Mitochondrial inefficiencies and anoxic ATP hydrolysis capacities in diabetic rat heart

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Pham T, Loiselle D, Power A, Hickey AJ. Mitochondrial inefficiencies and anoxic ATP hydrolysis capacities in diabetic rat heart. Am J Physiol Cell Physiol 307:C499–C507, 2014. First published June 11, 2014; doi:10.1152/ajpcell.00006.2014.—As ~80% of diabetic patients die from heart failure, an understanding of diabetic cardiomyopathy is crucial. Mitochondria occupy 35–40% of the mammalian cardiomyocyte volume and supply 95% of the heart’s ATP, and diabetic heart mitochondria show impaired structure, arrangement, and function. We predict that bioenergetic inefficiencies are present in diabetic heart mitochondria; therefore, we explored mitochondrial proton and electron handling by linking oxygen flux to steady-state ATP synthesis, reactive oxygen species (ROS) production, and mitochondrial membrane potential (ΔΨ) within rat heart tissues. Sprague-Dawley rats were injected with streptozotocin (STZ, 55 mg/kg) to induce type 1 diabetes or an equivalent volume of saline (control, n = 12) and fed standard rat chow for 8 wk. By coupling high-resolution respirometers with purpose-built fluorometers, we followed Magnesium Green (ATP synthesis), Amplex UltraRed (ROS production), and safranin-O (ΔΨ). Relative to control rats, the mass-specific respiration of STZ-diabetic hearts was depressed in oxidative phosphorylation (OXPHOS) states. Steady-state ATP synthesis capacity was almost one-third lower in STZ-diabetic heart, which, relative to oxygen flux, equates to an estimated 12% depression in OXPHOS efficiency. However, with anoxic transition, STZ-diabetic and control heart tissues showed similar ATP hydrolysis capacities through reversal of the F1F0 ATP synthase. STZ-diabetic cardiac mitochondria also produced more net ROS relative to oxygen flux (ROS/O) in OXPHOS. While ΔΨ did not differ between groups, the time to develop ΔΨ with the onset of OXPHOS was protracted in STZ-diabetic mitochondria. ROS/O is higher in lifelike OXPHOS states, and potential delays in the time to develop ΔΨ may delay ATP synthesis with interbeat fluctuations in ADP concentrations. Whereas diabetic cardiac mitochondria produce less ATP in normoxia, they consume as much ATP in anoxic infarct-like states.

Diabetes mellitus constitutes a global epidemic and is most prevalent in developed countries, with incidence in adults predicted to increase over time (19, 37, 45). Clinical and experimental studies have associated diabetes with atherosclerosis of the arterial tree (including coronary arteries), which increases the risk of limb loss through gangrene, stroke, and myocardial infarction, leading to the overall likelihood of developing heart failure (1, 8, 10, 19, 26). Mechanisms that are independent of coronary artery disease and hypertension appear to promote heart failure in diabetic patients. Rubler et al. (64) coined the term “diabetic cardiomyopathy,” noting that while diabetic patients had apparently normal coronary vasculature, they had abnormal ventricle structure and function independent of coronary artery disease and hypertension (22). Diabetic cardiomyopathy manifests with left ventricular (LV) hypertrophy, an increased susceptibility to ischemic injury, and altered myocardial structure, Ca2+ homeostasis, and cardiac substrate metabolism (2, 5, 7, 11, 17, 40).

Sustained heart function is dependent on aerobiologically derived ATP to fuel contraction. Approximately 95% of this ATP comes from mitochondrial oxidative phosphorylation (OXPHOS) systems. Diabetic hearts show progressive declines in cardiac contractile efficiency along with changes in metabolism (13). However, reports on changes in terms of metabolic efficiency, i.e., the conservation of energy from substrate oxidation to ATP production, are varied, with some studies reporting increased proton leak in diabetic rat heart mitochondria (decreased efficiency) (1, 11, 12, 57, 60), yet phosphorus-to-oxygen ratio (P/O) similar to nondiabetic mitochondria (57). We note that these efficiency measures were conducted with standard equipment and protocols that may not have fully stressed mitochondria or may not have had the resolution to detect differences in efficiencies in steady-state respiring mitochondria.

We predict that there are three points of potential energy loss: 1) ATP synthesis efficiency (tested by following ATP output and oxygen flux), 2) mitochondrial membrane potential (ΔΨ) and resulting dynamics with transitions between levels or degrees of phosphorylating respiration, and 3) the loss of electrons from the electron transport system (ETS) through the production of reactive oxygen species (ROS). Here we explore streptozotocin (STZ)-induced diabetes (STZ-diabetes) in Sprague-Dawley rat heart homogenates and real-time measurements of these three parameters simultaneously with respiratory flux by coupling purpose-built fluorometers to high-resolution respirometers.1

METHODS

Animals. Male Sprague-Dawley rats (250–300 g) were randomly assigned to two groups: STZ-diabetic rats were injected with 55 mg STZ/kg, and control rats were injected with an equivalent volume of saline (n = 12 in each group). Animals were housed in pairs in a 12:12-h light-dark cycle with 50–70% humidity at 19–21°C and fed standard rat chow and tap water ad libitum for 8 wk. Blood glucose levels and body weights were monitored daily in the 1st wk and weekly thereafter. All studies were approved by the Animal Ethics Committee of the University of Auckland.

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Committee of the University of Auckland (R925). All chemicals were sourced from Sigma-Aldrich (St. Louis, MO).

Sample preparation. Animals were anesthetized with isoflurane until they did not respond when their hindfeet were pinched. After cardiectomy, the LV was cut into small pieces and transferred into 1 ml of cold histidine-tryptophan-ketoglutarate buffer (Custodial, Koehler Chemie, Alsbach Hähnlein, Germany). Of the three distinct experimental preparations available (isolated mitochondria, permeabilized fibers, and tissue homogenates), we utilize homogenates to test cardiac mitochondrial function for reasons discussed extensively elsewhere (38).

LV tissue (~20 mg) was weighed and transferred to 500 µl of ice-cold incubation assay medium (for details see Ref. 68), homogenized for 15 s using a tissue homogenizer (Omni International, Kennesaw, GA), and loaded immediately into an oxygraph (1 mg/ml).

High-resolution respirometry. Three oxygraphs (O2k, Oroboros Instruments, Innsbruck, Austria) were employed for all measurements of mitochondrial respiratory fluxes (34). The O2k consists of two independent 2-ml chambers with polarographic oxygen sensors and stoppers that allow substrate inhibitor titrations. A purpose-built detachable fluorometer was inserted into each of the front two windows of the O2k chambers to measure fluorescence of different fluorophores (42). The oxygen concentration of the assay medium was 195 mmol/ml at 95 kPa barometric pressure. All experiments were performed at 37°C.

Mitochondrial respiration assays. Titration protocols of multiple substrates and inhibitors were used to assess mitochondrial function in terms of different respiration states. Complex I (CI)-mediated “leak” respiration was determined using malate, glutamate, and pyruvate. Succinate was then added to reduce FAD at complex II (CII, via succinate dehydrogenase), and saturating ADP was added to stimulate OXPHOS. The leak rate with CI and CII was determined before ADP addition and after ADP addition with oligomycin to block the F1F0-ATPase, which was followed with repeated carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (FCCP) titrations to uncouple and depolarize the mitochondria (43). Addition of antimycin A inhibited complex III (CIII), resulting in nonmitochondrial respiration with small contributions from electron leak in the uncoupled state. Further details of protocols are described by Goo et al. (38).

Protocol 1: measurement of ATP production in cardiac homogenates. ATP-ADP exchange was measured by following Magnesium Green (MgG) fluorescence using excitation and emission wavelengths at 503 and 530 nm, respectively (20). ATP assays were conducted using our standard respiration buffer MiRO5, which contains 3 mM MgCl2, 50 mM KCl, and 0.50% (vol/vol) Triton X-100. The tissue homogenate containing 2 mg of LV tissue. CI and CII substrates (malate, glutamate, pyruvate, and succinate, as described for protocol 1) were supplied for mitochondrial respiration. Once the safranin signal had stabilized, excess ADP (2.5 mM) was added to stimulate OXPHOS. The leak state (CI and CII) was determined by addition of oligomycin (5 µM) followed by repeated titrations of FCCP (0.5 µM) to uncouple and depolarize mitochondria (43). Addition of antimycin A (5 µM) inhibited CIII and respiration. Addition of further safranin (1 µM) to the chamber, in the presence of the tissue sample and all titrated compounds, was used to normalize the safranin signal.

While the calibration of the safranin signal can be performed by clamping and titration of KCl in the presence of the K+-ionophore valinomycin, MiRO5 (the buffer used in the present study) contains high K+. We used an alternative approach to estimate ∆Ψ. A near-linear relationship between change in safranin fluorescence and ∆Ψ has been reported, thereby allowing the estimation of ∆Ψ (28). Approximately 35–40% of the cardiomyocyte volume is occupied by mitochondria (6). The mitochondrial matrix volume in transitions between OXPHOS and leak states is dynamic, accounting for 29% and 44% of the mitochondrial volume, respectively (47). We therefore estimate matrix volumes to be 0.2 and 0.31 µl/mg tissue in respective OXPHOS and leak states. Thus the fluorescence from a known safranin concentration can be used to estimate the safranin in the medium and that imported into the mitochondrial matrix [as is conducted using tetraphenylphosphonium (TPP+)]. Using the Nernst equation, ∆Ψ = RT/2F ln[safranin]out/[safranin]in, where R is the gas constant, F is the Faraday constant, T is temperature (K), z is the valence state of the ion (+1), and [safranin]out and [safranin]in are safranin concentrations outside and inside the mitochondrial matrix.

Protocol 3: net production of ROS. The superoxide radical (O2·−) is formed from molecular oxygen as a by-product of oxidation at mitochondrial CI and CIII. In vivo, it is normally degraded by superoxide dismutases (SOD) to H2O2 or consumed by antioxidants and/or reductive systems and SOD-der SOD-de- der H2O2 were then linked to horseradish peroxidase (HRP), which in turn reacts with AUR to form a fluorescent product with excitation and emission wavelengths of 530 and 590 nm, respectively.

The net ROS production was measured simultaneously with respiratory flux using Amplex UltraRed (AUR) dye. O2·− released from mitochondria was reduced to form H2O2 by addition of exogenous SOD. The combined mitochondrial H2O2 and exogenous SOD-derived H2O2 were then linked to horseradish peroxidase (HRP), which in turn reacts with AUR to form a fluorescent product with excitation and emission wavelengths of 530 and 590 nm, respectively.

AUR (5 µM), SOD (10 U), and HRP (10 U) were added to the chambers, followed by H2O2 (330 nM), to calibrate the ROS signal. Homogenate (2 mg of wet tissue) was added to the chambers and allowed to equilibrate. CI and CII substrates were supplied to initiate the leak state. Addition of ADP (2.5 mM) stimulated OXPHOS. The leak rate with CI and CII was again determined by addition of oligomycin (5 µM) followed by repeated titrations of FCCP (0.5 µM) to uncouple mitochondria. Antimycin A (5 µM) was added, and the activity of cytochrome c oxidase (CCO) was measured using the electron donor substrate N,N,N′,N′-tetramethyl-p-phenylenediamine (0.5 mM) and ascorbate (2 mM) as ATP production.

Citrate synthase assay. Citrate synthase (CS) was used to provide an estimate of mitochondrial mass. Frozen (~80°C) tissues were thawed, minced, weighed, and homogenized in 1:10 (wt/vol) ice-cold buffer consisting of 25 mM Tris-HCl at pH 7.8, 1 mM EDTA, 2 mM MgCl2, 50 mM KCl, and 0.50% (vol/vol) Triton X-100. The tissue homogenates were centrifuged (model 5417R, Eppendorf) at 14,000 g for 10 min at 4°C, and the supernatant was frozen for CS assays (55). CS activities were determined following the procedure described by
Table 1. Average heart dimensions and functional parameters of control and diabetic rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt, g</td>
<td>482 ± 7</td>
<td>299 ± 13†</td>
</tr>
<tr>
<td>Heart wt, g</td>
<td>1.21 ± 0.04</td>
<td>0.96 ± 0.03‡</td>
</tr>
<tr>
<td>Lung wt, g</td>
<td>1.66 ± 0.06</td>
<td>1.40 ± 0.05‡</td>
</tr>
<tr>
<td>Heart wt/body wt, %</td>
<td>0.250 ± 0.007</td>
<td>0.326 ± 0.013‡</td>
</tr>
<tr>
<td>Lung wt/body wt, %</td>
<td>0.343 ± 0.010</td>
<td>0.471 ± 0.013‡</td>
</tr>
<tr>
<td>LV wall thickness, mm</td>
<td>3.63 ± 0.16</td>
<td>3.54 ± 0.14</td>
</tr>
<tr>
<td>RV wall thickness, mm</td>
<td>1.13 ± 0.01</td>
<td>1.03 ± 0.02*</td>
</tr>
<tr>
<td>LV/RV</td>
<td>3.23 ± 0.16</td>
<td>3.45 ± 0.16</td>
</tr>
<tr>
<td>LV thickness/heart wt, mm/g</td>
<td>3.02 ± 0.13</td>
<td>3.73 ± 0.17†</td>
</tr>
<tr>
<td>Blood glucose, mM</td>
<td>6.8 ± 0.2</td>
<td>28.3 ± 1.0‡</td>
</tr>
<tr>
<td>Tibia length, mm</td>
<td>46.1 ± 0.3</td>
<td>42.0 ± 0.5‡</td>
</tr>
<tr>
<td>LV thickness/tibia length, %</td>
<td>8.0 ± 0.4</td>
<td>8.4 ± 0.3</td>
</tr>
</tbody>
</table>

Values are means ± SE (n = 12 per group). LV, left ventricle; RV, right ventricle. *P < 0.05, †P < 0.01, ‡P < 0.005 (by Student’s t-test).

Sere (67), based on the slope (Δabsorbance/Δmin), and activities were standardized to purified CS.

Data analysis. Values are means ± SE. Student’s t-tests (2-tailed) were performed using Excel or Sigma Plot version 11.0 (Systat Software, San Jose, CA) to test the differences between diabetic and control groups. P < 0.05 was considered to be statistically significant. Each data point arose from replicated independent experiments.

RESULTS

Physiological parameters of the diabetic model. Blood glucose levels and body weights of all animals were measured weekly. At 2 days after STZ injection, the rats developed diabetic symptoms, such as high blood glucose (>20 mM), polydipsia, and polyuria. On average, the blood glucose levels remained almost four times higher and the average body weight was significantly lower in the diabetic group at 8 wk postinjection (Table 1). No statistical difference was detected in the average LV free wall thickness, but when expressed as a ratio of heart weight, the STZ-diabetic rats had a greater relative LV thickness.

Mitochondrial respiration. Respirational flux in CI + CII (CI+II) leak, OXPHOS, and ETS states in the ROS, ΨΔ, and ATP assays was consistently higher in the control than diabetic group (Fig. 1A), indicating an overall depression of respiration capacities in diabetes. The diabetic tissue homogenates showed no significant difference in respiratory control ratio (RCR CI+II) relative to control LV homogenates. Addition of the uncoupling agent FCCP led to a higher relative maximal flux rate and higher uncoupling control ratio [UCR (ETS/OXPHOS)] in the control than diabetic group (Table 2), indicating that the untreated mitochondria were more tightly controlled by the phosphorylation system and had greater reserve ETS capacities. CCO flux was 16% lower in the diabetic group (P = 0.07).

The mitochondrial enzyme CS was assayed in homogenates as a marker for mitochondrial content. CS activities did not differ significantly between the control and diabetic groups; 9.30 ± 0.79 and 8.29 ± 0.37 μmol-min⁻¹-mg wet wt⁻¹, respectively (P > 0.05). The trends of respirational fluxes normalized to CS (Fig. 1B) resembled those determined relative to mass, indicating that the depression of mitochondrial respirational flux results mostly from qualitative differences in mitochondria and is less attributable to decreased mitochondrial mass.

Steady-state ATP synthesis measurements. The diabetic group also showed a 40% depression in mass-specific ATP production rates (Fig. 2B). When presented relative to steady-state oxygen flux, we observed a significantly lower P/O as a measure of mitochondrial phosphorylation efficiency (Fig. 2C). In this experiment we permitted respiration to continue into anoxia and then observed a reversal of the ATP flow signal (Fig. 2A). After addition of oligomycin, ATP hydrolysis was mostly inhibited, indicating that hydrolysis was occurring at the FoF₁-ATP synthase. In contrast to ATP production rates, ATP consumption rates in anoxia were equivalent in diabetic and control groups (Fig. 2B).

Membrane potential. ΔΨ in the leak state reached similar estimated resting voltages of −197 ± 6 mV (n = 8) in the control group and −207 ± 3 mV (n = 8) in the diabetic group and, with the onset of OXPHOS, reached similar voltages...
170 mV (static) to 180 mV (dynamic) in both groups, depending on matrix volume model (Fig. 3A). Diabetic mitochondria, however, took longer to depolarize with the initiation of OXPHOS, indicating a slower response to elevated OXPHOS and, therefore, a decreased rate of ATP synthesis in diabetic hearts (Fig. 3B).

**DISCUSSION**

Our collective evidence indicates that mitochondria from diabetic hearts are less efficient than those from untreated hearts. They produce less ATP in normoxic steady-state respiration states and release marginally more ROS per oxygen consumed in OXPHOS states. They also take longer to modulate with fluctuations in ADP (i.e., as may occur between heartbeats). Our most important observation is that, despite substantial depression of ATP synthesis capacity in oxygen-saturated states, mitochondria from diabetic hearts appear to consume as much ATP in anoxic infarct-like states as mitochondria from healthy hearts.

With development of diabetic cardiomyopathy, structural and metabolic alterations impact the myocardium and contribute to cardiac dysfunction (8, 56). A relative increase of 24%...
in the LV thickness-to-heart weight ratio (Table 1) was apparent, and this is consistent with published data for STZ-induced diabetic rat hearts, which progressively show depressed pumping capacities (52), most likely through decreasing LV compliance (4). We note that contemporaneous work found decreased pumping efficiencies at elevated afterloads in rats from the same source as those used in the present study (39). A significant decrease in heart rate (15%) and increase in systolic duration (24%) also occur in 8-wk STZ-diabetic Wistar rats (52) and have been reported in diabetic men and women (22).

Multiple substrate and inhibitor titrations were used to maximize the flux in various respiration states in an attempt to mirror electron flow in substrate-saturated cardiomyocytes at high workloads. Mass-specific, ADP-stimulated, coupled respiration (OXPHOS) with parallel CI and CII electron inputs was decreased by 24% in the diabetic group when expressed per unit of tissue mass (Fig. 1A), which is consistent with reports of decreased mitochondrial activities in diabetic heart (44). Normalization of mitochondrial respiration to CS activity to distinguish changes in mitochondrial density (49) showed no statistical difference in CS activities between groups. This indicates that depression of mitochondrial function likely results from decreased capacities of the diabetic mitochondria.

The OXPHOS uncoupler FCCP, which dissipates $\Delta\psi$, generally maximizes respiration through the ETS (59). We note a slight depression of ETS flux relative to OXPHOS and contend that the loss of $\Psi\Delta$ may result from decreased electrogenic substrate import. Here we present the UCR as ETS capacity normalized to OXPHOS. Assuming that the influence of FCCP is equal between the two groups, we note that the UCR is 19% more depressed in diabetic samples. This depression may result from one or more of the following factors: a relative insufficiency of the ETS, a proportionate increase in ATP synthase capacity, or increased proton leak (29, 51).

The contribution of CII-derived flux to leak respiration was 26% higher in the control than diabetic group, indicating an intrinsic difference in ETS function (Table 2). Some investigators contend that CII-mediated respiration may elevate ATP output rates at a cost to efficiency (30). The lower fraction of CII contribution to leak respiration in diabetic mitochondria would predict greater phosphorylation efficiencies in OXPHOS; however, we detected lower ATP production rates in the diabetic group.

In accord with previous work (52), our findings show that respiration function is depressed in the diabetic heart. This also results in a depression of CCO activity, the terminal step of the ETS where oxygen is reduced to H$_2$O. CCO flux generally appears to be excessive relative to the ETS flux in most mitochondria (35) and is more excessive in cardiac mitochondria than in other tissues such as liver (9). While mass-specific CCO flux was substantially higher (16%) in control than diabetic rats (Fig. 1A), the CCO flux presented as that relative to OXPHOS and ETS (i.e., CCO/OXPHOS and CCO/ETS)
was conserved between groups; therefore, differences in CCO flux relate to differences in mitochondrial mass (35). Lower overall capacities of CCO in diabetic mitochondria may decrease oxygen uptake at low oxygen tensions and/or enhance the effect of NO, a potent inhibitor of CCO (33), which is known to increase with hyperglycemia (21).

The RCR, which is the inverse of the leak ratio, is widely used to evaluate the structural integrity of the inner mitochondrial membrane (IMM) and OXPHOS efficiency (30). The RCR is perhaps less informative, since the leak-to-OXPHOS ratio provides a more direct index of the fraction of leak respiration not contributing to OXPHOS. The leak control ratios as CI+II leak/CI+II OXPHOS in the ROS assay were ~20–25%. While this appears high relative to leak fluxes for CI-fueled respiration (7–10%) (32, 61), it reflects the lower proton-pumping capacity of CII electron transport chains. The RCR values did not differ between groups in the present study. However, phosphorylation efficiencies may be dynamic, as mitochondria with high apparent leak rates (or low RCRs) can show P/O values similar to those with low leak ratios (high RCRs) (59). This may result from experimental variability and/or the estimation of P/O values in non-steady states from single substrates.

**Steady-state ATP synthesis measurements.** Here we present P/O in steady-state respiration states in an attempt to maximize the loading on mitochondria through use of multiple electron inputs. Diabetic mitochondria were less efficient. ATP synthesis efficiency can be altered due to proton leakage through adenine nucleotide translocase and uncoupling proteins (UCPs) and directly through the IMM (14). The steady-state P/O derived in this study contrasts with those measured using isolated mitochondria and traditional extrapolation of traces for deriving OXPHOS efficiencies (57). We consider that these differences can be attributed to our real-time measurements of ATP synthesis in steady states at sustained high flux rates and the use of multiple electron inputs in the titration protocol.

The stoichiometry of proton translocation across the IMM can be used to determine the steady-state P/O. The maximum P/O for CI and CII substrates are predicted to be ~2.5–2.8 and 1.5, respectively (50). The steady-state P/O is calculated from the following identity: \[ P/O = (P/O)_{\text{max}} J_P/J_s \text{, where } J_P \text{ and } J_s \text{ represent flux through the phosphorylation and substrate modules, respectively (15). Proton leakage and the addition of less OXPHOS-coupled CII substrates to CI substrates should decrease } P/O (25). \]

The steady-state P/O with CI and CII substrates in this study provides a useful understanding of ATP synthesis and turnover. With CI and CII assayed together, the P/O should be between 2.5–2.8 and 1.5, and we observed a P/O of ~2 in healthy heart. This approximates the P/O of Langendorff-perfused hearts using 31P-NMR supported by pyruvate (~2.1) (46). Steady-state P/O was 18% lower in diabetic rats (Fig. 2C), indicating decreased mitochondrial coupling, which associates with decreased cardiac efficiency (17). While standard methods predicted decreased P/O in db/db type 2 diabetic mice (13) and UCP-DTA mice (27), Herlein et al. (41) also demonstrated unchanged, if not improved, P/O in mitochondria isolated from 8-wk STZ-diabetic rats. Although diabetic exposure in the study of Herlein et al. was similar to that in the present study, we did not isolate mitochondria, with the aim to retain all mitochondrial populations. Importantly, we measured ATP synthesis in real time with abundant ADP and both CI and CII electron inputs, as must occur in vivo in hard-working hearts. The absolute ATP output is 35% less per gram in diabetic hearts (Fig. 2B), which should decrease high-energy phosphate supplies at high workloads in diabetic hearts.

By measuring ATP in real time we could also measure adenylyte dynamics in anoxia. An imbalance between ATP production and consumption was apparent in diabetic mitochondria. Diabetic human hearts are more susceptible to ischemic insults, as are rodent hearts following prolonged diabetes (58). Under anoxia, mitochondria stop electron transport and decrease proton pumping across the IMM. When the \( \Delta \Psi \) is thought to dissipate to approximately –100 mV, the ATP synthase activity reverses and ATP hydrolyase activity dominates (24). While the rate of ATP production was lower in diabetic tissues, there was no significant difference in oligomycin-sensitive ATP consumption between diabetic and normal rat homogenates (Fig. 2B). Oligomycin addition shows that the hydrolytic activity of the diabetic rat heart ATP synthase can consume similar amounts of ATP in anoxia but cannot make the same amount of ATP in normoxia. These data provide a potential mechanism for the poor recovery of diabetic hearts from ischemic insults.

**Mitochondrial membrane potential.** On the basis of a known safranin fluorescence signal, we estimated \( \Delta \Psi \). As safranin is a lipophilic cationic dye, it may bind within the mitochondrial matrix independent of \( \Delta \Psi \). This binding coefficient is unavailable and may lead to overestimates of \( \Delta \Psi \) in the vicinity of ~25 mV (63). In accordance, the maximal \( \Delta \Psi \) reported in this study was around ~205 mV (Fig. 3A). An overestimate of ~25 mV would place this value at ~180 mV, which is consistent with results from studies in which tetraphenylphosphonium was used (31).

The mitochondrial volume is ~40% of that of a cardiomyocyte. The matrix volume, which is not static, occupies 29% of the mitochondrial volume in the OXPHOS state and can swell by 44% in leak states and in hypoxia (47). \( \Delta \Psi \) was similar for both groups [~200 mV (leak) and ~170 mV (OXPHOS)]. While these estimates are higher than those reported by others using different methods, the difference between states is similar (i.e., ~30 mV) (28, 69). While it makes no apparent difference for comparisons between treatment groups, a dynamic matrix volume predicts shrinkage on transition to OXPHOS from the leak state, and this increases \( \Delta \Psi \) by 10 mV relative to OXPHOS in a static matrix model. This impacts current views of ROS production, which is assumed to be mediated by high \( \Delta \Psi \) (54).

While \( \Delta \Psi \) values did not differ between the groups, our \( \Delta \Psi \) assays were able to show that the time required for \( \Delta \Psi \) to reach a steady state was protracted in diabetic samples with the transition from leak to OXPHOS (Fig. 3B). Protracted time required for \( \Delta \Psi \) to reach a steady state may reflect depressed respiratory flux rates in diabetes, which impedes \( \Delta \Psi \) development. The development of \( \Delta \Psi \) not only mediates ATP production but also drives substrate import and Ca\(^{2+}\) uptake, which also stimulate tricarboxylic acid cycle dehydrogenases (16, 23). Delays in the establishment of \( \Delta \Psi \) may therefore influence ATP synthesis, substrate import/turnover, and Ca\(^{2+}\) buffering and ROS production.

**ROS production in diabetic cardiac mitochondria.** One of the methodological advantages achieved in this study was the
simultaneous measurement of ROS output (pmol s⁻¹·mg tissue wet wt⁻¹) and respirational flux, measured also in leak andOXPHOS states. ROS generation from the ETS is assumed to be 0.1–4% of oxygen consumption (66). Our observations are consistent with those reported by others (41), as we demonstrate that, in control specimens, H₂O₂ production is greater in the CI+II leak state than in diabetic specimens, as was the percent ROS/O (1.48%). However, while the H₂O₂ production rate was low in absolute terms, ROS/O in the OXPHOS state was 47% higher in the diabetic group (Fig. 4B), and this state, unlike the leak state, is a physiologically relevant respiration state, with relevant electron inputs. Studies using a type 1 diabetes Akita mouse model and STZ-diabetic rats showed no evidence for increased mitochondrial ROS generation (18, 41); the range of ROS/O in OXPHOS was 0.015–0.03%, which was also observed in a hypertensive, nondiabetic rat heart failure model (43). While elevated relative to the control animals, whether this low amount of ROS can mediate damage in diabetic cardiomyopathy remains to be tested.

The CI and CII substrate combination elevates the electron inputs into ETS at ubiquinone and represents more realistic scenarios of ATP synthesis and ROS production. The high ΔΨ generated by CI+II leak is also thought to promote reverse electron transfer (RET) from CII back to CI through ubiquinone (3). While mechanistically unclear, electron accumulation at the semi-quinone formation site within CI results in O₂⁻ evolution (3). Whether RET occurs in vivo remains unanswered; however, RET is dependent on mitochondrial coupling and ADP concentration [as shown in the current study and elsewhere (62)], and ROS production clearly declines in OXPHOS states. Mitochondria of healthy working hearts in normoxia are likely never completely ADP-limited, and ROS measurement in the OXPHOS state is likely to be informative. However, as matrix ADP concentration fluctuates (16), so may ROS outputs. Moreover, mitochondrial creatine kinase decreases with diabetes (44), and this should impede ADP return to the matrix and potentially elevate ROS. Our study shows that while ΔΨ is maintained at similar apparent levels in control and diabetic samples, protracted transitions of ΔΨ from the high “near-leak” to the lower OXPHOS ΔΨ in diabetic groups could act to raise ROS.

Our measure of ROS in the leak and OXPHOS states is net ROS production, i.e., the sum of production and removal. The elevated ROS/O in the OXPHOS state in the diabetic group may be due to altered ETS function and/or impaired ROS defense systems. Electron leakage from the ETS as O₂⁻ is converted by SOD to H₂O₂ and further reduced to H₂O by systems such as glutathione peroxidase (3). While SOD2 concentrations can increase by 45% in diabetic hearts (44), decreased reduced glutathione concentrations have been reported in diabetic hearts (65), suggesting that downstream consequences of increased ROS production and challenged detoxification systems occur in diabetic mitochondria (18).

Conclusions. Diabetic cardiac mitochondria respires and phosphorylate with less capacity and efficiency (ATP produced per mole of oxygen) than mitochondria from healthy hearts. ROS production relative to oxygen is higher in diabetic samples in more lifelike OXPHOS states. While minimal, this ROS production represents a loss of electrons that otherwise could contribute to ATP synthesis. The protracted development of ΔΨ may impair activation of ATP synthesis and elevate ROS, with interbeat fluctuations in ADP concentration. Perhaps most importantly, during anoxic infarct-like states, mitochondria from diabetic hearts appear to consume as much ATP as those from healthy hearts, and this will possibly produce a greater ATP deficit on reoxygenation. This scenario presents a possible mechanistic explanation for the susceptibility of diabetic hearts to infarct.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

T.P., D.L., A.P., and A.J.R.H. are responsible for conception and design of the research; T.P., A.P., and A.J.R.H. performed the experiments; T.P., A.P., and A.J.R.H. analyzed the data; T.P., D.L., and A.J.R.H. interpreted the results of the experiments; T.P. and A.J.R.H. prepared the figures; T.P., D.L., A.P., and A.J.R.H. drafted the manuscript; T.P., D.L., A.P., and A.J.R.H. edited and revised the manuscript; A.J.R.H. approved the final version of the manuscript.

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