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Genetic Loss of Insulin Receptors Worsens Cardiac Efficiency in Diabetes

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Abstract

Aims—To determine the contribution of insulin signaling versus systemic metabolism to metabolic and mitochondrial alterations in type 1 diabetic hearts and test the hypothesis that antecedent mitochondrial dysfunction contributes to impaired cardiac efficiency (CE) in diabetes.

Methods and Results—Control mice (WT) and mice with cardiomyocyte-restricted deletion of insulin receptors (CIRKO) were rendered diabetic with streptozotocin (WT-STZ and CIRKO-STZ, respectively), non-diabetic controls received vehicle (citrate buffer). Cardiac function was determined by echocardiography; myocardial metabolism, oxygen consumption (MVO₂) and CE were determined in isolated perfused hearts; mitochondrial function was determined in permeabilized cardiac fibers and mitochondrial proteomics by liquid chromatography mass spectrometry. Pyruvate supported respiration and ATP synthesis were equivalently reduced by diabetes and genotype, with synergistic impairment in ATP synthesis in CIRKO-STZ. In contrast, fatty acid delivery and utilization was increased by diabetes irrespective of genotype, but not in non-diabetic CIRKO. Diabetes and genotype synergistically increased MVO₂ in CIRKO-STZ, leading to reduced CE. Irrespective of diabetes, genotype impaired ATP/O ratios in mitochondria exposed to palmitoyl carnitine, consistent with mitochondrial uncoupling. Proteomics revealed reduced content of fatty acid oxidation proteins in CIRKO mitochondria, which were induced by

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Conflict of interest

None declared

diabetes, whereas tricarboxylic acid cycle and oxidative phosphorylation proteins were reduced both in CIRKO mitochondria and by diabetes.

Conclusions—Deficient insulin signaling and diabetes mediate distinct effects on cardiac mitochondria. Antecedent loss of insulin signaling markedly impairs CE when diabetes is induced, via mechanisms that may be secondary to mitochondrial uncoupling and increased FA utilization.

Keywords

Insulin signaling; cardiac efficiency; mitochondria; diabetes

1 Introduction

In diabetes, the cardiac dysfunction that occurs independently of coronary heart disease and hypertension [1, 2] has been termed ‘diabetic cardiomyopathy’ [3]. The degree of cardiac dysfunction ranges from diastolic dysfunction, which is often the only manifestation to progressive left ventricular (LV) systolic dysfunction particularly in animal models of longstanding diabetes. Potential molecular mechanisms include oxidative stress, impaired intracellular Ca^{2+} handling, altered substrate utilization, impaired mitochondrial energetics, reduced cardiac efficiency, and increased fibrosis [4–10]. Although many studies in experimental models of diabetes reveal impaired cardiac contractility [4, 5, 9–11], there is strong evidence that defects in substrate metabolism and mitochondrial dysfunction may precede overt (LV) systolic dysfunction [4–6, 12]. Because poorly controlled diabetes is associated with complex neurohumoral changes in addition to hyperglycemia, such as increased concentrations of free fatty acids and triglycerides, insulin deficiency or hyperinsulinemia, catecholamine excess, activation of the renin-angiotensin system and increased circulating concentrations of inflammatory cytokines, it has been challenging to determine the specific relationship between these abnormalities and specific pathophysiological events in the heart [3, 13]. Transgenic mouse models with changes in the activity of specific signaling pathways that might be implicated in the pathophysiology of diabetic cardiomyopathy have shed some insights [14, 15]. For example overexpression of PPAR or Acyl CoA Synthase have provided some insights into the contribution of lipotoxicity to diabetic cardiomyopathy [16, 17] and cardiomyocyte deletion of insulin receptors (CIRKO) have implicated impaired insulin action or absence of insulin signaling in the pathophysiology of certain aspects of mitochondrial dysfunction [18, 19]. However, these models only partially recapitulate the pathophysiology of diabetic cardiomyopathy and only recently have studies began to address the potential roles of synergistic interactions between signaling pathways [20–22].

Others and we have previously shown that diabetic cardiomyopathy is associated with mitochondrial dysfunction, although the specific changes are dependent on the model and the nature of the diabetes [23]. Thus in models of type 1 diabetes, mitochondrial dysfunction is characterized by reduced mitochondrial respiratory capacity and ATP generation that is associated with remodeling of the mitochondrial proteome leading to induction of fatty acid oxidation pathways and repression of oxidative phosphorylation pathways [6, 24]. Interestingly, in type 1 diabetic (insulin deficient) models, there is little evidence for mitochondrial ROS overproduction, mitochondrial uncoupling or reduced cardiac efficiency [6, 25]. By contrast, in obese and insulin resistant mouse models such as *ob/ob* and *db/db* mice, mitochondrial dysfunction is accompanied by ROS overproduction, mitochondrial dysfunction and decreased cardiac efficiency [4, 5]. Indeed the mitochondrial adaptations in these models were recently shown to be due in part to leptin resistance or deficiency at the level of the hypothalamus [26]. However, the mechanisms for the divergent mitochondrial

phenotypes between models of type 1 and type 2 diabetes, and in particular differences in cardiac efficiency, remain incompletely understood.

In contrast to type 1 diabetes, with a relatively abrupt onset of hyperglycemia, type 2 diabetes is often preceded by a period of significant hyperinsulinemia and insulin resistance, which has recently been shown to independently impact mitochondrial function in the heart [27]. Thus we hypothesized that antecedent mitochondrial dysfunction that precedes the onset of hyperglycemia could potentially contribute to the greater degree of mitochondrial uncoupling and impairment in cardiac efficiency that characterizes type 2 diabetes. We utilized CIRKO mice, in which cardiomyocyte-restricted deletion of insulin receptors induces mitochondrial dysfunction that is characterized at the molecular level by reduced content of FAO proteins, TCA cycle enzymes and certain OXPHOS subunits [19]. ROS overproduction leads to mitochondrial uncoupling and although FAO and MVO₂ were increased in young mice, this was not sustained as the animals aged [18, 19]. We therefore rendered CIRKO mice diabetic by treating them with streptozotocin (STZ). Diabetes induced FAO proteins and led to a dramatic increase in myocardial MVO₂ and a reduction in cardiac efficiency that was not observed in diabetic wildtype controls or non-diabetic CIRKO mice. These findings support the hypothesis that antecedent mitochondrial dysfunction in a model of myocardial insulin resistance dramatically reduces myocardial cardiac efficiency when exposed to the abnormal metabolic milieu of diabetes.

2 Material and Methods

2.1 Animals

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996) and was approved by the Institutional Animal Care and Use Committee of the University of Utah. Male CIRKO and control littermates [18] were housed at 22°C with free access to water and food with a light cycle of 12h light and 12h dark. 5–6 week-old CIRKO or control littermates were injected intraperitoneally with 50mg/kg streptozotocin (Sigma-Aldrich, St. Louis, MO) or vehicle (100mmol/L sodium citrate, pH 4.5) for 5 days, according to the Animal Models of Diabetes Complications (AMDCC) protocol (<http://www.amdcc.org>). Streptozotocin-treated mice became hyperglycemic 10–14 days after the first injection and were used 4 weeks following onset of hyperglycemia (= 11–12 weeks of age).

2.2 Contractile function and substrate oxidation

Contractile function, substrate oxidation rates and myocardial oxygen consumption (MVO₂) in isolated working hearts were measured as described before [28]. In brief, hearts were isolated and perfused in the working mode with a preload of 15 mmHg, with systolic and diastolic pressures measured in the aortic outflow with a Millar catheter. Hearts were perfused with Krebs Henseleit buffer containing (in mmol/l) 118.5 NaCl, 25 NaHCO₃, 4.7 KCl, 1.2 MgSO₄, 1.2 KH₂PO₄, 2.5 CaCl₂, 0.5 EDTA, and 5 glucose, gassed with 95% O₂ and 5% CO₂ and supplemented with 0.4 mmol/l palmitate bound to 3% BSA with no added insulin. Glycolytic flux was determined by measuring ³H₂O released from the metabolism of exogenous [5-³H] glucose. Glucose oxidation was determined by trapping and measuring ¹⁴CO₂ released by the metabolism of [U-¹⁴C] glucose. Palmitate oxidation was determined in separate perfused hearts by measuring ³H₂O released from [9,10-³H] palmitate. In the Langendorff mode, hearts were perfused retrogradely at constant pressure of 60 mmHg with Krebs buffer containing (in mmol/L) 118 NaCl, 4.7 KCl, 25 NaHCO₃, 1.2 MgSO₄, 1.2 KH₂PO₄, 2 CaCl₂, 11 glucose, and 0.5 palmitate prebound to 3% BSA, gassed with 95% O₂ and 5% CO₂ [4]. Rate pressure product was calculated from left ventricular

pressure which was monitored from a water-filled balloon placed through the left atrial appendage and connected to a Millar transducer (Millar Instruments). The balloon was inflated to achieve an end-diastolic pressure of 7 to 10 mmHg.

2.3 Myocardial Oxygen Consumption and Cardiac Efficiency

In working hearts, oxygen consumption was measured in the pulmonary arterial effluent as previously described by us [28]. Myocardial oxygen consumption (MVO_2), cardiac hydraulic work and cardiac efficiency were calculated as follows: MVO_2 ($\mu\text{l/min/g WHW}$) = $((\text{PaO}_2 - \text{PvO}_2)/100) \times (\text{Coronary flow/WHW}) \times (725/760) \times (1000 \times C)$; where C = Bunsen Coefficient for plasma i.e. 0.0212, PaO_2 = Arterial partial pressure of oxygen, and PvO_2 = Venous partial pressure of oxygen (both in mmHg), 725 and 760 are atmospheric pressures at the University of Utah and at sea level respectively (mmHg) and WHW is the wet heart weight in grams (g). **Cardiac hydraulic work** [J/min/g WHW] = $\text{CO (ml/min)} \times \text{DevP (mm Hg)} \times 1.33 \times 10^{-4}/\text{g WHW}$ where CO = Cardiac output and DevP = Developed pressure. DevP is the difference between aortic systolic and diastolic pressure. CO is the sum of aortic and coronary flow. **Cardiac efficiency (%) (working hearts)** = $\text{Hydraulic work / MVO}_2 \times 100$. MVO_2 (ml/min) was converted to $\mu\text{mol/min}$ by multiplying by the conversion factor 0.0393, and then to Joules (J/min) using the conversion of $1\mu\text{mol O}_2 = 0.4478 \text{ J}$ as described by Suga [29]. In Langendorff preparations, MVO_2 ($\mu\text{mol/min/g}$) was calculated from the difference in oxygen content of incoming (aortic) and outgoing (pulmonary artery) perfusate with the formula: $\text{MVO}_2 = (\% \text{O}_2 \text{ perfusate} - \% \text{O}_2 \text{ pulmonary artery}) \times \text{coronary flow} \times \text{atmospheric pressure}/760 \times \text{O}_2 \text{ solubility} \times \text{O}_2 \text{ density}$, where O_2 solubility = 23.9 L/mL and O_2 density = 0.03933 mol/L, respectively, at 37°C. **Cardiac efficiency (Langendorff hearts) (%)** was calculated by relating rate pressure product to MVO_2 .

2.4 Mitochondrial function

Mitochondrial oxygen consumption and ATP synthesis rates were measured in saponin-permeabilized fibers of freshly excised hearts using pyruvate/malate (5mmol/L, 2mmol/L) and palmitoyl-carnitine/malate (20 $\mu\text{mol/L}$, 2mmol/L) as described before [6]. Oxygen consumption was measured in the presence of substrate alone (V_0), after addition of ADP (1mmol/L; = V_{ADP}), and following addition of the F_0F_1 -ATPase inhibitor oligomycin (1 $\mu\text{g/mL}$, = V_{Oligo}). ATP synthesis rates were measured using a bioluminometric assay, as described before [6]. Mitochondrial H_2O_2 generation was measured with succinate as published previously [5].

2.5 Electron microscopy

Samples were collected from freshly excised left ventricular myocardium and processed as described before [6]. Mitochondrial volume density, number of mitochondria, and number of lipid droplets were analyzed by stereology in a blinded fashion using the point counting method [6].

2.6 Comparative Mitochondrial Proteomics

Mitochondria were isolated, purified, and fractionated into matrix and membrane fractions as described before [24]. Proteins were typically digested and subjected to liquid chromatography mass spectrometry (LC-MS/MS; for detailed description see Supplementary Materials). Mass spectrometry data were analyzed by Waters ProteinLynx Global Server (PLGS) 2.3. Canonical pathway analysis was performed using the Ingenuity Pathways Analysis (IPA) software (Redwood City, CA).

2.7 Western blot analysis

Whole heart lysates were generated as described before [4]. Western blots were performed as described before, using a UCP3 antibody (Affinity BioReagents, Golden, CO) with a 1:1000 primary antibody and 1:5000 secondary antibody dilution [4].

2.8 Metabolite Analyses

Serum samples were obtained from random fed mice and stored at -80°C . Triglycerides (TG) were measured using the L-type TG M assay (Wako Chemicals, Richmond, VA). Free fatty acids (FFAs) were measured using the FFA half-micro test kit (Roche, Indianapolis, IN). Lactate concentrations were measured using the lactate assay kit (BioVision, Inc., Mountain View, CA). β -hydroxybutyrate (ketone body) was measured using the -HB assay kit (Cayman Chemical, Ann Arbor, MI). Triglyceride concentrations in heart tissue were measured as described previously [6].

2.9 Statistical analysis

Results are presented as means $\pm$ SEM. Data were analyzed using 2-way analysis of variance (2-way ANOVA), using the GraphPad Prism 5 software package (La Jolla, CA). A Bonferroni posthoc analysis was performed when significant interactions occurred or when appropriate. The proteomic data set was analyzed using Waters ProteinLynx Global Server (PLGS) 2.3. For all statistical analyses, significant difference was accepted when $p < 0.05$.

3 Results

3.1 Metabolites and cardiac size

Mice were sacrificed for experiments 4 weeks following onset of diabetes (= 11–12 weeks of age). Streptozotocin injections induced similar increases in serum glucose, free fatty acid, lactate and β -hydroxybutyrate concentrations irrespective of genotype (Table 1). Serum triglycerides were increased by diabetes or genotype, and were synergistically increased in CIRKO-STZ. Both diabetes and genotype reduced heart weight. Since body weights were reduced due to diabetes, heart weights were normalized to tibia length (heart weight-to-tibia length ratio; HW-TL ratio). HW-TL ratios were reduced by diabetes or genotype (Supplementary Table 2).

3.2 Cardiac function

Left ventricular (LV) wall thickness, chamber dimensions and cardiac function were determined by echocardiography (Supplementary Table 3). Genotype decreased LV septum diameter irrespective of diabetes. LV internal dimension at diastole, stroke volume (SV) and cardiac output (CO) were reduced by diabetes irrespective of genotype. Since diabetic animals had lower body weights, differences in SV and CO were not present when normalized to body weight. Ejection fraction and fractional shortening were unaffected by genotype or diabetes.

To determine cardiac function independent of *in vivo* neurohumoral factors, we determined contractile function *in vitro* in isolated working hearts. Developed pressure (aortic) was reduced by genotype irrespective of diabetes, and by the combination of genotype and diabetes in CIRKO-STZ (Fig. 1 A). Cardiac output was reduced by diabetes irrespective of genotype, and was synergistically decreased by the combination of diabetes and genotype in CIRKO-STZ (Fig. 1 B). Cardiac power, which is normalized to dry heart weight, was unaffected by diabetes or genotype (Fig. 1 C).

3.3 Myocardial substrate oxidation and oxygen consumption

Myocardial substrate oxidation rates were measured in isolated working hearts perfused with 0.4mmol/L palmitate, 5mmol/L glucose, and no insulin. Rates of palmitate oxidation were increased by diabetes irrespective of genotype, but were decreased by genotype irrespective of diabetes (Fig. 1 D). Glycolysis rates were decreased in CIRKO irrespective of treatment and diabetes reduced rates of glycolysis and glucose oxidation irrespective of genotype (Fig. 1 E, F). Cardiac glycogen levels obtained immediately post-mortem were not increased in non-diabetic CIRKO but were increased by diabetes irrespective of genotype (Supplementary Fig. 1). The glycogen results in non-diabetic CIRKO contrast with our previous study in which glycogen concentrations were measured in older mice after 60 minutes of perfusion in isolated perfused hearts ex vivo [18]. In contrast to CIRKO mice that exhibit metabolic insulin resistance and fail to increase Akt phosphorylation following insulin stimulation [18], diabetic WT hearts remain insulin sensitive (Supplementary Fig. 2).

While diabetes and genotype alone had no effect on MVO_2 , there was a marked increase in MVO_2 by the combination of diabetes and genotype (Fig. 1G, interaction $p < 0.0001$). Thus, cardiac efficiency (CE; = cardiac work/ MVO_2) was significantly lower in CIRKO-STZ relative to non-diabetic mice (Fig. 1 H, interaction $p < 0.001$). The increase in MVO_2 was independently confirmed in Langendorff-perfused hearts (0.5mmol/L palmitate, 11mmol/L glucose), in which a similar synergistic increase in MVO_2 occurred in CIRKO-STZ (interaction $p < 0.01$), which correlated with lower mean values of cardiac efficiency in these mice (Fig. 1 I, J, K).

3.4 Mitochondrial Function and Structure

To gain additional insights into potential mechanisms that were responsible for the differences in substrate utilization and the increase in MVO_2 in isolated hearts studies, we determined mitochondrial function in saponin permeabilized cardiac fibers using pyruvate and palmitoyl-carnitine as substrates. With pyruvate, diabetes or genotype impaired ADP-stimulated O_2 consumption (V_{ADP} , Fig. 2A). ATP synthesis rates were reduced by diabetes or genotype and were synergistically impaired by diabetes and genotype (Fig. 2B, interaction $p < 0.05$). ATP/O ratios did not differ between groups (Fig. 2 C). Following incubation with the long chain fatty acid palmitoyl-carnitine (which bypasses CPT-1), V_{ADP} was unaffected by diabetes or genotype (Fig. 2 D). However, while diabetes did not affect V_{oligo} or ATP/O ratios, CIRKO genotype increased V_{oligo} and decreased ATP/O ratios irrespective of diabetes, suggesting mitochondrial uncoupling (Fig. 2 D, F). Mitochondrial structure and myocardial ultrastructure was evaluated by electron microscopy. Cristae density was decreased in CIRKO hearts irrespective of diabetes and was equivalently reduced in WT-STZ (Fig. 3 A). Mitochondrial volume density was increased by diabetes irrespective of genotype, but mitochondrial number was unaffected (Fig. 3 B, C). Diabetes increased lipid droplet accumulation in the heart irrespective of genotype, although total cardiac triglyceride content was not different between groups (Fig. 3 D, E, F).

3.5 Uncoupling proteins and markers of oxidative stress

To further explore mechanisms that could account for mitochondrial uncoupling and decreased cardiac efficiency in diabetic CIRKO hearts, we measured cardiac UCP3 protein content and mitochondrial ROS generation. Diabetes increased cardiac UCP3 content irrespective of genotype (Fig. 4 A, B). While diabetes or genotype did not affect succinate-stimulated mitochondrial H_2O_2 generation when analyzed by 2-way ANOVA (Figure 4C), mitochondrial H_2O_2 generation was increased in nondiabetic CIRKO relative to WT (Bonferroni post-hoc analysis, $p < 0.05$), which supports our previously published comparison of WT and CIRKO mitochondria [19]. H_2O_2 generation was of intermediate value in the two diabetic groups (Fig. 4 C). By contrast, diabetes increased and genotype

decreased total cellular levels of the oxidative stress marker, 4-hydroxy-2-nonenal (HNE) (Supplementary Figure 3).

3.6 Proteomic and genomic adaptations to impaired insulin signaling versus diabetes

The data presented thus far supports a model in which the increased MVO_2 in CIRKO–STZ hearts could be the consequence of the synergistic interaction of uncoupled mitochondria in CIRKO with excess substrate delivery when diabetes is superimposed. To further elucidate the molecular basis for these mitochondrial adaptations we undertook an unbiased comparative proteomics approach to compare WT and CIRKO mitochondria in the absence or presence of diabetes. Mitochondria were isolated, purified, fractionated into matrix and membrane fractions to increase protein detection, and directly subjected to LC-MS/MS, as published before [19]. 56 matrix and 144 membrane proteins were detected in all groups and sorted by canonical pathways using Ingenuity Pathways Analysis (IPA) software. As summarized in Fig. 5, fatty acid oxidation (FAO) proteins abundance was repressed in mitochondria from non-diabetic CIRKO. In contrast, FAO proteins were generally increased in mitochondria of WT–STZ and CIRKO–STZ, with higher levels in WT–STZ (For fold changes of each protein see Supplementary table 4). By contrast, tricarboxylic acid (TCA) cycle enzymes were repressed in CIRKO and by diabetes in WT hearts, and this pattern persisted in CIRKO–STZ. Oxidative phosphorylation (OXPHOS) subunits were either unchanged or reduced by deletion of insulin receptors and by diabetes. A detailed list of detected proteins and their relative quantification is presented in Supplementary table 4. Taken together these data suggest that decreased insulin signaling reduces TCA and OXPHOS protein content in type 1 diabetes whereas the induction of FAO proteins is independent of insulin signaling. Of interest, the proteomic remodeling observed could not be fully accounted for by transcriptional mechanisms as representative mRNAs encoding FAO and OXPHOS genes were generally little affected by diabetes or genotype (Supplementary Figures 4 and 5). However, mean transcript levels of FAO genes in WT diabetic mice were generally increased relative to non-diabetic CIRKO.

4 Discussion

The present study was undertaken to determine respective contributions of impaired insulin action versus secondary disturbances in metabolism to the metabolic and mitochondrial adaptations of the heart in type 1 diabetes. We also tested the hypothesis that impaired myocardial insulin action could contribute to mitochondrial uncoupling and decreased cardiac efficiency (CE), which characterizes the hearts of some rodent models of obesity and insulin resistance but has not been consistently observed in the hearts of mice with type 1 (insulin deficient) diabetes [4–6, 23, 25, 30]. We took advantage of mice with genetic absence of insulin receptors in cardiomyocytes (CIRKO), which is a model of impaired myocardial insulin action in the absence of systemic metabolic disturbances [18] to determine myocardial changes that were more likely a consequence of impaired insulin action versus those associated with changes in systemic metabolism that accompany diabetes. The major findings of this study were that pre-existing mitochondrial dysfunction and uncoupling in CIRKO hearts were associated with a dramatic increase in myocardial oxygen consumption (MVO_2) and reduction in CE when diabetes was induced. We posit that this may be a consequence of increased delivery of fatty acids as suggested by diabetes-induced proteomic remodeling that increased beta-oxidation capacity, in the absence of a coordinate increase in oxidative phosphorylation. Our findings also support the hypothesis that decreased CE in the diabetic heart is more likely to occur when co-existent mitochondrial defects accompany the onset of hyperglycemia. For example in mice and humans, particularly those with type 2 diabetes, obesity and insulin resistance; evidence exists that mitochondrial dysfunction may occur independently of significant changes in

circulating concentrations of glucose [4, 26] arising potentially from lipotoxicity or oxidative stress that may predate significant hyperglycemia [26, 27].

CIRKO mice have a complex phenotype characterized by progressive mitochondrial dysfunction and changes in myocardial substrate metabolism and cardiac efficiency as a function of age [18, 19]. Thus in young CIRKO mice (< 8- weeks of age), FA oxidative capacity and MVO₂ are increased, and CE is decreased. This decrease in CE is likely due to increased FAO, as mitochondria are not uncoupled. However, as these mice age there is a progressive decline in FA oxidation such that FAO is reduced by 16–20 weeks of age [18] and mitochondria are significantly uncoupled at 24-weeks of age [19]. In these earlier studies MVO₂ was not measured in older mice. In the present study mice were studied at the age of 12-weeks. Interestingly, relative to 8-week-old CIRKO mice, FAO had already declined and mitochondrial uncoupling was present indicating that FAO capacity and mitochondrial function rapidly declines in CIRKO hearts between 8 and 12 weeks of age. Thus by 12-weeks of age MVO₂ was no longer increased in perfused CIRKO hearts, reflecting the net effect of reduced FAO capacity and mitochondrial dysfunction, despite intrinsic mitochondrial uncoupling.

We therefore postulate that a major mechanism for the increase in MVO₂ and decline in CE in CIRKO-STZ mice is mitochondrial uncoupling, driven in part by a propensity of CIRKO mitochondria to overproduce ROS, coupled with increased delivery of FA substrates when diabetes was induced. We confirmed our prior observations that non-diabetic CIRKO mitochondria are uncoupled, and overproduce H₂O₂ [19]. In that study we also showed that scavenging of this ROS by treating CIRKO mice with MnTBAP could normalize mitochondrial coupling [19]. FAO proteins are repressed in the hearts of non-diabetic CIRKO mice. When diabetes is superimposed on CIRKO hearts there is an expansion of the FAO proteome, which coupled with increased myocardial utilization of FA, increases the delivery of reducing equivalents to the electron transport chain, subunits of which are repressed. We postulate that this increases local superoxide generation that activates uncoupling proteins whose content is increased by diabetes. Increased uncoupling might limit mitochondrial H₂O₂ release that could contribute to the reduction in total tissue ROS in CIRKO-STZ. So why was mitochondrial uncoupling not observed in diabetic WT mice despite increased UCP3 and a similar expansion of the FAO proteome? This could be partially accounted for by the fact that mitochondria in WT hearts were not defective prior to the onset of diabetes and lacked ROS-mediated mitochondrial uncoupling at baseline. Our study also examined fairly short-term diabetes, thus it remains possible that longer-term insulin deficiency could ultimately precipitate sufficient mitochondrial dysfunction to uncouple mitochondria and decrease CE. We think that this is unlikely based on studies in Akita mice with > 20-weeks of hyperglycemia in which mitochondrial uncoupling was not observed [6]. Thus the presence of mitochondrial uncoupling observed in mouse models of type 2 diabetes [4, 5, 26] might reflect the impact of additional metabolic disturbances apart from hyperglycemia that impair mitochondrial function. The CIRKO model may or may not recapitulate these specific mechanisms, but supports the general notion that antecedent or concurrent mitochondrial dysfunction via mechanisms that could be independent of hyperglycemia, might be necessary to promote the mitochondrial uncoupling and reduced CE that characterizes these models of obesity and insulin resistance.

The present study provides interesting insights into the relative contributions of insulin signaling versus diabetes associated metabolic disturbances to the pathophysiology of diabetic cardiomyopathy. A reduction in cardiac size is a well-described characteristic of poorly controlled type 1 diabetes [6, 15]. Both diabetes and genotype independently decreased HW-TL ratios, which suggests that impaired insulin signaling and the catabolic state associated with poorly controlled type 1 diabetes contribute to a reduction in cardiac

mass. It is of interest, that *in vivo* cardiac function in non-stressed hearts was not severely impaired by loss of insulin receptors or by short-term diabetes, despite significant changes in mitochondrial function, substrate utilization and cardiac efficiency. We have previously reported similar discrepancies between mitochondrial dysfunction and *in vivo* cardiac function in mouse models of type 1 and 2 diabetes and in 12-week-old CIRKO mice [4–6, 12, 31]. These observations suggest that metabolic changes and mitochondrial dysfunction may precede overt contractile dysfunction in many animal models of diabetic cardiomyopathy. However, it is likely that these defects will predispose to adverse cardiac responses to hemodynamic stressors. For example, CIRKO hearts exhibit impaired functional responses to pathophysiological stressors such as pressure overload and ischemia [31–33]. Therefore, future studies will be required to determine if the markedly impaired CE in CIRKO-STZ mice will further increase the susceptibility of these mice to maladaptive responses to hemodynamic stress.

Loss of insulin signaling and diabetes equivalently reduced pyruvate supported mitochondrial metabolism. However, there was a synergistic impairment in ATP synthesis in CIRKO-STZ that indicates that insulin deficiency only partially accounts for the decrease in pyruvate utilization in diabetes. Loss of insulin signaling reduced the levels of FAO proteins. The mechanisms for this might be partially due to transcriptional repression. Diabetes increases the FAO proteome in WT and CIRKO hearts indicating that additional insulin-independent signaling mechanisms that are induced by diabetes are the key mechanisms for expanding FAO capacity. Although increased PPAR signaling likely contributes in part to differences in FAO capacity between non-diabetic CIRKO and WT–STZ, the relatively small differences in mRNA levels for PPAR target genes such as MCAD, Acaa2 and Hadhb, despite increased protein concentrations suggest the existence of important post-transcriptional mechanisms. By contrast, TCA cycle capacity and OXPHOS protein content are reduced by impaired insulin signaling or diabetes without clear evidence of synergy, which implies that impaired insulin action might mediate the reduction in TCA and OXPHOS capacity in diabetes. Finally, diabetes increased the number of myocardial lipid droplets irrespective of genotype, as similarly observed in other models of Type 1 diabetes [6]. In contrast, total myocardial triglyceride content was unaffected by diabetes or genotype in the current study. This observation may reflect differences in the intracellular distribution or partitioning of lipids occurring in concert with changes in FA utilization.

Diabetic cardiomyopathy has a complex pathophysiology including perturbations of various cellular pathways such as: oxidative stress, impaired intracellular Ca^{2+} handling, altered substrate utilization, impaired mitochondrial energetics, reduced cardiac efficiency, increased apoptosis, and increased fibrosis [4–8, 10]. Studies suggest that these molecular changes in diabetic hearts may result from hyperglycemia, increased serum lipid levels, and/or impaired insulin action [3, 19, 34, 35]. However, the relative contribution of each of these metabolic derangements is incompletely understood. The present study sheds light on metabolic pathways that might be differentially or synergistically regulated by the metabolic disturbances that accompany type 1 diabetes versus the consequences of decreased insulin availability. Moreover, our findings suggest that antecedent mitochondrial dysfunction may be required for diabetes to impair myocardial efficiency.

Highlights

- MVO_2 increases and cardiac efficiency (CE) decreases in obese mice but not in T1DM
- Cardiomyocyte KO of Insulin Receptors (CIRKO) reduces MVO_2 and FA oxidation with aging

- CIRKO mice exhibit mitochondrial dysfunction
- Hyperglycemia in CIRKO mice dramatically increases MVO₂ and reduces CE
- Antecedent mitochondrial dysfunction predisposes to reduced CE with diabetes

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Contractile function and substrate oxidation in perfused hearts

2-way ANOVA was performed to analyze differences by treatment and genotype, including a Bonferroni post-hoc analysis when significant interaction occurred. (A) Aortic developed pressure (DevP) ($p < 0.05$ for genotype, and interaction of genotype and treatment; Bonferroni: * $p < 0.05$ for WT-Veh vs. all other groups respectively), (B) cardiac output ($p < 0.05$ for treatment, and interaction of treatment and genotype; Bonferroni: * $p < 0.05$ for CIRKO-STZ vs. all other groups respectively), (C) cardiac power, (D) palmitate oxidation ($p < 0.05$ for treatment and genotype), (E) glycolysis ($p < 0.05$ for treatment and $p = 0.05$ for genotype), (F) glucose oxidation ($p < 0.05$ for treatment), (G) myocardial oxygen consumption (MVO₂) ($p < 0.05$ for treatment, genotype, and their interaction; Bonferroni: *

p<0.05 for CIRKO-STZ vs. all other groups respectively), and (H) cardiac efficiency (p<0.05 for interaction of genotype and treatment; Bonferroni: § p<0.05 for WT-Veh vs. CIRKO-Veh; # p<0.05 for CIRKO-Veh vs. CIRKO-STZ) in isolated working hearts of non-diabetic WT and CIRKO (WT-Veh, CIRKO-Veh), WT-STZ, and CIRKO-STZ mice; n=4–7. (I) Myocardial oxygen consumption (MVO₂) (p<0.05 for treatment, genotype, and their interaction; Bonferroni: * p<0.05 for CIRKO-STZ vs. all other groups respectively), (J) rate pressure product, and (K) cardiac efficiency in Langendorff perfused hearts of non-diabetic WT and CIRKO (WT-Veh, CIRKO-Veh), WT-STZ, and CIRKO-STZ mice; n=3–5.

Figure 2. Mitochondrial dysfunction in CIRKO or diabetic hearts

2-way ANOVA was performed to analyze differences by treatment and genotype, including a Bonferroni post-hoc analysis when significant interaction occurred. (A) Pyruvate-supported respiration rates (V_{ADP} : $p < 0.05$ for genotype and treatment), (B) Pyruvate-supported ATP synthesis ($p < 0.05$ for genotype, treatment, and their interaction; Bonferroni: * $p < 0.05$ for WT-Veh vs. all other groups respectively), (C) Pyruvate-supported ATP/O ratios, (D) Palmitoyl-carnitine-supported respiration rates (V_{Oligo} : $p < 0.05$ for genotype), (E) Palmitoyl-carnitine-supported ATP synthesis, and (F) Palmitoyl-carnitine-supported ATP/O ratios ($p < 0.05$ for genotype) in saponin-permeabilized cardiac fibers from non-diabetic WT and CIRKO (WT-Veh, CIRKO-Veh), WT-STZ, and CIRKO-STZ hearts; $n = 4-8$.

Figure 3. Mitochondrial ultrastructure and triglyceride accumulation in CIRKO or diabetic hearts

2-way ANOVA was performed to analyze differences by treatment and genotype. (A) Representative electron micrographs ($\times 10,000$ or $\times 40,000$), and stereological quantification of (B) mitochondrial volume density ($p < 0.05$ for treatment) and (C) mitochondrial number. (D) Representative electron microscopy images ($\times 2,000$), (E) quantification of lipid droplets ($p < 0.05$ for treatment), and (F) cardiac triglyceride content of non-diabetic WT and CIRKO (WT-Veh, CIRKO-Veh), WT-STZ, and CIRKO-STZ hearts ($n = 4-5$). Arrows indicate lipid droplets.

Figure 4. Expression of uncoupling proteins and mitochondrial superoxide production
2-way ANOVA was performed to analyze differences by treatment and genotype, including a Bonferroni post-hoc analysis for mitochondrial superoxide production. (A) Representative images and (B) quantification of UCP3 Western blots in heart homogenates ($p < 0.05$ for treatment), expressed as fold change relative to WT-Veh, which was assigned as one; $n = 3-4$. (C) Mitochondrial superoxide production in isolated mitochondria using succinate as a substrate (Bonferroni: * $p < 0.05$ vs. WTVeh); $n = 5-8$.

Model	Fatty acid oxidation	TCA cycle	OXPHOS
CIRKO-Veh	Increased: 0 Decreased: 7	Increased: 0 Decreased: 3	Increased: 0 Decreased: 10
WT-STZ	Increased: 9 Decreased: 1	Increased: 1 Decreased: 5	Increased: 0 Decreased: 10
CIRKO-STZ	Increased: 6 Decreased: 0	Increased: 0 Decreased: 5	Increased: 2 Decreased: 5

Figure 5. Mitochondrial proteomic remodeling in CIRKO or diabetic hearts

Summary of changes in abundance of proteins involved in fatty acid oxidation, tricarboxylic acid cycle, or oxidative phosphorylation illustrating the number of proteins exhibiting increased or decreased abundance in mitochondria of hearts from CIRKO – Veh, WT – STZ, and CIRKO – STZ mice relative to WT-Veh.

Table 1

Serum metabolites (n=4–5).

	WT-Veh	CIRKO-Veh	WT-STZ	CIRKO-STZ
Serum glucose [mg/dl] [#]	125 ± 17	126 ± 20	470 ± 89	488 ± 57
Serum triglycerides [mg/dl] ^{##} \$	47.4 ± 5.1	53.9 ± 6.7	226.1 ± 19.5	342.8 ± 55.3
Serum free fatty acids [mmol/l] [#]	0.54 ± 0.02	0.67 ± 0.03	1.08 ± 0.17	1.05 ± 0.08
Serum lactate [mmol/l] [#]	54.5 ± 2.5	56.9 ± 9.6	74.6 ± 4.1	69.0 ± 8.7
Serum β -hydroxybutyrate [mmol/l] [#]	0.34 ± 0.04	0.36 ± 0.05	1.07 ± 0.12	1.09 ± 0.17

2-way ANOVA was performed to analyze differences by treatment and genotype, including a Bonferroni post-hoc analysis when significant interaction occurred.

2-way ANOVA

[#] Effect of treatment (p<0.05),

^{*} Effect of genotype (p<0.05),

^{\$} Interaction of genotype and treatment (p<0.05)

Bonferroni post-hoc analysis for serum triglycerides: p<0.05 for WT-Veh vs. WT-STZ and CIRKO-STZ respectively and for CIRKO-Veh vs. WT-STZ, and CIRKO-STZ respectively.