Assessment of metformin induced changes in cardiac and hepatic redox state using hyperpolarized[1-^{13}C]pyruvate.

Running title: Hyperpolarized MR assessment of metformin

Andrew JM Lewis[1, 3], Jack JJ Miller[1,2], Chloe McCallum[1], Oliver J Rider[3], Stefan Neubauer[3], Lisa C Heather[1] and Damian J Tyler[1]

[1]Department of Physiology, Anatomy and Genetics, University of Oxford, OX1 3PT

[2]Department of Physics, Clarendon Laboratory, University of Oxford, OX1 3PU

[3]Oxford Centre for Clinical Magnetic Resonance Research, Radcliffe Department of Medicine, University of Oxford, OX3 9DU

Correspondence to:
Professor Damian Tyler
Department of Physiology, Anatomy & Genetics
Sherrington Building, University of Oxford
Parks Road, Oxford. OX1 3PT
Tel: ++44 (0)1865 282252
Fax: ++44 (0)1865 282272
damian.tyler@dpag.ox.ac.uk

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Abstract

Metformin improves cardiovascular outcomes in type 2 diabetes, but its exact mechanisms of action remain controversial. We used hyperpolarized [1-^{13}C]pyruvate magnetic resonance spectroscopy to determine the effects of metformin treatment upon heart and liver pyruvate metabolism in rats *in vivo*. Both oral treatment for four weeks and a single intravenous metformin infusion significantly increased the cardiac [1-^{13}C]lactate:[1-^{13}C]pyruvate ratio, but had no effect on the [1-^{13}C]bicarbonate + $^{13}CO_2$:[1-^{13}C]pyruvate ratio, an index of pyruvate dehydrogenase flux. These changes were paralleled by a significant increase in the heart and liver cytosolic redox state, estimated from the [lactate]:[pyruvate] ratio, but not whole cell [NAD^+]/[NADH] ratio. Hyperpolarized magnetic resonance imaging localised the increase in cardiac lactate to the left ventricular myocardium, implying a direct myocardial effect, though metformin had no effect on systolic or diastolic cardiac function. These findings demonstrate the ability of hyperpolarized pyruvate magnetic resonance spectroscopy to detect metformin induced changes in cytosolic redox biology, suggest that metformin has a previously unrecognised effect upon cardiac redox state and help to refine the design of impending hyperpolarized magnetic resonance studies in humans.

Introduction

Metformin, a biguanide, is the most commonly prescribed antihyperglycaemic drug for patients with type 2 diabetes and is associated with a lower risk of weight gain or hypoglycaemia than other oral antihyperglycaemic therapies(1). Despite long experience with this drug, the mechanisms by which metformin improves hyperglycaemia have been uncertain, but have been thought to involve suppression of hepatic gluconeogenesis either by activation of the AMPK signalling pathway(2) or by inhibition of mitochondrial complex 1 leading to a decreased cellular energy charge(3). More recently, metformin was also shown to inhibit the mitochondrial form of the redox shuttle glycerophosphate dehydrogenase (mGPD) in the liver(4). One effect of the inhibition of mGPD is to increase the cytosolic redox state, thus inhibiting the redox coupled gluconeogenic conversion of glycerol and lactate into glucose and lowering plasma glucose concentration.

mGPD is one of two major mitochondrial redox shuttles (the other being the malate-aspartate shuttle) and is expressed systemically, including in the heart(5). The cardiac effects of metformin are incompletely understood, but are of interest because metformin is more effective at reducing the risk of cardiovascular complications from type 2 diabetes than other antihyperglycaemic therapies(6) and is considered an investigational treatment for heart failure even in the absence of diabetes(7). Whether metformin alters cardiac redox state is unknown, in part because conventional imaging techniques cannot assess redox shifts *in vivo*.

The lactate dehydrogenase (LDH) catalysed interconversion of pyruvate and lactate is an important nicotinamide adenine dinucleotide (NAD) coupled reaction which can be assessed non-invasively using hyperpolarized [^{13}C]pyruvate magnetic resonance (MR) technology(8). Following injection, enzymatic conversion of hyperpolarized [^{13}C]pyruvate into lactate results in incorporation of the hyperpolarized ^{13}C label into the [^{13}C]lactate pool. The other major fate of hyperpolarized [1-^{13}C]pyruvate is decarboxylation via the pyruvate dehydrogenase (PDH) enzyme complex leading to the production of $^{13}CO_2$ and [^{13}C]bicarbonate, which provides a novel index of overall carbohydrate metabolism(9). Although hyperpolarized MR technology has been used to study redox state both in *in vitro* cancer cell lines(10) and tumours *in vivo*(11; 12), its sensitivity to changes in cardiac and hepatic

redox state is unknown. Hyperpolarized MR shows promise as a clinical tool in diabetes related heart disease in humans, as pharmacological restoration of PDH flux was associated with improved cardiac function in a rodent model(13).

In this study, we used hyperpolarized [^{13}C]pyruvate MR spectroscopy (MRS) to determine whether metformin caused changes in pyruvate metabolism in rodents using a sequential spectroscopic acquisition sequence to acquire information from both the heart and liver.

Research design and methods

Animal model

Male Wistar rats were used in this study and all procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986. Rats were provided with either 250mg/kg/day metformin in drinking water (22 mM) or water without metformin for 4 weeks. Both metformin treated and untreated water was flavoured with the same berry flavoured concentrate (SodaStream Waters Zero – Cranberry, Raspberry, SodaStream, Lod, Israel) to mask any flavour of the metformin, and water consumption was not different between the two groups (control 23.9±1 ml/rat/day versus 26.3±0.9 ml/rat/day in metformin treated group, P=ns by unpaired t-test). For subsequent studies of the acute effects of metformin, an intravenous infusion of either 50mg metformin dissolved in 0.9% saline or a saline infusion of the same volume was administered via a tail vein catheter 45 minutes prior to the administration of hyperpolarized pyruvate.

Hyperpolarized MRS and MRI

Animals were anaesthetized with vaporised 3.5% isoflurane (using 2.5 L/min O_2 as a carrier gas), reduced to 2% isoflurane for anaesthesia maintenance. A custom built MR cradle incorporating a warm air blower system maintained body temperature at 37°C throughout the experiments; electrocardiographic monitoring was performed throughout to allow observation of stable heart and respiration rates during the experiments.

[1-^{13}C]pyruvic acid was hyperpolarized in a prototype hyperpolarizer as described previously(8). Pre-polarised pyruvate was administered as a bolus of 1 ml of an 80 mM solution over 10 s. Slice selective spectra were acquired interleaved over the following two minutes from two axial slabs covering the heart and the liver (350 μs sinc pulse, 1 cm thick excitation, 8 kHz bandwidth, 15° flip angle, repetition time 1s, both slices ECG gated). Experiments were performed on an Agilent 7 T preclinical horizontal bore scanner with a volume transmit/two channel surface receive array. For

imaging experiments, a specifically designed MR sequence with spectral-spatial radiofrequency pulses with echo planar imaging gradients was used, as previously described(14).

Echocardiography

Under light isoflurane anaesthesia, systolic function was assessed from parasternal M-mode views whilst diastolic function was assessed using pulsed wave Doppler examination directed to the mitral valve inflow (E wave) and tissue Doppler imaging of the medial mitral annulus (to derive E').

Tissue and plasma analysis

Heart and liver tissue was harvested under isoflurane anaesthesia and rapidly snap frozen using aluminium tongs cooled to the temperature of liquid nitrogen. Blood samples were taken and placed in heparinized tubes before centrifugation at 13,000rpm for 10 minutes at 4°C. The plasma was then frozen in liquid nitrogen for later biochemical analyses. In order to avoid any potentially confounding effect of the hyperpolarized pyruvate administration upon systemic redox state, all samples were obtained at least two days following the administration of hyperpolarized pyruvate (with either continued administration of oral metformin or a second metformin infusion depending upon the study group). Because plasma levels of pyruvate have been shown to normalise within 10 minutes of administration of 1ml 80mM hyperpolarized pyruvate(9), it is highly unlikely that any residual effect upon systemic redox state would be present at this point.

Approximately 100mg frozen tissue was homogenised using a rotor stator homogeniser and metabolites extracted in 6% perchloric acid. To maximise the stability of metabolites, the perchloric acid extract supernatants were neutralised prior to deproteination using 10 kDa molecular weight cut off filtration and samples were maintained on ice whenever possible. [Lactate] and [β-hydroxybutyrate] concentrations were determined using an automated assay system (ABX Pentra 400, Horiba Medical), pyruvate concentration was determined using a fluorescent assay kit (Cambridge Bioscience Ltd, UK), free [NAD^+]:[NADH] ratio was determined using a colorimetric assay based on

the enzyme cycling method (Cambridge Bioscience Ltd, UK), and [acetoacetate] was derived using a colorimetric assay kit (Abcam, Cambridge, UK). Plasma lactate levels were determined using an automated assay system (ABX Pentra 400, Horiba Medical).

Quantitative polymerase chain reaction

Approximately 30mg frozen heart or liver tissue was homogenised in lysis buffer and RNA extracted using RNeasy mini columns (Qiagen). The integrity of the extracted RNA was confirmed by Nanodrop spectrophotometry. RNA was reverse transcribed using a high-capacity cDNA reverse transcription kit (Thermo Fisher). Taqman primers for rodent Slc22a1 (Rn00562250_m1), Ldha (Rn00820751_g1) and GPD2 (Rn00562472_m1) genes were used in 20µl qPCR reactions run in duplicate using a StepOnePlus qPCR system. Gene expression was normalised to expression of 45s pre-ribosomal RNA (Rn03928990_g1) and expressed according to the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

Data are reported as mean±standard error of the mean (SEM). Statistical significance was determined as $p \leq 0.05$ and as all differences were compared to independent control groups, statistical significance was calculated using unpaired two-tailed student t-tests.

Results

Treatment with oral metformin for four weeks had no effect upon either cardiac or hepatic pyruvate dehydrogenase flux (cardiac [1-^{13}C]bicarbonate + $^{13}CO_2$:[1-^{13}C]pyruvate ratio 0.04±0.01 versus 0.057±0.006, P=0.19 and hepatic [1-^{13}C]bicarbonate + $^{13}CO_2$:[1-^{13}C]pyruvate ratio 0.054±0.007 versus 0.045±0.006, P=0.29, Figure 1c). However metformin increased the [1-^{13}C]lactate:[1-^{13}C]pyruvate ratio in both the heart (0.27±0.06 versus 0.10±0.01, P=0.02, Figure 1b) and liver (0.87±0.21 versus 0.36±0.04, P=0.04) and also modestly increased the plasma lactate concentration without leading to lactic acidosis (4.1±0.3 versus 2.4±0.3 mM, P=0.002). Despite the change in lactate metabolism, metformin had no effect upon either systolic or diastolic cardiac function (left ventricular ejection fraction 75±2% versus 74±3%, P=0.74 and E/E' ratio 15±1 versus 16±1, P=0.47, Figure 1d).

In order to determine whether the same effect of metformin upon the cardiac and hepatic [1-^{13}C]lactate:[1-^{13}C]pyruvate ratio could be recapitulated acutely, we next performed hyperpolarized [1-^{13}C]pyruvate spectroscopy 45 minutes following a single intravenous infusion of either metformin (50mg) or an equal volume of saline. Metformin again increased the [1-^{13}C]lactate:[1-^{13}C]pyruvate ratio in both the heart (0.30±0.04 versus 0.14±0.02, P=0.002, Figure 2a) and liver (0.65±0.06 versus 0.40±0.04, P=0.003) and again increased the plasma lactate concentration (2.9±0.6 versus 1.0±0.2 mM, P=0.02). Acute metformin infusion also had no effect upon either cardiac or hepatic pyruvate dehydrogenase flux (cardiac [1-^{13}C]bicarbonate + $^{13}CO_2$:[1-^{13}C]pyruvate ratio 0.043±0.009 versus 0.058±0.005, P=0.15 and hepatic [1-^{13}C] bicarbonate + $^{13}CO_2$:[1-^{13}C]pyruvate ratio 0.044±0.006 versus 0.056±0.006, P=0.18).

In order to exclude the possibility that the increase in cardiac [1-^{13}C]lactate signal measured by spectroscopy might reflect metabolic shifts in the adjacent blood pool or skeletal muscle, we next investigated the effects of metformin using a hyperpolarized magnetic resonance imaging sequence to produce a metabolic 'map'. Almost all of the lactate signal localised to the left ventricular myocardium, whereas the large majority of the pyruvate signal localised to the ventricular cavity, supporting a direct effect of metformin upon the heart (Figure 2c).

In the liver(15) and kidney(16), organic cation transporters (OCTs) are the major mediators of cellular uptake of metformin. We confirmed that genes encoding both OCT1 (Slc22a1) and mGPD (Gpd2) were expressed in the heart as well as the liver by quantitative polymerase chain reaction (qPCR) and that expression of these genes was unchanged by metformin infusion (Figure 2d). Similarly, expression of the Ldha gene encoding lactate dehydrogenase was also unaffected by metformin infusion. These findings, together with the rapidity of the effect, suggest that non-transcriptional mechanisms explain the increase in hyperpolarized [1-^{13}C]lactate signal.

Because redox shuttle inhibition is a mechanism by which metformin reduces hepatic gluconeogenesis, we next questioned whether the increase in heart and liver [1-^{13}C]lactate:[1-^{13}C]pyruvate ratio caused by metformin might reflect a shift in cellular redox state(4). The [NAD^+]:[NADH] ratios in both heart and liver whole cell lysates were unchanged by either longer term or acute metformin treatment (cardiac and hepatic [NAD^+]:[NADH] ratios 5.7±0.6 versus 8±2, P=0.18 and 0.8±0.1 versus 0.7±0.1, P=0.64 respectively following 4 week treatment and 6.3±0.1 versus 7.5±0.8, P=0.14 and 1.9±0.2 versus 2.7±0.6, P=0.14 following single infusion Figure 3a). However, metformin can cause a shift in the cytosolic redox state with either an increase(17) or decrease(4) in the mitochondrial redox state depending upon the dosing strategy, and the total cellular [NAD^+]:[NADH] ratio may not be a reliable measure of compartmental redox shift. We therefore measured intracellular [lactate]:[pyruvate] ratios as a surrogate for cytoplasmic redox state(18) and identified that metformin increased the intracellular [lactate]:[pyruvate] ratio, consistent with a shift in heart and liver cytosolic redox state and paralleling the changes in the hyperpolarized [1-^{13}C]lactate:[1-^{13}C]pyruvate ratio (cardiac and hepatic [lactate]:[pyruvate] ratios 46±6 versus 30±6, P=0.04 and 60±9 versus 27±3, P=0.002 respectively following 4 week treatment and 50±8 versus 25±7, P=0.02 and 72±12 versus 16±8, P=0.002 following single infusion, Figure 3b). Metformin in this study caused no change in the [β-hydroxybutyrate]:[acetoacetate] ratio, an index of mitochondrial redox state, in cardiac or hepatic tissue following a single dose, or in cardiac tissue following longer term dosing, though it did increase the [β-hydroxybutyrate]:[acetoacetate] ratio in liver tissue following longer term dosing (online supplemental data).

Discussion

Cellular redox state is an important determinant of metabolism and function as numerous enzyme catalysed reactions exist in redox couples. This study demonstrated that metformin treatment significantly increased the production of [1-^{13}C]lactate following a hyperpolarized [1-^{13}C]pyruvate infusion in both the heart and liver, a finding which was paralleled by an increase in the cytosolic, but not whole cell, redox state. In addition to demonstrating the ability of hyperpolarized magnetic resonance to detect cytosolic redox shifts in the heart and liver, the subcellular compartmentalisation of metformin induced redox shifts is consistent with the view that the interconversion of hyperpolarized [1-^{13}C]pyruvate and [1-^{13}C]lactate primarily reflects a cytosolic reaction, despite the recognition of mitochondrial capacity for lactate and pyruvate oxidation(19).

Metformin caused changes in the [lactate]:[pyruvate] ratio within 45 minutes of administration without upregulating LDH, supporting a rapid, redox dependent mechanism(4). However, metformin has a complex pharmacokinetic profile and crosses plasma membranes slowly due to a net positive charge(20). As a result, metformin achieves higher hepatic concentrations following longer term oral treatment than are possible with a single intravenous treatment at physiological doses(21) and leads to higher hepatic concentrations than are reached in the heart(16; 22). The magnitude of the increase in the hyperpolarized [^{13}C]lactate:[^{13}C]pyruvate ratio in the liver was greater with longer term treatment, and it is tempting to speculate that this may reflect higher bioavailability, although the magnitude of the increase in liver [lactate]:[pyruvate] ratio was similar for both routes of metformin administration.

Although previously described in the liver, a metformin induced increase in cardiac cytosolic redox state not been previously recognised. Metformin has conventionally been thought to improve cardiovascular outcomes in type 2 diabetes primarily by systemic reduction of hyperglycaemia, although the elucidation of direct cardiac effects is of interest as the risks and benefits of metformin treatment in heart failure with or without diabetes remain contentious(23). Although the cardiac redox shift was not associated with a change in either systolic or diastolic function in this study, future work should be directed to understanding the effect of metformin in disease states including models of type II diabetes.

Our finding that metformin does not alter either cardiac or hepatic pyruvate dehydrogenase flux is consistent with the finding that the major mechanism by which metformin reduces hyperglycaemia is through inhibition of gluconeogenesis rather than potentiation of peripheral glucose utilisation(24). The finding is also consistent with data from [^{18}F]-2-fluoro-2-deoxy-D-glucose positron emission tomography studies demonstrating that metformin does not increase myocardial(25) or skeletal muscle(26) glucose uptake. PDH is a mitochondrial enzyme complex, which is partially regulated by redox state, though a phosphorylation/dephosphorylation cycle is also important(27). With the exception of livers following longer term dosing, mitochondrial redox states were generally unaffected by metformin in this study which is consistent with the understanding that both redox-dependent and redox-independent mechanisms determine PDH flux *in vivo*.

Hyperpolarized magnetic resonance has high translational potential with early human studies already underway(28). The ability to non-invasively assess metformin induced redox shifts *in vivo* in humans would be an important advance, as animal studies using metformin have been limited by differences in inter-species dosing and bioavailability profiles(21) and it remains unclear to what extent inhibition of mGPD would be sufficient to cause cellular redox shifts in humans, where the malate-aspartate shuttle is the quantitatively dominant shuttle mechanism(29). Such an approach would also allow an assessment of cellular energy charge by combining ^{31}P spectroscopy with hyperpolarized [1-^{13}C]pyruvate.

In summary, metformin treatment led to significant increases in the hyperpolarized [^{13}C]lactate: [^{13}C]pyruvate ratios in both the heart and liver, a finding which appears to reflect a shift in the cytosolic redox state in these organs. These findings suggest that hyperpolarized pyruvate magnetic resonance spectroscopy is sensitive to metformin induced changes in redox biology, suggest that metformin has a previously unrecognised effect upon cardiac redox state and may influence the design of future human studies using hyperpolarized ^{13}C magnetic resonance.

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DJT 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 the accuracy of the data analysis.

There are no conflicts of interest to report in relation to this work.

AJML, JJM and CM researched data; AJML wrote the manuscript. OJR, SN and LH reviewed and edited the manuscript and contributed to discussions. DJT conceived of and designed the study and reviewed and edited the manuscript. The authors are grateful to Mrs Vicky Ball (Department of Physiology, Anatomy and Genetics, University of Oxford, OX1 3PT) for excellent technical assistance. AJML and SN acknowledge funding from the British Heart Foundation Oxford Centre for Research Excellence, JJM acknowledges funding from EPSRC Doctoral Training Centre and Prize Fellowship (EP/M508111/1) grants, LCH acknowledges funding from Diabetes UK (grant number 11/0004175) and DJT acknowledges funding from the British Heart Foundation (grant numbers FS/14/17/30634 & RG/11/9/28921).

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Figure legends

Figure 1

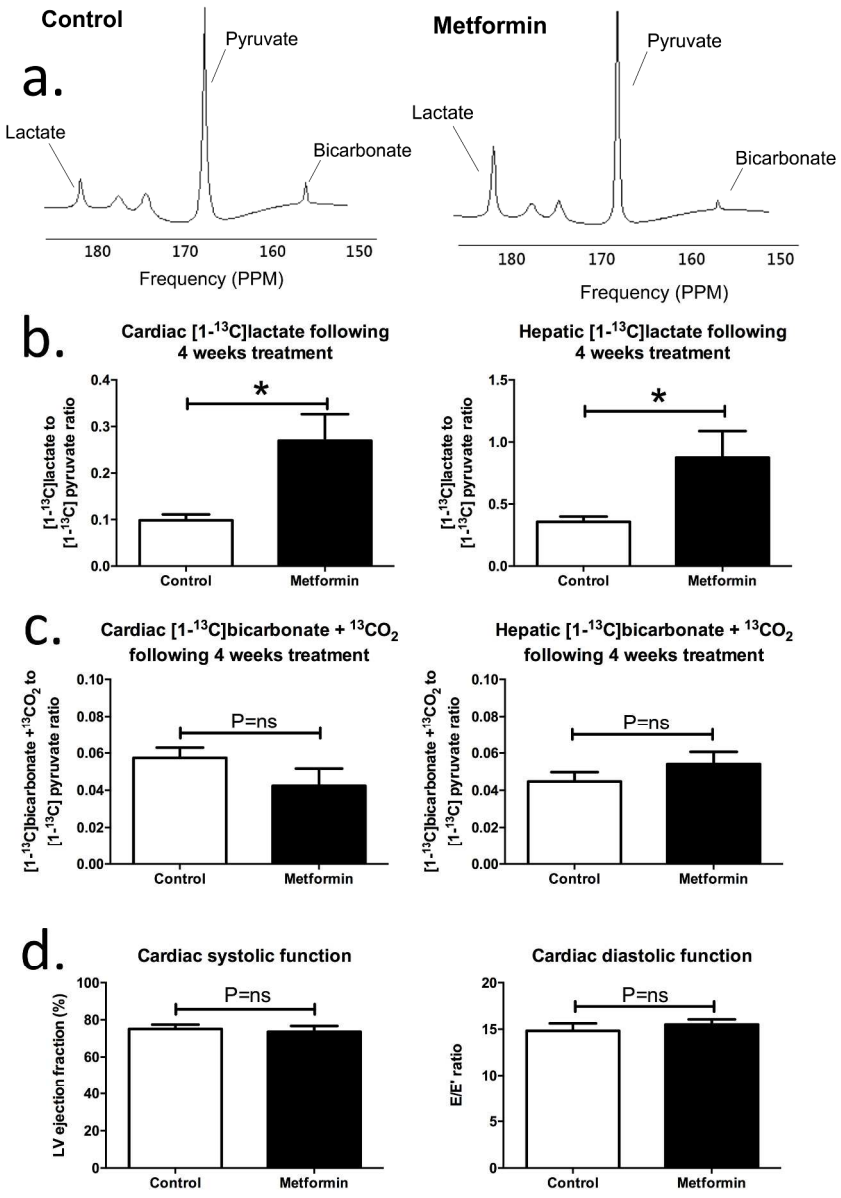
Panel a; representative spectra acquired from slabs covering the liver from control and metformin treated rats. Panels B and C; oral metformin treatment increases the hyperpolarized [1-^{13}C]lactate:[1-^{13}C]pyruvate ratio but not [1-^{13}C]bicarbonate + $^{13}CO_2$:[1-^{13}C]pyruvate ratio. Panel D; metformin did not change either systolic or diastolic cardiac function (n=6 per group, mean±SEM, *p<0.05).

Figure 2

Panels A and B; a single metformin infusion increases the hyperpolarized [1-^{13}C]lactate:[1-^{13}C]pyruvate ratio but not [1-^{13}C]bicarbonate + $^{13}CO_2$:[1-^{13}C]pyruvate ratio (n=10 per group, mean±SEM, **p<0.01). Panel C; hyperpolarized magnetic resonance imaging demonstrates that almost the entirety of the lactate signal localises to the left ventricular myocardium. Panel D; metformin had no effect upon the expression of the gene encoding lactate dehydrogenase; genes encoding both mGPD and OCT1 were confirmed to be expressed in the heart as well as the liver and were likewise unchanged by metformin infusion (n=4 biological replicates, mean ± SEM).

Figure 3

Panels A; neither oral metformin treatment nor a single metformin infusion change the whole cell [NAD^+]/[NADH] ratio. Panel B; both oral metformin treatment and a single metformin infusion treatment increased the cellular [lactate]:[pyruvate] ratio, implying an increase in the cytosolic redox state (n=5-6 per group, mean±SEM, *p<0.05, **p<0.01).



Panel a; representative spectra acquired from slabs covering the liver from control and metformin treated rats. Panels B and C; oral metformin treatment increases the hyperpolarized [1-13C]lactate:[1-13C]pyruvate ratio but not [1-13C]bicarbonate + 13CO2:[1-13C]pyruvate ratio. Panel D; metformin did not change either systolic or diastolic cardiac function (n=6 per group, mean±SEM, *p<0.05).

Figure 1

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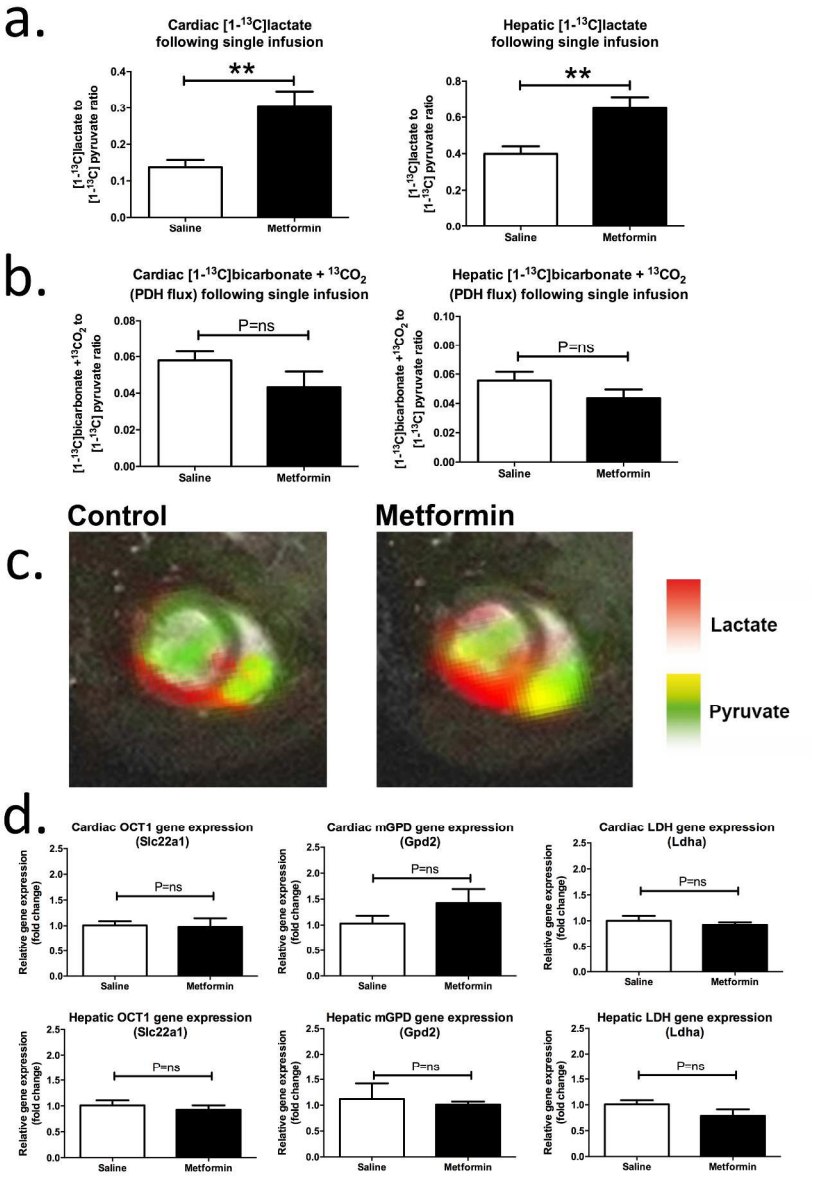

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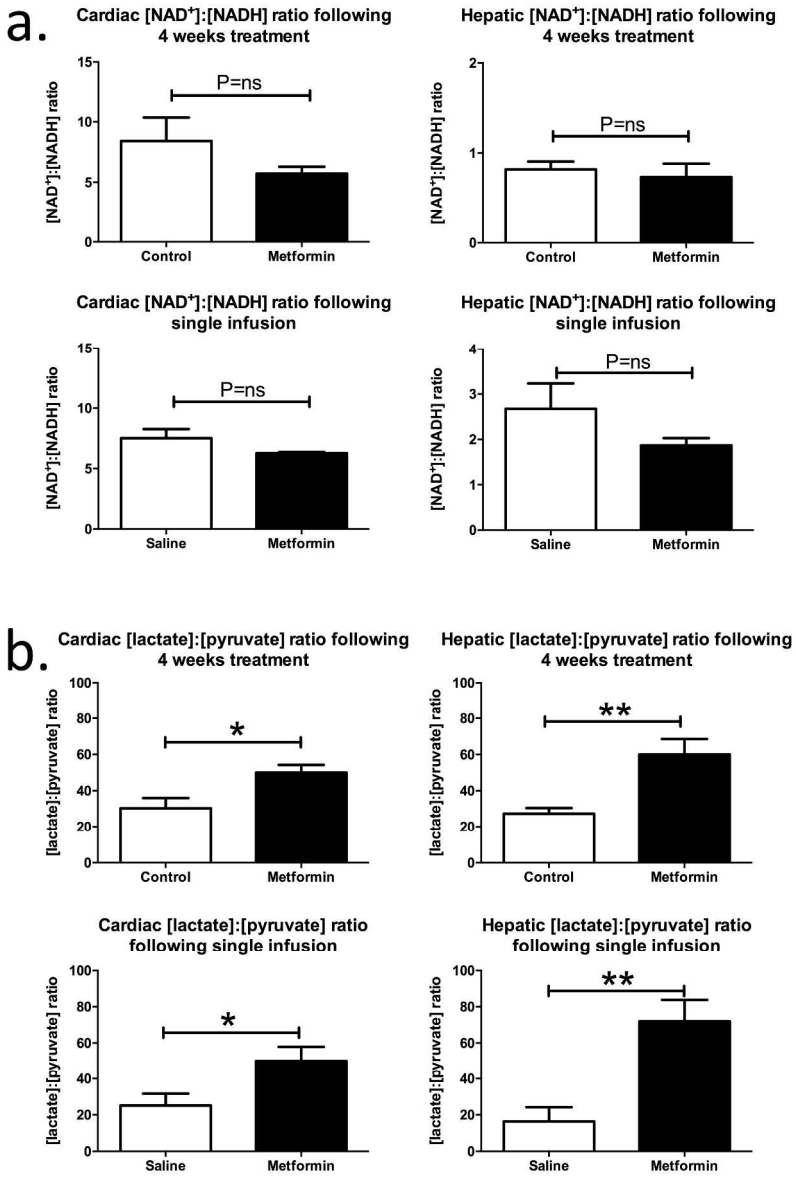
Figure 2

275x397mm (300 x 300 DPI)



Panels A; neither oral metformin treatment nor a single metformin infusion change the whole cell [NAD+]/[NADH] ratio. Panel B; both oral metformin treatment and a single metformin infusion treatment increased the cellular [lactate]:[pyruvate] ratio, implying an increase in the cytosolic redox state (n=5-6 per group, mean±SEM, *p<0.05, **p<0.01).

Figure 3
275x397mm (300 x 300 DPI)

Online Supplemental Data

Plasma glucose and insulin levels

Plasma glucose and insulin concentrations were measured in post-mortem plasma samples obtained from rats killed in the fed state and were not significantly different between groups. Plasma lactate and glucose levels were determined using an automated assay system (ABX Pentra 400, Horiba Medical) and plasma insulin levels were assessed using an insulin ELISA kit (Mercodia, Sweden).

	Single infusion			Longer term treatment		
	Control	Metformin	P value	Control	Metformin	P value
Glucose (mmol/L)	7.0 ± 0.5	8.5 ± 0.7	0.1	7.0 ± 0.3	6.7 ± 0.4	0.57
Insulin (µg/L)	4.9 ± 2	5.6 ± 1	0.75	6.2 ± 2	3.4 ± 2	0.25

β-hydroxybutyrate and acetoacetate measurement

Metformin caused no change in the [β-hydroxybutyrate]:[acetoacetate] ratio, an index of mitochondrial redox state, in cardiac or hepatic tissue following a single dose, or in cardiac tissue following longer term dosing, however, it increased the [β-hydroxybutyrate]:[acetoacetate] ratio in liver tissue following longer term dosing (Figure S1). β-hydroxybutyrate concentrations were determined using an automated assay system (ABX Pentra 400, Horiba Medical) and acetoacetate concentrations were derived using a colorimetric assay kit (Abcam, Cambridge, UK).

AMPK phosphorylation

Metformin caused no change in the phosphorylation level of AMPK in either cardiac or hepatic tissue following longer term dosing (Figure S2). Frozen tissue was crushed and lysis buffer added before tissue was homogenised; a protein assay established the protein concentration of each lysate. The same concentration of protein from each sample was loaded on to 12.5% SDS-PAGE gels and separated by electrophoresis(1). Primary antibodies raised against AMPK and phospho-AMPK (Thr172) were purchased from Cell Signaling Technology (Beverly, MA). Even protein loading and transfer were confirmed by Ponceau staining (0.1% w/v in 5% v/v acetic acid, Sigma-Aldrich), and

internal standards were used to ensure homogeneity between samples and gels. 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.

Supplemental figures

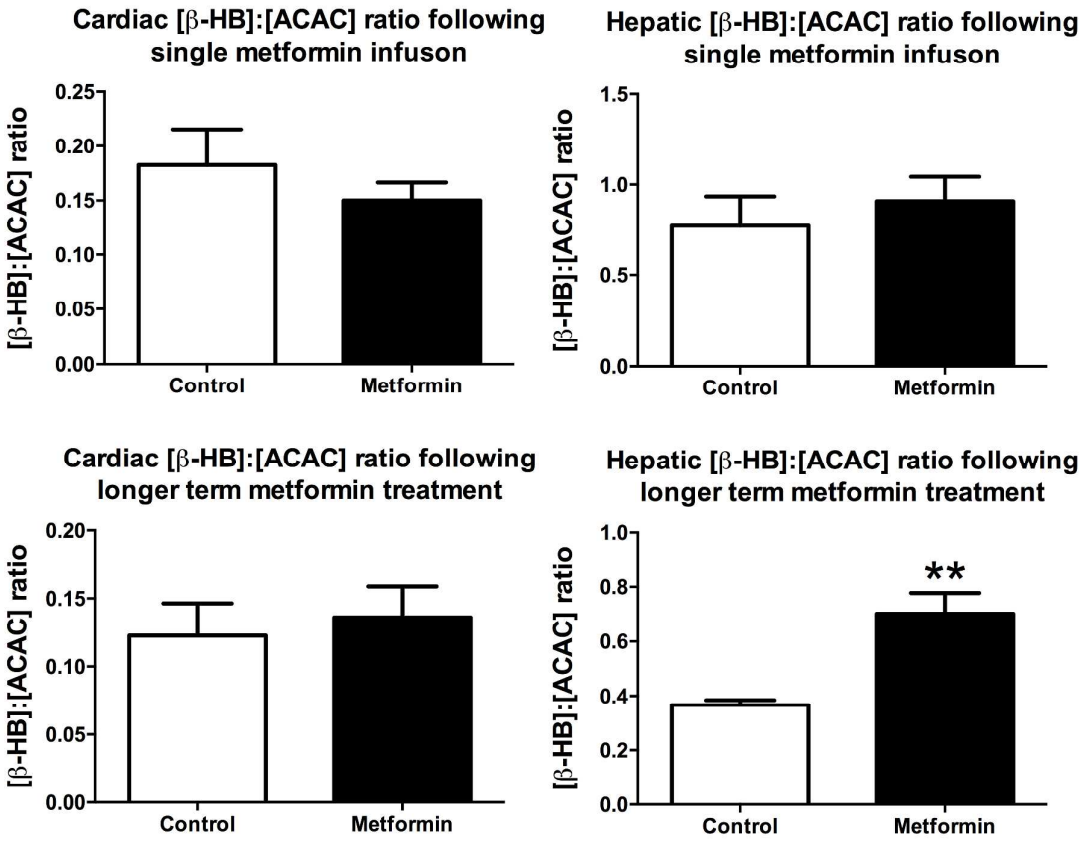


Figure S1: Ratios of cardiac and hepatic β-hydroxybutyrate and acetoacetate measured in tissue following a single metformin injection and following longer-term metformin treatment (n=5-6 per group, mean ± SEM, **p<0.01).

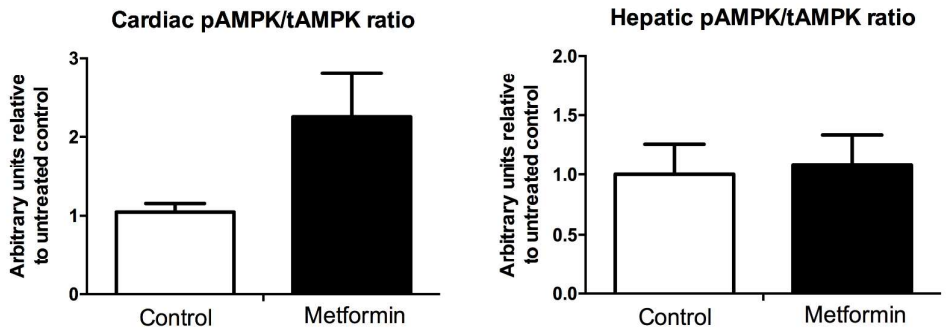


Figure S2: Ratios of cardiac and hepatic protein expression of phospho-AMPK and total-AMPK measured in tissue following longer-term metformin treatment (n=5-6 per group, mean ± SEM).

References

1. Boehm EA, Jones BE, Radda GK, Veech RL, Clarke K: Increased uncoupling proteins and decreased efficiency in palmitate-perfused hyperthyroid rat heart. Am J Physiol Heart Circ Physiol 2001;280:H977-983