Increased oxidative metabolism following hypoxia in the type 2 diabetic heart, despite normal hypoxia signalling and metabolic adaptation.

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Key Points

- Adaptation to hypoxia makes the heart more oxygen efficient, by metabolising more glucose. In contrast, type 2 diabetes does the opposite, making the heart metabolise more fatty acids.

- Diabetes increases the chances of the heart being exposed to hypoxia, but whether the diabetic heart can respond is unknown.

- In this study we show that diabetic hearts retain the ability to adapt their metabolism in response to hypoxia, with functional hypoxia signalling pathways.

- However, the hypoxia-induced changes in metabolism are additive to abnormal baseline metabolism, resulting in hypoxic diabetic hearts metabolising more fat and less glucose than controls. This stops the diabetic heart being able to recover its function when stressed.

- These results demonstrate the diabetic heart retains metabolic flexibility to adapt to hypoxia, but is hindered by the baseline effects of the disease. This increases our understanding of how the diabetic heart is affected by hypoxia-associated complications of the disease.

Abstract

Hypoxia activates the hypoxia-inducible factor (HIF), promoting glycolysis and suppressing mitochondrial respiration. In the type 2 diabetic heart, glycolysis is suppressed whereas fatty acid metabolism is promoted. The diabetic heart experiences chronic hypoxia as a consequence of increased obstructive sleep apnoea and cardiovascular disease. Given the opposing metabolic effects of hypoxia and diabetes, we questioned whether diabetes affects cardiac metabolic adaptation to hypoxia.

Control and type 2 diabetic rats were housed for three weeks in normoxia or 11% oxygen. Metabolism and function were measured in the isolated perfused heart using radiolabelled substrates. Following chronic hypoxia, both control and diabetic hearts upregulated glycolysis, lactate efflux and glycogen content and decreased fatty acid oxidation rates, with similar activation of HIF signalling pathways. However, hypoxia-induced changes were superimposed on diabetic hearts that were metabolically abnormal in normoxia, resulting in glycolytic rates 30% lower, and fatty acid oxidation 36% higher in hypoxic diabetic hearts than hypoxic controls. Peroxisome proliferator-activated receptor α target proteins were suppressed by hypoxia but activated by diabetes. Mitochondrial respiration in diabetic hearts was divergently activated following hypoxia compared with controls. These differences in metabolism were associated with decreased contractile recovery of the hypoxic diabetic heart following an acute hypoxic insult.

In conclusion, type 2 diabetic hearts retain metabolic flexibility to adapt to hypoxia, with normal HIF signalling pathways. However, they are more dependent on oxidative metabolism following hypoxia due to abnormal normoxic metabolism, which was associated with a functional deficit in response to stress.

Abbreviations: FAT/CD36, fatty acid translocase; GLUT, glucose transporter; HIF, hypoxia-inducible factor; MCAD, medium chain acyl-coenzyme A dehydrogenase; PDK, pyruvate dehydrogenase kinase; PPARα, peroxisome proliferator-activated receptor α; UCP, uncoupling protein

Introduction

The healthy heart can metabolise a range of substrates to meet its high energy requirements, including fatty acids, glucose, lactate, ketone bodies and amino acids (Taegtmeyer *et al.*, 2004). Fatty acids are a more energy dense fuel, providing more ATP per carbon, and account for 60-70% of ATP production, whereas glucose is a more oxygen-efficient fuel able to provide ATP via anaerobic glycolysis (Bing *et al.*, 1954; van der Vusse *et al.*, 1992). In contrast to the healthy heart, in type 2 diabetes the heart becomes metabolically abnormal, oxidising more fatty acids and metabolising less glucose, which is associated with increased myocardial oxygen consumption independent of contraction (Jagasia *et al.*, 2001; Peterson *et al.*, 2004; Boardman *et al.*, 2009).

A fine balance exists between the supply of and the demand for oxygen within the heart. A decrease in cellular oxygen availability, hypoxia, causes the transcription factor hypoxia-inducible factor (HIF)1α to escape degradation, translocate to the nucleus and induces transcription of HIF-target genes, which over a period of days to weeks regulate a plethora of cellular processes aimed at optimising utilisation of the available oxygen and restoring oxygen supply (Semenza, 2009). Hypoxia and HIF activation have profound effects on cardiac metabolism, increasing anaerobic glycolysis and suppressing mitochondrial oxidative metabolism (Semenza *et al.*, 1994; Ebert *et al.*, 1995; Kim *et al.*, 2006; Fukuda *et al.*, 2007; Ambrose *et al.*, 2014). Thus, hypoxia and HIF activation are major metabolic regulators of substrate selection in the heart, making the heart more oxygen efficient under hypoxic conditions. From a metabolic perspective, type 2 diabetes and hypoxia have opposite effects on cardiac metabolism. Type 2 diabetes promotes fatty acid metabolism and mitochondrial oxygen consumption, whereas HIF activation promotes glycolysis and suppresses mitochondrial respiration (Semenza *et al.*, 1994; Boardman *et al.*, 2009; Ambrose *et al.*, 2014). Therefore, we questioned what would happen when a diabetic heart was exposed to hypoxia, and whether the diabetic heart retains the ability to adapt its metabolism to hypoxia given its preference for fatty acids.

Type 2 diabetes is accompanied by a number of hypoxia-associated complications, which result in cardiomyocytes being exposed directly or indirectly to hypoxia. Systemically, type 2 diabetes increases the

incidence of obstructive sleep apnoea, which is a risk factor for cardiovascular disease and mortality, and there is a negative relationship between the severity of sleep apnoea and insulin sensitivity (Meslier *et al.*, 2003; West *et al.*, 2006; Tasali *et al.*, 2008). The Sleep Heart Health Study identified an increased incidence of periodic breathing during sleep in type 2 diabetics, and a systematic review identified an association between type 2 diabetes and decreased lung function (Resnick *et al.*, 2003; Klein *et al.*, 2010). Type 2 diabetes increases the risk of having a myocardial infarction and developing heart failure, and following myocardial infarction mortality rates are increased among diabetic patients (Rytter *et al.*, 1985; Almdal *et al.*, 2004; Shah *et al.*, 2015). Following myocardial infarction, cardiac ischemia reduces cellular oxygen availability within the cardiomyocyte and activates hypoxic signalling pathways within the myocardium, and HIF signalling persists chronically in the peri-infarct region of the heart (Lee *et al.*, 2000; Willam *et al.*, 2006). Thus, diabetes is associated with a greater incidence of the heart experiencing chronic hypoxia, and, whether this is via a direct or indirect mechanisms, it is associated with negative consequences for the diabetic heart. Therefore, important questions remain as to how the diabetic heart adapts when challenged by chronic hypoxia.

Conflicting data has been generated on the effects of diabetes on the cardiac HIF signalling pathway, with some studies showing HIF1α as increased, decreased or unchanged (Marfella *et al.*, 2002; Marfella *et al.*, 2004; Park *et al.*, 2009; Xue *et al.*, 2012). However, these studies have looked at HIF1α in normoxia or ischemia, studies have not been performed looking at HIF signalling and downstream effects using the transcription factors endogenous activator, hypoxia. Using a normobaric hypoxia chamber we can study how the diabetic heart metabolically adapts to chronic hypoxia and the effects of diabetes on activation of the HIF signalling pathway, which would not be possible in the isolated organ or tissue culture. Here, we demonstrate that type 2 diabetic hearts retain the capacity to upregulate glycolysis and glycogen content by the same percentage as control hearts, and that the HIF signalling pathway is not compromised. However, the adaptation to hypoxia in diabetes is superimposed on an abnormal metabolic fuel preference at normoxic baseline. This result in the diabetic heart having lower rates of anaerobic glycolysis and higher rates of

oxidative metabolism then control hearts following chronic hypoxia, and is associated with a decreased recovery following an acute hypoxic insult.

Methods

In vivo rat model of type 2 diabetes and chronic hypoxia

Ethical approval for experiments was granted by the United Kingdom Home Office guidelines under The Animal (Scientific Procedures) Act, 1986 after approval by the University of Oxford local ethics committee. Male Wistar rats (n = 73) were obtained from a commercial breeder (Harlan, UK). Control rats were fed for 42 days *ad libitum* on a standard chow diet (Harlan Laboratories). To induce type 2 diabetes, rats were fed a high-fat diet *ad libitum* (Special Diet Services) for 42 days, according to our previously published protocol (Mansor *et al.*, 2013a). On day 14 of 42, diabetic rats were given a single intraperitoneal injection of low dose streptozotocin (STZ, 25mg/kg bodyweight *w/w* in citrate buffer, pH 4). We have previously demonstrated that it is the combination of high-fat diet and STZ that induces diabetes, and that either factor in isolation is not sufficient to induce disease (Mansor *et al.*, 2013a). On day 20, control and diabetic rats had fasting blood collected from the saphenous vein for blood glucose analysis (Accu-check Aviva blood glucose testing system) and for plasma insulin concentrations (ELISA, R&D systems), to confirm hyperglycaemia and hyperinsulinemia in the diabetic animals prior to hypoxia. On day 21, subgroups of control and diabetic rats were transferred to a normobaric hypoxia chamber (Biospherix) (Heather *et al.*, 2012), while the remaining rats continued to be housed in normoxia. From days 21 to 28, the oxygen content in the hypoxia chamber was reduced from 21% to 11% in daily steps, which produced physiological hypoxic adaptation and prevented weight loss. From days 28 to 42, the oxygen content was maintained at 11%. Following hypoxia or normoxia, rats were terminally anaesthetised under normoxia, using an intraperitoneal injection of sodium pentobarbital, hearts were excised and blood collected for analysis. Due to Home Office restrictions, hearts could not be excised whilst the animals were still hypoxic.

Isolated heart perfusion

Hearts were perfused at a constant pressure of 100 mmHg and an end-diastolic pressure of 4-8 mmHg, according to our published protocol (Heather *et al.*, 2013). Hearts were perfused with Krebs-Henseleit buffer containing 11 mM glucose, 0.3 U/L insulin and 1.5% (w/v) fatty acid-free bovine serum albumin bound to

0.4 mM palmitate (gassed with 95% O_2 and 5% CO_2, at 37^{o}C). For measurement of palmitate oxidation rates, buffer was supplemented with 0.2 μCi.ml^{-1} [9,10-^{3}H] palmitate, and for measurement of glycolytic rates buffer was supplemented with 0.2 μCi.ml^{-1} [5-^{3}H]-glucose. Lactate efflux rates were measured in timed aliquots using lactate dehydrogenase. Metabolic rates were expressed per gram wet weight of tissue (gww). In a separate group of chronically hypoxic hearts, the oxygen partial pressure (pO_2) of the buffer was reduced from 413 ± 15 mmHg to 90 ± 10 mmHg for 32 minutes, by replacing the gas supply with 95% N_2 and 5% CO_2. This was followed by 8 minutes of reoxygenation at the original oxygen partial pressure, to study the recovery of function following an acute hypoxic insult.

Mitochondrial isolation and respiration

Mitochondria were isolated from hearts, according to our previously published protocol (Heather *et al.*, 2012). Mitochondria were incubated in respiratory media (100 mM KCl, 50 mM MOPS, 1 mM EGTA, 5 mM KH_2PO_4 and 1 mg/ml BSA, pH 7.4), and respiration was measured using a Clark-type oxygen electrode with a range of substrates (Heather *et al.*, 2012). State 3 (100 nmol ADP-stimulated) respiration, state 4 (ADP-limited) respiration, maximal ADP-stimulated (1000 nmol) respiration (max ADP), and respiratory control ratios (RCR, state 3/state 4 respiration rates) were measured at 30^{o}C.

Tissue analysis

Tissue assays were performed on freeze clamped tissue. Glycogen content was determined by the conversion of glycogen to glycosyl units, using amyloglucosidase. Triglyceride content was measured following Folch extraction. Citrate synthase activity, a marker of mitochondrial number, aconitase activity and medium chain acyl-coenzyme A dehydrogenase (MCAD) activity were measured according to established protocols (Srere, 1969; Lehman *et al.*, 1990).

Metabolomics

Aqueous metabolites were extracted using methanol/water/chloroform extraction protocol from freeze-clamped hearts and quantified using high resolution ^{1}H nuclear magnetic resonance spectroscopy (Mayr *et*

al., 2008). Chemical shifts and metabolite peak areas were calibrated against trimethylsilyl propanoic acid reference peak (3 µM) at pH 6.5 and normalized for gww of tissue extracted (Tyagi *et al.*, 1996).

Western blotting

Protein lysates were prepared, 30µg of protein was loaded onto 12.5% SDS-PAGE gels and separated by electrophoresis (Heather *et al.*, 2006). Even protein loading and transfer were confirmed by Ponceau staining and using cyclophilin B as an internal standard. 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. For measurement of HIF1α protein levels, tissue lysates were compared to HL1 cardiomyocytes cultured in 2% oxygen for 6 hours (Ambrose *et al.*, 2014), to act as a positive control.

Real-time quantitative PCR

Left ventricular tissue was immersed in RNAlater (Qiagen, Germany), and total RNA was extracted according to the RNeasy Fibrous Tissue kit Protocol (Qiagen, Norway). Real-time PCR (qPCR) was performed in an ABI PRISM 7900 HT fast real-time thermal cycler as previously described (Hafstad *et al.*, 2009). Housekeeping genes were selected on the basis of the average expression stability determined with Genorm from a pool of five candidate genes (Vandesompele *et al.*, 2002), and mRNA expression of the genes of interest was adjusted to the geomean of the three most stable housekeeping genes. Real-time quantitative PCr was performed for heme oxygenase 1 (NM_012580.2), vascular endothelial growth factor (VEGF; NM_031836.2), prolyl hydroxylase 3 (NM_019371.1), HIF1α (NM_024359.1), glyceraldehyde-3-phosphate dehydrogenase (NM_017008), ribosomal protein L13A (NM_173340.2) and hydroxymethylbilane synthase (NM_013168.2).

Statistics

Results are presented as means ± SEM, and were considered significant at $p < 0.05$ (SPSS Statistics 18). Differences were investigated using a two-way ANOVA (two factors were diabetic status and oxygen level), and interactions are reported where identified. Only if statistical significance was obtained at the

ANOVA level were subsequent post-hoc individual comparison between groups performed using unpaired t-tests.

Results

Physical characteristics and plasma metabolites

There were no significant differences in body weights between groups (Table 1). Hypoxia had no effects on heart weights or epididymal fat pad weights, but both measurements were increased by diabetes. Fasting plasma metabolites were measured in control and diabetic rats prior to entry into the hypoxia chamber, to confirm their diabetic status. Type 2 diabetic rats had 31% higher fasting blood glucose concentrations and 42% higher fasting plasma insulin concentrations compared with controls. Plasma metabolites were also measured following a three weeks in normoxia or hypoxia. In response to hypoxia, glucose concentrations were reduced by 18-20% in controls and diabetics, resulting in the amelioration of the hyperglycaemia in the diabetic rats. NEFA and TAG were increased by hypoxia in both groups, with the former also increased by diabetes. There was a significant effect of hypoxia on plasma insulin concentrations, with hypoxic diabetic rats having 41% lower insulin concentrations than normoxic diabetic rats. Interestingly, there was a significant interaction between diabetes and hypoxia on plasma β-OHB concentrations, which were elevated to a greater extent by hypoxia in diabetic rats than in controls.

Cardiac function

There was a significant interaction between hypoxia and diabetes for their effects on coronary flow rates, with diabetic rat hearts having significantly greater coronary flow rates following hypoxia, 25% higher than controls (Table 2). Rate pressure product, the multiple of developed pressure and heart rate, was not significantly different between groups, demonstrating that overall cardiac systolic function was not modified by diabetes or adaptation to chronic hypoxia.

Glucose metabolism

Exposure to chronic hypoxia increased cardiac glycolytic rates by 25% in both control and type 2 diabetic rats. However, diabetes had a significant opposing effect on glycolysis reducing rates by a third, resulting in a 30% lower rate of anaerobic glycolysis in hypoxic diabetics compared with hypoxic controls (Figure 1). The changes in cardiac glycolytic rates were reflected by changes in net lactate efflux from these hearts.

Hypoxia increased cardiac glycogen content by 24 and 20% in control and diabetic rats, respectively, but diabetes had a significant negative effect on glycogen content, resulting in hypoxic diabetic hearts having 18% lower glycogen content compared with hypoxic controls, mirroring the changes in glycolytic flux.

Fatty acid metabolism

Hypoxia and diabetes had opposing effects on fatty acid oxidation, with hypoxia decreasing and diabetes increasing cardiac fatty acid oxidation rates. As a consequence, diabetic rats oxidised 31% more fatty acid than controls in normoxia, and 36% more fatty acids than controls following hypoxia (Figure 2). These changes in fat oxidation were independent of significant changes in intracellular triglycerides. Citrate synthase activity, a marker of mitochondrial content, showed a similar profile to fatty acid oxidation rates, remaining significantly increased in hypoxic diabetic rats compared with hypoxic controls.

Changes in intracellular energy metabolism and substrate availability were further investigated using metabolomics (Table 3). Hypoxia decreased total creatine concentrations, with hypoxic diabetic hearts having 20% less creatine compared with normoxic diabetics. Krebs cycle intermediates, multiple amino acids and NAD^+, a marker of the redox state of the heart, were unchanged between groups. However, aspartate concentrations were increased by diabetes, with hypoxic diabetic hearts having 77% more aspartate compared with hypoxic controls.

Mitochondrial respiration

The higher coronary flow rates and fatty acid oxidation rates in hypoxic diabetic rat hearts compared with hypoxic control hearts would suggest increased rates of mitochondrial oxygen consumption. To test this hypothesis, oxygen consumption was measured in isolated interfibrillar (IFM) and subsarcolemmal (SSM) mitochondria. There was a significant interaction between diabetes and hypoxia on oxygen consumption in IFM and SSM when metabolising the fatty acid substrate palmitoyl CoA (Figure 3). Chronic hypoxia decreased state 3 respiration in mitochondria from control hearts by 28 and 25%, in IFM and SSM respectively. In contrast, this effect of chronic hypoxia was absent in mitochondria from diabetic hearts, as diabetes prevented the decrease in state 3 respiration in response to hypoxia. State 4 respiration, a measure

of oxidative phosphorylation-independent oxygen consumption, also displayed a significant interaction between diabetes and hypoxia. Whereas hypoxia had no effect on succinate state 4 respiration in IFM from control hearts, in diabetic hearts hypoxia significantly increased state 4 respiration rates by 53% compared with hypoxic controls. Thus, diabetes prevented the fatty acid-dependent decrease in state 3 respiration, and exacerbated the ATP-independent consumption of oxygen by the mitochondria following hypoxia.

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

The adaptation to chronic hypoxia is regulated by the HIF1α pathway, and there have been reports that HIF signalling is perturbed in diabetes. However, we hypothesised that the HIF signalling pathway was not disrupted in our model of type 2 diabetes, given that our diabetic animals were able to increase their cardiac glycolytic and lactate efflux rates by the same percentage as controls following hypoxia.

Haematocrits were increased by hypoxia to the same extent in control and diabetic animals, demonstrating no systemic defect in hypoxic sensing or signalling in diabetes (Figure 4). Cardiac HIF-1α mRNA was not different between any of the groups. Downstream targets of HIF signalling, prolyl hydroxylase 3 and heme oxygenase 1 were both increased in hypoxia to a similar extent in control and diabetic rat hearts. In addition, aconitase activity, a metabolic HIF target (Ambrose *et al.*, 2014), was decreased to the same extent by hypoxia in control and diabetic hearts. Cardiac VEGF mRNA showed no difference between control and diabetic rats. Finally, HIF-1α protein was not detected in normoxic diabetic or control hearts. Taken as a whole, these data demonstrate no prior abnormality in the HIF signalling pathway due to type 2 diabetes, and no abnormalities in the HIF-induced signals following hypoxia.

Overactivation of PPARα signalling in type 2 diabetes following hypoxia

Despite seeing similar percentage changes in fatty acid oxidation and glycolysis in response to hypoxia in diabetic and control rats, the absolute rates of metabolism through these two pathways remained significantly different between controls and diabetics following hypoxia. We hypothesised that this was due to the abnormal baseline normoxic metabolism, onto which the hypoxic response was superimposed. We questioned whether there was inappropriate activation of the key metabolic transcription factor, peroxisome

proliferator-activated receptor (PPAR) α, which maintained the hypoxic diabetic heart in a more fatty acid oxidative state and requiring greater oxygen consumption than the hypoxic control heart. To test this hypothesis we measured PPARα-targets: pyruvate dehydrogenase kinase 4 (PDK4), uncoupling protein 3 (UCP3), medium chain acyl-coenzyme A dehydrogenase (MCAD) and fatty acid transport protein 1 (FATP1).

Hypoxia and diabetes had opposing effects on PPARα targets, with hypoxia decreasing and diabetes increasing PPARα target proteins. PPARα targets were decreased by hypoxia, with significant decreases ranging from 20 - 34% in control hearts (Figure 5), and this was independent of a decrease in PPARα mRNA. PPARα targets PDK4, UCP3 and MCAD were all significantly upregulated by diabetes, ranging from a 30% to a 2 fold increase compared with controls. As a result, PPARα target proteins in hypoxic diabetic hearts were between 24% higher and 3 fold higher than in hypoxic control hearts. To confirm that these changes were specific to PPARα targets we measured protein levels of two metabolic proteins that are not direct PPARα targets, and found both GLUT4 and FAT/CD36 were modified by diabetes but not by hypoxia. Increased PPARα target enzymes would facilitate the increased fatty acid oxidation and mitochondrial oxygen consumption in hypoxic diabetic rat hearts, and via the Randle cycle would suppress glucose uptake and limit glycolysis, glycogenesis and lactate efflux (Randle *et al.*, 1963), providing a mechanism to account for metabolic changes following hypoxia in our type 2 diabetic hearts.

Decreased recovery of the diabetic heart following acute hypoxia

Diabetic hearts retained the ability to adapt to hypoxia, but the metabolic profile was more oxidative and less glycolytic than control heart following hypoxia. Therefore, we questioned whether this adaptation to hypoxia in the diabetic heart was sufficient for the heart to tolerate an acute hypoxic insult to the same extent as a hypoxic control heart. Cardiac function was the same in both groups when perfused in normoxia (Table 2). Cardiac function decreased to a greater percentage in diabetic hearts at the start of the acute hypoxic insult, and did not recovery to the same percentage following reoxygenation (Figure 6). Whereas control hearts recovered 101% of their pre-hypoxic rate pressure product, diabetic hearts only recovered 85%.

Therefore, diabetic hearts had an impaired functional response to acute hypoxia when compared to control hearts, when both had been acclimatised to chronic hypoxia.

Discussion

In this study we demonstrate that in response to chronic hypoxia, type 2 diabetic rats retain the ability to adapt their cardiac metabolism, by increasing anaerobic glycolysis and glycogen content by a similar percentage to control hearts. In addition, the HIF1α signalling pathway was preserved in diabetic hearts, and similarly activated by hypoxia in control and diabetic rats. However, in diabetes the hypoxia-induced changes in energy metabolism were superimposed on the abnormal metabolic state present in normoxia. This resulted in lower absolute rates of glycolysis, glycogen content and higher absolute rates of fatty acid oxidation and mitochondrial oxygen consumption following hypoxia in diabetic hearts compared with control hearts. Hypoxia and diabetes had opposing effects on PPARα target proteins, resulting in proteins involved in oxidative fatty acid metabolism being higher in hypoxic diabetic hearts than in controls. Mitochondrial respiration rates and coronary flow rates showed divergent responses in controls and diabetic hearts when exposed to chronic hypoxia. These differences in energy metabolism between control and diabetics following chronic hypoxia were associated with negative functional outcomes when challenged with an acute hypoxic insult, with diabetic hearts recovering less contractile function than controls.

Diabetes and hypoxia have opposing effects on cardiac substrate metabolism, as diabetes promotes fatty acid oxidation and suppresses glycolysis, whereas adaptation to hypoxia suppresses fatty acid oxidation and promotes glycolysis. Thus, diabetes and hypoxia sit at either end of a metabolic "see-saw", and it was important to understand what happens metabolically when both diabetes and hypoxia stimuli are present. From a clinical perspective being able to adapt to hypoxia is important for the diabetic heart, as diabetes makes the heart to be more likely to experience hypoxia, either indirectly due to obstructive sleep apnoea, impaired lung function, or directly via chronic cardiac disease (West *et al.*, 2006; Klein *et al.*, 2010; Shah *et al.*, 2015). Therefore, understanding if the diabetic heart can adapt to hypoxia and the metabolic consequences, are important for understanding the effects and consequences of these diseases.

Adaptation to hypoxia involves manipulating metabolism from a more fatty acid oxidative to a more glycolytic phenotype to ensure essential ATP generation can continue in oxygen-restricted conditions. The

percentage increases in anaerobic glycolysis, lactate efflux and glycogen content were similar in control and diabetic hearts in response to hypoxia, suggesting that diabetes does not impair the ability to upregulate glucose metabolism and glucose uptake in response to hypoxia. There have been conflicting reports on whether diabetes impairs cardiac metabolic flexibility, with some studies demonstrating metabolic inflexibility in response to glucose infusion (Oakes *et al.*, 2006), whereas other studies have reported metabolic responsiveness to insulin (Hafstad *et al.*, 2006). One advantage of probing metabolic flexibility using hypoxia is that this stimuli is a key metabolic regulator that operates via a different mechanism, acting through different pathways, to glucose and insulin. Our data would support that the diabetic heart retains metabolic plasticity and can metabolically adapt to a chronic hypoxia stimulus. This is an important finding in light of the growing interest in metabolic modulators for the treatment of the diabetic heart, as capacity to change metabolism in diabetes is essential if these compounds have any potential to produce measureable benefits. Thus, metabolic flexibility in diabetes may be a stimuli-specific phenomena, and targeting metabolism pharmacologically may have to take into account which signalling pathways retain their function in diabetes.

While the majority of metabolic parameters showed a hypoxic response of a similar direction or magnitude between controls and diabetics, a few measurements displayed a significant interaction between the effects of diabetes and hypoxia. Coronary flow, state 3-dependent mitochondrial respiration and state 4 oxidative phosphorylation-independent oxygen consumption all showed more pronounced rates in diabetics following hypoxia than in controls. This would suggest that, for these oxygen supplying and consuming pathways, that diabetics have an abnormal response to hypoxia. Given that these parameters were normal in normoxic diabetic hearts, our data demonstrates an as yet unidentified interplay between diabetes and hypoxic adaptation that promotes the use of oxygen under hypoxic conditions.

In contrast to other studies (Marfella *et al.*, 2002; Xue *et al.*, 2012), we found no pre-existing differences in HIF-1α mRNA or HIF targets genes or proteins in our diabetic hearts in normoxia. Due to technical limitations we were unable to measure HIFα protein during hypoxia in our diabetic and control rats, however,

HIF target genes were not differentially activated in response to hypoxia in type 2 diabetic hearts compared with controls, demonstrating that activation of this pathway in response to changes in oxygen concentration were not modified in diabetes. Thus, changes in metabolism where interactions between diabetes and hypoxia were found cannot be attributed to difference in activation of HIF1α signalling. The contrast between other studies and our data may lie in the degree of hyperglycaemia in these animal models. It has been reported in type 1 diabetic and *db/db* hearts, with plasma glucose concentrations ranging from 22-58 mmol/l, that normoxic HIF-1α mRNA was increased whereas downstream targets were decreased (Marfella *et al.*, 2002; Jesmin *et al.*, 2007; Park *et al.*, 2009). In cell culture experiments, incubating dermal fibroblasts with 5.5 – 11 mmol/l glucose concentrations did not affect HIF activation in hypoxia, however, concentrations of 25 – 30 mmol/l glucose suppressed the hypoxia-induced HIF accumulation (Catrina *et al.*, 2004). Thus, it is likely that the severity of diabetes and hyperglycaemia may play a key role in influencing HIF signalling in the heart. Our model of type 2 diabetes was chosen to mimic human type 2 diabetes, with mild hyperglycaemia, hyperinsulinaemia and hyperlipidaemia, and because it mimics the developmental process of the disease (Mansor *et al.*, 2013b). Therefore, in type 2 diabetes if adequate glucose control can be maintained, defects in HIF and downstream signalling may not be present, and the ability to adapt to hypoxia can be preserved.

While diabetic hearts retain the ability to adapt to hypoxia, in absolute terms they metabolise less glucose anaerobically, store less glycogen and are more dependent on fatty acid and oxidative metabolism following hypoxia than control hearts. This may have profound consequences if the hypoxia became more severe, either acutely, as occurs in an infarct, or chronically, as can occur during post-infarction structural remodelling or in sleep apnoea (Willam *et al.*, 2006). Increased myocardial glycogen content would protect the heart by providing an on-site glycolytic substrate during hypoxia, and higher glycogen reserves have been associated with increased tolerance of ischemia (Cross *et al.*, 1996). Similarly, myocardial work-independent oxygen consumption has been shown to decrease in diabetic hearts when fatty acid oxidation is suppressed (Boardman *et al.*, 2009). Thus, the diabetic heart may be less metabolically prepared if the

hypoxia were to escalate. Our data demonstrate that in response to an acute hypoxic insult the diabetic hearts chronically adapted to hypoxia did significantly worse than the control hearts adapted to hypoxia. The diabetic hearts decreased their function more rapidly and recovered it to a much less extent, demonstrating a reduced tolerance of acute hypoxia. While a direct causal connection cannot be made by the current data, a lower rate of glycolysis, lower glycogen content and elevated respiration rates may well contribute to this functional deficit.

Control rats following chronic hypoxia had decreased glucose concentrations accompanied by elevated lipid metabolites: NEFA, β-OHB and TAG. This profile is in agreement with the changes in systemic metabolism, shifting towards metabolising more glucose and less fat in hypoxia, shown to occur at the whole body level in both animal and humans (Stanley *et al.*, 1990; Jun *et al.*, 2012; Yao *et al.*, 2013). Diabetic animals became normoglycaemic in response to chronic hypoxia, likely a consequence of hypoxia-induced increase in systemic glucose metabolism, however, they became more hyperlipidemic, hyperketonaemic and hypoinsulinaemic than controls. Activation of HIF-1α in pancreatic β-cells decreases insulin secretion (Cantley *et al.*, 2009), and this may be more profound in our hyperinsulinaemic diabetic animals in which the pancreas is already working harder. Thus, the more extreme lipid metabolite profile in diabetic rats following hypoxia is likely the consequence of multiple factors: – the pre-existing hyperlipidaemic state of diabetes, the hypoxia-induced suppression of systemic fat oxidation, and the hypoxia-induced decrease in insulin secretion.

Myocardial fatty acid utilisation is promoted by the nuclear transcription factor PPARα, which is activated by elevated long chain fatty acid ligands, as occurs in the hyperlipidaemic state associated with diabetes and insulin resistance. In response to hypoxia, PPARα activation and transactivation of its targets genes are decreased (Huss *et al.*, 2001; Belanger *et al.*, 2007). Our data demonstrate that the diabetic hearts retains the ability for hypoxia to downregulate PPARα targets, but that these genes are so highly activated under baseline normoxic conditions, that the hypoxia-induced change in most instances only brings down the targets to the levels we find in a normoxic control heart. Hypoxia and diabetes are operating at either end of

a PPARα "see-saw", with hypoxia decreasing and diabetes increasing PPARα target proteins. Overactivation of PPARα has been shown to impair recovery following ischemia-reperfusion (Dewald *et al.*, 2005; Sambandam *et al.*, 2006), and the ability to decrease PPARα signalling pathways is part of the response to cardiac disease, therefore, preventing this may be deleterious in the long term.

In conclusion, type 2 diabetic hearts retain metabolic flexibility to adapt to chronic hypoxia by increasing glycolysis and decreasing fatty acid oxidation, associated with a normal HIF signalling system. However, these hypoxia-induced changes were superimposed on a diabetic heart that was metabolically abnormal at baseline, resulting in lower absolute rates of glycolysis, higher rates of fatty acid oxidation and oxygen consumption in hypoxic diabetic hearts compared with hypoxic controls. These differences in metabolism following adaptation to chronic hypoxia were associated with an impaired functional recovery of the diabetic heart when exposed to an acute hypoxic insult.

Competing Interests

None to declare

Author Contributions

L.S.M, M.A.C, L.C.H designed the experiments. K.M, C.A.C, D.A, T.L, L.L.P, MdL.S.F, M.J.S collected and analysed the data. L.S.M, D,A, D.J.T and L.C.H interpreted the data. D.J.T. K.C, E.A and L.C.H prepared the manuscript. All authors approved the manuscript for submission

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Figure Legends

Figure 1. Glycolytic rates, lactate efflux rates and glycogen content in control and diabetic hearts following adaptation to chronic hypoxia or normoxia. * $p < 0.05$ vs. normoxia within same disease state, # $p < 0.05$ vs. control under same oxygen condition, n = 5 – 9 per group.

Figure 2. Fatty acid oxidation rates, myocardial triglycerides and citrate synthase activities in control and diabetic hearts following adaptation to chronic hypoxia or normoxia. * $p < 0.05$ vs. normoxia within same disease state, # $p < 0.05$ vs. control under same oxygen condition, n = 5 – 9 per group.

Figure 3. Mitochondrial respiration rates under ADP-stimulated state 3 and ADP-depleted state 4 conditions in interfibrillar and subsarcolemmal mitochondria, from control and diabetic hearts following adaptation to chronic hypoxia or normoxia. * $p < 0.05$ vs. normoxia within same disease state, # $p < 0.05$ vs. control under same oxygen condition, † $p < 0.05$ interaction between hypoxia and diabetes, n = 4 – 8 per group.

Figure 4. Haematocrit, HIF and HIF-target genes, HIF-target enzymes and HIF protein, from control and diabetic hearts following adaptation to chronic hypoxia or normoxia. * $p < 0.05$ vs. normoxia within same disease state, # $p < 0.05$ vs. control under same oxygen condition, n = 5 – 12 per group.

Figure 5. PPARα targets pyruvate dehydrogenase kinase 4, uncoupling protein 3, medium chain acyl-coenzyme A dehydrogenase and fatty acid transport protein 1 protein and activity levels, from control and diabetic hearts following adaptation to chronic hypoxia or normoxia. Protein levels of substrate transporters GLUT4 and FAT/CD36, and PPARα mRNA from control and diabetic hearts following adaptation to chronic hypoxia or normoxia. * $p < 0.05$ vs. normoxia within same disease state, # $p < 0.05$ vs. control under same oxygen condition, n = 5 – 8 per group.

Figure 6. Percentage recovery of rate pressure product following 32 minutes of acute hypoxia and 8 minutes of reoxygenation, in chronically hypoxic control and diabetic hearts. # $p < 0.05$ vs hypoxic control, n = 3 – 4 per group.

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Table 1. Physical characteristics and plasma metabolites from control and diabetic rats housed in normoxia or hypoxia

	Normoxic Control	Hypoxic Control	Normoxic Diabetic	Hypoxic Diabetic
Starting body weight (g)	270 ± 13	270 ± 6	270 ± 8	265 ± 7
Terminal body weight (g)	380 ± 13	366 ± 5	395 ± 8	376 ± 10
Heart weight (g)	1.21± 0.09	1.34 ± 0.09	1.47 ± 0.06 #	1.52 ± 0.05
Heart weight to body weight ratio ($x10^3$)	3.14 ± 0.14	3.56 ± 0.19	3.66 ± 0.18 #	4.01 ± 0.10
Epididymal fat pad weight (g)	5.91 ± 0.56	5.65 ± 0.75	10.26 ± 0.32 #	8.40 ± 0.37 #
Fat pad to body weight ratio ($x\ 10^2$)	1.46 ± 0.13	1.60 ± 0.19	2.59 ± 0.09 #	2.30 ± 0.12 #
Fasting plasma metabolites				
Glucose (mmol/l)	6.18 ± 0.43		8.08 ± 0.61 #	
Insulin (ug/l)	0.31 ± 0.03		0.44 ± 0.04 #	
Plasma metabolites in the fed state				
Glucose (mmol/l)	12.7 ± 0.3	10.1 ± 0.5 *	14.1 ± 0.3 #	11.5 ± 0.7 *
NEFA (mmol/l)	0.05 ± 0.01	0.11 ± 0.02 *	0.09 ± 0.01 #	0.15 ± 0.02 *
TAG (mmol/l)	1.44 ± 0.18	2.15 ± 0.27 *	1.18 ± 0.11	1.81 ± 0.19 *
Insulin (ug/l)	1.90 ± 0.26	1.56 ± 0.21	1.88 ± 0.22	1.11 ± 0.16 *
β-OHB (mmol/l)	0.32 ± 0.02	0.39 ± 0.01 *	0.57 ± 0.03 #	0.78 ± 0.04 *#†

* $p < 0.05$ vs. normoxia within same disease state, # $p < 0.05$ vs. control under same oxygen condition, † $p < 0.05$ interaction between hypoxia and diabetes. Body weights n = 12 – 16, heart weights n = 8 – 10, fat pads n = 4 – 8. Fasting metabolites obtained prior to entry into the hypoxia chamber after 3 weeks (n = 5 – 9), fed metabolites after 6 weeks (n = 12 – 17). NEFA, non-esterified fatty acids; β-OHB, β-hydroxybutyrate; TAG, triacylglycerol.

Table 2. Cardiac function from control and diabetic rats housed in normoxia or hypoxia.

	Normoxic Control	Hypoxic Control	Normoxic Diabetic	Hypoxic Diabetic
Coronary flow rates (ml/min)	18 ± 1	16 ± 1	18 ± 1	20 ± 1 #†
Developed pressure (mmHg)	134 ± 2	149 ± 6 *	136 ± 6	149 ± 7
Heart rate (beats/min)	280 ± 8	258 ± 8	276 ± 10	277 ± 11
Rate pressure product (mmHg/min x 10^3)	38 ± 2	38 ± 1	38 ± 3	41 ± 1

* $p < 0.05$ vs. normoxia within same disease state, # $p < 0.05$ vs. control under same oxygen condition, † $p < 0.05$ interaction between hypoxia and diabetes, n = 7 – 10.

Table 3. Cardiac metabolites from control and diabetic rats housed in normoxia or hypoxia.

	Norxmoxic Control	Hypoxic Control	Normoxic Diabetic	Hypoxic Diabetic
ATP/ADP pool	2.54 ± 0.38	3.26 ± 0.77	2.45 ± 0.36	2.69 ± 1.17
Creatine	13.2 ± 1.4	11.2 ± 1.1	12.2 ± 0.8	9.7 ± 0.5 *
Succinate	0.61 ± 0.22	0.43 ± 0.38	0.63 ± 0.34	0.44 ± 0.23
Fumarate	0.04 ± 0.03	0.03 ± 0.01	0.03 ± 0.02	0.04 ± 0.01
Alanine	1.56 ± 0.18	1.59 ± 0.31	1.71 ± 0.31	1.45 ± 0.27
Glutamate	5.96 ± 0.65	6.43 ± 1.00	6.52 ± 0.39	6.23 ± 0.80
Glutamine	6.32 ± 0.55	6.58 ± 0.93	6.96 ± 0.77	7.59 ± 2.16
Glycine	0.44 ± 0.06	0.51 ± 0.06	0.56 ± 0.12	0.60 ± 0.12
Aspartate	1.35 ± 0.17	1.36 ± 0.08	1.87 ± 0.33	2.41 ± 0.45 #
NAD^+	0.49 ± 0.04	0.63 ± 0.10	0.58 ± 0.06	0.81 ± 0.15

Units are μmol/gww. * $p < 0.05$ vs. normoxia within same disease state, # $p < 0.05$ vs. control under same oxygen condition, $n = 4 – 5$.

Table 1

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Insulin (ug/l)	1.90 ± 0.26	1.56 ± 0.21	1.88 ± 0.22	1.11 ± 0.16 *
β-OHB (mmol/l)	0.32 ± 0.02	0.39 ± 0.01 *	0.57 ± 0.03 #	0.78 ± 0.04 *#†

* $p < 0.05$ vs. normoxia within same disease state, # $p < 0.05$ vs. control under same oxygen condition, † $p < 0.05$ interaction between hypoxia and diabetes. Body weights n = 12 – 16, heart weights n = 8 – 10, fat pads n = 4 – 8. Fasting metabolites obtained prior to entry into the hypoxia chamber after 3 weeks (n = 5 – 9), fed metabolites after 6 weeks (n = 12 – 17). NEFA, non-esterified fatty acids; β-OHB, β-hydroxybutyrate; TAG, triacylglycerol.

Table 2

Table 2. Cardiac function from control and diabetic rats housed in normoxia or hypoxia.

	Normoxic Control	Hypoxic Control	Normoxic Diabetic	Hypoxic Diabetic
Coronary flow rates (ml/min)	18 ± 1	16 ± 1	18 ± 1	20 ± 1 #†
Developed pressure (mmHg)	134 ± 2	149 ± 6 *	136 ± 6	149 ± 7
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Fumarate	0.04 ± 0.03	0.03 ± 0.01	0.03 ± 0.02	0.04 ± 0.01
Alanine	1.56 ± 0.18	1.59 ± 0.31	1.71 ± 0.31	1.45 ± 0.27
Glutamate	5.96 ± 0.65	6.43 ± 1.00	6.52 ± 0.39	6.23 ± 0.80
Glutamine	6.32 ± 0.55	6.58 ± 0.93	6.96 ± 0.77	7.59 ± 2.16
Glycine	0.44 ± 0.06	0.51 ± 0.06	0.56 ± 0.12	0.60 ± 0.12
Aspartate	1.35 ± 0.17	1.36 ± 0.08	1.87 ± 0.33	2.41 ± 0.45 #
NAD^+	0.49 ± 0.04	0.63 ± 0.10	0.58 ± 0.06	0.81 ± 0.15

Units are µmol/gww. * $p < 0.05$ vs. normoxia within same disease state, # $p < 0.05$ vs. control under same oxygen condition, $n = 4 – 5$.

Figure 1

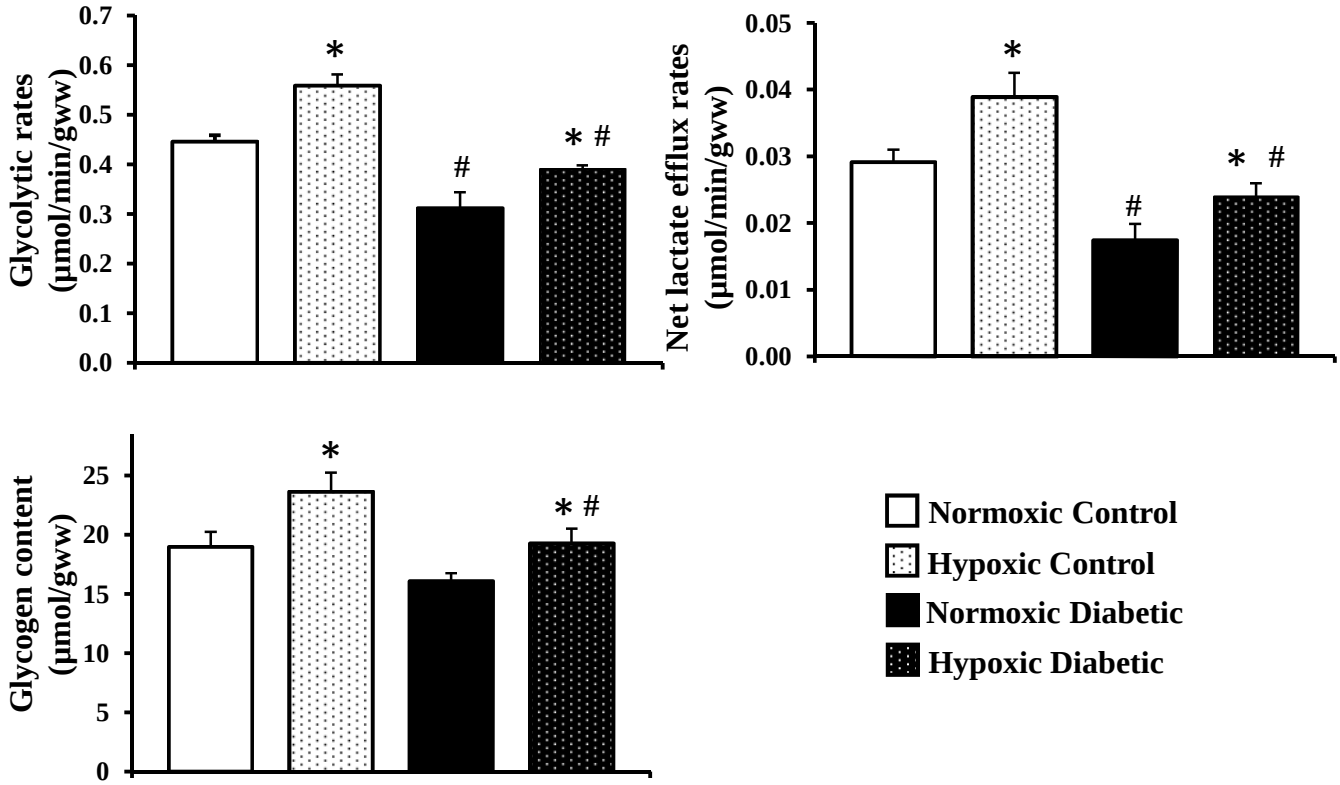

Glycolytic rates (µmol/min/gww)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
#
* #
Net lactate efflux rates (µmol/min/gww)
0.05
0.04
0.03
0.02
0.01
0.00
*
#
* #
Glycogen content (µmol/gww)
25
20
15
10
5
0
*
* #
Normoxic Control
Hypoxic Control
Normoxic Diabetic
Hypoxic Diabetic

Figure 2

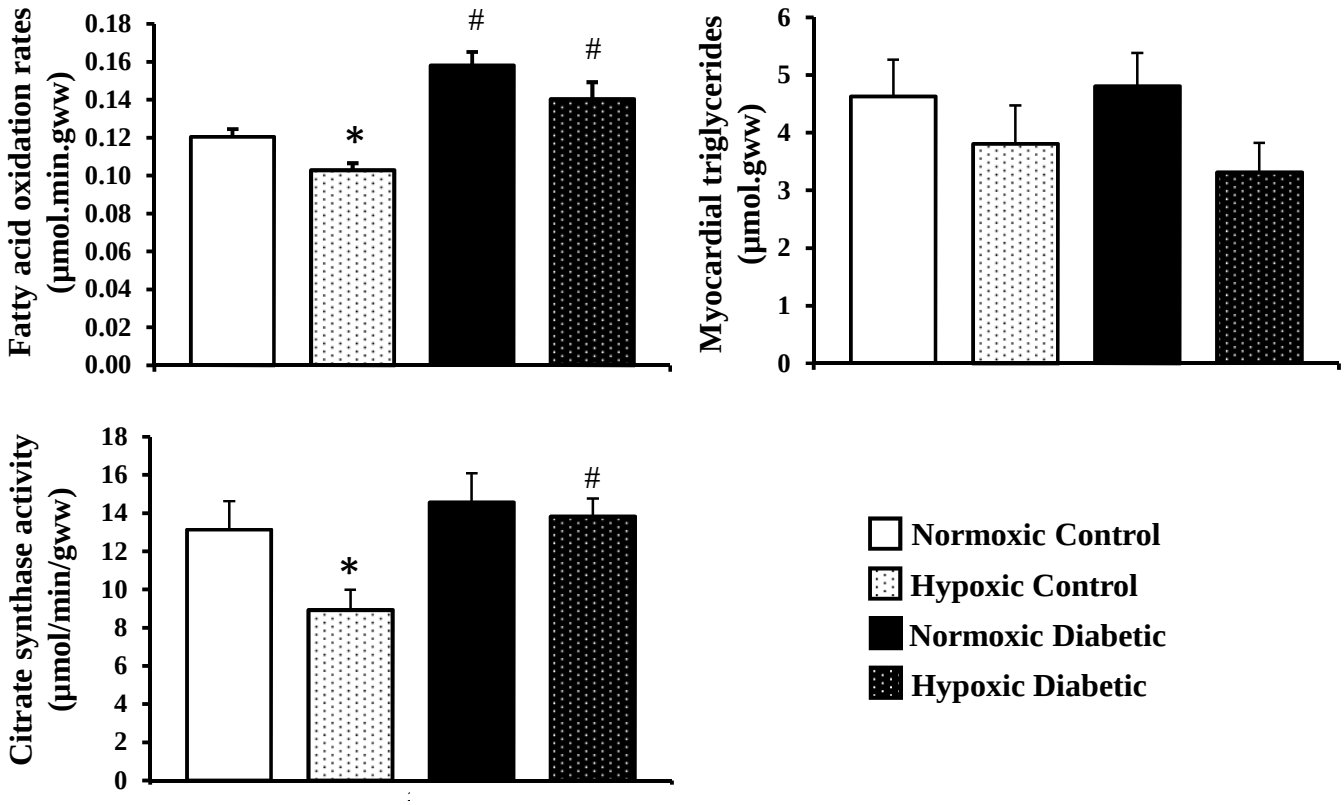

Fatty acid oxidation rates (µmol.min.gww)
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
*
#
#
Myocardial triglycerides (µmol.gww)
6
5
4
3
2
1
0
Citrate synthase activity (µmol/min/gww)
18
16
14
12
10
8
6
4
2
0
*
#
Normoxic Control
Hypoxic Control
Normoxic Diabetic
Hypoxic Diabetic

Figure 3

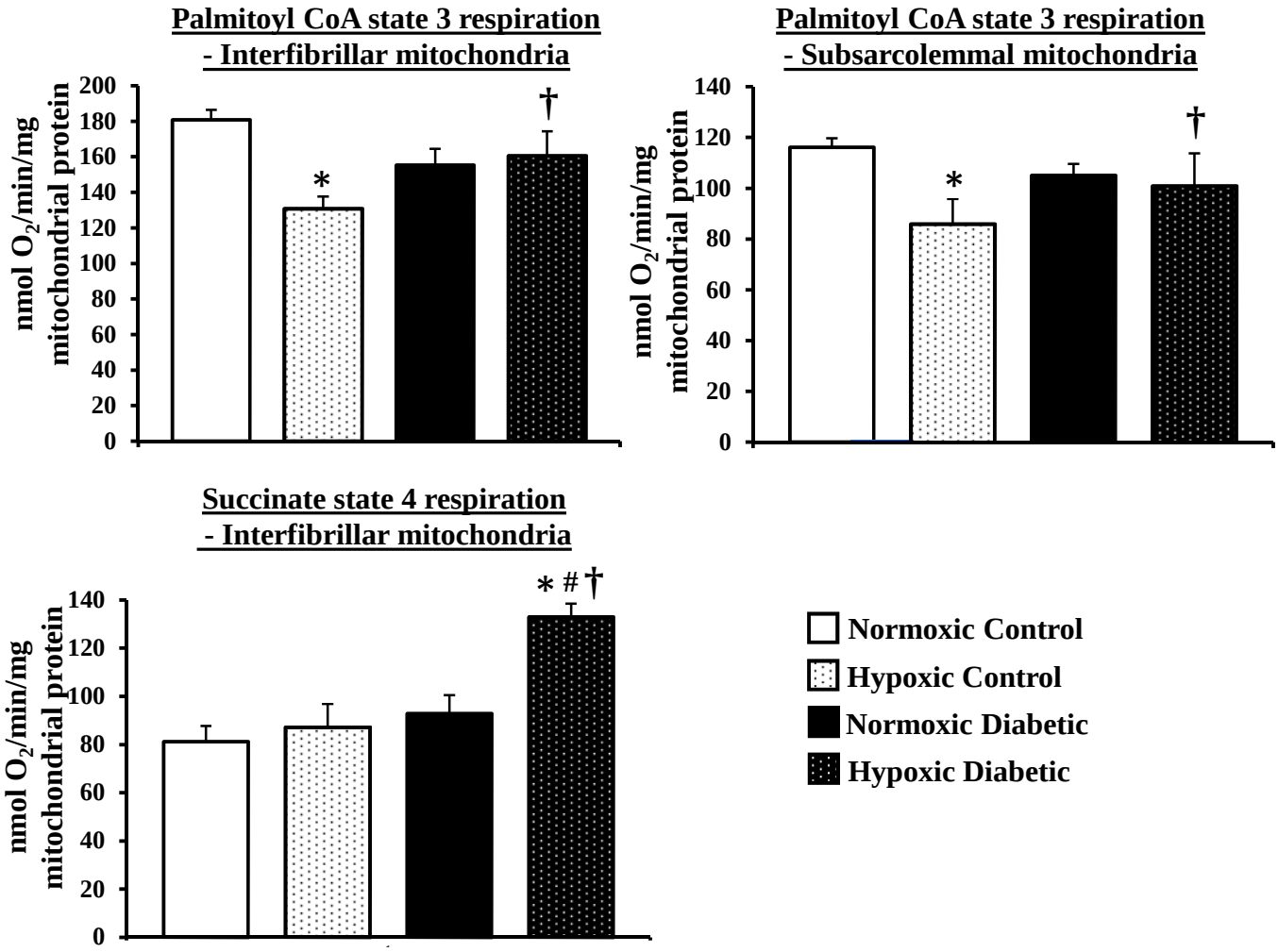

Palmitoyl CoA state 3 respiration
- Interfibrillar mitochondria
nmol O2/min/mg mitochondrial protein
0
20
40
60
80
100
120
140
160
180
200
*
†
Palmitoyl CoA state 3 respiration
- Subsarcolemmal mitochondria
nmol O2/min/mg mitochondrial protein
0
20
40
60
80
100
120
140
*
†
Succinate state 4 respiration
- Interfibrillar mitochondria
nmol O2/min/mg mitochondrial protein
0
20
40
60
80
100
120
140
* # †
Normoxic Control
Hypoxic Control
Normoxic Diabetic
Hypoxic Diabetic

Figure 4

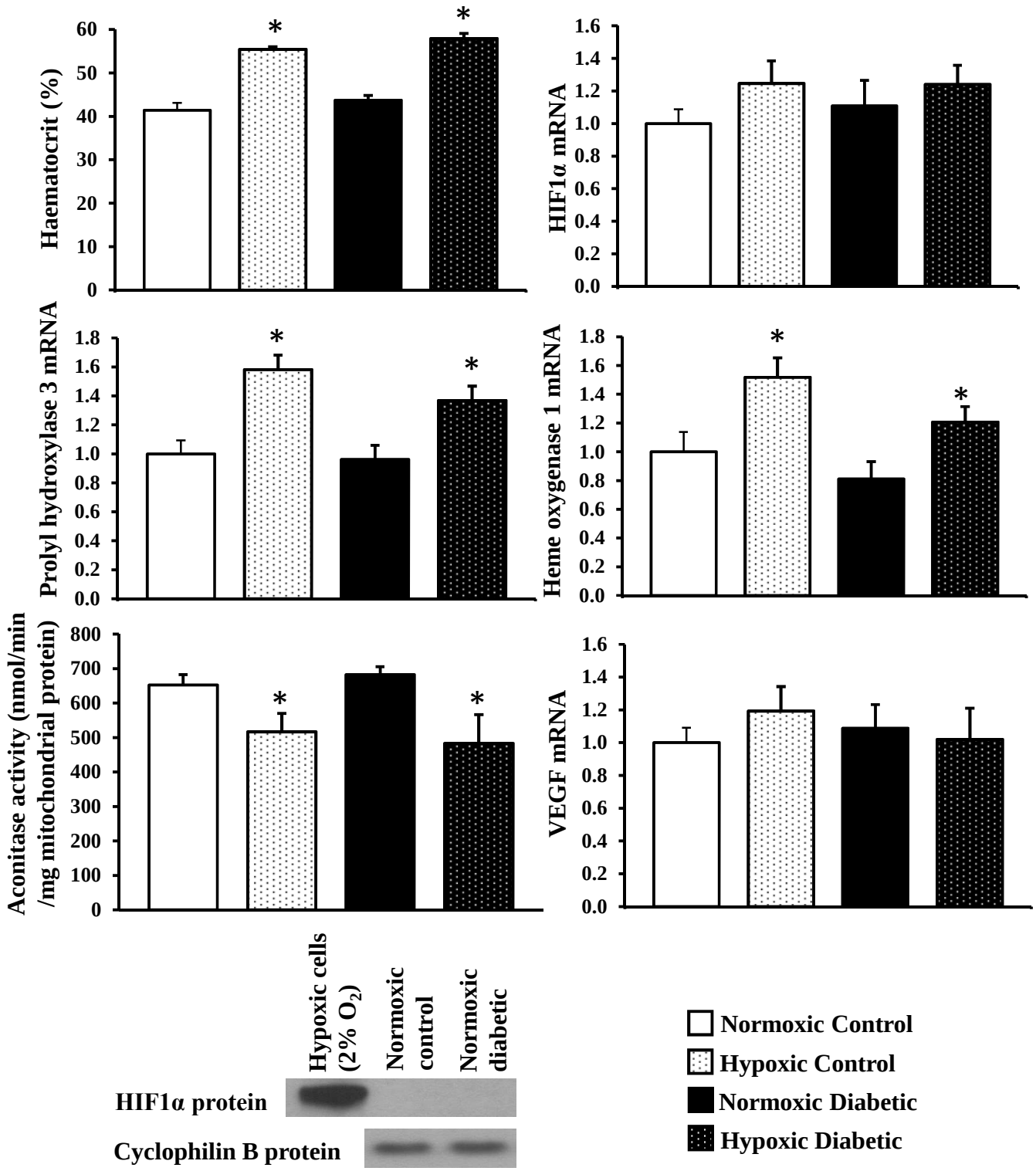

Haematocrit (%)
HIF1α mRNA
Prolyl hydroxylase 3 mRNA
Heme oxygenase 1 mRNA
Aconitase activity (nmol/min /mg mitochondrial protein)
VEGF mRNA
Hypoxic cells (2% O2)
Normoxic control
Normoxic diabetic
HIF1α protein
Cyclophilin B protein
Normoxic Control
Hypoxic Control
Normoxic Diabetic
Hypoxic Diabetic

Figure 5

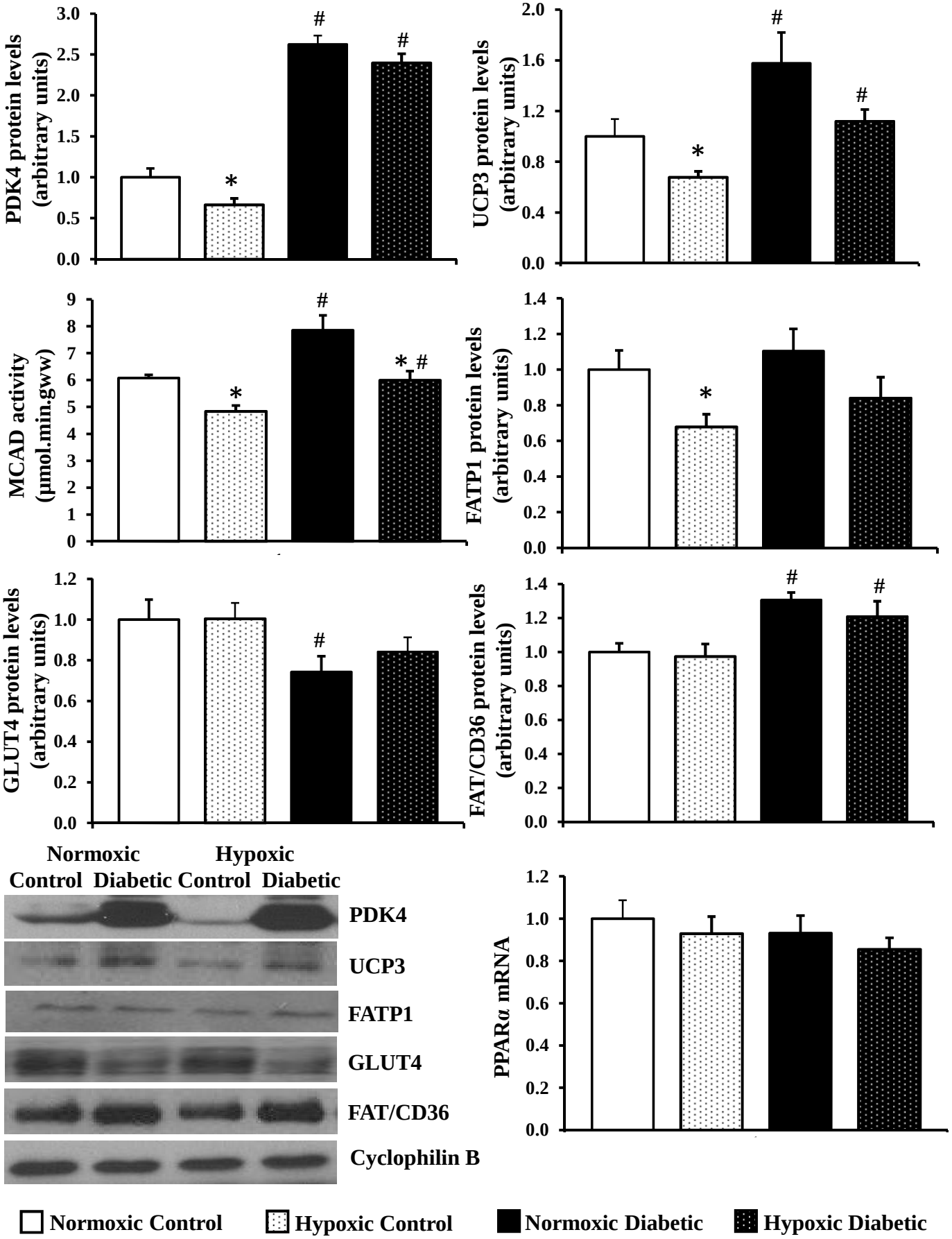
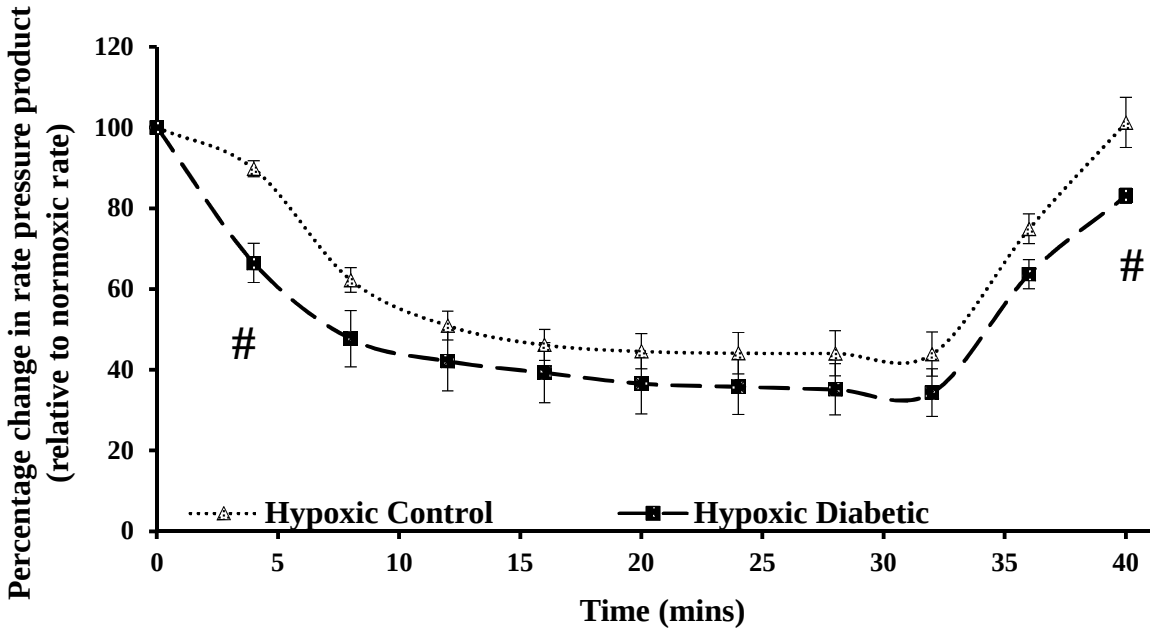

PDK4 protein levels (arbitrary units)
UCP3 protein levels (arbitrary units)
MCAD activity (µmol.min.gww)
FATP1 protein levels (arbitrary units)
GLUT4 protein levels (arbitrary units)
FAT/CD36 protein levels (arbitrary units)
Normoxic Control
Diabetic
Hypoxic Control
Diabetic
PDK4
UCP3
FATP1
GLUT4
FAT/CD36
Cyclophilin B
PPARα mRNA
Normoxic Control
Hypoxic Control
Normoxic Diabetic
Hypoxic Diabetic

Figure 6


Percentage change in rate pressure product (relative to normoxic rate)
120
100
80
60
40
20
0
0
5
10
15
20
25
30
35
40
Time (mins)
Hypoxic Control
Hypoxic Diabetic
#
#

Supplementary Table 1

Supplementary Table1. Real-time quantitative PCR primers and western blotting primary antibodies

Real-time quantitative PCR primers		
Target	NM	Primer sequence
Heme oxygenase 1	NM_012580.2	Fp: GTC-AGG-TGT-CCA-GGG-AAG-G
		Rp: CTC-TTC-CAG-GGC-CGT-ATA-GA
VEGF	NM_031836.2	Fp: CAA-GCC-AAG-GCG-GTG-AGC-CA
		Rp: TCT-GCC-GGA-GTC-TCG-CCC-TC
PHD3	NM_019371.1	Fp: CTA-TGT-CAA-GGA-GCG-GTC-CAA
		Rp: GTC-CAC-ATG-GCG-AAC-ATA-ACC
HIF1α	NM_024359.1	Fp: TGC-TTG-GTG-CTG-ATT-TGT-GA
		Pp: GGT-CAG-ATG-ATC-AGA-GTC-CA
GAPDH	NM_017008	FP: TGG-GAA-GCT-GGT-CAT-CAA-C
		Rp: GCA-TCA-CCC-CAT-TTG-ATG-TT
Cyclophilin A	NM_017101.1	Fp: CTG-ATG-GCG-AGC-CCT-TG
		Rp: TCT-GCT-GTC-TTT-GGA-ACT-TTG-TC
RPL13A	NM_173340.2	Fp: CCC-TCC-ACC-CTA-TGA-CAA
		Rp: GGT-ACT-TCC-ACC-CGA-CCT-C
HMBS	NM_013168.2	Fp: TCC-CTG-AAG-GAT-GTG-CCT-AC
		Rp: ACA-AGG-GTT-TTC-CCG-TTT-G
HPRT1	NM_012583.2	Fp: GAC-CGG-TTC-TGT-CAT-GTC-G
		Rp: ACC-TGG-TTC-ATC-ATC-ACT-AAT-CAC
Western blotting antibodies		
Target	Supplied by:	
GLUT4	Prof. Geoff Holman, University of Bath, UK	
PDK4	Prof. Mary Sugden, Queen Mary's, University of London, UK	
FAT/CD36	Dr Narendra Tandon, Otsuka Maryland Medicinal Labs, USA	
Cyclophilin B	Abcam	
UCP3	Abcam	
FATP1	Santa Cruz Biotechnology	
HIF-1α	Novus Biologicals	

GAPDH; glyceraldehyde-3-phosphate dehydrogenase, RP13A; ribosomal protein L13A, HMBS; hydroxymethylbilane synthase, HPRT1; hypoxanthine phosphoribosyltransferase 1.

Supplementary Table 2. Two way ANOVA p values for the effects of hypoxia, diabetes and interactions between the two, for metabolic measurements.

	Significant effect of hypoxia	Significant effect of diabetes	Interaction
Heart weight		0.003	
Heart to body weight ratio	0.017	0.003	
Fat pad weight		0.000	
Fat pad to body weight ratio		0.000	
Glucose	0.000	0.004	
NEFA	0.000	0.01	
TAG	0.000		
Insulin		0.013	
B-OHB	0.000	0.000	0.000
Coronary flow rates		0.025	0.004
Developed pressure	0.013		
Glycolytic rates	0.000	0.000	
Lactate efflux rates	0.002	0.000	
Glycogen	0.002	0.003	
Fatty acid oxidation rates	0.004	0.000	
Myocardial triglycerides	0.049		
Citrate synthase activity	0.048	0.014	
Creatine	0.043		
Aspartate		0.004	
State 3 IFM respiration	0.015		0.004
State 3 SSM respiration	0.013		0.045
State 4 IFM respiration	0.005	0.001	0.03
Haematocrit	0.000		
Prolyl hydroxylase 3 mRNA	0.000		
Heme oxygenase 1 mRNA	0.000		
Aconitase activity	0.000		
PDK4 protein	0.024	0.000	
UCP3 protein	0.013	0.024	
MCAD activity	0.000	0.000	
FATP1 protein	0.008		
GLUT4 protein		0.032	
FAT/CD36 protein		0.001	

Supplementary Table 3

Supplementary Table 3. Mitochondrial state 4 and maximal ADP-stimulated respiration in cardiac interfibrillar mitochondria from control and diabetic rats housed in normoxia or hypoxia.

	Normoxic Control	Hypoxic Control	Normoxic Diabetic	Hypoxic Diabetic
Palmitoyl CoA respiration (nmol O_2/min/mg mitochondrial protein)				
State 3	181 ± 6	131 ± 7 *	155 ± 9	161 ± 14 †
State 4	43 ± 3	40 ± 7	39 ± 4	36 ± 4
Max ADP	244 ± 8	181 ± 18 *	228 ± 17	205 ± 19
RCR	4.4 ± 0.3	3.5 ± 0.5	4.1 ± 0.3	4.6 ± 0.3
Pyruvate respiration (nmol O_2/min/mg mitochondrial protein)				
State 3	260 ± 9	210 ± 12 *	213 ± 11 #	216 ± 10 †
State 4	48 ± 4	42 ± 6	36 ± 3	39 ± 2
Max ADP	229 ± 15	219 ± 18	213 ± 18	210 ± 11
RCR	5.5 ± 0.4	5.2 ± 0.5	6.1 ± 0.6	5.6 ± 0.6
Glutamate respiration (nmol O_2/min/mg mitochondrial protein)				
State 3	207 ± 11	204 ± 15	188 ± 8	202 ± 13
State 4	24 ± 1	27 ± 4	23 ± 2	24 ± 3
Max ADP	241 ± 7	222 ± 29	209 ± 9	219 ± 12
RCR	8.7 ± 0.3	7.9 ± 1.1	8.5 ± 1.0	8.5 ± 0.9
Succinate respiration (nmol O_2/min/mg mitochondrial protein)				
State 3	311 ± 21	256 ± 23	296 ± 17	264 ± 7
State 4	81 ± 7	87 ± 10	93 ± 8	133 ± 6 *#†
RCR	3.9 ± 0.2	3.0 ± 0.3 *	3.3 ± 0.3	2.0 ± 0.1 *#
FCCP-uncoupled	248 ± 9	194 ± 15 *	239 ± 15	203 ± 3 *

* $p < 0.05$ vs. normoxia within same disease state, # $p < 0.05$ vs. control under same oxygen condition, † $p < 0.05$ interaction between hypoxia and diabetes. RCR, respiratory control ratio, n = 4 - 8.

Supplementary Table 4

Supplementary Table 4. Mitochondrial state 3, state 4 and maximal ADP-stimulated respiration in cardiac subsarcolemmal mitochondria from control and diabetic rats housed in normoxia or hypoxia.

	Normoxic Control	Hypoxic Control	Normoxic Diabetic	Hypoxic Diabetic
Palmitoyl CoA respiration (nmol O_2/min/mg mitochondrial protein)				
State 3	116 ± 4	86 ± 10 *	105 ± 5	101 ± 13 †
State 4	25 ± 3	35 ± 3	27 ± 1	29 ± 4
Max ADP	138 ± 4	120 ± 7	133 ± 5	123 ± 7
RCR	4.2 ± 0.2	3.0 ± 0.3	3.9 ± 0.2	4.0 ± 0.8
Pyruvate respiration (nmol O_2/min/mg mitochondrial protein)				
State 3	162 ± 7	148 ± 10	139 ± 3 #	145 ± 11
State 4	35 ± 2	38 ± 3	29 ± 3	32 ± 4
Max ADP	134 ± 9	140 ± 8	139 ± 6	143 ± 7
RCR	4.6 ± 0.2	3.9 ± 0.1	5.0 ± 0.5	4.5 ± 0.3
Glutamate respiration (nmol O_2/min/mg mitochondrial protein)				
State 3	112 ± 6	113 ± 12	94 ± 3	109 ± 8
State 4	19 ± 2	22 ± 2	17 ± 1	21 ± 1
Max ADP	140 ± 6	139 ± 16	118 ± 3 #	133 ± 7
RCR	6.2 ± 0.5	5.1 ± 0.4	5.7 ± 0.3	5.2 ± 0.3
Succinate respiration (nmol O_2/min/mg mitochondrial protein)				
State 3	193 ± 13	178 ± 14	179 ± 9	168 ± 6
State 4	54 ± 3	63 ± 6	62 ± 7	75 ± 7
RCR	3.5 ± 0.1	2.9 ± 0.3 *	2.8 ± 0.3 #	2.3 ± 0.3
Maximal FCCP-uncoupled	155 ± 6	134 ± 11	146 ± 9	123 ± 2 *

* $p < 0.05$ vs. normoxia within same disease state, # $p < 0.05$ vs. control under same oxygen condition, † $p < 0.05$ interaction between hypoxia and diabetes, RCR, respiratory control ratio, n = 4 - 8.