Activation of HIF1α Rescues the Hypoxic Response and Reverses Metabolic Dysfunction in the Diabetic Heart

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Non-standard abbreviations: HIF; Hypoxia-Inducible Factor, T2D; type 2 diabetes, PHD; prolyl hydroxylase domain, UCP; uncoupling protein, FAT/CD36: fatty acid translocase, GLUT; glucose transporter, BNIP; BCL2/adenovirus E1B 19 kDa protein-interacting protein, FIH; factor inhibiting HIF, PGK; phosphoglycerate kinase; VEGF; vascular endothelial growth factor, LDH; lactate dehydrogenase, MCAD; medium chain acyl co-enzyme A dehydrogenase.

Abstract

Type 2 diabetes (T2D) impairs Hypoxia-Inducible Factor (HIF)1α activation, a master transcription factor that drives cellular adaptation to hypoxia. Reduced activation of HIF1α contributes to the impaired post-ischaemic remodelling observed following myocardial infarction in T2D. Molidustat is a HIF stabiliser currently undergoing clinical trials for the treatment of renal anaemia associated with chronic kidney disease, however, it may provide a route to pharmacologically activate HIF1α in the T2D heart.

In human cardiomyocytes, molidustat stabilised HIF1α and downstream HIF target genes, promoting anaerobic glucose metabolism. In hypoxia, insulin resistance blunted HIF1α activation and downstream signalling, but this was reversed by molidustat. In T2D rats, oral treatment with molidustat rescued the cardiac metabolic dysfunction caused by T2D, promoting glucose metabolism and mitochondrial function, whilst suppressing fatty acid oxidation and lipid accumulation. This resulted in beneficial effects on post-ischemic cardiac function, with the impaired contractile recovery in T2D heart reversed by molidustat treatment.

In conclusion, pharmacological HIF1α stabilisation can overcome the blunted hypoxic response induced by insulin resistance. *In vivo* this corrected the abnormal metabolic phenotype and impaired post-ischaemic recovery of the diabetic heart. Therefore, molidustat may be an effective compound to further explore the clinical translatability of HIF1α activation in the diabetic heart.

Introduction

Hypoxia-inducible factor 1 (HIF1) is a heterodimeric transcription factor considered to be a master regulator of oxygen homeostasis, as it is key to the cellular and systemic adaptive responses to low oxygen availability (hypoxia) (1-3). HIF1 consists of a constitutively expressed and stable β subunit (HIF1β), and an α subunit whose regulation is oxygen-dependent (HIF1α) (2). In normoxia, HIF1α is targeted for degradation by the proteasome via hydroxylation of proline residues, mediated by the oxygen-dependent prolyl hydroxylase domain (PHD) family of enzymes (4). When oxygen delivery is compromised, as occurs during myocardial ischaemia, the PHDs are inhibited and HIF1α escapes hydroxylation, allowing it to migrate to the nucleus and induce transcription of HIF-target genes. HIF regulates many hundreds of different genes involved in metabolism, cell growth, angiogenesis and erythropoiesis, allowing the tissue/organism to survive during hypoxia (5). Optimal HIF activation is essential for the heart to maintain its contractile function following myocardial infarction (MI) and pressure-overload hypertrophy (6-8). Recognition of HIFs central role in physiology and medicine was recently demonstrated by the Nobel Prize award in 2019 to William Kaelin, Jr, Sir Peter Ratcliffe, and Gregg Semenza.

A key element of HIF activation is the promotion of glycolysis and suppression of fatty acid oxidation (9-14), reprogramming cardiac substrate metabolism (15). Interestingly, this HIF-mediated metabolic shift is in the opposite direction to that caused by diabetes, in which glucose metabolism is decreased and fatty acid oxidation is increased (16; 17). Thus, from a metabolic perspective, HIF and diabetes have opposing effects on cardiac metabolism (15). In type 2 diabetes the shift towards fatty acid metabolism is proportional to the severity of insulin resistance (18; 19). In addition, this abnormal metabolism is associated with abnormal cardiac function in type 2 diabetes (20). Thus, activating HIF in diabetes may provide a mechanism to shift metabolism back towards greater glucose use and suppress fatty acid metabolism, resulting in improved cardiac function (21).

We have previously shown that in diabetes HIF1α stabilisation is blunted, and downstream activation of the adaptive hypoxic response is reduced in insulin resistant cardiomyocytes (22). This reduction in HIF activation is mediated by the increased fatty acids present within the myocardium. In hypoxia, fatty acids suppress the accumulation of succinate, which is needed for optimal HIF stabilisation via inhibition of the PHD enzymes (23). This results in diabetic hearts being less able to tolerate hypoxia and less able to adapt in the longer term (15). The clinical consequence for people with type 2 diabetes is a more rapid progression into heart failure following MI (24), as the decreased collateral vessel development present post-MI in patients with diabetes is due to decreased activation of HIF-dependent processes (25; 26). Thus, strategies that pharmacologically activate HIF may correct the blunted HIF activation caused by increased fatty acids in type 2 diabetes.

Cardiovascular disease is the leading cause of mortality in diabetes, and even with optimal managed risk factors (glucose, blood pressure, cholesterol), people with type 2 diabetes still have a 21% increased risk of cardiovascular disease (27). Therefore, there is an unmet need for new therapies for the diabetic heart. Molidustat (BAY85-3934) is an orally bioavailable PHD inhibitor that successfully stabilises HIF-1α, and is undergoing phase III clinical trials to treat anaemia in patients with chronic kidney disease (28-30). In cancer cells, it has been shown that molidustat stabilises HIF1α, and induces downstream targets involved in angiogenesis and erythropoiesis (31; 32). We therefore questioned whether molidustat could be repurposed for use in the heart, to overcome the blunted HIF activation and correct the abnormal cardiac metabolism induced by diabetes. Using both human cardiomyocytes and animal models we demonstrate here that molidustat can improve cardiac metabolism and HIF signalling in diabetes, resulting in improved recovery of the heart post-ischaemia.

Methods

Human induced pluripotent stem cell-derived cardiomyocyte (hiPSC-CM) differentiation and maturation

Healthy human induced pluripotent stem cell (hiPSCs) lines IMR90 and M180 were cultured in mTeSR1 medium on Matrigel-coated plates and were dissociated using ReLeSR. The cells were then transferred onto Reduced Growth Factor (RF) Matrigel-coated plates for differentiation into hiPSC-CM. Differentiation was initiated on Day 0 using Differentiation Medium (Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco) supplemented with 1% B27 minus insulin (Gibco) and 6 μM CHIR99201 (Tocris Bioscience)) for 2 days. On day 3, the Differentiation Medium was supplemented with 2.5 μM Wnt-C59 (Tocris Bioscience). The glucose depletion method was performed on days 11 and 13 by changing medium to no glucose RPMI with 1% B27 minus insulin (33). hiPSC-CMs maturation was initiated from day 16 by re-platting cells onto RF-Matrigel-coated plates and culturing in DMEM containing 5 mM glucose and supplemented with 0.4 mM oleic acid conjugated to BSA, for 1 week (34). Results from the IMR90 line are presented in Figures 1 and 2, with key findings confirmed in the M180 line presented in Supplementary Figure 1.

Insulin resistance in hiPSC-CM

Insulin resistance was induced by culturing mature hiPSC-CM in 'glucose-free IR-media' comprising DMEM without glucose, 0.3 mM palmitic acid:BSA (bound 6:1), 50 nM insulin and 2 mM non-essential amino acids for 3 days. On day 4, the media was switched to 'IR media' as above but also containing 12 mM glucose for a further 3 days.

Rat model of type 2 diabetes

All animal experiments conformed to the Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act, 1986 and were approved by local ethics committee. Type 2 diabetes was

induced as previously described (35; 36), generating an early-stage model of the disease presenting with mild hyperglycaemia, hyperinsulinemia and hyperlipidaemia. Briefly, male Wistar rats (Envigo – starting body weight ~ 300g) were fed a high-fat diet (Special Diet Services 829197, 60% calories from fats) for 5 weeks, and on day 14 they received a single low-dose intraperitoneal (i.p.) injection of streptozotocin (25 mg/kg body weight, in citrate buffer, pH 4) in the fasted state. Control rats were fed a standard chow diet for 5 weeks.

Molidustat administration

Human Cardiomyocytes – Mature hiPSC-CMs were cultured in the presence or absence of 50 μM molidustat (ADV465750852, Merck and R16009, Advanced ChemBlocks) for 16 h. A subgroup of control and insulin resistant hiPSC-CMs were exposed to hypoxia (2% O_2, 5% CO_2) for 16 h, with molidustat added just prior to the insulin resistant cells entering the hypoxic incubator. Cell media was collected and assayed spectrophotometrically for glucose and lactate concentrations, by measuring the increase in absorbance at 340nm following incubation with glucose assay reagent (Merck) for the former, or lactate dehydrogenase and NAD for the latter. Glucose consumption rates and lactate production rates were normalised to cell number.

Type 2 diabetic rats – Molidustat was given orally (5 mg/kg body weight) to type 2 diabetic and control rats, dissolved in sugar-free jelly (to ensure the precise dose was administered, yet avoiding the daily stress of gavaging). Molidustat was given daily for the final 5 days of the 5-week protocol. Untreated rats were given an equivalent amount of drug-free jelly. Rats were terminally anaesthetised in the fed state 24 hours after the final dose of molidustat, using an i.p. injection of pentobarbital sodium (0.7 ml of 200 mg/ml Euthatal). Blood was collected for measurement of haematocrit (HemoCue 201+ System meter), and glucose concentrations were measured from a sample of blood using an Accu-chek Aviva meter, following heart removal. Epididymal fat pads were collected and weighed to assess adiposity. Hearts

were either perfused for measurement of function and metabolic flux, used for mitochondrial isolation, or freeze-clamped in liquid nitrogen for subsequent molecular biology analyses.

Isolated heart perfusion

Hearts were isolated and arrested in ice cold Krebs-Henseleit buffer, rapidly cannulated via the aorta and then perfused in retrograde Langendorff mode according to our published protocol (35; 37). Hearts contracted against a constant afterload pressure of 100 mmHg (to represent the arterial blood pressure (38)). A fluid-filled PVC balloon connected to a pressure transducer was inserted into the left ventricle, and inflated to give an end-diastolic pressure of 4-8 mmHg. This balloon allowed measurement of intraventricular pressure during systole and diastole, and the subsequent calculation of heart rate (systolic pressure peaks per minute). Left ventricular developed pressure was calculated as peak systolic pressure minus end-diastolic pressure for each contraction cycle, and expressed as mmHg. Rate pressure product was calculated as the developed pressure multiplied by the heart rate, and expressed as mmHg per minute. Hearts were perfused with recirculating Krebs-Henseleit buffer containing 11 mM glucose, 0.4 mM palmitate (bound to BSA) and insulin (3 U/l), gassed with 95% O_2 and 5% CO_2, at 37ºC. Hearts were perfused for 20 mins (baseline pre-ischaemia), followed by 30 mins of low-flow ischaemia (0.3 ml/min/gww), and then 25 mins of reperfusion.

^{3}H-perfusion and metabolic rates

For measurement of glycolytic rates, buffer was supplemented with 0.2 μCi/ml of [5-^{3}H]-glucose. For measurement of palmitate oxidation rates, buffer was supplemented with 0.2 μCi/ml [9,10-^{3}H] palmitate. Buffer aliquots were collected at 4-minute intervals throughout the baseline perfusion, and ^{3}H metabolic rates calculated according to published protocols (15). Lactate efflux rates were calculated spectrophotometrically at 340nm using these timed aliquots, following addition of lactate dehydrogenase and NAD.

Tissue analyses

Cardiac and hepatic triglyceride concentrations were measured spectrophotometrically using a Randox triglyceride assay kit, following Folch extraction. Glycogen content was measured using amyloglucosidase to convert glycogen to glucose, which was subsequently quantified spectrophotometrically using glucose assay reagents (Merck). Medium chain acyl-coenzyme A dehydrogenase (MCAD) activity was measured spectrophotometrically according to the previously published protocol (37).

Mitochondrial isolation and respiration

Mitochondria were isolated as previously described, and respired with pyruvate (10 mM) and malate (5 mM) as carbohydrate substrates (39). Respiration was measured under ADP-stimulated (0.2 mM) state 3 conditions, and maximally stimulated with the uncoupling agent FCCP (20 μM).

Western Blotting

Tissue and cells were lysed in ice-cold lysis buffer (22), a protein assay was used to calculate protein concentration, and 10 μg of cell protein or 25 μg of tissue protein was loaded on to SDS-PAGE gels and separated by electrophoresis. For measurement of sarcolemmal transporters, separation of the sarcolemmal membrane fraction was carried out using density centrifugation according to the established protocol of Luiken *et al.* (40). Primary antibodies to HIF1α (Cayman 10006421), lactate dehydrogenase (LDH, Abcam Ab47010), glucose transporter 1 (GLUT1, Abcam ab652), glucose transporter 4 (GLUT4, a kind gift from Prof Geoff Holman), uncoupling protein 3 (UCP3, Abcam Ab180643), glyceraldehyde 3 phosphate dehydrogenase (GAPDH, Proteintech, 60004-1-Ig), pyruvate dehydrogenase kinase 1 (PDK1, Cell Signalling, 3062) and fatty acid translocase (FAT/CD36, MO25 a gift from Dr Narendra Tandon) were used, in combination with the relevant secondary antibodies (Abcam). Even protein loading and transfer were confirmed by ponceau S total protein staining as a housekeeping loading control for tissue,

and normalised to GAPDH for molidustat-treated cells. Bands were quantified using LI-COR C-Digit chemiluminescent detection system (LI-COR Biotechnology) and Image Studio Software Version 5.2.5.

RNA isolation and qPCR

RNA was extracted from cells and tissue using a RNeasy mini kit (Qiagen), with cDNA conversion carried out with a high-capacity RNA-to-cDNA kit (Applied Biosystems) using a SensoQuest Labcycler (GeneFlow). Quantitative polymerase chain reaction amplification was performed using Power SYBR Green PCR Master Mix (Thermofisher) with 15 ng per well of cDNA, using a StepOne Plus Real-Time PCR systems machine (Applied Biosystems). Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method, normalised to the housekeeper gene (SDHA for hearts, UBC for cells). Primer sequences are located in the Supplementary Table.

Data and Resource Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author upon reasonable request. No applicable resources were generated or analysed during the current study.

Statistics

Results are presented as mean ± SEM, and considered significant at p values of less than 0.05, analysed using GraphPad Prism version 8. Data sets containing two groups were analysed using a 2-tailed parametric unpaired t-test with Welch's correction (Figure 1). Data sets containing multiple groups were analysed using a 1-way ANOVA, with Tukey's post hoc correction for multiple comparisons (Figure 2). Data sets with two groups (control vs. diabetic) and two variables (vehicle vs. molidustat) were analysed using a 2-way ANOVA, with Sidak's post hoc correction for multiple comparisons (Figures 3-6).

Results

Molidustat stabilises HIF1α signalling and downstream metabolic adaptation in human cardiomyocytes

Firstly we set out to determine whether molidustat could regulate HIF-dependent pathways in human iPSC-derived cardiomyocytes. In control cardiomyocytes, HIF1α protein was barely detectable, however, following 16 hours exposure to molidustat there was robust 30-fold stabilisation of HIF1α protein (Figure 1A). Molidustat increased the expression of multiple genes involved in glycolysis, increasing glucose transporter GLUT1 7-fold, and increasing by over 2-fold the expression of aldolase A, enolase 1 and phosphoglycerate kinase (PGK) 1 (Figure 1B-E). This resulted in a 2-fold increase in glucose metabolism and a 36% increase in lactate release, in molidustat-treated human cardiomyocytes (Figure 1F-G). Other downstream targets of HIF1α also changed accordingly, with molidustat increasing the expression of the mitophagy gene BNIP3 6-fold and prolyl hydroxylase domain 2 (PHD2) 2-fold (Figure 1H-I). Molidustat decreased HIF1α mRNA by 33%, demonstrating a feedback loop from HIF1α protein stabilisation (Figure 1K). These key findings in the IMR90 human iPSC-CM line were confirmed in the M180 iPSC-CM line (Supplementary Figure 1).

Molidustat overrides the blunted HIF signalling caused by insulin resistance.

Exposure of control cardiomyocytes to hypoxia stabilised HIF1α protein, and induced transcription of downstream HIF target genes BNIP3, GLUT1, aldolase A, and PHD2 (Figure 2 and Supplementary Figure 2A). In contrast, when insulin resistant human cardiomyocytes were exposed to the same hypoxic challenge, stabilisation of HIF1α protein and induction of downstream gene targets were all suppressed, demonstrating a blunted adaptive response to hypoxia in insulin resistance (Figure 2). Addition of molidustat to insulin resistant cells just prior to hypoxia was able to improve hypoxic signalling. Molidustat was able to increase HIF1α protein (Figure 2A) and induce transcription of the HIF target

genes (BNIP3, GLUT1, aldolase A, PGK1 and PHD2, Figure 2B-F), overriding the inhibitory effect of insulin resistance on hypoxic adaptation. Thus, molidustat provides a pharmacological approach to restore cardiac HIF signalling and hypoxic adaptation in insulin resistance.

Molidustat increases systemic HIF targets in the type 2 diabetic rat

We next administered molidustat orally to control and type 2 diabetic rats. Molidustat upregulated haemoglobin content, by 14% and 10%, respectively, in control and diabetic rats (Figure 3A), but did not affect body weight or heart weight in any group (Figure 3B-C). Molidustat did not correct the adiposity or hepatic triglycerides, which remained elevated in our diabetic rats, thus, molidustat did not correct the systemic diabetic phenotype (Figure 3D-E). Similarly, molidustat did not correct the hyperglycaemia in the diabetic rats (Figure 3F and Supplementary Figure 3A).

In agreement with published literature (31), the HIF1α protein did not remain elevated once the drug was metabolised from the circulation (Supplementary Figure 2B), but the downstream genes and products remained elevated 24 hours post-final dose. The HIF target gene, VEGF, was decreased by 29% in type 2 diabetic hearts (Figure 3G). *In vivo* treatment with molidustat increased VEGF mRNA by 42% in the diabetic hearts, back to that found in control hearts (Figure 3G). Similarly, lactate dehydrogenase (LDH), another cardiac HIF target, showed a significant interaction between diabetes and molidustat (Figure 3H).

Molidustat improves cardiac glucose metabolism in diabetes

Diabetes impairs normal cardiac metabolism, forcing the heart to metabolise less glucose and more fatty acid. Given that molidustat was able to promote glucose metabolism in the human IPS-derived cardiomyocytes, we questioned whether pharmacological HIF1α activation may provide a route to correct cardiac metabolism *in vivo* in type 2 diabetes. Type 2 diabetic hearts had impaired glycolytic rates, lactate efflux rates and mitochondrial pyruvate oxidation (Figure 4A,B,G,H). *In vivo* treatment with molidustat increased cardiac glycolytic rates by 77% in type 2 diabetic rats (Figure 4A). Similarly, lactate efflux

rates were increased by 70% following molidustat treatment of type 2 diabetic rats (Figure 4B). We investigated whether changes in glucose transporters were associated with the increased glycolysis and lactate production with molidustat treatment. Changes in total GLUT1 protein in the heart did not reach significance (Figure 4C), however, when we specifically looked at GLUT1 at the sarcolemma we found a significant increase with molidustat treatment (Figure 4E). This contrasted with GLUT4, which was decreased in the diabetic heart and was not upregulated with molidustat treatment (Figure 4D and F), thus, the increase in glucose uptake was driven by GLUT1 and not GLUT4. The improvement in glycolysis was accompanied by improvements in mitochondrial pyruvate oxidation. Mitochondria from diabetic hearts had impaired respiration when oxidising pyruvate, but when treated with molidustat this resulted in a 34% increase in maximal respiration (Figure 4G-H). In contrast to glucose catabolism, cardiac glycogen content was not modified by molidustat (Supplementary Figure 3B).

Molidustat decreases cardiac fatty acid metabolism in diabetes

Type 2 diabetic hearts had elevated rates of fatty acid oxidation, concentrations of myocardial triglycerides, and activity/expression of lipid metabolism proteins (Figure 5A-E). In type 2 diabetic hearts, treatment with molidustat decreased fatty acid oxidation rates by 15%, compared with untreated type 2 diabetic rats (Figure 5A). Myocardial triglyceride concentrations were significantly decreased by 70% with molidustat treatment in type 2 diabetes (Figure 5B). Thus, both aspects of fatty acid metabolism, oxidation and esterification, were downregulated in the heart by *in vivo* molidustat treatment. PPARα is the predominant regulator of fatty acid metabolism in the heart and has been shown to be under the control of HIF, therefore, we measured activity and protein expression of several PPARα targets. Medium chain acyl co-enzyme A dehydrogenase (MCAD) activity was increased 2-fold in type 2 diabetic hearts compared with control hearts, but molidustat treatment decreased MCAD activity by 30% in type 2 diabetes (Figure 5C). Uncoupling protein 3 (UCP3) protein levels were increased 5-fold in type 2 diabetic

hearts compared with control hearts, but molidustat treatment decreased UCP3 levels by 88% in type 2 diabetes (Figure 5D). There was a significant interaction term for fatty acid translocase (FAT/CD36) protein levels between diabetes and molidustat treatment (Figure 5E), decreasing in type 2 diabetic hearts treated with molidustat, which mirrored the changes in the other PPARα responsive proteins.

Molidustat improves post-ischaemic function in diabetes

Molidustat treatment had no effect on baseline cardiac function, with no change in rate pressure product (Figure 6A), heart rate or developed pressure (Supplementary Figure 3C-D) under normal aerobic perfusion conditions. Hearts were then challenged with 30 mins of low-flow ischaemia, followed by 25 mins of reperfusion. Type 2 diabetic hearts had significantly decreased post-ischemic recovery compared with control hearts, as shown by a 20% decrease in rate pressure product during reperfusion (Figure 6B). Molidustat treatment significantly increased reperfusion recovery by 27% in type 2 diabetic rats, back to control levels (Figure 6B-C). Molidustat caused small increases in heart rate and developed pressure during reperfusion, which culminated in the significant improvement in rate pressure product in type 2 diabetic rats (Figure 6D-E). Thus, molidustat was able to restore blunted HIF stabilisation and downstream signalling in both human cardiomyocytes and rodent hearts, to improve cardiac substrate metabolism and post-ischaemic functional recovery in type 2 diabetes.

Discussion

The diabetic heart has impaired HIF1α stabilisation, which subsequently blunts critical metabolic adaptations to hypoxia, contributing to decreased cardiac function following myocardial ischaemia. Molidustat, a HIF stabiliser developed for the treatment of anaemia associated with chronic kidney disease, provides a potential pharmacological route to promote HIF signalling in the diabetic heart, thereby improving metabolism and post-ischaemic function. Here we show for the first time that molidustat can stabilise HIF1α in human iPSC-derived cardiomyocytes, and can overcome the inhibitory effect of insulin resistance on hypoxic signalling. In a rodent model of type 2 diabetes we further show that molidustat can correct cardiac metabolism, by improving glucose metabolism and suppressing fatty acid metabolism. This metabolic remodelling results in improved cardiac recovery following ischaemia in type 2 diabetes. Thus, repurposing molidustat may be beneficial for metabolism and function in the diabetic heart.

Suppression of HIF signalling by diabetes

We have previously shown that insulin resistance suppresses HIF1α stabilisation and downstream hypoxic adaptation in rodent cardiomyocytes (22). This is due to elevated fatty acids in diabetes suppressing myocardial succinate concentrations, as succinate and fumarate inhibit the PHD enzymes necessary to promote HIF stabilisation (22). Here we demonstrate impaired HIF stabilisation and hypoxic signalling in human insulin resistant cardiomyocytes, demonstrating translation to the human disease state. We have shown in the heart, in contrast to fibroblasts and retinal cells (41; 42), that the driver for HIF dysfunction is specifically the hyperlipidaemia, rather than the hyperinsulinaemic or hyperglycaemic state in diabetes (22). As highlighted by Catrina and Zheng (43), this divergence between tissues is likely due to the differential effects of diabetes on glycolysis in these tissues, with the type 2 diabetic heart presenting with decreased glucose metabolism and decreased intracellular glucose concentrations (18; 35).

Impaired adaptation to hypoxia has profound effects in cardiac disease, as genetic models of HIF1α deletion exhibited impaired function during pressure overload hypertrophy (8), whereas HIF1α overexpression resulted in improved post-MI function and reduced infarct size (6; 7). Determining that insulin resistance blunts HIF activation provides a mechanism to explain decreased VEGF and collateral vessel development seen in patients with diabetes (25; 26), with the former identified as a seminal event in the development of diabetic cardiomyopathy (44). However, given the far-reaching effects of the HIF transcription factor, regulating many hundreds of different genes, the effects of impaired HIF stabilisation in response to hypoxia would be far reaching, affecting metabolism, cell growth and death, oxygen homeostasis, iron homeostasis and vasomotor regulation. Thus, impaired HIF signalling in diabetes would impair the ability of the heart to adapt following MI and would result in accelerated progression into heart failure. In agreement, Stone *et al.* reported patients with diabetes have a 72% increase in the progression to heart failure 3 months after MI (24).

Molidustat can restore HIF signalling in human cardiomyocytes

Molidustat (BAY85-3934) is an orally-bioavailable triazole based PHD inhibitor that is undergoing phase III clinical trials to treat anaemia in patients with chronic kidney disease (28; 29; 31). Molidustat confers various benefits over other clinical PHD inhibitors in development, as it has a higher stability upon binding, increased potency and greater selectivity for PHD enzymes (30; 32). Despite its development to target the kidney, we show here that molidustat can robustly stabilise HIF1α in human cardiomyocytes and induce downstream signalling across several HIF targets in the heart. Furthermore, molidustat can overcome the inhibition of HIF signalling induced by insulin resistance, thus providing a mechanism to correct the hypoxic signalling defects associated with diabetes. Therefore, molidustat is an effective compound to further explore the clinical translatability of HIF stabilisation in the diabetic heart.

Molidustat reprograms substrate metabolism in diabetes

Cardiac metabolism is dysregulated in type 2 diabetes, with decreased dependence on glucose and increased dependence on fatty acid metabolism. This metabolic shift towards greater fat use is associated with contractile dysfunction and impaired recovery post-ischaemia (20; 35). Here we show that molidustat treatment restores the diabetes-induced suppression of glucose metabolism in the heart. HIF1α upregulates enzymes and proteins involved in glucose uptake, glycolysis and lactate production, which resulted in an increase in both glycolytic flux within the heart and lactate efflux from the heart in the molidustat-treated diabetic animals. Concomitantly, molidustat decreased fatty acid oxidation and triglyceride accumulation within the myocardium in diabetic rats, reducing the dependency on fatty acids for ATP generation in diabetes. PPARα, the master transcription factor controlling fatty acid metabolism, has been shown to be downregulated in response to HIF stabilisation (12; 13), decreasing the transcription of PPARα target genes in hypoxia. The benefit of targeting PPARα in diabetes is that it corrects both fatty acid oxidation and also the lipotoxicity associated with lipid overload within the cytosol, which are hallmarks of the pathophysiology of the diabetic heart. Molidustat also restored the impaired mitochondrial pyruvate oxidation observed in diabetic hearts, which was initially counterintuitive given that in cells HIF1α increases pyruvate dehydrogenase kinase (PDK) 1, the suppressor of pyruvate dehydrogenase (PDH) (45). However, we have shown previously that PDK1 is not regulated by hypoxia in the heart (46) (Supplementary Figure 3D). Therefore, the increased pyruvate oxidation is most likely due to the correction of fatty acid metabolism, removing the allosteric inhibition on PDH activity.

Several pre-clinical metabolic therapies have been proposed to treat the diabetic heart, specifically targeting glucose or fatty acid metabolic pathways. The reciprocal relationship between these two substrate pathways was described by Randle *et al*. (47), whereby as metabolism of one fuel is increased the other is decreased due to intracellular crosstalk between pathways. Targeting HIF as an approach to correct metabolism has an additional advantage over these alternative metabolic therapies, as it

transcriptionally regulates both glucose and fatty acid metabolism simultaneously, directly upregulating glucose and downregulating fatty acid metabolism. Thus, molidustat-dependent HIF stabilisation has the added advantage as a metabolic regulator by directly changing both metabolic pathways, in addition to the endogenous Randle cycle which will further operate between pathways.

Molidustat improves recovery post-ischaemia

Here we have shown that molidustat treatment shows functional benefit for the diabetic heart in the setting of ischaemia, by improving recovery at reperfusion. We postulate that improved glycolytic flux in the diabetic heart from molidustat treatment primes the heart to be able to tolerate ischaemia to a greater extent, due to its ability to generate ATP under oxygen-restricted conditions. However, given the far-reaching targets for HIF and the integrated nature of metabolism, it is likely that the benefit is the culmination of multiple pathways. We have previously shown a similar functional improvement in diabetes using the non-specific HIF stabiliser dimethyloxallyl glycine, corroborating our findings here with a more selective and clinically relevant compound (22). The work presented here highlights the potential to repurpose molidustat as a cardiac therapy for patients with diabetes. Given that cardiovascular disease is the leading cause of mortality in individuals with diabetes, and even with optimal managed risk factors (glucose, blood pressure, cholesterol) people with type 2 diabetes still have a 21% increased risk of cardiovascular disease (27), there is an unmet need for treatments for the heart in diabetes.

Molidustat did not modify systemic markers of diabetes in our early stage type 2 diabetic model, as animals remained hyperglycaemic, with increased adiposity and elevated hepatic triglycerides (as a marker for fatty liver disease). However, it did raise blood haematocrit in the diabetic animals, in line with the drugs development as a treatment for anaemia. Though anaemia is not a facet of our early-stage model of type 2 diabetes, people with diabetes commonly have anaemia associated with diabetic kidney disease, estimated at 1 in 4 patients with diabetes also being anaemic (48). Thus, while this study was

designed to assess molidustat for the treatment of the diabetic heart, there is potential that this compound could serve as a pan-complication therapy for diabetes.

In conclusion, pharmacological HIF1α stabilisation with molidustat corrects the blunted HIF response to hypoxia caused by insulin resistance in both human and rodent cardiomyocytes. *In vivo* this corrects the abnormal metabolic phenotype of the diabetic heart, resulting in improved post-ischaemic contractile recovery.

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Conflict of Interest

None to declare

Author Contributions

MdLSF, UP, KMJHD, CNMA, MCG, EM and LCH researched data, KMJH, CNMA and LCH wrote the manuscript, DJT, CAC and LCH contributed to discussion and edited the manuscript.

Guarantor Statement

LCH is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and accuracy of the data analysis.

Figure Legends

Figure 1 – Molidustat increases HIF signalling in human cardiomyocytes.
Hypoxia-Inducible Factor (HIF)1α protein levels in control and molidustat-treated (Moli) human IMR90 inducible pluripotent stem cell-derived cardiomyocytes (**A**). Expression of HIF1α target genes in control and molidustat-treated cardiomyocytes, including glucose transporter 1 (GLUT1), aldolase A, enolase 1, phosphoglycerate kinase 1 (PGK1), bcl2/adenovirus E1B 19kDa interacting protein 3 (BNIP3), prolyl hydroxylase domain (PHD)2, factor inhibiting HIF (FIH) and HIF1α mRNA (**B-E, H-K**). Glucose consumption by and lactate release from control and molidustat-treated cardiomyocytes (**F-G**). * $p < 0.05$ vs. control.

Figure 2 - Molidustat overrides the impaired HIF signalling caused by insulin resistance.
Hypoxia-Inducible Factor (HIF)1α protein levels in control (Con) and insulin resistant (IR) human cardiomyocytes, cultured in normoxia (Nx) or hypoxia (Hx) for 16 hrs. Molidustat (Moli) was added to IR cells just prior to entry into hypoxic chamber (**A**). Expression of HIF1α target genes under the respective conditions, including bcl2/adenovirus E1B 19kDa interacting protein 3 (BNIP3), glucose transporter 1 (GLUT1), aldolase A, phosphoglycerate kinase 1 (PGK1) and prolyl hydroxylase domain (PHD)2 (**B-F**). * $p < 0.05$ vs. respective control group, # $p < 0.05$ vs. respective insulin resistant group.

Figure 3 – Molidustat increases systemic HIF targets in type 2 diabetes
Haemoglobin content, body weight, heart weight, epididymal fat pad weight in control and diabetic rats, with and without molidustat treatment (**A-D**). Hepatic triglycerides and fed blood glucose concentrations in control and diabetic rats, with and without molidustat treatment (**E-F**). Cardiac VEGF expression and lactate dehydrogenase (LDH) protein levels in control and diabetic rats, with and without molidustat treatment (**G-H**). * $p < 0.05$ vs. respective control group, # $p < 0.05$ vs. diabetic untreated, γ $p < 0.05$ significant interaction between disease and drug treatment.

Figure 4 – Molidustat increases cardiac glucose metabolism in the type 2 diabetes
Cardiac glycolytic rates and lactate efflux rates in control and diabetic rats, with and without molidustat treatment (**A-B**). Glucose transporter 1 (GLUT1) and 4 (GLUT4) protein levels in control and diabetic rats, with and without molidustat treatment (**C-D**). Sarcolemmal GLUT1 and GLUT4 protein levels in control and diabetic rats, with and without molidustat treatment (**E-F**). State 3 ADP-stimulated and maximally FCCP-stimulated pyruvate respiration rates in isolated mitochondria from control and diabetic rats, with and without molidustat treatment (**G-H**). * $p < 0.05$ vs. control, # $p < 0.05$ vs. diabetic untreated, † $p < 0.05$ significant effect of molidustat treatment at 2-way ANOVA.

Figure 5 – Molidustat decreases cardiac fatty acid metabolism in type 2 diabetes
Cardiac fatty acid oxidation rates in control and diabetic rats, with and without molidustat treatment (**A**). Myocardial triglyceride concentrations, medium chain acyl Co-enzyme A dehydrogenase (MCAD) activity, uncoupling protein 3 (UCP3) protein and fatty acid translocase (FAT/CD36) protein within the myocardium of control and diabetic rats, with and without molidustat treatment (**B-E**). * $p < 0.05$ vs. control, # $p < 0.05$ vs. diabetic untreated, γ $p < 0.05$ significant interaction between disease and drug treatment.

Figure 6 – Molidustat improves post-ischaemic recovery in type 2 diabetes

Cardiac function was measured as rate pressure product (the multiple of heart rate and developed pressure) in control and diabetic rats, with and without molidustat treatment. Hearts were perfused at baseline (**A**), subjected to 30 mins of low-flow ischaemia followed by 25 mins of reperfusion (**B-E**). Average RPP traces are shown from the time course of the perfusion experiment (**C**). * $p < 0.05$ vs. control, # $p < 0.05$ vs. diabetic untreated.

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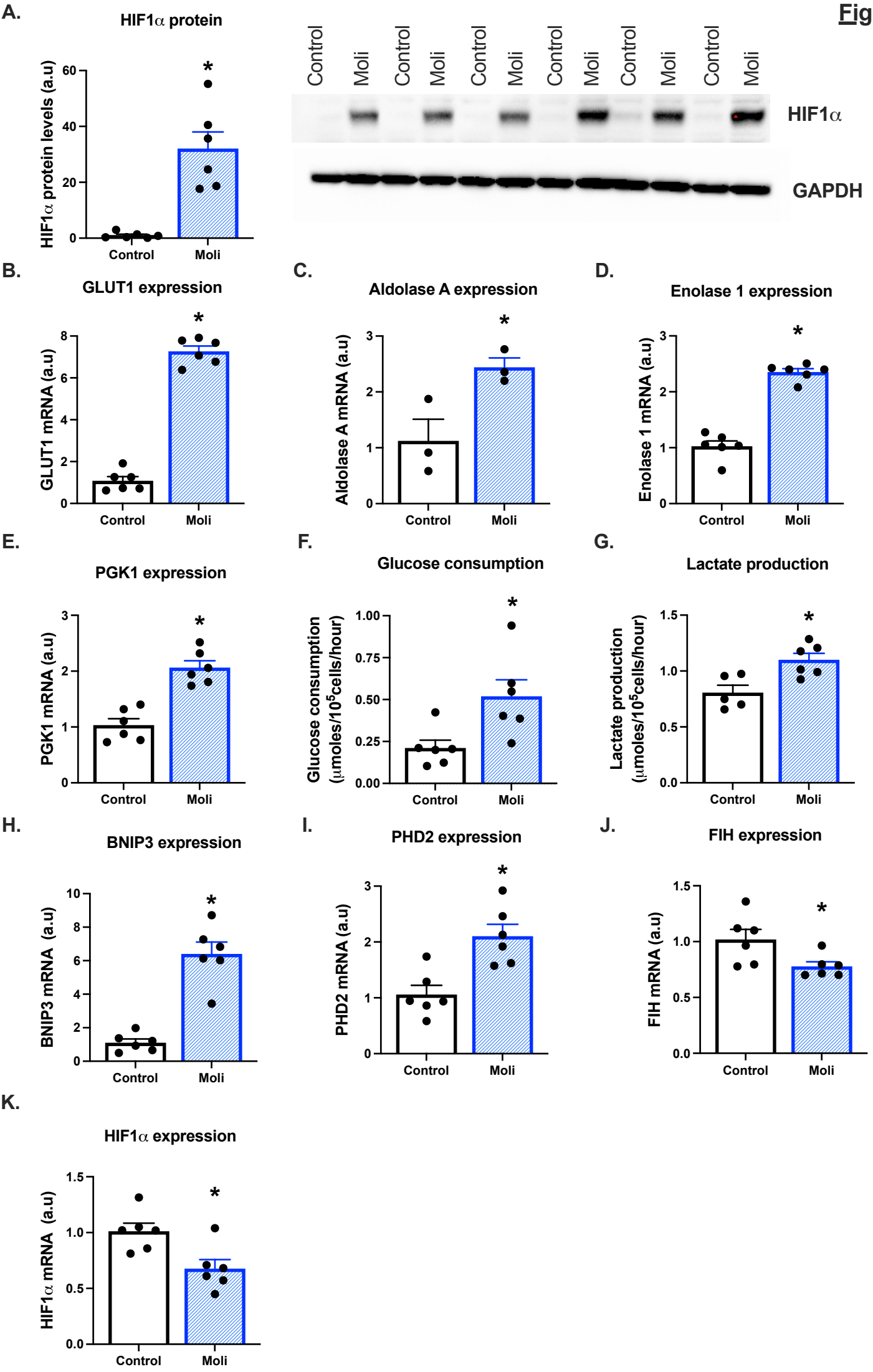

A.
HIF1α protein
HIF1α protein levels (a.u)
60
40
20
0
Control
Moli
*
Control
Moli
Control
Moli
Control
Moli
Control
Moli
Control
Moli
Control
Moli
HIF1α
GAPDH
B.
GLUT1 expression
GLUT1 mRNA (a.u)
8
6
4
2
0
Control
Moli
*
C.
Aldolase A expression
Aldolase A mRNA (a.u)
3
2
1
0
Control
Moli
*
D.
Enolase 1 expression
Enolase 1 mRNA (a.u)
3
2
1
0
Control
Moli
*
E.
PGK1 expression
PGK1 mRNA (a.u)
3
2
1
0
Control
Moli
*
F.
Glucose consumption
Glucose consumption (μmoles/10^5cells/hour)
1.00
0.75
0.50
0.25
0.00
Control
Moli
*
G.
Lactate production
Lactate production (μmoles/10^5cells/hour)
1.5
1.0
0.5
0.0
Control
Moli
*
H.
BNIP3 expression
BNIP3 mRNA (a.u)
10
8
6
4
2
0
Control
Moli
*
I.
PHD2 expression
PHD2 mRNA (a.u)
3
2
1
0
Control
Moli
*
J.
FIH expression
FIH mRNA (a.u)
1.5
1.0
0.5
0.0
Control
Moli
*
K.
HIF1α expression
HIF1α mRNA (a.u)
1.5
1.0
0.5
0.0
Control
Moli
*

Figure 2

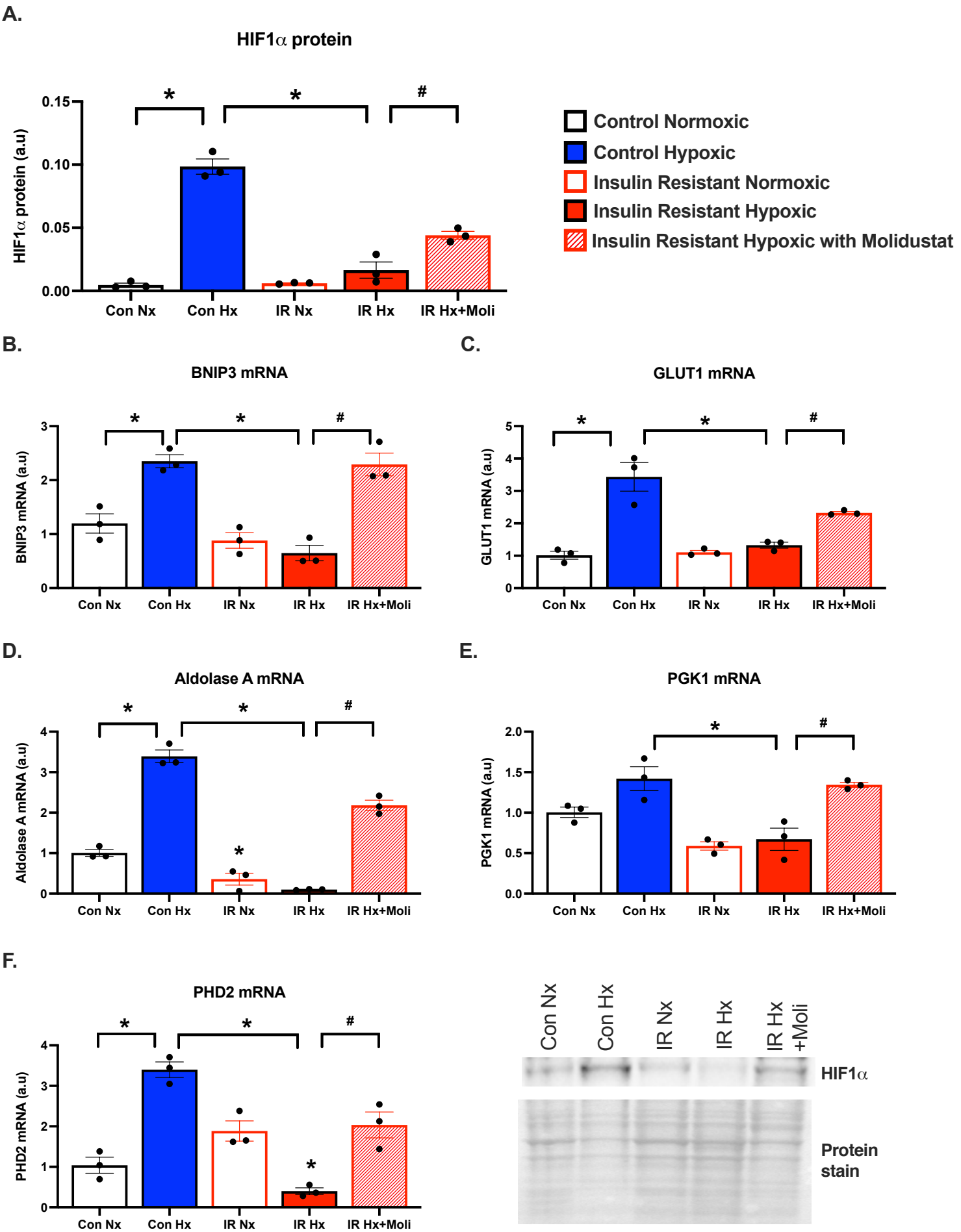

A.
HIF1α protein
HIF1α protein (a.u)
0.15
0.10
0.05
0.00
*
*
#
Con Nx
Con Hx
IR Nx
IR Hx
IR Hx+Moli
Control Normoxic
Control Hypoxic
Insulin Resistant Normoxic
Insulin Resistant Hypoxic
Insulin Resistant Hypoxic with Molidustat
B.
BNIP3 mRNA
BNIP3 mRNA (a.u)
C.
GLUT1 mRNA
GLUT1 mRNA (a.u)
D.
Aldolase A mRNA
Aldolase A mRNA (a.u)
E.
PGK1 mRNA
PGK1 mRNA (a.u)
F.
PHD2 mRNA
PHD2 mRNA (a.u)
Con Nx
Con Hx
IR Nx
IR Hx
IR Hx +Moli
HIF1α
Protein stain

Figure 3

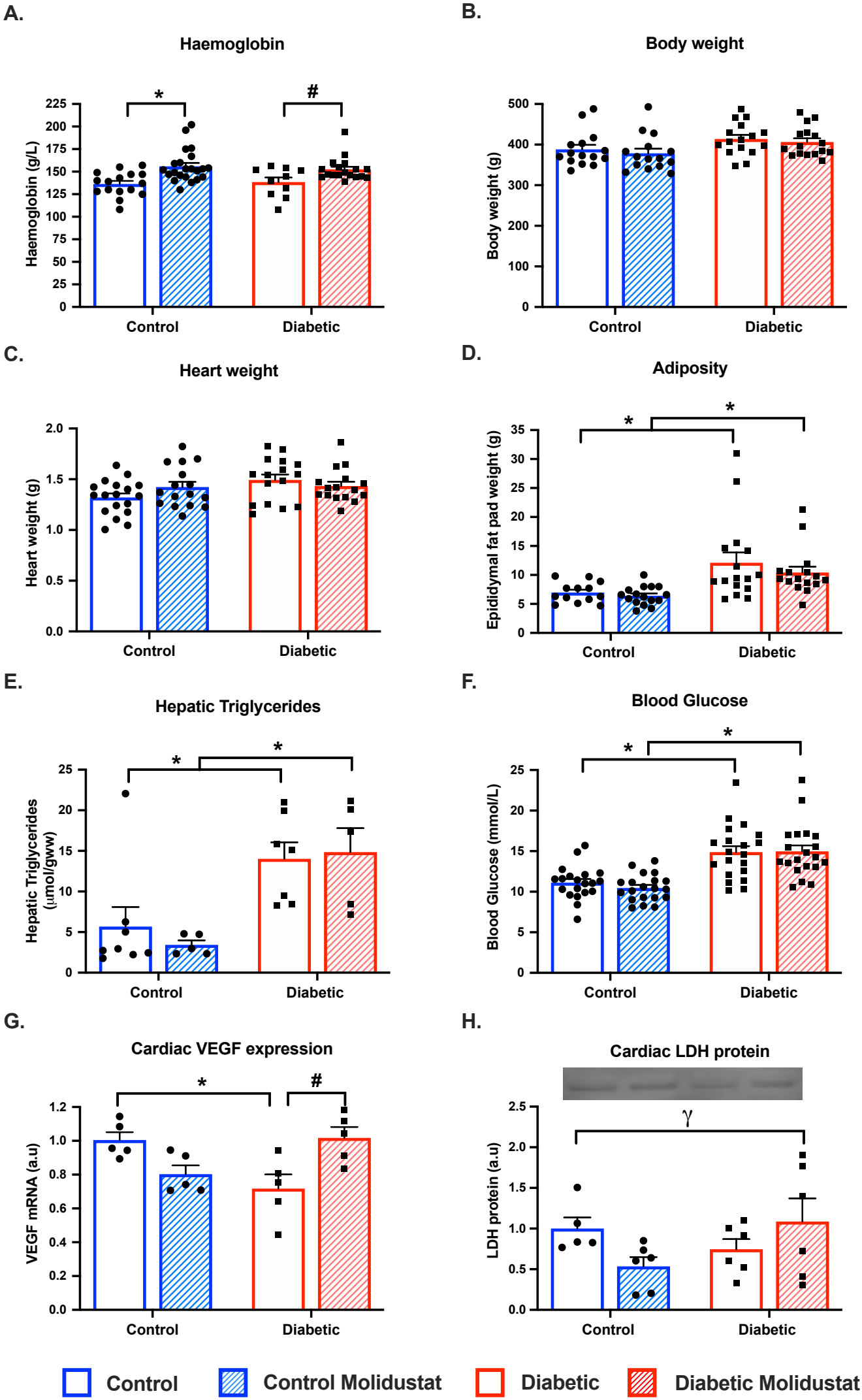

A.
Haemoglobin
Haemoglobin (g/L)
225
200
175
150
125
100
75
50
25
0
*
#
Control
Diabetic
B.
Body weight
Body weight (g)
500
400
300
200
100
0
Control
Diabetic
C.
Heart weight
Heart weight (g)
2.0
1.5
1.0
0.5
0.0
Control
Diabetic
D.
Adiposity
Epididymal fat pad weight (g)
35
30
25
20
15
10
5
0
*
*
Control
Diabetic
E.
Hepatic Triglycerides
Hepatic Triglycerides (µmol/gww)
25
20
15
10
5
0
*
*
Control
Diabetic
F.
Blood Glucose
Blood Glucose (mmol/L)
25
20
15
10
5
0
*
*
Control
Diabetic
G.
Cardiac VEGF expression
VEGF mRNA (a.u)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
#
Control
Diabetic
H.
Cardiac LDH protein
LDH protein (a.u)
2.5
2.0
1.5
1.0
0.5
0.0
γ
Control
Diabetic
Control
Control Molidustat
Diabetic
Diabetic Molidustat

Figure 4

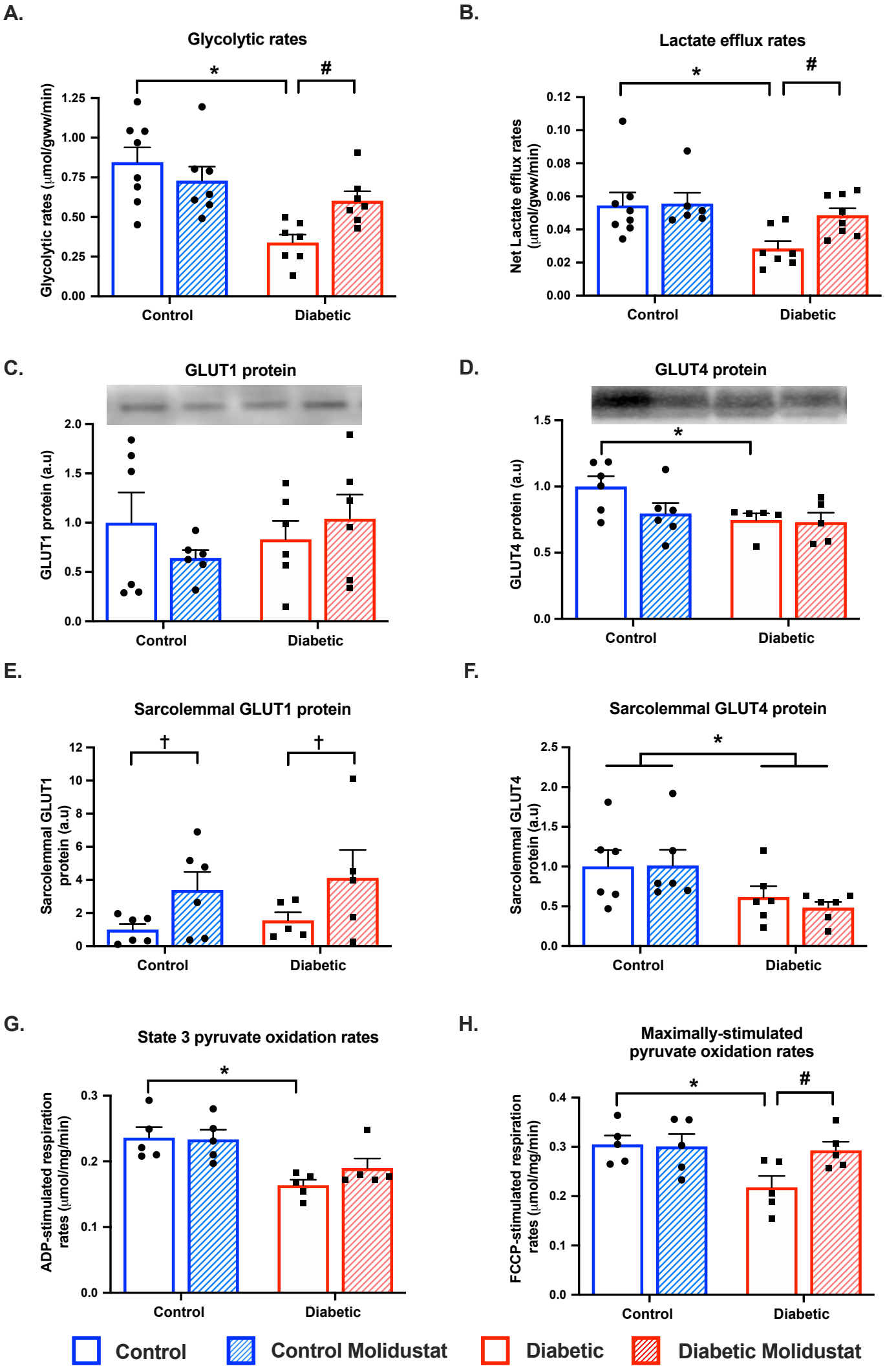

A.
Glycolytic rates
Glycolytic rates (µmol/gww/min)
1.25
1.00
0.75
0.50
0.25
0.00
*
#
Control
Diabetic
B.
Lactate efflux rates
Net Lactate efflux rates (µmol/gww/min)
0.12
0.10
0.08
0.06
0.04
0.02
0.00
*
#
Control
Diabetic
C.
GLUT1 protein
GLUT1 protein (a.u)
2.0
1.5
1.0
0.5
0.0
Control
Diabetic
D.
GLUT4 protein
GLUT4 protein (a.u)
1.5
1.0
0.5
0.0
*
Control
Diabetic
E.
Sarcolemmal GLUT1 protein
Sarcolemmal GLUT1 protein (a.u)
12
10
8
6
4
2
0
†
†
Control
Diabetic
F.
Sarcolemmal GLUT4 protein
Sarcolemmal GLUT4 protein (a.u)
2.5
2.0
1.5
1.0
0.5
0.0
*
Control
Diabetic
G.
State 3 pyruvate oxidation rates
ADP-stimulated respiration rates (µmol/mg/min)
0.3
0.2
0.1
0.0
*
Control
Diabetic
H.
Maximally-stimulated pyruvate oxidation rates
FCCP-stimulated respiration rates (µmol/mg/min)
0.4
0.3
0.2
0.1
0.0
*
#
Control
Diabetic
Control
Control Molidustat
Diabetic
Diabetic Molidustat

Figure 5

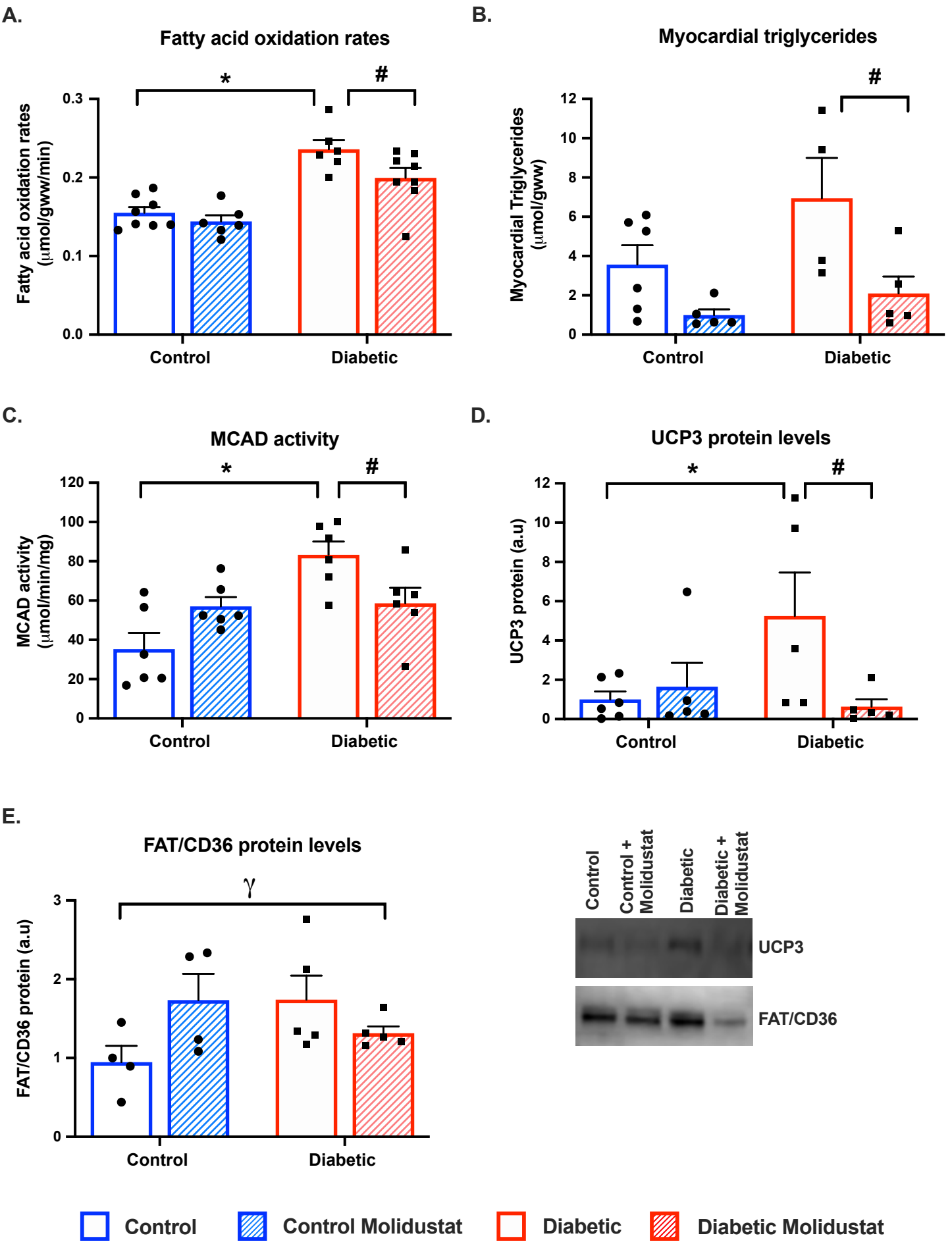
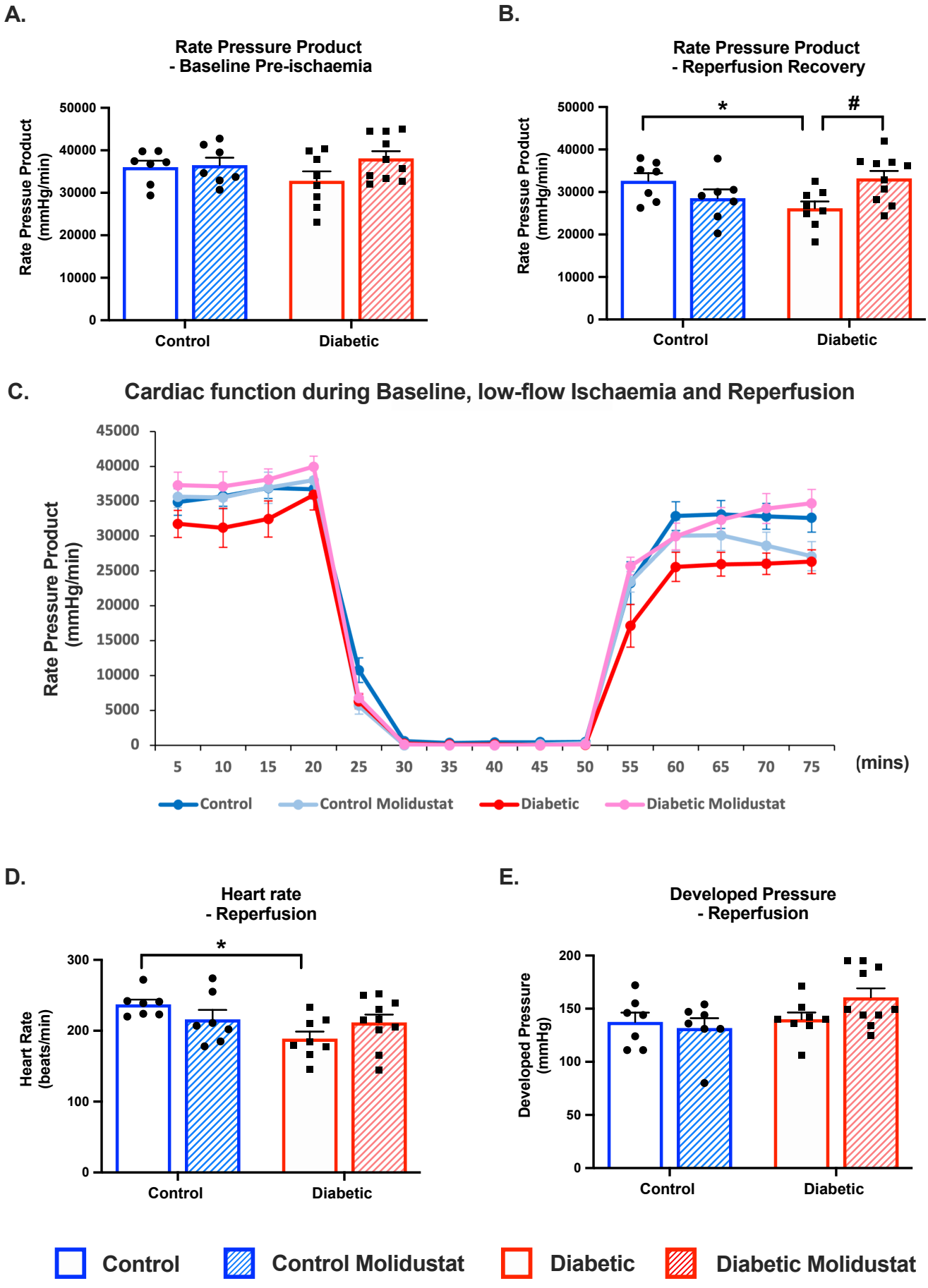

A.
Fatty acid oxidation rates
Fatty acid oxidation rates (µmol/gww/min)
0.3
0.2
0.1
0.0
*
#
Control
Diabetic
B.
Myocardial triglycerides
Myocardial Triglycerides (µmol/gww)
12
10
8
6
4
2
0
#
Control
Diabetic
C.
MCAD activity
MCAD activity (µmol/min/mg)
120
100
80
60
40
20
0
*
#
Control
Diabetic
D.
UCP3 protein levels
UCP3 protein (a.u)
12
10
8
6
4
2
0
*
#
Control
Diabetic
E.
FAT/CD36 protein levels
FAT/CD36 protein (a.u)
3
2
1
0
γ
Control
Diabetic
Control
Control + Molidustat
Diabetic
Diabetic + Molidustat
UCP3
FAT/CD36
Control
Control Molidustat
Diabetic
Diabetic Molidustat

Figure 6


A.
Rate Pressure Product
- Baseline Pre-ischaemia
Rate Pressure Product (mmHg/min)
50000
40000
30000
20000
10000
0
Control
Diabetic
B.
Rate Pressure Product
- Reperfusion Recovery
*
#
Rate Pressure Product (mmHg/min)
50000
40000
30000
20000
10000
0
Control
Diabetic
C.
Cardiac function during Baseline, low-flow Ischaemia and Reperfusion
Rate Pressure Product (mmHg/min)
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
(mins)
Control
Control Molidustat
Diabetic
Diabetic Molidustat
D.
Heart rate
- Reperfusion
*
Heart Rate (beats/min)
300
200
100
0
Control
Diabetic
E.
Developed Pressure
- Reperfusion
Developed Pressure (mmHg)
200
150
100
50
0
Control
Diabetic
Control
Control Molidustat
Diabetic
Diabetic Molidustat