Mitochondrial complex I defect and increased fatty acid oxidation enhance protein lysine acetylation in the diabetic heart

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Aims
Cardiomyopathy is a major complication of diabetes. Our study was aimed to identify the sites of mitochondrial dysfunction and delineate its consequences on mitochondrial metabolism in a model of type 1 diabetes.

Methods and results
Diabetes was induced by streptozotocin injection to male Lewis rats. We found a decrease in mitochondrial biogenesis pathway and electron transport chain complex assembly that targets Complex I. Oxidation of Complex II and long-chain fatty acid substrates support the electron leak and superoxide production. Mitochondrial defects do not limit fatty acid oxidation as the heart’s preferred energy source indicating that the diabetic heart has a significant reserve in Complex I- and II-supported ATP production. Both mitochondrial fatty acid oxidation and Complex I defect are responsible for increased protein lysine acetylation despite an unchanged amount of the NAD⁺-dependent mitochondrial deacetylase sirt3. We quantitatively analysed mitochondrial lysine acetylation post-translational modifications and identified that the extent of lysine acetylation on 54 sites in 22 mitochondrial proteins is higher in diabetes compared with the same sites in the control. The increased lysine acetylation of the mitochondrial trifunctional protein subunit α may be responsible for the increased fatty acid oxidation in the diabetic heart.

Conclusion
We identified the specific defective sites in the electron transport chain responsible for the decreased mitochondrial oxidative phosphorylation in the diabetic heart. Mitochondrial protein lysine acetylation is the common consequence of both increased fatty acid oxidation and mitochondrial Complex I defect, and may be responsible for the metabolic inflexibility of the diabetic heart.

Keywords
Diabetes • Heart • Mitochondria • Oxidative phosphorylation • Lysine acetylation

1. Introduction
Cardiac dysfunction in diabetes can develop independent of diabetes-induced co-morbidities (hypertension, coronary atherosclerosis), a condition called ‘diabetic cardiomyopathy’. Cardiomyopathy induced by either type 1 or 2 diabetes in humans and animal models is characterized by similar cardiac abnormalities including decreased glucose oxidation, mitochondrial bioenergetics, and cardiac function and increased fatty acid (FA) oxidation, lipid storage, and myocardial fibrosis. The diabetic heart is almost completely reliant on mitochondrial FA oxidation for ATP production with decreased insulin-dependent glucose uptake, a condition called metabolic inflexibility. Recent data show that although this substrate selection is a common event, its consequences on mitochondrial metabolism and cardiac function are different in type 1 and type 2 diabetes. The increase in FA oxidation is associated with higher oxygen consumption with unchanged contractile force and decreased cardiac efficiency in mice with type 2 diabetes and obese humans as a result of mitochondrial
uncoupling. In contrast, the increase in FA oxidation is not accom-panied by mitochondrial uncoupling and decreased cardiac efficiency in 2-week streptozotocin-induced and Ins2/−/− Akita mouse models of type 1 diabetes.

The pathogenic mechanism of metabolic inflexibility in the diabetic heart is unknown. The decreased glucose uptake and increased reliance on FA oxidation are an early event upon conditions of increased FA availability such as streptozotocin-induced diabetes and precede insulin resistance in obese human subjects and rodents. It is suggested that both early substrate selection and insulin resistance may be induced by increased lysine acetylation of mitochondrial proteins and components of the insulin signalling. Conflicting data are reported regarding the effect of lysine acetylation on mitochondrial FA oxidation. Individual FA oxidation enzymes are targets of the NAD+-dependent mitochondrial deacetylase sirt3, which deacetylates and activates them. In contrast, in response to a high fat diet, the decrease in sirt3 amount and hyperacetylation of FA oxidation enzymes is associated with an increased FA oxidation in the heart. The Hsp10–Hsp60 chaperone complex-dependent protein folding was identified as a mechanism that controls FA oxidation disregarding the decrease in sirt3.

In addition to this metabolic remodelling, a defect in mitochondrial bioenergetics has been hypothesized to precede cardiac dysfunction in diabetic human subjects. Anderson et al. report a decreased mitochondrial capacity to oxidize NADH-generating fuel substrates in atria of type 2 diabetic patients. However, animal model studies provide conflicting evidence about the mitochondrial dysfunction in the diabetic heart. Conflicting data have been reported about the mitochondrial biogenesis signalling that was found either amplified or decreased. Mitochondrial function in the diabetic heart was reported either unchanged or decreased in type 1 and 2 diabetes in rodents. The decrease in mitochondrial function was revealed by a decline in oxidative phosphorylation when mitochondrial oxidize substrates generating NADH and FADH2 to be oxidized by Complexes I and II. Because both electron routes merge to coenzyme Q and further reduce Complex III, cytochrome c and Complex IV, the defect responsible for mitochondrial dysfunction may be located at the level of any of the downstream electron transport chain (ETC) components. The specific site of the ETC defect has not been investigated, nor were the molecular mechanisms responsible for this defect or its consequences on mitochondrial metabolism delineated. It is also not known whether these mitochondrial defects result in cardiac energy deficit in diabetes. Intriguingly, a genetic defect in mitochondrial complex I in mice did not lead to cardiac dysfunction and energy deficit indicating that Complex I is not limiting for the ATP generation and cardiac function in rodents. The Complex I defect led to a decrease in NAD+/NADH ratio and inhibition of the NAD+-dependent deacetylase sirt3 with an increased lysine acetylation of mitochondrial proteins.

Recently, therapeutic strategies targeted to specific mitochondrial pathways have been proposed to correct mitochondrial dysfunction.

The goal of this work was to better inform potential therapeutic approaches by identifying the ETC defects as pharmacological targets in mitochondria to improve mitochondrial bioenergetics and cardiac function. We report a decrease in mitochondrial biogenesis and ETC assembly that targets Complex I. Defects in Complexes I and II do not limit FA oxidation as the major metabolic fuel in diabetes and therefore are unlikely to decrease the mitochondrial energy production. The lack of Complex I control on FA oxidation contributes to the metabolic inflexibility in the diabetic heart. Mitochondrial complex II and FA oxidation support ROS generation. Both Complex I defect and increased FA oxidation lead to increased protein lysine acetylation despite the unchanged sirt3 amount. By using a proteomic comparison approach, we identified specific lysine residues and peptides that show increased acetylation in diabetic mitochondria. The increased lysine acetylation of the mitochondrial trifunctional protein subunit α may be responsible for the increased FA oxidation and metabolic inflexibility of the diabetic heart.

2. Methods

2.1 Reagents

Unless otherwise specified, all reagents were purchased from Sigma-Aldrich and were of the highest purity grade.

2.2 Animal model

Experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 2011) and approved by the Case Western Reserve University and Central Michigan University Institutional Animal Care and Use Committees. Diabetes was induced by a single intra-peritoneal injection of streptozotocin (55 mg/kg body weight) to 2-month-old male Lewis rats. The duration of diabetes was assessed from the moment the rats develop diabetic hyperglycaemia (~1–2 weeks after the streptozotocin injection). Low doses of NPH insulin were given subcutaneously to avoid severe hypercatabolic state and ketosis. Rats were euthanized with pentobarbital (100 mg/kg). Cardiac tissue was collected from the left ventricle (LV). Control tissue was taken from normal rats that were not injected with streptozotocin.

2.3 Cardiac function

Cardiac function was assessed by two-dimensional guided M-mode, two-dimensional and Doppler flow echocardiography, as described. Systolic and diastolic haemodynamic parameters were evaluated using a Millar pressure transducer catheter introduced into the LV via the right carotid artery.

2.4 Mitochondrial respiratory studies

Cardiac subsarcolemmal (SSM) and interfibrillar (IFM) mitochondria were isolated from the LV using a previously published protocol. Mitochondrial protein concentration was determined by the Lowry method. Mitochondria were investigated for respiratory properties as described.

2.5 Citrate synthase, ETC complexes, and cytochromes

Citrate synthase activity was measured spectrophotometrically at 412 nm. ETC complex activities were measured as specific donor–acceptor oxidoreductase activities, and individual complexes were extracted with β-o-maltose and separated on 6–13 acrylamide–bisacrylamide gradient gel. The amount of the reducible heme in cytochrome c was measured.

2.6 Mitochondrial DNA copy number

Mitochondrial DNA copy number was measured by quantitative real-time PCR (details are provided in the Supplementary material online).

2.7 Measurement of mitochondrial hydrogen peroxide production

Hydrogen peroxide (H2O2) production by intact mitochondria was measured as the oxidation of the fluoroegenic indicator, amplex red, in the presence of substrate concentrations similar to those indicated for oxidative phosphorylation.
2.8 Statistical analysis
The results were analysed using a two-tailed Student’s t-test. Data are reported as mean ± SEM. Significance was established at P < 0.05.

3. Results

3.1 Clinical, biochemical, echocardiographic, and haemodynamic characteristics of control vs. diabetic rats
Diabetic rats did not experience weight loss indicating the absence of a hypercatabolic state. However, the body weight of the diabetic rats was lower than that of the control at the end of the study. Also, diabetic rats experience severe hyperglycaemia over the duration of the study and 10 weeks after the onset of diabetes (see Supplementary material online, Table S1). Heart rate was decreased in the diabetic group leading to a prolongation of the ejection time and of contraction and relaxation times. Diabetes caused a decrease in the end-systolic and end-diastolic dimensions associated with an increase in the LV mass when normalized per body weight, indicating ventricular remodelling. Cardiac output was significantly decreased in the diabetic heart with no change when normalized to body weight. The ejection fraction and fractional shortening were not changed. In vivo LV haemodynamic function showed that peak rates of pressure rise (dP/dt) and fall (−dP/dt) are significantly lower in the diabetic group compared with control, indicating systolic and diastolic dysfunctions.

3.2 Diabetic heart does not exhibit changes in mitochondrial content despite alterations in mitochondrial biogenesis signalling
The activity of mitochondrial marker enzyme, citrate synthase, was not altered by diabetes, and no significant changes in heart SSM and IFM yields were observed (19.2 ± 2.0 vs. 23.0 ± 1.1 mg/g wet weight and 18.0 ± 2.5 vs. 14.0 ± 2.1 mg/g wet weight for SSM and IFM, respectively). Mitochondrial density (milligrams mitochondrial proteins per gram LV tissue) was unchanged by diabetes. Mitochondrial recovery (citrate synthase activity in isolated mitochondria per whole tissue citrate synthase activity) also was similar in control and diabetic hearts (Figure 1A). These data indicate that there are no diabetes-induced changes in cardiac mitochondrial content. In contrast to citrate synthase, mitochondrial DNA copy number was decreased in the diabetic hearts (Figure 1B). The linked activity of Complexes I and III (NADH cytochrome c reductase, NCR), was decreased in the diabetic cardiac tissue homogenate. This deficiency was not due to a decline in the activity of Complex III, suggesting that Complex I may be the cause of the decreased NADH cytochrome c reductase activity (Figure 1C).

The decrease in mitochondrial DNA suggests a dysregulation in mitochondrial biogenesis. The gene expression of mitochondrial biogenesis regulators—the peroxisome proliferator-activated receptor γ co-activator α (PGC-1α), estrogen-related receptor α (ESRRA), and mitochondrial transcription factor A (TFAM)—is not altered in the diabetic heart; nuclear respiratory factor 1 (NRF1) mRNA tends to be decreased but is not significant (see Supplementary material online, Figure S1). In contrast with gene expression, the PGC-1α and TFAM proteins are decreased in diabetes, with no change in the NRF1 (Figure 1D).

3.3 Diabetic heart mitochondria show a decrease in oxidative phosphorylation through Complexes I and II with specific substrates except FAs
State 3 respiratory rate (ADP-dependent) with glutamate is decreased in both SSM and IFM isolated from the diabetic hearts. State 4 respiratory rates (ADP-limited) were unchanged, as were the respiratory control ratios and ADP/O ratios, indicating that the coupling ability is preserved in diabetic mitochondria (see Supplementary material online, Table S2). The decrease in glutamate oxidation may be due to a decrease in either mitochondrial glutamate uptake, NADH formation via glutamate dehydrogenase, NADH oxidation and electron transport via ETC or the ADP phosphorylation via the phosphorylation apparatus. The decrease in glutamate oxidation was insensitive to the experimental collapse of mitochondrial potential with an uncoupler, dinitrophenol (DNP), excluding the phosphorylation system as the cause of the defect (data not shown). The decrease in pyruvate oxidation in diabetic mitochondria (Figure 2A and B) may be due to alterations in pyruvate transporter and dehydrogenase, ETC, and the phosphorylation apparatus. Since the oxidation of both substrates donates reducing equivalents to Complex I via the generation of NADH, the data suggest that NADH oxidation by Complex I rather than NADH formation is affected in diabetes.

Succinate donates electrons to FAD in Complex II and generates significantly lower respiratory state 3 rates in both cardiac SSM and IFM from the diabetic hearts compared with the control; this decrease also was insensitive to the uncoupler, suggesting that either the succinate transporter or components of the ETC including Complex II and downstream sites (Complex III, cytochrome c, Complex IV) are defective in diabetes. However, respiratory state 3 rates supported by duroquinol (Complex III electron donor) and the cytochrome c electron donor TMPD-ascorbate were not significantly changed, indicating that Complexes III and IV are functionally intact (Figure 2A and B). These data narrow the potential sites of ETC defect to Complex I, II, or coenzyme Q.

To investigate the changes in mitochondrial substrate preference in the diabetic heart, we assessed respiratory rates with a long-chain FA substrate. Cardiac SSM from diabetic animals shows an increase in state 3 respiration with palmitoylcarnitine (Figure 2A), whereas IFM capacity to oxidize palmitoylcarnitine is unchanged (see Supplementary material online, Figure S2A). In addition, the specific activities of the medium- and long-chain acylCoA dehydrogenases (MCAD, LCAD) as well as the MCAD amount are increased in SSM (see Supplementary material online, Figure S2B).

3.4 Mitochondria from diabetic hearts exhibit Complex I and II defects
The specific activity of Complex I (rotenone-sensitive NADH coenzyme Q oxidoreductase) as well as the Complex I dehydrogenase activity (NADH ferricyanide reductase, NFR) was decreased in both populations of diabetic heart mitochondria (Figure 3A and B). The specific activity of Complex II also was decreased in cardiac mitochondria in diabetes and was not improved by the addition of exogenous coenzyme Q (+Q), indicating that coenzyme Q deficiency is not responsible for the mitochondrial defect. The specific activities of Complexes III and IV were not changed (data not shown).

To summarize, both SSM and IFM from the diabetic hearts show a decrease in oxidative phosphorylation through Complexes I and II induced by the decline in Complex I- and II-specific activities. The
Figure 1 Mitochondrial biogenesis in the diabetic heart. (A) Citrate synthase activity, mitochondrial density (mg mitochondrial protein/g heart tissue) and recovery (percentage of citrate synthase activity in mitochondria reported to that in the heart tissue). (B) Mitochondrial DNA. (C) Electron transport chain complex activities measured spectrophotometrically as oxidoreductase activities and expressed as micromoles/min/g cardiac tissue except Complex IV (cytochrome c oxidase) which is expressed as the first-order rate constant (k = 1/min/g cardiac tissue). (D) Mitochondrial biogenesis regulators. NCR, NADH Cytochrome c Reductase; C III, Complex III; C IV, Complex IV; PGC-1α, peroxisome proliferator-activated receptor γ co-activator α; TFAM, mitochondrial transcription factor A; NRF-1, nuclear respiratory factor 1; GAPDH, glyceraldehyde 3 phosphate dehydrogenase (the lower band was used for densitometry). *P < 0.05 control (n = 4–7) vs. diabetic (n = 3–7). Mean ± SEM.
oxidation of FA substrates is not limited by these specific mitochondrial defects. Given that cardiac SSM and IFM do not differ in their response to diabetes, we performed the subsequent analysis on SSM only.

3.5 The amount and assembly of the Complex I subunits are decreased in the diabetic heart

Mitochondrial ETC defects may be due to either decreased amount of protein subunits, lack of their assembly in complexes or supercomplexes, or post-translational modifications.

We reported that mitochondrial biogenesis is altered in the diabetic heart (Figure 1). We also found a decreased amount of the nuclear-encoded Complex I subunit NDUFB8 in both cardiac tissue and isolated SSM. The amount of Complex II SDHB was unchanged. There were no changes in Complex III subunit UQCRC2, ATPase subunit A, as well as in Complex IV Subunit IV (Figure 4A). The fully assembled monomeric complex I (Figure 4B) as well as Complex I-containing supercomplexes (see Supplementary material online, Figure S4) were reduced in mitochondria from the diabetic heart. We found that the mitochondrial apoptosis-inducing factor (AIF)—one of the factors involved in the assembly of Complex I—also was reduced relative to the amount in the whole LV homogenate, suggesting that the redistribution of AIF from mitochondria to extramitochondrial sites may contribute to the decreased complex assembly (Figure 4C).

3.6 Increased ROS-generating capacity of diabetic mitochondria is supported by oxidation of FA and Complex II substrates

Diabetic heart mitochondria oxidizing palmitoylcarnitine (Figure 5) produce a larger amount of H$_2$O$_2$ than the control. Rotenone limits the electron flux from Complexes I to III and leads to an increase in H$_2$O$_2$ generation in both groups. Antimycin A induces a complete reduction of centres in the ETC known to be responsible for electron leakage (the Complex III Qo site and the upstream Complex I centres) and caused similar electron leakage by control and diabetic mitochondria. The electron leak did not decrease when inhibiting the electron supply from Complex I to III with rotenone. When oxidizing succinate at Complex II and the reverse electron flow towards Complex I was inhibited with rotenone, diabetic cardiac mitochondria produced more H$_2$O$_2$ than the control. H$_2$O$_2$ was dissipated by catalase to a similar extent, indicating that the increase in the measured fluorescence was because of H$_2$O$_2$ and not induced by the chemical reaction of Amplex red with FA hydroperoxides.

A potential loss of cytochrome c by the diabetic mitochondria may be responsible for the decrease in the oxidative phosphorylation and increased superoxide generation since oxidized cytochrome c is a superoxide removal mechanism operating in the intermembrane space. The superoxide generated within the mitochondrial matrix and intermembrane space is converted into H$_2$O$_2$ by the
manganese (Mn) and zinc (Zn) superoxide dismutase (SOD) enzymes, respectively. We show that the amount of mitochondrial cytochrome c (both protein and heme) is unchanged by diabetes (see Supplementary material online, Figure S5A and B). The amounts of Mn-and Zn-SOD are also unchanged (see Supplementary material online, Figure S5C and D) indicating that changes in superoxide defense mechanisms are not responsible for mitochondrial dysfunction or ROS generation in the diabetic heart.

3.7 The protein lysine acetylation is enhanced in the diabetic hearts

We found that lysine acetylation in both mitochondria (Figure 6A) and LV homogenate (Figure 6B) is increased in diabetes. We compared the level of lysine acetylation modifications in mitochondria oxidizing the long-chain FA, palmitoylCoA (+-carnitine + malate) vs. glutamate, and observed that oxidation of palmitoylCoA leads to increased acetylation of mitochondrial proteins (Figure 6C). The amount of the NAD\(^+\)-dependent mitochondrial lysine deacetylase, sirt3, is unchanged (Figure 6D).

Lysine acetylated proteins (Figure 6A) were identified by proteomic analysis, and comparison of the level of acetylation was performed between the control and diabetic group. We identified 850 peptides belonging to 111 proteins that were collected in both control and diabetic mitochondria (see Supplementary material online, Table S3 for the full proteomic survey). We identified 54 lysine acetylation sites in 22 mitochondrial proteins (Table 1), indicating that mitochondria are important targets of this post-translational modifications in diabetes. The extent of acetylation on these sites in diabetic mitochondria is higher compared with the same sites in normal mitochondria. No difference was observed in the expression levels of those 22 proteins (see Supplementary material online, Table S3) indicating that the increased lysine acetylation is not due to their increased expression. Similar proteins were identified in the heart homogenates (see Supplementary material online, Table S4).

4. Discussion

Diabetic cardiomyopathy develops in both absence (type 1 diabetes) and resistance (type 2 diabetes) to insulin.\(^2\) In humans, type 2 diabetes is more frequent than type 1 diabetes and is usually preceded by obesity-induced insulin resistance and hyperinsulinaemia. Upon insulin resistance, although glucose uptake is impaired, intact parts of the

Figure 3 Specific activities of mitochondrial electron transport chain Complexes I and II in isolated SSM (A) and IFM (B). Mitochondrial complexes were assessed spectrophotometrically as specific donor–acceptor oxidoreductase activities. C I, Complex I; NFR, NADH ferricyanide reductase; C II, Complex II; Q, Q-coenzyme. *P < 0.05 control (n = 7) vs. diabetic (n = 7). Mean ± SEM.
insulin signalling (i.e. Akt) may facilitate the anabolic effect of hyperinsulinaemia and cause cardiac hypertrophy.\textsuperscript{32} The use of a type 1 diabetes model in our study eliminates the effect of hyperinsulinaemia on left ventricular changes.

To appropriately assess the full extent of cardiac remodelling and contractile dysfunction in our model, we incorporated evaluations by both echocardiography and pressure–volume catheterization. We report decreased heart rates in the diabetic rats, a finding consistent with

**Figure 4** Fully assembled mitochondrial complex I is decreased in the diabetic heart. (A) Amount of mitochondrial electron transport chain complex subunits in the heart homogenate \((n = 5)\). (B) Amount of the fully assembled electron transport chain complexes was assessed after the separation of complexes by Blue Native PAGE electrophoresis \((n = 4)\). The densitometric analysis is shown as the percentage of the specific complex band reported to the sum of all complex band intensity. (C) AIF in mitochondria vs. cardiac tissue \((n = 5)\). The band marked with the arrow in the heart tissue was used for densitometry. *\(P < 0.05\) control vs. diabetic. Mean ± SEM.
other studies, and proposed to be caused by autonomic cardiac neuropathy with decreased adrenergic stimulation.\textsuperscript{33} The systolic and diastolic dysfunctions are mild, whereas signs of ventricular remodelling predominate in our model; these data are not consistent with those reported by Choi et al.\textsuperscript{34} who reported cardiac dysfunction in diabetes without changes in ventricular chamber and wall dimensions. This discrepancy may be reconciled by the lack of insulin treatment in the latter study compared with ours, which may be responsible for the hypercatabolic state that limits protein synthesis and heart hypertrophy. The heart rate affects the contractile function by the Bowditch effect. In contrast with other animal species in which the contractile force–heart rate relationship is positive,\textsuperscript{35} in rat the force–frequency relationship has a bell shape. At low non-physiological heart rates, the relationship is positive. However, at a heart rate within the physiological range (200–500 bpm), the slope is slightly negative meaning that the contractile force is higher at lower heart rate. In our study, the heart rate in diabetes is in the normal range and lower than the control; therefore, the real diabetic-induced decrease in dp/dt is slightly larger than that measured, and cardiac dysfunction is greater than that assessed by our method.

We show that the mild LV dysfunction and remodelling are associated with severe Complex I and II defects, and the impairment of cardiac mitochondrial function develops early during the evolution of type 1 diabetes before heart failure develops. Our results showing a decrease in Complex I-dependent cardiac mitochondrial respiration in a model of type 1 diabetes are similar to those of Anderson’s et al.\textsuperscript{16} who also observed a decrease in NADH-dependent cardiac mitochondrial respiration in atrial tissue of 45- to 65-year-old patients with type 2 diabetes. This similarity suggests that the presence of mitochondrial dysfunction and the site of the defect do not depend on the type or duration of diabetes.

Our study identifies the decreased NADH coenzyme Q oxidoreductase and succinate-coenzyme Q oxidoreductase of Complexes I and II, respectively, as the mechanisms for the decreased mitochondrial oxidative phosphorylation in the diabetic heart. The pathogenesis of mitochondrial electron transport defects in diabetes is complex and involves either decreased amount (decreased synthesis or increased degradation) or loss of catalytic activity of their subunits. Synthesis of ETC protein subunits is part of mitochondrial biogenesis that is governed by a hierarchical set of transcription factors. NRF1 is activated by PGC-1α through direct physical interaction and induces the expression of genes encoding for both ETC protein subunits and nuclear transcription factors involved in mitochondrial DNA transcription and replication (TFAM). We found a decrease in mitochondrial biogenesis manifested as a decrease in PGC-1α and TFAM, and mitochondrial DNA with no change in NRF1. Changes in the biogenesis factor amounts in our rat model are consistent with the type 1 diabetic Akita mice\textsuperscript{18} but do not match with their gene expression level possibly because of specific post-translational modifications induced by chronic diabetes leading to protein degradation.

Due to its complexity (46 protein subunits), Complex I is the major target of the mitochondrial biogenesis deficiency. Specific alterations in mitochondrial proteome also occur in type 1 diabetes.\textsuperscript{36} While the amount of specific FA oxidation enzymes is increased, protein subunits of electron transport, specifically Complex I, are decreased.\textsuperscript{18} In addition to the mitochondrial proteome remodelling, protein post-translational modifications have been reported in the diabetic hearts including nitration,\textsuperscript{37} oxidation,\textsuperscript{38} and acetylation\textsuperscript{12} that may cause mitochondrial dysfunction. Multiple lysine acetylation sites were reported in Complex I and II in the heart in basal conditions.\textsuperscript{39} Lysine acetylation has also been reported to inhibit the activity of Complexes I\textsuperscript{40} and II\textsuperscript{41} in sirt3-deficient models. We did not find Complex I or II with different level of lysine acetylation in the control vs. diabetic mitochondria, indicating that a change in lysine acetylation is not responsible for Complex I and II defects in the diabetic hearts in our model.

Despite the depressed mitochondrial biogenesis pathway and mtDNA, there is no decrease in mitochondrial density. PGC-1α deletion does not change mitochondrial density whereas it leads to impaired expression of particular oxidative phosphorylation genes in the adult life.\textsuperscript{42,43} Similarly, TFAM deficiency does not change mitochondrial density until advanced stages, whereas mitochondrial function is severely impaired at Complex I.\textsuperscript{44} These data support our finding that mtDNA and Complex I are critical targets of mitochondrial biogenesis signalling alterations.
Protein lysine acetylation in mitochondria and heart homogenate. (A) Lysine acetylation modifications on proteins extracted from isolated mitochondria from control and diabetic hearts. (B) Lysine acetylation modifications on protein extracts from control and diabetic heart homogenates. Densitometric analysis is shown as lysine acetylation reported to actin as a loading control. (C) Regulation of protein lysine acetylation by mitochondrial long-chain fatty acid β-oxidation. Lysine acetylation modifications in control mitochondrial oxidizing 20 mM glutamate (1) or 0.04 mM palmitoylCoA + carnitine + malate for 2 min (2). (D) The amount of mitochondrial NAD⁺-dependent deacetylase, sirt3. *P < 0.05 control (n = 3–5) vs. diabetic (n = 3–7). Mean ± SEM.
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<tr>
<td>ATP synthase subunit b</td>
<td>K.AQQLVOKK.R.H</td>
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<td>R.KEKAQQLVOKK.R</td>
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<td>Acetyl-CoA acetyltransferase</td>
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<td></td>
<td>R.GATPYGGVKLEDLIVKD.D</td>
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<td>Enoyl-CoA delta isomerase 1</td>
<td>K.LENDQKR.G</td>
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<td>Fumarate hydratase, mitochondrial</td>
<td>K.KVHPHNPDHNK.S</td>
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<tr>
<td></td>
<td>R.GFRAKVAK.V</td>
<td>9.8</td>
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Mitochondrial bioenergetics in the diabetic heart

We show that diabetes causes a Complex I defect that is reflected as a decrease in the NADH dehydrogenase activity in Complex I, as reflected by the decrease in oxidation of ferricyanide (electron acceptor) and a larger decrease in Complex I-specific activity (oxidation of NADH and reduction of coenzyme Q); these data indicate the presence of additional defects within the Complex I electron route downstream of the NADH dehydrogenase. The 50% decrease in Complex I-specific activity causes a decrease of a similar extent in the glutamate- and pyruvate-supported mitochondrial oxidative phosphorylation but is not limiting for FA-supported respiration. There is an apparent excess of ETC complex activities relative to the need for the overall oxidative phosphorylation, and a decrease in specific activity of an individual complex may not lead to a decrease of similar extent in oxidative phosphorylation. In the heart, mitochondrial FA β-oxidation is the major source of acetylCoA and reducing equivalents, FADH2 and NADH, that are donated to the ETC at coenzyme Q (via electron transfer flavoprotein) and Complex I, respectively. Due to this functional link, it is expected that the inhibition of either of these connecting steps leads to a decrease in FA oxidation. However, humans with <50% Complex I activity and amount do not experience defects in oxidation of fatty acids in skin fibroblasts. A similar decrease in non-lipid substrate-supported mitochondrial respiration was reported in a OVE26 mouse model of type 1 diabetes, suggesting that Complex I has limited control on FA β-oxidation during diabetes. We also observed that in diabetes the control of Complex I activity on the overall mitochondrial oxidative phosphorylation differs when mitochondria are energized with non-lipid substrates. The parallel decrease in glutamate-supported oxidative phosphorylation is due to the inhibition of mitochondrial glutamate dehydrogenase by NADH that is accumulated secondary to the Complex I defect. Mitochondrial β-oxidation flux is also dependent on the recycling of cofactors NADH and NAD+ as there is a requirement for NAD+ by the 3-hydroxyacyl-CoA dehydrogenase. In parallel, the NAD+ /NADH ratio also regulates mitochondrial FA oxidation by controlling the level of lysine acetylation of the enzyme components. In contrast with the concept that the decrease in NAD+ limits FA oxidation, under increased FA availability (high fat diet, fasting) A decreased NAD+ -dependent sirtuin 3 lysine deacytlation is associated with an increase in FA oxidation via regulating the mitochondrial protein folding. Our data indicate that the limiting step of glutamate- and pyruvate-supported respiration is located at the level of Complex I, whereas fatty acid-supported mitochondrial oxidative phosphorylation is not limited by Complex I. Because the ADP-dependent oxygen consumption rates supported by long-chain FA (the major energetic fuel for the diabetic heart) are not limited by the Complex I defect, and the oxygen consumption is coupled with ATP production, we conclude that mitochondrial Complex I and II defects do not affect mitochondrial ATP production. Similar observations were reported by Karamanlidis et al. in the heart in a murine model of cardiac Complex I defect.

We also report an increased capacity of diabetic cardiac mitochondria to produce H2O2 which is supported by oxidation of FA and Complex II substrates. Because FA supply electrons to both Complex I and III, multiple sites of electron leakage may be revealed upon their oxidation. Rotenone limits the electron flux from Complex I to III. The full reduction of electron leakage sites in Complex I (FMN-containing NADH binding site and the ubiquinone reduction site) with rotenone strongly limits FA oxidation, under increased FA availability. Antimycin A fully reduces the proximal Qo site in Complex III and leads to similar electron leakage indicating that the control and diabetic heart mitochondria do not differ in their maximal capacity to generate ROS. The Qo site is supplied with electrons both from Complex I and the FA β-oxidation. The electron leak does not decrease when inhibiting the electron supply from Complex I to III with rotenone, indicating that the electrons responsible for leakage are supplied by FA β-oxidation. When oxidizing

**Table I** Continued

<table>
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<th>Entry</th>
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<td>sp</td>
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<td>SRBP1_RAT</td>
</tr>
</tbody>
</table>

Bold: acetylation at K.

The exact value is not shown, because this quantitative method cannot provide accurate value when the peptide ratios exceeds >10. Exact value can be seen in Supplementary material online, Table S1.
succinate at Complex II and the reverse electron flow towards Complex I is inhibited with rotenone, diabetic cardiac mitochondria produce more H₂O₂ than the control. The site of electron leak may be Complex II or Qo site in Complex III. When Qo site in Complex III is fully reduced with antimycin A, the maximal capacity of the Qo site to leak electrons is equal in control and diabetic heart mitochondria. There is no Complex III defect in diabetes, and therefore, there is no antimycin-like inhibition. Antimycin inhibition is also not a physiological condition, the full reduction of Qo site is never obtained in vivo, and the maximal capacity of the Qo site to leak electrons is not reached in our diabetic model. These data suggest that Complex II components may be sites of electron leak in diabetic heart mitochondria.

We found a dramatic increase in the amount of protein lysine acetylation in the diabetic heart tissue and mitochondria, and propose that this phenomenon is due to both increased acetylation and decreased deacetylation reactions in the diabetic heart. Acetyl-CoA is the limiting factor for lysine acetylation. Because the Complex I defect does not limit mitochondrial FA β-oxidation, which remains even higher than the control in diabetic SSM, we propose that the continuous generation of acetyl-CoA from mitochondrial FA β-oxidation facilitates lysine acetylation. In support of our proposal, mitochondrial oxidation of the long-chain acyl-CoA derivative, palmitoyl-CoA, increases the level of lysine acetylation on mitochondrial proteins (Figure 6B). The amount of NAD⁺-dependent sirt3 (the master mitochondrial deacetylase) is unchanged, indicating that the decrease in sirt3 amount is not required for the increased lysine acetylation and activation of FA oxidation enzymes in the diabetic heart. This observation linked with the knowledge that fat oxidation provides a superior net production of acetyl-CoA (donor for lysine acetylation) and consumes NAD⁺ (cofactor for lysine deacetylation), sirtuins indicates that the increased FA oxidation is responsible for increased lysine acetylation. In addition, a recent report supports our observation that the Complex I defect limits the NAD⁺-dependent deacetylation rather than leading to energy deficit. These data indicate that both Complex I defect and increased FA oxidation leads to increased lysine acetylation in the diabetic heart.

Although a large number of proteins have been reported to be acetylated in the liver and heart in basal conditions and upon high fat diet, our study is the first that uses a screening proteomic approach to compare the intensity of acetylation of specific lysine residues on mitochondrial proteins in the control vs. diabetic hearts. We found that one of the lysine acetylation targets is the mitochondrial trifunctional protein complex subunit α, including the short- and long-chain 3-hydroxyacyl-CoA dehydrogenase and 2.3 enoyl-CoA hydratase, which mediates critical steps in FA oxidation. Recent studies show conflicting data regarding the effect of lysine acetylation on FA oxidation. For example, medium- and long-chain acyl-CoA dehydrogenases and 3-hydroxyacyl-CoA are sirt3 targets and activated by deacetylation. In contrast, high fat diet and fasting are associated with increased lysine acetylation of mitochondrial FA oxidation enzymes and activation of the FA oxidation pathway via decreased sirt3 amount. A recent report advances the understanding of this paradox by showing that the decrease in sirt3 amount favors the correct folding and integrity of FA oxidation enzymes and increases fat oxidation during increased FA supply. Our study includes the diabetic heart in this paradigm and shows that mechanisms other than decreased sirt3 amount are responsible for increased lysine acetylation (Figure 7). The increase in lysine acetylation of enzymes of FA oxidation pathway may be responsible for the metabolic inflexibility in the diabetic heart.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

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**References**


8. Bugger H, Boudina S, Hu XX, Turner J, Zaha VG, Theobald HA, Yun UJ, McQueen AP, Wayment B, Litwin SE, Abel ED. Type 1 diabetic akita mouse hearts are insulin sensitive.

![Figure 7](image-url)
but manifest structurally abnormal mitochondria that remain coupled despite increased uncoupling protein 3. Diabetes 2008;57:2924–2932.


