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On the pivotal role of PPARα in adaptation of the heart to hypoxia and why fat in the diet increases hypoxic injury

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Non-standard abbreviations

ARNT	Aryl hydrocarbon nuclear translocator
CPT	Carnitine palmitoyltransferase
GLUT	Glucose transporter
HIF	Hypoxia inducible factor
MCAD	medium-chain acyl-CoA dehydrogenase
MRI	Magnetic resonance imaging
MRS	Magnetic resonance spectroscopy
PCr	Phosphocreatine
PDK	pyruvate dehydrogenase kinase
PPAR	Peroxisome proliferator-activated receptor
PHD	prolyl hydroxylase domain
UCP	Uncoupling protein
VHL	von Hippel-Lindau

Abstract

The role of peroxisome proliferator activated alpha (PPARα) -mediated metabolic remodeling in cardiac adaptation to hypoxia has yet to be defined. Here, mice were housed in hypoxia for 3 weeks before *in vivo* contractile function was measured using cine magnetic resonance (MR) imaging. In isolated, perfused hearts, energetics were measured using ^{31}P MR spectroscopy and glycolysis and fatty acid oxidation were measured using ^{3}H labelling. Compared with normoxic, chow-fed control mouse heart, hypoxia decreased PPARα expression, fatty acid oxidation and mitochondrial UCP3 levels, while increasing glycolysis, all of which served to maintain normal ATP concentrations and thereby ejection fractions. A high-fat diet increased cardiac PPARα expression, fatty acid oxidation and UCP3 levels, with decreased glycolysis. Hypoxia was unable to alter the high PPARα expression or reverse the metabolic changes caused by the high fat diet, with the result that ATP concentrations and contractile function decreased significantly. The adaptive metabolic changes caused by hypoxia in control mouse hearts were found to have already occurred in PPARα$^{-/-}$ mouse hearts, and sustained function in hypoxia despite an inability for further metabolic remodelling. We conclude that decreased cardiac PPARα expression is essential for adaptive metabolic remodelling in hypoxia, but is prevented by dietary fat.

Keywords: Cardiac contractile function; Cine magnetic resonance imaging; Hypoxia inducible factor (HIF); Myocardial energy metabolism; ^{31}P Magnetic resonance spectroscopy; Substrate metabolism

Introduction

Each day, the human heart beats about 100,000 times and pumps about 10 tons of blood through the body, so requiring around 6 kg of ATP.(1) To produce such a large quantity of ATP, the heart relies on mitochondrial oxidative phosphorylation of a variety of metabolic fuels, including free fatty acids (FFA) and glucose. In theory, FFAs require ~13% more oxygen than glucose to generate the same amount of ATP, although hearts metabolising FFAs may require far more oxygen owing to increased mitochondrial uncoupling.(2, 3) ATP is transported from the mitochondria to be used for contractile function via the creatine kinase energy shuttle, in which phosphate is transferred from ATP to creatine with the formation of phosphocreatine and ADP, in a reaction catalysed by mitochondrial creatine kinase:(1)

$$ATP + Cr \leftrightarrow PCr + ADP + H^{+}$$

The creatine kinase system acts to keep ATP levels constant via a fall in phosphocreatine and a rise in free ADP, which controls mitochondrial oxidative phosphorylation when oxygen is not limiting.(4)

In hypoxia, when oxygen availability limits oxidative phosphorylation, the heart moves towards more oxygen-efficient carbohydrate, away from fatty acid metabolism.(5) Thus, cardiac glucose uptake was increased in hypoxic rats(6) and in high altitude-adapted humans(7), and fatty acid metabolising enzyme (carnitine palmitoyltransferase 1 (CPT1) and medium-chain acyl-CoA dehydrogenase (MCAD)), expression was decreased by hypoxia.(8-10) Magnetic resonance (MR) studies have shown that cardiac PCr/ATP ratios are lower in high altitude-adapted humans(11) and in lowlanders returning to sea level following a trek to Mt Everest Base Camp.(12) However, the links between limited oxygen availability, changes in substrate metabolism, ATP generation and cardiac function have not been well defined.

The balance between fatty acid and glucose metabolism may be regulated, at least in part, by the nuclear peroxisome proliferator activated receptors (PPARs). Of the three receptor isoforms, α, δ and γ, PPARα and PPARδ are highly expressed in heart.(13) PPARα regulates several genes encoding for proteins that control fatty acid metabolism, including MCAD,(14) and glucose metabolism, including pyruvate dehydrogenase kinase 4 (PDK4) and GLUT4.(15)

Hypoxia is a potential driver of metabolic reprogramming, with the oxygen-sensitive transcriptional activator, hypoxia-inducible factor 1α (HIF-1α), being elevated in ischemic cardiac myocytes(16) and in infarcted hearts.(17) In normoxia, HIFα subunits are polyubiquitinated for proteasomal degradation, the constant degradation of HIFα subunits being mediated by hydroxylation via the prolyl hydroxylase domain (PHD or EGLN) oxygenase family. In hypoxia, PHD activity is reduced, stabilizing HIFα, which translocates to the nucleus and dimerizes with HIF1β. The dimer transcriptionally activates ~200 genes, including those involved in erythropoiesis, angiogenesis and energy metabolism.(18) In hypoxia-adapted Tibetans, variants of the genes *EGLN1* and *EPAS1*, encoding for PHD2 (the most important of the three human PHDs) and the HIF-2α subunit,

respectively, associated with the *PPARA* gene in genome-wide scans,(19) and serum fatty acid concentrations in this group correlated with the PPARA haplotype.(20) These findings suggest that PPARα may control cardiac substrate metabolism, ATP generation and thereby cardiac function in hypoxia (Figure 1).

Recent studies using cultured cells, knockout or transgenic mice seemingly demonstrate that alterations in the HIF system, whether decreases or increases in ARNT (HIF1β)(21), HIF1α(22–24), HIF2α(21, 25), PHD1(25), or VHL(26) levels, increase either PPARα or PPARγ(22) in heart or skeletal muscle. Such apparently contradictory studies necessitate a deeper understanding of the cellular mechanisms whereby a decrease in PPARα is absolutely required to maintain ATP levels for the contractile function of the hypoxic heart. Here, we show that hypoxia causes the heart to undergo a series of metabolic changes, coordinated by PPARα and designed to use the limited available oxygen as efficiently as possible. We demonstrate in normal mice how a high fat diet increases PPARα expression, which prevents the metabolic changes required for acclimation to hypoxia, thereby significantly decreasing ATP concentrations and myocardial contractile function. We also demonstrate that the lack of myocardial PPARα prevents any metabolic response to hypoxia and that chemical induction of HIF, by inhibition of HIF hydroxylases in mechanically active cardiomyocytes, has the same effect on PPARα as hypoxia. In short, for adaptation to hypoxia, it is essential that PPARα expression decreases in order to increase the efficiency of oxygen use, via orchestrated changes in substrate metabolism and mitochondrial respiration, so that normoxic ATP concentrations and cardiac function can be maintained.

Methods

Animals

Male wild-type 129Ev/Sv (n = 106, 14 mo) and littermate PPARα$^{-/-}$ mice (n = 72) were housed on a 12 h light/dark cycle and fed ad libitum. The mice, a kind gift from Dr Frank J. Gonzalez (National Cancer Institute, Bethesda, MD, USA), were bred in-house on a pure 129Ev/Sv background with 10 backcrosses. All procedures conformed to ethical regulations of the UK Home Office.

Hypoxia

Mice were housed in a glass-fronted hypoxia chamber (Biospherix, USA) in which N_2 replaced O_2. Chamber O_2, monitored continuously, was used to regulate chamber N_2 levels via a feedback system. Mice were fed laboratory chow (7.5% fat) or a high fat (55% fat) diet. In order to acclimatize mice to hypoxia, chamber O_2 was reduced in daily steps over a 7 day period, to be maintained at 11% (v:v) for a further 12 days. Following hypoxia, animals were re-oxygenated for 1 h to ameliorate any short term effects of re-oxygenation on cardiac function.

Cardiac magnetic resonance imaging

Magnetic resonance imaging (MRI) was carried out on an 11.7T (500 MHz) vertical bore (123 mmø) magnet (Magnex Scientific, Oxon, UK) as described previously.(27)

Heart perfusion

Mice were anesthetised using 60 mg.kgbw^{-1} i.p. sodium pentobarbitone and hearts were excised and arrested in ice-cold Krebs-Henseleit buffer. Hearts were perfused in Langendorff mode at 80 mmHg perfusion pressure and at 37 ºC with modified Krebs-Henseleit recirculating buffer gassed with 95% O_2/5% CO_2 containing 11 mM glucose and 0.4 mM palmitate pre-bound to 1.5% (w:v) albumin. A polyethylene balloon connected to a pressure transducer was inserted in the left ventricle and inflated to an end-diastolic pressure of 4-8 mmHg to measure pressures and heart rates.

Cardiac substrate metabolism

Glycolytic flux and palmitate oxidation were measured in perfused hearts using 25 μCi [5-^{3}H]-glucose or [9,10-^{3}H]-palmitate, respectively, in recirculating perfusion buffer, as previously described.(3, 28) Buffer samples were taken every 5 min for the measurement of [^{3}H] label conversion to ^3H_2O using Dowex anion separation or Folch extraction. In a subset of experiments, insulin (500 μU) was added to the buffer to measure maximal insulin-stimulated response. Hearts were snap-frozen in liquid N_2. Cardiac lactate efflux was determined by measuring lactate concentrations, using lactate dehydrogenase, in timed buffer collections.

Cardiac high energy phosphate metabolism

Perfused hearts were inserted into the same NMR system as used for imaging (see above), but using a 10 mm diameter probe (Rapid, Wolfsburg, Germany). ^{31}P MR spectra were collected using a 60 μs pulse, TR 10 s and 60 averages in a total acquisition time of 5 min. Data were analysed using jMRUI software and 4 spectra were summed to determine ATP, PCr and P_i peak areas. Intracellular pH, cytosolic free ADP, and the free energy of ATP hydrolysis, ΔG_{ATP}, were calculated as previously described.(4)

HL-1 cardiomyocyte culture

HL-1 murine cardiomyocytes, a kind gift from Dr William C. Claycomb (Louisiana State University Medical Center, New Orleans, LA, USA), were maintained in Claycomb medium (Sigma, UK). Cells were supplemented with 10% fetal bovine serum, ascorbic acid (0.3 mM), norepinephrine (0.1 mM) and L-glutamine (4 mM). Cells were cultured on plates pre-coated with 5 $\mu g.ml^{-1}$ fibronectin and 0.02% gelatin and incubated in 5% CO_2 at 37 ºC and 95% humidity. All experiments were performed on confluent, beating cells. Cells were exposed to 2% hypoxia or normoxia for 24 h. In separate experiments, normoxic HL-1 cells were incubated for 24 h with a specific PHD inhibitor, 50 μM 2-(1-chloro-4-hydroxyisoquinoline-3-carboxamido) acetic acid, IOX3,(29, 30) which was synthesized specifically for the experiment.(31) Cells were aspirated with PBS and harvested for protein analysis or RTqPCR.

RTqPCR analysis of cardiac and skeletal muscle and HL-1 cells

Primers were designed using Primer3 software based on GenBank or Ensembl Genome Browser searches and obtained from Eurofins MWG Operon (UK). References to primer sequences were; PPARα, NM_011144.6; PPARβ/δ, NM_011145.3; PPARγ, NM_011146.3; MCAD, NM_007382.4; UCP3, NM_009464.3; PDK4, NM_013743.2; VEGF, NM_009505.4; β-Actin, NM_007393.3. Total RNA was extracted and purified from frozen heart and skeletal muscle tissues using a Qiagen® Kit (UK). For cells, total RNA was extracted and purified from ≤ $5x10^6$ confluent HL-1 cells using a Qiagen® kit (UK). Complementary DNA (cDNA) was synthesized from the RNA template using a high capacity transcriptase kit (Applied Biosystems). Real time PCR amplification was performed using the Applied Biosystems RTqPCR System (CA). The PCR program had an initial heat activation step of 95°C for 10min. Forty cycles of thermocycling were performed with a denaturation step at 95°C for 15 s, an annealing and extension step at 60 ºC for 1 min. Fluorescence was measured following each extension step. After amplification, a melting curve was acquired and used to determine the PCR product specificity. Relative quantification of target gene expression was normalized to the housekeeping gene, β-actin and performed using a $2^{-\Delta\Delta Ct}$ method.

Tissue and cell lysate preparation and immunoblotting

As previously described,(3, 28) powdered frozen cardiac or skeletal muscle was added to 300 μl of lysis buffer and homogenised for 30 s. Confluent HL-1 cardiomyocytes in 6-well plates were washed with PBS and added

to 150 µl of lysis buffer containing protease inhibitor. Lysates were boiled for 5 min and centrifuged at 13,000 rpm for 10 min, supernatant was saved and protein concentrations determined using a BCA protein assay kit (Perbio, UK). Samples were stored at -80 ºC following addition of 5% β-mercaptoethanol (v/v) and boiling for 5 min.

Equal concentrations of protein from cardiac and skeletal muscle samples were loaded onto 12% SDS-PAGE gels, separated using electrophoresis and transferred onto Immobilon-P membranes (Millipore, UK). Cardiac and skeletal muscle proteins were detected using the following polyclonal antibodies and dilutions, in 5% milk; PGC1α and MCAD, Santa Cruz, CA, USA, 1:500; GLUT1, Abcam, UK, 1:1000; GLUT4, Kind gift G. Holman, Bath, UK, 1:4000; PDK4 and UCP3, Abgent, UK, 1:500 and 1:2500; MTE-1, Kind gift, Dr S. Alexson, Karolinska Institute, Stockholm, Sweden, 1:2000; RXRα, Santa Cruz, UK, 1:1000; HIF-1 and 2α, Novus Biologicals, 1:2000 and 1:500.

Secondary antibodies were horseradish peroxidase conjugate polyclonal with goat anti-rabbit specificity (Autogen Bioclear, Wiltshire, UK), all diluted to 1:3500, with the exception of GLUT1,4 and HIF-1α (1:2000), HIF-2α (1:1000) and MCAD (Donkey anti-goat). Consistent protein loading and transfer were confirmed by Ponceau staining, and protein levels related to internal standards to ensure homogeneity between samples and gels. Bands were quantified using UN-SCAN-IT software (Silk Scientific, USA), and all samples run in duplicate on separate gels to confirm results.

Glycolytic Enzyme Activities

The myocardial enzymatic activities of 3-phosphoglycerate kinase (PGK) and pyruvate kinase (PK) were measured using coupled enzyme assays.(32) Frozen, ground heart tissue (1 mg/ml) was extracted with the buffer containing 150 mM NaCl, 60 mM Tris-HCl, 5 mM EDTA, 0.2% Triton-X 100, 1 mM PMSF, 10 ug/ml leupeptin, 1 ug/ml aprotinin, pH 7.5 The pyruvate kinase activity was assayed at 30 °C, 340 nm in media containing 50 mM imidazole (pH 7.6), 20 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM NADH, 1 mM ADP, 4.5 U/ml lactate dehydrogenase and 1 mM phosphoenolpyruvate. The 3-phosphoglycerate kinase activity was recorded at 25 °C, 340 nm in an assay medium containing 50 mM imidazole buffer (pH 7.6), 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM ATP, 5 mM 3-phosphoglycerate, and 0.2 mM NADH.

Myocardial triacylglycerol (TAG) assay

As previously described,(3, 28) cardiac lipids were extracted from freeze-clamped tissue (25-50 mg) using 10 ml of 2:1 chloroform:methanol solution. After mixing and phase separation, the lower phase was air dried at 50^{o}C and resuspended in ethanol. Using a kit (RANDOX, UK), cardiac triacylglycerol (TAG) content was measured spectrophotometrically at 500 nm.

Statistics

Grouped data (means ± SEM) were analysed using three factor ANOVAs, with individual comparisons subsequently performed using t-tests. Results were considered significantly different at $p < 0.05$.

Results

Effects of hypoxia on mouse physiology and cardiac morphology

In order to investigate functional and metabolic responses of the *in vivo* mouse heart to hypoxia, we used a 3-week protocol that began with 7 d of lowering ambient oxygen concentration from 21 to 11% in daily increments, to produce a graded, physiological hypoxia (Figure 2). Hypoxia in wild-type, chow-fed mice increased whole blood hemoglobin by 51% ($p<0.001$) and hematocrit by 33% ($p<0.001$), but did not alter body weight. Established HIF-1α downstream targets, cardiac VEGF and PDK1 protein increased 3.6-fold ($p<0.001$) and 1.8-fold ($p<0.01$) respectively. Left ventricular (LV) mass decreased by 19% ($p<0.001$) following hypoxia, whereas right ventricular mass was unaltered. Plasma metabolites in wild-type mice were unchanged by hypoxia (Figure S1).

Effects of hypoxia on cardiac function and metabolism (chow-fed wild-type mice)

Hypoxia in chow-fed, wild-type mice decreased cardiac PPARα mRNA levels by 36% ($p<0.05$), with PPARδ mRNA, and PPARγ, RXR and PGC-1α protein levels remaining unchanged (Figures 3A and S2). In parallel with lower PPARα mRNA levels, hypoxia decreased protein levels of downstream targets of PPARα: UCP3, MTE-1 and PDK4 (Figure 3B). MCAD protein levels were 24% ($p<0.05$) lower, but proteins involved in mitochondrial respiration were unchanged, following hypoxia (Figure S2).

Hypoxia increased cardiac glycolysis 2.3-fold ($p<0.01$) and lactate efflux 2.4-fold ($p<0.01$), measured in isolated, beating perfused hearts using ^{3}H substrate labelling (Figure 3C). Hypoxia did not alter total cardiac glucose transporter-4 (GLUT-4) protein or activities of glycolytic intermediates (Figure S3). Fatty acid oxidation was 26% ($p<0.02$) lower in hearts from hypoxic mice compared to normoxic controls (Figure 3D), whilst myocardial TAG levels were 28% ($p<0.05$) higher in hypoxic mice. In isolated, perfused hearts, [PCr] was 31% ($p<0.001$) lower, but [ATP] was unaltered by hypoxia, measured using ^{31}P NMR spectroscopy and HPLC (Figure 3E); total creatine was 28% ($p<0.05$) lower following hypoxia. Although free cytosolic [ADP] increased 2-fold ($p<0.02$), the calculated free energy available from ATP hydrolysis, ΔG_{ATP}, was preserved. *In vivo* cine MRI showed that 3 weeks of hypoxia did not alter cardiac output, cardiac index or ejection fraction, despite an 18% ($p<0.02$) lower stroke volume (SV), resulting from a 17% ($p<0.05$) lower end-diastolic volume (EDV) in hypoxic mice (Figure 3F).

Effect of hypoxia on cardiac function and metabolism in high fat-fed, wild-type mice

The cardiac metabolic and energetic changes observed in hypoxic mice may have been caused by decreased PPARα expression. To investigate whether PPARα expression is central to hypoxic adaptation, we prevented PPARα down-regulation by increasing PPARα expression using high (55%) fat feeding. Feeding mice a high fat diet for 3 weeks in normoxia did not alter body weight, but with hypoxia caused a 10% ($p<0.01$) loss of body weight and a 19% ($p<0.01$) decrease in LV mass (Table S1). High fat feeding increased PPARα mRNA expression in both normoxia and hypoxia ($p<0.05$), preventing any hypoxia-induced decrease (Figure 4A).

PPARδ mRNA, and PPARγ, RXR and PGC-1α protein levels were unchanged by high fat feeding. Downstream targets of PPARα, UCP3 and MTE-1 proteins, increased 2-fold ($p<0.001$), and MCAD protein increased by 39% ($p<0.01$) with high fat feeding under both normoxia and hypoxia (Figures 4B and S2). High fat feeding did not significantly increase cardiac PDK4 protein, although it prevented the decrease caused by hypoxia in chow-fed mice.

High fat feeding prevented the increase in cardiac glycolysis and lactate efflux that occurred in chow-fed mice in hypoxia, although it decreased GLUT-4 protein by 59% ($p<0.05$), which remained unaltered by hypoxia (Figure 4C). High fat feeding increased cardiac fatty acid oxidation by 22% ($p<0.05$, Figure 4D) and myocardial TAG content by 82% ($p<0.01$), both being unaffected by hypoxia. High fat feeding decreased cardiac [PCr] by 25% ($p<0.05$) in normoxic mice, but [PCr] did not decrease further with hypoxia (Figure 4E). [ATP] was normal in normoxic high fat-fed mouse hearts, but was 31% ($p<0.05$) lower in hearts from hypoxic high fat-fed mice compared to chow-fed normoxic mice. Total creatine was unaltered in normoxic high fat-fed hearts, but was 37% ($p<0.05$) lower in hearts from high fat-fed mice exposed to hypoxia. Free cytosolic [ADP] was 55% ($p<0.05$) higher in hearts from normoxic high fat-fed mice compared to normoxic chow-fed mice, and increased by a further 38% with hypoxia ($p<0.05$). High fat feeding significantly lowered cardiac ΔG_{ATP} in all mouse hearts.

High fat feeding under normoxia had no effect on cardiac function (Figure 4F), whereas feeding a high fat diet under hypoxia decreased *in vivo* cardiac output by 24% compared to normoxic chow ($p<0.01$) or high fat-fed ($p<0.05$) mice. This was due to a 35% ($p<0.001$) lower SV, accounted for by a 28% ($p<0.01$) lower EDV and despite a significantly higher heart rate. The cardiac index was decreased by 15% ($p<0.05$) and ejection fractions were 12% ($p<0.02$) lower than in normoxic chow-fed mice. Hence, high fat feeding prevented the adaptive molecular and metabolic responses to hypoxia that were observed in chow-fed mouse hearts, thereby causing cardiac dysfunction.

Effect of hypoxia on cardiac function and metabolism in PPARα$^{-/-}$ mice

Having demonstrated that increased PPARα was associated with impaired function of the hypoxic heart, we next determined whether loss of PPARα altered cardiac metabolic and functional responses to hypoxia. PPARα$^{-/-}$ mice were fed a chow diet and housed under hypoxia for 3wks. PPARα$^{-/-}$ mice had 13% ($p<0.05$) lower body weight and 18% ($p<0.05$) lower LV mass than wild-type mice, with neither altered by hypoxia (Table S1). Cardiac PPARα mRNA levels in PPARα$^{-/-}$ mice were negligible, although PPARδ mRNA, and PPARγ, RXR and PGC-1α protein levels were normal and unchanged by hypoxia (Figures 5A and S2). Cardiac UCP3 protein (Figure 5B) was 71% ($p<0.001$) lower and MTE-1 was 49% ($p<0.02$) lower in PPARα$^{-/-}$ mouse hearts compared with wild-type mice, and both were unchanged by hypoxia. Other key mitochondrial proteins were unaltered relative to wild-type mice (Figure S2). PDK4 was 45% lower ($p<0.01$) and MCAD protein was 43% ($p<0.001$) lower in normoxic and hypoxic PPARα$^{-/-}$ mouse hearts.

PPARα$^{-/-}$ mouse hearts had 3-fold ($p<0.01$) higher glycolytic rates than normoxic wild-type mouse hearts and were unaffected by hypoxia (Figure 5C). Glycolysis was not maximal following hypoxia, as stimulation with insulin doubled glycolytic rates in both normoxic and hypoxic PPARα$^{-/-}$ mouse hearts (Figure S4). Lactate efflux from hypoxic PPARα$^{-/-}$ mouse hearts was 2-fold ($p<0.01$) higher than from normoxic wild-type hearts, and not different from normoxic PPARα$^{-/-}$ hearts. Cardiac GLUT-4 protein was similar in normoxic and hypoxic PPARα$^{-/-}$ mice. Consistent with PPARα being a regulator of fatty acid metabolism, PPARα$^{-/-}$ mice had 24% lower ($p<0.05$) cardiac fatty acid oxidation (Figure 5D) than wild-type mice, and this was unaltered by hypoxia. Cardiac TAG content was increased by 45% ($p<0.05$) in normoxic and hypoxic PPARα$^{-/-}$ mouse hearts. Thus, the lack of PPARα prevented the metabolic changes that were observed in hypoxic wild-type mouse hearts.

In PPARα$^{-/-}$ mice, [PCr] was decreased by 43% ($p<0.001$) although [ATP] was unaltered compared to normoxic wild-type mouse hearts (Figure 5E). Hypoxic cardiac [PCr] and [ATP] were decreased by 51% ($p<0.001$) and 34% ($p<0.05$), respectively, in PPARα$^{-/-}$ mice. Total creatine was decreased by 20% ($p<0.001$) in PPARα$^{-/-}$ mouse hearts, with no effect of hypoxia. As a consequence, cytosolic free [ADP] was 2.7-fold ($p<0.05$) higher and ΔG_{ATP} significantly lower in all PPARα$^{-/-}$ mouse hearts, being unaltered by hypoxia.

Normoxic PPARα$^{-/-}$ mice had normal cardiac function, but hypoxic PPARα$^{-/-}$ mice had 23% lower EDV ($p<0.01$), 19% lower ESV ($p<0.05$), 29% ($p<0.01$) lower SV and therefore 20% lower cardiac output than normoxic wild-type mice (Figure 5F). Therefore, deletion of PPARα significantly altered myocardial substrate and energy metabolism and prevented any metabolic response to hypoxia, thereby impairing cardiac function.

Effect of hypoxia on cardiac function and metabolism in PPARα$^{-/-}$ mice fed a high fat diet

In order to test whether high fat feeding was operating via an alternative mechanism to PPARα regulation, PPARα$^{-/-}$ mice were fed a high fat diet, and housed in either normoxia or hypoxia. High fat feeding had no effect on body weight or LV mass compared to chow fed PPARα$^{-/-}$ mice (Table S1). Cardiac PPARα mRNA levels were unaffected by high fat feeding, and PPARδ mRNA, and PPARγ and RXR proteins were unaltered compared to chow fed PPARα$^{-/-}$ mice, with no effect of hypoxia (Figure 6A). Cardiac UCP3, MTE-1, PDK 4 and MCAD proteins were unaltered in PPARα$^{-/-}$ mice fed a high fat diet compared to a chow diet, again unaffected by chronic hypoxia (Figure 6B). Cardiac metabolism in PPARα$^{-/-}$ mice was also unaltered by either high fat feeding, or the addition of hypoxia (Figure 6C,D). Cardiac output in PPARα$^{-/-}$ mice fed a high fat diet was 29% lower than chow fed PPARα$^{-/-}$ mice ($p<0.01$), and 18% lower with hypoxia ($p < 0.01$), similar to chow fed PPARα$^{-/-}$ mice exposed to chronic hypoxia (Figure 6E). Cardiac index was 29% lower in PPARα$^{-/-}$ mice fed a high fat diet vs chow, with no effect of hypoxia ($p < 0.01$). Ejection fraction was 6% lower in high fat fed PPARα$^{-/-}$ mice, and unaltered by hypoxia ($p < 0.05$). Therefore PPARα ablation nullified the metabolic flexibility seen in healthy mice fed a high fat diet, resulting in impaired cardiac function and unaltered by hypoxia.

Effect of hypoxia and a high fat diet on whole body physiology and PPARα-regulated proteins in skeletal muscle

In order to investigate the weight loss observed in wild-type hypoxic mice fed a high-fat diet (Table S1), energy intake was measured during the 3wk protocol. Food consumption was not affected by hypoxia, or by the high-fat diet (Figure 7A). However, due to higher calorific value, there was a 28% ($p<0.05$) higher energy intake on high fat diet, which again was unaffected by hypoxia. Cardiac levels of UCP3 and MTE-1 were elevated by high fat feeding (Figure 4B), suggesting that skeletal muscle uncoupling proteins responded to the high fat diet in a similar manner, thereby contributing to body weight loss in hypoxia. UCP3 protein levels in skeletal (gastrocnemius) muscle were 26% ($p<0.05$) lower after 3wks of hypoxia (Figures 7B and S5), a similar response to that in cardiac muscle (Figure 3B). As found in cardiac muscle, UCP3 levels in skeletal muscle increased with high fat feeding compared to chow-fed controls ($p<0.001$), and were not altered by hypoxia. Skeletal muscle MTE-1 protein levels showed identical responses to those of UCP3, being 34% ($p<0.05$) lower in hypoxic chow-fed mice, and 71% ($p<0.001$) higher after high fat feeding with/without hypoxia. PDK4 protein levels increased by 55% ($p<0.01$) during high fat feeding, which again prevented the adaptive responses to hypoxia.

Effect of hypoxia or direct HIF activation on HL-1 cardiac cells

Having established that adaptation to cardiac hypoxia was regulated by PPARα, we used confluent, beating HL-1 cardiac cells to investigate whether such adaptations were directly linked to the HIF system. Cells were exposed to hypoxia (2% O_2) for 24 h, or the HIF system was chemically induced using 24 h incubation with the selective prolyl hydroxylase inhibitor, IOX3.(33) Hypoxia caused a 5-fold increase in HIF-1α ($p<0.001$) and a 72% increase in HIF-2α ($p<0.05$) protein levels (Figures 8A and S5). IOX3 was a more potent activator of the HIF system than hypoxia, with a 9-fold increase in HIF-1α ($p<0.001$) and doubling of HIF-2α ($p<0.01$) protein levels after 24 h incubation. Under HIF-1α control, VEGF mRNA increased by 37% ($p<0.05$) in hypoxia and 59% ($p<0.001$) with IOX3. PDK1 protein levels increased 34% in hypoxia ($p<0.05$) and 86% with IOX3 treatment ($p<0.01$). GLUT-1 protein levels increased by 48% ($p<0.02$) with hypoxia and 3-fold ($p<0.01$) with IOX3.

Hypoxia decreased PPARα mRNA by 23% ($p<0.01$) in HL-1 cells (Figure 8B), similar to that caused by *in vivo* hypoxia in the intact heart. IOX3 produced a similar, 25% decrease in PPARα mRNA ($p<0.001$). PPARδ mRNA levels were unaffected by hypoxia or IOX3. PPARγ mRNA levels were not altered by hypoxia, but were doubled ($p<0.001$) after IOX3 incubation.

In cultured cardiac cells, PPARα controlled mRNA expression was altered by hypoxia in a similar manner as the respective protein in the *in vivo* hypoxic heart (Figure 8C). UCP3 levels were lowered by 27% ($p<0.01$) by hypoxia, but were not altered by IOX3. MCAD levels were 31% ($p<0.001$) lower after hypoxia and 17% ($p<0.01$) lower after IOX3. PDK4 levels were unaffected by either hypoxia or IOX3.

Discussion

In this study, we have demonstrated that normobaric hypoxia in mice decreased myocardial PPARα expression, fatty acid oxidation and mitochondrial UCP3, and increased carbohydrate metabolism; such changes allowed normoxic ATP concentrations and contractile function to be maintained. Genetic ablation of PPARα in mice caused metabolic changes in the normoxic mouse heart that were already at the extreme of hypoxic adaptation and therefore could not be altered by hypoxia. A high fat diet increased mouse heart PPARα expression and prevented PPARα down-regulation by hypoxia. Thus, with high fat feeding, the cardiac proteins under PPARα control, UCP3, MTE-1, PDK4 and MCAD, and fatty acid oxidation, increased, while GLUT4 levels and glycolysis decreased: metabolic changes that remained in hypoxia. Consequently, the high fat diet plus 3 weeks exposure to hypoxia significantly decreased myocardial ATP concentrations, cardiac index and ejection fraction. To our knowledge, this is the first time that an increase in a dietary constituent, fat, has been shown to cause myocardial dysfunction in hypoxia, a finding that may have implications for altitude exposure(34) or heart failure (in which similar metabolic changes, the "fetal gene program", occur).(1, 28, 35, 36) However, the human diet typically contains ~30% fat and probably seldom the 7.5% or 55% fat used in the mouse diets.

PPARα down-regulation and substrate switching is essential for the maintenance of contractile function of the hypertrophied heart(37) and there have been reports of decreased PPARα expression in the heart following *in vivo* hypoxia.[24, 26-28] In our mice, PPARα appeared to be the sole PPAR isoform involved in the myocardial metabolic changes, as neither PPARδ nor PPARγ levels changed. In agreement with findings in human skeletal muscle at altitude,(38) there were no changes in PGC1α, nor were cardiac RXR protein levels decreased significantly by hypoxia. In cultured cardiomyocytes, however, others have demonstrated decreased hypoxia-induced PPARα/RXR DNA binding due to decreased RXR(39) or increased HIF1α(24) levels. PPARα has been linked to hypoxic adaptation in high altitude tolerant humans(19) and simulated hypoxia, using cobalt chloride to inhibit prolyl hydroxylases(40), decreased PPARα mRNA(9), suggesting regulation of PPARα via the HIF system. Certainly, in beating cardiomyocytes, we found that either hypoxia or prolyl hydroxylase inhibition increased HIF1α and HIF2α, whilst decreasing PPARα, MCAD and UCP3 expression, decreased UCP3 levels being a beneficial adaptation to hypoxia.(41)

Yet studies of the HIF complex in transgenic(23) or knock-out mouse hearts(21, 22, 26) or skeletal muscle(25) have yielded apparently conflicting (and confusing) results, in that decreased HIF1β,(21) HIF1α(22, 23) or HIF2α(21), or increased HIF1α(26) or HIF2α(25), are reported to increase,(21, 23, 25) or not alter,(22) PPARα, or decrease PPARγ(22), expression with increased(21) or no change(25) in fatty acid oxidation. Of course, these are not studies on the metabolic effects of hypoxia, but on the contribution of individual constituents of the HIF pathway to changes in metabolism via the PPAR transcription factors. So, both increased(25) or decreased(21) HIF2α increased PPARα expression, whereas decreased HIF1α increased either PPARα(23) or PPARγ(22) expression. It is difficult to compare such studies as few, and rarely the same, measures of myocardial metabolism or function have been made and seldom using the same techniques.

However, most studies reported the deposition of lipids in the heart(21-23, 26) or cardiomyocytes(24) whether HIF1α was increased(24, 26) or decreased.(22, 23) We also found increased triacylglycerol in all hypoxic mouse hearts, whether or not PPARα levels and fatty acid oxidation were increased or decreased and unrelated to the effects of hypoxia on contractile function. Indeed, both the high fat-fed and the PPARα$^{-/-}$ mouse hearts had high triacylglycerol levels in normoxia that were unaltered by hypoxia, suggesting that more fatty acids were taken up than could be oxidised and that lipid deposits per se do not cause cardiac injury.

Carbohydrate metabolism was increased with hypoxia, but high fat feeding prevented this potentially adaptive mechanism. GLUT4 was downregulated with activation of PPARα via high fat feeding and was unaltered by chronic hypoxia. Consistent with this finding, PPARα overexpression is known to repress GLUT4 mRNA (42), and we have shown that cardiac GLUT4 content is reduced in obesity (43). Lower GLUT-4 levels may have contributed to cardiac dysfunction when hypoxia was combined with a high fat diet, preventing increased carbohydrate metabolism and resulting in lower cardiac ATP concentrations in hypoxic mice.

Ventricular mass was lower in chronically hypoxic mice, resulting in cardiac hypotrophy. This reflects human studies of altitude exposure in sea-level natives (12). The stimulus for such reduction in LV mass, in the absence of loss of body weight may be a requirement to improve oxygen diffusion distances in tissue with a sustained, high oxygen demand (44). The molecular mechanisms associated with such loss of cardiac muscle are incompletely understood, but may involve a reversal of the hypertrophic mechanisms associated with physiological hypertrophy (45). In hypoxic mice fed a high fat diet, whilst absolute left ventricular mass fell in hypoxia, cardiac hypotrophy was not evident, as hypoxic high fat fed animals also lost body weight. The molecular mechanisms for this weight loss is unclear - however all groups of PPARα$^{-/-}$ mice in this study were smaller than wild type littermates, which potentially implicates PPARα in the regulation of body weight.

Not only did hypoxia decrease PPARα mRNA levels and PPARα downstream targets, UCP3, MTE-1 and MCAD protein levels in heart, but also in skeletal muscle. In contrast to the increased PPARα observed in skeletal muscle in the PHD1-deficient mouse,(25) the decrease in PPARα expression is in agreement with findings in human skeletal muscle at altitude in which UCP3 levels and β-hydroxyacyl-CoA dehydrogenase, a β-oxidation enzyme and PPARα target, were downregulated.(38) The adaptation to hypoxia via changes in PPARα expression, observed here in heart and skeletal muscle, may be relevant to other organs that have high PPARα expression, such as liver, kidney and intestine.(46) As in the heart, we found that the high fat diet increased (and prevented any hypoxia-induced decrease in) UCP3, MTE-1 and PDK4 in mouse skeletal muscle. Although the high-fat fed mice consumed 28% more calories than those on the chow diet, they did not gain weight, possibly owing to energy wastage via increased mitochondrial uncoupling.(3)

The normobaric hypoxia-induced decrease in cardiac PCr in our mice has been shown in human heart as a decrease in PCr/ATP, measured using *in vivo* MRS: in low-landers following a trek to Mt Everest Base Camp,(12) and in Sherpa volunteers, even after 4 weeks of de-acclimation at low altitudes.(11) The decreased cardiac PCr/ATP in the Sherpa hearts was attributed to the requirement for a higher $[ADP]_{free}$, to be closer to the Km of 110 μM(47) for ADP-requiring pyruvate kinase activity in glycolysis, reflecting the elevated contribution of carbohydrate to myocardial energy needs.(7, 11) Such a mechanism may be related to a decrease in PPARα levels via high altitude adaptation of the *PPARA* gene in Tibetans(19) and could explain why the highest free ADP concentrations occurred in the highly glycolytic PPARα$^{-/-}$ mouse hearts in both normoxia and hypoxia. However, a genetic adaptation cannot explain the 18% decrease in myocardial PCr/ATP and diastolic dysfunction in human lowlanders after a trek to Mt Everest,(12) which may have been exacerbated by the hypobaria and due to the requirement for increased $[ADP]_{free}$ plus the insulin resistance and elevated epinephrine observed in the same cohort.(48)

The cytosolic free ADP concentration is a key metabolite controlling oxidative phosphorylation,(4) so it is possible that the decrease in PCr in the hypoxic mouse hearts was due to the need for higher $[ADP]_{free}$ to stimulate mitochondrial respiration and to increase glycolytic flux. The requirement for oxygen would have been decreased in the hypoxic hearts by the decrease in fatty acid oxidation and mitochondrial uncoupling, with increased flux through PDH and thereby increased glucose oxidation, all of which allowed the ATP concentrations and contractile function to remain at normoxic levels.

In the mice fed a high fat diet in normoxia, myocardial PCr decreased with no loss of total creatine, in order to maintain normal ATP levels and cardiac function despite the increased oxygen required for fatty acid oxidation and increased oxygen wastage via mitochondrial uncoupling. Such changes with high fat diets have been observed in human(49) and rat hearts, associated with decreased efficiency.(3) However, when the oxygen supply was restricted, the combination of greater oxygen requirement for fatty acid oxidation, plus oxygen wastage via increased mitochondrial UCP3 proteins, significantly decreased ATP, cardiac index and ejection fraction. Similarly, mice with a cardiac-specific ARNT ablation had increased PPARα expression and fatty acid oxidation with decreased ejection fraction.(21)

The metabolic changes caused by hypoxia in wild-type mouse hearts were found to have already occurred in normoxic PPARα$^{-/-}$ mouse hearts, and did not change further with hypoxia. PPARα$^{-/-}$ mouse hearts maintained normal cardiac index and ejection fractions in hypoxia owing to high $[ADP]_{free}$, high glycolytic flux, low PDK4 (suggesting increased PDH flux and glucose oxidation), low fatty acid oxidation and low mitochondrial UCP3 and MTE-1 levels, all of which would have resulted in the most efficient use of any available oxygen. Thus metabolism in the normoxic PPARα$^{-/-}$ mouse heart may be viewed as at the extreme of hypoxic adaptation, which increases resistance to ischemia,(50, 51) but may not support a high workload(52) with greater hypertrophy and functional impairment following aortic constriction.(53) It is possible that the PPARα$^{-/-}$ mouse hearts may have had decreased ejection fractions, in addition to the lower cardiac output and higher heart rates,

with a longer duration of hypoxia. Because metabolism in PPARα$^{-/-}$ mouse hearts was at the limit of hypoxic adaptation during normoxia, with no response to hypoxia, the use of PPARα$^{-/-}$ mice to validate PPARα as a HIF target is questionable. For example, knockout mice with decreased HIF2α (HIF1β$^{-/-}$)(21), increased HIF2α (Phd1$^{-/-}$)(25) and decreased HIF1α (Tg Nox$^{-/-}$)(22) had their metabolic phenotypes reversed in a double knockout made by crossing with PPARα$^{-/-}$ mice.

In summary, we propose that decreased myocardial PPARα expression is central to the metabolic changes required to maintain contractile function in hypoxia. A high fat diet increased PPARα expression, fatty acid oxidation and mitochondrial uncoupling protein levels - changes that were tolerated in normoxia, but decreased ATP and impaired cardiac function in hypoxia. Finally, the metabolic changes in normoxic PPARα$^{-/-}$ mouse heart can be viewed as at the extreme of hypoxic metabolic adaptation, in that they have no metabolic response to hypoxia.

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Disclosures

P.J. Ratcliffe and C.J. Schofield are scientific co-founders of, and hold equity in, ReOx Ltd, a University of Oxford spin-out company that is developing HIF hydroxylase inhibitors. The other authors have nothing to disclose.

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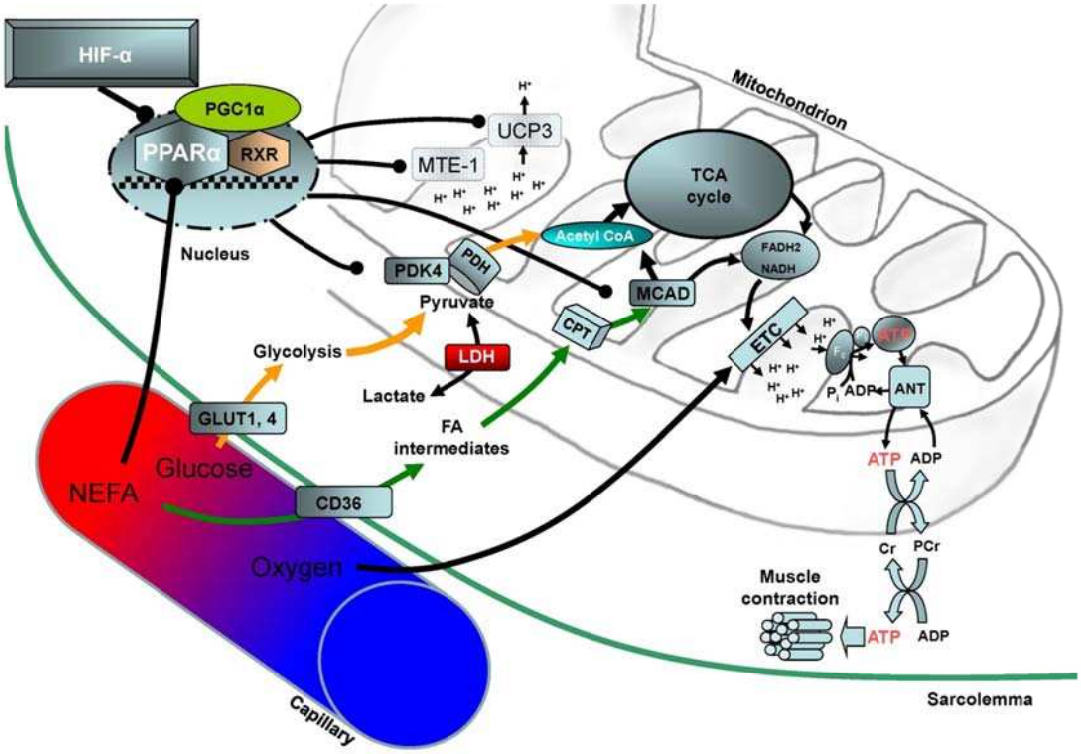

Figure 1. Putative mechanism for the control of cardiac substrate metabolism and function in hypoxia. Decreased oxygen tension in the blood inhibits PHD activity, stabilizing HIF-α subunits and down-regulating the nuclear hormone receptor, PPARα. In turn, PPARα regulates fatty acid and glucose metabolism via changes in several proteins, including MCAD and PDK4, and the mitochondrial inner membrane potential, via UCP3 and MTE-1 proteins. Increased glycolytic flux and decreased mitochondrial uncoupling increases the efficiency of ATP production, the ATP being consumed primarily by myocardial contraction.

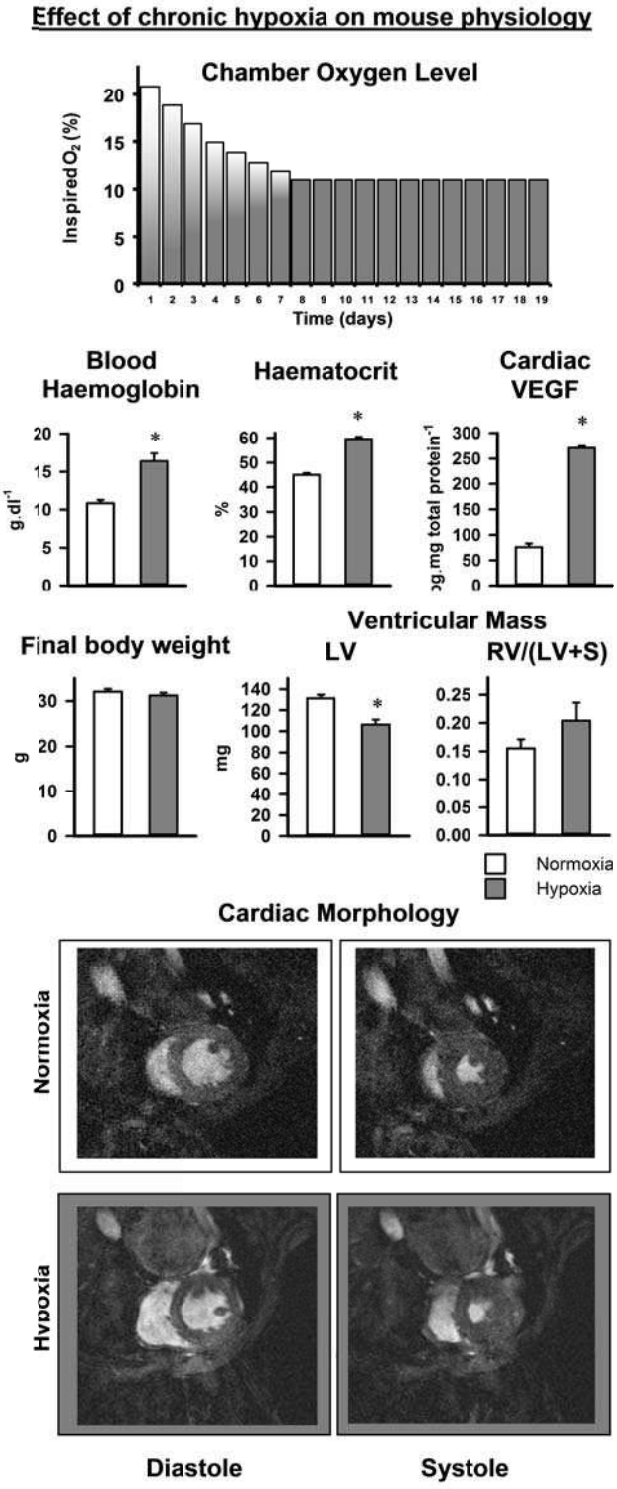

Figure 2. Effect of 3 weeks hypoxia on whole body physiology and *in vivo* cardiac morphology in wild-type mice fed a chow diet, n=6. VEGF, vascular endothelial growth factor; LV, left ventricle; RV, right ventricle; S, septum. * $p<0.05$ vs. normoxic chow-fed control.

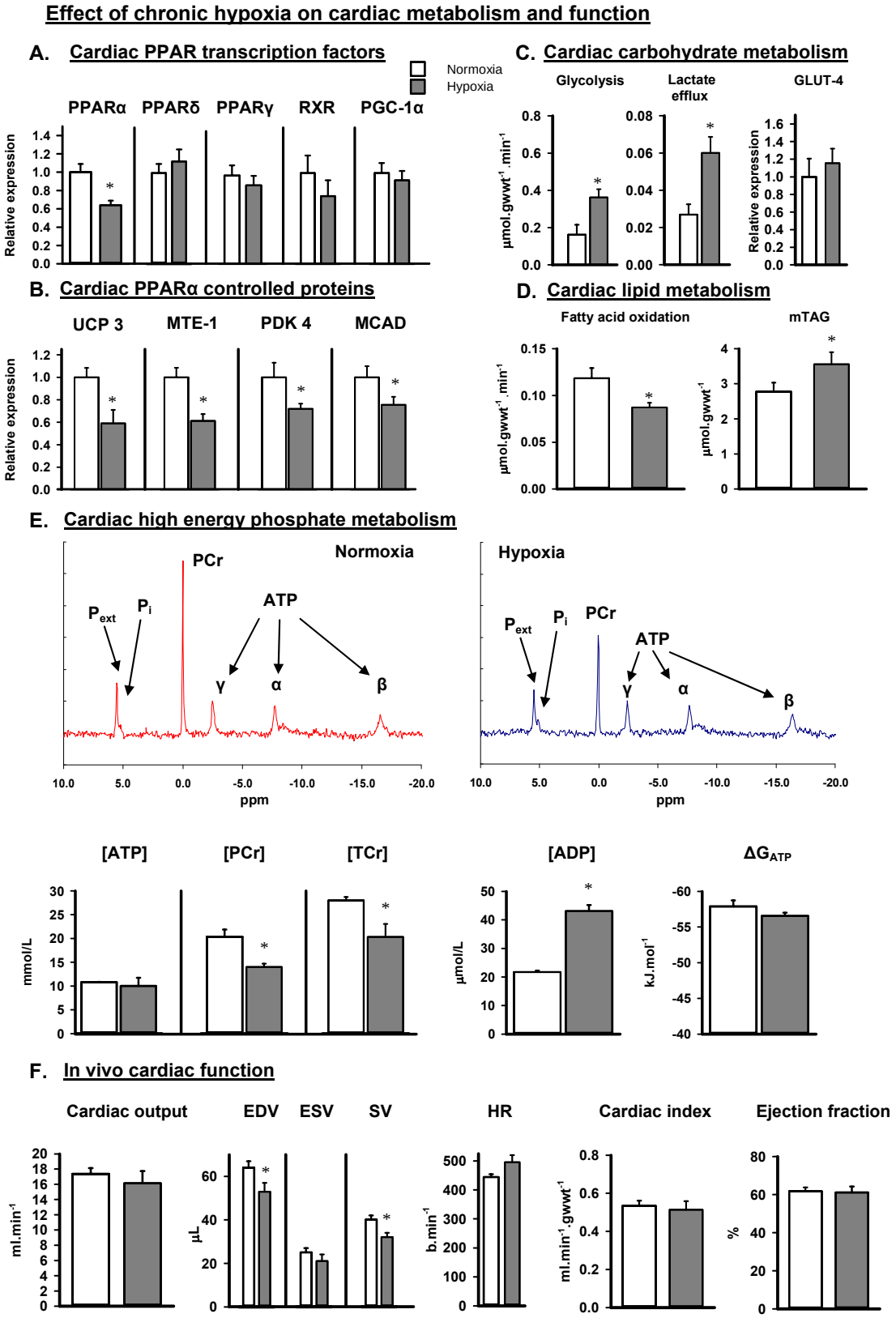

Figure 3. Effects of 3 weeks of hypoxia on cardiac PPARα and PPARδ mRNA, plus PPARγ, RXR and PGC-1α proteins (A), PPARα-controlled proteins (B), myocardial carbohydrate and lipid metabolism (C and D), ^{31}P MR spectra and high energy phosphate metabolism (E) and *in vivo* cardiac function (F) in wild-type mice fed a (7.5% fat) chow diet, min n=6. * $p<0.05$ vs. normoxic chow-fed control, min n=6.

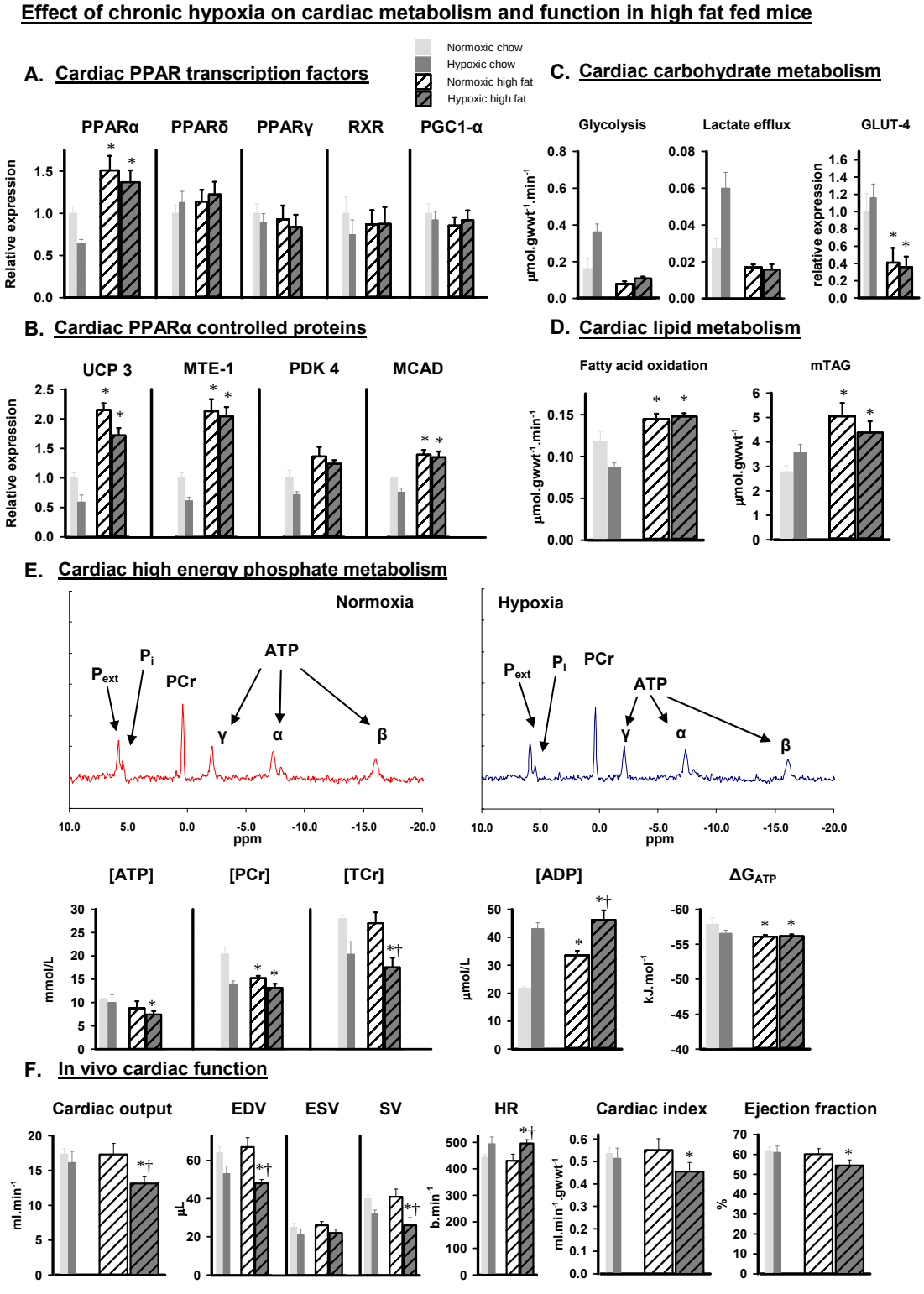

Figure 4. Effects of a high (55%) fat diet plus 3 weeks of hypoxia in wild-type mice on cardiac PPARα and PPARδ mRNA, plus PPARγ, RXR and PGC-1α proteins (A), PPARα controlled proteins (B), myocardial carbohydrate and lipid metabolism (C and D), ^{31}P MR spectra and high energy phosphate metabolism (E) and *in vivo* cardiac function (F), min n=6. Data in gray shows hypoxic response of chow-fed wild-type mice for comparison. * $p<0.05$ vs. normoxic chow-fed control, † $p<0.05$ vs. normoxic high fat-fed control.

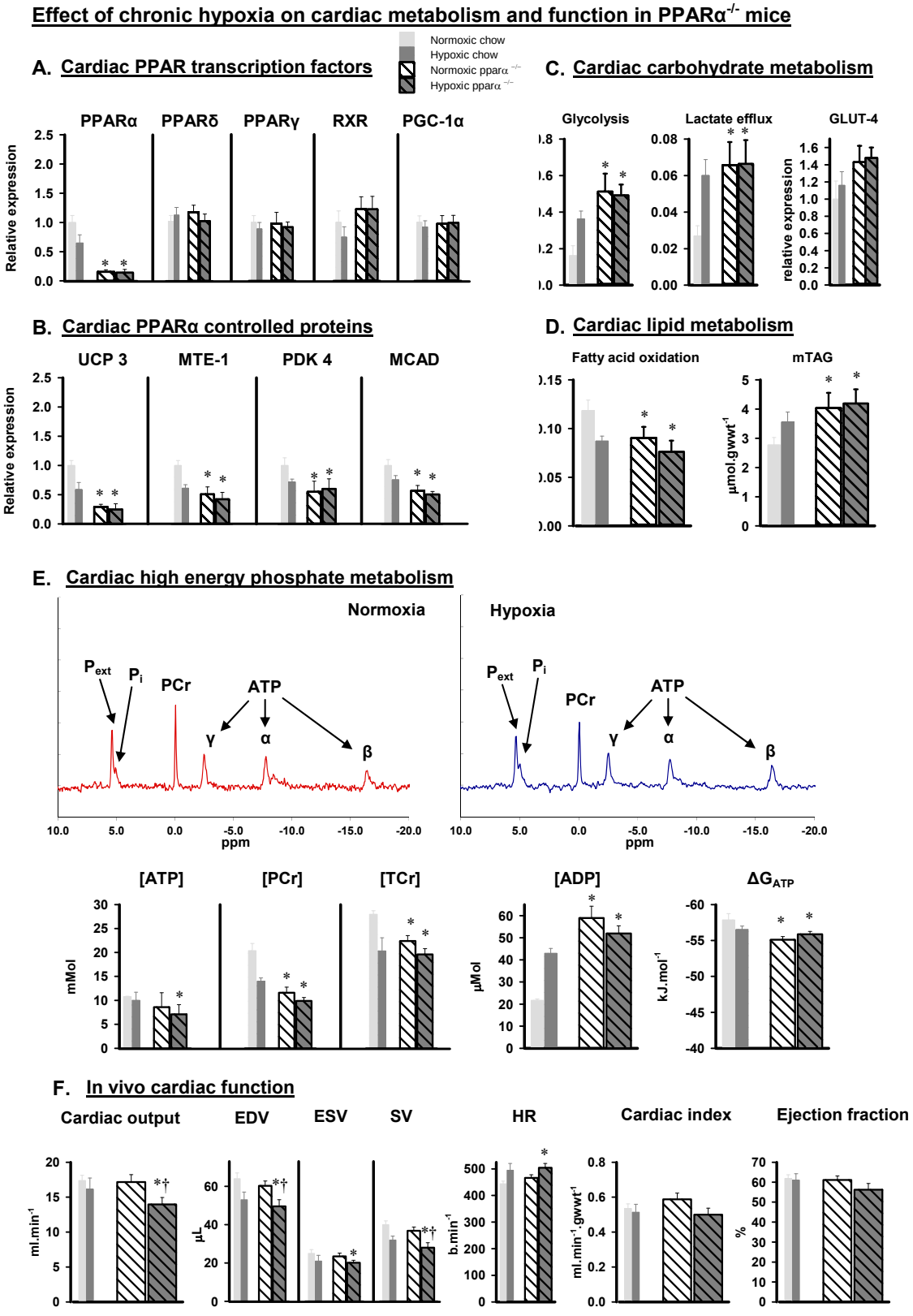

Figure 5. Effects of 3 weeks of hypoxia in PPARα$^{-/-}$ mice, fed a chow (7.5% fat) diet, on cardiac PPARα and PPARδ mRNA, plus PPARγ, RXR and PGC-1α proteins (A), PPARα controlled proteins (B), myocardial carbohydrate and lipid metabolism (C and D), ^{31}P MR spectra and high energy phosphate metabolism (E) and *in vivo* cardiac function (F), min n=6. Data in gray shows the hypoxic response of chow-fed wild-type mice for comparison. * $p<0.05$ vs normoxic chow-fed control, ‡ $p<0.05$ vs. normoxic PPARα$^{-/-}$ chow-fed.

Effect of high fat feeding and chronic hypoxia on cardiac metabolism and function in PPARα$^{-/-}$ mice

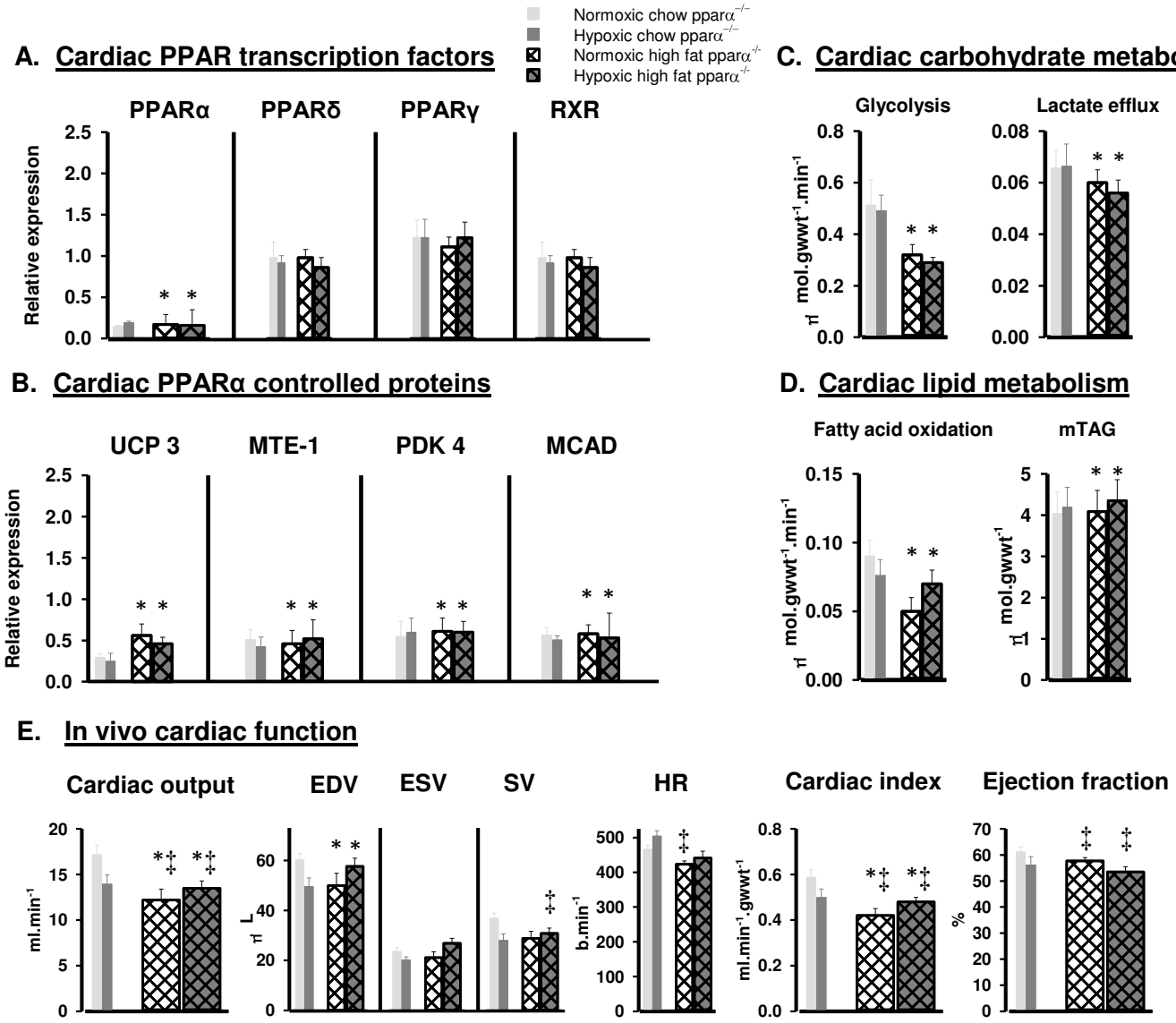

Figure 6. Effects of 3 weeks of hypoxia on PPARα$^{-/-}$ mice, fed a high fat (55% fat) diet, on cardiac PPARα mRNA, plus PPARγ and RXR proteins (A), PPARα controlled proteins (B), myocardial carbohydrate and lipid metabolism (C and D) and in vivo cardiac function (F), min n=6. Data in gray shows the hypoxic response of chow-fed pparα$^{-/-}$ mice for comparison. * $p < 0.05$ vs chow fed control, ‡ $p < 0.05$ vs chow fed PPARα$^{-/-}$.

Effect of chronic hypoxia on energy balance and skeletal muscle

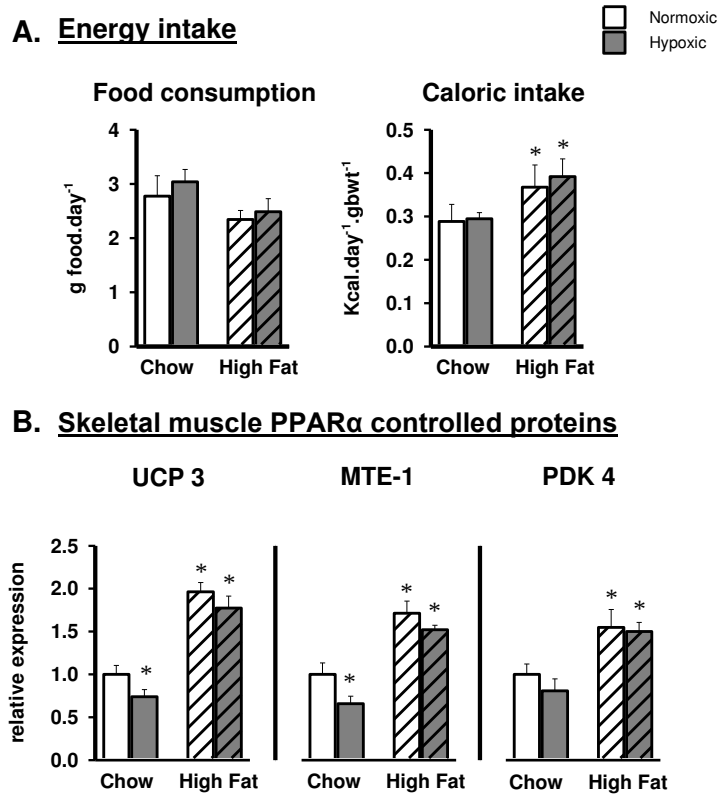

Figure 7. Effect of 3 weeks of hypoxia on energy intake (A, n=4), and skeletal muscle proteins (B, n=6), under PPARα control in wild-type mice fed chow (7.5% fat) or a high (55%) fat diet. * $p<0.05$ vs. normoxic chow-fed control.

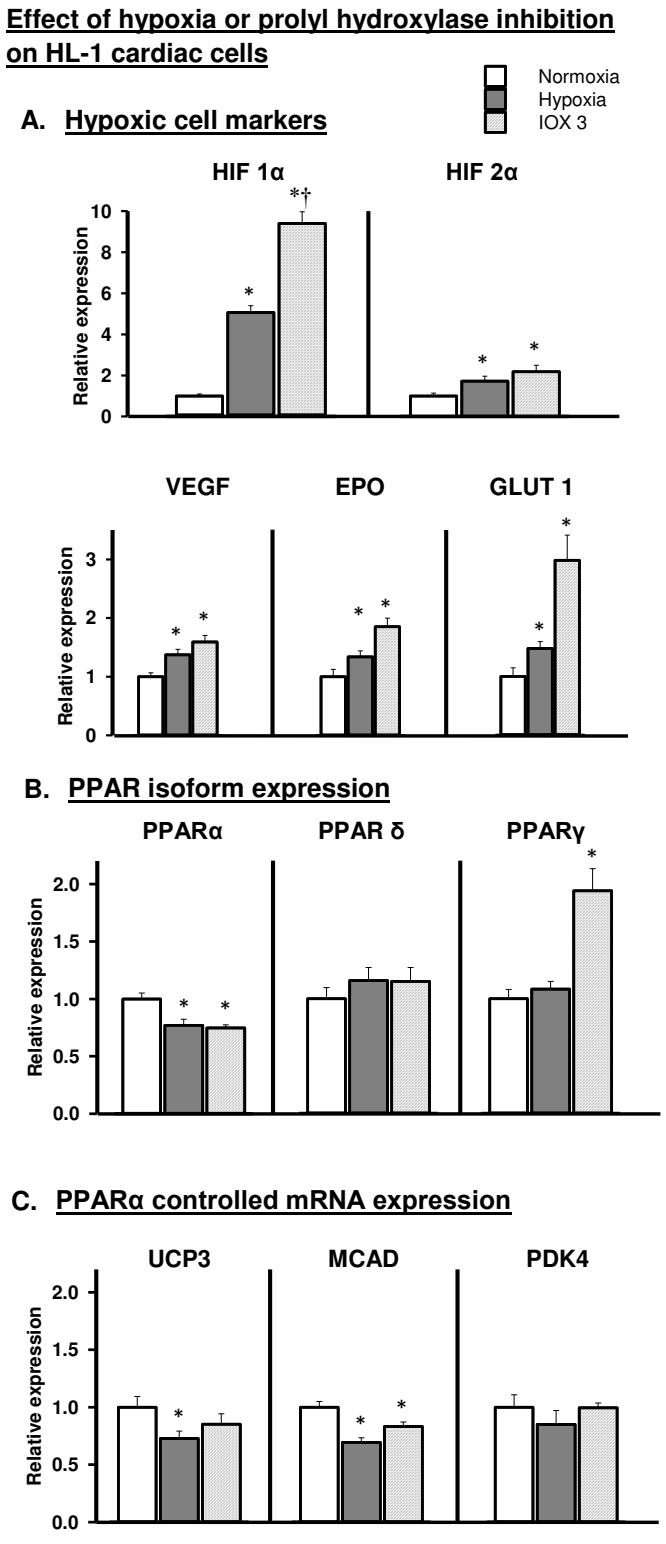

Figure 8. Effect of 24 h hypoxia or a PHD inhibitor, IOX3, on HL1 cardiomyocytes, n=4. * $p<0.05$ vs normoxic cells, † $p<0.05$ vs hypoxic cells.