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Impaired cytosolic NADH shuttling and elevated UCP3 contribute to inefficient citric acid cycle flux support of postischemic cardiac work in diabetic hearts

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ABSTRACT

Diabetic hearts are subject to more extensive ischemia/reperfusion (ISC/REP) damage. This study examined the efficiency of citric acid cycle (CAC) flux and the transfer of cytosolic reducing equivalents into the mitochondria for oxidative support of cardiac work following ISC/REP in hearts of c57bl/6 (NORM) and type 2 diabetic, db/db mouse hearts. Flux through the CAC and malate–aspartate shuttle (MA) were monitored via dynamic ^{13}C NMR of isolated hearts perfused with ^{13}C palmitate + glucose. MA flux was lower in db/db than NORM. Oxoglutarate malate carrier (OMC) was elevated in the db/db heart, suggesting a compensatory response to low NADHc. Baseline CAC flux per unit work (rate-pressure-product, RPP) was similar between NORM and db/db, but ISC/REP reduced the efficiency of CAC flux/RPP by 20% in db/db. ISC/REP also increased UCP3 transcription, indicating potential for greater uncoupling. Therefore, ISC/REP induces inefficient carbon utilization through the CAC in hearts of diabetic mice due to the combined inefficiencies in NADHc transfer per OMC content and increased uncoupling via UCP3. Ischemia and reperfusion exacerbated pre-existing mitochondrial defects and metabolic limitations in the cytosol of diabetic hearts. These limitations and defects render diabetic hearts more susceptible to inefficient carbon fuel utilization for oxidative energy metabolism.

1. Introduction

Patients with type II diabetes mellitus (DM2) are at an increased risk for cardiovascular disease. In fact, cardiovascular complications are the leading cause of diabetes related morbidity and mortality [1]. While some controversy exists as to the whether diabetic hearts are more susceptible to injury, most in vivo animal studies suggest that the diabetic myocardium is more sensitive to dysfunction following ischemic injury [2]. Both the diabetic heart and the metabolically related cardiac phenotype of the PPARα over-expressing mouse heart (MHC-PPARα), show exacerbated post-ischemic dysfunction [3]. Yet, the distinctions in post-ischemic metabolic recovery of diabetic versus normal myocardium are at best, only superficially understood, and mechanisms regulating the production and oxidation of reducing equivalents for mitochondrial energy production have not been directly studied in the intact, post-ischemic diabetic hearts. This study probed the efficiency of carbon flux in the production of NADH by citric acid cycle (CAC) flux in the support of contractile recovery of intact, post-ischemic diabetic hearts. The protocols enabled a focus on mechanisms linking CAC flux to energy dissipation and the transfer and oxidation of cytosolic NADH into mitochondria.

Characterized by a decrease in glucose oxidation and an increase in fatty acid oxidation (FAO), DM2 is related to not only an increase in the delivery of long chain fatty acids (LCFA) into the myocardium but also decreased insulin signaling and activation of peroxisome proliferator-activated receptor-α signaling [4–6]. Changes in myocardial fuel metabolism, a decrease in glucose oxidation and an increase in fatty acid oxidation, drive diabetic cardiomyopathy [4]. The DM2 heart is characterized by substrate inflexibility due to insulin responsiveness; reduced glucose uptake and oxidation with increased long chain fatty acid (LCFA) delivery and oxidation [4,7]. As glucose availability becomes limited, the diabetic myocardium relies nearly completely on fatty acid oxidation (FAO) for energy production [3,8].

Cardiac inefficiency, the ratio of cardiac work to myocardial O_2 consumption (MVO_2), is considered to be an underlying cause of cardiac dysfunction in both type I and type II diabetic myocardium due to consequences of impaired mitochondrial function [7,9]. While previous work has shown both high insulin and high glucose enhance the

Abbreviations: CAC, citric acid cycle; DM2, type 2 diabetes mellitus; db/db, db/db mouse; FAO, fatty acid oxidation; ISC/REP, ischemia/reperfusion; LCFA, long chain fatty acid; MA, malate–aspartate shuttle; MHC-PPARα, myosin heavy chain-peroxisome proliferator activated receptor alpha; MVO_2, myocardial oxygen consumption; NADHc, cytosolic NADH; NMR, nuclear magnetic resonance; NORM, normal c57bl/6 heart; OMC, oxoglutarate malate carrier; RPP, rate pressure product; UCP3, uncoupling protein 3; UCP2, uncoupling protein 2.

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pressure–volume area–MVO_2 relationship during post-ischemic recovery in the db/db mouse heart, the relationship between reducing equivalent production from carbon-based fuels and cardiac function in the postischemic db/db heart remains unclear [10,11]. To date, only oxygen consumption rates and substrate oxidation rates have been measured in diabetic hearts with studies of respiratory function in isolated mitochondria. This study provides the first detailed analysis of the efficiency of carbon flux through the CAC for oxidative, mitochondrial NADH production and the coordination of CAC flux with the transfer of cytosolic NADH following ischemia/reperfusion in hearts of diabetic animals.

We hypothesized that the contributions of cytosolic NADH to mitochondrial oxidative energy production is limited, due to impaired ability for glucose uptake in the diabetic heart, rendering oxidative metabolism increasingly reliant on NADH production via the CAC. Published studies suggest the increase in MVO_2 is attributable to the increase in UCP3 [12,13], but no direct measurements of the actual NADH producing pathways have been performed beyond the indirect substrate preference or substrate oxidation rates. The current studied explores how the contributions of both cytosolic and mitochondrial NADH to oxidative energy metabolism in the mitochondria are linked to the demand for oxidative carbon flux through the CAC in postischemic, diabetic hearts.

Recently, changes UCP3 were shown to contribute to cardiac inefficiency through mitochondrial uncoupling in the post-ischemic, reperfused state [14]. Previous work in normal hearts has demonstrated the sensitivity of NMR detection of ^{13}C enrichment rates of intramyocellular glutamate to the coupled processes of transfer of reducing equivalents produced in the cytosol into the mitochondria via exchange of malate for α-ketoglutarate within the malate–aspartate shuttle and flux through the citric acid cycle [15–20]. Here, we exploit the ^{13}C NMR detection of these intracellular events in the intact myocardium to investigate changes in CAC flux as an oxidative source of NADH and its balance with transfer of reducing equivalents from cytosolic NADH into mitochondria through the oxaloacetate–malate carrier (OMC) [15–20]. The findings suggest that despite adaptive responses in mitochondrial OMC expression, the availability of cytosolic NADH remains limiting, and together with elevated expression of uncoupling proteins, exacerbate the metabolic inefficiencies of postischemic contractile dysfunction in the diabetic heart.

2. Material and methods

2.1. Animal model

Male db/db, BKS.Cg- + $Lepr^{db}$/+$Lepr^{db}$/OlaHsd obtained from Harlan, were studied at 12 weeks of age. The background strain, c57bl/6, was obtained from Harlan and studied at 12 weeks of age [21,22]. At this age, db/db mice were consistently and severely hyperglycemic and exhibit altered substrate metabolism and cardiac efficiency [13]. Blood glucose from both db/db and c57bl/6 was monitored. Mice had free access to food and water while being housed under controlled temperature and lighting. All experimental procedures were approved by the University of Illinois at Chicago Animal Care and Use Committee.

2.2. Isolated heart protocols

12-week old animals were heparinized (50 U/10 g, i.p.) and anesthetized with ketamine (80 mg/kg, i.p.) plus xylazine (12 mg/kg, i.p.). Hearts were excised and retrogradely perfused (80 cm H_2O) with modified Krebs–Henseleit buffer (118.5 mM NaCl, 4.7 mM KCl, 1.5 mM $CaCl_2$, 1.2 mM $MgSO_4$ and 1.2 mM KH_2PO_4) equilibrated with 95% O_2/5% CO_2, at 37 °C, and containing 0.4 mM ^{12}C palmitate/fatty acid free albumin complex (3:1 molar ratio) and 10 mM glucose. Perfusion media contained 0.4 mM palmitate for both c57bl/6 and db/db. The use of a physiologically normal palmitate concentration allowed for direct comparison of normal and diabetic hearts for elucidating fundamental metabolic mechanisms within the cardiomyocyte in the absence of confounding variables introduced by high exogenous lipids and glucose [7,23–26]. Previous findings indicate that in the functioning, whole heart, elevated fat content influences lipid storage in the cardiomyocyte but cannot override the metabolic demand of mechanical work and does not result in elevated LCFA oxidation [27]. Protocols were designed to use similar substrates for isolated heart perfusions, as per previously published studies examining oxygen consumption and mitochondrial uncoupling in the diabetic, postischemic heart. As thusly indicated hearts were perfused with LCFA plus glucose and no lactate [7,23–26].

Hearts undergoing ISC/REP were subjected to 8 min no flow ischemia followed by 10 min of reperfusion. After 10 min of reperfusion, hearts were switched to ^{13}C-enriched media ([4,6,8,10,12,14,16,-$^{13}C_7$] palmitate) for 30 min at baseline workload for mice on a regular chow diet (db/db, N = 9; c57bl/6, N = 6) and undergoing 8 min no flow ischemia/reperfusion (ISC/REP) (db/db, N = 8; c57bl/6, N = 6). [4,6,8,10,12,14,16,-$^{13}C_7$] palmitate was used for ease of analysis for the C4 carbon of glutamate in the ^{13}C NMR spectra due to overlapping resonances from the C4 position of glutamate and C2 position of acyl intermediates. Sequential ^{13}C NMR spectra were collected and hearts were frozen in liquid N_2 cooled tongs for biochemical analysis [28,29]. Mouse hearts are more susceptible to ISC/REP than other species, and small changes in time of global ischemia have resulted in large reductions in recovery contractile recovery [6]. For the purposes of this study, we subjected hearts to 8 min of ischemia, which produced a 50% reduction in the recovery of RPP throughout reperfusion in the db/db model.

A water-filled latex balloon was fitted into the left ventricle and set to a diastolic pressure of 5 mm Hg. Left ventricular developed pressure (LVDP) and heart rate (HR) were continuously recorded with a pressure transducer and digital recording system (Powerlab, AD Instruments, Colorado Springs, CO). Rate-pressure product (RPP) was calculated as the product of heart rate and developed pressure. Temperature was maintained at 37 °C.

2.3. NMR spectroscopy and tissue chemistry

Using previously established methods, dynamic ^{13}C-spectra from intact, beating hearts were collected as previously reported [30–33]. Sequential, proton-decoupled ^{13}C NMR spectra were acquired (2 min each) with natural ^{13}C abundance correction using previously reported NMR methods (Fig. 1) [30,31]. Magnetic field homogeneity was optimized by shimming to a proton line width of 10–20 Hz.

Tissue metabolites were extracted from frozen heart tissue using 7% perchloric acid and neutralized with KOH. Tissue extracts were analyzed by enzymatic assay either spectrophotometrically or fluorometrically for metabolite content (aspartate, α-ketoglutarate, citrate) using previously described methods [34–36]. Glutamate concentration was determined with glutamate dehydrogenase and diaphorase (Roche L-Glutamic acid colorimetric kit). In vitro high-resolution ^{1}H and ^{13}C NMR spectra of tissue extracts reconstituted in 0.5 mL of D_2O were collected with a 5 mm ^{13}C probe (Fig. 1) (Bruker Instruments, Billerica, MA). Analysis of ^{13}C spectra was performed to determine fractional enrichment of [2-^{13}C] acetyl CoA [37,38].

2.4. Kinetic analysis of isotope enrichment and oxidative rates

Kinetic analysis of isotopic enrichment rates provided quantitative measures of flux through the CAC, rates of LCFA oxidation, and the rate of cytosolic NADH transfer through malate/α-ketoglutarate exchange between the cytosol and mitochondria [15–17,19,20]. Briefly, the oxidation of ^{13}C enriched LCFA to form [2-^{13}C] acetyl CoA results in enrichment of the first span of the CAC to form [4-^{13}C] α-ketoglutarate within the mitochondria. Competition for [4-^{13}C] α-ketoglutarate, as a substrate for either oxidation via α-ketoglutarate dehydrogenase or efflux via OMC from the mitochondria in exchange for cytosolic malate, determines the extent and rate of the production