

Infarct-remodelled hearts with limited oxidative capacity boost fatty acid oxidation after conditioning against ischaemia/reperfusion injury

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Aims Infarct-remodelled hearts are less amenable to protection against ischaemia/reperfusion. Understanding preservation of energy metabolism in diseased vs. healthy hearts may help to develop anti-ischaemic strategies effective also in jeopardized myocardium.

Methods and results Isolated infarct-remodelled/sham Sprague–Dawley rat hearts were perfused in the working mode and subjected to 15 min of ischaemia and 30 min of reperfusion. Protection of post-ischaemic ventricular work was achieved by pharmacological conditioning with sevoflurane. Oxidative metabolism was measured by substrate flux in fatty acid and glucose oxidation using [³H]palmitate and [¹⁴C]glucose. Mitochondrial oxygen consumption was measured in saponin-permeabilized left ventricular muscle fibres. Activity assays of citric acid synthase, hydroxyacyl-CoA dehydrogenase, and pyruvate dehydrogenase and mass spectrometry for acylcarnitine profiling were also performed. Six weeks after coronary artery ligation, the hearts exhibited macroscopic and molecular signs of hypertrophy consistent with remodelling and limited respiratory chain and citric acid cycle capacity. Unprotected remodelled hearts showed a marked decline in palmitate oxidation and acetyl-CoA energy production after ischaemia/reperfusion, which normalized in sevoflurane-protected remodelled hearts. Protected remodelled hearts also showed higher β -oxidation flux as determined by increased oxygen consumption with palmitoylcarnitine/malate in isolated fibres and a lower ratio of C16:1+C16OH/C14 carnitine species, indicative of a higher long-chain hydroxyacyl-CoA dehydrogenase activity. Remodelled hearts exhibited higher PPAR α -PGC-1 α but defective HIF-1 α signalling, and conditioning enabled them to mobilize fatty acids from endogenous triglyceride stores, which closely correlated with improved recovery.

Conclusions Protected infarct-remodelled hearts secure post-ischaemic energy production by activation of β -oxidation and mobilization of fatty acids from endogenous triglyceride stores.

Keywords Remodelling • Ischaemia • Reperfusion • Metabolism

1. Introduction

An iconic principle in cardioprotection by metabolic interventions stipulates that increasing fatty acid oxidation under ischaemia/reperfusion conditions is detrimental, while increasing glucose oxidation and concomitantly reducing fatty acid oxidation ('Randle cycle') is beneficial.^{1–3} This concept gained support from many experimental

studies mainly using healthy hearts and is based on seemingly convincing rationales. A number of mechanisms may underlie the benefit of a reduced reliance on fatty acid oxidation as an energy source and include oxygen sparing due to the more energetically favourable glucose (3.17 ATP/O₂) relative to fatty acid oxidation (2.83 ATP/O₂), an effect that may be specifically advantageous in stressed hearts with limited oxygen supply. In addition, fatty acid oxidation is

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known to uncouple electron from proton flux in mitochondria, increasing the formation of reactive oxygen species⁴ and to uncouple glycolysis from glucose oxidation, enhancing detrimental proton production leading to dysregulation of ionic homeostasis.⁵ Moreover, studies with preconditioning, the most effective cardioprotective strategy, show increased glucose oxidation and decreased fatty acid oxidation accompanied by improved functional recovery and cell survival in conditioned hearts, suggesting a causal relationship between this oxygen-sparing metabolic phenotype and protection.^{6,7} We have recently demonstrated that enhanced glucose uptake via GLUT4 fuels recovery from Ca^{2+} overload after ischaemia/reperfusion in rat hearts treated with the preconditioning-mimicking anaesthetic sevoflurane.⁸

Post-infarct hypertrophy accounts for a large part of the clinically relevant cases of left ventricular remodelling in a growing population of elderly patients.⁹ The hearts from these patients would benefit most from protective anti-ischaemic strategies, but unfortunately appear less amenable to therapy.¹⁰ Maladaptive post-infarct remodelling dramatically reduces oxidative capacity in these hearts¹¹ and induces a foetal gene programme resulting in a metabolic phenotype closer to the immature heart, where glycolysis is more important for energy production relative to substrate oxidation.^{12–14} Nevertheless, in what would appear to be in contrast to the adverse effects of fatty acids in adult hearts, Ito et al.¹⁵ recently demonstrated that high levels of fatty acids in the perfusate are capable of enhancing post-ischaemic energy production and increasing contractile function in isolated working neonatal rabbit hearts as opposed to adult hearts. That study provides evidence that in hearts with limited oxidative capacity increasing exogenous energy substrate supply and boosting fatty acid oxidation, which generates ~20% more ATP per metabolized C-atom than glucose oxidation, quickly normalizes energy production. It further raises the possibility that fatty acid oxidation may be also essential in improving post-ischaemic recovery in the conditioned infarct-remodelled myocardium. However, this correlate has not been explored directly so far. Hence, we set out to determine oxidative metabolism in working rat hearts 6 weeks after coronary artery ligation. We hypothesized that infarct-remodelled hearts because of their limited oxidative capacity would enhance post-ischaemic energy production after conditioning by selective up-regulation of fatty acid rather than glucose oxidation. Conditioning of the hearts was achieved using the preconditioning-mimicking anaesthetic sevoflurane, an agent previously shown to be effective in normal and infarct-remodelled myocardium.^{8,16}

Our study now shows for the first time that conditioned infarct-remodelled hearts with marked limitations in oxidative capacity preserve post-ischaemic energy metabolism by activation of fatty acid and not glucose oxidation and mobilization of surplus energy from accumulated endogenous triglyceride stores, which closely correlated with recovery.

2. Methods

The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011) and was approved by the University of Alberta Animal Policy and Welfare Committee. All the chemicals were from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada), unless otherwise stated.

2.1 Coronary artery ligation to promote ventricular remodelling and echocardiography

Ligation of the left anterior descending coronary artery (CAL) and sham operations were performed in 8-week-old 200 g male Sprague–Dawley rats, as previously described.¹⁷ Ligation was performed under inhalational anaesthesia with isoflurane. Adequacy of anaesthesia was confirmed by the loss of the righting reflex during surgery. Buprenorphine (0.05 mg/kg) was administered sc every 12 h for the first three post-operative days. The rats were kept for additional 6 weeks fed with normal diet, and cardiac function was assessed using echocardiography under light isoflurane anaesthesia, using a Vevo 770 high-resolution imaging system equipped with a 30 MHz transducer (RMV-707B, VisualSonics, Toronto, Canada). The function of all the hearts was further evaluated ex vivo in the isolated working heart mode. Morphological and biochemical markers of ventricular remodelling were also determined.

2.2 Working heart perfusions

Six weeks after ligation or sham operation, the rats were euthanized with pentobarbital (150 mg/kg, ip). Each heart was rapidly removed and perfused in the working mode with Krebs–Henseleit solution containing glucose (11 mM), palmitate (1.2 mM, pre-bound to 3% bovine serum albumin), and insulin 100 mU/L.⁸ The hearts were subjected to 15 min of no-flow ischaemia and 30 min of reperfusion (Supplementary material online, Figure S1). Sevoflurane (0.5 mM) bubbled into the perfusate was used to induce protection. The protocols are detailed in the Supplementary material online.

2.3 Metabolic flux measurements of glucose and fatty acid oxidation

Glucose and fatty acid oxidation were determined by perfusing the hearts with [^{14}C]glucose and [$^{9,10-3}\text{H}$]palmitate, respectively.⁸ Total myocardial $^{14}\text{CO}_2$ production and $^3\text{H}_2\text{O}$ production were determined every 10 min. Rates expressed as $\mu\text{mol/g dry wt/min}$ were calculated for each time interval and were averaged for pre- and post-ischaemic periods. Differences between averaged pre-ischaemic and averaged post-ischaemic values were computed for each heart.

2.4 Citrate synthase activity

To determine the capacity of the tricarboxylic acid cycle, the activity of the mitochondrial matrix marker enzyme citrate synthase (CS) was measured at 412 nm by monitoring the formation of thionitrobenzoate, as detailed in the Supplementary material online.

2.5 Pyruvate dehydrogenase complex activity

Pyruvate dehydrogenase activity was measured by a radioisotopic-coupled enzyme assay, which determines the ratio PDHactive/PDHtotal. This ratio gives an indication of the phosphorylation state of pyruvate dehydrogenase E1 α subunit. Details are provided in the Supplementary material online.

2.6 Hydroxyacyl-coenzyme A dehydrogenase activity

Enzyme activity was measured under standardized optimal conditions using continuous kinetic determination. One unit of activity is defined as enzyme activity converting 1.0 μmol of acetoacetyl-CoA into β -OH-butyryl-CoA in the presence of NADH per minute per mg protein at 37°C (pH 7.3).

2.7 Triglyceride hydrolase activity

Frozen ventricular tissue was homogenized in ice-cold lysis buffer containing 0.25 M sucrose, 1 mM EDTA, 1 mM dithiothreitol, 20 $\mu\text{g/mL}$ leupeptin, 2 $\mu\text{g/mL}$ antipain, and 1 $\mu\text{g/mL}$ pepstatin (pH 7.0) and spun at 20 000 g at 4°C for 30 min.¹⁸ The infranatant (100 μg of protein in 100 μL lysis buffer) was incubated with 100 μL of substrate in a water

bath for 60 min at 37°C. The reaction was terminated by the addition of 3.25 mL of methanolchloroform-heptane (10:9:7 [vol/vol/vol]) and 1 mL of 0.1 M potassium carbonate—0.1 M boric acid, pH 10.5, and the samples were vigorously mixed for extraction. After centrifugation (800 g, 20 min), radioactivity in 1 mL of the upper phase was determined by liquid scintillation counting. The triglyceride substrate contained 33 nmol of glycerol trioleate/assay (glycerol tri[9,10(*n*)-³H]oleate 40 000 cpm/nmol) in 50 mM potassium phosphate buffer, pH 7.0, and 2.5% defatted bovine serum albumin and was prepared by sonication on ice.

2.8 High-resolution respirometry in permeabilized cardiac fibres

Respiration measurements were performed in saponin-permeabilized fibres prepared from freshly excised left ventricular apex of perfused hearts, using the Oroboros Oxygraph 2K system (Oroboros, Innsbruck, Austria). Characterization of the mitochondrial respiratory complexes was obtained using the following substrates in the presence of 5 mM ADP (State 3) and the absence of ADP (State 2): pyruvate (5 mM)/malate (2 mM), glutamate (10 mM)/malate (2 mM), succinate (10 mM), ascorbate (2 mM)/tetramethyl-phenylenediamine dihydrochloride (TMPD; 0.5 mM), and palmitoylcarnitine (20 μM)/malate (2 mM). The respirometry data were normalized to mitochondrial content (CS activity). The protocols are provided in the Supplementary material online in detail.

2.9 Mass spectrometry for acylcarnitine profiling

From all the hearts, tissue levels of thirty-two acylcarnitine species were measured using electrospray ionization tandem mass spectrometry.¹⁹ Acylcarnitines were extracted from heart tissue with methanol and quantified using eight isotopically labelled internal standards (Cambridge Isotopes Laboratories, Andover, MA, USA). Precursor ions of an *m/z* of 85 in the mass range *m/z* 150 to 450 were acquired on a PE SCIEX API 365 LC-ESI-MS/MS instrument (AppliedBiosystems, Foster City, CA, USA).

2.10 HPLC-fluorescence spectrometry for short-chain CoA ester determinations

Frozen rat heart tissue was homogenized in a 6% perchloric acid/1 mM 1,4-dithiothreitol solution.¹⁹ The homogenate was incubated on ice for 20 min and centrifuged at 12 000 g for 15 min. The supernatant was collected and incubated on ice for 4–5 h. Short-chain CoA (acetyl-CoA, malonyl-CoA, succinyl-CoA, and free CoA) concentrations were determined using Waters AcQuity with PDA eλDetector, sample manager, and Binary solvent manager by high-performance liquid chromatography (Waters, ON, Canada).

2.11 Determination of tissue triglyceride content and incorporation of [9,10-³H]palmitate into triglycerides

After chloroform/methanol extraction of lipids from cardiac tissue, triglyceride content was quantified colorimetrically with the enzymatic assay kit L-Type Triglyceride M (Wako Pure Chemical Industries, Richmond, VA, USA).¹⁹ Incorporation of [9,10-³H]palmitate into triglycerides was counted.

2.12 Determination of tissue glycogen content and incorporation of [U-¹⁴C]glucose into glycogen

Myocardial glycogen content (μmol glucosyl units/g dry wt) was determined by powdering heart tissue samples and subjecting them to alkaline extraction by 30% KOH followed by ethanol precipitation and acid hydrolysis (2N H₂SO₄).¹⁹ This was followed by measurement of glucose

content in these extracts. The amount of radiolabelled glucose in glycogen extracts was also determined to evaluate the degree of incorporation of radiolabelled glucose into glycogen corresponding to the rate of glycogen synthesis.

2.13 Immunoblotting and ELISA

Immunoblots were performed as detailed in the Supplementary material online. For sirtuin-1 (Sirt1), PPARα and PGC-1α immunoblots, nuclear fractions using nuclear extraction kit NE-PER® Nuclear Thermo Scientific (Rockford, IL, USA), and for Sirt3 immunoblots crude mitochondrial fractions were prepared. The antibodies used, and their sources, are provided in the Supplementary material online. Nuclear presence of PPARα (CSB-E09755r, Cusabio Biotech Co Ltd, Wuhan, Hubei, China) and PGC-1α (CSB-EL018426RA, Cusabio Biotech Co Ltd, Wuhan, Hubei, China) was measured by ELISA following the manufacturer's instructions.

2.14 HIF1α transcription factor assay

Nuclear extracts were prepared from frozen tissue using the NE-PER Nuclear and Cytoplasmic Extraction Reagent Kit (Product No. 78833; Thermo Scientific). Electrophoretic mobility shift assay was performed using the HIF1α LightShift Chemiluminescent electrophoretic mobility shift assay kit (10006910, Cayman Chemical Company, MI, USA)

2.15 Statistical analysis

Values are given as mean (SD) or median (25th, 75th percentile) depending on the underlying data distribution for the indicated number of independent observations (*n*). The significance of differences in haemodynamic and metabolic variables among groups was determined by Student's *t*-test (two groups) or by analysis of variance (ANOVA) followed by the Student–Newman–Keuls method for *post hoc* analysis or by non-parametric methods (the Mann–Whitney rank sum test or the Kruskal–Wallis test), depending on the underlying data distribution. To test the association between haemodynamic changes and triglyceride and glycogen tissue contents and the association between Sirt1 and PGC-1α, linear regression analyses were performed. The correlation coefficient *R* and the corresponding *P*-value are reported. Differences are considered significant if *P* < 0.05. SigmaStat (version 3.5; Systat Software, Inc., Chicago, IL, USA) was used for the analyses.

3. Results

3.1 Infarct-remodelled hearts exhibit metabolic deficits in tricarboxylic acid cycle capacity and mitochondrial respiration

Six weeks after ligation of the coronary artery, the ratio of heart weight to body weight and the molecular markers of hypertrophy and remodelling were significantly increased (Table 1 and Supplementary material online, Figure S2). Echocardiography revealed reduced ejection fraction and fractional shortening, and increased wall thickness and diastolic and systolic inner diameters of the left ventricle (Table 1). Measurement of metabolic capacities showed marked reduction in tricarboxylic acid cycle capacity (Table 1) and in mitochondrial respiration with various substrates, including glutamate/pyruvate/malate (complex I), succinate (complex II), palmitoylcarnitine/malate (β-oxidation), and ascorbate/TMPD (complex IV) (Table 2). Oxygen consumption in the absence of ADP ('leak respiration') was elevated and respiratory control ratio ('oxidative capacity') was accordingly reduced in infarct-remodelled hearts. Pyruvate dehydrogenase activity and hydroxyacyl-CoA dehydrogenase activity were unchanged

Table 1 Model characteristics

	Sham	CAL	P-value
Measures of cardiac remodelling			
Heart weight/body weight	0.380 (0.037)	0.426 (0.051)	0.02
ANP protein level	0.09 (0.07)	0.44 (0.16)	<0.001
α -skeletal actin protein level	1.14 (0.31)	1.79 (0.57)	0.01
Measures of metabolic capacity			
Citrate synthase activity ($\mu\text{mol mL}^{-1} \text{min}^{-1} \mu\text{g}^{-1}$)	24.5 (3.0)	17.5 (2.8)	<0.001
Total pyruvate dehydrogenase activity ($\mu\text{mol/g protein/min}$)	1.19 (0.28)	1.25 (0.53)	0.83
Pyruvate dehydrogenase activity (ratio active/total)	0.74 (0.13)	0.74 (0.14)	0.95
Hydroxyacyl-CoA dehydrogenase activity ($\text{mmol/g protein/min}$)	7.22 (3.33)	6.26 (0.62)	0.59
Echocardiography data			
M-mode measures			
Ejection fraction (EF, %)	73.8 (66.8; 76.0)	56.9 (46.4; 60.1)	0.001
Fractional shortening (ES, %)	44.2 (38.7; 46.7)	31.4 (24.9; 33.67)	0.002
Average cardiac output (CO, mL/min)	100 (21)	80 (17)	0.07
Wall measurements			
Interventricular septum thickness, diastole (mm)	1.9 (0.1)	2.1 (0.2)	0.009
Interventricular septum thickness, systole (mm)	3.1 (0.3)	3.2 (0.3)	0.84
Left ventricular inner diameter, diastole (mm)	8.6 (0.6)	9.8 (0.8)	0.004
Left ventricular inner diameter, systole (mm)	4.9 (0.7)	7.0 (0.9)	<0.001
Left ventricular posterior wall thickness, diastole (mm)	1.9 (0.1)	2.1 (0.2)	0.023
Left ventricular posterior wall thickness, systole (mm)	3.1 (0.3)	3.2 (0.3)	0.61
Tissue Doppler data			
TEI index	0.80 (0.09)	0.89 (0.17)	0.21
E/E'	17.5 (3.8)	25.0 (7.6)	0.03
E'/A'	1.0 (0.3)	0.9 (0.2)	0.45

Data are presented as mean (SD) or median (25th; 75th percentile).

ANP, atrial natriuretic peptide; E/E', ratio of mitral velocity to velocity of the mitral annulus in early diastole; E'/A', early (E') to late (A') diastolic velocity ratio of mitral annulus; Sham, sham-operated hearts ($n = 8$); CAL, infarct-remodelled hearts ($n = 8$).

between infarct-remodelled and sham hearts under aerobic baseline conditions (Table 1).

3.2 Metabolic responses to ischaemic injury are different in protected infarct-remodelled vs. sham hearts

Consistent with echocardiography, *ex vivo*-remodelled working hearts exhibited reduced left ventricular work at baseline (Figure 1A). Fifteen minutes of ischaemia followed by 30 min of reperfusion reduced peak systolic pressure, cardiac output, and left ventricular work in untreated sham and infarct-remodelled hearts (Figure 1B, Supplementary material online, Table S1). None of the hearts experienced sustained ventricular fibrillation and thus, all the hearts could be used for metabolic analysis. Sevoflurane markedly increased functional recovery in both sham and infarct-remodelled hearts (Figure 1). Baseline glucose and fatty acid oxidation and acetyl-CoA turnover (TCA cycle activity) were similar in sham and infarct-remodelled hearts (Table 3). However, fatty acid oxidation was markedly reduced after ischaemia/reperfusion in infarct-remodelled but not in sham hearts. Protection with sevoflurane restored fatty acid oxidation to 84% of baseline values in infarct-remodelled hearts, securing energy production as evidenced by increased acetyl-CoA turnover (Table 3). Consistent with

this notion, acetyl-CoA tissue levels also significantly increased in protected infarct-remodelled hearts (Table 4).

3.3 Infarct-remodelled hearts secure post-ischaemic energy production by activation of β -oxidation and mobilization of fatty acids from endogenous triglyceride stores

In accordance with the metabolic flux measurements in isolated hearts, high-resolution respirometry revealed increased oxygen flux in permeabilized conditioned remodelled left ventricular muscle fibres when using palmitoylcarnitine/malate as substrate providing evidence for improved β -oxidation (Figure 2A, Table 2). Since oxygen consumption is measured in these experiments under unloaded resting conditions, it can be concluded that increased β -oxidation is a cause and not a consequence of the observed improved cardiac function. Interestingly, increased β -oxidation in protected infarct-remodelled hearts occurred independently of malonyl-CoA tissue levels (Table 4). While total hydroxyacyl-CoA dehydrogenase activity, as measured under *in vitro* conditions, was unchanged after ischaemia/reperfusion between protected and unprotected sham and infarct-remodelled hearts (Figure 2B), acylcarnitine profiling revealed a lower ratio of C16:1+C16OH/C14 carnitine species, a

Table 2 High-resolution respirometry in cardiac fibres

	Sham/aerobic	CAL/aerobic	Sham/IR	Sham + SEV/IR	CAL/IR	CAL + SEV/IR
Oxygen consumption in the presence of ADP						
Glutamate/malate	9.17 (1.13)	6.17 (2.04)	5.10 (1.75)	3.27 (1.39)	7.19 (1.62)	5.34 (1.14)
P-value	0.026		0.104		0.039	
Pyruvate/malate	10.33 (1.62)	6.86 (1.52)	6.51 (1.57)	6.03 (1.67)	8.86 (2.37)	8.67 (1.89)
P-value	0.014		0.656		0.878	
Glutamate/malate/succinate	18.34 (2.70)	10.83 (3.04)	8.63 (1.23)	6.72 (1.10)	12.12 (3.20)	9.53 (2.51)
P-value	0.006		0.033		0.137	
Succinate	11.30 (1.48)	7.34 (2.14)	5.80 (1.01)	4.81 (0.85)	7.96 (2.13)	6.26 (0.92)
P-value	0.013		0.135		0.098	
Palmitoylcarnitine/malate	3.72 (1.44)	2.06 (0.95)	2.13 (0.39)	2.38 (0.54)	2.37 (0.60)	3.34 (0.92)
P-value	0.089		0.416		0.042	
Ascorbate/TMPD	26.24 (4.99)	19.34 (6.49)	16.69 (11.1;21.6)	12.49 (11.7;14.9)	22.87 (7.31)	24.29 (7.66)
P-value	0.112		0.548 (Mann–Whitney)		0.739	
Oxygen consumption in the absence of ADP						
Glutamate/malate	0.81 (0.13)	1.71 (0.64)	1.41 (1.26;1.84)	1.26 (1.18;1.47)	2.32 (0.42)	2.08 (0.36)
P-value	0.017		0.310 (Mann–Whitney)		0.305	
Pyruvate/malate	0.91 (0.73;1.31)	2.34 (1.99;2.64)	2.39 (0.52)	1.98 (0.78)	3.04 (0.78)	3.12 (1.11)
P-value	0.016 (Mann–Whitney)		0.365		0.885	
Palmitoylcarnitine/malate	0.78 (0.13)	1.20 (0.26)	1.11 (0.28)	1.13 (0.22)	1.41 (0.39)	1.80 (0.51)
P-value	0.017		0.935		0.154	
Respiratory control ratio						
Glutamate/malate	11.58 (2.37)	3.72 (0.50)	3.22 (0.99)	2.31 (0.45)	3.09 (0.29)	2.55 (0.22)
P-value	<0.001		0.097		0.003	
Pyruvate/malate	10.93 (2.41)	2.97 (0.36)	2.74 (0.46)	3.15 (0.43)	3.16 (0.34)	2.91 (0.51)
P-value	<0.001		0.179		0.313	
Palmitoylcarnitine/malate	4.85 (1.97)	1.68 (0.58)	1.97 (0.38)	2.02 (0.13)	1.72 (0.35)	1.89 (0.33)
P-value	0.018		0.821		0.386	

The measured oxygen flux (normalized to citrate synthase activity) is expressed as $\text{nmol O}_2 \cdot \text{s}^{-1} / \text{CS}$. Data are presented as mean (SD) or median (25th percentile; 75th percentile). $N = 5$ in all sham groups (aerobic and ischaemia/reperfusion), $n = 4$ in CAL/aerobic, $n = 7$ in CAL/IR, $n = 6$ in CAL + SEV/IR.

sham/IR, sham hearts exposed to ischaemia/reperfusion without treatment; Sham + SEV/IR, sham hearts exposed to ischaemia/reperfusion with sevoflurane; CAL/IR, infarct-remodelled hearts exposed to ischaemia/reperfusion without treatment; CAL + SEV/IR, infarct-remodelled hearts exposed to ischaemia/reperfusion with sevoflurane.

protein-independent measure of long-chain hydroxyacyl-CoA dehydrogenase activity, in protected infarct-remodelled hearts (Figure 2C, Supplementary material online, Table S2). The ratio of C0/C16 + C18 carnitine species, an index of reduced fatty acid transport into mitochondria by the carnitine palmitoyl transferase system, was significantly increased in protected sham but not in infarct-remodelled hearts (Figure 2D). Sevoflurane protection also reduced accumulation of C18, OH-, and unsaturated acylcarnitine intermediates in sham and OH-acylcarnitine species in infarct-remodelled hearts. It further reduced complex II activity in sham hearts and complex I activity in infarct-remodelled hearts (Table 2). Consistent with previous reports,²⁰ unprotected infarct-remodelled hearts exhibited higher baseline triglyceride contents (cold triglycerides) (Figure 3A), which were normalized after ischaemia/reperfusion in protected infarct-remodelled hearts with improved flux in β -oxidation. Incorporation of radioactive palmitate into triglyceride was slightly (but significantly $P < 0.004$) reduced in protected sham hearts, but unaffected in infarct-remodelled hearts (Figure 3A). Sevoflurane protection also preserved glycogen tissue contents and enhanced incorporation of radioactive glucose into glycogen in both sham and infarct-remodelled hearts (Figure 3B).

Importantly, triglyceride tissue content inversely and closely correlated with changes in left ventricular work in infarct-remodelled but not in sham hearts, consistent with the concept that additional fatty acids were mobilized from endogenous triglyceride stores boosting energy production (Figure 4A). However, triglyceride hydrolase activity was unchanged in protected remodelled hearts and phosphorylation of hormone-sensitive lipase at Ser660, a well-known activity controlling site,²¹ was not increased (Supplementary material online, Figure S3). To further elucidate the protective role of fatty acid mobilization in remodelled hearts, additional experiments were performed using 2.4 mM palmitate in the perfusate mimicking increased supply of fatty acids from triglyceride stores. This higher concentration of palmitate (2.4 mM) in the perfusate increased post-ischaemic recovery from 22 to 73% ($P = 0.004$) in remodelled hearts (Supplementary material online, Figure S4), suggesting that mobilization of fatty acids indeed contributes to protection. In contrast to triglyceride tissue contents, glycogen tissue levels directly correlated with changes in left ventricular work in sham and in infarct-remodelled hearts. This is in accordance with their role of an ischaemic injury marker rather than an efficient energy source under ischaemic conditions (Figure 4B).

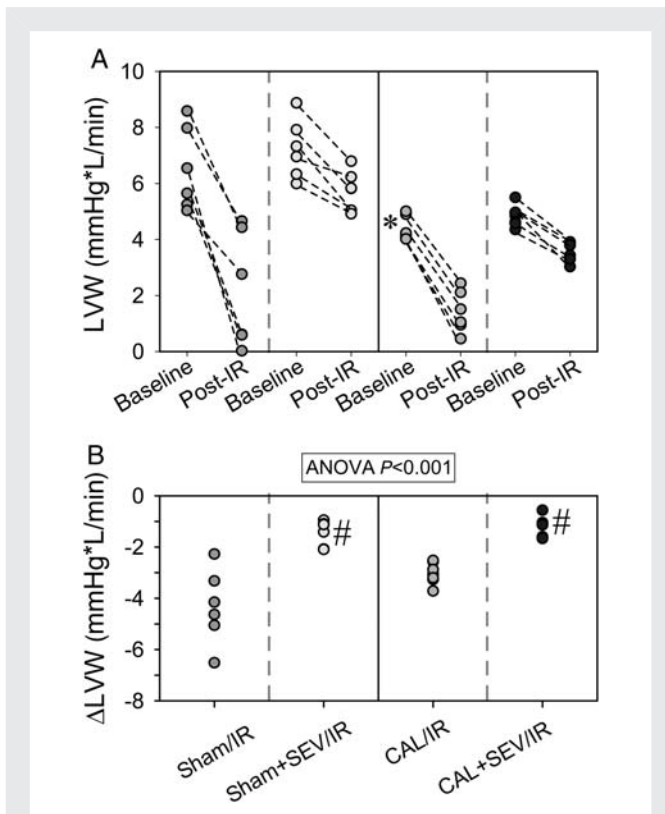


Figure 1 Functional recovery in sham and remodelled hearts. (A) LVW, left ventricular work. (B) Δ LVW, corresponding change in LVW; Sham/IR, unprotected sham hearts exposed to ischaemia/reperfusion alone without treatment; Sham + SEV/IR, protected sham hearts exposed to sevoflurane and ischaemia/reperfusion; CAL/IR, unprotected remodelled hearts exposed to ischaemia/reperfusion alone without treatment; CAL + SEV/IR, protected remodelled hearts exposed to sevoflurane and ischaemia/reperfusion. * $P < 0.001$ baseline sham vs. baseline CAL (aerobic). # $P < 0.001$ Sham + SEV/IR or CAL + SEV/IR vs. Sham/IR or CAL/IR.

3.4 Infarct-remodelled hearts exhibit higher nuclear levels of PPAR α and PGC-1 α and a deficit in the HIF-1 α hypoxic response indicating a loss of metabolic flexibility

To dissect the regulation of energy metabolism in sham vs. infarct-remodelled hearts, we determined nuclear PPAR α and PGC-1 α protein levels. Infarct-remodelled hearts exhibited markedly higher nuclear PPAR α and PGC-1 α levels (Figure 5A) than sham hearts, while no regulation in response to sevoflurane conditioning or ischaemia/reperfusion was evident (data not shown). HIF-1 α , a transcription factor that regulates glycolytic gene expression, was reduced in infarct-remodelled hearts compared with sham hearts (Figure 5A). Remodelled hearts exposed to ischaemia/reperfusion did not exhibit HIF-1 α regulation as opposed to sham hearts (Figure 5B). We further determined nuclear Sirt1 and mitochondrial Sirt3 expression in infarct-remodelled and sham hearts, since mitochondrial Sirt3 was previously reported to deacetylate and activate hydroxyacyl-CoA dehydrogenase,²² while nuclear Sirt1 was reported to deacetylate and inactivate HIF-1 α .²³ Sirt1 was increased in infarct-remodelled vs. sham hearts, and there was only a minor regulation in response to conditioning (Supplementary material online, Figure S5). Sirt3 levels were not different between infarct-remodelled and sham hearts. Sirt3 decreased more in infarct-remodelled hearts after ischaemia/reperfusion (Supplementary material online, Figure S5), but there was no regulation in response to conditioning. Consistent with previous reports on PGC-1 α deacetylation and activation by Sirt1,²⁴ Sirt1 levels correlated closely with nuclear PGC-1 α (Figure 5C). Together, the observed metabolic phenotype in remodelled hearts is consistent with a loss of metabolic flexibility and indicates a shift in energy metabolism towards fatty acid oxidation.

4. Discussion

Our study examined the bioenergetics in infarct-remodelled hearts in the presence and the absence of pharmacological protection, which

Table 3 Rates of glucose oxidation, fatty acids oxidation, and acetyl-CoA production

		Sham/IR	Sham + SEV/IR	CAL/IR	CAL + SEV/IR
GOX ($\mu\text{mol/g dry wt min}$)	Before ischaemia	0.748 (0.243)	0.771 (0.316)	0.628 (0.162)	0.698 (0.168)
	Reperfusion	0.471 (0.179)	0.639 (0.115)	0.662 (0.221)	0.579 (0.212)
Δ GOX ($\mu\text{mol/g dry wt min}$)		-0.276 (0.300)	-0.131 (0.268)	0.034 (0.301)	-0.119 (0.088)
P-value		0.399		0.259	
FOX ($\mu\text{mol/g dry wt min}$)	Before ischaemia	0.898 (0.236)	0.990 (0.264)	0.878 (0.142)	0.749 (0.141)
	Reperfusion	0.555 (0.473)	0.901 (0.207)	0.296 (0.254)	0.629 (0.230)
Δ FOX ($\mu\text{mol/g dry wt min}$)		-0.343 (0.358)	-0.089 (0.124)	-0.571 (0.227)	-0.120 (0.152)
P-value		0.131		0.002	
acCoA ($\mu\text{mol/g dry wt min}$)	Before ischaemia	8.680 (2.004)	9.397 (2.108)	8.333 (1.350)	7.311 (1.245)
	Reperfusion	5.344 (4.168)	8.488 (1.807)	3.616 (2.405)	6.186 (1.970)
Δ acCoA ($\mu\text{mol/g dry wt min}$)		-3.337 (3.389)	-0.909 (0.698)	-4.717 (2.312)	-1.125 (1.374)
P-value		0.116		0.008	

Data are presented as mean (SD). $N = 6$ in all groups.

GOX, glucose oxidation rate ($\mu\text{mol/g dry wt min}$); FOX, fatty acids oxidation rate ($\mu\text{mol/g dry wt min}$); acCoA, acetyl coenzyme A production rate ($\mu\text{mol/g dry wt min}$); Sham/IR, sham hearts exposed to ischaemia/reperfusion without treatment; sham + SEV/IR, sham hearts exposed to ischaemia/reperfusion with sevoflurane; CAL/IR, infarct-remodelled hearts exposed to ischaemia/reperfusion without treatment; CAL + SEV/IR, infarct-remodelled hearts exposed to ischaemia/reperfusion with sevoflurane.

Table 4 Short-chain acyl-CoA tissue levels

	Sham/IR	Sham + SEV/IR	CAL/IR	CAL + SEV/IR
Free CoA (nmol/g dry wt)	864 (181)	1098 (138)	754 (206)	974 (178)
P-value	0.030		0.067	
Malonyl-CoA (nmol/g dry wt)	12.0 (3.1)	15.9 (2.4)	10.2 (2.0)	12.5 (2.5)
P-value	0.039		0.086	
Acetyl-CoA (nmol/g dry wt)	134 (53)	174 (56)	91 (25)	128 (31)
P-value	0.236		0.032	
Succinyl-CoA (nmol/g dry wt)	216 (71)	308 (81)	175 (94)	231 (62)
	0.064		0.241	

Data are presented as mean (SD). $N = 6$ in all groups except CAL/IR ($n = 7$).

Sham/IR, sham hearts exposed to ischaemia/reperfusion without treatment; Sham + SEV/IR, sham hearts exposed to ischaemia/reperfusion with sevoflurane; CAL/IR, infarct-remodelled hearts exposed to ischaemia/reperfusion without treatment; CAL + SEV/IR, infarct-remodelled hearts exposed to ischaemia/reperfusion with sevoflurane.

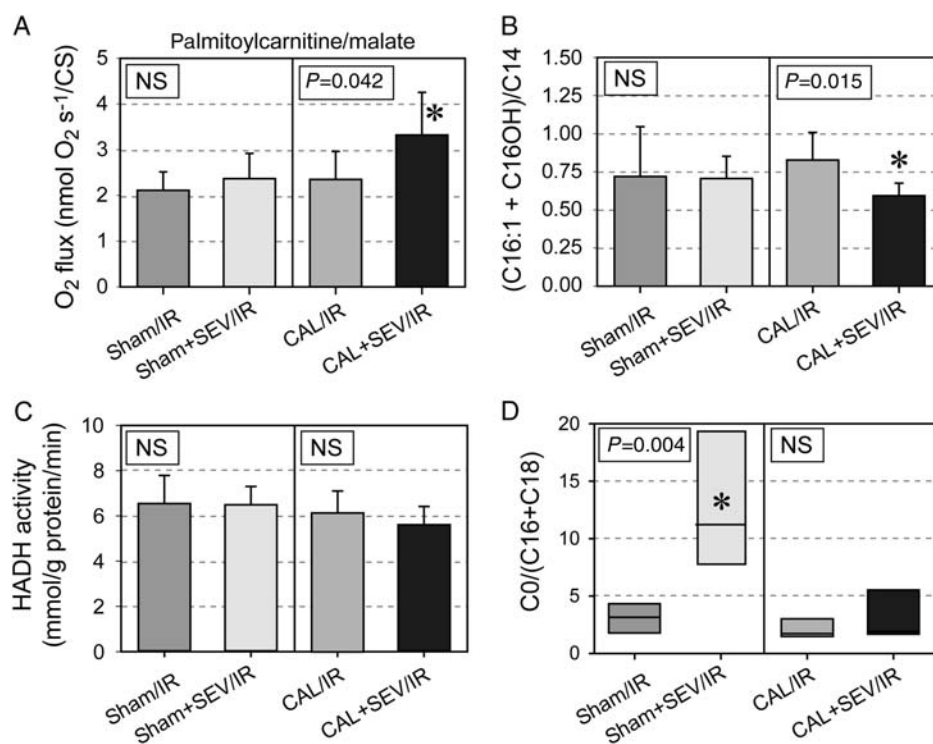


Figure 2 β -Oxidation in sham and remodelled hearts. (A) Oxygen flux in sham and remodelled cardiac fibres as measured by high-resolution respirometry. (B) hydroxyacyl-CoA dehydrogenase (HADH) activity as determined by the *in vitro* assay. (C) Protein-independent measure of hydroxyacyl-CoA dehydrogenase activity reflecting enzyme activity under physiological conditions using acylcarnitines profiling. (D) Ratio of C0/C16 + C18 carnitine species, an index of the carnitine palmitoyl transferase system. Data are mean (SD) or median (25th, 75th percentile). Sham/IR, unprotected sham hearts exposed to ischaemia/reperfusion without treatment; Sham + SEV/IR, protected sham hearts exposed to sevoflurane and ischaemia/reperfusion; CAL/IR, unprotected remodelled hearts exposed to ischaemia/reperfusion alone without treatment; CAL + SEV/IR, protected remodelled hearts exposed to sevoflurane and ischaemia/reperfusion.

was achieved by administration of a preconditioning-mimicking anaesthetic known to be effective in the infarct-remodelled myocardium.^{17,25} Since cardiac remodelling markedly changes gene and protein expression promoting a metabolic phenotype more similar to the foetal or immature heart,^{11–14} we hypothesized that conditioned remodelled hearts would adapt to ischaemia/reperfusion differentially at metabolic level. The novel finding of this study is that

conditioned remodelled hearts with limited oxidative capacity secure post-ischaemic energy production by activation of fatty acid and not glucose oxidation and by mobilization of surplus fuel from accumulated intracellular triglyceride stores. We previously showed that sevoflurane rather decreases fatty acid oxidation excluding the possibility that the observed metabolic shift is caused by an intrinsic metabolic effect of sevoflurane itself.¹⁹ Since we determined

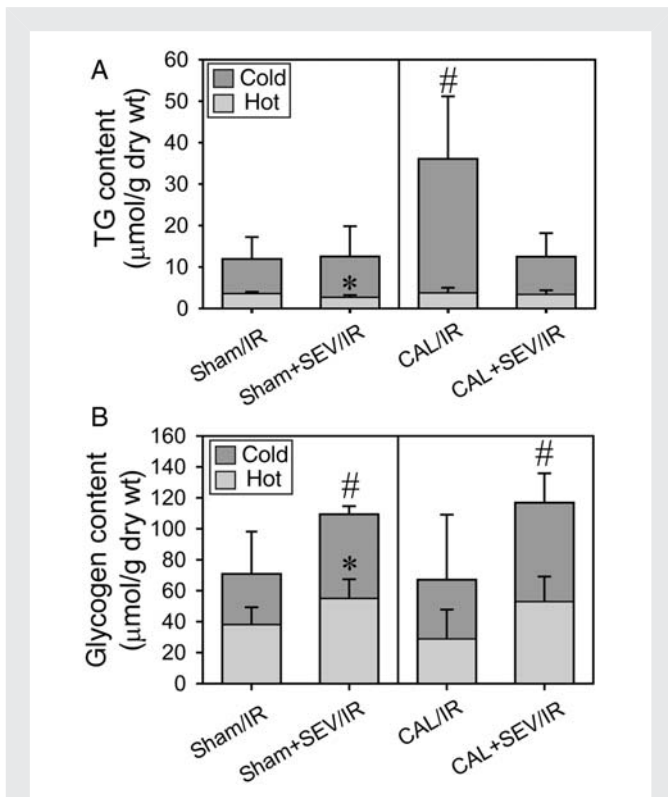


Figure 3 Hot and cold constituents of triglycerides (TG) and glycogen in sham and remodelled hearts. (A) Higher cold TG levels in remodelled hearts, which are reduced to sham heart levels in protected remodelled hearts ($\#P = 0.005$). Reduced incorporation of hot palmitate into TG was observed in sham + SEV/IR vs. sham/IR ($*P = 0.004$). (B) Higher glycogen levels in protected sham (cold $\#P = 0.007$, hot $*P = 0.03$) and remodelled hearts (cold $\#P = 0.025$). Data are mean (SD). Sham/IR, unprotected sham hearts exposed to ischaemia/reperfusion without treatment; Sham + SEV/IR, protected sham hearts exposed to sevoflurane and ischaemia/reperfusion; CAL/IR, unprotected remodelled hearts exposed to ischaemia/reperfusion alone without treatment; CAL + SEV/IR, protected remodelled hearts exposed to sevoflurane and ischaemia/reperfusion.

stress, i.e. favouring glucose over fatty acids as fuel. Collectively, the metabolism in infarct-remodelled hearts clearly mirrors the loss of metabolic flexibility in energy production and fundamentally differs from healthy hearts.

A better understanding of the relationships between energy metabolism, contractility, and anti-ischaemic tolerance in infarct-remodelled hearts could help identify novel approaches to limiting cardiac injury or preventing the progression to heart failure. Energy metabolism was previously examined in hearts remodelled chronically.²⁶ Whether abnormal metabolism is an adaptive response that slows progression to failure or whether it is a maladaptive response that accelerates the development of heart failure has not been resolved. However, in failing hearts, low rates of fatty acid oxidation may underlie the observed increased reliance on glucose as an energy source, which potentially helps maintain rates of acetyl-CoA production. Our study now shows for the first time that infarct-remodelled hearts with significant deficits in oxidative phosphorylation and counterregulatory PPAR α -PGC-1 α up-regulation but reduced and defective hypoxic HIF-1 α signalling boost fatty acid and not glucose oxidation after conditioning against ischaemia/reperfusion injury. It is remarkable that under aerobic baseline conditions infarct-remodelled hearts in our study did not exhibit changes in partitioning of glucose and fatty acid oxidation compared with sham hearts despite their deficits in energy production. This is more striking so as PPAR α -PGC-1 α , which promotes fatty acid oxidation, was significantly up-regulated and HIF-1 α activity and its hypoxic response were blunted. Inhibition of HIF-1 α signalling because of p53 accumulation with subsequent cardiac dysfunction was previously reported in pressure-overloaded hearts progressing from hypertrophy to heart failure.²⁷ Alternatively, increased nuclear Sirt1 may have deacetylated and inactivated HIF-1 α ,²³ which is however at odds with other reports showing that Sirt1 does not affect HIF-1 α but deacetylates and activates HIF-2 α .²⁸ On the other hand, reduced HIF-1 α activity in infarct-remodelled hearts may be regarded as an endogenous protective strategy of the heart since chronic HIF-1 α activation is observed in patients with end-stage heart failure and reportedly accelerates progression to heart failure.²⁹ It is noteworthy that in our study coupling between PGC-1 α and HIF-1 α signaling, which is normally functional in healthy oxidative tissue,³⁰ was absent in infarct-remodelled hearts. However, this is consistent with previous reports increased Sirt1 closely correlated with nuclear PGC-1 α levels.³¹ Nonetheless, upon exposure to ischaemia/reperfusion, conditioned remodelled hearts replenished their citric acid cycle with C₂-bodies from activated fatty acid oxidation despite its oxygen-wasting effects, uncovering their intrinsic preference in substrate oxidation. Restoration of energy production was neither at the expense of glucose oxidation, which remained unchanged, nor regulated by malonyl-CoA, the key inhibitor of fatty acid transport into mitochondria, implying that this metabolic change occurred independently of the 'Randle cycle'.³² Since hydroxyacyl-CoA dehydrogenase is the critical rate-limiting enzyme in fatty acid oxidation and its enhanced activity was previously reported to foster mitochondrial respiration and improve contractile function in failing hearts,³³ we determined its activity in sham and infarct-remodelled hearts. However, we were unable to detect increased activity in conditioned remodelled hearts, which clearly exhibited increased fatty acid oxidation, most probably because the assay used measures only enzyme activity under non-physiological conditions. Hence, we employed a protein-independent method using acyl-carnitine profiling and calculated the ratio of C16:1+

mitochondrial function under unloaded resting conditions, we also provide evidence that increased β -oxidation is a cause and not a consequence of the observed improved cardiac function in conditioned remodelled hearts. Our results are to a certain extent in contrast to those of previous studies,^{12,14} but may be related to the unique metabolic profile of infarct-remodelled hearts, which exhibit increased PPAR α -PGC-1 α but reduced and defective HIF-1 α signaling. In the current study, protected sham hearts only showed minor metabolic changes with some recovery of glucose and fatty acid oxidation in response to 15 min of ischaemia, but clearly exhibited reduced uptake of fatty acids into mitochondria. This is consistent with the previous studies from our laboratory with more sustained ischaemia (20 min) using the same experimental model and protocol,⁸ which have demonstrated a significant reduction in fatty acid and a concomitant increase in glucose oxidation in non-remodelled healthy myocardium. We believe that these and the present findings highlight a key concept in cardiac metabolism whereby diseased hearts with reduced or limited oxidative capacity are unable to switch to the 'oxygen-sparing' metabolic programme under metabolic

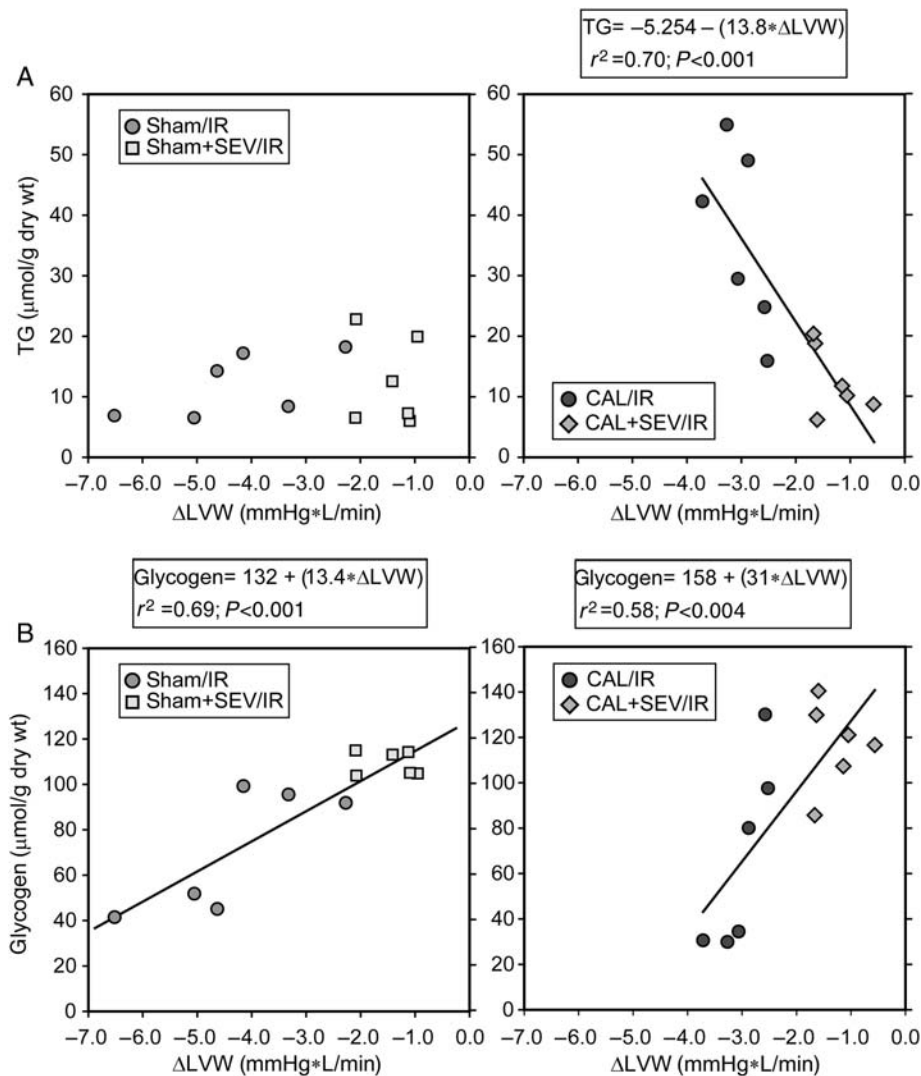


Figure 4 Correlation between triglyceride (TG) and glycogen tissue contents at the end of perfusion with loss in left ventricular work (ΔLVW). (A) TG levels do not correlate in sham hearts but show close inverse correlation with the loss of function in infarct-remodelled hearts. (B) Glycogen levels directly correlate with the loss of function in sham and remodelled hearts. Sham/IR, unprotected sham hearts exposed to ischaemia/reperfusion without treatment. Sham + SEV/IR, protected sham hearts exposed to sevoflurane and ischaemia/reperfusion; CAL/IR, unprotected remodelled hearts exposed to ischaemia/reperfusion alone without treatment; CAL + SEV/IR, protected remodelled hearts exposed to sevoflurane and ischaemia/reperfusion.

C16OH/C14 carnitine species, which inversely correlates with the activity of long-chain hydroxyacyl-CoA dehydrogenase, the initial step of palmitate oxidation.³⁴ Consistent with our data from high-resolution respirometry, these measurements provide evidence for an increased activity of the long-chain hydroxyacyl-CoA dehydrogenase in conditioned vs. non-conditioned remodelled hearts. Sirt3, a mitochondrial NAD⁺-dependent protein deacetylase, was recently reported to activate long-chain hydroxyacyl-CoA dehydrogenase.²² Although we did not observe changes in Sirt3 expression, it is possible that changes in NAD⁺ levels in conditioned remodelled hearts activated Sirt3 and subsequently deacetylated hydroxyacyl-CoA dehydrogenase. Alternatively, since long-chain hydroxyacyl-CoA dehydrogenase activity was increased despite complex I inhibition in conditioned remodelled hearts and inhibition of the respiratory chain should rather decelerate than accelerate β -oxidation,³⁵ we suspect that the observed increased long-chain hydroxyacyl-CoA dehydrogenase activity in conditioned

remodelled hearts might be a direct effect of the conditioning-induced protection on the integrity of the inner mitochondrial membrane,³⁶ where the four enzymes of β -oxidation (acyl-CoA dehydrogenase, enoyl-CoA hydratase, hydroxyacyl-CoA dehydrogenase, and thiolase) form a highly efficient multi-functional complex for fatty acid oxidation.

Myocardial triglycerides form a critical energy reservoir. In fact, fatty acids released by hydrolysis significantly contribute to myocardial ATP generation.³⁷ Conversely, increased triglyceride lipid content ('fatty hearts') was previously found to be associated with cardiac dysfunction.³⁸ In our study, we detected triglyceride accumulation in infarct-remodelled hearts, consistent with previous reports.²⁰ But lipid-loaded infarct-remodelled hearts in our study showed lower HIF-1 α activity compared with sham hearts although most studies linked increased triglyceride accumulation in diseased myocardium to enhanced rather than reduced HIF-1 α activity.²⁰ Nonetheless, lipid accumulation in infarct-remodelled hearts might also occur in a

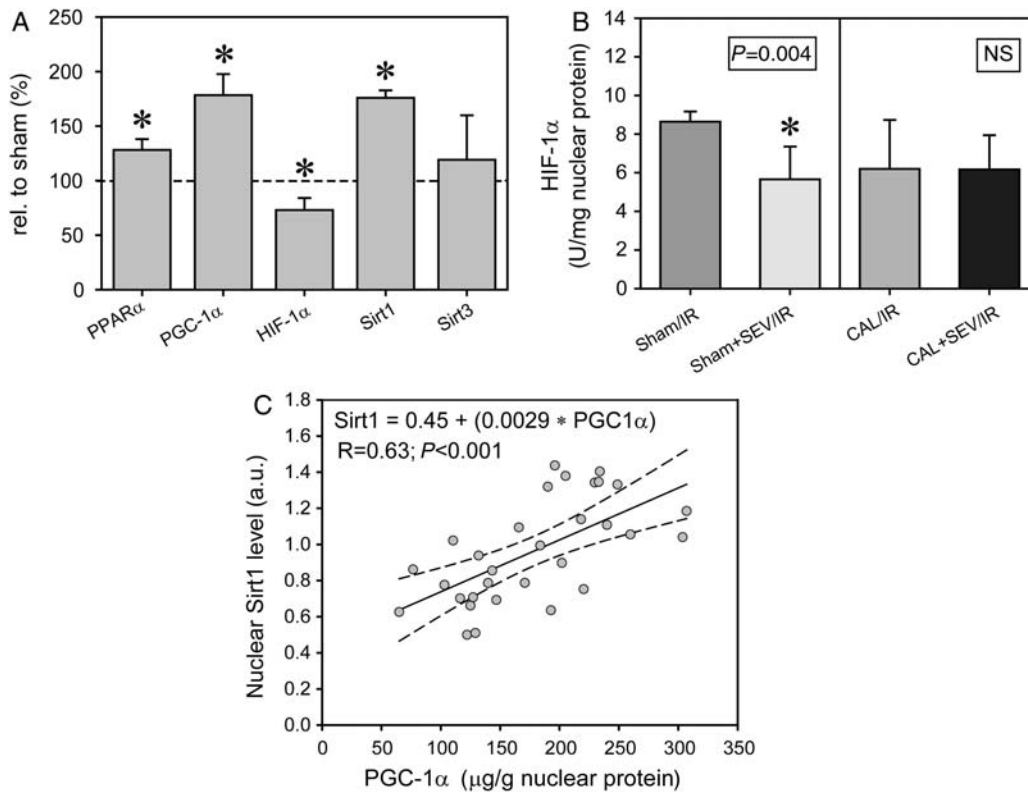


Figure 5 Regulation of energy metabolism in sham and infarct-remodelled hearts. (A) Key regulators in remodelled hearts relative to sham hearts (aerobic). (B) HIF-1 α activity in conditioned and non-conditioned sham and remodelled hearts after ischaemia/reperfusion. (C) Correlation between Sirt1 and PGC-1 α . Data are mean (SD) in (A) and (B). * $P < 0.05$ infarct-remodelled vs. sham hearts (aerobic). Sham/IR, unprotected sham hearts exposed to ischaemia/reperfusion without treatment. Sham + SEV/IR, protected sham hearts exposed to sevoflurane and ischaemia/reperfusion; CAL/IR, unprotected remodelled hearts exposed to ischaemia/reperfusion alone without treatment; CAL + SEV/IR, protected remodelled hearts exposed to sevoflurane and ischaemia/reperfusion.

HIF-1 α -independent manner because of lacking interaction between rarified mitochondria and lipid droplets, potentially reducing fatty acid metabolism. We here report that conditioned remodelled as opposed to sham hearts mobilize these accumulated lipid deposits for energy production under metabolic stress. Interestingly, reduced capacity to recruit triglycerides for oxidation was reported in PGC-1 α ^{-/-} mice,³⁹ and remodelled hearts in our study exhibited markedly increased PPAR α -PGC-1 α levels, suggesting that this metabolic phenotype specifically promotes triglyceride mobilization for energy production.³⁷ A simple calculation reveals that reduction of the triglyceride content by 20 μ mol/g wet weight, as observed in our study, generates 480 μ mol C₂-bodies/g wet weight (assuming that three palmitate molecules per glycerol would be completely metabolized) in addition to the energy provided from exogenous perfusate-borne fatty acids. This amount is substantial and two times higher than what a healthy heart requires under aerobic conditions in our experimental protocol (~240 μ mol C₂-bodies for 30 min of reperfusion). It appears that mobilization of fatty acids from triglyceride stores in conditioned remodelled hearts is a passive stoichiometric process whereby triglyceride hydrolysis is accelerated by reduced product feedback inhibition due to increased mitochondrial β -oxidation in protected vs. unprotected remodelled hearts.⁴⁰ Moreover, our experiments in remodelled hearts perfused with 2.4 mM palmitate suggest that increased substrate availability at least partly compensates for mitochondrial dysfunction and improves post-

ischaemic left ventricular work. Regardless, our results demonstrate a close inverse correlation between loss of function and triglyceride content, implying a causal relationship between the generation of energy from endogenous triglycerides and improved post-ischaemic functional recovery.

Although the majority of studies suggest that fatty acids and fatty acid oxidation are detrimental in the context of ischaemia/reperfusion, some findings support the opposite view.^{15,41–44} Reconciling the results of these studies and our own findings leads to the concept that whenever the heart needs a rapid replenishment of critically depleted energy stores in the presence of apparent (immature or failing heart) or anticipated (second window of protection) limitations in oxidative phosphorylation capacity, as reflected by PGC-1 α up-regulation, myocardial energy metabolism will be restored by boosting fatty acid rather than glucose oxidation. The absence of functional HIF-1 α signalling may further promote this fuel switch from glucose to fatty acid metabolism in the diseased myocardium. Despite its oxygen-wasting effects, fatty acid oxidation has the potential for rapidly normalizing energy production (8 ATP/C-atom). Hence, it will be interesting to test in future experiments whether metabolic support in the form of fatty acid provision may be salutary to limit the ischaemic damage in energy-depleted infarct-remodelled hearts. We believe that our findings will be of relevance when targeting novel anti-ischaemic metabolic therapies to diseased hearts that have lost their metabolic flexibility.

In summary, chronically infarct-remodelled conditioned hearts with limited citric acid cycle capacity and marked deficits in respiratory chain activities secure post-ischaemic energy production by activation of fatty acid and not glucose oxidation and by mobilization of fatty acids from accumulated endogenous triglyceride stores.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Conflict of interest: none declared.

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