Differential Translocation of FAT/CD36 and GLUT4 Coordinates Changes

in Cardiac Substrate Metabolism During Ischemia and Reperfusion

Heather et al: Sarcolemmal Transporter Relocation in Ischemia

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Abstract

Background—Fatty acid and glucose transporters translocate between the sarcolemma and intracellular compartments to acutely regulate substrate metabolism. We hypothesised that, during ischemia, fatty acid translocase (FAT/CD36) would translocate away from the sarcolemma, to limit fatty acid uptake when fatty acid oxidation is inhibited.

Methods and Results—Wistar rat hearts were perfused during pre-ischemia, low-flow ischemia and reperfusion, using ³H-substrates for measurement of metabolic rates, followed by metabolomic analysis and subcellular fractionation. During ischemia, there was a 32% decrease in sarcolemmal FAT/CD36 accompanied by a 95% decrease in fatty acid oxidation rates, with no change in intramyocardial lipids. Concomitantly, the sarcolemmal content of the glucose transporter *GLUT4*, increased by 90% during ischemia, associated with an 86% increase in glycolytic rates, 45% decrease in glycogen content and a 3-fold increase in phosphorylated AMP-activated protein kinase. Following reperfusion, decreased sarcolemmal FAT/CD36 persisted, but fatty acid oxidation rates returned to pre-ischemic levels, resulting in a 35% decrease in myocardial triglyceride content. Elevated sarcolemmal GLUT4 persisted during reperfusion, in contrast, glycolytic rates decreased to 30% of pre-ischemic rates, accompanied by a 5-fold increase in intracellular citrate levels and restoration of glycogen content.

Conclusions—During ischemia FAT/CD36 moved away from the sarcolemma as GLUT4 moved towards the sarcolemma, associated with a shift from fatty acid oxidation to glycolysis, whilst intramyocardial lipid accumulation was prevented. This relocation was maintained during reperfusion, which was associated with replenishing glycogen stores as a priority, occurring at the expense of glycolysis and mediated by an increase in citrate levels.

Key Words: fatty acid metabolism, glucose metabolism, ischemia reperfusion, acute metabolic changes

Fatty acids are the predominant fuel used by the adult heart under aerobic conditions ^{1, 2}, providing ATP via mitochondrial β-oxidation and oxidative phosphorylation. During ischemia, cardiac metabolism must rapidly adapt to the limited oxygen supply, to optimise anaerobic ATP generation in order to ensure cell survival. Ischemia upregulates glucose uptake and glycogenolysis, which increases glycolysis and oxygen-independent ATP generation, accompanied by downregulation of mitochondrial fatty acid oxidation and pyruvate oxidation ^{3, 4}. These acute metabolic changes are facilitated by rapid changes in enzyme activity and protein localisation, and changes may persist during the early reperfusion phase, having a subsequent effect once the oxygen supply is reinstated ⁵. It is important to understand the metabolic changes that occur during ischemia and reperfusion, as these influence the metabolic phenotype that may subsequently develop post-reperfusion.

Substrate transport across the sarcolemma is the initial regulated ^Istep^{an}in^{Liv}cardiomyocyte substrate metabolism. Cardiac glucose uptake is regulated by the glucose transporters, GLUT1 and GLUT4, which can translocate from intracellular vesicles to the sarcolemma to acutely increase glucose uptake ^{6, 7}. In a Heart Failure similar manner, fatty acid uptake occurs predominantly via a protein-mediated process by a family of transporters; fatty acid translocase (FAT/CD36), plasma membrane fatty acid binding protein (FABPpm) and fatty acid transport protein 1 (FATP1)⁸⁻¹⁰. These fatty acid transporters are located at different positions in relation to the sarcolemma: FABPpm is associated with the sarcolemmal extracellular surface, FAT/CD36 lies within the transmembrane region, and FATP1 is associated with the sarcolemmal intracellular surface. Similar to the glucose transporters relocating to regulate glucose uptake, fatty acid transporters can move to the sarcolemma to acutely modify the rate of fatty acid uptake. FAT/CD36 is hypothesised to be the key regulated step in this process ¹¹, translocating to the sarcolemma to increase fatty acid uptake in response to insulin, contraction, and AMP-activated protein kinase (AMPK) phosphorylation and activation ¹²⁻¹⁴. Reverse translocation away from the sarcolemma, to decrease fatty acid uptake, has received far less attention and has not been demonstrated in response to acute physiological stimuli. During ischemia, GLUT4 translocates to the sarcolemma to increase glucose uptake via an AMPK-dependent

mechanism^{7, 15}. However, movement of the fatty acid transporters in response to cardiac ischemia has not been investigated, and their relationship to fatty acid metabolism during reperfusion has not been studied. We hypothesised that acute movement of fatty acid transporters away from the sarcolemma during ischemia would contribute to decreased fatty acid metabolism, in an attempt to match fatty acid uptake to fatty acid utilization and prevent lipid accumulation. Therefore, we investigated the effect of ischemia and reperfusion on fatty acid and glucose transporter subcellular localisation and the downstream effects on glucose and fatty acid utilization and storage in the isolated perfused heart. In addition, we used metabolomic profiling to identify changes in metabolic intermediates, which may accumulate or become depleted, due to changes in flux through their respective metabolic pathways during cardiac ischemia and reperfusion.

Methods

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Isolated heart perfusion

Male Wistar rats (n = 50, 291 \pm 5 g) were obtained from a commercial breeder (Harlan, UK). All procedures were in accordance with Home Office (UK) guidelines under The Animals (Scientific Procedures) Act, 1986, following approval by the Institutional Review Board and in accordance with institutional guidelines. Following terminal anaesthesia with sodium pentobarbital, hearts were rapidly excised and placed in ice-cold Krebs-Henseleit (KH) buffer. Hearts were cannulated via the aorta and perfused in Langendorff mode at a constant perfusion pressure of 100 mmHg at 37 °C. Hearts were perfused with 200 ml recirculating KH buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO₄, 1.3 mM CaCl₂, 0.5 mM EDTA, 25 mM NaHCO₃, 1.2 mM KH₂PO₄, pH 7.4) containing 11 mM glucose and 0.4 mM palmitate (bound to 1.5% fatty acid-free bovine serum albumin), gassed with 95% O₂ and 5% CO₂¹⁶

To measure functional changes during the perfusion protocol, a fluid filled, PVC balloon was inserted into the left ventricle, inflated to achieve an end-diastolic pressure of 4 mmHg, and attached via a polyethylene tube to a bridge amplifier and PowerLab data acquisition system (ADInstruments, Oxfordshire, UK). Left ventricular developed pressure was determined as systolic pressure minus end-diastolic pressure. Rate pressure product (RPP) was calculated as the product of developed pressure and heart rate.

Perfusion protocol

Hearts were initially perfused under aerobic conditions for 30 min, termed "pre-ischemia", with typical flow rates of 16 ml/min/gww ¹⁶. Hearts subsequently underwent 30 min of low-flow ischemia at a constant flow rate of 0.4 ml/min/gww, followed by 30 min of aerobic reperfusion under conditions identical to pre-ischemia. Hearts were perfused with ³H-labelled substrates for measurement of metabolic rates throughout pre-ischemia, ischemia and reperfusion. A separate group of hearts were perfused without radiolabel and were freeze clamped on the cannula using Wollenberger clamps pre-chilled in liquid nitrogen, at the end of pre-ischemia, at the end of ischemia, or at the end of reperfusion.

Measurement of metabolic rates



For measurement of palmitate oxidation rates, 0.2 μ Ci.ml⁻¹[9,10-³H] palmitate was bound to albumin in the recirculating KH buffer. For measurement of glycolytic rates, a separate group of hearts was perfused with KH buffer supplemented with 0.2 μ Ci.ml⁻¹[5-³H]-glucose ¹⁷. Timed aliquots of perfusate were collected during the perfusion protocol. Palmitate oxidation rates were determined from the conversion of ³H-palmitate to ³H₂O and glycolytic rates were determined from the conversion of ³H-glucose to ³H₂O in the timed aliquots ¹⁸. Lactate efflux was measured in aliquots using lactate dehydrogenase, as described ¹⁹. Steady state metabolic rates were calculated from the linear increase in ³H₂O or lactate.

Metabolomics

Metabolites from cardiac tissue were extracted using a methanol/chloroform/water extraction procedure ²⁰. Tissue was pulverised in methanol/chloroform (2:1 v/v) solution, water and chloroform were added, the resulting aqueous and organic layers were separated and the organic layer was dried. Aqueous samples were derivatised using the procedure reported by Gullberg *et al* ²¹. Methoxyamine hydrochloride (20 mg/ml in pyridine) was added to a quarter of the total aqueous fraction. The samples were derivatised at room temperature for 17 h, followed by silylation with 30 μ l of N-methyl-N-trimethylsilyltrifluoroacetamide for 1

h. The derivatised samples were diluted (1:10) with hexane prior to Gas Chromatography-Mass Spectrometry (GC-MS) analysis. One quarter of the total organic-phase metabolites were derivatised by acid-catalysed esterification ²². Lipids were dissolved in chloroform/methanol (1:1 v/v). An aliquot of BF3/methanol was added and the vials were incubated at 80 °C for 90 min. Once cool, 0.3 ml H₂O and 0.6 ml hexane were added to each vial, and the organic layer was isolated and evaporated to dryness before reconstitution in 1 ml hexane for analysis.

The derivatised aqueous samples were injected into a Thermo Electron Trace GC Ultra equipped with a 30 m x 0.25 mm ID 5% phenyl polysilphenylene-siloxane column with a chemically bonded 25 mm TR-5MS stationary phase (Thermo Electron Corporation; injector temperature 220 °C, helium carrier gas flow rate 1.2 ml/min). The initial column temperature was 70 °C which was held for 2 min, then increased by 5 °C/min up to a temperature of 230 °C, and then increased at a rate of 201°C/min to 310 °C. The derivatised organic metabolites were injected onto a ZB-WAX column (30 m x 0.25 mm ID; 100% polyethylene glycol). The initial column temperature was 60 °C; this was held for 2 min and then increased by 10 °C/min Heart Failure to 150 °C and then by 4 °C/min up to a temperature of 230 °C where it was held for 7 min. The column eluent was introduced into a DSQ quadrupole mass spectrometer (Thermo Electron Corporation) (transfer line temperature 310 °C for aqueous metabolites and 230 °C for lipid metabolites, ion source temperature 250 °C, electron beam 70 eV). The detector was turned on after a solvent delay of 240 s and data were collected in full-scan mode using three scans per second across a mass range of 50-650 m/z. Data were normalised to internal standard or total integral area, corrected for tissue weight, and expressed relative to the pre-ischemic control group. In total 75 metabolites were identified and analysed (Supplementary Tables 1 and 2 for list of aqueous metabolites).

For specific analysis of diacylglycerols, chloroform:methanol lipid extracts were further diluted in isopropanol:acetonitrile:water (2:1:1) containing 20 μ M C17 phosphatidylcholine. Mass spectra were collected in positive and negative mode using a Xevo QToF mass spectrometer (MS) interfaced with an ultra performance liquid chromatography (UPLC) unit. Spectra were acquired from 50 *m/z* to 1200 *m/z* with

a capillary voltage and cone voltage of 3.0kV and 30V for positive ion mode and 2.5kV and 25V for negative ion mode, respectively. Lipids were separated using a CSH C18 column, with mobile phases consisting of A: 10mM ammonium formate in 3:2 acetonitile:water and B: 10mM ammonium formate in 9:1 *iso*-propanol: acetonitrile. Lipid species were identified by MS/MS and reference to on-line databases. Data was imported into SIMCA software for processing using multivariate statistics including principal components analysis (PCA), partial least squares discriminate analysis (PLS-DA) and orthogonal partial least squares discriminate analysis (PLS-DA). Diacylglycerides were identified by a combination of retention time and mass matching, with a dataset created from features detected between 9-11 min retention time and 568.51-650.53 amu.

Subcellular fractionation

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Separation of the sarcolemmal membrane fraction from intracellular endosomes (low-density microsomal fraction) was carried out according to the established method of Luiken *et al* ¹³. Briefly, cardiac tissue was incubated in a high salt solution (20 mM HEPES, 2 M NaCl and 5 mM NaN₃), followed by centrifugation, resuspension in fractionation buffer (20 mM HEPES, 250 mM sucrose, 2 mM EDTA, 1 mM MgCl₂, 5 mM NaN₃) and homogenisation in a glass hand-held homogeniser. Differential centrifugation was used to separate the membrane fractions, and, as described previously, sarcolemmal fractions had 3 fold enrichment of Na⁺K⁺-ATPase protein levels, whereas microsomal fractions had 9-fold enrichment of Rab4 protein levels ¹⁹.

Tissue analysis

The glycogen content of cardiac tissue was determined by the breakdown of glycogen to glucose units, using amyloglucosidase. Glycogenolysis and glycogenesis rates were calculated by taking the difference in glycogen content at the end of each time period, and dividing this by the time between samples ²³. The active fraction of pyruvate dehydrogenase was assayed according to the protocol of Seymour *et al* ²⁴. Myocardial triglyceride content was measured in crushed tissue, following Folch extraction, using a kit from Randox, UK.

Western blotting

Equal concentrations of protein were loaded and separated on 12.5% SDS-PAGE gels and transferred onto immobilon-p membranes (Millipore, UK) ¹⁶. FAT/CD36 was detected with an antibody kindly donated by Dr Narendra Tandon (Otsuka Maryland Medicinal Laboratories, USA). FABPpm was detected using an antibody kindly donated by Dr Jorge Calles-Escandon (Wake Forest University School of Medicine, USA). Prof. Geoff Holman (University of Bath, UK) kindly donated the GLUT4 antibody. Antibodies against GLUT1 and FATP1 were purchased from Abcam, UK and Santa Cruz, USA, respectively. Antibodies raised against AMPK and phospho-AMPK (Thr172) were purchased from Cell Signalling, USA. Even protein loading and transfer were confirmed by Ponceau staining, and protein levels were related to internal standards to ensure homogeneity between gels.

Statistical analysis



Results are presented as means \pm SEM, and were considered significant at p < 0.05 (PASW Statistics 18). Metabolic rates and cardiac function were measured in the same heart under the three conditions, and were analysed using repeated measures general linear model with Bonferroni correction. All other data were from separate hearts collected at either the end of pre-ischemia, end of ischemia or end of reperfusion, and were analysed using an un-paired one way ANOVA with Tukey correction. Exact p-values are given in Supplementary Table 3.

Results

Cardiac function

Cardiac function was stable throughout the 30 min pre-ischemic period of aerobic perfusion. During lowflow ischemia, left ventricular developed pressure and heart rate dropped to 16% and 10% of pre-ischemic values, respectively, but recovered to pre-ischemic levels during reperfusion (Table 1). Despite the 98% decrease in rate pressure product (RPP) during ischemia, RPP returned to 86% of initial values during reperfusion. Thus, in agreement with previous studies, this perfusion protocol of 30 min of mild low-flow ischemia did not impair functional recovery of the heart, therefore, metabolic changes during reperfusion were unlikely to be due to decreased contractile function ²⁵.

Fatty acid metabolism

Fatty acids are the predominant substrate used by the heart to fuel contraction under aerobic conditions ^{1, 2}. During ischemia, palmitate oxidation rates decreased by 95% compared with pre-ischemic aerobic rates (Figure 1). During reperfusion, palmitate oxidation rates fully recovered to pre-ischemic rates, in line with contractile function. Fatty acids taken up by the cardiomyocyte can either be oxidised or stored, therefore, measurement of both pathways is necessary to give an indirect assessment of fatty acid uptake ^{16, 26}. The large decrease in fatty acid oxidation during ischemia was not accompanied by a change in myocardial triglyceride content. In contrast, the recovery of fatty acid oxidation during reperfusion was associated with a 35% decrease in myocardial triglyceride content. The total fatty acid pool in these hearts was assessed by GC-MS. There were no significant differences in the individual fatty acids or the fatty acid classes between pre-ischemic, ischemic and reperfused hearts (Table 2). Similarly, diacylglyerides were detected but Heart Failure multivariate models revealed that no discrimination was achievable between the three perfusion conditions (Supplementary Figure 1). Thus, during ischemia, the decrease in fatty acid oxidation was not accompanied by an accumulation of myocardial lipids, suggesting that fatty acid uptake decreased in line with oxidation. In contrast, the full recovery of fatty acid oxidation rates during reperfusion occurred at the expense of the myocardial triglyceride stores, indicating that exogenous fatty acid uptake rates were not sufficient to fulfil oxidation rates in the reperfused heart.

Fatty acid transporters in ischemia/reperfusion

Fatty acid uptake can be acutely regulated by translocating fatty acid transporters between the sarcolemma and intracellular membrane compartments (endosomes) ¹³. During ischemia, sarcolemmal FAT/CD36 protein levels decreased by 32% compared with pre-ischemia due to movement of FAT/CD36 to the endosomes, as shown by the increase in FAT/CD36 in the microsomal fraction (Figure 2). During reperfusion, this relocation away from the sarcolemma was maintained, with sarcolemmal FAT/CD36

content remaining significantly decreased compared with pre-ischemia. In contract, the subcellular location of the other fatty acid transporters, FABPpm and FATP1, did not change following low-flow ischemia and reperfusion. There was no change in total FAT/CD36 protein levels under the different perfusion conditions (Supplementary Figure 2). Thus, this selective relocation of FAT/CD36 away from the sarcolemma would decrease the transporter-mediated capacity for fatty acid uptake, likely contributing to the changes in fatty acid metabolism during ischemia and reperfusion.

Glucose transporters in ischemia/reperfusion

In contrast to the fatty acid transporters, movement of the glucose transporters showed the opposite profile (Figure 3). Sarcolemmal GLUT4 content increased by 90% during ischemia due to movement of the transporters away from the endosomal stores, as shown by the decrease in GLUT4 in the microsomal fraction. During reperfusion, GLUT4 remained significantly increased at the sarcolemma compared with pre-ischemia. Changes in the sarcolemmal content of GLUT4 occurred independent of changes in total GLUT4 in the heart (Supplementary Figure 2). GLUT1 localisation did not change in the isolated perfused hearts during 30 min of low-flow ischemia or subsequent reperfusion (Figure 3).

Glucose metabolism

Glycolytic rates under aerobic conditions were 0.27 µmol/min/gww, and during low-flow ischemia increased by 86% compared with pre-ischemic rates (Figure 4). During reperfusion, glycolytic rates did not return to pre-ischemic levels, but instead significantly decreased to only 30% of pre-ischemic rates. Glycolytically-derived pyruvate can be reduced to lactate, transaminated to alanine or oxidised in the mitochondria by pyruvate dehydrogenase. During ischemia, net lactate efflux rates increased 3-fold compared with pre-ischemic rates, and during reperfusion decreased to significantly lower than pre-ischemic levels, mirroring the changes in glycolytic rates. Intracellular lactate and alanine levels, as determined by metabolomic analysis of cardiac tissue, increased 7-fold and 4-fold, respectively, during ischemia. Pyruvate dehydrogenase activity did not change during ischemia, indicating that mitochondrial oxidation was no restricted at the level of pyruvate decarboxylation. However, pyruvate dehydrogenase

activity was decreased by 63% in reperfused hearts compared with ischemic hearts, concomitant with the decrease in glycolysis and lactate efflux during reperfusion. Thus, GLUT4 translocation to the sarcolemma was associated with increased glycolysis during ischemia, but not during reperfusion.

Glycogen metabolism

As glycolysis was decreased during reperfusion, despite the increased sarcolemmal GLUT4, we investigated whether glucose was being diverted into glycogen storage. Intracellular glucose levels decreased by 33% during ischemia and returned to pre-ischemic levels during reperfusion, indicating that glucose uptake was maintained despite the decrease in glycolysis (Figure 5). By the end of 30 min of low-flow ischemia, myocardial glycogen content had decreased by 45% compared with pre-ischemia. The decrease of 4.15 µmol glucosyl units per gww during ischemia was equivalent to a net rate of glycogenolysis of 0.138 Association ²³. During 30 min of reperfusion, glycogen content increased ²² fold, back to pre-ischemic levels, resulting in a net rate of glycogenesis of 0.17 µmol/gww/min. Thus, during reperfusion, the increased sarcolemmal GLUT4 content was associated with increased glycogenesis and replenishment of **HEATH Failure**

Metabolic intermediates and regulation

Total protein levels of AMPK did not differ between groups (Figure 5). However, phosphorylated AMPK (p-AMPK) was 3-fold higher in ischemic hearts, concomitant with the translocation of GLUT4 to the sarcolemma, increased glycolytic rates and increased glycogenolysis. In contrast, after 30 minutes of reperfusion, p-AMPK returned to pre-ischemic levels.

Using metabolomic analysis, Krebs cycle intermediates were measured in cardiac tissue from the end of preischemia, low-flow ischemia and reperfusion (Figure 6 and Supplementary Tables 1 and 2). During ischemia, citrate levels decreased by 59% compared with pre-ischemia. Succinate levels increased 5-fold during ischemia, however, levels of fumarate, the next metabolite in the cycle, malate and cis-aconitate were unchanged. The accumulation of succinate relative to the unchanged subsequent metabolites indicated decreased flux through succinate dehydrogenase during ischemia. During reperfusion, citrate levels increased nearly 2-fold compared with pre-ischemia. Citrate is a potent inhibitor of phosphofructokinase activity and hence glycolysis, therefore, likely contributed to the redirection of glucose away from glycolysis and towards glycogenesis at reperfusion. In addition, cis-aconitate levels were increased by 83% compared with pre-ischemia, mirroring the changes in its precursor citrate. However, levels of succinate, fumarate and malate were not significantly different following reperfusion. Citrate and cis-aconitate are metabolites prior to two dehydrogenase reactions (isocitrate and α -ketoglutarate dehydrogenase), and accumulation of these two metabolites relative to the unchanged subsequent metabolites indicates decreased flux through the Krebs cycle at the level of these enzymes. Thus, reduced flux through the dehydrogenase reactions would account for the increased citrate levels, the inhibition of glycolysis and preferential replenishment of glycogen during reperfusion.

We are the first to demonstrate FAT/CD36 relocation away from the sarcolemma, occurring in response to **Heart Failure** cardiac ischemia. In this study we show that the ischemia-induced movement of FAT/CD36 away from the sarcolemma occurs concomitantly with the translocation of GLUT4 to the sarcolemma, demonstrating divergent regulation of these two transporters. The subcellular reorganisation of substrate transporters in ischemia was associated with an acute shift from fatty acid oxidation to more oxygen-efficient glycolysis, in the absence of cellular lipid overload. During reperfusion, the decreased sarcolemmal FAT/CD36 was associated with depletion of triglycerides, as fatty acid oxidation rates were restored to pre-ischemic levels. Concomitantly, the increased sarcolemmal GLUT4 facilitated the repletion of glycogen, associated with a citrate-mediated inhibition of glycolysis.

Discussion

FAT/CD36 is the predominant regulatory site for fatty acid uptake, with strong positive relationships between sarcolemmal FAT/CD36 content and sarcolemmal fatty acid uptake rates ^{27, 28}. The relocation of FAT/CD36 away from the sarcolemma during ischemia and early reperfusion would be expected to directly decrease the rate of and capacity for fatty acid uptake, and subsequently decrease fatty acid utilization by the

heart. Nickerson *et al* demonstrated that FAT/CD36 has far greater transporter efficiency than the other fatty acid transporters ²⁹, and our findings in ischemia demonstrate a FAT/CD36-specific relocation, with no movement of the other fatty acid transporters FABPpm and FATP1 under the same conditions. Thus, the changes in sarcolemmal fatty acid uptake capacity in ischemia are likely a FAT/CD36-specific event.

The ischemia-induced near cessation of fatty acid oxidation was accompanied by only a 32% decrease in sarcolemmal FAT/CD36, which at first appearance seems a small change to be associated with such a large decrease in oxidation. However, in ischemia there are two factors that have to be considered, not only the decrease in FAT/CD36 but also the cessation of contraction, the primary consumer of the fatty acid-derived ATP. Thus, in ischemia, it may be that the combined contribution of decreased sarcolemmal FAT/CD36 and decreased contraction that would decrease overall fatty acid uptake_{n H}If_T considered in light of the reperfusion findings, in which contraction and fatty acid oxidation are normalised, whereas FAT/CD36 and triglycerides are reduced by 32% and 35%, respectively, these results may more closely elucidate the independent contribution of FAT/CD36 relocation to changes in fatty acid metabolism.

Decreased fatty acid oxidation during ischemia was not associated with a change in intramyocardial triglycerides or free fatty acids, indicating that fatty acid uptake likely decreased with ischemia. This is in line with a study showing decreased fatty acid uptake in an ischemic open chest swine model ³⁰. It has been proposed for many years that the primary destination of exogenous fatty acids following uptake is the myocardial triglyceride pool, that fatty acids transported from the circulation are first esterified into triglycerides, then hydrolysed from this intermediary lipid pool for oxidation in the mitochondria when required ³¹. This would explain our observations at reperfusion, that, despite decreased sarcolemmal FAT/CD36, fatty acid oxidation rates were restored, occurring at the expense of intramyocardial triglycerides. Studies by Saddik *et al*, using pulse-chase perfusion, showed that triglyceride lipolysis rates were not different between reperfusion and pre-ischemia ³², and that rates of exogenous fatty acid oxidation also did not differ between the two ^{32, 33}. Therefore, at reperfusion, the decreased triglycerides were most

likely due to decreased esterification of fatty acids into triglyceride, as a consequence of reduced FAT/CD36-mediated uptake of fatty acids across the sarcolemma.

The mechanism underpinning the relocation of FAT/CD36 away from the sarcolemma is currently unknown. Whereas many signalling pathways have been shown to induce movement to the sarcolemma, to the authors best knowledge we are the first to show movement away. Forward transport to the sarcolemma and increased fatty acid uptake can be induced by AMPK, fatty acids, reactive oxygen species, insulin and contraction ^{13, 14, 34, 35}, therefore, these can all be ruled out as mediating our changes in ischemia, and contraction can be ruled out at reperfusion. The location of FAT/CD36 is determined by the balance between forward movement to and reverse movement away from the sarcolemma ³⁶, thus it could be that either pathway is affected in ischemia. A potential mechanism that may explain our findings is related to pH changes during ischemia. Steinbusch *et al* showed that increasing the pH in the endosomes induced translocation of FAT/CD36 to the sarcolemma ³⁷, therefore, it is possible that the ischemia-induced decrease in pH due to lactate accumulation may decrease trafficking to the sarcolemma, resulting in an overall **HEAT FAILURE**

From a physiological point of view, having an ischemia-induced mechanism to restrict cytosolic fatty acid accumulation would be advantageous, as high intracellular concentrations of fatty acids decrease functional recovery of the ischemic heart during reperfusion ³⁸. Thus, the decrease in sarcolemmal FAT/CD36 likely occurs to limit fatty acid uptake under conditions in which there is insufficient oxygen available for oxidation, thereby protecting against lipid accumulation and contractile impairment. This would be even more significant *in vivo*, as the ischemia-induced catecholamine surge would induce adipose lipolysis and elevate plasma free fatty acid concentrations ³⁹, thereby increasing the fatty acid concentration gradient across the sarcolemma.

During ischemia the majority of glycolytic flux is from exogenous glucose ⁴⁰, facilitated by translocation of GLUT4 to the sarcolemma ⁷, mediated by AMPK-dependent mechanisms ¹⁵. This suggests that, during ischemia, not only are GLUT4 and FAT/CD36 travelling in different directions relative to the sarcolemma,

but they are also regulated by different signalling pathways. The increased sarcolemmal GLUT4 ensured that sufficient intracellular substrate was available for ATP generation, and was independent of a change in GLUT1 localisation. Using pulse-chase experiments, Wambolt *et al* demonstrated that glycogen-derived glycolytic rates accounted for 29% of the total glycolytic rate during low-flow ischemia, ⁴⁰, which was comparable to our 27% net rate of glycogenolysis in ischemic hearts. Glycogen turnover is regulated by the activity of both glycogen phosphorylase and glycogen synthase, with glycogen phosphorylase activity increasing during ischemia and glycogen synthase activity increasing during reperfusion, glucose uptake is not primarily for energy generation, but for restoration of glycogen stores ^{41, 42}. It has been shown that if insufficient glycogen is available to fuel glycolysis during ischemia then functional recovery of the heart is Association and increasing the severity of contracture ^{43, 44}. Thus, replenishing glycogen would be a priority for the heart during reperfusion, to optimise cell survival if a second ischemic period should follow.

Metabolomic analysis of ischemic and reperfused hearts allowed identification of multiple modified metabolites and indicated where flux through specific enzymes was modified. The ischemia-induced increase in glycolytic flux was likely mediated by two complimentary factors converging at the same enzyme; AMPK activation of phosphofructokinase 2 activity ⁴⁵, assisted by a decrease in citrate levels, a potent inhibitor of phosphofructokinase activity ⁴⁶. In contrast, during reperfusion the large increase in citrate would be expected to inhibit phosphofructokinase activity, accounting for the decrease in glycolysis and diversion of glucose-6-phosphate into the glycogen pool. Thus, changes in citrate levels are likely to be a key signal behind the catabolic to anabolic shift in glucose metabolism that occurs during reperfusion. Measuring the relative changes in Krebs cycle intermediates indicated changes in flux through specific enzymes, and pointed towards reduced flux through succinate dehydrogenase during ischemia and through isocitrate and α-ketoglutarate dehydrogenase during reperfusion. These enzymes are key regulated steps in

the Krebs cycle, with their activity influenced by the redox state of the mitochondria, and likely underpin the metabolic switch between pathways that occurs during ischemia and reperfusion.

In conclusion, during ischemia FAT/CD36 moved away as GLUT4 moved towards the sarcolemma, associated with a downregulation of fatty acid oxidation and an increase in glycolysis. During reperfusion, the substrate transporters remained in their ischemic location, which, in the case of GLUT4, was associated with replenishing glycogen stores at the expense of glycolysis, mediated by increased citrate levels. In contrast, restoration of fatty acid oxidation rates during reperfusion occurred despite decreased sarcolemmal FAT/CD36, associated with depletion of triglyceride stores. Thus, sarcolemmal translocation of the fatty acid transporters FAT/CD36 contributed to the acute cardiac metabolic remodelling that occurs during ischemia and reperfusion.



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Disclosures

None.

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	Pre-ischemia	Ischemia	Reperfusion
Developed pressure (mmHg)	112 ± 8	18 ± 4 *	100 ± 9 †
Heart rate (bpm)	262 ± 10	26 ± 17 *	252 ± 7 †
Rate pressure product (mmHg/min x 10 ³)	29.3 ± 1.9	0.7±0.4 *	25.3 ± 2.5 †

Table 1. Cardiac function in isolated perfused hearts during pre-ischemia, low-flow ischemia and reperfusion

* p = 0.000 vs. pre-ischemia, † p = 0.000 vs. low-flow ischemia. n = 8 per group



Pre-ischemia	Ischemia	Reperfusion
1.0 ± 0.2	1.3 ± 0.2	1.1 ± 0.2
1.0 ± 0.3	1.2 ± 0.2	0.9 ± 0.3
1.0 ± 0.1	1.3 ± 0.2	1.1 ± 0.1
1.0 ± 0.3	1.1 ± 0.2	0.9 ± 0.2
1.0 ± 0.2	1.7 ± 0.6	1.4 ± 0.7
1.0 ± 0.1	1.3 ± 0.2	1.1 ± 0.1
1.0 ± 0.1	1.5 ± 0.3	1.3 ± 0.1
	А	Merican Heart Association
1.0 ± 0.2	1.3 ± 0.2	Learn and Live. 1.1 ± 0.1
1.0 ± 0.2	1.3 ± 0.2	1:1 ± 0.1
1.0 ± 0.2	1.2 ± 0.3	1.1 ± 0.3
1.0 ± 0.2	Fail.3±0.2	1.1 ± 0.2
l of1 :0 ± 0.3eri	can H <u>F.3f±0,2</u> sc	0.9 ± 0.3
1.0 ± 0.1	1.3 ± 0.3	1.2 ± 0.1
1.0 ± 0.1	1.4 ± 0.3	1.3 ± 0.1
1.0 ± 0.3	1.2 ± 0.2	1.0 ± 0.1
	Pre-ischemia 1.0 ± 0.2 1.0 ± 0.3 1.0 ± 0.1 1.0 ± 0.3 1.0 ± 0.2 1.0 ± 0.1 1.0 ± 0.1 1.0 ± 0.1 1.0 ± 0.2 1.0 ± 0.3 EVALUATE: 100 =	Pre-ischemiaIschemia 1.0 ± 0.2 1.3 ± 0.2 1.0 ± 0.3 1.2 ± 0.2 1.0 ± 0.1 1.3 ± 0.2 1.0 ± 0.3 1.1 ± 0.2 1.0 ± 0.2 1.7 ± 0.6 1.0 ± 0.1 1.3 ± 0.2 1.0 ± 0.1 1.5 ± 0.3 1.0 ± 0.2 1.3 ± 0.2 1.0 ± 0.1 1.3 ± 0.3 1.0 ± 0.1 1.4 ± 0.3 1.0 ± 0.3 1.2 ± 0.2

Table 2. Metabolomic profile of predominant fatty acids and fatty acid classes extracted from pre-ischemic, ischemic and reperfused hearts.

Changes are expressed relative to the pre-ischemic values, n = 5 per group

Figure Legends

Figure 1. Fatty acid oxidation rates and myocardial triglyceride concentrations in isolated perfused hearts following pre-ischemia, low-flow ischemia and reperfusion. * p < 0.05 vs. pre-ischemia, † p < 0.05 vs. ischemia. For fatty acid oxidation rates, n = 6 per group, and for myocardial triglycerides, n = 5 per group.

Figure 2. Sarcolemmal and microsomal FAT/CD36, FABPpm and FATP1 localisation, in isolated perfused hearts following pre-ischemia, low-flow ischemia and reperfusion. * p < 0.05 vs. pre-ischemia. For pre-ischemia, n = 6 per group, and for ischemia and reperfusion, n = 5 per group.

Figure 3. Sarcolemmal and microsomal GLUT4 and GLUT1 localisation, on isolated perfused hearts *Learn and Live*. following pre-ischemia, low-flow ischemia and reperfusion. * p < 0.05 vs. pre-ischemia, n = 5 per group.

Figure 4. Glycolytic rates, lactate efflux rates, intracellular lactate, intracellular alanine and pyruvate dehydrogenase activity in isolated perfused hearts following pre-ischemia, low-flow ischemia and reperfusion. * p < 0.05 vs. pre-ischemia, † p < 0.05 vs. ischemia, n = 5 per group.

Figure 5. Intracellular glucose levels, glycogen content and phosphorylated and total AMPK protein levels in isolated perfused hearts following pre-ischemia, low-flow ischemia and reperfusion. * p < 0.05 vs. pre-ischemia, † p < 0.05 vs. ischemia, n = 5 per group.

Figure 6. Myocardial levels of citrate, cis-aconitate, succinate, fumarate, and malate in isolated perfused hearts following pre-ischemia, low-flow ischemia and reperfusion. * p < 0.05 vs. pre-ischemia, † p < 0.05 vs. pre-ischemia, † p < 0.05 vs. ischemia, n = 5 per group.



Figure 2















Differential Translocation of FAT/CD36 and GLUT4 Coordinates Changes in Cardiac Substrate Metabolism During Ischemia and Reperfusion

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SUPPLEMENTAL MATERIAL

	Pre-ischemia	Ischemia	Reperfusion
Adenine	1.00 ± 0.13	1.32 ± 0.22	0.83 ± 0.25
Butanoate	1.00 ± 0.13	$2.50 \pm 0.47 *$	1.25 ± 0.20 †
Cis-aconitate	1.00 ± 0.18	0.59 ± 0.12	1.83 ± 0.35 *†
Citrate	1.00 ± 0.07	0.41 ± 0.07 *	1.98 ± 0.11 *†
Creatine	1.00 ± 0.15	1.48 ± 0.11	1.23 ± 0.38
Fructose	1.00 ± 0.14	0.82 ± 0.28	3.93 ± 1.27 *†
Fumarate	1.00 ± 0.25	1.36 ± 0.05	2.12 ± 0.67
Glucose	1.00 ± 0.06	$0.67 \pm 0.06 *$	0.99 ± 0.10 †
Glutaric acid	1.00 ± 0.29	0.38 ± 0.08	2.79 ± 1.08 †
Glyceric acid	1.00 ± 0.06	0.87 ± 0.17	0.97 ± 0.14
Glycerophosphoric acid	1.00 ± 0.23	9.25 ± 1.49 *	1.46 ± 0.14 †
Lactate	1.00 ± 0.19	6.95 ± 1.47 *	0.99 ± 0.13 †
Malate	1.00 ± 0.22	1.30 ± 0.14	2.10 ± 0.58
Nicotinamide	1.00 ± 0.11	1.08 ± 0.03	1.17 ± 0.12
Ornithine	1.00 ± 0.22	0.79 ± 0.20	0.87 ± 0.15
2-oxo-proline	1.00 ± 0.10	0.83 ± 0.05	0.90 ± 0.06
Pantothenic acid	1.00 ± 0.19	1.15 ± 0.34	1.27 ± 0.29
Propanoate	1.00 ± 0.28	0.79 ± 0.10	2.06 ± 0.84
Succinate	1.00 ± 0.24	5.65 ± 1.30 *	1.41 ± 0.46 †
Threonate	1.00 ± 0.25	1.13 ± 0.31	0.84 ± 0.26

Supplementary Table 1. Aqueous metabolites from isolated perfused hearts following preischemia, ischemia and reperfusion

* p < 0.05 vs. pre-ischemia, † p < 0.05 vs. low-flow ischemia. n = 5 per group

,	1		
	Pre-ischemia	Ischemia	Reperfusion
Alanine	1.00 ± 0.11	3.67 ± 0.51 *	0.90 ± 0.07 †
Glycine	1.00 ± 0.05	1.27 ± 0.07	1.08 ± 0.12
Valine	1.00 ± 0.05	3.31 ± 0.19 *	1.13 ± 0.05 †
Leucine	1.00 ± 0.04	3.47 ± 0.17 *	1.01 ± 0.04 †
Proline	1.00 ± 0.06	2.87 ± 0.17 *	1.30 ± 0.09 †
Serine	1.00 ± 0.06	1.79 ± 0.23 *	1.63 ± 0.11 *
Threonine	1.00 ± 0.09	1.51 ± 0.11 *	1.51 ± 0.10 *
Aspartate	1.00 ± 0.09	2.30 ± 0.63 *	1.02 ± 0.17 †
Phenylalanine	1.00 ± 0.28	1.78 ± 0.51	1.33 ± 0.10
Glutamate	1.00 ± 0.09	0.75 ± 0.08	0.97 ± 0.10
Glutamine	1.00 ± 0.13	1.88 ± 1.11	1.35 ± 0.32
Lysine	1.00 ± 0.18	1.61 ± 0.24	2.04 ± 0.13 *

Supplementary Table 2. Aqueous metabolites from isolated perfused hearts following preischemia, ischemia and reperfusion

* p < 0.05 vs. pre-ischemia, † p < 0.05 vs. low-flow ischemia. n = 5 per group

Figure	Data	Ischemia vs. pre-ischemia	Reperfusion vs. pre-ischemia	Reperfusion vs. ischemia
Figure 1	Palmitate oxidation rates	Ischemia vs. pre-ischemia, p = 0.001		Reperfusion vs.ischemia, p = 0.002
	Myocardial triglycerides			Reperfusion vs. ischemia, p = 0.046
Figure 2	FAT/CD36	Ischemia vs. pre-ischemia, p = 0.015	Reperfusion vs. pre-ischemia, p = 0.011	
Figure 3	GLUT4	Ischemia vs. pre-ischemia, p = 0.001	Reperfusion vs. pre-ischemia, p = 0.014	
Figure 4	Glycolytic rates	Ischemia vs. pre-ischemia, p = 0.015	Reperfusion vs. pre-ischemia, p = 0.01	Reperfusion vs. ischemia, p = 0.003
	Lactate efflux rates	Ischemia vs. pre-ischemia, p = 0.000	Reperfusion vs. pre-ischemia, p = 0.000	Reperfusion vs. ischemia, p = 0.003
	Intracellular lactate	Ischemia vs. pre-ischemia, p = 0.000		Reperfusion vs. ischemia, p = 0.000
	Intracellular alanine	Ischemia vs. pre-ischemia, p = 0.000		Reperfusion vs. ischemia, p = 0.000
	Pyruvate dehydrogenase activity			Reperfusion vs. ischemia, p = 0.008
Figure 5	Intracellular glucose	Ischemia vs. pre-ischemia, p = 0.017		Reperfusion vs. ischemia, p = 0.021
	Glycogen	Ischemia vs. pre-ischemia, p = 0.011		Reperfusion vs. ischemia, p = 0.003
	Phospho-AMPK	Ischemia vs. pre-ischemia, p = 0.005		Reperfusion vs. ischemia, p = 0.036

Supplementary Table 3. Specific p-values for presented data.

Figure	Data	Ischemia vs. pre-ischemia	Reperfusion vs. pre-ischemia	Reperfusion vs. ischemia
Figure 6	Citrate	Ischemia vs. pre-ischemia, p = 0.000	Reperfusion vs. pre-ischemia, p = 0.000	Reperfusion vs.ischemia, p = 0.000
	Cis-aconitate		Reperfusion vs. pre-ischemia, p = 0.04	Reperfusion vs.ischemia, p = 0.004
	Succinate	Ischemia vs. pre-ischemia, p = 0.002		Reperfusion vs.ischemia, p = 0.004

Supplementary Table 3 continued. Specific p-values for presented data.



Top panel – Extracted ion chromatogram for 1,2-dihexadecanoyl-glycerol (568.51). Middle panel – Extracted ion chromatogram between 550 and 650 amu showing the localisation of diacylglycerides to 9.5-10.5 min.

Bottom panel - Total ion chromatogram of intact lipid extract analysed by LC-MS.

Supplementary Figure 1. No difference in diacylglycerides in isolated perfused hearts following pre-ischemia, low-flow ischemia and reperfusion. n = 5 per group



Supplementary Figure 2. Total GLUT4 and FAT/CD36 protein levels in isolated perfused hearts following pre-ischemia, low-flow ischemia and reperfusion. n = 5 per group