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Mitochondrial superoxide and coenzyme Q in insulin-deficient rats: increased electron leak

Judith A. Herlein,1 Brian D. Fink,1 Dorylne M. Henry,1 Mark A. Yorek,1 Lynn M. Teesch,2 and William I. Sivitz1

1Departments of Internal Medicine/Endocrinology and 2Analytic Chemistry, University of Iowa and the Iowa City Veterans Affairs Medical Center, Iowa City, Iowa

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Herlein JA, Fink BD, Henry DM, Yorek MA, Teesch LM, Sivitz WI. Mitochondrial superoxide and coenzyme Q in insulin-deficient rats: increased electron leak. Am J Physiol Regul Integr Comp Physiol 301: R1616–R1624, 2011. First published September 21, 2011; doi:10.1152/ajpregu.00395.2011.—Mitochondrial superoxide is important in the pathogenesis of diabetes and its complications. However, there is uncertainty regarding the intrinsic propensity of mitochondria to generate this radical. Studies to date suggest that superoxide production by mitochondria of insulin-sensitive target tissues of insulin-deficient rodents is reduced or unchanged. Moreover, little is known of the role of the Coenzyme Q (CoQ), whose semiquinone form reacts with molecular oxygen to generate superoxide. We measured reactive oxygen species (ROS) production, respiratory parameters, and CoQ content from gastrocnemius muscle of control and streptozotocin (STZ)-diabetic rats. CoQ content did not differ between mitochondria isolated from vehicle- or STZ-treated animals. CoQ also was unaffected by weight loss in the absence of diabetes (induced by caloric restriction). Under state 4 or state 3 conditions, both respiration and ROS release were reduced in diabetic mitochondria fueled with succinate, glutamate plus malate, or with all three substrates (continuous TCA cycle). However, H2O2 and directly measured superoxide production were substantially increased in gastrocnemius mitochondria of diabetic rats when expressed per unit oxygen consumed. On the basis of substrate and inhibitor effects, the mechanism involved multiple electron transport sites. More limited results using heart mitochondria were similar. ROS per unit respiration was greater in muscle mitochondria from diabetic compared with control rats during state 3, as well as state 4, while the reduction in ROS per unit respiration on transition to state 3 was less for diabetic mitochondria. In summary, ROS production is, in fact, increased in mitochondria from insulin-deficient muscle when considered relative to electron transport. This is evident on multiple energy substrates and in different respiratory states. CoQ is not reduced in diabetic mitochondria or with weight loss due to food restriction. The implications of these findings are discussed.

Address for reprint requests and other correspondence: W. Sivitz, Dept. of Internal Medicine, Div. of Endocrinology and Metabolism, The Univ. of Iowa Hospitals and Clinics, 422GH, 200 Hawkins Dr., Iowa City, IA 52242 (e-mail: william-sivitz@uiowa.edu).

HIGH NUTRIENT SUPPLY TO CELLS as typified by glucose and fatty acids in diabetes and obesity is posited to increase substrate delivery to mitochondria, resulting in increased production of reactive oxygen species (ROS) (6, 14, 34). Consequent oxidative damage is believed to contribute to diabetic complications as well as to progressive pancreatic β-cell damage and worsening insulin sensitivity among insulin target cells. A less studied issue is whether mitochondria of cells exposed to the in vivo diabetic milieu are intrinsically altered in a way leading to excess ROS production. If this were the case, then these mitochondria, when isolated and incubated, in vivo would be expected to manifest increased ROS production. However, existing data do not support that expectation. We recently found that skeletal muscle mitochondria from insulin-deficient streptozotocin diabetic rats generated less H2O2 and unchanged amounts of superoxide [directly assessed by electron paramagnetic spectroscopy (EPR)] compared with control mitochondria (15). Moreover, a recent study (7) showed that heart mitochondria of an insulin-deficient type 1 model, the Akita mouse, manifest no greater ROS production than nondiabetic controls.

Although the above data seem to imply that superoxide production by insulin-deficient diabetic mitochondria is reduced or unchanged, the issue is actually more complex when viewed in terms of superoxide produced per unit oxygen consumed, an index of overall activity of the electron transport system (ETS), wherein electron leaks occur. In other words, ROS production by diabetic mitochondria could be confounded by concurrent changes in respiration or membrane potential, both of which might be affected in diabetes.

Another factor that might alter mitochondrial ROS production is the content of mitochondrial coenzyme Q (ubiquinone/ubiquinol or CoQ). In fact, the large part of the electron leak to molecular oxygen results from the semiquinone form of CoQ generated during the Q-cycle in complex III or by a similar, less defined mechanism in complex I (5, 8, 21). Hypothetically, CoQ might be reduced in insulin-deficient states since the first step in CoQ biosynthesis, like cholesterol biosynthesis, depends on hydroxy-methylglutaryl-CoA (HMG-CoA) reductase (CoQ synthesis subsequently branches from the cholesterol pathway). At least in the liver, HMG-CoA reductase transcription is enhanced by insulin and reduced in streptozotocin (STZ)-diabetic rats (20). However, mitochondrial CoQ concentrations in diabetic states are not well defined, and most studies have involved small numbers of animals. Moreover, CoQ content and content within skeletal muscle mitochondria have not been reported to our knowledge.

In the current work, we examined the interrelations between mitochondrial function and ROS production, as well as gastro-
nemius muscle mitochondrial CoQ content. Functional parameters and ROS were assessed in parallel under different substrate conditions, and ROS were assessed both as H₂O₂ measured by fluorescence and superoxide assessed directly by EPR. Prior work in our laboratory and others (16, 26, 32) have shown that the fluorescent method (when applied to isolated mitochondria) detects superoxide indirectly after release to the matrix, conversion by manganese superoxide dismutase (MnSOD), and release externally as H₂O₂, while EPR detects superoxide as directly released, largely from complex III (26).

Here, we provide novel information regarding the relationship of ROS to mitochondrial functional parameters and CoQ. As will be discussed, our results have mechanistic and physiological implications.

MATERIALS AND METHODS

**Materials.** Reagents were purchased as indicated or from standard sources.

**Animal studies.** Male Sprague-Dawley rats were obtained from Harlan (Indianapolis, IN). Animals were fed standard rat chow (Harlan Teklad; #7001) and maintained according to standard National Institutes of Health guidelines. The protocol was approved by our Institutional Animal Care and Use Committee. Rats were fed standard chow ad libitum and euthanized by intraperitoneal injection of 25 mg/kg pentobarbital sodium followed by incision of the left ventricle. Data have shown that doses of pentobarbital sodium up to 4-fold more than we used does not affect mitochondrial respiration or membrane potential (33). Moreover, euthanasia was carried out in the same way for control and diabetic groups.

Rats were made diabetic with STZ, 60 mg/kg ip. Controls received vehicle (saline). Glucose was determined on tail vein blood using a reagent strip and meter (OneTouch Ultra). Rats were killed at ~1000 h, 2 h after removal of food. Gastrocnemius muscle and heart tissues were removed, washed, blotted, and weighed before preparation of mitochondria.

A subgroup of rats were subject to either STZ or vehicle treatment or to food restriction (n = 8 per group). Food restriction was carried out by limiting food intake to 11 g (half of what the control group ate) per day and continued until the rats lost 20% of their initial weight (maximum allowed by our animal care unit).

The data presented herein derive from mitochondrial studies carried out over five separate groups of experiments, each involving STZ-diabetic and vehicle-treated control animals. Data for ROS production, proton conductance, and oxygen consumption from 34 rats (17 control and 17 STZ-diabetic), comprising groups II and III of a previous publication (15), has previously been reported in that manuscript. Here, we combine these results with 57 newly studied animals (26 control and 31 STZ-diabetic), increasing the numbers for data comparison and enabling adequate numbers to assess ROS in relation to multiple mitochondrial functional parameters assessed under different substrate and inhibitor conditions. Along with these additional studies, we included a group of eight food-restricted rats, as previously described (15). Under these conditions, H⁺ flux is proton leak-dependent (27). Proton conductance is given by hydrogen transfer/potential (nmol H⁻ min⁻¹ mg⁻¹ mV⁻¹). In some experiments, we determined state 3 and state 4 respiration by measuring oxygen consumption in the presence or absence of ADP (0.2 mM), respectively.

**Mitochondrial ROS production by fluorescent measurement.** H₂O₂ production was assessed using the fluorescent probe, 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red; Invitrogen, Carlsbad, CA), a highly sensitive and stable substrate for horseradish peroxidase and a well-established probe for isolated mitochondria (28). Samples were prepared in 96-well plates containing 0.06 ml well respiratory buffer identical to that used for respiration and membrane potential, except for the absence of nigericin, which has been reported to alter mito-

**Isolation of mitochondria.** Muscle and heart tissues were minced for 1 min prior to homogenization. Mitochondria were then isolated and washed three times, as previously described (11, 15, 17). Mitochondria prepared in this fashion were highly pure, as indicated by the distribution of glyceraldehyde-3-phosphate dehydrogenase and porin in whole tissue and mitochondrial extracts (15). Moreover, these characteristics, as well as mitochondria yields (mg/g tissue), did not differ between control and diabetic rats (15).

**Determination of CoQ by HPLC.** CoQ is predominantly present as CoQ9 or CoQ10 (dependent of the number of 5-carbon prenyl units in the side chain) in rodents or larger mammals, including humans, respectively. Mitochondrial CoQ9 and CoQ10 were determined by HPLC using an Ascentis C-18 (25 cm × 4.6 mm) silica-based reverse-phase column with ultraviolet detection at 275 nm and C18 precolumn using a mobile phase consisting of 90% ethanol/10% methanol. Isolated mitochondria in double-distilled water were extracted with hexane:ethanol, 5:2. Samples were vortexed and centrifuged, and the upper phase was dried under nitrogen and dissolved in ethanol at 50°C for injection. Figure 1 depicts representative HPLC spectra. Spectra were determined without and with added (spiked) CoQ10. The identity of the eluted material corresponding to the CoQ9 peak was confirmed by mass spectroscopy (data not shown). Since CoQ10 makes up only a very small portion of the total CoQ content, we spiked samples with known amounts of CoQ10 for determination of extraction efficiency and corrected for the small portion of endogenous CoQ10. CoQ9 and CoQ10 were quantified against standard curves, which were linear over the range of values assayed.

**Respiration, membrane potential, and proton conductance.** Respiration and mitochondrial inner membrane potential were determined, as we previously described (10, 26). Membrane potential was calculated using the Nernst equation based on the distribution (inside and external to the mitochondrial matrix) of the lipophilic cation, tetraphenyl phosphonium. Mitochondria (0.5 mg/ml) were incubated in ionic respiratory buffer (120 mM KCl, 5 mM KH₂PO₄, 2 mM MgCl₂, 1 mM EGTA, 3 mM HEPES, pH 7.2 with 0.3% fatty acid free BSA). In experiments that assessed membrane potential, respiration was assessed simultaneously, and the buffer included 2 μM oligomycin to inhibit ATP synthase, and 0.1 μM nigericin to abolish the Δψ (23) across the mitochondrial membrane, as we have previously described (10). Under these conditions, H⁺ flux is proton leak-dependent (27). Proton conductance is given by hydrogen transfer/potential (nmol H⁻ min⁻¹ mg⁻¹ mV⁻¹). In some experiments, we determined state 3 and state 4 respiration by measuring oxygen consumption in the presence or absence of ADP (0.2 mM), respectively.
Table 1. Characteristics of the control (vehicle-treated) and diabetic (STZ-treated) rats

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<th>Control (Vehicle)</th>
<th>Diabetes (STZ)</th>
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<tr>
<td>n</td>
<td>43</td>
<td>48</td>
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<tr>
<td>Age at sacrifice, days</td>
<td>149 ± 3.5</td>
<td>150 ± 3.4</td>
</tr>
<tr>
<td>Days post-STZ† or vehicle</td>
<td>52.2 ± 3.3</td>
<td>52.8 ± 3.1</td>
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<tr>
<td>Weight, g</td>
<td>444 ± 4</td>
<td>266 ± 8*</td>
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<tr>
<td>Weight loss or gain, g</td>
<td>91.1 ± 6.4</td>
<td>-91.4 ± 8.3*</td>
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<tr>
<td>Glucose, mg/dl††</td>
<td>108 ± 2</td>
<td>585 ± 6*</td>
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Data are expressed as means ± SE. †Diabetes diagnosed on day 3 after streptozotocin (STZ) administration. ††Values above upper limit of detection reported as 600 mg/dl. *P < 0.001 compared to control.

Mitochondrial superoxide production by abolition of the pH gradient across the inner membrane (22). Fluorescence was measured, as we previously described (26), once every 44 s for 50 cycles. For quantification, a H2O2 standard curve ranging from 0 to 12 µM was prepared and included on each plate. The addition of catalase, 500 units/ml, reduced fluorescence to below the detectable limit indicating specificity for H2O2. The addition of substrates to respiratory buffer without mitochondria did not affect fluorescence. The addition of the ETS inhibitor rotenone with mitochondria in the absence of substrate altered fluorescence only about 5%.

To calculate H2O2 production per unit respiration, the H2O2 production rate (pmol H2O2 per mg mitochondria per minute) was divided by the respiration rate (nmol O2 per mg per min) to determine H2O2 produced per nmol oxygen consumed.

ERS spectroscopy: ROS were also measured by EPR, as we have previously described (11, 26). Mitochondria were studied during state 4 respiration in 0.2 ml of respiratory buffer with 0.069 M 5,5-dimethyl-1-pyrroline-N-oxide, and 0.09 mg mitochondria. Respiration was initiated with the addition of substrate and samples incubated for 5 min at 37°C before transfer to a flat aqueous EPR cell. Spectra were then recorded at room temperature using the following instrument settings: microwave power 40 mW, modulation amplitude 2G, receiver gain 2 × 105, conversion time 40.96 ms, time constant 81.92 ms, and scan rate 80G/41.92 s. Spectra show the average of seven scans. Superoxide production for diabetic and control mitochondria was expressed as relative signal intensity calculated as signal intensity per milligram mitochondria of the sample in question normalized to the average value for mitochondria of control samples (assigned 100). That value was divided by the respiration rate to determine superoxide per nanomoles per minute oxygen consumed.

Whole animal gas exchange. O2 consumption was determined using a PhysioScan Metabolic System to assess gas exchange in small animals. Diabetic and control rats were examined in side-by-side fashion.

Parallel measurements. For all results comparing ROS (either as H2O2 fluorescence or by EPR), respiratory parameters, or CoQ, the results reported represent measurements made on mitochondria from control and STZ-treated rats followed and treated in side-by-side fashion from the day of initial administration or STZ or vehicle to the time of death. One or two control and one or two STZ rats of the same age were killed on each individual day of study.

Statistics. Data were analyzed by two-tailed, unpaired t-test, ANOVA, or by Pearson correlation, as indicated in the figure legends, tables, or text.

RESULTS

Animal characteristics. As indicated in Table 1, the diabetic rats manifest the expected changes in body weight and glucose and were closely matched with respect to age at death and duration of treatment with STZ or vehicle. A smaller number of rats were subject to calorie restriction. These rats lost 20% of their initial body weight, equal to the amount allowable by our Animal Care and Use Committee.

The data in Table 1 include all animals. In actuality, since all individual parameters could not be assessed in mitochondria from all animals, most parameters were determined in subgroups. However, for each subgroup, diabetes was induced in the same way, and the diabetic and control animals studies were followed over the same time periods in side-by-side fashion. Hence, the differences or lack of differences between the diabetic and control animals within any subgroup were essentially as indicated in Table 1.

O2 consumption was determined in a subset of the animals. Whole body O2 consumption determined one day prior to death was 28.2 ± 1.1 ml·kg−1·min−1 (n = 12) in diabetic rats compared with 18.5 ± 0.9 (n = 6) in control rats (P < 0.001).

CoQ content. CoQ content did not differ between the diabetic and control rats (Fig. 2), even though the diabetic rats lost body mass (Table 1). There was also no significant correlation between CoQ content and weight loss among the diabetic animals. Moreover, in a subset of the control and diabetic animals depicted in Fig. 2, we included a group of 22-day food-restricted rats (age 140 ± 1 days, weight 314 g after an average loss of 77 ± 2 g). CoQ content (means ± SE) in the food-restricted rats compared with STZ and vehicle-treated rats followed at the same time (n = 8 for each group) did not differ (5.41 ± 0.80, 5.55 ± 0.64, and 5.97 ± 0.47 for the food-restricted, vehicle, and STZ-treated rats, respectively). CoQ content was also not different if compared with the mean values for all 31 of the vehicle and all 35 of the STZ-treated rats using one-way ANOVA.

Respiration, membrane potential, and proton conductance in gastrocnemius mitochondria of vehicle and STZ-treated rats. As shown in Table 2, respiration and proton conductance under state 4 conditions on glutamate plus malate or on succinate were substantially reduced in mitochondria of the diabetic compared with control rats. We saw no significant differences in membrane potential between control and diabetic mitochondria.
ROS production by gastrocnemius mitochondria of vehicle and STZ-treated rats. Like respiration, H$_2$O$_2$ production was reduced in mitochondria of diabetic compared with control rats (Table 2). This was the case for H$_2$O$_2$ generated under either of the substrate conditions and in the presence or absence of rotenone. The only exception was H$_2$O$_2$ production in mitochondria fueled by succinate in the absence of rotenone, wherein the reduction was not statistically significant. Superoxide directly assessed by EPR did not differ between mito-

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<th>Ctrl Glutamate 3.2 mM + Malate</th>
<th>16 mM Succinate</th>
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<tr>
<td>Respiration, nmol O·min$^{-1}$·mg$^{-1}$</td>
<td>45 ± 4 (18)</td>
<td>147 ± 6 (24)</td>
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<tr>
<td>Proton conductance, pmol H$^+$·min$^{-1}$·mg$^{-1}$·mV$^{-1}$</td>
<td>161 ± 30 (6)</td>
<td>831 ± 33 (10)</td>
</tr>
<tr>
<td>Potential, mV</td>
<td>229 ± 5 (6)</td>
<td>191 ± 2 (10)</td>
</tr>
<tr>
<td>H$_2$O$_2$, pmol·min$^{-1}$·mg$^{-1}$</td>
<td>183 ± 9 (28)</td>
<td>1935 ± 144 (33)</td>
</tr>
<tr>
<td>H$_2$O$_2$, pmol·min$^{-1}$·mg$^{-1}$ in presence of 5 μM rotenone</td>
<td>460 ± 24 (16)</td>
<td>225 ± 16 (17)</td>
</tr>
<tr>
<td>Superoxide, EPR signal intensity#</td>
<td>nd</td>
<td>100 (9)</td>
</tr>
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Data are expressed as means ± SE; values in parentheses indicate number of repetitions. ROS, reactive oxygen species; STZ-D, STZ-diabetic; Ctrl, control; nd, not determined. *P < 0.05, **P < 0.01, ***P < 0.001, compared to Ctrl by unpaired t-test. #Signal intensity is expressed in diabetic mitochondria relative to control mitochondria isolated and assayed under the same EPR conditions in side-by-side fashion on the same day.

In a subset of animals used to generate the data in Fig. 3, we measured mitochondrial respiration and H$_2$O$_2$ production (Fig. 4, A–C) in control and diabetic mitochondria in the absence or presence of ADP (state 3 and state 4, respectively). Here, we found that the propensity of the diabetic mitochondria to generate more ROS per unit respiration (Fig. 4C) extends to state 3 respiration. As expected, for both control and diabetic mitochondria, ROS production was reduced during state 3 respiration, an effect felt to be a consequence of reduced membrane potential, a well-known consequence of diverting charge to ATP formation. However, of note, the extent of reduction was less in the diabetic mitochondria (Fig. 5).

We also measured ROS, respiration, and ROS per unit respiration in mitochondria fueled by substrates donating electrons both to complex I and II, i.e., glutamate and malate plus succinate (Fig. 4). Respiration on complex I or complex II substrates alone does not generate continuous turnover of the TCA cycle due to loss of intermediates through shuttle path-

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**Fig. 3.** Reactive oxygen species production by gastrocnemius mitochondria of STZ-D and control rats. A: H$_2$O$_2$ production expressed per unit oxygen consumed by mitochondria fueled by 16 mM succinate (for vehicle, n = 24; for STZ, n = 24). B: H$_2$O$_2$ production expressed per unit oxygen consumed by mitochondria fueled by 16 mM glutamate plus 3.2 mM malate (for vehicle, n = 18; for STZ, n = 24). C: Superoxide EPR signal intensity expressed per unit oxygen consumed by mitochondria fueled by 5 mM succinate; for vehicle, n = 10; for STZ, n = 9. Data are expressed as means ± SE. P values were determined by unpaired, 2-tailed Mann-Whitney test.
However, this is not the case when combined substrates are supplied. We found that the increase in ROS per unit respiration in the diabetic mitochondria exposed to complex I or complex II substrates extends to respiration with electron donation at both complexes.

Respiration and ROS production by heart mitochondria of vehicle and STZ-treated rats. Respiration was decreased and H2O2 production was unchanged in heart mitochondria of STZ-diabetic rats compared with controls (Fig. 6). However, H2O2 production was greater ($P = 0.05$) in the diabetic mitochondria when expressed per unit oxygen consumed. Unlike the data for gastrocnemius muscle mitochondria, we did not observe an increase in the superoxide EPR signal when expressed per unit oxygen consumed.

**DISCUSSION**

We report several novel findings. Contrary to prior reports that examined absolute mitochondrial ROS production from insulin-deficient rodents (7, 15), we show that ROS production is, in fact, increased when considered in relation to electron transport activity. Moreover, our data show that this is evident during state 3, as well as state 4, that the reduction in ROS when mitochondria transition to state 3 is less in diabetic mitochondria, that increased ROS production relative to respiration is also evident in mitochondria respiring during a continuous TCA cycle, and that CoQ is not reduced in diabetic mitochondria or with the weight loss of food restriction. These findings have important mechanistic and physiological implications.

Isolation of mitochondria remains the only way to accurately assess the intrinsic capacity of these organelles for ROS production free of cytoplasmic energy flux or cytoplasmic antioxidant activity. Prior studies of superoxide production by mitochondria of insulin-deficient rodents by our laboratory and others (7, 15) revealed a decrease for gastrocnemius muscle mitochondria (15) or no change for mitochondria isolated from heart (7). Our current data confirm this over a larger number of animals.
ROS production and that diabetes in humans and rodents is associated with oxidative damage (14). Moreover, as opposed to insulin-deficient rodents, muscle or heart mitochondria from type 2 rodent models show no reduction or an increase in ROS production (2, 16). So, why is it that mitochondria of insulin-deficient diabetes appear to generate less ROS?

Our current data provide important new light on this issue. Compared with prior studies, we examined larger sample sizes and examined respiration in parallel to ROS under varied substrate conditions. We show that the reduction in mitochondrial ROS production by muscle mitochondria of insulin-deficient rats is more apparent than real when ROS production (2, 16). So, why is it that mitochondria of insulin-deficient diabetes appear to generate less ROS?

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Our current data provide important new light on this issue. Compared with prior studies, we examined larger sample sizes and examined respiration in parallel to ROS under varied substrate conditions. We show that the reduction in mitochondrial ROS production by muscle mitochondria of insulin-deficient rats is more apparent than real when ROS production is proportional to electron transport in a manner dependent on the fuel consumed for energy, glutamate plus malate, or succinate. This is aside from a small amount of oxygen consumed in the very process of generating the oxygen radicals. But, this latter amount is of the order of about 1% or less of all oxygen consumed by mitochondria (4); that is, not enough to appreciably impact the overall quantitative relationship between oxygen use and electrons transported. Thus, as shown in Figs. 3 and 4, normalizing superoxide production (measured either as H$_2$O$_2$ fluorescence or EPR) is not decreased, but actually increased, in diabetic mitochondria when considered per unit oxygen consumed. Hence, our current study, performed with isolated mitochondria, shows ROS corrected in this way confirms predictions of mitochondrial ROS generation in whole cells exposed to conditions mimicking the diabetic milieu (6, 14, 34).

Although ROS production normalized to oxygen consumption appears useful for comparing physiological states, a limitation is that this parameter looks only at mitochondrial function as a whole. That is, we do not suggest that perturbations in respiration or ROS arise from the same or from any single site within the electron transport system. Respiration in the diabetic mitochondria is likely due to a failing at one or more particular sites, and this site(s) may well be different from the site(s) where most ROS are generated.

If absolute ROS production is reduced in diabetes, does the ratio to respiration have in vivo implications? We suggest that it does. Although defects in the ETS will decrease respiration in isolated mitochondria, any reduction in vivo would be met with a compensatory drive, due to a decrease in ATP:ADP. Hence, if the difference in the ratio of ROS to respiration between diabetic and control mitochondria were maintained or increased as diabetic mitochondria were driven to respire in vivo (at a rate matching, exceeding, or even approaching respiration by nondiabetic mitochondria), then we expect that absolute mitochondrial ROS production would be greater in the diabetic state. On the other hand, we cannot be sure this difference in ratios would be maintained as respiratory rates change in vivo, since accurate measurement would be difficult, and the ratio per se would be sensitive to factors beyond respiration, including ATP production, membrane potential, respiratory coupling, and substrate utilization. We do point out that whole body oxygen use is actually increased in insulin-deficient diabetes as our data (see text under Animal characteristics) and other reports (1, 25, 29, 31) indicate.

Our data provide information suggesting that the ratio of ROS per unit respiration is greater or unchanged in diabetic mitochondria as conditions are perturbed, at least, in vitro. This ratio is greater (Figs. 4 and 5) in the diabetic mitochondria relative to control when examined under state 3 conditions, as well as state 4, suggesting that ATP synthesis does not abrogate this difference between control and diabetic mitochondria. In theory, the increased ROS per unit respiration seen in our diabetic mitochondria could be explained by higher membrane potential. However, at least under the state 4 conditions examined here, this was not the case, since membrane potential was unchanged by diabetes (Table 2). Respiratory uncoupling is also felt to decrease ROS, so a decrease in uncoupling might explain higher ROS. In fact, proton conductance, a measure of uncoupling, was decreased in the diabetic mitochondria. However, this interpretation does not seem to hold in this situation. Classically, uncoupling increases respiration, as mitochondria try to compensate for a decrease in membrane potential due to the proton leak. But, our data show that the difference in proton conductance was essentially due to the decrease in respiration in the diabetic mitochondria, even though the membrane potential was unchanged or lower, not higher. Finally, we point out that absolute ROS production in diabetic mitochondria could be increased in vivo, even if the ratio of ROS to...
respiration were to decrease—simply if muscle mitochondrial respiration were increased enough.

Our data imply that some property (or properties) of the ETS is (are) altered in diabetic mitochondria, leading to an intrinsic tendency to leak electrons to molecular oxygen, thereby, generating superoxide. Our findings suggest that a large part, but not all, of the electron leak occurs at complex I. This follows from the effect of rotenone on H$_2$O$_2$ release by mitochondria respiring on complex I substrates (Table 2). Rotenone actually enhances glutamate plus malate-induced electron transport from complex I, a phenomenon explained by the effect of rotenone to block the quinone-binding site of complex I, resulting in backup of electrons and full reduction of upstream redox centers (4). Second, rotenone inhibited H$_2$O$_2$ production by mitochondria respiring on succinate (Table 2). The large proportion of H$_2$O$_2$ production on succinate is generated through reverse electron transport to complex I, a process blocked by rotenone (4). However, complex I is not likely the only site involved in the electron leak. This follows since we observed an increase in ROS per unit respiration in the diabetic mitochondria when superoxide was directly detected by EPR (Fig. 3). As we have shown previously for both muscle and endothelial cell mitochondria (15, 26), EPR spectroscopy, as applied here, largely measures superoxide released externally from complex III, while H$_2$O$_2$ production largely measures superoxide released to the matrix from complex I, followed by conversion to H$_2$O$_2$ by SOD and diffusion outward from mitochondria.

Our findings are not likely explained by alterations in antioxidant enzymes. In past studies, we measured the content of the major antioxidant enzymes, MnSOD, and glutathione peroxidase, in gastrocnemius mitochondria from diabetic and control animals and found no difference (15). We were not able to detect mitochondrial catalase, which is consistent with the classical view that this is not a mitochondrial enzyme in skeletal muscle (30). We did see a decrease in peroxiredoxin III in the diabetic mitochondria. Theoretically, this deficiency might increase H$_2$O$_2$ detection from the diabetic mitochondria. However, this would not affect the observed difference in ROS per unit oxygen consumption compared with ROS normalized only to mitochondrial protein. In fact, this would make our findings even more relevant since an increase in H$_2$O$_2$ production was not evident in the diabetic (vs. control) mitochondria (in spite of this potential effect of reduced detoxification) unless expressed per unit oxygen consumed.

As shown in Fig. 4, B and C, ROS production was markedly less under state 3 compared with state 4 conditions for both the diabetic and normal rats. This was expected since ROS production is charge sensitive and the membrane potential is strongly reduced under state 3 conditions, wherein the gradient is harnessed to drive ATP synthase (chemiosmotic mechanism). However, it is of interest to note that the reduction in ROS by state 3 conditions was less in the diabetic compared with control mitochondria (Fig. 5). There is evidence that ROS production may limit respiratory coupling (9) and, thus, efficiency of ATP synthesis; so we speculate that this process may, in part, explain the reduction in ATP production rates reported in insulin-deficient diabetes (18).

We also showed that increased ROS production relative to electron transport is evident in mitochondria respiring on glutamate and malate plus succinate (Fig. 4), as well as under conditions of pure complex I or complex II fuels. This observation has mechanistic implications. Most ROS generated by mitochondria respiring on succinate occurs through reverse electron transport (4), as evident by the marked reduction in ROS production when rotenone is added (Table 2); however, as shown in Fig. 4, respiration on glutamate plus malate plus succinate generates ROS in amounts comparable to that on succinate alone and far more than on the complex I substrates alone. This suggests that reverse transport may actually contribute a substantial amount of ROS, even during a continuous (more physiological) cycle, offering support for the concept that reverse transport may have physiological meaning, a controversial issue (4). To be thorough, we should point out that the continuous cycle studied here generates electron donation to both complex I and complex II, but it is not necessarily complete since transaminase will catalyze the reaction of oxaalacetate to 2-oxoglutarate, which can then establish a cycle without generation of citrate (13).

We considered the possibility that changes in mitochondrial CoQ content could affect the relationship between mitochondrial ROS production and respiration. This large inner membrane molecule, often considered an antioxidant, displays prooxidant properties as well. This is thought to be due to the semiquinone form, which leaks electrons to form superoxide during electron transport through the Q-cycle in complex III and by a similar, less defined, mechanism in complex I (5, 8, 21). Studies of mitochondrial CoQ content in rodent models of insulin-deficient diabetic states have produced variable results, showing an increase (3) or decrease (19) in liver mitochondria, a decrease in renal mitochondria (19, 24), and a decrease (19) or increase (24) in heart mitochondria. Our current work, which included a much larger number of repetitions than these past studies, revealed no difference for gastrocnemius mitochondria. Thus, we cannot attribute the diabetes-induced increase in superoxide per unit oxygen consumed to perturbed content of CoQ.

There are some additional limitations to our findings. Notably, the studies reported here were done under contrived state 3 and 4 conditions, whereas mitochondria in vivo generally function somewhere between state 3 and state 4. Our measurements of membrane potential and proton conductance reported here refer only to mitochondria respiring under uninhibited state 4 conditions. In prior work (15), we carried out kinetic analysis using muscle and heart mitochondria of a subgroup of the animals reported here. When respiration on succinate was titrated using malonate, the data revealed that the membrane potential was higher for the diabetic mitochondria at any given respiratory rate, indicating greater respiratory coupling (less uncoupled). Our studies of food restriction were limited in that it is essentially impossible to accurately match weight loss to streptozotocin diabetic rats. Pair feeding cannot be done since the diabetic rats are actually hyperphagic, and weight loss in diabetic rats is, by nature, more variable. Moreover, weight loss by food restriction is limited in extent by institutional animal care regulations.

In summary, our current results suggest viewing mitochondrial superoxide production in the context of electron transport activity. As opposed to past reports addressing superoxide production per se, superoxide production is substantially in-
increased in mitochondria of insulin-deficient diabetic rats when viewed relative to the electron transport process. Our data further imply that the increased electron leak in diabetic mitochondria likely occurs at more than one site within the ETS. We also show that the increased electron leak is evident under state 3, as well as state 4 conditions, that the reduction in ROS when mitochondria respire under state 3 conditions compared with state 4 is less in diabetic mitochondria, and that increased ROS production relative to respiration is evident during a continuous TCA cycle. Finally, we show that muscle mitochondrial CoQ content is not altered by insulin-deficient diabetes, so that perturbed CoQ does not explain the increase in superoxide per unit respiration seen in the diabetic mitochondria.

Perspectives and Significance

Our data imply an intrinsic defect in muscle mitochondria of diabetic rats. When ROS production is viewed relative to O2 consumption (thus, substrate utilization for electron transport), this is associated with an increase in formation of these radicals. As discussed, the implications for absolute in vivo ROS production depend on whether respiration is increased in vivo, whether the difference in ratio of ROS to respiration between diabetic and control mitochondria is maintained at the same or higher level in vivo, and (if not maintained at the same or higher level) the extent to which respiration may actually increase. Perturbations in CoQ content were not evident in diabetic muscle mitochondria, so they do not explain changes in parameters of mitochondrial function.

GRANTS

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DISCLOSURES

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

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