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Transmural differences in respiratory capacity across the rat left ventricle in health, aging, and streptozotocin-induced diabetes mellitus: evidence that mitochondrial dysfunction begins in the subepicardium

J. R. MacDonald, M. Oellermann, S. Rynbeck, G. Chang, K. Ruggiero, G. J. S. Cooper, and A. J. R. Hickey

School of Biological Sciences and Centre for Molecular Biodiscovery, University of Auckland, Auckland, New Zealand

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MacDonald JR, Oellermann M, Rynbeck S, Chang G, Ruggiero K, Cooper GJ, Hickey AJ. Transmural differences in respiratory capacity across the rat left ventricle in health, aging, and streptozotocin-induced diabetes mellitus: evidence that mitochondrial dysfunction begins in the subepicardium. Am J Physiol Cell Physiol 300: C246–C255, 2011. First published November 17, 2010; doi:10.1152/ajpcell.00294.2010.—In diabetic cardiomyopathy, ventricular dysfunction occurs in the absence of hypertension or atherosclerosis and is accompanied by altered myocardial substrate utilization and depressed mitochondrial respiration. It is not known if mitochondrial function differs across the left ventricular (LV) wall in diabetes. In the healthy heart, the inner subendocardial region demonstrates higher rates of blood flow, oxygen consumption, and ATP turnover compared with the outer subepicardial region, but published transmural respirometric measurements have not demonstrated differences. We aim to measure mitochondrial function in Wistar rat LV to determine the effects of age, streptozotocin-diabetes, and LV layer. High-resolution respirometry measured indexes of respiration in saponin-permeabilized fibers dissected from the LV subendocardium and subepicardium of 3-mo-old rats after 1 mo of streptozotocin-induced diabetes and 4-mo-old rats following 2 mo of diabetes. Heart rate and heartbeat duration were measured under isoflurane-anesthesia using a fetal-Doppler, and transmission electron microscopy was employed to observe ultrastructural differences. Heart rate decreased with age and diabetes, whereas heartbeat duration increased with diabetes. While there were no transmural respiration differences in young healthy rat hearts, both myocardial layers showed a respiratory depression with age (30–40%). In 1-mo diabetic rat hearts only subepicardial respiration was depressed, whereas after 2 mo diabetes, respiration in subendocardial and subepicardial layers was depressed and showed elevated leak (state 2) respiration. These data provide evidence that mitochondrial dysfunction is first detectable in the subepicardium of diabetic rat LV, whereas there are measurable changes in LV mitochondria after only 4 mo of aging.

For its mass the human heart is the most metabolically active organ in the body and cycles approximately 6 kg of ATP daily to pump 10 tons of blood (51). The thick-walled left ventricle (LV) generates the greatest pressure to supply the body with oxygenated blood, and it is this region that suffers the most in the face of pathology (45). Diabetes mellitus (DM) is a disease associated with a specific cardiomyopathy (diabetic cardiomyopathy), involving LV dysfunction in the absence of coronary artery disease or hypertension (58).

The LV wall can be divided into five layers where the innermost endocardium covers the luminal side of the myocardium and the outer epicardium interfaces with the pericardium. The myocardium can be further subdivided into the innermost subendocardium (EN), midmyocardium, and outermost subepicardium (EP).

The regions of the LV differ in their susceptibility to pathological stressors such as ischemia. The EN is the most susceptible to global ischemic injury, where global ischemia produces a “wave front of cell death” that progresses from the EN toward the EP (60). Relative to the EP, increased necrosis in the EN coincides with a decline in blood flow, the adenylates ATP, and phosphocreatine and mitochondrial respiration, with a more rapid accumulation of lactate and inorganic phosphate (Pi) and a more prolonged action potential (1). These observations point to inherent regional differences across the LV. Indeed, electrophysiological properties, blood flow (13), oxygen consumption (8), oxidative phosphorylation capacity (4, 9, 18, 36, 41, 62), adenylate and phosphogen levels (61), and metabolic-marker enzyme activities (18, 63, 64) have been explored in healthy hearts in attempts to explain apparent differences in metabolic capacity between the EP and EN layers.

The LV EN appears to be more highly perfused, potentially due to a greater coronary supply and also the presence of freshly oxygenated blood in the LV lumen. The EN and EP also exhibit distinct electrophysiological properties in terms of the action potential characteristics. Ion channel expression and the density of ion currents also differ regionally, and the EN shows a higher oxygen consumption and ATP turnover than the LV EP (8). This suggests differences in mitochondria or their regulation. However, activities of enzymes of the Kreb’s cycle [citrate synthase, malate dehydrogenase, and isocitrate dehydrogenase (37, 42)], glycolysis [hexokinase, phosphoglycerate kinase, and lactate dehydrogenase (63, 64)], the electron transport system [cytochrome c-oxidase, succinic dehydrogenase (17, 18)], and phosphofructokinase [creatine kinase (63)] generally appear to show minor differences or are similar. In addition, respiratory activity measured in isolated mitochondria or in those contained in saponin-permeabilized fibers appear to show little difference between the EN and EP (62). Transmural differences are less well documented in diabetic mellitus but similar to health include higher EN perfusion (27) and longer action potential duration relative to the EP (6).

However, the most recent respirometric study that compared the EN and EP layers was conducted more than a decade ago with simple protocols and underdeveloped respirometers. Here we re-explore mitochondrial functional differences between the EN and EP using high-resolution respirometry combined with a more complex titration protocol that permits better...
dissection of the oxidative phosphorylation and electron transport systems (19, 21). We also used permeabilized fibers because these permitted sampling from defined regions of the LV. Although there may well be few differences between layers in health, we questioned whether functional differences occur across these two general regions in the face of a diseased diabetic state, such as diabetic cardiomyopathy, and whether there are differences with age.

**METHODS**

**Animals.** All studies were approved by the University of Auckland ethics committee. While under isoflurane anesthesia, male Wistar rats (2 mo of age, 250–300 g) were made diabetic by injection of streptozotocin (STZ, 60 mg/kg in saline) into the tail vein. Controls were injected with an equivalent volume of saline. Rats were kept under a constant 12 h light:dark cycle and fed standard rat chow and tap water ad libitum for 3 mo (3 mo old with 1 mo diabetes, 3m-1mDM, n = 9) or 4 mo (4 mo old with 2 mo diabetes, 4m-2mDM, n = 8). Blood glucose levels and body weights were measured weekly.

**Doppler ultrasonography.** Body weight and blood glucose levels were measured in isoflurane-anesthetized rats. The right-hand side of the thorax was shaved and an 8-MHz ultrasonic Doppler probe (Sonotrax A, Contec Medical Systems) was used with lubricating gel to record heart rate and heartbeat duration under constant flow rates of 3 l/min of 3% vol/vol isoflurane. Sound files were generated by attachment of the ultrasound to a personal computer with the software package Audacity v1.2.6., which permitted background noise filtration, and beat rate and duration determination. The heart was then excised following 1 min of heparin injection (1,000 IU/kg) and thoracic puncture. Hearts were immediately immersed in ice-cold Krebs-Henseleit bicarbonate buffer (KHB) containing (in mmol/l) 4.7 KCl, 2.3 CaCl₂, 1.2 KH₂PO₄, 1.2 MgSO₄, 118 NaCl, 24 NaHCO₃, and 11 glucose. KHB (30 ml) was then perfused retrograde through the aorta to wash blood from the hearts.

Heart weight was recorded after trimming the major vessels and blot drying. The heart was transected midway between the base and apex, and the wall thickness of the right ventricle (RV), septum, and left ventricle (LV) were measured using electronic micrometers. The atria and septum were removed and myocardium separately trimmed from the (inner) subendocardial (EN) and (outer) subepicardial (EP) layers of the LV. Approximately 25 mg of myocardium from each layer was frozen at −80°C for citrate synthase assay.

**Preparation of skinned myofibers.** Approximately 25 mg of EN and EP were dissected into fiber bundles 0.5 × 1 mm after placement in 1 ml ice-cold, high-energy relaxing solution (in mmol/l, 10 Ca-EGTA buffer, 0.1 free calcium, 20 imidazole, 20 taurine, 50 K-MES [potassium 2-(N-morpholinoo)ethanesulfonic acid], 0.5 dithiothreitol, 6.56 MgCl₂, 5.77 ATP, 15 phosphocreatine, at pH 7.1). To permeabilize fibers, fiber bundles were transferred into 2 ml fresh high-energy relaxing solution with saponin (50 μg) and agitated for 30 min at 4°C, as described previously (70). Saponin perforates the sarcolemma of cardiac muscle fibers by targeting cholesterol, leaving intracellular structures such as mitochondria intact, due to their lower cholesterol content. To remove saponin and adenine nucleotides, fibers were washed three times for 10 min in ice-cold incubation assay medium (in mmol/l, 0.5 EGTA, 3 MgCl₂, 60 K-lactobionate, 20 taurine, 10 KH₂PO₄, 110 sucrose, and 1 mg/ml BSA in 20 mmol/l HEPES, pH 7.1 at 30°C). Fiber bundles were blot dried on lint-free lens paper, and ~2 mg of EN and EP were weighed for respiration assays.

**Respiration assays.** A multiple substrate-inhibitor titration protocol (Fig. 1) was employed to better reflect respiratory flux in vivo and to explore relative capacity of the electron transport system (ETS) and phosphorylation system components (20). Two OROBOROS Oxynograph-2K (Anton Paar, Graz, Austria) were employed for oxygraphy, respiratory measurements of fiber bundles were performed at 30°C in 2 ml incubation assay medium, and the oxygen concentration at air saturation of the medium was 215 mmol O₂/ml at 95 kPa barometric pressure. Respiration was measured as weight-specific oxygen flux (pmol O₂ s⁻¹·mg wet wt⁻¹) calculated as the time derivative of oxygen concentration using the DatLab 4 Analysis Software, OROBOROS (Innsbruck, Austria).

Approximately 2–3 mg of fibers were added to chambers and allowed to equilibrate. Oxygen was then added to chambers and maintained above 280 mmol/ml to ensure saturation (19). Complex 1 leak (C₁ leak, state 2 respiration) was measured by the addition of Complex 1 substrates alone (10 mmol/l glutamate, 2 mmol/l malate, and 5 mmol/l pyruvate). Excess ADP (1.25 mmol/l) stimulated oxidative phosphorylation (C₁ Oxphos, state 3 respiration), and by then adding cytochrome c (10 μmol/l), the functional integrity of mitochondria was measured. Phosphorylating respiration with Complex 1 and 2 substrates (C₁₂ Oxphos) was assessed by addition of succinate (10 mmol/l). The “leak rate” with Complex 1 and 2 substrates (C₁₂ leak or state 4 respiration) was measured by addition of atractyloside (1 μmol/l), followed with repeated titrations of carbonyl cyanide p-(trifluoromethoxy)phenyl-hydrazone (FCCP, 0.5 μmol/l) to uncouple mitochondria. Complex I and then Complex III activity were selectively inhibited by the addition of rotenone (1 μmol/l) and antimycin a (1 μmol/l), respectively. Finally, the activity of cytochrome c-oxidase (CCO, Complex IV) was measured by the addition of the electron donor couple N₃N₅,N₂,N₄-tetramethyl-p-phenylenediamine (TMPD, 0.5 mmol/l) and ascorbate (2 mmol/l). Activities were determined relative to wet mass of tissue and to the mitochondrial marker enzyme citrate synthase (CS). CS activity was measured according to Ref. 52.

Flux-control ratios (see Fig. 4) were calculated and include two different respiratory control ratios; RCR₁ (C₁ Oxphos/C₁ leak) and RCR₁₂ (C₁₂ Oxphos/C₁₂ leak).

**Electron microscopy.** Approximately 25 mg of myocardium from both LV layers of control and diabetic rat hearts were placed in fixative buffer (10 mmol/l HEPES, 250 mmol/l sucrose, 2.5% glutaraldehyde, pH 7.1) and stored at 4°C. After three 10-min washes in Sörenson’s phosphate buffer (100 mmol/l), samples were postfixed for 1 h in 1% osmium tetroxide in 100 mmol/l Sörenson’s phosphate buffer. In a series of 10-min ethanol washes (30–100%), followed by two acetone (100%) washes, samples were dehydrated. The myocardium was infiltrated for 1 h using 1:1 812 epoxy resin:aceton following by 100% aceton for 24 h.
after both 1 and 2 mo of diabetes and a 10% drop in heart rate. There was a drop in heart rate and extension of beat duration after 2 mo DM. Doppler ultrasound revealed clear differences in LV relative to heart mass (although this was only significant significantly thicker LV relative to body mass, as well as thicker diabetic groups show evidence of LV hypertrophy, with significant differences in respect to left ventricular (LV)

Table 1. Blood glucose, heart and body weights, and heart dimensions of animals

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 9)</th>
<th>STZ (n = 9)</th>
<th>Control (n = 8)</th>
<th>STZ (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood glucose, mmol/l</td>
<td>5.2 ± 0.2</td>
<td>28.7 ± 1.2†</td>
<td>5.2 ± 0.1</td>
<td>29.5 ± 1.3†</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>439.8 ± 8.5</td>
<td>358.6 ± 10.4*</td>
<td>452.0 ± 20.2</td>
<td>331.1 ± 8.9*</td>
</tr>
<tr>
<td>Heart weight, g</td>
<td>1.22 ± 0.03</td>
<td>1.08 ± 0.02</td>
<td>1.12 ± 0.05</td>
<td>1.01 ± 0.03</td>
</tr>
<tr>
<td>% Heart of body mass</td>
<td>0.277 ± 0.005</td>
<td>0.302 ± 0.007</td>
<td>0.251 ± 0.014</td>
<td>0.305 ± 0.010*</td>
</tr>
<tr>
<td>LV, mm</td>
<td>4.25 ± 0.20</td>
<td>4.35 ± 0.26</td>
<td>4.04 ± 0.22</td>
<td>4.54 ± 0.21</td>
</tr>
<tr>
<td>Septum, mm</td>
<td>3.81 ± 0.22</td>
<td>3.52 ± 0.26</td>
<td>2.98 ± 0.13‡</td>
<td>3.63 ± 0.20</td>
</tr>
<tr>
<td>RV, mm</td>
<td>1.74 ± 0.13</td>
<td>1.48 ± 0.15</td>
<td>1.19 ± 0.06</td>
<td>1.38 ± 0.09</td>
</tr>
<tr>
<td>LV, mm/RV, mm</td>
<td>2.56 ± 0.23</td>
<td>3.21 ± 0.43</td>
<td>3.43 ± 0.18</td>
<td>3.43 ± 0.30</td>
</tr>
<tr>
<td>LV, mm/body mass, g</td>
<td>9.66 ± 0.45</td>
<td>10.76 ± 1.45*</td>
<td>9.16 ± 0.70</td>
<td>13.71 ± 0.05*</td>
</tr>
<tr>
<td>LV, mm/heart mass, g</td>
<td>3.48 ± 0.15</td>
<td>4.01 ± 0.21</td>
<td>3.65 ± 0.22</td>
<td>4.52 ± 0.52*</td>
</tr>
</tbody>
</table>

Values are means ± SE. STZ, streptozotocin; LV, left ventricle; RV, right ventricle *; †Significant difference between STZ and age-matched control (P < 0.05, P < 0.001), respectively; ‡significant difference between 3- and 4-mo-old animals.

Samples were then embedded into fresh resin moulds and cured for 48 h at 60°C. Semithin sections of 1–5 μM were prepared and further sectioned to 70 nm. These ultrathin sections were mounted on copper mesh grids, stained with uranyl acetate and lead citrate, and scanned using a Philips CM12 at 120 kV.

Statistical tests. All data are presented as means ± SE, and analyses were performed using the statistical package JMP 8.0. Characteristics of the animal model as well as Doppler measurements were analyzed using two-way ANOVA with post hoc Tukey-Kramer tests. Hierarchical mixed models were fitted (according to a split-plot design) to the fiber respiration and citrate synthase activity data using residual maximum likelihood (REML) to estimate age, disease, and LV layer main effects and their interactions. Log transformations were applied to the data, where appropriate, before model fitting. Post hoc Tukey-Kramer tests were then used for pair-wise comparisons and differences were considered significant at P < 0.05.

RESULTS

General parameters and heart function. An acute form of diabetes manifested in STZ-injected animals in 2–3 days (blood glucose >11 mmol/l; polyuria and polydipsia). When compared with control animals, body weight in the diabetic group was significantly lower at time of death (Table 1). Both diabetic groups show evidence of LV hypertrophy, with significantly thicker LV relative to body mass, as well as thicker LV relative to heart mass (although this was only significant after 2mDM). Doppler ultrasound revealed clear differences in heart function in isoflurane-anesthetized animals with diabetes. There was a drop in heart rate and extension of beat duration after both 1 and 2 mo of diabetes and a 10% drop in heart rate with age (Fig. 2, A and B).

Respiratory measurements. When looking at the main effects of age, disease, and LV layer, age was a significant contributor to respiratory-flux depression (P < 0.001 for all states measured), as was diabetes (P < 0.001 for C1 Oxphos, C1,2 Oxphos, ETS, and CCO and P < 0.01 in C1,2 leak and C2 ETS); however, there were significant interactions among these three effects. For example, the phosphorylating (C1 and C1,2 Oxphos) depression in EP relative to EN is dependent on age; only 3m-1mDM animals exhibit a layer difference, whereas 4m-2mDM phosphorylating flux is not different when comparing layers (Fig. 3, C, E, and G).

Within the two control groups little difference was apparent between EN and EP layers (Fig. 3). However, comparison across the age groups showed a depression in overall phosphorylation, Complex 1 and 2 leak, ETS capacity, C2 ETS and CCO (Fig. 3). In contrast, C1 leak increased with age (Fig. 3).

A similar pattern is seen in the respiratory control ratio with Complex 1 substrates (RCR1; C1 Oxphos/ C1 leak, Fig. 4A); both age and disease lead to a depressed ability to phosphorylate Complex 1 substrates, the most affected layer was EP in 3m-1mDM animals (Fig. 4A). There were few differences between groups in the RCR1,2. Similarly, in other ratios (CCO-stimulated respiration; ETS capacity and ETS capacity: C1,2 oxphos) the groups varied little and do not significantly differ from each other (Fig. 4, C and D).

Flux was also normalized to CS activity, as this provides an estimate of mitochondrial mass (48). CS was significantly elevated in the EP layer of 3m-1mDM compared with all other groups (with the exception of EN in control animals) revealed by post hoc tests (Fig. 5). Therefore for 3m-1mDM the de-
Fig. 3. Respiratory flux per milligram cardiac skinned-fiber in subendocardial (EN) and subepicardial (EP) LV layers of 3-mo-old animals subject to 1 mo streptozotocin (STZ) diabetes and 4-mo-old animals subject to 2 mo diabetes and their age-matched controls. Complex 1 leak (A,B), Complex 1 Oxphos (C,D), Complex 1+2 Oxphos (E,F), Complex 1+2 leak (G,H), ETS (I,J), Complex 2 ETS (K,L), CCO (M,N). Restricted Maximum Likelihood (REML) analysis reveals the effects of LV layer, disease, and age, and interactions between these factors are shown at the bottom left of each graph (*P < 0.05, **P < 0.01, ***P < 0.0001). Brackets indicate different groups in respect to LV layer, age, or disease analyzed using post hoc Tukey's.
pressed respiratory capacity in EP relative to EN was amplified when normalized to CS activity (pmol O$_2$·s$^{-1}$·U CS$^{-1}$, Fig. 3, B, D, F, H, J, L, N).

Electron microscopy. There were clear morphological differences between healthy and diabetic hearts (Fig. 6, a, b, e, f vs. c, d, g, h, respectively). There were no obvious differences between the EN and EP layers in either healthy or diabetic hearts (Fig. 6, left [EN] vs. right [EP]). The Z-bands between adjacent sarcomeres are poorly aligned and mitochondria appear much less well organized when compared with age-matched controls (Fig. 6, c, d, EN and EP in 3m-1mDM vs. a, b in age-matched controls, 6, g, h, EN and EP in 4m-2mDM vs. 6, e, f in age-matched controls), and this arrangement appears to worsen with diabetes duration.

DISCUSSION

Clear differences were apparent in whole cardiac and mitochondrial function between age groups and diabetic groups. Although we report no substantial differences between the EN and EP regions in healthy animals, the EP showed a depression in respirational function early in diabetes. Although this regional difference was no longer apparent following 2 mo of diabetes mellitus (4m-2mDM), respirational flux dropped substantially over this brief period in control and diabetic fibers. Perhaps most surprising was the substantial drop in respirational flux in healthy rats over only 1 mo.

Using a simple Doppler ultrasound, we confirmed that diabetes induced clear differences in heart rate and extended the heartbeat...
duration under isoflurane anesthesia after 1mDM and 2mDM. In 1- and 2-mo diabetic animals, there was a significant decrease in heart rate (18% and 15%, respectively) and increase in heartbeat duration (17% and 24%, respectively) when compared with age-matched controls. Although heartbeat duration remained constant, heartbeat rate also declined in 4-mo old control hearts relative to 3-mo old control hearts. STZ induced changes in cardiac function, which may be suggestive of diabetic cardiomyopathy in both age groups, although this was most evident in 4m-2mDM animals. Bradycardia (29, 43) and a depressed relaxation (but not contraction) rate in the isoprotenol-maximal state (16) has been reported following 1 mo STZ-diabetes, and echocardiography indicates contractile dysfunction at this stage (71), however, other studies did not find this effect until at least 6 wk of STZ-induced diabetes (29, 72). Furthermore, cardiac function measurements in the present study were taken during isoflurane anesthesia, which is known to depress cardiac contractility in a dose-dependent manner (30). However, available evidence suggests that isoflurane-anesthesia affects STZ-diabetic and healthy animals similarly in respect to heart rate depression (67) and contraction magnitude depression [in ventricular myocytes (22); though this is debated in an earlier study on papillary muscle (25)].

Changes in heart function are well associated with shifts in the cardiomyocyte contractile machinery, in particular with myosin heavy chain isoform switching from $\beta$-myosin to $\alpha$-myosin, which has a lower ATPase activity (24). Between 2 and 4 mo of age, the contraction and relaxation rate of papillary muscle can drop by 32% and 40%, respectively in Wistar rats (38). The expression of $\beta$-myosin relative to $\alpha$-myosin in LV myocardium also increases in aged rats (65), and increase occurs between 2 and 5 mo in Wistar rats (5, 56). $\beta$-Myosin is a myosin heavy-chain isoform and has a threefold lower ATPase activity relative to $\alpha$-myosin (23), and isoform-switching has been shown to

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**Fig. 4.** Ratios of respiratory flux measurements in the subendocardial (EN) and subepicardial (EP) LV layers of 3-mo-old animals subject to 1 mo STZ diabetes and 4-mo-old animals subject to 2 mo diabetes and their age-matched controls. RCR1 (C1 Oxphos/C1 leak) decreases with age in both EN and EP layers in control and diabetic animals and in EP of diabetic animals vs. EP of control animals (A), whereas other respiratory flux ratios do not change (B–D). REML analysis reveals the effects of LV layer, disease, and age, and interactions between these factors are shown at the bottom left of each graph ($*P < 0.05$, **$P < 0.01$, ***$P < 0.0001$). Brackets indicate different groups with respect to LV layer, age, or disease analyzed using post hoc Tukey’s.

**Fig. 5.** Citrate synthase (CS) activity ($\text{U•mg wetwt}^{-1} \text{•s}^{-1}$) in the subendocardium (EN) and subepicardium (EP) of 3-mo-old animals subject to 1 mo STZ diabetes and 4-mo-old animals subject to 2 mo diabetes and their age-matched controls. REML analysis reveals the effects of LV layer, disease, and age, and interactions between these factors are shown at the bottom left of each graph ($*P < 0.05$, **$P < 0.01$, ***$P < 0.0001$). Brackets indicate different groups with respect to LV layer, age, or disease analyzed using post hoc Tukey’s.
depress the contractile rate and force of cardiac muscle with increasing age (10, 29, 40). Ongoing proteomic analyses of the 3m-1mDM animals in our study demonstrated that myosin isoforms have switched in these animals, with a 36% drop in H251–myosin and 320% increase in H252–myosin relative to control animals (supplemental data). These changes should also result in a corresponding decline in cardiac ATP requirements and therefore may match the lower mitochondrial respiratory fluxes in older rats.

This study employed high-resolution respirometry to measure mitochondrial function in the subepicardium (EP) and subendocardium (EN) of 1- and 2-mo diabetic animals and their age-matched controls, a comparison that has not previously been studied using saponin-skinned fibers. This permitted finer-scale comparisons across the left ventricle, which would have most likely been missed using isolated mitochondria because these would have sampled all LV mitochondria and most likely introduced bias by selecting for more robust mitochondria (35). The multisubstrate-inhibitor protocol also permitted us to explore changes among different respiratory complexes (26, 35).

In healthy rats, the decrease in mitochondrial function and drop in heart rate with age was surprisingly large, especially given that this occurred in only 1 mo. This is particularly pertinent as senescence studies generally classify “young” and “old” rodents as 6 and 24 mo of age (34). Mitochondrial dysfunction in the heart has been associated with aging in rodents, with decreased Complex I and IV activity (49), loss of cardiolipin (57), and loss of interfibrillar mitochondria (12); however, respirational results are conflicting, with some reporting decreased respiratory flux in mixed populations of subsarcolemmal (SSM) and interfibrillar mitochondria (IFM) (57) or IFM alone (12, 33), and others finding no change in IFM (28) or SS plus IFM (44). Although permeabilized fiber-based methods cannot differentiate mitochondrial subpopulations, sampling fibers permitted regional comparisons across the LV wall as only a few milligrams are required to measure flux.

As with studies using isolated mitochondria, we also show decreased Complex I and IV activities, as we found depressed OXPHOS flux on complex I substrates (C1 Oxphos) and depressed complex IV (CCO) flux. However, using the multisubstrate-inhibitor titration protocol, we show that ratios of Complex I/Complex II were unchanged with age. This suggests that aging, at least in these hearts, was associated with an overall depression of the electron transport system (ETS), and this conclusion was supported by depression of flux when reported relative to CS. A depressed RCR1 also indicates intrinsic mitochondrial alterations in the older animals.

Our data suggest that a respiratory flux depression occurs at an early life stage in rodents, as we note a 30–40% drop between 3 and 4 mo of age. Although only a 10% drop in heart rate occurs over this period, heart rates were measured in a submaximal state, i.e., under isoflurane anesthetic. The contractile machinery of the heart is known to change at similar ages to the rats used in our study. Although a depression of respirational flux may be an ontogenic effect, hypobaric hypoxia (38) and training (7) can attenuate losses of papillary muscle force generation, and training impedes the aging-related increase in β-myosin and decrease in α-myosin (31). This suggests that the early drop in contractile performance may result from the lack of physical activity, which most likely occurs in caged animals. Exercise improves mitochondrial function (19) and attenuates the drop in cardiac output with age (53) and STZ diabetes (3). It also retards the α- to β-myosin switch with aging (31) and hypertension (11) [though this is debated in diabetes (55, 66)]. Exercise also impedes age-dependent declines in ETS activity, as trained rats show higher Complex...
I activity (50) as well as higher OXPHOS and leak respiration relative to sedentary animals (2, 32).

Respiratory flux depression following 1 mo of STZ-induced diabetes has previously been shown in isolated myocardial mitochondria (39, 68); however, this study provides evidence that this depression is initially restricted to the EP layer after 1mDM, and this then occurs in the EN and EP after 2mDM. The increase in CS activity in 3m-1mDM indicates a compensatory response to decreased respiratory flux and/or an increase in mitochondrial mass with decreased flux for a given mitochondrial mass. Given that we saw limited changes in mitochondria by transmission electron microscopy increased CS activity may better reflect compensation at the level of the tricarboxylic acid cycle.

These data illustrate differences between acute and chronic insults, as global ischemic models drive necrosis and dysfunction in the EN (59). An explanation for this difference is required.

Though few studies have investigated transmural differences in STZ diabetes, there is evidence that the EN layer is more compromised, electrophysiologically (prolonged AP and a delayed rectifier potassium current [fK] (6)) and structurally greater number of collagen cross-links (hydroxylysylpyridinoline) indicating more fibrosis compared with EP (46). During ischemia in STZ-diabetic animals, the EN layer is most easily impaired. Paradoxically, the acute STZ model of diabetes appears to protect against ischemia-reperfusion injury, with restoration of transmural perfusion following ischemia in diabetic animals and subendocardial perfusion absent in control animals (54). This effect may be mediated by defective cation channels in diabetes, which may retard calcium accumulation that precedes cell death (47). In accordance with this, STZ-diabetic rats are resistant to calcium overload (69), and the subepicardium is reportedly more greatly affected (ultrastructural damage and enzyme activities). Furthermore, mitochondrial transcription factors were not measured in the current study, which may vary between EN and EP. Increased levels of peroxisome proliferator-activated receptor-α (PPAR-α) and PGC-1α mRNA have been found in 6 wk STZ mice myocardium, along with upregulation of fatty acid oxidation (FAO) enzyme mRNA (14). As PPAR-α overexpression induces increased hallmarks of diabetic cardiomyopathy [expression of fatty acid oxidation enzymes and a dilated cardiomyopathy (15)], future study should determine its levels in EN and EP of 4 wk diabetic rats and measure fatty acid oxidation, because this may have been a compensatory mechanism in EP not measured in the present study.

In conclusion, this study has provided insight into the time course of myocardial decline in STZ-induced diabetes. At an early stage (3m-1mDM), mitochondrial function in only the outer EP layer is depressed, and 1 mo later this depression is transmurally uniform. Additionally, we have found an early age-related decline in mitochondrial function in healthy rats. Further study should investigate other transmural differences 3m-1mDM to understand this region-dependent effect.

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REFERENCES


