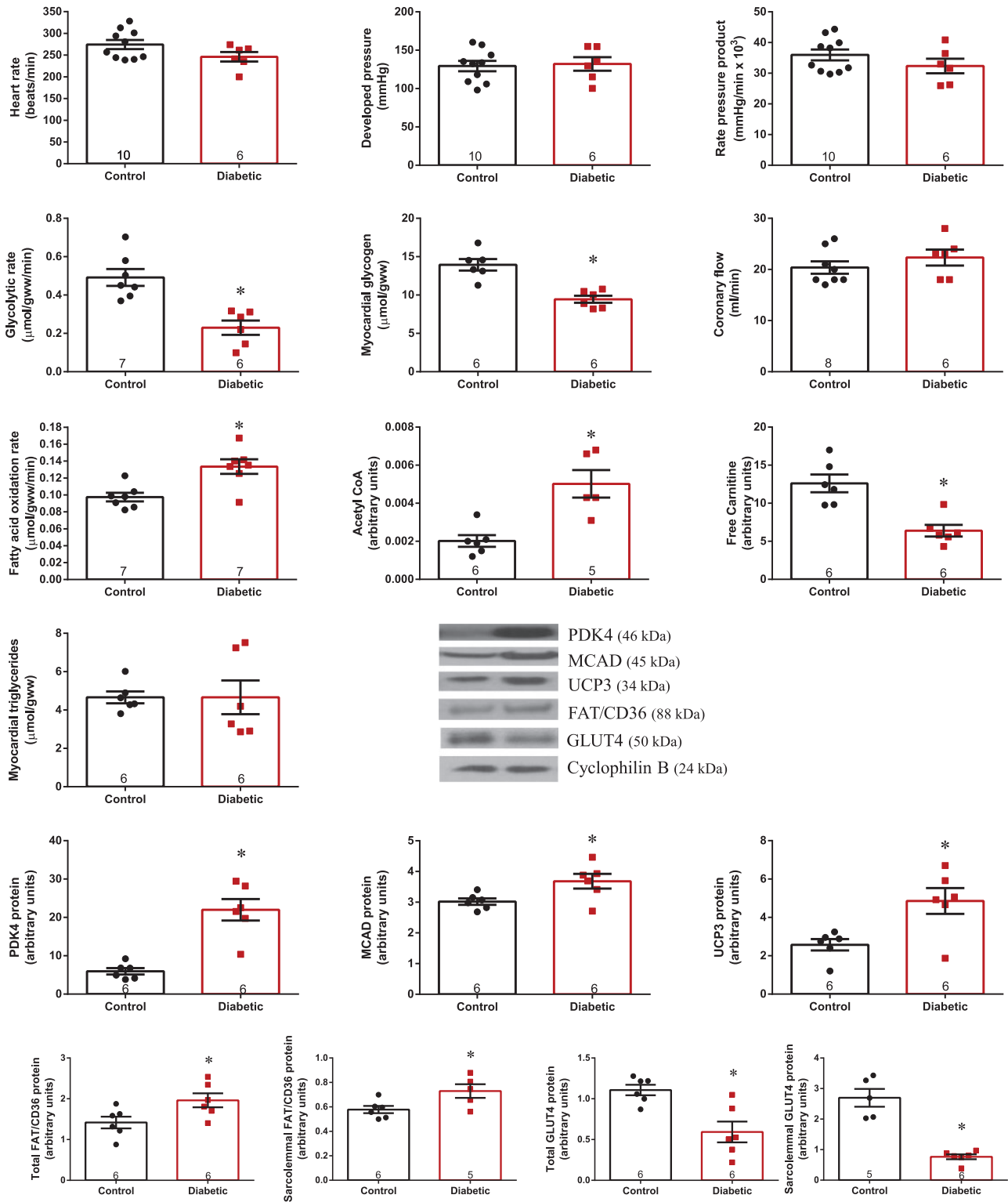


Figure 1 Cardiac function and substrate metabolism in control and diabetic hearts under normoxic baseline perfusion. * $P < 0.05$ vs. control. Acetyl CoA and free carnitine expressed as peak area ratio, relative to internal standard.

enzyme A dehydrogenase (MCAD), and uncoupling protein 3 (UCP3) were all significantly increased in the diabetic heart. In line with flux measurements and PPAR α activation, whole tissue level of the fatty acid transporter FAT/CD36 was increased by 38%, and sarcolemmal content of

FAT/CD36 was increased by 26% in diabetic hearts compared with control hearts. In contrast, whole tissue level of the glucose transporter GLUT4 was decreased by 46%, and sarcolemmal content of GLUT4 was decreased by 72% in diabetic hearts compared with controls.

3.2 Infusion of the FAT/CD36 inhibitor, SSO, into control hearts rapidly modified metabolism without compromising function

We questioned whether SSO, an inhibitor of FAT/CD36, would rapidly decrease fatty acid metabolism and promote glycolysis in the perfused heart, without having deleterious effects on function. We optimized the protocol in aerobically perfused control hearts, and found that 0.5 mM SSO decreased fatty acid oxidation rate by 28% and increased glycolytic rate by 43%, occurring within minutes of SSO addition (Figure 2). This metabolic effect of SSO occurred without compromising cardiac function, with no change in RPP (Figure 2), heart rate (279 ± 11 vs. 287 ± 9 bpm before and after SSO infusion, respectively) and developed pressure (132 ± 7 vs. 125 ± 6 mmHg before and after SSO infusion, respectively). No additional decrease in fatty acid oxidation was seen with a higher dose of SSO, and we confirmed that the DMSO-vehicle was not affecting fatty acid metabolism (see Supplementary material online, Tables S1 and S2).

3.3 Inhibition of FAT/CD36 in diabetic hearts normalized substrate metabolism and prevented lipid overload following hypoxic stress

It has long been recognized that diabetic hearts do worse functionally when challenged with stress, and that abnormal metabolism plays a key role in this dysfunction. Given that diabetic hearts have elevated sarcolemmal FAT/CD36 and increased fatty acid metabolism, we questioned whether moderate inhibition of FAT/CD36 with SSO would show benefit for the diabetic heart during exposure to hypoxic stress. Therefore, we infused SSO into diabetic and control hearts 4 min prior to acute hypoxia, and recirculated it throughout the subsequent reoxygenation period. Hypoxia significantly decreased the fatty acid oxidation rate, with no difference between control and diabetic hearts, with or without SSO present (Figure 3), demonstrating this metabolic response was likely driven by low oxygen availability. In contrast, following reoxygenation SSO treatment of diabetic hearts normalized the fatty acid oxidation rate back to that found in control hearts, decreasing fatty acid oxidation by 29% compared with untreated diabetic hearts. This improvement in fatty acid oxidation at reoxygenation occurred concomitantly with an improvement of myocardial triglycerides. Whereas in untreated diabetic hearts myocardial triglyceride concentration was 73% greater than in control hearts at reoxygenation, this was restored to control levels by SSO infusion. Hypoxia significantly increased glycolytic rates in all hearts, however, in diabetic hearts glycolytic rate remained 55% lower than in

control hearts, demonstrating impaired upregulating of glycolysis in response to hypoxia. This was not due to impaired p-AMPK activation and AMPK signalling in diabetic hearts during hypoxia (see Supplementary material online, Figure S1). SSO treatment of diabetic hearts was able to increase glycolytic rate by 46% compared with untreated diabetic hearts during hypoxia (Figure 3). However, SSO had no significant effect on glycolytic rate at reoxygenation or on myocardial glycogen concentration, SSO increased PDH activity in diabetic hearts by 53%, so that it was no longer significantly different to controls. This SSO-mediated increase in PDH flux in diabetic hearts was accompanied by a significant decrease in lactate efflux rate following reoxygenation, indicating that more pyruvate was being oxidized in the mitochondria following SSO treatment of diabetic hearts. Glucose-1-phosphate level was restored to control levels by SSO-treatment of diabetic hearts.

3.4 FAT/CD36 inhibition modulated Krebs cycle intermediates in diabetic hearts, predominantly within the second span of the cycle

Next, we questioned whether metabolic intermediates were being changed by hypoxic stress, and whether these changes were abrogated following reoxygenation by SSO. In normoxia, there were no significant differences in any Krebs cycle intermediate between control and diabetic hearts (Figure 4). In response to hypoxia-reoxygenation, intermediates within the first span of the Krebs cycle, namely citrate, aconitate, and isocitrate, were decreased in control and diabetic hearts, with citrate significantly lower in diabetic hearts at reoxygenation, and isocitrate significantly lower in diabetic hearts after hypoxia. Sequential intermediates in the second span of the Krebs cycle, namely succinate, fumarate, and malate, were increased in control hearts, by between two- and five-fold, following hypoxia. There were significant interactions for succinate, fumarate, and malate between diabetes and oxygenation, demonstrating that in diabetic hearts these intermediates were differently modified by hypoxia-reoxygenation. In diabetic hearts, succinate, fumarate, and malate were 28–54% lower following hypoxia compared with control hearts. The total FAD pool (FAD and FADH₂), essential for the oxidation of succinate to fumarate by succinate dehydrogenase, (in addition to branched chain amino acid catabolism and fatty acid oxidation), was selectively depleted by 31% in diabetic hearts following reoxygenation compared with controls. This decrease in the FAD total pool in diabetic hearts occurred independently of changes in NAD total pool, phosphocreatine or ATP, when compared with control hearts (Table 1).

SSO treatment of diabetic hearts had an anaplerotic effect on selective steps within the Krebs cycle (Figure 5). SSO treatment of diabetic hearts increased Krebs cycle intermediates in the second span of the cycle,

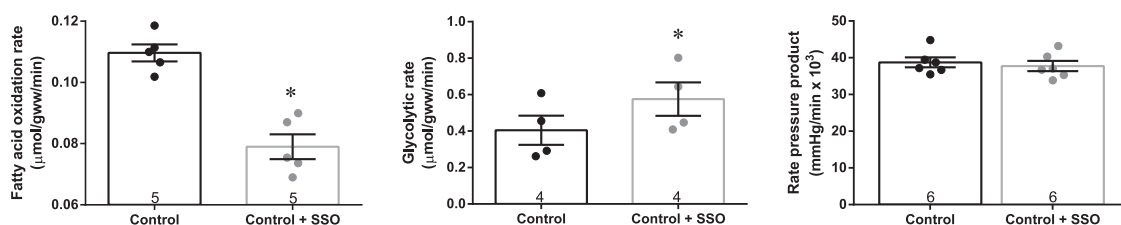


Figure 2 Substrate metabolism and cardiac function in control normoxic perfused hearts, before and after infusion of 0.5 mM SSO. * $P < 0.05$ vs. control without SSO.

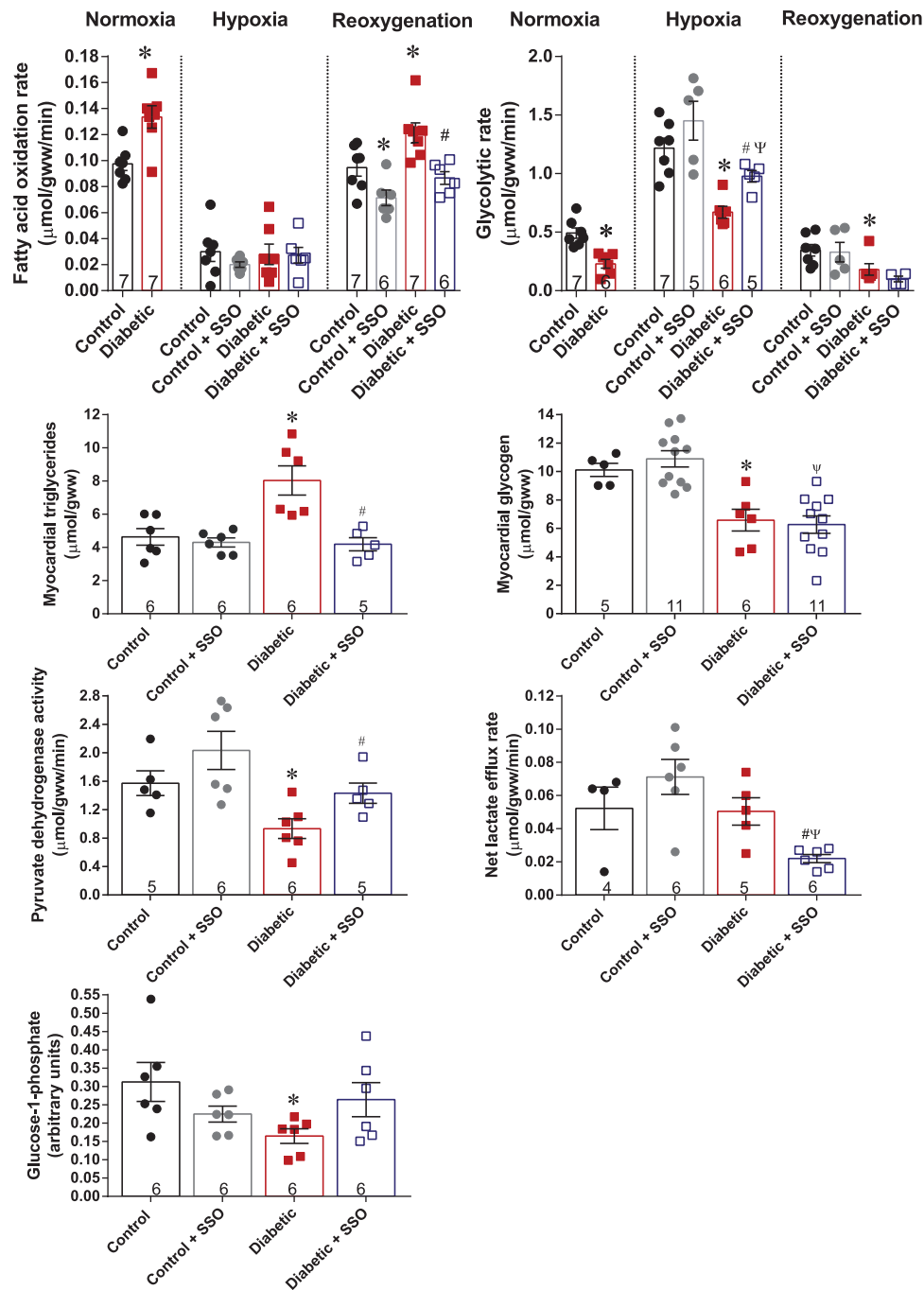


Figure 3 Substrate metabolism in control and diabetic hearts, with and without FAT/CD36 inhibitor SSO, during hypoxia and reoxygenation. * $P < 0.05$ vs. control under same oxygen condition, # $P < 0.05$ vs. diabetic, $\Psi P < 0.05$ vs. control + SSO. Glucose-1-phosphate expressed as peak area ratio, relative to internal standard. Fatty acid oxidation and glycolytic rate n numbers are the same in hypoxia and reoxygenation.

doubling the level of fumarate and increasing oxaloacetate by 61% compared to untreated diabetic hearts. The total FAD pool, which was depleted at reoxygenation in diabetic hearts, was significantly increased by 67% by SSO treatment of diabetic hearts. Citrate level was marginally increased, so that it was no longer significantly different to controls (Figure 5).

A number of amino acids were modulated in diabetic hearts exposed to hypoxia/reoxygenation (Table 1 and see Supplementary material

online, Table S3). Lysine, aspartate, and asparagine were increased in diabetic hearts in normoxia, leucine, isoleucine, and phenylalanine were decreased following hypoxia, and histidine and phenylalanine were decreased following reoxygenation in diabetic hearts compared with controls. Markers of the antioxidant system, reduced and oxidized glutathione, were not different between controls and diabetic hearts. None of these metabolites were modulated by SSO treatment (see Supplementary materials online, Table S4).

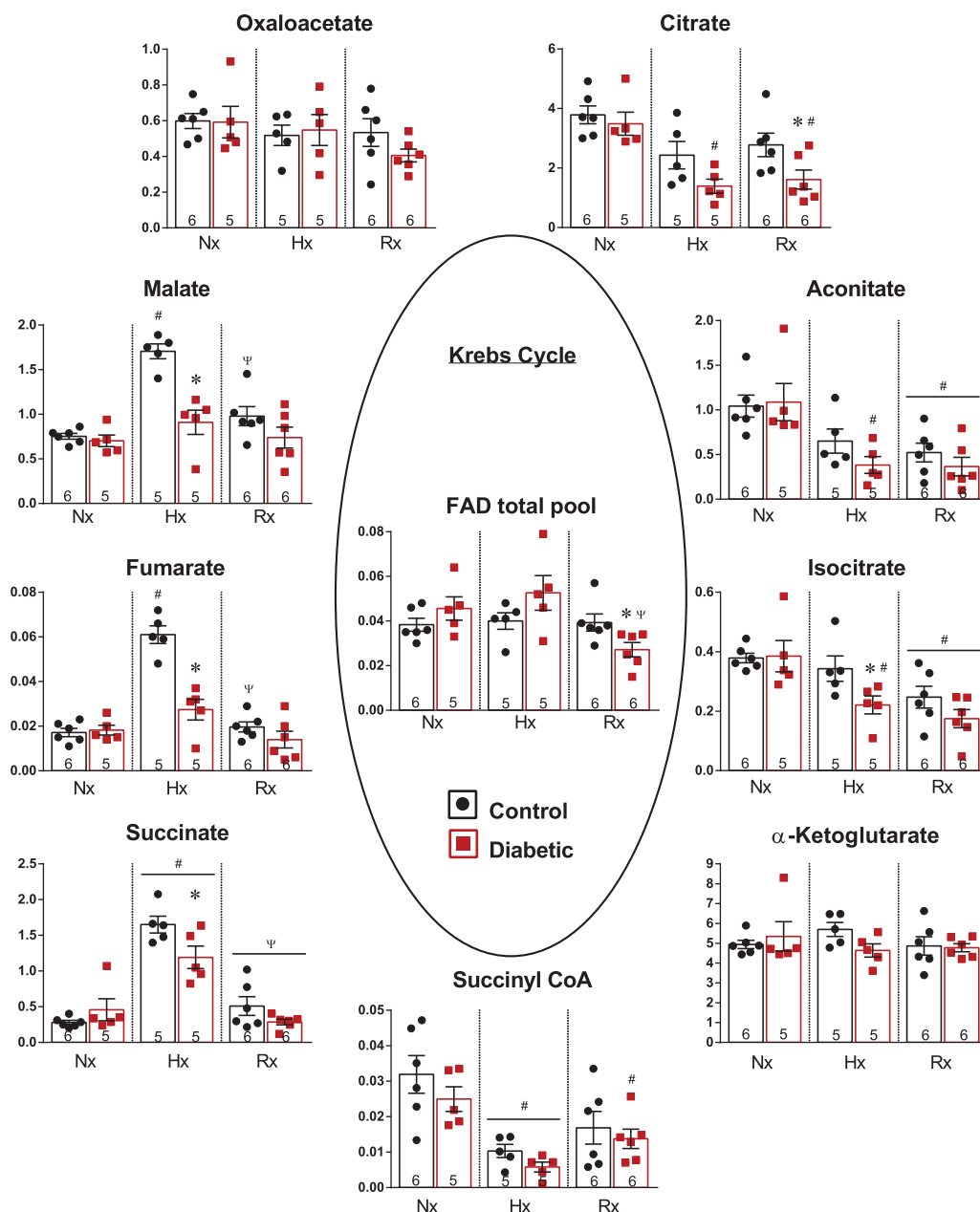


Figure 4 Myocardial levels of Krebs cycle intermediates from control and diabetic hearts under normoxia (Nx), hypoxia (Hx), and reoxygenation (Rx). * $P < 0.05$ vs. control under same oxygen condition, # $P < 0.05$ vs. normoxia within same disease state, $\Psi P < 0.05$ vs. hypoxia within same disease state. Data expressed as peak area ratio, relative to internal standard.

3.5 Inhibition of FAT/CD36 corrected cardiac dysfunction in diabetes

Finally, we questioned whether the beneficial metabolic effects of SSO would translate to a functional benefit during hypoxia and reoxygenation in the diabetic heart (Figure 6). At reoxygenation, RPP and developed pressure were significantly lower ($P < 0.05$) in both control and diabetic hearts compared with in normoxia, whereas other parameters returning to their normoxic levels. SSO had an overall beneficial effect on RPP in diabetic hearts both during hypoxia and reoxygenation. In diabetic hearts, SSO treatment increased RPP during hypoxia by 60% and during

reoxygenation by 32% compared with untreated diabetic hearts. This resulted in average percentage recovery of 73% in control hearts, 67% in control hearts treated with SSO, 55% in diabetic hearts, and 69% in diabetic hearts treated with SSO. Following SSO treatment end-diastolic pressure was normalized in diabetic hearts during hypoxia, and was no longer significantly different to control hearts. SSO treatment significantly increased heart rate in diabetic hearts during hypoxia, and following reoxygenation restored heart rate in diabetic hearts to control levels. SSO had no significant effects on developed pressure, positive or negative dP/dt max, or coronary flow rates in any group.

Table 1 Metabolic intermediates from control and diabetic hearts following normoxia, hypoxia and reoxygenation

	Normoxia		Hypoxia		Reoxygenation	
	Control (n = 6)	Diabetic (n = 5)	Control (n = 5)	Diabetic (n = 5)	Control (n = 6)	Diabetic (n = 6)
NAD total pool	0.93 ± 0.05	0.96 ± 0.11	0.95 ± 0.05	0.89 ± 0.07	0.82 ± 0.12	0.60 ± 0.05 [#]
Phosphocreatine	36.2 ± 2.5	32.4 ± 4.7	16.3 ± 2.9 [#]	13.3 ± 3.5 [#]	38.9 ± 5.2 [†]	28.5 ± 3.2 [†]
ATP	8.0 ± 0.4	9.2 ± 0.9	5.4 ± 0.9	4.1 ± 0.7 [#]	4.7 ± 1.1 [#]	5.5 ± 0.5 [#]
Adenine	0.013 ± 0.001	0.013 ± 0.002	0.016 ± 0.000	0.013 ± 0.001*	0.016 ± 0.001	0.011 ± 0.001*
Acetyl CoA	0.0020 ± 0.0003	0.0050 ± 0.0007*	0.0013 ± 0.0003	0.0021 ± 0.0003	0.0011 ± 0.0009 [#]	0.0006 ± 0.0002 [#]
Glutathione	0.00070 ± 0.00032	0.00157 ± 0.00126	0.00020 ± 0.00003	0.00018 ± 0.00005	0.00016 ± 0.00003	0.00096 ± 0.00045
Oxidized glutathione	0.202 ± 0.006	0.226 ± 0.015	0.229 ± 0.010	0.229 ± 0.011	0.184 ± 0.014 [#]	0.153 ± 0.011 ^{#,†}
Leucine	0.072 ± 0.004	0.097 ± 0.012	0.188 ± 0.011 [#]	0.146 ± 0.012 ^{*,#}	0.108 ± 0.013 [†]	0.079 ± 0.008 [†]
Isoleucine	0.060 ± 0.005	0.083 ± 0.011	0.170 ± 0.007 [#]	0.135 ± 0.013 ^{*,#}	0.088 ± 0.014 [†]	0.060 ± 0.006 [†]
Phenylalanine	0.052 ± 0.004	0.055 ± 0.006	0.080 ± 0.010	0.053 ± 0.005*	0.076 ± 0.006	0.054 ± 0.006*
Lysine	0.16 ± 0.01	0.32 ± 0.04*	0.17 ± 0.02	0.25 ± 0.03	0.22 ± 0.02	0.22 ± 0.02
Aspartate	0.11 ± 0.01	0.16 ± 0.01*	0.09 ± 0.01	0.14 ± 0.03	0.09 ± 0.01	0.09 ± 0.01 [#]
Asparagine	0.015 ± 0.001	0.027 ± 0.002*	0.017 ± 0.001	0.018 ± 0.005	0.014 ± 0.002	0.015 ± 0.002
Glutamate	17.6 ± 1.3	19.3 ± 2.9	10.2 ± 0.7 [#]	12.2 ± 0.9 [#]	9.6 ± 1.4 [#]	7.7 ± 0.8 [#]
Glutamine	6.2 ± 0.3	6.6 ± 0.9	4.0 ± 0.3 [#]	4.8 ± 0.1	3.8 ± 0.4 [#]	2.6 ± 0.2 ^{*,†}
Histidine	0.28 ± 0.01	0.25 ± 0.01	0.25 ± 0.03	0.20 ± 0.02 [#]	0.20 ± 0.02 [#]	0.13 ± 0.01 ^{*,#}
Alanine	0.19 ± 0.02	0.22 ± 0.06	0.42 ± 0.03 [#]	0.37 ± 0.06	0.16 ± 0.03 [†]	0.11 ± 0.02 [†]

Data expressed as peak area ratio, relative to internal standard.

**P* < 0.05 vs. control at same oxygenation (highlighted in bold);

[#]*P* < 0.05 vs. normoxia within same disease state;

[†]*P* < 0.05 vs. hypoxia within same disease state.

4. Discussion

The type 2 diabetic heart had an abnormal metabolic and functional response when challenged with acute hypoxia-reoxygenation. In this study we demonstrate for the first time that moderate inhibition of FAT/CD36 can be achieved rapidly in the intact perfused heart using SSO. During hypoxic stress, SSO can promote glycolysis, decrease myocardial triglyceride deposition, and increase PDH flux in diabetic hearts. In addition, using metabolomics we were able to identify unexpected changes in metabolic intermediates, which demonstrated that SSO treatment had an anaplerotic effect to increase Krebs cycle intermediates within the second span of the cycle. These metabolic changes culminated in improved cardiac function during hypoxia and after reoxygenation, restoring function to levels found in control hearts. Thus, pharmacological inhibition of FAT/CD36 provides a mechanism to improve substrate metabolism, prevent lipid overload and restore cardiac function in diabetes.

SSO was used in the initial identification of FAT/CD36 as the plasma membrane protein responsible for fatty acid uptake,⁴⁰ and has subsequently been used to define the functional significance of FAT/CD36.^{41–44} By administering SSO to the perfused heart we addressed two questions: whether pharmacological inhibition of FAT/CD36 was feasible and, secondly, whether rapidly suppressing fatty acid metabolism is sufficient to restore cardiac function following stress. Genetic studies have shown that FAT/CD36 deficiency can rescue cardiac lipotoxicity associated with PPAR α overexpression,¹⁷ thus, translation of a genetic finding to a therapeutic angle was of great interest. As therapeutic proof of concept, incubating primary cardiomyocytes with an antibody raised to FAT/CD36 improved sarcomere shortening following exposure to a high palmitate culture media.⁴⁵ In this study, we demonstrated that SSO infusion rapidly restored fatty acid oxidation rates in diabetic hearts back to control levels. In addition, SSO had the added advantage

of also preventing the accumulation of intracellular triglycerides, a benefit that does not occur with compounds that target mitochondrial fatty acid oxidation, such as etomoxir, where fatty acid import and esterification may continue unhindered.¹⁶ One potential concern was that the degree of FAT/CD36 inhibition may be too great, which would result in substrate starvation and contractile dysfunction given that fatty acids are the main fuel for ATP generation. However, we have shown that SSO can be used to induce small changes in metabolism without compromising cardiac function.

In diabetic hearts, functional defects only arose when the myocardium was stressed by hypoxia, and could be reversed in minutes by changing metabolism, demonstrating that the functional deficit was not a fixed irreversible effect of the disease. In diabetic and GLUT4-deficient mice where glucose metabolism is suppressed, hypoxia induces abnormalities in electrical activity and action potential duration.^{46,47} This documented association between metabolism and electrical activity in the heart may contribute to the changes in heart rate in our hypoxic diabetic animals and with acute metabolic therapy. SSO treatment improved end-diastolic pressure during hypoxia in diabetic hearts, an indicator of improved relaxation and compliance following metabolic modulation.

Glucose uptake and metabolism are regulated by multiple signalling pathways, both at baseline and in response to stress.^{22,48} Glycolytic rates were increased to a much lesser extent in diabetic hearts than controls in response to hypoxia, which we show was not attributable to abnormal p-AMPK activation. This raised the question of whether hypoxia had already reached the maximal glycolytic response in the diabetic heart. We found this was not the case, and inhibition of FAT/CD36 provided an additional stimulus to increase glycolytic rates further in diabetic hearts. This demonstrated that increased fatty acid metabolism was responsible for suppressing anaerobic metabolism, in accordance with the Randle cycle,¹⁰ and as highlighted by the restoration of PDH activity. That SSO

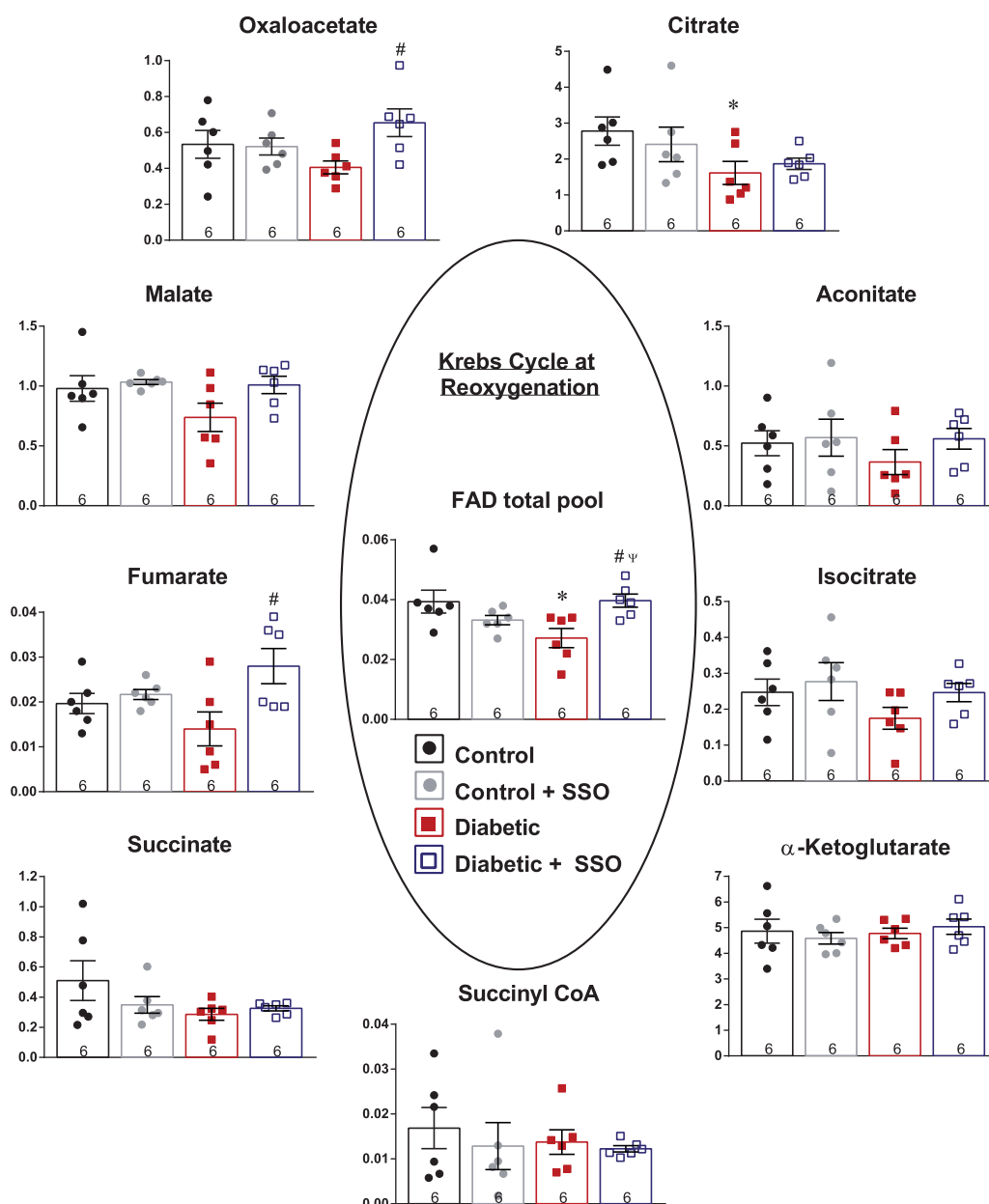


Figure 5 Myocardial levels of Krebs cycle intermediates at reoxygenation from control and diabetic hearts, with and without FAT/CD36 inhibitor SSO. * $P < 0.05$ vs. control, # $P < 0.05$ vs. diabetic, $\Psi P < 0.05$ vs. control + SSO. Data expressed as peak area ratio, relative to internal standard.

did not confer the same benefits to control hearts may be due to metabolic flexibility already being optimal in the healthy heart. Thus, there is inherent metabolic flexibility in the diabetic heart; but it requires simultaneous activation of multiple pathways—via the intrinsic hypoxic response, including AMPK signalling, and via extrinsic metabolic modulation. The combined effects of decreasing fatty acid metabolism and increasing glucose metabolism by SSO make it difficult to assign which of these two factors is responsible for improving functional outcomes in the diabetic heart. Given the reciprocal regulation between metabolic pathways, any process that changes one of these pathways will always modify the other, and, therefore, it is most likely that overall benefit is gained by the combination of the two.

Metabolomics is a non-hypothesis driven experimental approach, which allowed us to determine if there were any additional effects of

short-term metabolic modulation, above and beyond those that would be predicted from traditional studies. Metabolomics identified that the effect of hypoxia on the Krebs cycle was not uniform, that intermediates within the first span were depleted, whereas intermediates within the second span accumulate. The enzymatic steps where these patterns change are all NAD-dependent dehydrogenase or substrate level phosphorylation steps, indicating this profile in hypoxia is likely due to mitochondrial redox-dependent regulation. The increase in succinate during hypoxia is consistent with Chouchani *et al.*,⁴⁹ who showed an increase in succinate during ischaemia. However, in contrast to our findings, they did not identify changes in other Krebs cycle intermediates, indicating differences in the myocardial response to different stressors. Hypoxia is a less severe stressor for the heart than global ischaemia, as waste product removal and substrate delivery can still continue, which may account for

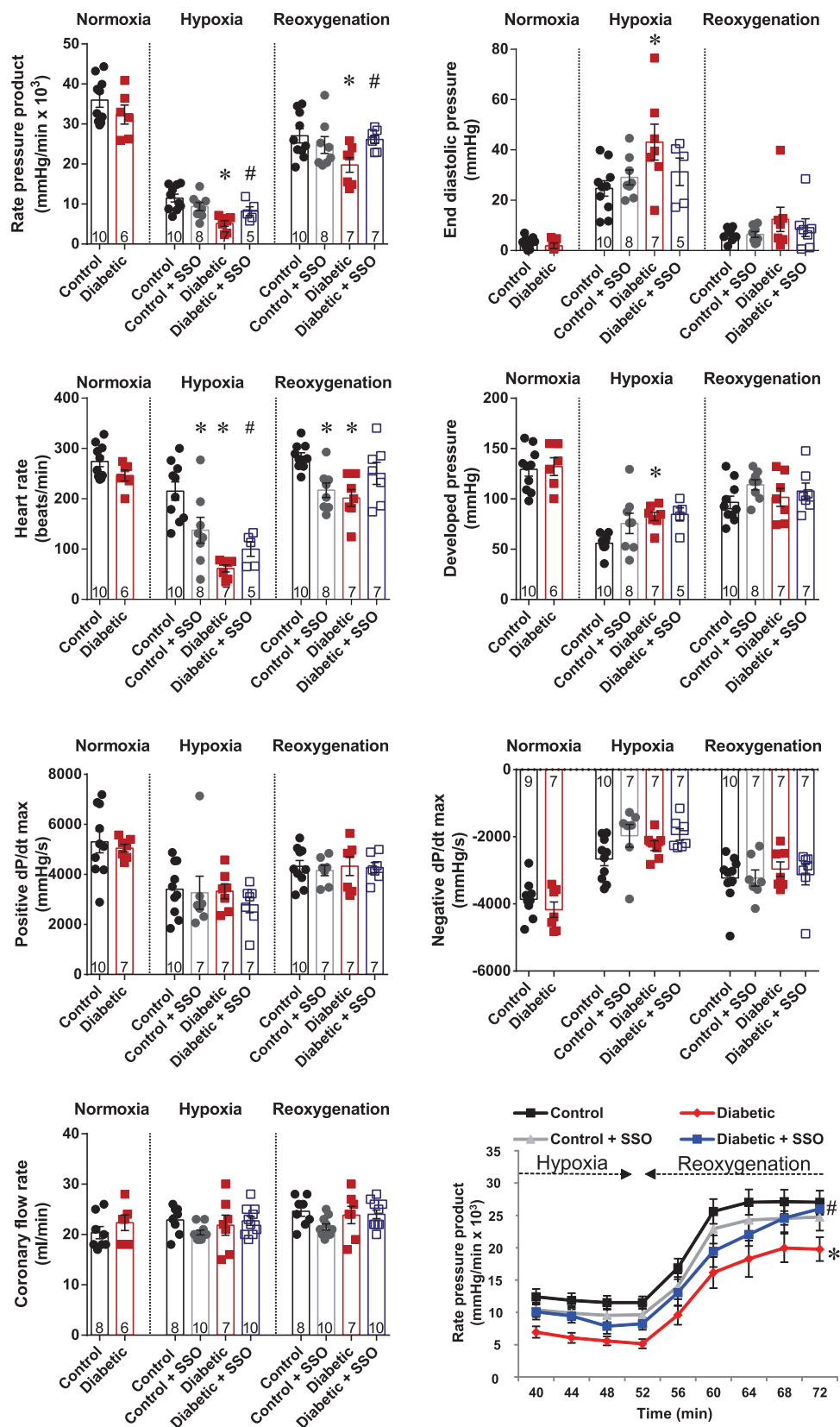


Figure 6 Cardiac function in control and diabetic hearts, with and without FAT/CD36 inhibitor SSO, during hypoxia and reoxygenation. * $P < 0.05$ vs. control, # $P < 0.05$ vs. diabetic. End diastolic pressure n numbers as per developed pressure.

these different responses. Diabetes decreased a number of intermediates within the Krebs cycle, namely citrate, isocitrate, succinate, fumarate, and malate. In agreement with our findings, Banke *et al.*⁵⁰ demonstrated abnormal Krebs cycle flux in diabetic hearts only when stressed by ischaemia. Of interest was the increase in fumarate, oxaloacetate and the FAD pool with SSO treatment of diabetic hearts, demonstrating an additional anaplerotic benefit from metabolic therapy in diabetes.

The current findings demonstrate beneficial metabolic and functional effects of SSO for the diabetic heart, and future *in vivo* studies are warranted. Any changes induced by acute SSO in this study were immediate via existing proteins, whereas chronic studies may exert additional effects via transcriptional/translational changes within the heart. Potentially SSO may also reduce the deleterious effects of intracellular lipids associated with insulin resistance and cell death.^{51,52} However, there are a number of critical hurdles to overcome, such as systemic delivery affecting metabolism and function of other tissues, such as skeletal muscle and adipose, as well as unknown longer term effects on cardiac function.

In conclusion, type 2 diabetes impairs the metabolic and functional response to hypoxia-reoxygenation stress. Pharmacological inhibition of the sarcolemmal fatty acid transporter, FAT/CD36, provides a rapid mechanism to suppress fatty acid oxidation and promote glycolysis. Targeting the primary transport step in the pathway also prevented excessive lipid deposition and rebalanced Krebs cycle intermediates resulting in improved cardiac function in diabetic hearts following hypoxic stress.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Acknowledgements

We acknowledge Peter Ratcliffe for intellectual input on the study, and Michael Dodd and Dunja Aksentijevic for technical assistance. We thank Evangelia Shaw, whose arrival prompted this study.

Conflict of interest: none declared.

Funding

This work was supported by grants from Diabetes UK (grant number 11/0004175) and the British Heart Foundation (FS/14/65/31292 and FS/15/68/32042). This publication arises from research funded by the John Fell Oxford University Press (OUP) Research Fund. J.L.G. and J.W. acknowledges the financial support of the Medical Research Council UK (Lipid profiling and signalling MC_UP_A90_1006).

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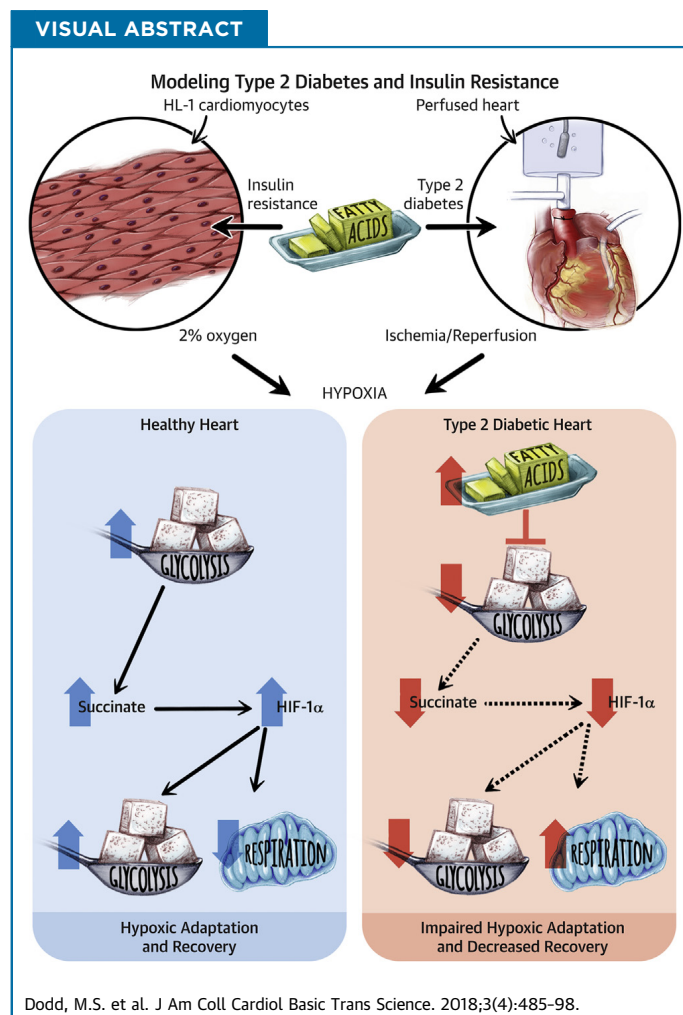
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PRECLINICAL RESEARCH

Fatty Acids Prevent Hypoxia-Inducible Factor-1 α Signaling Through Decreased Succinate in Diabetes



Michael S. Dodd, PhD,^a Maria da Luz Sousa Fialho, MSc,^a Claudia N. Montes Aparicio, MSc,^a Matthew Kerr, MSc,^a Kerstin N. Timm, PhD,^a Julian L. Griffin, PhD,^b Joost J.F.P. Luiken, PhD,^c Jan F.C. Glatz, PhD,^c Damian J. Tyler, PhD,^a Lisa C. Heather, PhD^a



HIGHLIGHTS

- HIF-1 α is activated following myocardial infarction, and is a critical transcription factor promoting survival in hypoxia.
- Type 2 diabetes blunts HIF-1 α activation in ischemia and downstream adaptation to hypoxia.
- This effect is mediated by increased long-chain fatty acids, which prevent HIF-1 α activation in hypoxia.
- Succinate promotes HIF-1 α activation by inhibiting the regulatory HIF hydroxylases. Fatty acids decrease succinate concentrations in hypoxia, by blocking increased glycolysis and malate-aspartate shuttle activity.
- Pharmacologically activating HIF-1 α or increasing succinate concentrations restores the hypoxic response and improves functional recovery post-ischemia in diabetes.

From the ^aDepartment of Physiology, Anatomy and Genetics, University of Oxford, Oxford, United Kingdom; ^bDepartment of Biochemistry and MRC Human Nutrition Research, University of Cambridge, Cambridge, United Kingdom; and the ^cDepartment of Molecular Genetics, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University, Maastricht, the Netherlands. Dr. Dodd is currently at the Faculty of Health and Life Sciences, Coventry University, Coventry, United Kingdom. This work was supported by fellowships from the British Heart Foundation (FS/17/58/33072, FS/14/65/31292, FS/15/68/32042) and Diabetes UK (11/0004175). Dr. Dodd has been supported by a Novo Nordisk Postdoctoral Fellowship run in partnership

ABBREVIATIONS AND ACRONYMS

ANOVA = analysis of variance

BSA = bovine serum albumin

DMF = dimethyl fumarate

DMOG = dimethylolxalylglycine

FA = fatty acid

FIH = factor inhibiting hypoxia-inducible factor

HIF = hypoxia-inducible factor

i.p. = intraperitoneal

IR = insulin resistance/resistant

MI = myocardial infarction

PHD = prolyl hydroxylase domain

SSO = sulfo-*N*-succinimidyl oleate

SUMMARY

Hypoxia-inducible factor (HIF)-1 α is essential following a myocardial infarction (MI), and diabetic patients have poorer prognosis post-MI. Could HIF-1 α activation be abnormal in the diabetic heart, and could metabolism be causing this? Diabetic hearts had decreased HIF-1 α protein following ischemia, and insulin-resistant cardiomyocytes had decreased HIF-1 α -mediated signaling and adaptation to hypoxia. This was due to elevated fatty acid (FA) metabolism preventing HIF-1 α protein stabilization. FAs exerted their effect by decreasing succinate concentrations, a HIF-1 α activator that inhibits the regulatory HIF hydroxylase enzymes. In vivo and in vitro pharmacological HIF hydroxylase inhibition restored HIF-1 α accumulation and improved post-ischemic functional recovery in diabetes. (J Am Coll Cardiol Basic Trans Science 2018;3:485–98) © 2018 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

Activation of hypoxic signaling pathways through stabilization of hypoxia-inducible factor (HIF)-1 α is vital for cardiac survival in hypoxia, as occurs post-myocardial infarction (MI) or in heart failure (1,2). In normoxia, HIF-1 α protein is hydroxylated by the oxygen-sensing HIF hydroxylase enzymes: prolyl hydroxylase domain (PHD) and factor inhibiting HIF (FIH), thereby targeting HIF-1 α for proteasomal degradation (3). During hypoxia, reduced oxygen availability inhibits these HIF hydroxylase enzymes, leading to HIF-1 α protein accumulation, which can translocate to the nucleus, bind to HIF1 β , and induce transcription of HIF target genes. HIF-1 α has many hundreds of gene targets, and broadly, these targets up-regulate oxygen sparing pathways, down-regulate oxygen consuming pathways, and increase oxygen delivery (4–7). HIF-1 α adapts metabolism to the oxygen-restricted environment, increasing anaerobic glycolysis while decreasing fatty acid (FA) oxidation and mitochondrial respiration. HIF-1 α plays a critical role in the cellular response to hypoxia in many cardiovascular diseases (1,2,8–10), and decreased HIF-1 α activation accelerates the progression into heart failure in genetic mouse models (1).

function post-ischemia has been associated with abnormal cardiac FA metabolism in diabetes. FAs are the predominant fuel used by the heart to power contraction, accounting for 60% to 70% of cardiac ATP generation. In diabetes, FA oxidation and storage are up-regulated, and lipid intermediates accumulate within the myocardium (14,15). We have shown that type 2 diabetes blunts the normal metabolic adaptation to hypoxia, resulting in impaired cardiac function (16). However, the explanation for why hypoxic adaptation is blunted in diabetes and the underlying mechanisms require investigation, as these may explain why diabetic patients have poorer long-term prognosis following MI and in heart failure. We questioned whether abnormal FA metabolism could drive abnormal HIF-1 α activation and downstream adaptation to hypoxia in diabetes.

Here, we show that type 2 diabetic hearts fail to accumulate HIF-1 α protein during ischemia, and this is due to elevated FAs. FAs exert their effects by decreasing succinate concentrations, an inhibitor of the HIF hydroxylase enzymes, and supplementing succinate restores HIF-1 α accumulation in insulin resistance (IR). Finally, we demonstrate that in vivo HIF hydroxylase inhibition improves post-ischemic recovery in diabetic rats.

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METHODS

ANIMALS. All animal experiments conformed to the Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act, 1986, and were

Cardiovascular disease is the leading cause of mortality in type 2 diabetic patients, with poorer long-term prognosis following MI and higher incidence of heart failure (11–13). Impaired cardiac

with the University of Oxford. The authors have reported that they have no relationships relevant to the contents of this paper to disclose.

All authors attest they are in compliance with human studies committees and animal welfare regulations of the authors' institutions and Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the JACC: Basic to Translational Science [author instructions page](#).

Manuscript received March 6, 2018; revised manuscript received April 23, 2018, accepted April 24, 2018.

approved by the local ethics committee. Type 2 diabetes was induced as previously described (17,18), generating a mild model of the disease presenting with hyperglycemia, hyperinsulinemia, and hyperlipidemia (19). Briefly, male Wistar rats were fed a high-fat diet ad libitum for 30 days, and on the 14th day, rats received a single low-dose, intraperitoneal (i.p.) injection of streptozotocin (25 mg $\cdot$ kg⁻¹ body weight, w/w in citrate buffer). On day 30, rats were terminally anesthetized using a 0.7-ml i.p. injection of pentobarbital sodium (200 mg/ml Euthatal, Merial Animal Health/Boehringer Ingelheim, Ingelheim, Germany). Blood glucose concentrations were significantly increased from 8.7 $\pm$ 0.4 mmol/l in control rats to 10.8 $\pm$ 0.5 mmol/l in diabetic rats. Hearts were removed and perfused, according to our published protocol (20), in retrograde Langendorff mode at 100 mm Hg constant pressure. Hearts were perfused with Krebs-Henseleit buffer containing 11 mmol/l glucose, 0.3 U $\cdot$ l⁻¹ insulin and 1.5% (w/v) FA-free bovine serum albumin (BSA) bound to 0.4 mmol/l palmitate (gassed with 95% O₂ and 5% CO₂, at 37°C). Cardiac function was measured using a polyvinyl chloride balloon inserted into the left ventricle, with rate-pressure product calculated as the multiple of heart rate and developed pressure. Hearts were perfused for 20 min at normal flow, followed by 25 min of low-flow ischemia (0.3 ml/min/gww). At the end of ischemia, hearts were freeze clamped on the cannula for tissue analysis.

For long-term in vivo dimethyloxalylglycine (DMOG) treatment of diabetic rats, on the penultimate week of high-fat feeding, animals were injected i.p. with DMOG (40 mg/kg) every other day over 5 days (totaling 3 injections). After these 5 days of pretreatment, hearts were perfused for 20 min at normal flow, challenged with 30 min of low-flow ischemia, followed by 15 min of reperfusion, for assessment of post-ischemic contractile recovery.

CELL CULTURE. HL-1 murine cardiomyocytes were cultured as previously described (21), and all experiments were performed on confluent, beating cells. For induction of IR, cells were washed twice with warm phosphate-buffered saline, before the addition of either control medium (Dulbecco's Modified Eagles' Medium) containing 1 g/l glucose, 2 mmol/l glutamine, 2 mmol/l nonessential amino acids, and 1 $\times$ penicillin/streptomycin or IR medium (control medium supplemented with 500 μ mol/l palmitate:BSA [bound 6:1] with 50 nmol/l insulin) (22,23). After 8 h, this medium was replenished, and cells were either maintained in normoxia or transferred into a hypoxic incubator (2% O₂, 5% CO₂) for a further 16 h. In specific experiments,

control medium was supplemented with only FAs (either oleate or palmitate at concentrations from 150 to 500 μ mol/l bound to BSA) or with only insulin (50 nmol/l). In other experiments, compounds were added to the media immediately before the cells entered hypoxia for 16 h, which included 1 mmol/l DMOG, 100 μ mol/l to 1 mmol/l dimethyl fumarate (DMF), 0.4 mmol/l sulfo-*N*-succinimidyl oleate (SSO), 5 mmol/l 2-deoxyglucose, 2 mmol/l phenylsuccinate, 2 mmol/l amino-oxyacetate, and 5 mmol/l dimethylmalonate. The cell-permeable fumarate ester, dimethyl fumarate, was used to elevate intracellular succinate levels, because this has been shown to be more efficient/cell permeable than the succinate ester, dimethyl succinate (24). For experiments that inhibited HIF degradation, the proteasomal inhibitor MG132 (25 μ mol/l) was added, and cells were maintained in hypoxia for 4 h rather than 16 h, due to toxicity effects.

WESTERN BLOTTING. Cells were washed twice with ice-cold phosphate-buffered saline, before being lysed with the addition of ice-cold lysis buffer. Frozen heart tissue was crushed and lysed in ice-cold lysis buffer. Protein (9 μ g for cells and 30 μ g for tissue) was loaded onto SDS-PAGE gels and separated by electrophoresis. Even protein loading and transfer were confirmed by either the loading control β -actin or Ponceau S staining. Bands were quantified using LI-COR C-Digit chemiluminescent detection system (LI-COR Biotechnology, Lincoln, Nebraska) and Image Studio software version 5.2.5 (LI-COR).

QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION. Total RNA was extracted from cells using mini-RNeasy kit (Qiagen, Hilden, Germany), and cDNA was synthesized from the RNA using a high-capacity RNA-to-cDNA kit (Thermo Fisher Scientific, Waltham, Massachusetts). Primers were generated by searching PrimerBank for accession numbers of the following genes: *Fih-1* NM_176958.3, *Hif1A* NM_001313919, *Hif1B* NM_001037737.2, *Hprt1* NM_013556.2, *Hmbs* NM_001110251.1, *Phd2* NM_053207.2, *Phd3* NM_028133.2, *Rpl13A* NM_173340.2, and *vhl* NM_009507.3 (PrimerBlast was used to check specificity, and sequences are listed in Supplemental Table 1). *Phd1* is expressed at low levels in the heart, and could not be reliably measured (9). cDNA (15 μ g per well) were loaded and quantitative polymerase chain reaction amplification was performed using Power SYBR Green PCR Master Mix and a Step-One Plus Real-Time PCR system (Thermo Fisher Scientific, Waltham, Massachusetts). Relative gene expression was normalized to the geometric mean of the housekeeping genes: *Rpl13a*, *Hmbs*, and *Hprt1*.

RESPIRATION MEASUREMENTS. Cells were harvested from flasks with 0.05% trypsin-EDTA at 37°C and counted (using a Countess 1, Thermo Fisher Scientific), and experiments were normalized to viable cell number, expressed per million cells. Respiration was measured using a Clark-type oxygen electrode in medium (100 mmol/l potassium chloride, 50 mmol/l MOPS, 1 mmol/l EGTA, 5 mmol/l potassium phosphate, and 1 mg/ml BSA) (25), supplemented with palmitate (500 μ mol/l bound to BSA) and malate (2.5 mmol/l) as substrates. Carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) (10 mmol/l) was used to measure maximal rates of oxygen consumption, and oligomycin (2 mmol/l) was used to measure ATP synthase-independent proton leak.

OIL-RED-O. Cells were washed and fixed with 4% paraformaldehyde, before being stained with oil-red-O (for neutral lipids) and DAPI (for nuclei) as described (26). Quantification was performed by eluting the oil-red-O in 100% isopropanol and measuring absorbance at 492 nm. To account for cell number, absorbance was normalized to protein content using a bicinchoninic acid assay.

MEDIA METABOLITE MEASUREMENTS. An ABX Pentra 400 (Horiba ABX Diagnostics, Irvine, California) was used to measure media glucose and lactate concentrations, and to calculate glucose consumption and lactate efflux rates. Plasma nonesterified fatty acids (NEFA) were measured using a Randox NEFA kit (Randox Laboratories, Crumlin, County Antrim, United Kingdom).

METABOLOMIC ANALYSIS. Metabolites were extracted from frozen cardiac tissue using methanol-chloroform-water, and the aqueous phase was analyzed as previously described using liquid chromatography tandem-mass spectrometry (19). Glycolytic and Krebs cycle metabolites were semiquantitatively assessed, by normalizing the peak intensity to a number of internal standards. Succinate concentrations were measured in cells using a colorimetric assay (Abcam, Cambridge, United Kingdom), normalized to protein.

STATISTICS. Results are presented as mean $\pm$ SEM (Figures 1 to 6). GraphPad Prism version 7 (GraphPad Software, La Jolla, California) was used for statistical tests, and results were considered significant at $p < 0.05$. Depending on the experiment, either Student's unpaired *t*-test (Figures 1, 3, and 5), 1-way analysis of variance (ANOVA) (Figures 4 to 6), or 2-way ANOVA (Figures 2 to 4) were performed. Pearson correlations were used for Figures 1 and 5. For the ANOVA analysis, where statistical significances between groups were observed, a Tukey post hoc comparison was performed and corrected for multiple comparisons.

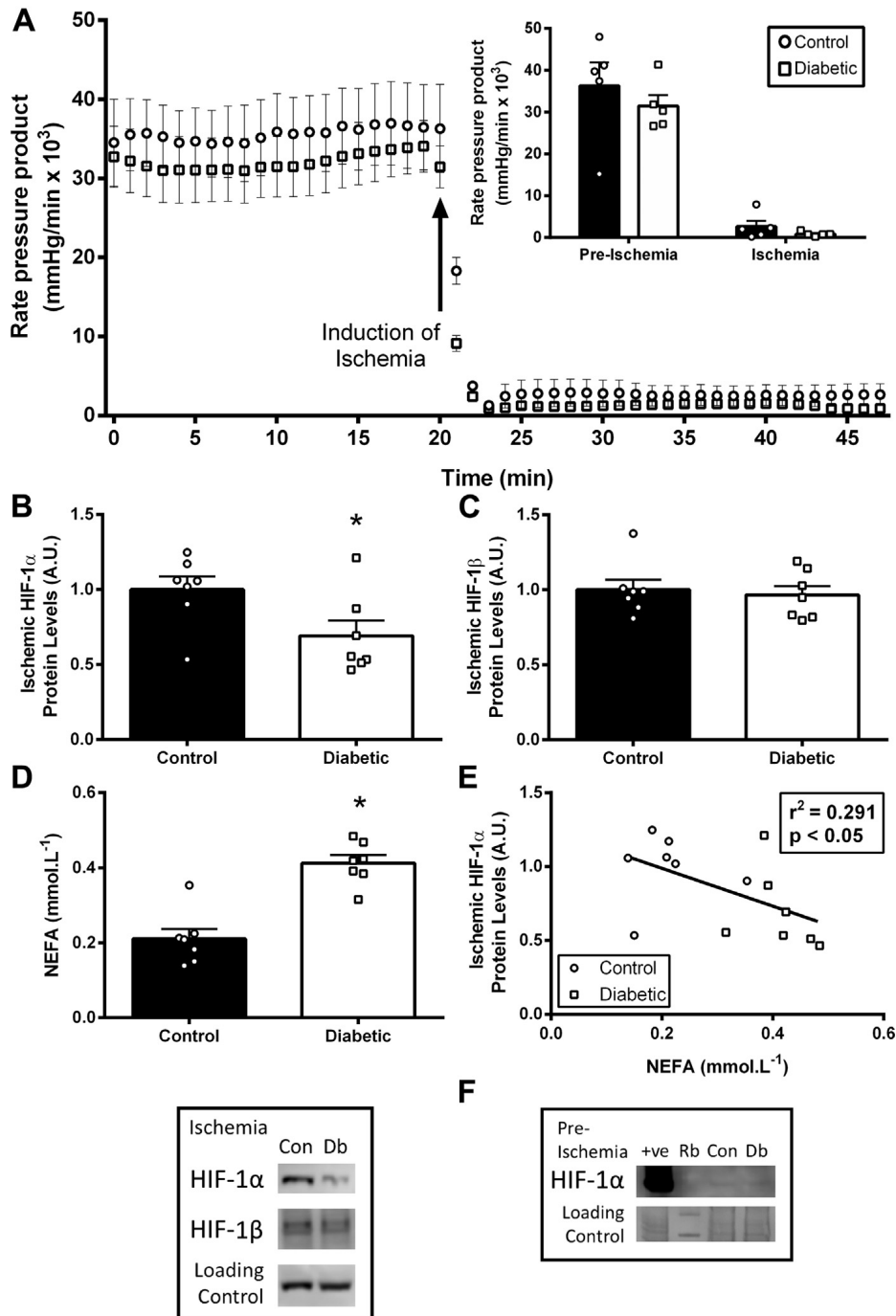
RESULTS

TYPE 2 DIABETIC HEARTS FAIL TO ACCUMULATE HIF-1 α DURING ISCHEMIA. Hearts from type 2 diabetic and control rats were subjected to 25 min of low-flow ischemia (Figure 1), and at the end of ischemia accumulation of HIF-1 α protein was measured. HIF-1 α protein levels were 21% lower in diabetic hearts challenged with ischemia compared with control hearts. The decreased HIF-1 α accumulation was independent of any change in HIF1 β protein levels. There was a 96% increase in plasma NEFA concentrations in diabetic rats, which correlated negatively with ischemic HIF-1 α protein levels. HIF-1 α in preischemia/normoxia was similarly absent in both control and diabetic hearts (Figure 1), in agreement with previous findings (16).

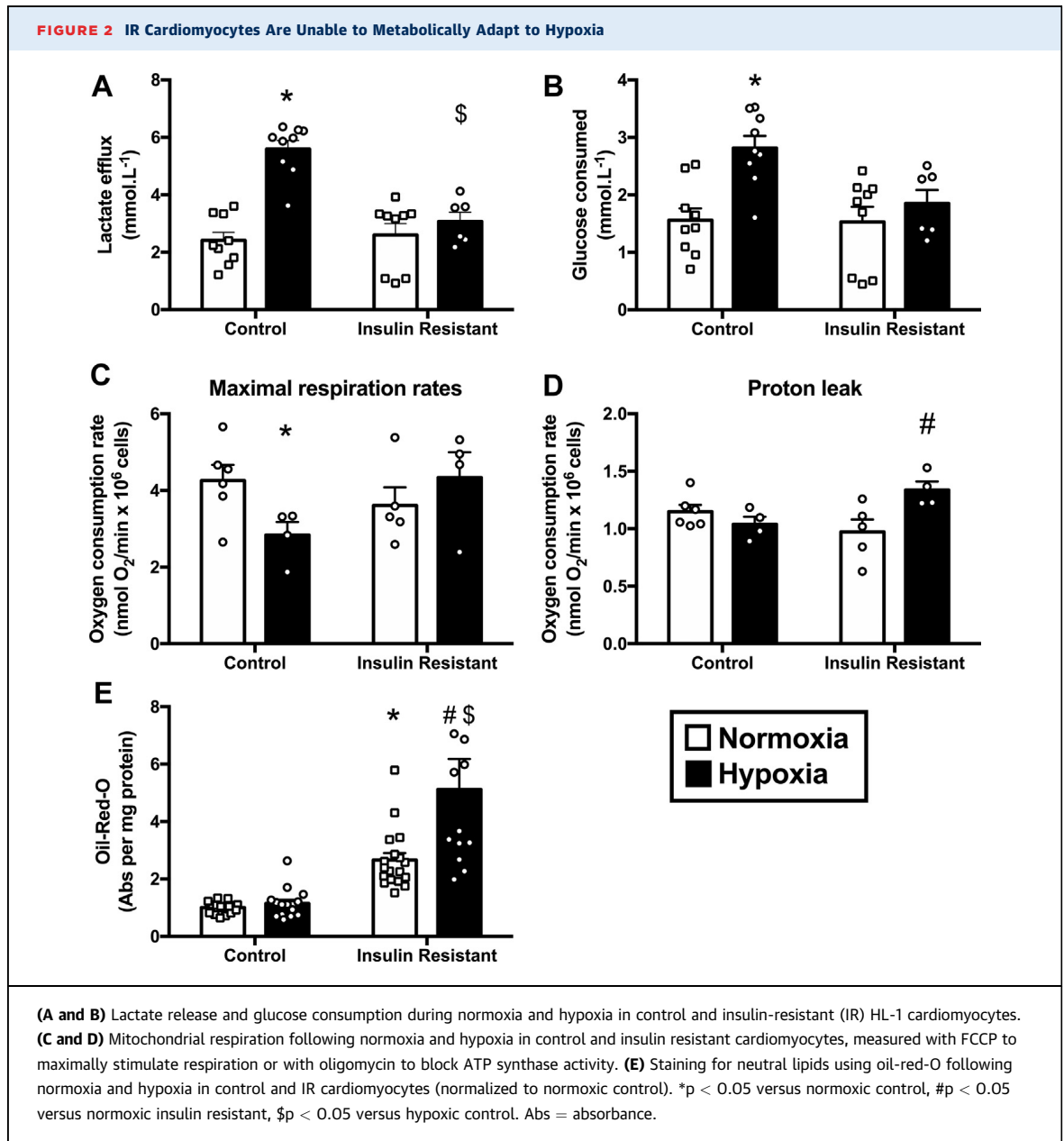
IR CARDIOMYOCYTES HAVE BLUNTED METABOLIC ADAPTATION TO HYPOXIA. To investigate mechanistically why diabetes suppresses cardiac HIF-1 α activation, an IR cardiac cell culture model was used (22,23) (Supplemental Figure 1). Control HL-1 cardiomyocytes exposed to 16 h of hypoxia exhibited the hallmarks of the hypoxic metabolic shift from oxidative to anaerobic metabolism, increasing lactate efflux by 132%, increasing glucose consumption by 81%, and decreasing maximal respiration rates by 33% (Figure 2). By contrast, these hypoxia-mediated metabolic responses were all blunted in IR cells, which failed to increase lactate efflux and glucose consumption. IR cells did not reduce maximal respiration following hypoxia, and instead increased ATP synthase-independent proton leak by 38%. In addition, hypoxia further increased intracellular lipid accumulation in the IR cardiomyocytes.

IR IMPAIRS HIF-1 α ACCUMULATION AND DOWNSTREAM SIGNALING IN HYPOXIA. HIF-1 α protein levels were minimal in control and IR cells cultured in normoxia (Figure 3), in agreement with previous findings (16). In control cells, hypoxia caused an $\sim$ 23-fold increase in HIF-1 α protein levels, causing downstream targets of HIF (GLUT1 and cyclophilin B) to be increased, GLUT1 increased 3-fold, and cyclophilin B increased by 67% (6,27). By contrast, IR cells failed to accumulate HIF-1 α protein in hypoxia, and downstream metabolic HIF targets, GLUT1, and cyclophilin B, did not increase in hypoxic IR cardiomyocytes. Whereas IR cells were unable to activate HIF-1 α signaling during hypoxia, there was activation of the hypoxia-sensitive activating transcription factor ATF1 (28). Phosphorylated-ATF1 (pATF1) was elevated by hypoxia, in both the controls and IR cells. This demonstrates that IR selectively impairs hypoxic signaling through HIF-1 α protein.

FIGURE 1 Type 2 Diabetes Decreases HIF-1 α Accumulation in Ischemia



(A) Cardiac function in control and diabetic rat hearts, at normal flow and during ischemia ($n = 5$ per group). **(B and C)** HIF-1 α and HIF-1 β protein levels in ischemic control and diabetic hearts. **(D and E)** Non-esterified fatty acid (NEFA) plasma concentrations in control (Con) and diabetic (Db) rats correlated with ischemic HIF-1 α protein accumulation. HIF-1 α protein in pre-ischemic control and diabetic hearts was minimal, and is shown adjacent to a positive control (hypoxic cells) and rainbow marker (Rb). * $p < 0.05$ versus control. A.U. = arbitrary units.

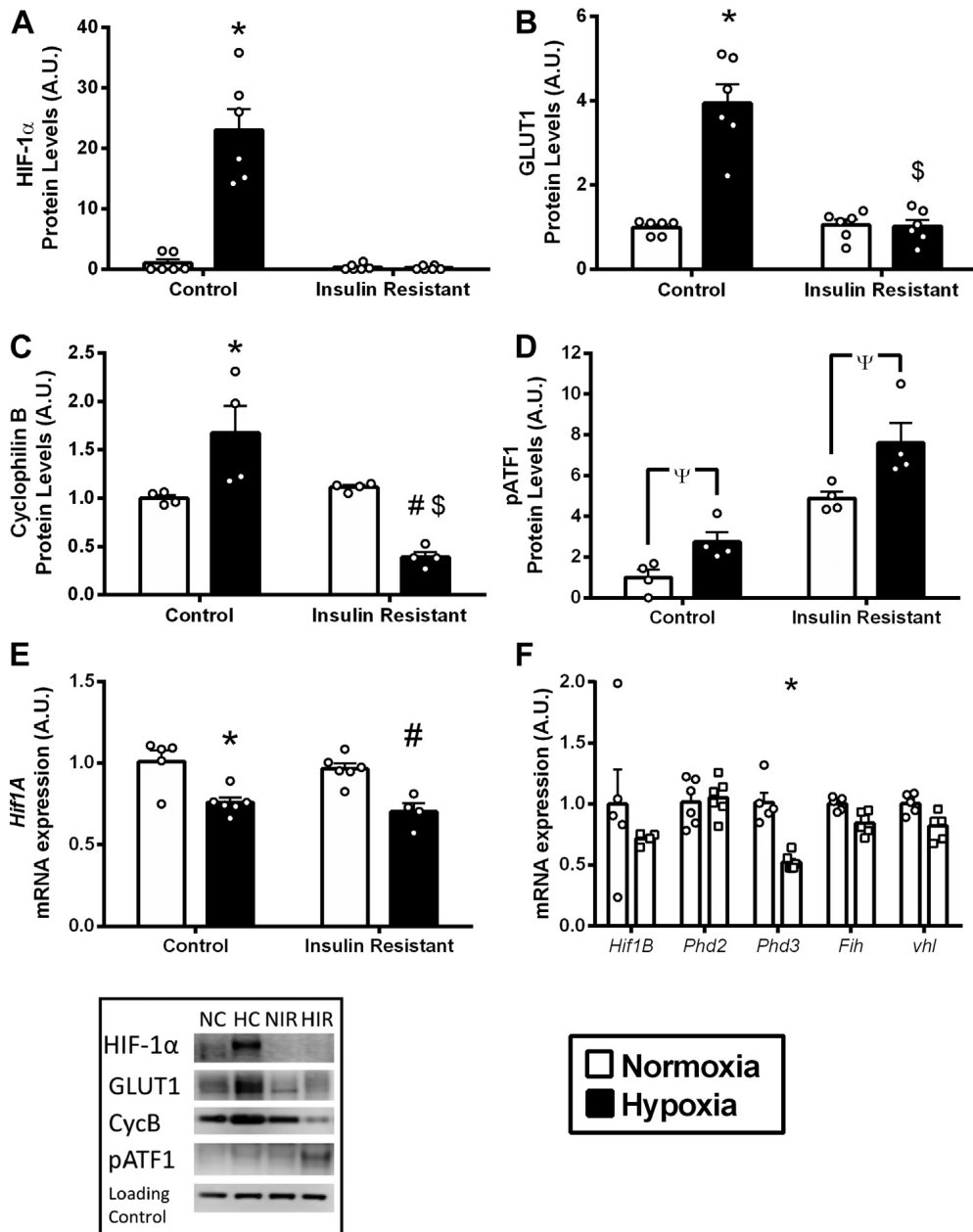


We investigated whether components of the HIF signaling pathway were changed in the IR cells, which would account for the lack of HIF-1 α protein stabilization in hypoxia. There was no difference in *Hif1A* mRNA expression between control and IR cells (Figure 3, Supplemental Figure 2). Components of the HIF regulatory pathway did not differ between IR and control cells in normoxia, including HIF-1 α heterodimer partner *Hif1B*, the oxygen-sensing enzymes prolyl hydroxylase 2 (*Phd2*) and asparaginyl hydroxylase factor inhibiting HIF (*Fih*), and the von Hippel-Lindau (*vhl*)-E3 ubiquitin ligase that targets HIF-1 α protein for proteasomal degradation. *Phd3* mRNA was

decreased in IR cells, but this would be predicted to increase HIF-1 α protein in hypoxia, not decrease it as observed in the IR cells.

FAs PREVENT HIF-1 α ACCUMULATION IN HYPOXIA IN A CONCENTRATION-DEPENDENT MANNER. IR was induced in our cells by a combination of hyperlipidemia and hyperinsulinemia. The component responsible for impaired HIF-1 α activation was investigated by treating cells with either 50 nmol/l insulin or 500 μ mol/l palmitate. Hyperinsulinemia alone did not affect HIF-1 α activation or the metabolic response to hypoxia (Figure 4). By contrast, hyperlipidemia suppressed HIF-1 α accumulation in

FIGURE 3 IR Impairs HIF-1 α Protein Accumulation and Downstream Signaling

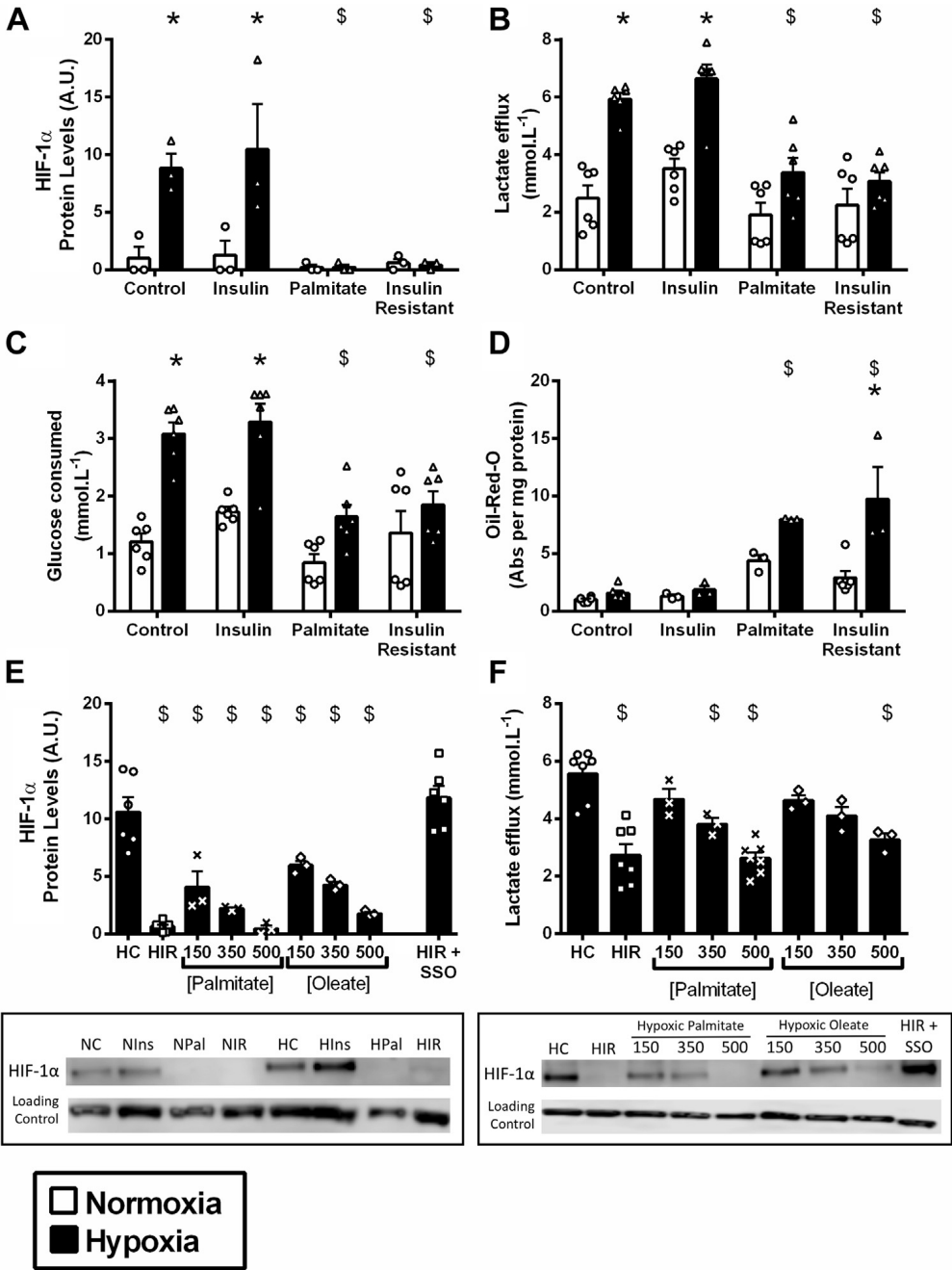


(A to C) HIF-1 α protein accumulation and downstream targets GLUT1 and cyclophilin B protein levels following normoxia and hypoxia, in control and insulin resistant cardiomyocytes. (D) Hypoxia-sensitive phospho-activating transcription factor (pATF) 1 protein levels following normoxia and hypoxia, in control and insulin-resistant (IR) cardiomyocytes. (E and F) mRNA expression of *Hif1A*, *Hif1B*, prolyl hydroxylase domain enzymes (*Phd*) 2 and 3, factor inhibiting HIF (*Fih*), and von-Hippel Lindau (*vhl*), in normoxic control and insulin resistant cardiomyocytes. * $p < 0.05$ versus normoxic control, # $p < 0.05$ versus normoxic insulin resistant, \$ $p < 0.05$ versus hypoxic control, $\Psi p < 0.05$ versus effect of oxygen at 2-way analysis of variance. A.U. = arbitrary units.

hypoxia, as exposure to palmitate alone reduced HIF-1 α to levels seen in IR cells. In addition, palmitate decreased the downstream HIF-mediated metabolic effects during hypoxia, decreasing lactate efflux,

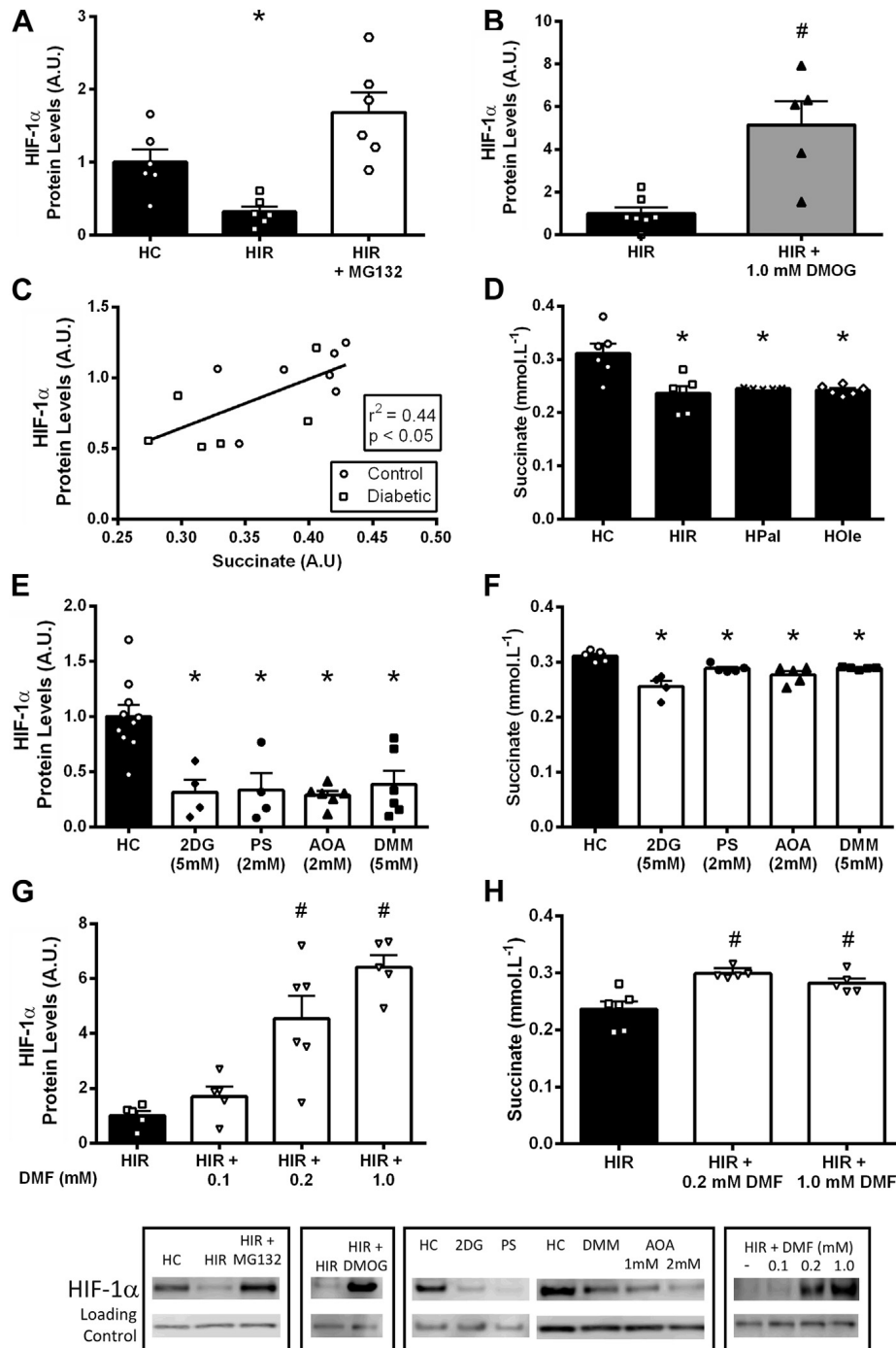
reducing glucose consumption and increasing lipid accumulation in hypoxia. To investigate whether changes were dependent on the concentration or saturation of the FA, cells were incubated with 150,

FIGURE 4 FAs Prevent HIF-1 α Accumulation in Hypoxia

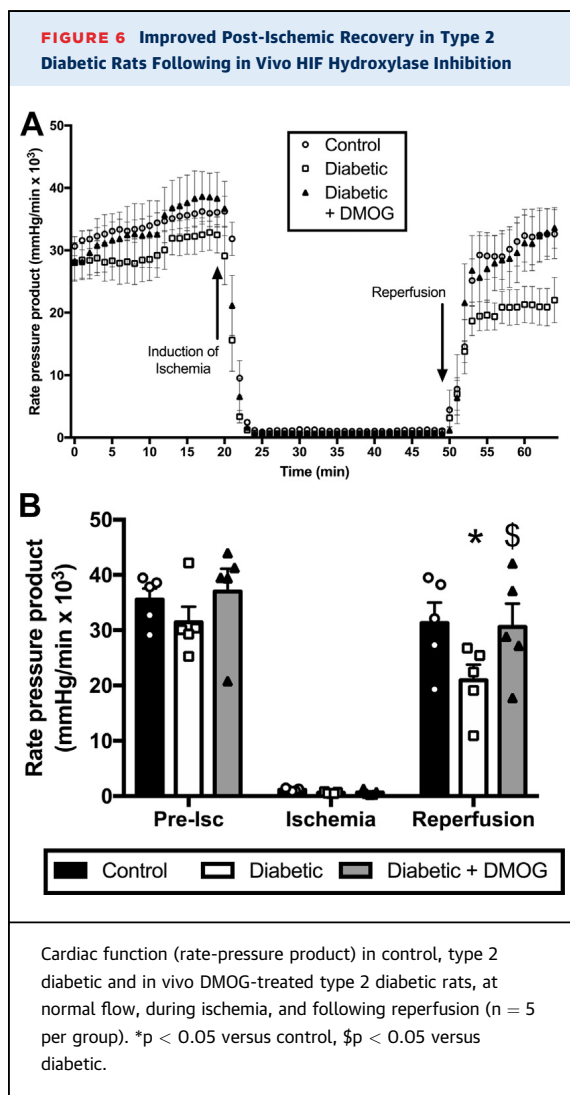


(A to D) HIF-1 α protein levels, lactate release, glucose consumption, and neutral lipid oil-red-O staining in normoxia and hypoxia in HL-1 cardiomyocytes cultured in control medium, insulin-resistant medium, and the 2 components that comprise the insulin-resistant medium: high insulin (50 nmol/l) and high palmitate (500 μ mol/l). (E and F) HIF-1 α protein levels and lactate release in hypoxic cells cultured in control medium (HC), insulin-resistant medium (HIR), and control medium supplemented with increasing concentrations of fatty acids (FAs) palmitate or oleate (bound to BSA). (E) HIF-1 α protein in hypoxic insulin-resistant cells when FA uptake was blocked with sulfo-*N*-succinimidyl oleate (SSO), immediately before the onset of hypoxia (HIR+SSO). **p* < 0.05 versus respective normoxic group, \$*p* < 0.05 versus hypoxic control. A.U. = arbitrary units.

FIGURE 5 Elevated FAs in IR Decrease Hypoxic Succinate Concentrations Required for HIF-1 α Stabilization



(A) HIF-1 α protein levels following acute hypoxia in control (HC) and IR (HIR) HL-1 cardiomyocytes with the proteasome inhibitor MG132. (B) HIF-1 α protein levels in hypoxic IR cells with or without the HIF hydroxylase inhibitor dimethylxylglycine (DMOG). (C) In ischemic perfused hearts, HIF-1 α protein levels correlate positively with myocardial succinate levels. (D) Succinate concentrations following hypoxia in cells cultured in control medium (HC), IR medium (HIR), and control medium supplemented with 500 μ mol/L palmitate (HPal) or 500 μ mol/L oleate (HOle). (E and F) HIF-1 α protein levels and succinate concentrations in hypoxic cells in the presence of the glycolysis inhibitor 2-deoxyglucose (2DG), the malate-aspartate shuttle inhibitors phenylsuccinate and amino-oxyacetate (PS and AOA), and the succinate dehydrogenase inhibitor dimethylmalonate (DMM). (G and H) HIF-1 α protein levels and succinate concentrations in hypoxia in IR cells, with or without the cell permeable succinate donor dimethyl fumarate (DMF). *p < 0.05 versus hypoxic control, #p < 0.05 versus hypoxic insulin resistant. Abbreviations as in Figure 4.



350, or 500 $\mu\text{mol/l}$ of palmitate or oleate, the 2 most abundant FAs in blood (29). The inhibition of HIF-1 α accumulation in hypoxia was proportional to the concentration of FA, and to the same extent whether palmitate or oleate were used. Consistent with the reduced HIF-1 α accumulation, there was a failure to increase glycolytic lactate efflux with FA concentrations of 350 $\mu\text{mol/l}$ and above. Finally, we added the sarcolemmal FA uptake inhibitor, SSO, to IR cells immediately prior to hypoxia. Blocking sarcolemmal fat uptake during hypoxia restored HIF-1 α accumulation (Figure 4), despite cells remaining IR (Supplemental Figure 1).

ELEVATED FAs DECREASE SUCCINATE CONCENTRATIONS, WHICH IS REQUIRED FOR HIF-1 α ACCUMULATION. To prevent HIF-1 α degradation, we inhibited the proteasome with MG132 in IR cells, and found that

proteasome inhibition restored HIF-1 α to control hypoxic levels (Figure 5), demonstrating the FA-induced defect was due to increased HIF-1 α targeting for degradation during hypoxia. HIF-1 α is targeted for degradation by the HIF hydroxylases, which are inhibited by low concentrations of oxygen. Pharmacologically inhibiting these HIF hydroxylases using DMOG during hypoxia significantly increased HIF-1 α accumulation in IR cells. Taken together, this demonstrates that in IR, HIF-1 α is being incorrectly targeted by the HIF hydroxylases for proteasomal degradation, which should be inhibited in hypoxia.

In cancer cells, in addition to low oxygen, HIF hydroxylases have also been shown to be inhibited by increased succinate concentrations, the product of their hydroxylation reaction (24,30). Returning to our ischemic hearts, myocardial levels of succinate correlated positively with HIF-1 α accumulation (control succinate 0.39 ± 0.02 , diabetic succinate 0.33 ± 0.03 ; p < 0.06) (Figure 5, Supplemental Table 2). In the hypoxic IR cells succinate concentrations were decreased by 24% compared with hypoxic controls, which could be replicated by culturing hypoxic cells with palmitate or oleate.

Succinate could be derived from the malate-aspartate shuttle utilizing glycolytic NADH, coupled to reverse Krebs cycle and succinate dehydrogenase activity (31). To investigate whether this pathway was responsible for regulating HIF-1 α stabilization in hypoxia, we pharmacologically inhibited multiple steps in this pathway. In hypoxia, inhibition of glycolysis using 2-deoxyglucose, inhibition of the malate-aspartate shuttle using phenylsuccinate or amino-oxycacetate, or inhibition of succinate dehydrogenase all decreased HIF-1 α stabilization to a similar extent. Thus, in hypoxia, succinate is derived from glycolysis driving malate-aspartate shuttle activity. FAs interfere with this process by suppressing glycolysis (Figure 4) and decreasing succinate concentrations (Figure 5). Culturing with the cell-permeable succinate donor, DMF (24), increased succinate concentrations in hypoxic IR cells. In addition, succinate supplementation with DMF increased HIF-1 α accumulation in hypoxic IR cells in a concentration-dependent manner, and at 1 mmol/l DMF to the same level as DMOG. Increasing succinate restored HIF-1 α accumulation in IR, overriding the inhibitory effects of FAs.

IN VIVO HIF HYDROXYLASE INHIBITION IS ABLE TO IMPROVE POST-ISCHEMIC RECOVERY IN TYPE 2 DIABETES. Finally, we questioned whether in vivo HIF hydroxylase inhibition could provide a

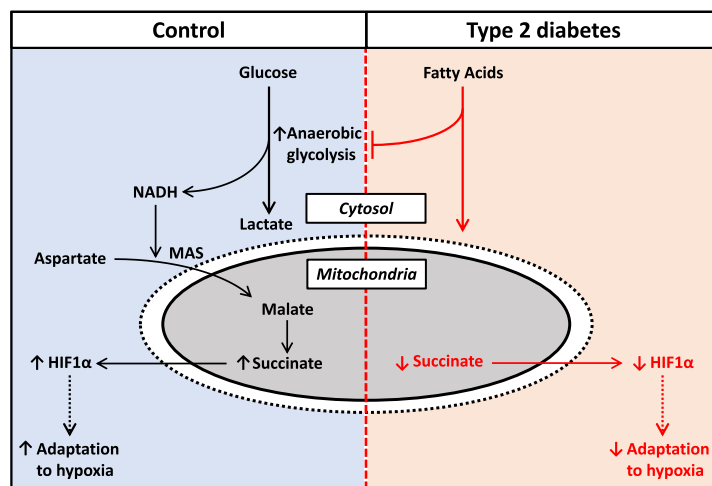
mechanism to improve post-ischemic recovery in type 2 diabetes. Type 2 diabetic rats were treated in vivo long term with the HIF hydroxylase inhibitor DMOG for 5 days, and after these 5 days, hearts were isolated, perfused, and challenged with ischemia (Figure 6). There were no differences in cardiac function between groups at normal flow or during low-flow ischemia. Untreated diabetic hearts had a 33% decrease in recovery of cardiac function following reperfusion compared with controls. By contrast, treating diabetic rats in vivo with DMOG improved cardiac function by 46% compared with untreated diabetic rats, restoring post-ischemic recovery to levels found in control hearts.

DISCUSSION

IR impairs cardiac HIF-1 α activation during ischemia and downstream adaptation to hypoxia. Here, we demonstrate that this is mediated by FA-induced inhibition of HIF-1 α protein accumulation. This is due to increased targeting of HIF-1 α for proteasomal degradation by the regulatory HIF hydroxylases. Mechanistically, this is mediated by decreased succinate concentrations from glycolytically driven malate-aspartate shuttle activity, with succinate supplementation restoring HIF-1 α signaling in IR. Finally, in vivo inhibition of the HIF hydroxylases restores post-ischemic recovery of cardiac function in diabetic rats.

Decreased HIF-1 α protein accumulation was evident in both diabetic hearts challenged with ischemia, and in IR cardiomyocytes exposed to hypoxia. HIF-1 α plays a vital role in the survival of cardiomyocytes following ischemia (2), and decreased HIF activation post-ischemia has been shown to accelerate the progression to heart failure in genetic mouse models (1), thus failure to fully activate HIF-1 α in diabetic hearts would translate to poorer long-term prognosis post-MI. FAs are the predominant fuel used by the heart to power contraction, accounting for 60% to 70% of cardiac ATP generation. In diabetes, FA oxidation and storage are up-regulated, and lipid intermediates accumulate within the myocardium (14,15). The IR-induced suppression of HIF-1 α accumulation was recapitulated by exposure to FAs. Palmitate and oleate are both long-chain FAs; the former is saturated and often implicated in oxidative damage and apoptosis, whereas the latter is unsaturated and does not induce these deleterious effects (32). Both palmitate and oleate induced the same degree of

FIGURE 7 Schematic Detailing the Proposed Mechanism for HIF-1 α Suppression in Diabetes



In control cells, hypoxia drives increased anaerobic glycolysis, malate-aspartate shuttle (MAS) activity, and reverse Krebs cycle activity. The increased succinate concentrations promote HIF-1 α protein stabilization (via inhibition of the HIF hydroxylase enzymes), driving optimal adaptation to hypoxia. In diabetic cells, increased fatty acids in hypoxia inhibits anaerobic glycolysis, resulting in suppressed succinate concentrations and decreased HIF-1 α protein stabilization, blunting the adaptation to hypoxia.

HIF-1 α suppression and in a concentration-dependent manner, demonstrating that this is a conserved response between the 2 most abundant FAs in blood. The relationship between FAs and HIF-1 α was further confirmed pharmacologically using the FA uptake inhibitor SSO, which was able to restore HIF-1 α accumulation in IR. This study shows for the first time that FAs can regulate HIF-1 α stabilization and downstream signaling, and although we demonstrate this effect in type 2 diabetes, this may also have relevance in any condition of increased circulating lipids, for example, cardiovascular disease, familial combined hyperlipidemia, or starvation.

In normoxia, HIF-1 α is translated and immediately targeted for proteasomal degradation by a family of HIF hydroxylases, which hydroxylate HIF making it a substrate for destruction by the von Hippel-Lindau ubiquitin-proteasome pathway. These HIF hydroxylases, PHDs and FIH, use oxygen as their substrate, thereby in hypoxia their activity is blunted, and HIF-1 α escapes hydroxylation and degradation, instead migrating to the nucleus. IR did not modify HIF-1 α mRNA, demonstrating the inhibitory effect of FAs was not mediated via changes in expression of this

transcription factor. Instead, HIF-1 α protein in IR could be rescued by inhibiting the proteasomal degradation pathway or by additional pharmacological inhibition of the HIF hydroxylases. These data pointed towards the fact that (at 2% oxygen) the HIF hydroxylases are inhibited by more than just low oxygen but also by an additional factor, which was absent in the IR cells.

The HIF hydroxylases are α -ketoglutarate-dependent dioxygenases, which generate CO₂ and succinate as products of their reaction. In cancer cells, mutations in Krebs cycle enzymes result in accumulation of succinate, which via product inhibition inhibits the HIF hydroxylases, causing HIF-1 α accumulation (30). Studies have shown that succinate can inhibit the HIF hydroxylases to promote HIF-1 α accumulation, independently of oxygen concentration (24,30,33). In healthy hearts, myocardial succinate levels increased 5-fold in response to acute hypoxia (19), and others have shown that in response to ischemia succinate levels increased 4-fold (31). By contrast, we have shown previously that in diabetic hearts, this hypoxia-induced succinate accumulation was blunted (19). In the present study, we demonstrate that this is due to FAs, because culturing cells with palmitate or oleate blunted succinate accumulation. This is further reinforced by findings that pharmacologically suppressing FA uptake in the diabetic heart increases multiple metabolites within the second span of the Krebs cycle (19), and restored HIF-1 α accumulation. Replenishing succinate was able to restore HIF-1 α accumulation in the presence of FAs, showing that the inhibitory effect of FAs on HIF-1 α accumulation could be overcome. This signaling role for succinate, linking metabolism to HIF regulation, occurs during the hypoxic challenge, and provides a mechanism to regulate the transcriptional response when oxygen is chronically restricted. There has been growing interest in the role of succinate, following its recognition as a key driver of reperfusion injury via reverse electron transport when oxygen supply is reinstated (31). These 2 roles for succinate are not mutually exclusive; when oxygen is limited, succinate is playing an adaptive HIF signaling role, but upon sudden restoration of oxygen the succinate is rapidly reoxidized, removing this metabolite signal (19) but with consequent effects on reactive oxygen species generation (31).

At low oxygen, increased glycolysis is coupled to increased malate-aspartate shuttle activity to accommodate the increased cytosolic NADH (31).

When oxygen availability is restricted, this mitochondrial malate is diverted backwards around the Krebs cycle resulting in the generation of succinate by reverse succinate dehydrogenase activity (Figure 7) (31). Here, we demonstrate that inhibition of any of these steps—glycolysis, malate-aspartate shuttle activity, or succinate dehydrogenase activity—is able to suppress HIF-1 α stabilization in hypoxia. In the present study and previous findings, we have shown that glycolysis and lactate efflux during hypoxia are blunted by diabetes and insulin resistance, facilitated by increased FA metabolism (16,19,21). This is mediated by FA-induced Randle cycle inhibition of key targets in glucose metabolism, which can be overcome by blocking FA metabolism (19,34). This FA-mediated decrease in glycolysis would result in decreased cytosolic NADH (35) and decreased malate-aspartate shuttle activity (36) as reported by others.

Phase 3 clinical trials are currently underway using HIF hydroxylase inhibitors for the treatment of anemia. Here, we demonstrate that this pharmacological approach, using the non-specific HIF hydroxylase inhibitor DMOG, was able to stabilize HIF-1 α in IR cells, and improve recovery of cardiac function post-ischemia in diabetic rats. Thus, by promoting HIF stabilization and by overriding the inhibitory effect of FAs on succinate, improvement in cardiac function could be achieved.

CONCLUSIONS

IR prevents cardiac HIF-1 α accumulation and downstream hypoxic adaptation in response to hypoxia. This is mediated by elevated FAs in diabetes suppressing succinate accumulation during hypoxia, and increasing succinate concentrations can override this inhibitory effect on HIF-1 α stabilization. Pharmacologically inhibiting the HIF hydroxylases promotes HIF-1 α accumulation and improves cardiac function following ischemia-reperfusion in diabetic rats.

ACKNOWLEDGMENTS The authors thank Prof. Kieran Clarke for continued mentorship. They thank Dr. Katja Gehmlich for assistance with the cell culture experiments.

ADDRESS FOR CORRESPONDENCE: Dr. Lisa Heather, Department of Physiology, Anatomy and Genetics, Sherrington Building, University of Oxford, Parks Road, Oxford OX1 3PT, United Kingdom. E-mail: lisa.heather@dpag.ox.ac.uk.

PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: Cardiovascular disease is the leading cause of death in patients with type 2 diabetes, with increased mortality and accelerated progression into heart failure following myocardial infarction. A greater understanding of why this occurs in diabetes and strategies to improve outcome are needed. We report that the lipid-enriched metabolic state of the diabetic heart prevents activation of the master transcription factor hypoxia-inducible factor, and the downstream adaptation to hypoxia needed for cardiomyocytes to survive in an ischemic/hypoxic environment. From a clinical standpoint, impaired activation of hypoxic pathways in diabetes would contribute to decreased recovery following a myocardial infarction.

TRANSLATIONAL OUTLOOK: For clinical translation, these observations in cells and animal models should be confirmed at the tissue level in humans with and without diabetes. Due to the exquisite oxygen sensitivity of hypoxia-inducible factor, a challenge for this translation will be how to obtain ischemic human tissue yet avoiding re-oxygenation of the sample. Pharmacologically activating hypoxia-inducible factor following myocardial infarction in diabetes is an exciting prospect from the present data, given that similar compounds are undergoing phase III clinical trials for the treatment of anemia. However, further basic research is required to confirm their repurposing for the diabetic heart.

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KEY WORDS cardiovascular disease, diabetes, fatty acids, metabolism, HIF-1 α , hypoxia

APPENDIX For supplemental tables and figures, please see the online version of this paper.



Contents lists available at ScienceDirect

BBA - Molecular Basis of Disease

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Review

Hypoxia-inducible factor 1 signalling, metabolism and its therapeutic potential in cardiovascular disease[☆]Maria da Luz Sousa Fialho^{a,*}, Amira H. Abd Jamil^b, George A. Stannard^a, Lisa C. Heather^a^a Department of Physiology, Anatomy and Genetics, University of Oxford, Oxford, UK^b Department of Pharmacy, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia

ARTICLE INFO

Keywords:

Metabolism
Hypoxia
HIF
Vascular
Diabetes
Infarction

ABSTRACT

Cardiovascular disease (CVD) accounts for the largest number of deaths worldwide, necessitating the development of novel treatments and prevention strategies. Given the huge energy demands placed on the heart, it is not surprising that changes in energy metabolism play a key role in the development of cardiac dysfunction in CVD. A reduction in oxygen delivery to the heart, hypoxia, is sensed and responded to by the hypoxia-inducible factor (HIF) and its family of proteins, by regulating the oxygen-dependent signalling cascade and subsequent response. Hypoxia is one of the main drivers of metabolic change in ischaemic disease and myocardial infarction, and we therefore suggest that HIF may be an attractive therapeutic target. In this review, we assess cardiac energy metabolism in health and disease, and how these can be regulated by HIF-1 α activation. We then present an overview of research in the field of hypoxia-mimetic drugs recently developed in other treatment fields, which provide insight into the potential of systemic HIF-1 α activation therapy for treating the heart.

1. Introduction

The healthy adult heart extracts between 8 and 70 ml of oxygen per minute per 100 g from the circulation, predominantly to power oxidative metabolism and energy generation. Oxygen is the terminal electron acceptor for oxidative phosphorylation, a process estimated to contribute 90% to cardiac energy generation. Under these normoxic conditions, optimal metabolism drives optimal function of the heart, with the average human adult heart pumping over 7000 l of blood per day.

Hypoxia, a decrease in oxygen availability, has the capacity to impact on metabolism, morphology and function of the heart. Hypoxia can arise as a consequence of both physiological and pathological stimuli. From a physiological perspective, the foetal heart develops in a

hypoxic environment, undergoing a hypoxic to normoxic transition at birth. The adult heart can equally be challenged physiologically by hypoxia, whether due to altitude exposure or in response to high intensity exercise. These stimuli induce whole body responses that can have a direct primary effect in the heart, as well as a secondary impact on the heart. From a pathological perspective, the heart may encounter hypoxia due to diseases of cardiac origin as well as diseases affecting peripheral tissues. Examples of the latter include situations as diverse as anaemia, lung diseases and sleep apnoea, which can all reduce oxygen delivery to the heart [1,2]. This review will focus on cardiovascular diseases that directly change oxygen availability for the cardiomyocytes, which induce regions of hypoxia within the myocardium, including myocardial infarction (MI) and heart failure (HF).

Abbreviations: 2-OG, 2-oxoglutarate; ADP, adenosine diphosphate; AMPK, AMP-activated protein kinase; ARNT, aryl hydrocarbon receptor nuclear translocator; ATP, adenosine triphosphate; C-TAD, C-terminal activation domain; CAT, carnitine acyltransferase; CBP, CREB binding protein; CODD, C-terminal oxygen-dependent degradation domain; CPT, carnitine palmitoyltransferase; DMOG, dimethylxalyl glycine; EPO, erythropoietin; ET1, endothelin 1; FABPm, fatty acid binding protein (membrane); FADH, flavin adenine dinucleotide H⁺; FAT, fatty acid translocase; FATP, fatty acid transporter protein; FIH, factor inhibiting HIF; GLUT, glucose transporter; HBS, HIF-binding sites; HIF, hypoxia-inducible factor; HLH, helix-loop-helix; HO1, haem oxygenase 1; HRE, HIF response elements; IFM, interfibrillar mitochondria; KO, knock-out; LCFA, long-chain fatty acids; LDH, lactate dehydrogenase; MCAD, medium chain acyl-CoA dehydrogenase; MCT, monocarboxylate transporter; N-TAD, N-terminal activation domain; NADH, nicotinamide adenine dinucleotide H⁺; NEFA, non-esterified fatty acid; NODD, N-terminal oxygen-dependent degradation domain; PAS, Per-ARNT-Sim; PDH, pyruvate dehydrogenase; PDK, pyruvate dehydrogenase kinase; PFK, phosphofructokinase; PGK, phosphoglycerate kinase; PHD, prolyl hydroxylase domain; PPAR, peroxisome proliferator-activated receptor; pVHL, von Hippel–Lindau tumor suppressor; ROS, reactive oxygen species; SSM, subsarcolemmal mitochondria; UCP, uncoupling protein; VEGF, vascular endothelial growth factor

[☆] “This article is part of a Special Issue entitled: The power of metabolism: Linking energy supply and demand to contractile function edited by Torsten Doenst, Michael Schwarzer and Christine Des Rosiers.”

* Corresponding author at: Department of Physiology, Anatomy and Genetics, University of Oxford, Parks Road, Oxford OX1 3PT, United Kingdom.

E-mail address: maria.sousafialho@dpag.ox.ac.uk (M.d.L. Sousa Fialho).

<https://doi.org/10.1016/j.bbadis.2018.09.024>

Received 30 May 2018; Received in revised form 24 August 2018; Accepted 18 September 2018

0925-4439/ © 2018 Published by Elsevier B.V.

The cellular mechanism that has evolved in response to hypoxia is highly evolutionarily conserved, present in organisms as diverse as Cnidarian sea anemones, cartilaginous fish and humans [3]. The major transcription factors responding to hypoxia and adapting the cell accordingly are the hypoxia-inducible factors (HIF). The hypoxia-inducible factors (HIFs) are a family of obligate heterodimeric transcription factors that play a key role in mediating cellular adaptation to hypoxia [4], first identified in erythropoietin-producing hepatoma cells [5]. Broadly, HIFs target genes adapt the cell to this new lower oxygen environment, by downregulating processes that consume oxygen, up-regulating anaerobic processes, and restoring normal oxygen delivery. In this review we will discuss the cellular mechanisms that sense and respond to hypoxia, the impact they have on metabolism, how this manifests in cardiovascular diseases, and the therapeutic potential for targeting hypoxia-inducible factor signalling in cardiac disease.

2. Hypoxia signalling

2.1. Hypoxia-inducible factor

HIFs are heterodimers composed of an unstable α -subunit (HIF α), of which levels and activity are tightly and acutely controlled by cellular oxygen concentrations, and a stable β -subunit (HIF β), which is a constitutively-expressed aryl hydrocarbon receptor nuclear translocator (ARNT) [6,7]. In hypoxia, protein levels of HIF- α are stabilized, and the protein travels to the nucleus to dimerise with its HIF- β partner (Fig. 1). The resultant HIF α/β complex binds to specific hypoxia response element (HRE) promoter regions of target genes, and regulates their expression [8].

Three HIF- α isoforms exist in humans: HIF-1 α , HIF-2 α and HIF-3 α . Of these, the first two are better characterised, forming the active

transcription factors HIF-1 and HIF-2 when bound to HIF- β subunits. While they share similar protein structures, the different isoforms serve distinct physiological roles, with unique target genes and mechanisms of action [9]. It has been shown in some cell lines that HIF-1 primarily mediates the acute hypoxic response (< 24 h), whereas HIF-2 drives the response during chronic hypoxic challenge (> 24 h) [10,11]. Thus the two isoforms can interact via a complex HIF 'switch' mechanism to regulate the cellular response to hypoxia, each with specific temporal and functional roles.

Both HIF- α and - β -subunits are bHLH/PAS transcription factors, with each comprising a bHLH (basic helix-loop-helix) motif and two hydrophobic PAS (Per-ARNT-Sim) motifs (PAS-A and PAS-B), which allow heterodimerisation, nuclear localization and DNA binding [7,12]. When HIF-1 α and - β -subunits bind and stabilise, this recruits transcriptional co-activators p300 and CREB-binding protein (CBP) [13,14] (Fig. 1). This is an additional oxygen-dependent interaction, which can be prevented by factor inhibiting HIF (FIH) [14]. HIF α contains a C-terminal transactivation domain (C-TAD) that recruits coactivator proteins to create a transcriptional complex (although some HIF-3 α splice variants have been described which lack a transactivation domain, instead possessing other inhibitory domains) [15,16]. In addition, HIF- α subunits contain a N-terminus transcriptional activation domain (N-TAD), as well as C- and N-terminal oxygen-dependent degradation domains (CDD and NDD, respectively), which allow oxygen-dependent hydroxylation and degradation [17].

2.2. Regulation of HIF

In normoxia, HIF- α protein subunits have a short half-life (< 5 min) since they are continually transcribed, translated and rapidly degraded [7]. This degradation is mediated by the post-translational

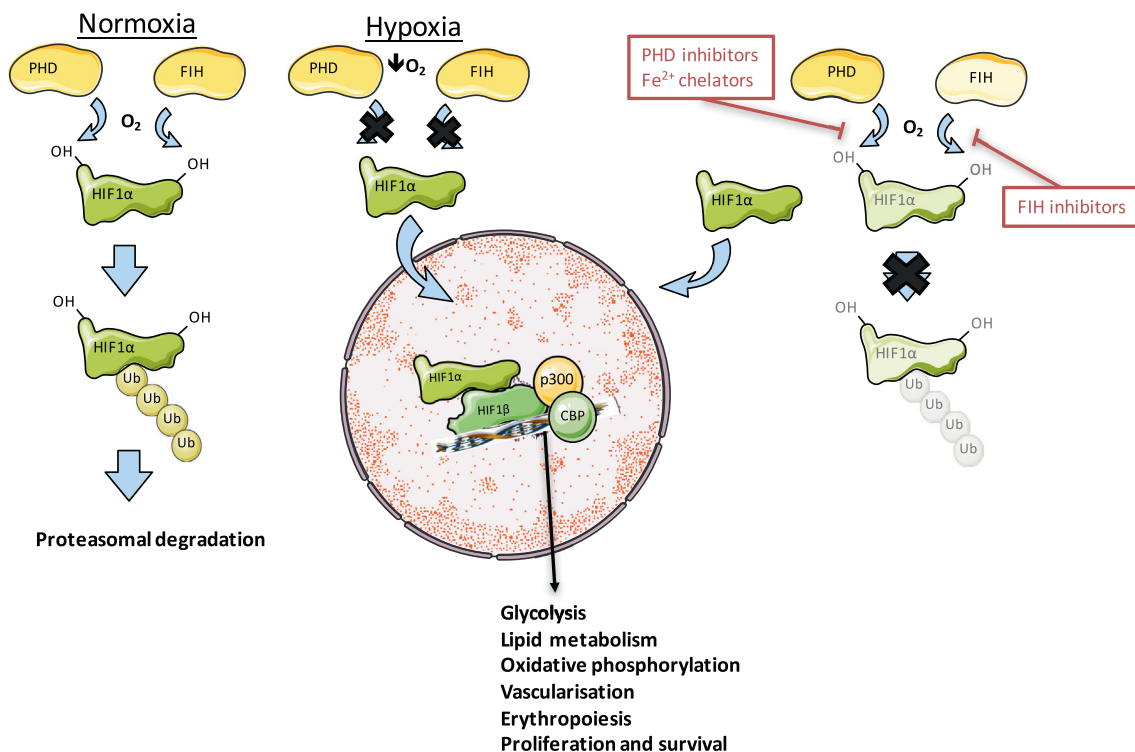


Fig. 1. The regulation of HIF-1 α . Under normoxic conditions, HIF-1 α is constantly synthesized, hydroxylated and degraded. This hydroxylation occurs through the action of Prolyl Hydroxylase Domain (PHD) and Factor Inhibiting HIF (FIH) enzymes, which are oxygen dependent. Once hydroxylated, HIF-1 α is then polyubiquitinated and undergoes subsequent proteasomal degradation. In hypoxia, the PHDs and FIH are inhibited by lack of their substrate oxygen so cannot hydroxylate HIF1 α , which is therefore stabilized and able to translocate to the nucleus, where it binds HIF1 β . This complex recruits co-activators of transcription p300 and CBP, which allows the initiation of transcription of target genes. Compounds developed as hypoxia-mimetics target either the PHDs or FIH, preventing hydroxylation of HIF-1 α and allowing for stabilisation and transcription of target genes under normoxic conditions.

hydroxylation of highly-conserved proline residues (Pro402 and Pro564 in HIF-1 α , Pro405 and Pro531 in HIF-2 α) within their NODD and CODD domains, by specific prolyl hydroxylase domain enzymes (PHDs) [18]. This hydroxylation forms a binding site for the von Hippel-Lindau tumor suppressor protein (pVHL), which forms the substrate recognition component of the multiprotein E3 ubiquitin ligase complex (alongside the transcription factors Elongin B and Elongin C, cullin-2 and ring-box 1) [19]. pVHL catalyses the polyubiquitination of specific lysine residues in HIF-1 α , marking it for proteasomal degradation [20]. Since the regulatory hydroxylases are oxygen-dependent, they are inhibited under hypoxic conditions, and hence pVHL does not bind to the HIF- α subunit in hypoxia to target it for degradation. This results in stabilisation and accumulation of HIF- α subunits, which can translocate to the nucleus to dimerise with HIF- β . Other signalling pathways have since been shown to interact with HIF, however the PHD-VHL-HIF axis is now widely-regarded as the primary control of the cellular hypoxic response [21–24].

The prolyl hydroxylase domain enzymes (also known as EGLN1-3) responsible for HIF prolyl hydroxylation exist as three different isoforms, PHD1-3. These three isoforms are near ubiquitously expressed, but with large differences in expression levels between tissues [25]. PHD1 is exclusively nuclear, with PHD2 predominantly located in the cytoplasm, while PHD3 can be found in both [11]. A fourth enzyme (P4H-TM), found in the endoplasmic reticulum, permits *in vitro* HIF- α hydroxylation [23]. On a tissue level, PHD2 and 3 are more highly expressed in the heart relative to other tissues [26]. The different PHD isoforms manifest different affinities and specificities for each HIF isoform. PHD2 and PHD3 mRNA levels are increased by hypoxia [26,27], and are transcriptional targets of HIF-1, forming a feedback loop in hypoxia [28]. The expression of multiple isoforms of PHDs in the same tissue has raised questions regarding differences in regulation and targets [29]. It has been postulated that PHD2 likely acts as the primary rate-limiting HIF prolyl hydroxylase in normoxia, while the other PHD isoforms affect HIF activity during chronic hypoxia. Interestingly, PHD2 silencing has been shown to impact to a greater extent on HIF-1 α , whereas PHD3 impacts on HIF2 α [29].

The prolyl hydroxylases form part of the non-heme Fe(II) and 2-oxoglutarate (2OG)-dependent dioxygenase superfamily, with a conserved two-histidine-one-carboxylate Fe(II) coordination motif, two positions for binding 2-OG and one for molecular oxygen. Each oxygen bound is split in two; one atom is used to catalyse the oxidative decarboxylation of 2-oxoglutarate (α -ketoglutarate) into succinate and CO₂ and the other is incorporated into the hydroxylated amino acid residue of HIF- α [30] (with a highly-reactive ferryl intermediate formed during the process). The unusually slow reaction of the hydroxylase with oxygen, as demonstrated kinetically, is thought to be a key factor in facilitating the enzyme's role as a hypoxia sensor [31].

HIF is also regulated by a second round of post-translational modification, which affects its transcriptional activity rather than its stability. Another independent Fe(II)- and 2-oxoglutarate dependent dioxygenase, FIH, catalyses the hydroxylation of conserved asparagine residues in the HIF- α CAD (N803 in HIF-1 α , N847 in HIF-2 α) under normoxic conditions. This asparagine hydroxylation of HIF α likely results in a direct steric clash with the coactivator protein p300 necessary for transcriptional expression of most HIF target genes. Like the PHDs, FIH is also inhibited by hypoxia, but to a lesser extent, so it may perform a 'fine-tuning' role, to inhibit HIF- α proteins not targeted by PHDs in moderate hypoxia [32,33]. Furthermore, FIH exerts a stronger inhibitory effect on HIF-1 α than it does on HIF-2 α , which suggests that some HIF target genes require a greater level of hypoxia than others to be expressed [34].

2.3. Regulatory control of HIF hydroxylases

Studies suggest that the prolyl hydroxylases and FIH may also be controlled at several levels independently of oxygen tension, allowing

greater flexibility within the hypoxic response. Nitric oxide donors may be used to competitively inhibit the HIF hydroxylases *in vitro* and increase their transcriptional activity [35], however, recent studies have produced conflicting results [36,37]. As the HIF hydroxylases are Fe-dependent dioxygenases, changes in Fe levels have been shown to modulate HIF- α stabilisation [38]. Of interest, levels of Krebs cycle intermediates, particularly succinate (the product of hydroxylase reaction) and fumarate, exert regulatory effects on the HIF hydroxylase enzymes. Mutations in Krebs cycle enzymes in cancer provide the strongest evidence to date: genetic defects in succinate dehydrogenase elevate succinate levels and upregulate HIF- α in normoxia, associated with oncogenesis [39]. Similarly, mutations in fumarate hydratase (fumarase) stabilise HIF-1 α , creating a state of pseudohypoxia [40,41]. We have recently shown this Krebs-cycle intermediate-dependent phenomenon in the heart, whereby changes in myocardial succinate in hypoxia regulate cardiac HIF-1 α stabilisation [42]. The proposed mechanism linking abnormal Krebs cycle metabolism to HIF hydroxylase inhibition is via feedback inhibition of the regulatory enzymes.

2.4. HIF target genes

HIF α / β complex binds to hypoxia response elements (HREs) at target gene loci, regulating expression of a network of genes involved in the hypoxic response on both a cellular and systemic level. The HREs share one or more common binding sites for HIF, known as HIF-binding sites (HBSs) [43], as well as neighbouring DNA binding sites for additional transcription factors, which must also be bound to allow gene transactivation. The mandatory core consensus sequence within the HBS of the HRE is NCGTG (where N is A or G). Epigenetic mechanisms, such as CpG methylation [44] and oxidative DNA damage caused by reactive oxygen species (ROS) also affect HIF binding to the HRE [45].

HIF has been shown to be a master transcription factor, regulating many hundreds of target genes in response to hypoxia [46]. Broadly, HIF targets are aimed at adapting the cell to this new lower oxygen environment, downregulating processes that consume oxygen, upregulating anaerobic processes, as well as aiming to restore normal oxygen delivery.

The hypoxia-responsive genes may respond exclusively to HIF-1, to HIF-2, or respond to both. HIF-1 preferentially promotes the expression of genes encoding enzymes involved in the glycolytic pathway such as phosphoglycerate kinase (PGK) and lactate dehydrogenase A (LDHA). HIF-2, on the other hand, preferentially targets genes that are involved in invasion, for example Oct-4 and TGF- α [47]. However, HIF-1 and -2 also share some overlapping target genes, including those that encode vascular endothelial growth factor A (VEGFA) and glucose transporter 1 (GLUT1) [47]. Moreover, the two isoforms demonstrate an ability to compensate for the absence of the other by performing each other's isoform-specific functions [48,49]. Given the tissue-specific isoform expression patterns of both the HIF transcription factors and their regulatory HIF hydroxylases, coupled with different degrees of hypoxia between various tissues, differences in gene expression have been identified between tissues and between hypoxia-associated diseases.

In addition to upregulating target genes, HIF activation has been shown to decrease expression of hundreds of genes. Examples of this include genes that drive oxygen consumption and oxidative metabolism [50,51]. In a recent study, there was no significant association between the presence of an HBS and downregulation of the gene by hypoxia, demonstrating that the downregulation of target proteins is via indirect mechanisms [46].

Metabolism is a major target for adaptation following HIF activation, as oxygen is the terminal electron acceptor in oxidative phosphorylation. During hypoxia, cellular metabolism is shifted away from the oxygen-dependent process of oxidative phosphorylation, to favour the oxygen-sparing glycolytic pathway [52]. This allows cells to generate ATP in conditions of lower oxygen tensions. HIF regulates the expression of multiple genes that promote oxygen delivery through

increased erythropoiesis (such as erythropoietin) and angiogenesis (such as VEGF), to try to restore the cell back to its previous normoxic environment [53,54].

3. Metabolism in the healthy heart

In order to maintain ceaseless contractile activity, the heart requires a constant supply of energy, in the form of ATP. The human heart synthesises approximately 6 kg of ATP daily [55], generated predominantly via mitochondrial oxidative phosphorylation [56]. The heart is a metabolic omnivore, capable of metabolising various substrates including fatty acids, glucose, pyruvate and lactate, to satisfy its energy demands [57,58]. Cardiac fuel substrate selection is dynamic and may be altered according to the prevailing physiological or pathophysiological conditions. This metabolic flexibility allows the heart to maintain ATP production under varying physiological conditions, and is primarily regulated by substrate concentration, hormone levels, coronary blood flow, oxygen supply and cardiac workload [59,60]. Fatty acids yield more ATP per carbon atom than glucose, pyruvate or lactate, as a result, the healthy heart preferentially uses fatty acid as a fuel source under steady state conditions [55]. In the healthy heart, fatty acid metabolism accounts for approximately 60–70% of energy metabolism in the fasted state [61]. However, in response to feeding, insulin-stimulated glucose metabolism becomes the predominant fuel. This metabolic flexibility between fuels allows the heart to match substrate use to the physiological conditions.

3.1. Fatty acid uptake and metabolism

Long chain fatty acids (LCFA) are hydrophobic and insoluble in aqueous environments, so they are transported to the heart bound to albumin as non-esterified fatty acids (NEFAs) or esterified to triacylglycerol and incorporated into lipoproteins, such as chylomicrons [62,63]. The uptake of NEFAs into myocardial cells can occur passively across cell membranes, but primarily uptake is mediated by transporter mechanisms involving fatty acid translocase (FAT/CD36), plasma membrane fatty acid binding protein (FABPpm) and fatty acid transport proteins (FATP1 and 6, Fig. 2). Cytosolic fatty acids are esterified by acyl-CoA synthetase on the mitochondrial membrane, forming long chain fatty acyl coenzyme A. Carnitine palmitoyltransferase-1 (CPT1) in the mitochondrial intermembrane space substitutes the CoA group of the long chain acyl-CoA with carnitine, and carnitine acyltransferase (CAT) shuttles fatty acyl-carnitine across the inner mitochondrial membrane in exchange for free carnitine. In the inner membrane, CPTII converts the fatty acyl-carnitine back to long chain fatty acyl-CoA in the mitochondrial matrix. CPT1 serves as a key regulatory step in mitochondrial fatty acid oxidation, due to its allosteric inhibition by malonyl CoA (derived from acetyl CoA carboxylase in the fatty acid synthetic pathway). The long chain fatty acyl-CoA undergoes β -oxidation in the mitochondrial matrix, resulting in the production of acetyl-CoA and reduced cofactors NADH + H^+ and FADH₂, which enter the electron transport chain to produce ATP [56,64].

3.2. Glucose uptake and metabolism

Glucose is transported into the cytosol by glucose transporters, including GLUT1 and GLUT4 (Fig. 3). GLUT1 is responsible for basal glucose uptake and constitutively present in the sarcolemma [65], whereas GLUT4 cycles between the sarcolemma and intracellular vesicles in response to insulin and AMP-activated protein kinase (AMPK) activation, and therefore serves as the predominant regulator of glucose uptake [66]. When glucose is taken up in cardiomyocytes, it is rapidly phosphorylated by the enzyme hexokinase to glucose-6-phosphate, which subsequently can enter many metabolic pathways including glycolysis, glycogenesis, the pentose phosphate and hexosamine biosynthetic pathways. Glycolysis represents a major pathway for glucose

utilization in the heart. Glycolysis generates pyruvate, NADH + H^+ and a small quantity of ATP via substrate level phosphorylation, through a series of cytosolic reactions that can operate under oxygen-independent conditions. PFK is a key regulatory enzyme in the glycolytic pathway, allosterically regulated by the cellular energy status and metabolite concentrations [67,68]. The end product of glycolysis, pyruvate, can either enter the mitochondria for oxidation or be reduced to lactate in the cytosol. Under aerobic conditions, pyruvate is decarboxylated to acetyl-CoA within the mitochondria, prior to entry into the Krebs cycle for glucose oxidation (Fig. 3). Mitochondrial pyruvate dehydrogenase (PDH) catalyses this oxidative decarboxylation of pyruvate, and is the key regulated enzyme complex in glucose metabolism [69]. PDH is phosphorylated and inhibited by pyruvate dehydrogenase kinase (PDK) enzymes 1–4, which are activated by the NADH + H^+ and acetyl-CoA. In contrast, PDH is dephosphorylated and activated by pyruvate dehydrogenase phosphatase (PDP), which is activated by insulin and calcium [70]. Under anaerobic conditions, pyruvate is reduced to lactate for export by the action of lactate dehydrogenase, utilizing glycolytically-derived NADH + H^+ .

3.3. Myocardial energetics

Glucose and fatty acid metabolic pathways converge at acetyl-CoA, which enters into the mitochondrial Krebs cycle, generating mitochondrial NADH + H^+ and FADH₂. These reducing equivalents donate electrons that are shuttled along the electron transport chain (ETC) in the inner mitochondrial membrane between complexes I to IV. The electrons are ultimately donated to oxygen at complex IV, producing H₂O. Concomitantly, complex I, III and IV of the ETC utilize the energy released in these redox reactions to pump protons into the mitochondrial intermembrane space, generating an electrochemical gradient across the inner membrane. This gradient is the driving force for the phosphorylation of ADP to ATP by F₁F₀ ATP synthase [71,72].

4. Metabolism in the hypoxic heart

Given that the majority of ATP required by the heart is generated by mitochondrial oxidative phosphorylation, oxygen plays a critical role in cardiac energy metabolism. When oxygen availability is limited, the heart is metabolically remodelled, and shifts away from oxygen-demanding substrates to more oxygen-efficient fuels. Although long chain fatty acids are more energy dense than glucose (ideal for making large amounts of ATP), they also need large amounts of oxygen to generate that ATP. In contrast, complete metabolism of glucose yields 11% more ATP per oxygen atom consumed than fatty acids [55]. Collectively, many studies demonstrated that chronic hypoxia triggers regulatory pathways that mediate cardiac metabolic remodelling, particularly at the transcriptional level, to sustain energy production and contractile function under oxygen-limited conditions [73,74]. This includes adaptation to hypoxia in the context of a myocardial infarction (MI), but also chronic disruptions of oxygen delivery, such as chronic intermittent hypoxia, sleep apnoea, or anaemia.

4.1. Fatty acid uptake and metabolism

Data on myocardial fuel substrate preferences in humans exposed to chronic hypoxia is limited, however low-landers acclimatised to high-altitude displayed decreased reliance on fatty acid metabolism [75,76]. The most frequently used experimental model in animal studies of hypoxia are simulated in normobaric or hypobaric chambers, which have been employed most commonly in a simulated altitude of approximately 5500 m (11% oxygen). Molecular findings in response to chronic hypoxia indicated that the putative regulatory steps orchestrating substrate switching away from fatty acid metabolism may include a) attenuated sarcolemmal fatty acid uptake, b) reduced mitochondrial fatty acid uptake and oxidation, and c) increased malonyl-

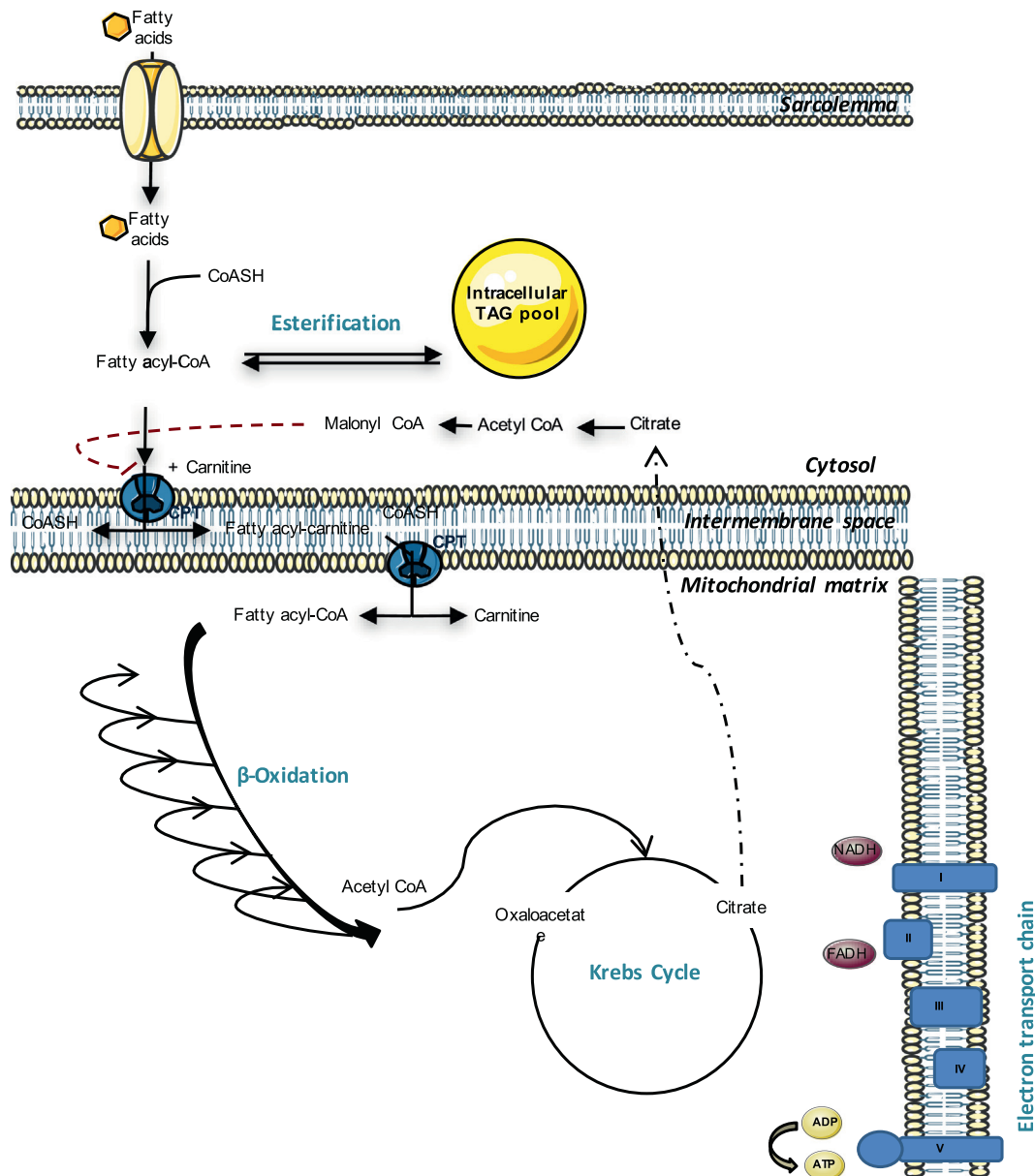


Fig. 2. Myocardial fatty acid utilization. Non-esterified fatty acids (NEFA) enter the cardiomyocyte either through passive mechanisms or through transporters such as FAT/CD36, FABPm and FATP. NEFA can then undergo esterification to fatty acyl-CoA and enter the cellular triacylglyceride pool or enter the mitochondria. Transportation into the mitochondria occurs through CPT1, using carnitine. This can be allosterically inhibited by malonyl-CoA, as a feedback inhibition mechanism. Once in the mitochondrial matrix, fatty acyl-CoA can undergo β -oxidation, producing acetyl-CoA to be used in the Krebs cycle, and reduced cofactors NADH + H⁺ and FADH₂, which enter the electron transport chain to produce ATP [56,64].

CoA levels, allosterically inhibiting CPT1 activity.

Chronically hypoxic rat hearts have decreased protein levels of FATP1 [77] which could potentially decrease the capacity for fatty acid uptake across the sarcolemma. Three weeks exposure to 11% oxygen, markedly decreased β -oxidation rates in both mouse [73] and rat [77] hearts. Cardiac mitochondria have decreased ADP-stimulated state 3 respiration with fatty acid substrates following chronic hypoxia [78]. This is in agreement with decreased enzyme activity for fatty acid oxidation enzymes: CPT1, β -hydroxy-acyl-CoA dehydrogenase [79,80] and medium-chain acyl coenzyme A dehydrogenase (MCAD) [81].

Hypoxia-induced cardiac fatty acid metabolic remodelling is tightly regulated by the nuclear transcription factor peroxisome proliferator-activated receptor alpha (PPAR α). PPAR α has been shown to negatively correlate with blood haemoglobin content in high altitude tolerant humans [82], and hypoxic animal models [81,83]. PPAR α transcriptionally regulates numerous genes that control cardiac fatty acid

mitochondrial import and oxidation, including FATP1, MCAD and CPT1 [84,85], which were shown to be down regulated in parallel with decreased PPAR α [73,81] expression following 3–5 weeks of chronic normobaric hypoxia (Fig. 4). Decreased PPAR α expression and CPT1 have also been reported in other models of systemic hypoxia, induced by chemical induction using cobalt chloride treatment and isovolaemic dilution [86]. In rat cardiomyocytes, HIF-1 was involved in hypoxia-induced suppression of fatty acid metabolism by decreasing the DNA binding activity of PPAR α and its coactivator retinoid X receptor (RXR) [87]. PHD1-deficient mice, a model with chronic HIF upregulation, have increased PPAR α expression in skeletal muscle [88]. In HL-1 cardiomyocytes, hypoxia or prolyl hydroxylase inhibition increased both HIF-1 α and HIF-2 α , and decreased PPAR α and its target genes [73]. Metabolic remodelling mediated by PPAR α downregulation was shown to be essential to maintain ATP production and cardiac function during chronic hypoxia; and the disruption of metabolic adaptation via

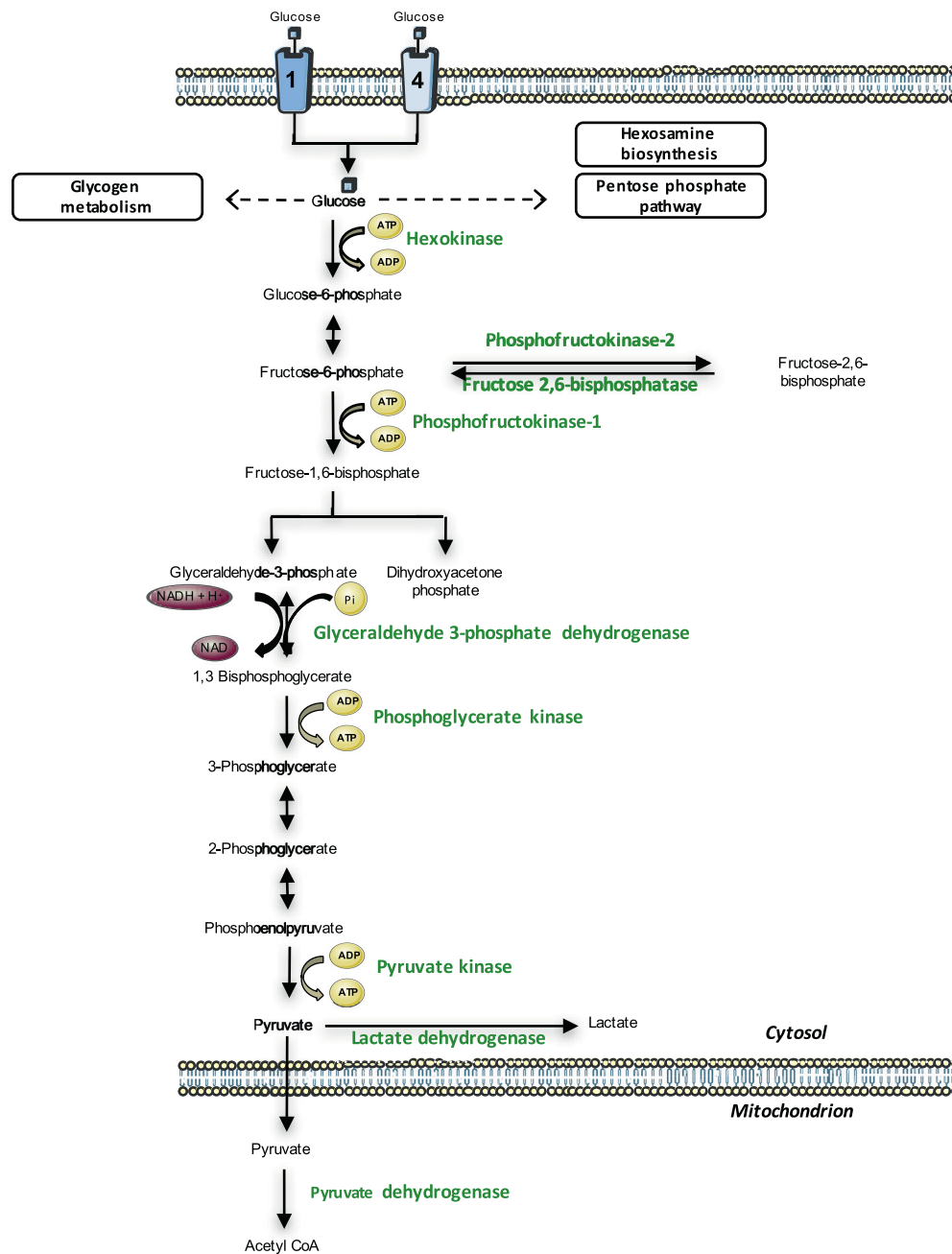


Fig. 3. Myocardial glucose utilization. Glucose enters the cardiomyocyte through glucose transporters GLUT1 and 4. It is then rapidly phosphorylated by the enzyme hexokinase to glucose-6-phosphate, which subsequently enters many metabolic pathways including glycolysis, glycogenesis, the pentose phosphate and hexosamine biosynthetic pathways, though glycolysis is the main pathway for glucose use in the heart. Glycolysis produces a small quantity of ATP anaerobically, as well as generating pyruvate, which can enter the mitochondria for oxidation. Mitochondrial pyruvate dehydrogenase (PDH) catalyses the oxidative decarboxylation of pyruvate, and is the key regulated enzyme complex in glucose metabolism.

manipulating PPAR α , achieved by high fat feeding and genetic ablation of PPAR α , impaired hypoxic adaptation and resulted in cardiac dysfunction [73].

4.2. Glucose uptake and metabolism

In human, both high-altitude adapted natives and low-landers exposed to altitude simulations displayed enhanced cardiac glucose uptake [89,90]. In rodents, exposure to chronic hypoxia increased glucose uptake [91] and the activities of cardiac glycolytic enzymes including hexokinase [79,92], pyruvate kinase, PFK (both PFK-1 and the inducible form of the enzyme, PFK-2) [79,93] and LDH [79,94], parallel to augmented glycolytic flux [73,77]. Many of these glycolytic targets have been demonstrated in non-cardiac tissue to be driven directly by the HIF system [95]. Findings on glucose transporters have been inconclusive in the heart; mRNA expression of GLUT1 was not altered after 4 weeks of chronic hypoxia [83] but at the protein level GLUT1

has been reported to increase after 2 weeks, while GLUT 4 protein levels remain unchanged [73,96,97].

Many studies have indicated that in non-cardiac tissues glycolysis is uncoupled from the Krebs cycle in hypoxia, via transcriptional activation of PDK1 driven by HIF [98,99]. PDK1 phosphorylates and inhibits PDH, directing the fate of pyruvate towards lactate production instead of oxidation. This hypoxia-induced metabolic switch shunts glucose derivatives away from the mitochondria. In heart this mechanism does not appear as clear-cut. Hypoxic HL1-cardiomyocytes have increased PDK1 protein levels [100], however, PDH complex activity was not changed in the heart in response to in vivo chronic hypoxia [101]. Cardiac PDK4 is consistently decreased in many hypoxia studies [83,86], as PDK4 is a downstream target of PPAR α .

Certainly, lactate efflux from the heart is increased in response to chronic hypoxia [73,97] (Fig. 4). This is supported by multiple studies which have shown higher expression of the plasma membrane lactate transporter, monocarboxylate transporter MCT4, as well as cardiac

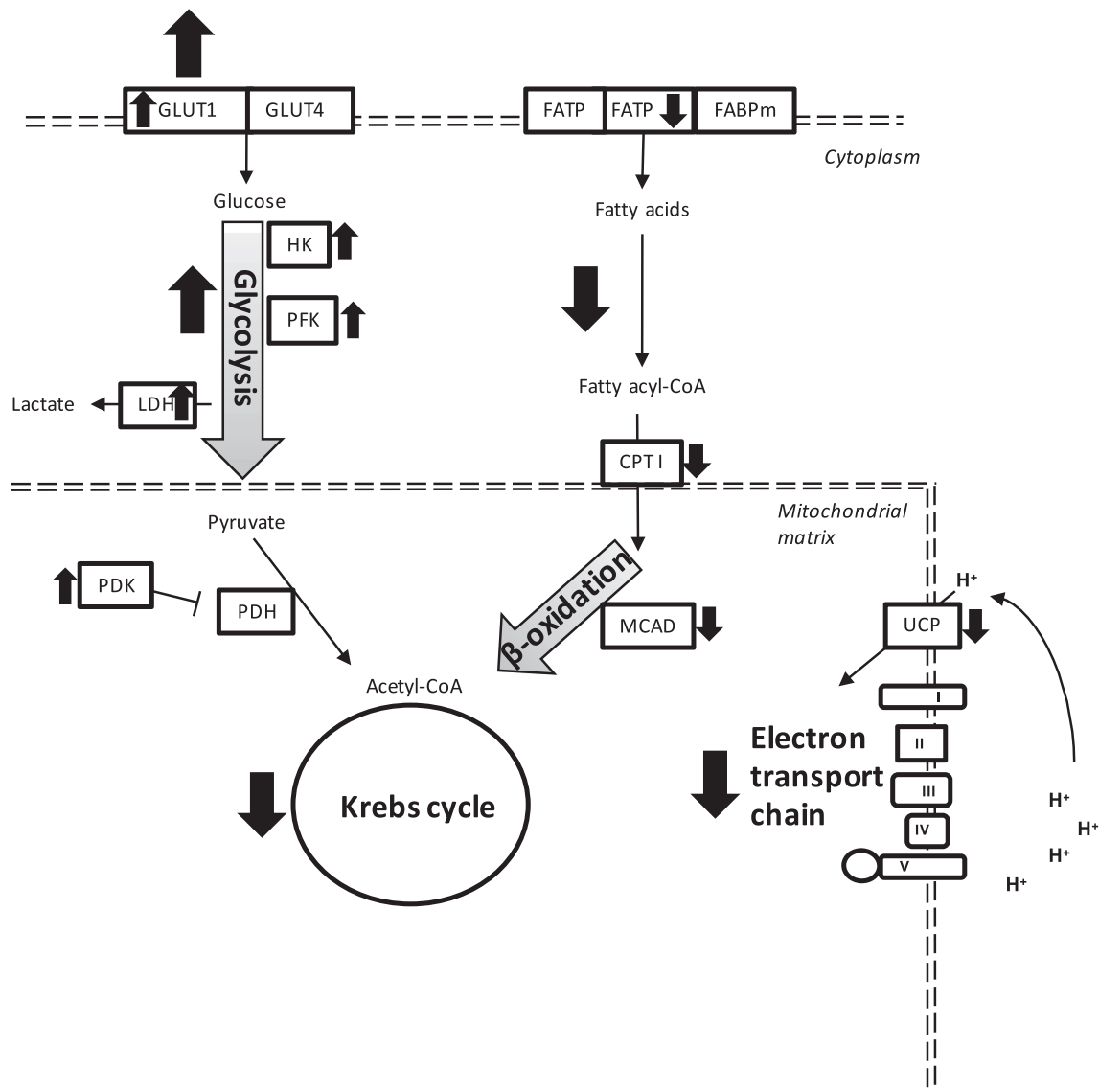


Fig. 4. Cellular metabolic effects of HIF-1. HIF-1 activation leads to the upregulation of a number of targets genes, many of which are involved in cellular metabolism. Key metabolic targets include glucose transporter 1 (GLUT1), hexokinase (HK), phosphofructokinase (PFK), lactate dehydrogenase (LDH), and pyruvate dehydrogenase kinase (PDK), all of which lead to an increase in glucose utilization, upregulating the glycolytic pathway. In addition, via activation of PPAR α , HIF-1 activation results in a decrease of proteins involved in fatty acid uptake and oxidation, namely fatty acid transport protein (FATP), carnitine palmitoyltransferase I (CPTI), medium chain acyl-CoA dehydrogenase (MCAD), and uncoupling proteins (UCP). Together, this results in an overall decrease in oxygen-demanding fatty acid oxidation. Consequently, the activity of the electron transport chain (ETC) is also downregulated by HIF-1 activation.

lactate dehydrogenase expression in response in vitro and in vivo hypoxia [102,103]. Increased net lactate efflux is proportional to the increase in glycolytic flux with hypoxia, thus the ratio of lactate production to glucose uptake was still maintained, indicative of unchanged pyruvate oxidation rate [73,77].

4.3. Mitochondrial respiration and myocardial energetics

Following chronic in vivo hypoxia, decreased isolated mitochondrial respiration with fatty acid and pyruvate substrates has been consistently reported [78,104,105]. We have shown previously that in response to hypoxia not all mitochondria within the heart adapt in the same way, and that glutamate respiration is decreased in subsarcolemmal mitochondria (SSM) but not in interfibrillar mitochondria (IFM) [78]. This is because SSM adapt to hypoxia by modulating their electron transport chain activity, whereas IFM modulate their Krebs cycle, upstream of where glutamate derivatives enter the Krebs cycle.

Hypoxia decreases enzymatic activities of electron transport chain complexes I, II and IV in SSM, and aconitase in IFM [106]. The decrease of complex I might serve to decrease ROS generation but potentially at the cost of decreased ATP synthesis [104]. The mitochondrial inner membrane uncoupling protein UCP3 was decreased by hypoxia [73,77,105], another PPAR α target gene [56] (Fig. 4). The reduction in UCP3 with hypoxia may serve to increase mitochondrial efficiency of oxidative phosphorylation, either directly or via its involvement in fatty acid oxidation. Indeed, mitochondrial changes may underlie the observed depression in cardiac energetics following hypoxia.

In non-cardiac tissue, HIF maintains respiratory efficiency by targeting other mitochondrial proteins, by inducing a complex IV cytochrome c oxidase (COX) 4-1 to COX4-2 subunit switch [50], by inhibiting expression of ISCU, an iron-sulfur cluster assembly factor that is required for activity of aconitase and electron transport complex I [109], and by inducing BNIP3, which triggers mitophagy following prolonged hypoxia [110].

In humans, changes in myocardial energetics have been reported in response to chronic hypoxia. Acclimatisation to a normobaric hypoxia chamber decreased cardiac phosphocreatine to ATP (PCr/ATP) ratios by 15% [111]. Similarly, 11 days at altitude of 5300 m at Everest Base Camp decreased PCr/ATP ratio by 18% [112]. In Sherpas indigenous to living at altitude, cardiac PCr/ATP ratios were significantly lower than native lowlanders, even after a month at low altitude [113]. In animal models, decreased cardiac PCr/ATP ratios in chronically hypoxic mouse hearts [73,114,115] was also observed as a result of decreased phosphocreatine levels, with a subsequent increase in cytosolic ADP. Therefore, metabolic remodelling in hypoxia and activation of the HIF system is a fundamental adaptation of the heart to ensure continuous ATP production, by guiding substrate switching towards anaerobic glycolysis. The need for this metabolic flexibility is particularly important in the context of the current epidemic of cardiovascular disease.

5. Cardiovascular diseases and metabolism

Cardiovascular disease is the leading cause of death worldwide, and accounts for approximately 17.3 million deaths per year [116]. Myocardial infarction occurs when a thrombus causes occlusion of the coronary circulation, resulting in ischaemia in the area no longer perfused by the affected artery [117]. The ischaemic tissue is no longer receiving oxygen or nutrients, and is not being cleared of waste products. During the period of ischaemia, the lack of supply to the affected tissue causes ischaemic injury, which includes failure of energy metabolism, dysregulated calcium homeostasis, generation of free radicals and mitochondrial dysfunction [118]. The survival of the myocardium is dependent on several factors, namely the severity of the occlusion, the duration of ischaemia, the size/location of the area affected by the occlusion, and how the cardiomyocyte adapts to the ischemic insult. It is already well known that promoting new blood vessel growth can improve survival of the myocardium [120]. This highlights the key role for collateral blood vessel formation and ischaemia-induced angiogenesis in response to myocardial infarction.

Taking into account the heart's high metabolic demand, it comes as no surprise that cardiovascular diseases involve marked changes in myocardial metabolism. Many studies have assessed the metabolic changes that take place in the heart during the progression to heart failure, assessing animal models of cardiac hypertrophy and failure, as well as patients with heart failure. Though the results have not always been consistent, the consensus is that fatty acid use is decreased in almost all models of hypertrophy and heart failure, and this is most often accompanied with an increase in glycolysis and glucose oxidation. Patients with idiopathic dilated cardiomyopathy, who exhibited systolic dysfunction, showed decreases in markers of fatty acid utilization and evidence for increased glucose use [121,122]. Similarly left ventricular hypertrophy, induced by aortic banding or by myocardial infarction, is associated with decreased fatty acid oxidation and increased glucose use [123,124]. It is important to note that the progression to heart failure is a slow, complex process and, for this reason, the metabolic changes observed in cardiac hypertrophy and heart failure will vary depending on aetiology, progression, and severity and stage of disease. In pathologic hypertrophy, hypoxia induces HIF-1 α expression [125], which plays an important role in reprogramming myocardial metabolism. The initial metabolic reprogramming, which involves an adaptive increase in glucose uptake and glycolysis, is likely to be due to the hypoxia that results from the mismatch between oxygen supply and demand [126]. The transition to heart failure involves changes in metabolic targets involved in glycolysis and PDH flux in patients at the later stages of hypertrophy [86]. Additionally, the transition to heart failure has also been associated with changes in fatty acid oxidation markers, namely PPAR α targets [127].

Diabetes is a growing concern worldwide and a systemic metabolic disease, which has been recognised as an independent risk factor for the development of heart failure, with a significant decrease in survival rate

following MI compared to non-diabetic patients [128–130]. Diabetic cardiomyopathy (DCM) is a specific pathology associated with diabetes mellitus, which is characterised by structural and functional cardiac changes, including fibrosis, ventricular dilation and diastolic dysfunction [131]. In diabetes mellitus, energy metabolism is greatly reshaped, due to a dysregulation of glucose metabolism and fatty acid metabolism. From a metabolic perspective, type 2 diabetes is characterised by increased cardiac fatty acid oxidation and an inability to use glucose due to insulin resistance [132], and as a consequence, cardiac metabolic flexibility is impaired [59]. It is likely that early in obesity, prior to type 2 diabetes, the changes in substrate use relate to substrate availability, namely the increase in circulating fatty acids and triglycerides. This, however, leads to an adaptation of the cardiac metabolic machinery in response to changing conditions, leading to decreased glucose use as a result of increased fatty acid use via the Randle cycle [129,133]. The resulting hyperglycaemia and hyperlipidaemia, together with insulin resistance, lead to changes in the expression and translocation of GLUT4 and FAT/CD36, with increased translocation of FAT/CD36 towards the sarcolemma and decreased sarcolemmal GLUT4 [134]. Increased fatty acid uptake is associated with increased fatty acid oxidation, activation of PPAR α and its fatty acid metabolism target genes and increased lipid storage. These fatty acid metabolic changes ultimately result in a decreased ability to use glucose, and an associated loss of diastolic function, which has been observed in rodent models of obesity with insulin resistance [135], diabetes [136] and in patients with type 2 diabetes with no concomitant cardiovascular symptoms [137].

6. HIF signalling in cardiovascular diseases

Ischaemia inevitably results in local hypoxia, which elicits a hypoxic response in the affected tissue and neighbouring region. HIF signalling is stimulated in these hypoxic conditions [125]. This allows increased expression of its target genes, which leads to a range of beneficial effects including angiogenesis and the aforementioned metabolic adaptations to hypoxia. This response is key in supporting the tissue to cope with the ischaemic insult and alleviate the subsequent deleterious longer-term effects of ischaemia-reperfusion. HIF-1 activity has been shown to increase early following MI, in ventricular biopsies from patients undergoing coronary bypass surgery [138]. Studies using coronary ligation in the rat heart showed increased expression of HIF-1 α protein and HIF-1 targets mRNA over the first week following infarction [26]. In addition, Wei et al. found that HIF-1 α conditional KO mice quickly developed cardiac hypertrophy and decompensation when subjected to pressure overload, and concluded that HIF-1 plays a crucial role in protecting the myocardium in the development of hypertrophy [139]. In line with this, Cai et al showed that protective effect of intermittent hypoxia was lost in HIF-1 α $^{+/-}$ mice [140]. In contrast, work in a transgenic mouse model of constitutively active HIF-1 α showed reduced infarct size and improved cardiac function four weeks after coronary ligation [141].

The activation of HIF-1 in endothelial cells during hypoxia or ischaemia is equally crucial to the subsequent recovery of the heart. As well as having metabolic oxygen sparing effects on the endothelium, HIF-1 mediates the angiogenic response to hypoxia by upregulating the expression of angiogenic factors. A genetic model of prolyl hydroxylase inhibition showed that activation of HIF-1 signalling in endothelial cells resulted in improved LV function and survival following ischaemia-reperfusion [142]. Furthermore, a lack of concordance between myocardial growth and endothelial angiogenesis has been shown to contribute to the development of heart failure [143]. Angiogenic HIF-1 targets such as VEGF are responsible for the fast growth of collateral vessels and have been implicated in cell survival following myocardial infarction [138,144]. Polymorphisms in the HIF-1 α locus have been associated with reduced collateral vessel formation, affecting the progression of ischaemic disease [145]. Furthermore, expression of target

Table 1
Classes and examples of HIF activators.

Class	Compound	Current use
2-OG mimetics	Dimethyloxalylglycine	Tool compound – PHD inhibitor
	<i>N</i> -oxalyl- <i>D</i> -phenylalanine	Tool compound – FIH inhibitor
Iron chelators	Desferrioxamine	Phase II clinical trials for use in brain haemorrhage
	Hydralazine	Licensed as an antihypertensive, recently found to also have a HIF mechanism of action
	AKB-4924	Phase I clinical trials for the treatment of inflammatory bowel diseases
PHD active-site blockers	GSK360A	Pre-clinical studies showed improved left-ventricular function and remodelling in rat model of established heart failure.
	Daprodustat (GSK1278863)	Phase III clinical trials for use in anaemia associated with chronic kidney disease. Also in Phase II for the treatment of perioperative ischaemia and diabetic foot ulcers.
	Roxadustat (FG-4592)	NDA filed in China for treatment in anaemia associated with chronic kidney disease. Also in Phase III clinical trials for use in anaemia associated with chronic kidney disease
	Molidustat (BAY85-3934)	Phase II clinical trials for use in anaemia associated with chronic kidney disease
	Vadadustat (AKB-6548)	Phase III clinical trials for use in anaemia associated with chronic kidney disease
	JTZ-951	Phase III clinical trials for use in anaemia associated with chronic kidney disease

genes VEGF and HO-1 have been shown to mediate the all-important improvement in cell survival following ischaemia-reperfusion [120,141,146]. Though the initial response mediated by hypoxic signalling protects the myocardium by improving oxygen delivery and preventing oxygen waste, it remains unclear whether the hypoxia response is beneficial in the longer term, as it has become apparent that angiogenic stimuli induce hypertrophy [147]. It is clear that HIF-1 signalling is increased in the initial stages of hypertrophy, but it may not be the case in prolonged hypoxia, as shown in models of pressure overload [146]. This mismatch between persistent hypoxia and decreased HIF-1 signalling could be at the core of the damage and dysfunction observed in the failing heart [147].

Although the role and effects of HIF-1 have been extensively studied over the past two decades, as it was the first identified of this family of transcription factors, the complimentary and contrasting roles of HIF-2 and HIF-3 remain relatively poorly understood to date. For instance, HIF-2 has been shown to have no effect on glycolysis, unlike HIF-1 [9]. Generally, HIF-2 has been found to have effects particularly on longer term adaptation to hypoxia, suggesting a sort of takeover from HIF-1 to HIF-2 with prolonged periods of hypoxia, which appears to be mostly focused on mitochondrial homeostasis [148]. Studies in KO mice suggest HIF-2 may also be important in affecting cell survival during I/R through the regulation of cellular antioxidant capacity [149]. HIF-3 poses a more complex problem to investigate, given that there are alternatively spliced variants of the HIF-3 α subunit, adding up to at least seven isoforms [150]. Very little is known about the effects of HIF-3 signalling in the heart. Importantly, HIF-3 has a role in attenuating the activity of HIF-1 α , also acting in the transition from HIF-1 to HIF-2 to HIF-3 in sustained [151] hypoxia.

There is a growing body of evidence for impaired HIF-1 activation in diabetes, though there is still some controversy. Specimens of human heart tissue in angina and biopsies from coronary bypass surgery showed decreased HIF-1 α and VEGF levels in type 2 diabetic patients compared to non-diabetics [152]. Work in streptozotocin-induced diabetic rats showed increased infarct size in hyperglycaemic rats, which correlated with decreased levels of HIF-1 α [153,154]. A negative correlation has been identified between cardiac HIF-1 α levels and glycaemic control in patients with type 2 diabetes and in STZ-induced diabetic rats [154,155]. We have recently demonstrated that the mechanism behind HIF-1 α impairment in diabetes is of metabolic origin, driven by increased fatty acids. This elevated dependence on fatty acid metabolism suppresses glycolysis, which results in downregulation of myocardial succinate in hypoxia, resulting in decreased HIF-1 α stabilisation [42]. It has also been suggested that HIF-1 β impairment could precede the development of type 2 diabetes [156].

Studies in both animal models and patients with type 2 diabetes have shown decreased glucose uptake, glycolytic rates, collateral blood vessel formation, and increased ROS production compared to healthy hearts in response to ischaemia [157–161]. We have shown that in

response to in vivo chronic hypoxia, type 2 diabetes decreases the up-regulation of glycolysis, limits downregulation of fatty acid oxidation, and prevents mitochondrial adaptation [97]. Together, these studies suggest abnormal HIF-1 α activity in the diabetic heart, correlating with impaired functional recovery when stressed [153]. One study showed that HIF-1 α overexpression in mice prevented cardiac remodelling in diabetic mice [162]. Gu et al. showed that the HIF-1 α Pro582Ser polymorphism could confer protection against diabetic nephropathy, by preventing the negative effect of glucose on HIF-1 signalling, suggesting a beneficial effect of HIF-1 on the microvasculature [163].

7. Therapeutic use of HIF activation

HIF-1 activation has already been proven to have therapeutic potential in the context of myocardial ischaemia, through the use of ischaemic pre-conditioning. When tissue is given short bursts of ischaemia-reperfusion before an ischaemic insult, survival is improved and this has been linked to upregulation of the HIF signalling pathway [164]. In a HIF-1 α deletion mouse study, when mice underwent pre-conditioning, the infarct size was reduced in control mice but this was not observed in mice with the HIF-1 α deletion [165]. Furthermore, genetic studies using gene silencing to prevent PHD activity led to attenuated ischaemia-reperfusion injury in the heart [166].

HIF-1 α -activating pharmacological compounds fall into one of several classes, all of which have their effect by preventing HIF-1 α degradation. Examples of these are listed in Table 1. 2-OG mimetics have been used as tool compounds in pre-clinical studies. Pre-treatment with dimethyloxalylglycine (DMOG) in a rabbit model of myocardial ischaemia-reperfusion resulted in a significant decrease in the resulting infarct size following reperfusion [167]. Similarly, hearts from rats pre-treated with DMOG showed improved functional recovery following ischaemia-reperfusion in an ex vivo perfused heart setup [168]. In most studies these compounds were administered before the onset of injury or ischaemia, which could put in question their usefulness in a clinical setting. They have also been criticised for their poor selectivity and risk of off-target effects due to their inhibition of other 2-OG dependent enzymes [38,169]. Iron chelators such as desferrioxamine and hydralazine, have been shown to have HIF-1 α stabilising properties through PHD inhibition. Desferrioxamine treatment was shown to improve brainstem blood flow and reduce vasospasm in a rat model of aneurysmal subarachnoid haemorrhage [170].

However, new generation PHD inhibitors have since been developed, several of which are undergoing clinical trials for use in conditions such as anaemia associated with renal disease, inflammatory disease, or brain haemorrhage (Table 1). With regards to potential for cardiovascular disease, chronic PHD inhibition strategies have already shown potential in rodent models of heart failure. Treatment with orally-active PHD inhibitor GSK360A resulted in increased expression of HIF targets, accompanied by improved recovery post MI in rats with

established heart failure, showing long term improvements in remodelling and left ventricular function [171]. Roxadustat (FG-4592) has completed Phase I and Phase II trials for use in anaemia, and showed good tolerability, improved circulating haemoglobin, as well as decreased total cholesterol levels, independent of the use of lipid-lowering agents [172–174]. Another orally-available PHD inhibitor [175], Molidustat (BAY85-3934), has completed Phase II clinical trials for treatment of anaemia associated with chronic kidney disease (DIALOGUE trial, NCT02021370), and Phase III clinical trials are currently recruiting (NCT03351166). Vadadustat (AKB-6548) has also undergone Phase II clinical trials, where it showed enhanced iron mobilisation and haemoglobin in patients with chronic kidney disease [176], and showed no significant changes in blood pressure, VEGF or total cholesterol levels [177]. JTZ-951, a recent clinical PHD inhibitor, has completed Phase II clinical trials (NCT01971164), showing safety and tolerability, with improved haemoglobin levels in patients with end-stage renal disease [178]. Some preliminary work has been done which suggests that HIF-1 α activation can also be beneficial in diabetes. The clinical PHD inhibitor, Daprodustat (GSK1278863), has now completed Phase I clinical trials for treatment of diabetic foot ulcer (NCT01831804), as well as perioperative ischaemia, suggesting that repurposing of these compounds for use in cardiovascular disease may not be far.

8. Conclusions

In conclusion, HIF-1 signalling plays a key role in the response to cardiovascular diseases, from a metabolic and angiogenic point of view. This evokes HIF-1 α as a potential therapeutic target in these diseases, and the recent advances in the development of PHD inhibitors are hopeful in this regard. However, there is still some concern that systemic treatment with hypoxia-mimetic drugs may lead to undesirable effects in healthy tissue, and it is important to note that HIF effects are tissue-specific. For instance, the Vadadustat trial reported several systemic side effects, as well as three deaths, in the treatment arm [176]. In the Roxadustat trial, five patients withdrew due to worsening side effects [174]. Despite being conserved targets for hypoxic adaptation in a number of organs, the regulation of many targets via HIF-1 activation is achieved via different organ-specific mechanisms. Whether sustained HIF-1 α upregulation is beneficial or deleterious for the heart is still under debate, and further investigation is needed to better understand the complex mechanisms at play. It may be that there is a cut-off point after which HIF signalling becomes detrimental to cardiac function [179,180]. Additionally, alternative hypoxic signalling mechanisms other than HIF, such as AMPK activation, may also provide a suitable alternative therapeutic target in cardiovascular diseases. HIF-targeting compounds developed so far have been designed with the kidney as their primary target. The current challenge is therefore the development of tissue-specific and PHD-selective inhibitors, which have so far been slow emerging. And although we have alluded to the potential of repurposing of compounds for use in cardiovascular diseases, this will have to be done on a compound-specific basis, with careful assessment of individual compounds.

Transparency document

The Transparency document associated with this article can be found, in online version.

Acknowledgements and funding

We would like to thank the British Heart Foundation (grants FS/17/58/33072, FS/14/65/31292) for funding our work.

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