Mitochondrial reactive oxygen species (ROS) cause kidney damage in diabetes. We investigated the source and site of ROS production by kidney cortical tubule mitochondria in streptozotocin-induced type 1 diabetes in rats. In diabetic mitochondria, the increased amounts and activities of selective fatty acid oxidation enzymes is associated with increased oxidative phosphorylation and net ROS production with fatty acid substrates (by 40% and 30%, respectively), whereas pyruvate oxidation is decreased and pyruvate-supported ROS production is unchanged. Oxidation of substrates that donate electrons at specific sites in the electron transport chain (ETC) is unchanged. The increased maximal production of ROS with fatty acid oxidation is not affected by limiting the electron flow from complex I into complex III. The maximal capacity of the ubiquinol oxidation site in complex III in generating ROS does not differ between the control and diabetic mitochondria. In conclusion, the mitochondrial ETC is neither the target nor the site of ROS generation in kidney cortical tubule mitochondria in short-term diabetes. Mitochondrial fatty acid oxidation is the source of the increased net ROS production, and the site of electron leakage is located proximal to coenzyme Q at the electron transfer flavoprotein that shuttles electrons from acyl-CoA dehydrogenases to coenzyme Q. Diabetes 61:2074–2083, 2012

Diabetic nephropathy (DN) is the leading cause of end-stage renal disease (ESRD) (1). Hyperlipidemia is an independent factor in renal injury in animals (2) and humans (3). Increased fatty acid (FA) synthesis enzymes and triglyceride deposition correlated with increased profibrotic factors were found in the kidney in diabetes in rats (4) and mice (5). Inhibition of lipid synthesis in humans (6) amelioration of dyslipidemia (7) protect against diabetic renal disease.

Glomerular disease has been considered the initial and cardial manifestation of DN. However, tubulointerstitial fibrosis is considered a strong predictor of the progressive loss of renal function leading to ESRD (8,9) and has been shown to determine the progression to ESRD (10).

The increase in plasma nonesterified FAs (NEFAs) is a major component of diabetic dyslipidemia. Tubular cells are exposed to blood and urine FAs present in free form or bound to albumin. The expression of cluster of differentiation 36 (CD36) involved in the transport of FA is induced by high glucose in proximal tubular cells and causes palmitylate-induced apoptosis only in human kidneys with diabetic tubular epithelial degeneration (11). Albumin-bound FA, rather than albumin, itself is a major mediator of tubulointerstitial lesions in various types of proteinuria-developing ESRD (12,13).

Proximal tubular cells (>90% of the kidney cortex) engage in active uptake and transepithelial transport of glucose, but only a small amount of glucose, if any, is used for ATP production (14). Moreover, diabetes causes a decrease in kidney glucose oxidation due to the inhibition of pyruvate dehydrogenase activity (15). Skin fibroblasts isolated from type 1 diabetic patients, with very fast rates in developing DN, have increased expression of genes involved in mitochondrial FA oxidation (16). The data suggest an energy fuel preference in diabetes favoring fat oxidation at the expense of glycolysis. The enzyme carnitine palmitoyltransferase 1 (CPT1) catalyzes the rate-controlling step in the overall mitochondrial FA β-oxidation pathway. Its expression is reported to be increased in liver and heart in diabetes, whereas its sensitivity to malonyl-CoA is reduced, favoring its activity.

Increased generation of ROS within the mitochondrial electron transport chain (ETC) has been considered to be an important contributor to the development of chronic diabetes complications (17). Increased availability of glucose-generated reducing equivalents in cultured aortic endothelial (18) and retinal (19) cells or defects in the ETC in epineural (20) and cardiac (21) mitochondria increase the electron pressure at specific mitochondrial sites. This perturbation prolongs the lifetime of partially reduced intermediates that donate electrons to molecular oxygen to form superoxide. In contrast, although mitochondrial-targeted antioxidant mitoquinone mesylate (mitoQ) is beneficial in the treatment of diabetic nephropathy, mitochondrial ETC is not the source of ROS generation in the kidney of diabetic Akita mice (22) or in the sensory neurons from diabetic rats (23), indicating that there are differences in the source and sites of ROS production in target organs of diabetes complications. In short-term diabetes, we found that mitochondrial ETC is neither the target nor the site of ROS generation in kidney cortical tubules. The source of increased ROS production is mitochondrial FA β-oxidation; the site of electron leakage is located proximal to CoQ at the electron transfer flavoprotein that shuttles electrons from acyl-CoA dehydrogenases to coenzyme Q.
Oxidative phosphorylation. Oxygen consumption was measured with a Clark-type electrode (Strathkelvin) in respiration buffer (100 mmol/L KCl, 50 mmol/L MOPS, and 0.5 mmol/L EGTA, pH 7.4). Phase-contrast microscopy was performed during each experiment to examine the purity of the glomerular material and ensure that the isolation was harvested within the tubulointerstitial compartment. Because the tubules represent the major component of the cortical tubulointerstitial material, the isolated mitochondria were designated as cortical tubule mitochondria. Tubule mitochondria were isolated (26) and suspended in buffer B (100 mmol/L KC1, 50 mmol/L MOPS, and 0.5 mmol/L EGTA, pH 7.4).

Oxidative phosphorylation. Oxygen consumption was measured with a Clark-type electrode (Strathkelvin) in respiration buffer (100 mmol/L KCl, 50 mmol/L MOPS, and 0.5 mmol/L EGTA, pH 7.4) (27) at 30°C with glutamate (20 mmol/L) + malate (5 mmol/L), pyruvate (10 mmol/L) + malate, succinate (20 mmol/L) + rotenone (3.75 μmol/L), durohydroquinone (DHQ, 1 mmol/L) + rotenone, N,N,N,N’-tetramethyl-p-phenylenediamine (TMPD) + ascorbate (1:10 mmol/L) + rotenone, palmitoyl-CoA (0.02 mmol/L) + carnitine (2 mmol/L) + malate, and palmitoylcarnitine (0.04 mmol/L) + carnitine (2 mmol/L) + malate, as substrates. State 3 respiration rate, which is ADP-dependent was measured with 200 μmol/L ADP. State 4 respiration (ADP-limited) was recorded after ADP consumption. Then, sequential additions of 2 mmol/L ADP and 200 μmol/L of the uncoupler dinitrophenol were made to determine the maximal ADP-stimulated and uncoupled respiratory rates, respectively. State 3 to state 4 respiratory control ratios (RCR) reflect the control of oxygen consumption by phosphorylation (*coupled state is ADP oxygen ratios number of ADP molecules added for each oxygen atom consumed). The turnover number of ADP molecules added for each oxygen atom consumed was (28) in an index of the efficiency of oxidative phosphorylation. In preliminary experiments, the complex IV inhibitor sodium azide (40 μmol/L) completely inhibited oxidative phosphorylation with complex IV substrates in both control and diabetic tubule mitochondria, showing that the azide-sensitive complex IV-supported respiration equals the TMPD + ascorbate-supported respiration and that there are no differences in azide sensitivity between control and diabetic tubule mitochondria. Therefore, the results are presented as TMPD + ascorbate-supported respiration. Rates of oxygen consumption are expressed in nanomoles (nA) oxygen per minute per milligram of mitochondrial proteins.

Measurement of CPTI activity. The activity of CPTI was determined using the modified radiolabelled forward assay by measuring the formation of [14C]-labeled palmitoylcarbonylamine from 14C-carboxylamine and palmitoyl-CoA at 37°C as described (29). CPTI activity was expressed as the activity inhibited by 200 μmol/L octanoyl carnitine per minute per milligram of protein. Malonyl-CoA sensitivity was determined at fixed palmitoyl-CoA concentrations by varying the concentration of malonyl-CoA.

Measurement of fatty acid oxidation enzymes. Frozen-thawed mitochondria were treated with 0.5% cholate and diluted to 0.1 mg/mL buffer A. The dehydrogenase assays used octanoyl-CoA for medium-chain acyl-CoA dehydrogenase (MCAD) and palmitoyl-CoA for long-chain acyl-CoA dehydrogenase (LCAD). ADP was added as an intermediate electron acceptor from flavin adenine dinucleotide (FADH2) and oxidized cytochrome c as the final electron acceptor. The reduction of cytochrome c was recorded spectrophotometrically at 550 nm.

Western blot analysis. Proteins (equal amounts of kidney tubule mitochondrial protein [18 μg/lane], heart mitochondrial protein [6.5 μg/lane], and liver outer membrane protein [0.5 μg/lane]) were subjected to SDS-PAGE, electroblotted onto polyvinylidene fluoride membranes, and probed with specific antibodies. Densitometric evaluation of the gels was carried out using Image software (National Institutes of Health) coupled with Multi Gauge V3.0 (FujiFilm Life Science).

RESULTS

Clinical and biochemical characteristics of diabetic rats. Diabetic rats did not lose weight during the study but showed decreased weight gain, hyperglycemia, polyuria, and increased glycated hemoglobin levels 8–9 weeks after the onset of diabetes (Table 1). The serum was macroscopically turbid, indicating secondary diabetic hyperlipidemia. Glycerol and NEFA levels were increased in sera of diabetic rats compared with the control rats. Kidney weight was significantly increased in the diabetic group, suggesting renal hypertrophy. Mitochondrial yield does not change in diabetes (20.1 ± 2.9 vs. 18.9 ± 1.2 mg mitochondrial protein/g wet cortex tissue in control and diabetic rats).

| TABLE 1 | Clinical and biochemical characteristics of control and diabetic rats |
|---------|---------------------------------------------------------------|
| Control rats | Diabetic rats |
| (n = 7) | (n = 6) |
| **Body weight (g)** | 310 ± 12 | 275 ± 15 |
| **Glycemia (mg/100 mL)** | 142.5 ± 10.5 | 255.1 ± 4.5* |
| **HbA1c (%)** | 2.9 ± 0.1 | 9.7 ± 0.3* |
| **Serum visual appearance** | Clear | Turbid |
| **Glycerol (mmol/L)** | 2.114 ± 0.169 | 19.208 ± 2.109* |
| **Total NEFA (mmol/L)** | 0.370 ± 0.007 | 1.256 ± 0.131* |
| **Palmitic acid (mmol/L)** | 0.156 ± 0.003 | 0.490 ± 0.046* |
| **Oleic acid (mmol/L)** | 0.095 ± 0.004 | 0.421 ± 0.046* |
| **Stearic acid (mmol/L)** | 0.119 ± 0.008 | 0.345 ± 0.034* |
| **Kidney weight (g)** | 1.5 ± 0.0 | 1.8 ± 0.1* |
| **Diuresis (mL/day)** | 11.8 ± 1.5 | 153.0 ± 3.6* |

Data are presented as mean ± SEM. *P < 0.05.
tubules, respectively), suggesting that there has been no change in mitochondrial content in kidney tubules.

**Substrate processing rather than the mitochondrial ETC is changed in kidney tubules in diabetes.** Diabetic tubule kidney mitochondriaoxidizing glutamate have higher state 3 respiratory rates compared with the control (Table 2). The ADP-to-oxygen ratios were unchanged, showing that the coupling efficiency of the tubule kidney mitochondria is preserved in diabetes. With the uncoupler, dihydrogen, the glutamate-supported respiratory rates of nondiabetic tubule mitochondria do not increase nor do they reach the level of respiratory rates of diabetic mitochondria (Table 2). The oxidation of glutamate + malate is not changed by diabetes in tubule mitochondria, whereas pyruvate + malate oxidation is lower in diabetic tubule mitochondria compared with the control (Fig. 1A). The oxidation of succinate, which donates electrons to FAD in complex II, was unchanged in diabetic tubule mitochondria, as were the state 3 respiratory rates with durohydroquinone (complex III electron donor) and those with the cytochrome c electron donor TMPD + ascorbate (Fig. 1A). The data show that the ETC complexes in tubule mitochondria are not the targets of diabetes-induced alterations in this experimental model.

**Mitochondrial FA oxidation is increased in kidney tubules in diabetes.** CPT1 is the rate-controlling enzyme in mitochondrial FA oxidation. We measured oxidative phosphorylation rates with CPT1-dependent and CPT1-independent substrates. Palmitoyl-CoA is converted to palmitoylcarbonyl by the outer membrane CPT1. Palmitoylcarbonyl and octanoylcarbonyl are transported into the mitochondrial matrix via the CACT bypassing CPT1, and react with CoA in the matrix in a reaction catalyzed by the inner membrane CPT2. Within the matrix, palmitoyl-CoA and octanoyl-CoA undergo sequential β oxidation, producing acetyl-CoA, NADH, and FADH2. Electrons from NADH enter the ETC at complex I, whereas those from the FADH2 of acyl-CoA dehydrogenases enter the ETC at complex III via electron transfer flavoprotein (ETF) and ETF-coenzyme Q oxidoreductase (ETF-QOR). As shown in Fig. 1B, CPT1-dependent and CPT1-independent lipid substrates were both oxidized at higher rates by diabetic tubule mitochondria.

CPT1 activity is not changed by diabetes despite the increased protein amount. CPT1 converts long-chain acyl-CoA to acylcarnitines and exists as two isoforms, the muscle (CPT1b) and the liver (CPT1a) isoforms, which differ with respect to their kinetic properties. The activity of CPT1 was not changed by diabetes in kidney tubule mitochondria (Fig. 2A). The in vivo flux through CPT1 depends on the sensitivity of the enzyme to malonyl-CoA inhibition. Liver mitochondria are significantly less sensitive to malonyl-CoA inhibition (half-maximal inhibitory concentration [IC50] ~5 μmol/L) compared with kidney tubular mitochondria (IC50 ~2 μmol/L; Fig. 2B). Figure 2C shows that a slight but significantly higher percentage of CPT1 is not inhibited by 2 μmol/L malonyl-CoA (equals the IC50 for nondiabetic mitochondria) in diabetic tubule mitochondria compared with the control, suggesting a decrease in the sensitivity of CPT1 to malonyl-CoA in kidney tubules in diabetes.

The two CPT1 isoforms show differences in migration (Fig. 2D), with slightly higher calculated size of CPT1a (~88 kDa) than CPT1b (~82 kDa), as reported (34). CPT1b protein predominates in the heart mitochondria, whereas only CPT1a is in present in liver mitochondrial outer membrane. CPT1a is the only isoform expressed in kidney tubule mitochondria, and the amount of CPT1a protein is increased in diabetes.

**The activities and amounts of selective FA β-oxidation enzymes are increased in kidney tubules in diabetes.** Figure 3A shows the increase in the activity of medium-chain and long-chain acyl-CoA dehydrogenases in tubule mitochondria isolated from the diabetic group. The specific activity of citrate synthase, a mitochondrial marker enzyme, does not differ in tubule mitochondria isolated from the control or diabetic group, indicating that the purity of both preparations was identical. The amounts of CACT and CPT2 proteins are increased in tubule mitochondria isolated from diabetic kidneys compared with the controls (Fig. 3B).

**Increased capacity of diabetic tubule mitochondria to produce ROS is supported by oxidation of FA substrates; the putative site of electron leakage is located proximal to coenzyme Q within the FA oxidation pathway.** When added to intact mitochondria, Amplex red detects the net mitochondrial release of H2O2 into the buffer when H2O2 reaction catalyzed by the inner membrane CPT2. Within the matrix, palmitoyl-CoA and octanoyl-CoA undergo sequential β oxidation, producing acetyl-CoA, NADH, and FADH2. Electrons from NADH enter the ETC at complex I, whereas those from the FADH2 of acyl-CoA dehydrogenases enter the ETC at complex III via electron transfer flavoprotein (ETF) and ETF-coenzyme Q oxidoreductase (ETF-QOR). As shown in Fig. 1B, CPT1-dependent and CPT1-independent lipid substrates were both oxidized at higher rates by diabetic tubule mitochondria.

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### TABLE 2

| Substrate processed | Control rats | Diabetic rats |
|---------------------|--------------|--------------|
| Fat                  | Activity (μmol/min/mg mitochondrial protein) | Activity (μmol/min/mg mitochondrial protein) |
| Palmitoyl-CoA        | 100.0 ± 8.3  | 154.0 ± 12.9* |
| Octanoyl-CoA         | 22.0 ± 3.7   | 20.0 ± 1.6   |
| RCR                  | 5.2 ± 0.7    | 8.1 ± 0.8*   |
| ADP-to-oxygen ratio  | 3.1 ± 0.2    | 3.0 ± 0.2    |
| Maximal ADP          | 11.0 ± 9.9   | 159.0 ± 12.8* |
| Dinitrophenol        | 80.0 ± 3.8   | 128.0 ± 14.8* |

Data are presented as mean ± SEM. State 3 was induced with 0.2 mM/L ADP. Respiratory rates are expressed as nA O/min/mg mitochondrial protein. Maximal ADP: State 3 with saturating 2 mM/L ADP. *P < 0.05.
release capacity with fat substrates. H$_2$O$_2$ released by control and diabetic tubule mitochondria oxidizing pyruvate or fat substrate was dissipated by catalase at similar extent, indicating that the increase in the measured fluorescence was because of H$_2$O$_2$ and not induced by the chemical reaction of Amplex red with FA hydroperoxides.

When oxidizing succinate at complex II (Fig. 4C), control and diabetic tubule mitochondria both produce H$_2$O$_2$, which was decreased in both by ~45% with rotenone, which inhibits the reversed electron transport toward complex I. The data indicate that a major source of superoxide in kidney tubule mitochondria oxidizing succinate is the electron back-flow to complex I and that this feature is unchanged by diabetes. The addition of antimycin A increases the production of H$_2$O$_2$ in control and diabetic tubule mitochondria oxidizing succinate.

This increase in the net release of H$_2$O$_2$ by diabetic mitochondrial with fat substrates indicates that the production surpasses its consumption by mitochondrial antioxidant defense mechanisms. The amount of both SODs determined by immunoblot analysis was not significantly changed by diabetes (Fig. 4D). These data indicate that it is the production of superoxide rather than its dismutation that leads to increased H$_2$O$_2$ release in the buffer by diabetic kidney tubule mitochondria.

**DISCUSSION**

This study shows that 8- to 9-week type 1 diabetes induces an increase in mitochondrial FA β-oxidation without defects in the electron transport and identifies oxidation of FA rather than glycolysis-derived substrate (pyruvate) oxidation as the source of mitochondrial ROS in diabetic tubules. Because more than 90% of kidney cortex consists of proximal tubules, our results can be attributed to proximal tubule mitochondria. These changes in mitochondria co-exist with renal structural and functional modifications consistent with the early stage of DN (35,36).

Diabetic cortical tubule mitochondria reach maximal respiratory rates with glutamate when oxidation is coupled with and depends on phosphorylation of ADP, and do not further increase when the control of oxidation by phosphorylation is eliminated by the uncoupler. These data show that the control site of oxidative phosphorylation is located at the level of glutamate oxidation to form NADH or electron transport rather than at the level of the phosphorylation system. When substrates that donate electrons at specific sites in the ETC were used, no change in respiratory rates was detected, showing that the formation of NADH from glutamate (glutamate transporter or dehydrogenase) rather than the electron transport is increased in diabetes.

The decrease in pyruvate + malate oxidation in diabetic tubule mitochondria suggests a decrease in either pyruvate transporter or pyruvate dehydrogenase that was reported inhibited in the diabetic kidney (15,37). However, the oxidation of pyruvate in isolated tubule mitochondria was performed with a concentration of pyruvate (10 mmol/L) that keeps pyruvate dehydrogenase in its active form.
FIG. 2. CPT1 in kidney cortical tubule mitochondria. A: Specific activity of CPT1 in tubule mitochondria from control and diabetic kidneys. B: Comparison of malonyl-CoA sensitivity (IC₅₀) of CPT1 in mitochondria from control rat liver and kidney cortical tubules. C: The percentage of CPT1 not inhibited by 2 μmol/L malonyl-CoA in tubule mitochondria from control and diabetic kidneys. D: Semiquantitative determination CPT1 protein with antibodies prepared against the amino acid sequence is shown. The bar graphs represent the densitometric quantitation of the immunoblots expressed in arbitrary units normalized per iron sulfur protein (ISP). H, heart subsarcolemmal mitochondria; L, liver mitochondrial outer membrane. *P < 0.05 control vs. diabetic. Mean ± SEM. (A high-quality color representation of this figure is available in the online issue.)
by inhibiting pyruvate kinase (38). Therefore, pyruvate transporter rather than pyruvate dehydrogenase seems to be responsible for the decrease in pyruvate oxidation in diabetic tubule mitochondria.

Diabetic tubule mitochondria oxidize FA substrates at higher rates than the controls. This is associated with an increase in the specific activities of both MCAD and LCAD without changes in the MCAD amount, suggesting...
FIG. 4. Kidney cortical tubule mitochondria production of H$_2$O$_2$ with pyruvate + malate (A) palmitoylcarnitine + malate (B), and succinate + rotenone (C) as the substrates. Rot, rotenone; AA, antimycin A; Cat, catalase. D: Semiquantitative determination of mitochondrial Mn-SOD and Cu/Zn-SOD. The bar graphs represent the densitometric quantitation of the immunoblots expressed in arbitrary units normalized per iron sulfur protein (ISP). H, heart subsarcolemmal mitochondria. *P < 0.05 compared with the indicated groups. Mean ± SEM. (A high-quality color representation of this figure is available in the online issue.)
post-translational modifications that stimulate its catalytic activity. In contrast, despite the increase in CPT1a protein amount that favors mitochondrial FA β-oxidation, the catalytic activity of CPT1a is unchanged in diabetes, suggesting that CPT1 has a low flux control coefficient for mitochondrial FA β-oxidation in kidney tubules in diabetes.

The twofold higher IC₅₀ for malonyl-CoA of CPT1 assessed in control tubule mitochondria compared with liver mitochondria is not due to the presence of the CPT1b isoform, as shown by the Western blot approach. We propose that this kinetic property of CPT1a is an intrinsic feature of this isoform in kidney tubule mitochondria due to differences in outer membrane lipid environment or to post-translational modifications.

Proximal kidney tubules (>90% of the kidney cortex) rely on FA rather than glucose to form ATP during normal metabolic conditions (14); we found that this feature is enhanced in diabetes. Peroxisome proliferator-activated receptors (PPAR) are potential candidates that induce the enhanced mitochondrial FA β-oxidation in kidney tubules in diabetes. All members of this subfamily of nuclear receptors were detected in kidney cortex (39), are activated by NEFA (40), which were found increased in the diabetic sera in our model (Table 1), induce expression of FA β-oxidation enzymes (41), and promote FA utilization.

Our data also show that diabetic tubule mitochondria produce more ROS than the controls under both basal and maximal conditions when oxidizing FA substrates compared with oxidation of pyruvate. Prior reports suggesting that pyruvate may be the source of ROS (42) were based on the association of the elevated inner membrane potential and ROS with the increased content of both pyruvate and the tricarboxylic cycle intermediates originating from the pyruvate carboxylase route in mitochondria isolated from the whole kidney tissue. Our results contradict this assumption. Owing to kidney cellular complexity, the current study was performed specifically on mitochondria isolated from cortical tubular structures (highly oxidative nephron segments that depend on the ATP generated from FA oxidation) free of medullary structures (highly glycolytic). The inhibition of the pyruvate dehydrogenase in the diabetic medulla may lead to the increased content of pyruvate within mitochondria isolated from the whole kidney.

Oxidation of FA substrates was reported to be a source of ROS in the normal heart (43) and skeletal muscle (44) mitochondria. Increased oxidation of FA in aortic endothelial cells was reported to increase superoxide production from sites located within the electron transport (45,46). Fatty acids are complex I and III substrates via NADH and FADHₑ ETF/ETF-QOR, respectively (Fig. 5). Rotenone blocks the distal part of complex I, increases the reduction of the NADH dehydrogenase site of complex I, and causes an electron leak toward the matrix in cardiac mitochondria (33,43). Because rotenone does not increase H₂O₂ production with lipid and nonlipid complex I substrates in control and diabetic tubule mitochondria, we propose that complex I and III are not the main site of ROS production in tubule mitochondria or that complex I-generated oxidants are directed toward the mitochondrial matrix and inactivated by matrix-antioxidant systems in both control and diabetic conditions. The equal decrease of ROS with rotenone indicates that an additional site rather than the reverse electron transport to complex I is responsible for the increased ROS generation by diabetic tubule mitochondria oxidizing fat substrates.

Complex III is a key site for ROS generation in cardiac mitochondria (33,43). Because the limitation of the electron flow from complex I into complex III with rotenone equally decreased the release of H₂O₂ by control and diabetic mitochondria oxidizing pyruvate, the data show that complex III is the sole contributor to the release of H₂O₂ by tubular mitochondria oxidizing pyruvate. In contrast with cultured cells (45,46), the rotenone-inhibited tubule mitochondria from diabetic kidney show a higher amount of H₂O₂ than the control mitochondria oxidizing fat substrates. These data also indicate that a site other than complex III significantly contributes to ROS generation when fatty acids are used as substrates in diabetes.

The ubiquinol oxidation (Qo) site in complex III is the main site of electron leak oriented equally toward the intermembrane space and mitochondrial matrix (47). Antimycin A binds to complex III at a site that overlaps the ubiquinone reduction (Qi) site, increases electron leak from the Qo site, and increases superoxide generation. The minimal increase in H₂O₂ production in control mitochondria with antimycin A indicates that the superoxide production is oriented mainly toward the matrix and scavenged by the matrix antioxidants. The different topology of the superoxide released by kidney tubule mitochondria compared with cardiac mitochondria may reside in differences in lipid structure of the inner membrane responsible for a different mobility of the semiquinone in the Q cycle.

Antimycin A increases H₂O₂ released by diabetic tubule mitochondria oxidizing only fat substrates, indicating that major changes in the topology of ROS production are induced by diabetes in kidney tubule mitochondria when oxidizing fat. With succinate + rotenone + antimycin, diabetic tubule mitochondria do not show a significantly higher maximal capacity to produce H₂O₂ compared with the control mitochondria (P = 0.081). Because complex II is not considered to be the major site of superoxide production, the data suggest that the maximal capacity of the Qo site in complex III to generate oxidants does not differ between the control and diabetic tubule mitochondria.

A more likely explanation is that an additional site within the ETF/ETF-QOR–Qo segment (Fig. 5) becomes a site of superoxide production. The first dehydrogenase reaction in FA β-oxidation generates FADHₑ, with the electron donated to ETF. The FAD prosthetic group of ETF is rapidly reduced to the semiquinone form and more slowly to the fully reduced form (48,49), suggesting that the semiquinone form of the ETF may be the electron donor for the univalent reduction of oxygen to form superoxide. Similarly, the FAD prosthetic group of ETF-QOR cycles between the oxidized and partially reduced (semiquinone) forms. However, crystallography studies of ETF-QOR show that the semiquinone is protected from reacting with molecular oxygen (50) and that ETF-QOR is unlikely to be a site of superoxide production. Specific inhibitors must be developed to evaluate the real contribution of ETF or ETF-QOR to oxyradical production.

The matrix-generated superoxide from the site located at the ETF is dismutated to H₂O₂ by the matrix Mn-SOD. This enzyme reroutes superoxide from combining with nitric oxide to form peroxynitrite, which may alter mitochondrial function via tyrosine nitration of the ETC protein subunits. Therefore, the fate of mitochondrial ETC in long-term diabetes is controlled by crosstalk between mitochondrial antioxidant defense mechanisms.
In conclusion, mitochondrial ETC is neither the target nor the ROS-generating site in short-term diabetes. This study identifies FA as the source of reducing equivalents responsible for increased ROS production by kidney tubule mitochondria in diabetes and shows that ETF is the major site of electron leakage. A future time course study will help to link the increase in ROS production originating from mitochondrial FA oxidation pathway with the ETC damage and the progression to advanced stages of diabetic kidney disease.

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M.G.R. formulated the hypothesis, researched and interpreted the data, and wrote the manuscript. E.J.V. and Q.C. researched data. J.K. researched data, contributed to discussion and interpretation of the data, and reviewed the manuscript. T.S.K. and C.L.H. contributed to discussion and interpretation of the data and reviewed the manuscript. M.G.R. and C.L.H. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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