Type 1 Diabetic Akita Mouse Hearts are Insulin Sensitive but Manifest Structurally Abnormal Mitochondria that Remain Coupled Despite Increased Uncoupling Protein 3

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ABSTRACT

Objective: Fatty acid (FA)-induced mitochondrial uncoupling and oxidative stress have been proposed to reduce cardiac efficiency and contribute to cardiac dysfunction in type 2 diabetes. We hypothesized that mitochondrial uncoupling may also contribute to reduced cardiac efficiency and contractile dysfunction in the type 1 diabetic Akita mouse model (Akita).

Research Design and Methods: Cardiac function and substrate utilization was determined in isolated working hearts, and in vivo function by echocardiography. Mitochondrial function and coupling were determined in saponin-permeabilized fibers and proton leak kinetics in isolated mitochondria. H$_2$O$_2$ production and aconitase activity were measured in isolated mitochondria, and total ROS in heart homogenates.

Results: Resting cardiac function was normal in Akita mice, and myocardial insulin sensitivity was preserved. Although Akita hearts oxidized more fatty acids, MVO$_2$ was not increased and cardiac efficiency was not reduced. ADP-stimulated mitochondrial oxygen consumption and ATP synthesis were decreased, and mitochondria showed grossly abnormal morphology in Akita. There was no evidence of oxidative stress and despite a 2-fold increase in UCP3 content, ATP/O ratios and proton leak kinetics were unchanged, even following perfusion of Akita hearts with 1mM palmitate.

Conclusions: Insulin-deficient Akita hearts do not exhibit FA-induced mitochondrial uncoupling, indicating important differences in the basis for mitochondrial dysfunction between insulin-responsive type 1 versus insulin resistant type 2 diabetic hearts. Increased UCP3 levels do not automatically increase mitochondrial uncoupling in the heart, which supports the hypothesis that FA-induced mitochondrial uncoupling as exists in type 2 diabetic hearts requires a concomitant increase in ROS generation.
Cardiac efficiency (CE) is the ratio of cardiac work to myocardial O₂ consumption (MVO₂). Impaired CE has been proposed to be an underlying mechanism leading to cardiac contractile dysfunction in type 2 diabetes (1; 2). In type 2 diabetic db/db and ob/ob mice, increased FA utilization and MVO₂ are not accompanied by a proportionate increase in contractile function, resulting in reduced CE (3; 4). We previously demonstrated that FA-induced mitochondrial uncoupling occurs in the hearts of these mice and likely results from activation of cardiac uncoupling proteins (5; 6). Thus, increased FA utilization in type 2 diabetic hearts is associated with mitochondrial uncoupling, which leads to decreased ATP production. This prevents a proportionate increase in cardiac work and results in reduced CE (1). The presence of increased mitochondrial ROS production in db/db mice, and the ability of ROS to activate uncoupling proteins, also suggested that FA-induced mitochondrial uncoupling in these hearts required the presence of increased ROS (6; 7). While FA-induced uncoupling may contribute to reduced CE in type 2 diabetic models, it is not known if mitochondrial uncoupling also contributes to impaired cardiac efficiency and contractile dysfunction in models of type 1 diabetes.

Most studies of type 1 diabetes in rodents have been performed after induction of diabetes with streptozotocin. In streptozotocin-diabetic mice, many cardiac alterations are similar to type 2 diabetic hearts, such as increased FA oxidation, reduced glucose oxidation, impaired mitochondrial respiration, oxidative stress, and impaired contractile function (8-11). In addition, it was reported that CE was reduced in streptozotocin-injected animals, and expression of UCP3 was increased (8; 12). These similarities to type 2 diabetic models prompted us to investigate in this study if mitochondrial uncoupling also contributes to impaired CE and contractile dysfunction in type 1 diabetic models. To date, measurements of cardiac state 4 respiration rates and ADP/O ratios performed in streptozotocin-injected animals have yielded conflicting results, making it difficult to make any conclusions about the presence or absence of mitochondrial uncoupling in this model of type 1 diabetes (8; 13-15).

A disadvantage of the streptozotocin model are potential extra-pancreatic toxic effects, in particular when high-dose streptozotocin treatment is used (16). In addition, the severity of diabetes can vary considerably in this model and mitochondrial dysfunction may or may not occur (17). To circumvent these concerns, genetic models of type 1 diabetes are increasingly used, and the Animal Models of Diabetic Complications Consortium (AMDCC) has proposed the Akita diabetic mouse as a useful model with which to study the chronic complications of type 1 diabetes (18). This mouse develops diabetes as a consequence of a single base pair substitution in the Ins2 gene, resulting in improper folding of proinsulin which leads to protein aggregate-induced endoplasmic reticulum stress in pancreatic islets (19; 20) and eventual β-cell failure (19). Akita mice are severely hyperglycemic by 5 to 6 weeks of age and develop typical chronic complications of diabetes, such as retinopathy, neuropathy, and nephropathy (21; 22).

In this study, we hypothesized that mitochondrial uncoupling may also contribute to reduced cardiac efficiency and contractile dysfunction in type 1 diabetes. To test our hypothesis, we measured cardiac contractile function, energy substrate metabolism, and mitochondrial function and coupling in the type 1 diabetic Akita mouse model (Akita). The main finding of this study was that Akita hearts are protected from FA-induced
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mitochondrial uncoupling despite increased UCP3 expression. Our data implicate that mechanisms for mitochondrial dysfunction differ importantly between insulin-deficient type 1 and insulin resistant type 2 diabetic hearts.

METHODS

Animals – Male Akita mice (C57BL/6) and C57BL/6 controls were obtained from Jackson Laboratories (Bar Harbor, ME), and housed at 22°C with free access to water and food with a light cycle of 12h light and 12h dark. Animals were studied in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of Utah. All studies were performed in random-fed animals.

Serum analyses: Free fatty acid concentrations, triacylglycerol levels and blood glucose levels were measured as previously described (4).

Echocardiography: Contractile function and left ventricular chamber dimensions were measured as described before (23).

Heart perfusions: Contractile function and substrate metabolism were measured in isolated working hearts perfused with Krebs Henseleit buffer (KHB) containing 5mM glucose and 0.4mM palmitate in the presence or absence of 1nM insulin as previously described (3). For some mitochondrial respiration studies, hearts were perfused in the Langendorff mode with KHB containing 11mM glucose and 1mM palmitate for 30min (5). The effect of a calcium-induced increase in workload on contractility was investigated in Langendorff perfused hearts with KHB buffer (5mM glucose, 0.4mM palmitate) (5). Hearts were perfused for 40min, and calcium was raised from 2mM to 4mM after 20min.

Mitochondrial function: Saponin-permeabilized fibers were prepared from freshly excised or Langendorff pre-perfused hearts as described before (5). Respiration and ATP synthesis were measured using palmitoyl-carnitine (20µM), pyruvate (10mM), or glutamate (5mM) as substrates, each combined with malate (2mM). Respiration was measured in the presence of substrate alone (V0), after addition of 1mM ADP (VADP), and following addition of 1µg/ml of the FoF1-ATPase inhibitor oligomycin (VOligo).

Proton leak kinetics: Proton leak measurements were performed in hearts that were pre-perfused for 20min with 11mM glucose and 1mM palmitate. Mitochondria were prepared from whole hearts by differential centrifugation, and proton leak kinetics measured in the presence of 20µM palmitoyl-carnitine as previously described (6; 24).

Electron microscopy: Hearts were freshly excised and immediately washed in ice-cold saline. Samples were collected from left ventricular myocardium and processed as described before (6). Mitochondrial volume density and number were analyzed by stereology in a blinded fashion using the point counting method (6; 25).

Hydrogen peroxide (H2O2) production: Mitochondrial H2O2 generation was measured using succinate as a substrate as described before (6). Rotenone (10 µmol/L) was added to the reaction to block H2O2 production at Complex I. Mitochondrial H2O2 production is widely accepted as a measure of mitochondrial superoxide production.

Oxidative stress: Tissue ROS levels were measured by the conversion of non-fluorescent 2’, 7’-dichlorofluorescein-diacetate (DCFDA) to the highly fluorescent 2’, 7’-dichlorofluorescein (DCF) in the presence of esterases and ROS (26). (For details see Supplementary Methods). Activity of mitochondrial aconitase was measured in isolated mitochondria as described before (27).

RNA extraction and quantitative RT-PCR: Total RNA was extracted from hearts with Trizol reagent (Invitrogen Corporation,
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Carlsbad, CA), purified with the RNEasy Kit (Qiagen Inc., Valencia, CA) and reverse transcribed (6). Data were normalized by expressing them relative to the levels of the invariant transcript 16S RNA. Primer sequences and accession numbers are presented in Supplementary Table 1.

**Western-blot analysis:** Isolated mitochondria or whole cell extracts were prepared as described before and resolved by SDS-PAGE (5; 6). (For antibody conditions see Supplementary Methods). Protein concentrations were determined using the Micro BCA Protein Assay Kit (Pierce, Rockford, IL), and equal protein loading was verified by Coomassie staining.

**Statistical analysis** – Data are presented as means ± SEM. When comparing two groups, significance was determined using a student’s t-test. Effects of calcium-induced increase in contractility were analyzed using a paired t-test. Analysis of working heart perfusions ± insulin was performed using 1-way ANOVA. Significance was assessed by Fisher’s protected least significant difference test. For all analyses, the Statview 5.0.1 software package was used (SAS Institute, Cary, NC), and significant difference was accepted when p<0.05.

**RESULTS**

**Serum metabolites and cardiac growth.** Akita diabetic mice develop type 1 diabetes as early as 5 to 6 weeks of age, show normal survival until 6 months of age, but then show a dramatic decrease in survival with almost no survivors by 12 months of age (19). To investigate the long-term effects of type 1 diabetes on the heart, we chose to investigate mice mainly at 24 weeks of age. Serum glucose levels were markedly increased in Akita mice at 24 weeks of age (138 ± 5 vs. 566 ± 21 mg/dl; p<0.05). Hyperglycemia was accompanied by increased serum fatty acid levels (261 ± 43 vs. 537 ± 72 μmol/l; p<0.05) and increased serum triacylglycerol levels (0.98 ± 0.11 vs. 2.40 ± 0.54 mmol/l; p<0.05). In contrast to WT, Akita mice showed no increase in body weights as they aged (Supplementary Table 2). Heart weights and tibia lengths were measured at 10, 24, and 54 weeks of age in Akita (Supplementary Table 2). While heart-to-tibia length-ratios were not different at 10 weeks, we observed reduced ratios at 24 and 54 weeks of age. Heart weight to body weight ratios were not reduced, indicating that the reduction in heart size parallels the general catabolic state of diabetic Akita mice.

**Cardiac Function.** Cardiac contractile function and chamber dimensions were examined in Akita diabetic mice by echocardiography at 20, 36, and 54 weeks of age. Fractional shortening and ejection fraction were not different at any age (Table 1), and there were no differences in chamber dimensions in diabetic and non-diabetic mice (Supplementary Table 3). Cardiac output was increased at 20 weeks of age but was lower at 54 weeks of age in Akita, which was likely due to a moderate but non-significant reduction in heart rate. Since adaptations of the neuro-humoral system can mask the presence of contractile dysfunction in vivo, we investigated contractile function in isolated working hearts in the absence and presence of insulin (Fig. 1 A-C). In the absence of insulin, left ventricular developed pressure was reduced in 24 week-old Akita and a trend towards increased cardiac output in Akita hearts led to no differences in cardiac power. Insulin had a positive inotropic effect in WT hearts, which was blunted in Akita mice. Heart rates (beats per minute) were not significantly changed from baseline by insulin (basal vs. insulin: WT 263 ± 8 vs. 281 ± 8; Akita 290 ± 5 vs. 310 ± 9). To further evaluate myocardial contractile reserve Langendorff perfused hearts were examined under normal and increased calcium concentrations. Contractile parameters were similar in 15 week-old WT and Akita mice.
under baseline conditions (2mM calcium). Changing perfusion conditions to 4mM calcium, significantly increased systolic pressure, developed pressure, and rate pressure product in WT and Akita mice by equivalent extents (Supplementary Table 4). Thus, despite persistent hyperglycemia, Akita mice exhibited modest LV dysfunction that was evident mainly in insulin perfused working hearts. In contrast to *ob/ob* mice (5), short-term inotropic reserves were maintained.

Substrate metabolism, insulin signaling and cardiac efficiency. Decreased CE is believed to be an important contributor to the development of contractile dysfunction in type 2 diabetic models and has been postulated to result from FA-induced mitochondrial uncoupling (1). We therefore measured cardiac substrate utilization and oxygen consumption in isolated working hearts of 24 week-old Akita mice in the absence and presence of insulin. Palmitate oxidation rates were increased and glucose oxidation rates were decreased in the presence or absence of 1nM insulin in Akita hearts compared to WT (Fig. 1 D, E). Despite increased FA oxidation, MVO$_2$ and CE were not different between WT and Akita (Fig. 1 G, H). Of interest, metabolic insulin responsiveness was retained in Akita hearts. Thus insulin suppressed palmitate oxidation increased glycolysis and glucose oxidation both in WT and Akita (Fig. 1 D, E, F). Consistent with intact metabolic insulin signaling, insulin-stimulated Akt phosphorylation on Ser$^{473}$ was maintained in hearts of 10 and 24-week-old mice (Figure 2 A, B). Thus despite increased FA utilization, Akita hearts maintained normal insulin sensitivity.

Mitochondrial oxygen consumption and respiratory coupling. The absence of reduced CE was unexpected and revealed an important difference between the type 1 diabetic Akita mouse and type 2 diabetic mouse models. Because mitochondrial uncoupling may underlie impaired CE in type 2 diabetic hearts (1; 5; 6), we hypothesized that mitochondrial coupling would be normal in Akita hearts. Mitochondrial function was initially evaluated in fibers that were prepared from freshly isolated mouse hearts. In hearts from 24-week-old mice, maximal ADP-stimulated mitochondrial oxygen consumption ($V_{ADP}$) and ATP synthesis were unchanged using palmitoyl-carnitine as a substrate (Fig. 3 A, E), but $V_{ADP}$ and ATP synthesis were significantly reduced in Akita mice using pyruvate (Fig. 3 B, F) or glutamate (Fig. 3 C, G) as substrates. Although a respiratory defect was evident, there was no evidence for mitochondrial uncoupling. Rates of oligomycin-insensitive respirations ($V_{oligo}$) and ATP/O ratios were unchanged with any substrate, indicating intact coupling of ATP synthesis to oxygen consumption. In 10-week old mice, similar reductions in glutamate-supported respirations ($V_{ADP}$) were observed (Figure 3 D). ATP synthesis rates were proportionately reduced, thus ATP/O ratios were not different (Figure 3 H).

We previously reported that mitochondrial uncoupling became evident in *ob/ob* and *db/db* mouse hearts after hearts were perfused with increased concentrations of fatty acids (5; 6). Thus palmitoyl-carnitine respirations were determined in permeabilized fibers from hearts of 10-week-old Akita and control mice after perfusion with 11mM glucose and 1mM palmitate (Figure 4 A, B). Under these conditions we observed no differences in $V_{ADP}$, $V_{oligo}$, ATP production rates or ATP/O ratios. Finally, to provide more definitive evidence for the absence of mitochondrial uncoupling in Akita hearts, we isolated mitochondria from hearts of 24-week-old Akita following perfusion of hearts with 11mM glucose and 1mM palmitate and measured proton leak kinetics (Figure 4 C). Proton leak kinetics curves were entirely overlapping between mitochondria from
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Akita and non-diabetic controls, indicating the absence of mitochondrial uncoupling. 

**Expression of mitochondrial genes and mitochondrial morphology.** To understand the molecular basis for impaired mitochondrial respiratory capacity with glutamate and pyruvate, we investigated gene expression in Akita and WT hearts at 24 weeks of age (Fig. 5 A). Transcription of genes encoding for subunits of the electron transport chain was reduced for 4 out of 5 genes examined in Akita. In contrast, PGC-1 coactivators (PGC-1α, PGC-1β) and their downstream mediators (NRF1, TFAm, ERRα) were not differentially expressed. Genes encoding for PPARα as well as enzymes and transporters of FA utilization did not show a uniform trend towards increased expression except for malonyl CoA decarboxylase (MCD). Expression of GLUT 1 and 4 was reduced, and expression of PDK4 was increased in Akita, consistent with impaired glucose oxidation in Akita. Interestingly, expression of UCP2 and UCP3 was significantly increased in Akita and these changes were accompanied by increased expression of mitochondrial thioesterase 1 (MTE1). Consistent with increased mRNA levels there was a 2-fold increase in UCP3 protein content (Fig. 5 B). This observation is of particular interest since we detected no evidence of mitochondrial uncoupling in mitochondria from Akita mice.

Electron microscopy revealed markedly reduced cristae density in Akita but not in WT mice (Fig. 6 A). 75% of the images evaluated showed lipid droplets in Akita mice, but no lipid droplets were observed in WT mice (arrows in Fig. 6 A). While mitochondrial volume density was increased in Akita, mitochondrial number was unchanged between the groups (Figure 6 B, C).

**No evidence for oxidative stress or increased ROS in Akita hearts.** Thus far we have shown that Akita mitochondria reveal defects in oxygen consumption in the absence of mitochondrial uncoupling (despite increased UCP3 content). We previously demonstrated that, in type 2 diabetic db/db mice, FA-induced mitochondrial uncoupling is mediated by activation of UCPs in the presence of increased ROS production and evidence of oxidative stress. These results suggested that increased ROS is required for activation of UCPs in diabetic hearts (6). We therefore hypothesized that the absence of mitochondrial uncoupling in Akita hearts despite increased UCP3 content was due to reduced ROS in Akita hearts. Consistent with this hypothesis, there was no increase in mitochondrial H2O2 production. Indeed, Akita mice showed a 38-46% decrease in mitochondrial H2O2 generation compared to WT at 10 or 24-weeks of age (Fig. 7 A, D). Addition of the complex I inhibitor rotenone abolished mitochondrial H2O2 production, suggesting that mitochondrial ROS production mainly arises from complex I in WT and Akita mice. Mitochondrial aconitase activity was not decreased in Akita mice at both ages (Fig. 7 B, E), and oxidation of DCFDA, a measure of tissue ROS levels, was unaltered in 24-week-old hearts (Fig. 7 C). In addition, there was no induction of antioxidant defense pathways. Transcript levels of glutathione peroxidase 1 (GPX1), mitochondrial manganese superoxide dismutase (SOD2) and peroxiredoxin 3 (PRDX3) were either unchanged or even reduced in Akita (Fig. 5 A). Similarly, protein levels of SOD2 and PRDX3 were unchanged between the groups (Fig. 7 F, G).

**DISCUSSION**

Reduced CE is a well-described characteristic of type 2 diabetic hearts in animal models and humans (3; 28). In this study, we addressed the question: does impaired CE and FA-induced mitochondrial uncoupling contribute to cardiac contractile dysfunction in type 1 diabetic Akita mice? Similar to type 2 diabetic Akita mice, substrate
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metabolism shifts towards increased FA metabolism, and mitochondrial oxidative capacity is impaired. However, in contrast to type 2 diabetic mouse models, Akita mice show relatively preserved contractile function under ambient conditions, show no impairment in CE and no evidence of mitochondrial uncoupling (normal V_{oligo}, ATP/O ratios and proton leak). Indeed perfusion of hearts with high FA concentrations was not sufficient to induce mitochondrial uncoupling despite the presence of increased uncoupling protein 3 (UCP3) levels. Moreover, the absence of oxidative stress distinguishes the Akita mouse from type 2 diabetic models. Thus, the molecular mechanisms for mitochondrial dysfunction importantly differ between insulin-deficient type 1 and insulin resistant type 2 diabetic mice. We also show that increased UCP3 does not invariably lead to increased mitochondrial uncoupling in the heart, supporting the hypothesis that FA-induced mitochondrial uncoupling in diabetic hearts may require a concomitant increase in ROS generation.

The absence of mitochondrial uncoupling in Akita hearts was associated with the absence of oxidative stress. Mitochondrial superoxide production, measured as H_2O_2 generation, was actually decreased and the activity of mitochondrial aconitase and oxidation of DCFDA, which are independent measures of oxidative damage, were unaltered in Akita hearts. The association of mitochondrial uncoupling with increased ROS and lipid peroxide generation in type 2 diabetic db/db hearts (6), and the lack of uncoupling and the absence of oxidative stress in type 1 diabetic Akita hearts suggests a causal interdependence of ROS production and mitochondrial uncoupling in diabetic hearts. Indeed, superoxide increases proton conductance by UCP3 in isolated rat skeletal muscle mitochondria, and lipid peroxidation products, such as hydroxynonenal, increase proton leak in isolated mitochondria from multiple tissues including the heart, via UCPs and ANT (7; 24). While FAs appear to be required for proton transport of UCP2 and UCP3 when reconstituted in liposomes, Echtay et al. showed that hydroxynonenal-induced proton conductance does not require the presence of FAs (24; 29). Thus, it appears that for FA-induced mitochondrial uncoupling, as observed in type 2 diabetic hearts to occur, an increase in ROS may be required in addition to increased FA availability and utilization.

The absence of oxidative stress or of any increase in ROS or H_2O_2 production in Akita hearts was unexpected. Inhibition of mitochondrial H_2O_2 production in the presence of rotenone suggested that complex I accounted for most of the ROS production in Akita and control hearts. This contrasts with similar studies in mitochondria isolated from db/db hearts in which additional mitochondrial complexes (likely complex III) also contributed to ROS generation (6). The reduction in ROS generation in Akita suggested that there might be a defect at the level of mitochondrial complex I. This was also supported by the observation that mitochondrial respirations in the presence of glutamate and pyruvate, which are complex I substrates, were also reduced while this was not the case with palmitoyl carnitine that delivers reducing equivalents to complex I and complex II. Thus a simple increase in substrate flux might not be sufficient to increase mitochondrial ROS generation in diabetic hearts in the absence of specific alterations in mitochondrial complex activities that amplify ROS generation.

Oxidative stress has been proposed to contribute to mitochondrial dysfunction in the hearts of type 1 diabetes mouse models (30; 31). In the present study we demonstrated impaired mitochondrial function and perturbed mitochondrial morphology in the absence of any evidence of oxidative stress.
Interestingly, impaired mitochondrial morphology in the type 1 diabetic OVE26 mouse can be normalized by overexpression of MnSOD or catalase in the heart, but MnSOD-deficient mice die from early dilated cardiomyopathy and have normal cardiac mitochondrial morphology (30-32). In other transgenic models, knockout of the mitochondrial transcription factor A leads to reduced mitochondrial DNA replication and transcription, and enlarged mitochondria with abnormal cristae morphology, and combined deletion of the cardiac insulin and IGF1 receptor results in reduced mitochondrial DNA replication and transcription, and enlarged mitochondria with abnormal cristae morphology, and combined deletion of the cardiac insulin and IGF1 receptor results in impaired OXPHOS gene transcription and decreased mitochondria that appeared to be less dense in electron micrographs (33-35). Thus in some contexts, changes in the content of electron transport chain subunits can be associated with changes in mitochondrial morphology. Since insulin is a positive regulator of OXPHOS gene expression, and OXPHOS gene expression is decreased in the Akita mouse heart, insulin deficiency as opposed to oxidative stress might be an important contributor to mitochondrial dysfunction and altered morphology in the Akita mouse model (36; 37). Defective insulin signaling has been reported to reduce expression levels of genes involved in beta-oxidation (23), yet FAO genes were unchanged or increased in Akita mice. We believe that this reflects the impact of PPARα activation in the face of increased FA availability.

An important observation in the present study is that increased content of UCP3 in the heart should not be taken to indicate increased mitochondrial uncoupling. Indeed we previously reported that increased mitochondrial uncoupling activity can occur in the absence of any changes in UCP3 content in hearts from ob/ob and db/db mice (5; 6). It is important to discuss what might drive UCP3 levels in Akita mouse hearts. Besides detoxification of ROS, UCP3 has been postulated to play a role in the regulation of FA metabolism. Conditions associated with increased FA availability, such as fasting, high-fat feeding, and diabetes, result in increased UCP3 expression, likely due to increased PPARα signaling (12; 38-40). In general, muscle UCP3 protein content is negatively related to FA oxidative capacity, and induction of UCP3 is most pronounced in glycolytic muscle upon fasting and high-fat feeding (41; 42). Thus increased UCP3 expression may reflect an adaptive response to FA overload (43). Based on the FA cycling model, it has been proposed that export of FA anions from the matrix into the intermembrane space via UCPs may help to lower intramitochondrial FA levels if increased FA availability exceeds mitochondrial oxidative capacity (43-45). Thereby, oxidation of intramitochondrial FAs to harmful lipid peroxides could be prevented. Expression of MTE1 often parallels that of UCP3, and this was also the case in the present study. MTE1 catalyzes conversion of acyl-CoAs back to FA anions, which can then be translocated out of the matrix by UCPs (13; 29). In the cytosol, they may be reesterified and oxidized in the mitochondria, stored as triglycerides, or used for other pathways. Nonesterified FAs can be generated within mitochondria and exported from the matrix, and these events are markedly increased in cardiac mitochondria from streptozotocin-induced type 1 diabetic rats (13). Despite increased basal FA oxidation, Akita mice also revealed increased lipid droplets, thus, it is tempting to speculate that fatty acyl-CoAs would be converted to FA anions by increased MTE1 activity, exported by UCP3, re-esterified and stored as triglycerides. Thus, UCPs would contribute to match FA availability with oxidation to prevent intramitochondrial lipotoxic effects.

A number of important differences between Akita mice and previously evaluated models of type 2 diabetes were observed (3-6; 8). There was a relative preservation of
cardiac function and preserved inotropic responses to a calcium-induced increase in workload. This likely reflects the more profound mitochondrial dysfunction in the type 2 models and a greater degree of mitochondrial uncoupling. Preliminary studies do suggest though, that cardiac dysfunction may develop in Akita mice following longer term hemodynamic challenge such as following isoproterenol infusion (Supplementary results and Supplementary table 5), but the mechanisms for this remains to be elucidated. Second, insulin sensitivity was preserved in Akita hearts in contrast to the insulin resistance reported in models of type 2 diabetes (2; 3). Although insulin levels in diabetic Akita are 40% of insulin levels in non-diabetic controls (19), these ambient insulin concentrations might be sufficient to maintain mitochondrial function to a greater extent than models in which insulin signaling is severely impaired. Finally, MVO₂ was not increased despite increased oxidation rates of exogenous palmitate. Although this may partly reflect the absence of mitochondrial uncoupling, it is also possible that reduced oxidation of glucose and endogenous triglycerides coupled with mitochondrial dysfunction, reduced MVO₂ in intact perfused hearts.

In conclusion, despite a significant increase in UCP3 content, insulin-deficient Akita hearts do not develop FA-induced mitochondrial uncoupling, suggesting that underlying mechanisms for mitochondrial dysfunction may importantly differ between insulin-responsive type 1 versus insulin resistant type 2 diabetic hearts. Increased UCP3 levels do not invariably lead to increased mitochondrial uncoupling in the heart, thereby supporting the hypothesis that FA-induced mitochondrial uncoupling in diabetic hearts requires a concomitant increase in ROS or lipid peroxide generation.

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Table 1: Echocardiographic evaluation of contractile function in WT and Akita mice at 20, 36 and 54 weeks of age.

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<tr>
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<th>FS [%] ± SD</th>
<th>EF [%] ± SD</th>
<th>HR [bpm] ± SD</th>
<th>SV [μl/gBW] ± SD</th>
<th>CO [μl/min/gBW] ± SD</th>
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<tr>
<td>WT 20wk (9)</td>
<td>38.6 ± 2.4</td>
<td>76 ± 3</td>
<td>415 ± 12</td>
<td>1.13 ± 0.03</td>
<td>467 ± 14</td>
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<tr>
<td>Akita 20wk (7)</td>
<td>38.7 ± 2.4</td>
<td>76 ± 3</td>
<td>472 ± 31</td>
<td>1.12 ± 0.07</td>
<td>527 ± 15</td>
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<tr>
<td>WT 36wk (6)</td>
<td>39.6 ± 1.2</td>
<td>78 ± 1</td>
<td>470 ± 41</td>
<td>0.97 ± 0.04</td>
<td>456 ± 25</td>
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<tr>
<td>Akita 36wk (5)</td>
<td>39.7 ± 2.7</td>
<td>78 ± 3</td>
<td>421 ± 29</td>
<td>1.13 ± 0.04</td>
<td>476 ± 33</td>
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<tr>
<td>WT 54wk (6)</td>
<td>24.6 ± 1.9</td>
<td>57 ± 3</td>
<td>523 ± 29</td>
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<tr>
<td>Akita 54wk (6)</td>
<td>27.6 ± 1.9</td>
<td>62 ± 3</td>
<td>448 ± 28</td>
<td>0.95 ± 0.04</td>
<td>426 ± 28</td>
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FS, Fractional shortening; EF, Ejection fraction; HR, Heart rate; SV, Stroke volume; CO, Cardiac output. *p*<0.05 vs. WT
Figure legends

FIG. 1: Substrate oxidation and contractile performance of Akita hearts. 24 week-old WT and Akita mouse hearts were perfused in the isolated working mode without insulin (basal) or in the presence of 1nM insulin (n=4-5). A, Left ventricular developed pressure (LVDevP); B, Cardiac output; C, Cardiac power; D, Palmitate oxidation; E, Glucose oxidation; F, Glycolysis; G, Oxygen consumption; H, Cardiac efficiency. * p<0.05 vs. WT, Ψ p<0.05 vs. without insulin.
FIG. 2: **Intact insulin signaling in Akita hearts.** Representative Western blot images showing cardiac protein levels of phosphorylated Akt (p-Akt) on Ser\(^{473}\) and total levels of Akt (t-Akt), and densitometric quantification of the ratio of p-Akt to t-Akt (p-Akt/t-Akt) following isolated working heart perfusions in the absence (basal) or presence of 1nM insulin at the age of 10 weeks (A) and 24 weeks (B). * p<0.05 vs. basal, Ψ p<0.05 vs. WT.

FIG. 3: **Preserved mitochondrial coupling and impaired mitochondrial oxidative capacity in Akita hearts.** Mitochondrial respiratory rates (A-C) and ATP synthesis and ATP/O ratios (E-G) of saponin-permeabilized cardiac fibers from 24 week-old WT and Akita mice using palmitoyl-carnitine (A, E), pyruvate (B, F) or glutamate (C, G) as a substrate (n=6). Similar data were also obtained in 10-week-old mice following incubation with glutamate (D, H), n=6. * p<0.05 vs. WT.
FIG. 4: **Fatty acid perfusion does not induce mitochondrial uncoupling.** Mitochondrial respiratory rates (A), and ATP synthesis and ATP/O ratios (B), of saponin-permeabilized cardiac fibers generated from 24 week-old WT and Akita mouse hearts that were perfused with Krebs Henseleit buffer containing 11mM glucose and 1mM palmitate. Palmitoyl-carnitine was used as respiratory substrate (n=6). C: Proton leak kinetics of mitochondria isolated from 24 week-old WT and Akita mouse hearts pre-perfused with Krebs Henseleit buffer containing 11mM glucose and 1mM palmitate. Palmitoyl-carnitine was used as respiratory substrate (n=6).
FIG. 5: **Reduced OXPHOS expression and increased UCP expression.** A: Myocardial gene expression in 24 week-old WT and Akita mice normalized to 16S RNA transcript levels (n=8). Values represent fold change in mRNA transcript levels relative to WT, which was assigned as 1 (dashed line). B: Representative Western blot images and densitometric quantification of uncoupling protein 3 (UCP3) protein levels in isolated mitochondria from 24 week-old WT and Akita mice (n=4). Coomassie blue staining was used as a loading control. * p<0.05 vs. WT
FIG. 6: Altered mitochondrial morphology in Akita hearts. Representative longitudinal electron microscopy images of left ventricular wall of 24 week-old WT and Akita mice (A), and stereological quantification of mitochondrial volume density (B) and mitochondrial number (C) (n=4). Magnifications (x 10,000, x 40,000) are shown on each image, and black arrows on the images indicate lipid droplets. * p<0.05 vs. WT
FIG. 7: **Oxidative stress is absent in Akita hearts.** H₂O₂ production in isolated mitochondria of WT and Akita mice at 24 weeks (A) and 10 weeks (D) of age (n=4). Mitochondrial aconitase activity of WT and Akita mice at 24 weeks (B) and 10 weeks (E) of age (n=4). C. Oxidation of DCFDA measured in cardiac whole tissue extracts from 24 week-old WT and Akita mice (n=5). Representative Western blot images and densitometric quantification of Manganese superoxide dismutase, MnSOD (F) and Peroxiredoxin 3, PRDX3 (G) in isolated mitochondria of 24 week-old WT and Akita mice (n=4). Coomassie blue staining was used as a loading control. * p<0.05 vs. WT.