Progression of type 2 diabetes in GK rats affects muscle and liver mitochondria differently: pronounced reduction of complex II flux is observed in liver only

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Impaired mitochondrial function is implicated in the development of type 2 diabetes mellitus (T2DM). This was investigated in mitochondria from skeletal muscle and liver of the Goto-Kakizaki (GK) rat, which spontaneously develops T2DM with age. The early and the manifest stage of T2DM was studied in 6- and 16-wk-old GK rats, respectively. In GK16 compared with GK6 animals, a decrease in state 3 respiration with palmitoyl carnitine (PC) as substrate was observed in muscle. Yet an increase was seen in liver. To test the complex II contribution to the state 3 respiration, succinate was added together with PC. In liver mitochondria, this resulted in an ~50% smaller respiratory increase in the GK6 group compared with control and no respiratory increase at all in the GK16 animals. Yet no difference between groups was seen in muscle mitochondria. RCR and P/O ratio was increased (P < 0.05) in liver but unchanged in muscle in both GK groups. We observed increased lipid peroxidation and decreased Akt phosphorylation in liver with the progression of T2DM but no change in muscle. We conclude that, during the progression of T2DM in GK rats, liver mitochondria are affected earlier and/or more severely than muscle mitochondria. Succinate dehydrogenase flux in the presence of fatty acids was reduced severely in liver but not in muscle mitochondria during manifest T2DM. The observations support the notion that T2DM pathogenesis is initiated in the liver and that only later are muscle mitochondria affected.

Goto-Kakizaki rat; type 2 diabetes mellitus; oxygen consumption; P/O ratio

The involvement of dysfunctional mitochondria in the pathogenesis of type 2 diabetes is substantiated by many studies (5, 26, 31, 27, 34, 42, 43), but it is debatable whether mitochondrial dysfunction is the cause or rather an effect of the metabolic derangements that lead to insulin resistance and eventually type 2 diabetes mellitus (T2DM) (25, 39, 55). Also, it has been shown that insulin resistance occurs in liver before skeletal muscle during the development of T2DM (27), but it is not known if this implies that the mitochondria in the liver are involved earlier or differently compared with muscle. Whereas most studies have found decreased mitochondrial respiratory capacity and oxidative phosphorylation in insulin-resistant skeletal muscle, some recent studies report unaffected mitochondrial respiratory capacity (9, 12) and unaffected ATP production in skeletal muscle (12, 53), perhaps suggesting a time course with mitochondrial derangement in skeletal muscle occurring only at a later stage of T2DM. The mitochondrial defect related to skeletal muscle insulin resistance has been correlated with decreased activity of one or more of all components of the electron transport chain, with complex II being the least studied (22, 46, 49, 57). Also, a reduced muscle mitochondrial mass has been observed in the presence of insulin resistance (27, 36). However, when considering respiratory complex activity in liver mitochondria, the information is not as extensive (16, 17, 28). Yet spectrophotometric analyses have shown decreased activity of complex I–IV and the ATPase in the liver of the GK rat (21). Similar findings have been reported in the liver of patients with nonalcoholic steatohepatitis (41), and this has relevance since the disease has been linked to hepatic insulin resistance and is speculated to involve a mitochondrial pathogenesis (48).

The development of the mitochondrial dysfunction and insulin resistance has frequently been suggested to involve increased production of reactive oxygen species (ROS) (1, 8, 26). The mitochondrial electron transport chain complexes I and III are believed to be the main sites for ROS generation, as observed in several tissues (54, 56, 57). There is evidence suggesting that this could be through reverse electron flow from complex II to I (4, 23, 29, 30) combined with increased inner membrane potential (3). Also, it has been shown that the antioxidant defense, and specifically the lon protease, is downregulated in liver of db/db mice (32), suggesting that the liver may be particularly vulnerable to oxidative stress. However, the details of this process, and in particular the threshold level of ROS production with pathological consequences for the cell, are currently unknown (38).

Insulin resistance develops in both muscle and liver during the development of T2DM, but it develops sooner in liver than in muscle (27), and the two tissues are only rarely considered in comparison in the same study. In the present study, we decided to use the Goto-Kakizaki (GK) rat to study mitochondrial function in both liver and skeletal muscle to evaluate whether mitochondria are affected differently in the two organs during the development of type 2 diabetes. The GK animal model is particularly useful for such a study since it displays a spontaneous onset of nonobese diabetes that progradiates with age (20) and exhibits all the characteristics of human T2DM, except hyperlipidemia (40). We used two groups of GK rats, 6 (GK6) and 16 wk of age (GK16), representing early- and late-stage type 2 diabetes, respectively (20).

Specifically, the aim of the study was to examine 1) whether the mitochondrial phenotype in liver and skeletal muscle was different in an early compared with a later stage of T2DM and 2) whether the mitochondria from the two tissues responded differently with the progression of the diabetic state.

METHODS

Chemicals and Solutions

Reagents. Unless otherwise stated, reagents were purchased from Sigma-Aldrich (St. Louis, MO) and were of analytical grade or better.
Animals

Goto-Kakizaki rats (GK rats) were used for this study as an animal model of lean spontaneous onset of type 2 diabetes. It is produced by selective breeding from a non-diabetic Wistar strain and exhibits hyperglycemia and hyperinsulinemia (20). Sixteen male GK rats and eight male Wistar rats were obtained from Taconic (Ejby, Denmark). The “Principles of Laboratory Animal Care” and national law were followed during all aspects of the experiment. All experimental procedures were approved by the National Committee on Animal Experimentation (Denmark). Rats were housed in pairs in a laboratory rat facility with 12:12-h light-dark cycle and were fed a standard rat chow (Altromin, Lage, Germany). The rats were euthanized in a fed state under general anesthesia (Hypnorm-Dormicium; 0.3 ml/100 g); the young rats were euthanized at 6 wk of age, the older rats at 16 wk, and Wistar at 16 wk as well.

Since there is no reason to believe that the mitochondrial functions, tested in the present study, are changing in normal rats between the ages 6 and 16 wk, we decided to save animals and operate only with a group of 16-wk-old Wistar rats as the control.

Oral Glucose Tolerance Test

Following an overnight fast (16–18 h), rats were weighed, and a tail vein was punctured using a lancet. Baseline plasma glucose was measured using plasma-calibrated strips (Accu-Chek Compact Plus; Roche, Hvidovre, Denmark) and a blood sample (~200 µl) collected in EDTA-coated tubes (Microvette CB 300; Sarstedt, Nümbrecht, Germany). Rats were gavaged a 2 g/kg body wt dose of glucose from 30, 60, 90, and 120 min after gavaging, plasma glucose was measured from fresh tail punctures obtained as indicated above. Fasted plasma insulin concentration was measured on the 0-time blood samples.

Preparation of Isolated Mitochondria

Muscle mitochondria were prepared from the quadriceps muscle as described in Ref. 18, and animals were anesthetized with Hypnorm/Dormicium (0.3 ml/100 g). In brief, the muscles were minced with scissors in ca. 10 mg of mitochondrial protein/ml for oxygraph measurements. Both the liver and muscle mitochondrial preparations were tested for outer membrane integrity by observing the changes of state 3 respiration by the addition of cytochrome c. Only preparations with negligible changes were included in the study.

Mitochondrial Respiration

Mitochondrial respiration was measured in Oroboros Oxygraph-2K instruments (Oroboros, Austria), six oxygraph chambers operated in parallel at 25°C, Ten micoliters of muscle mitochondria suspension or 5 µl of liver mitochondria suspension was added to 2 ml of medium in each chamber. Stirrer speed was 600 rpm. Approximately 5 min after the mitochondria were introduced into the chambers, malate (2 mM) as well as either pyruvate (1.5 mM), palmitoylcarnitine (PC) (10 µM), or pyruvate plus PC was added. Once a steady-state 4 respiration was observed, 3 mM ADP was added to obtain a state 3 respiration. After a steady state was achieved, 6 mM succinate was added. Between experiments, the chambers were washed twice with ethanol and water.

The respiratory coupling ratio (RCR) was calculated as the state 3/state 4 respiration ratio. The Vmax of O2 was taken as the state 3 respiration with addition of 6 mM succinate so that both complex I and complex II substrates contributed to respiration. The P/O ratio was obtained by measuring the amount of oxygen needed to consume 0.1 µmol of ADP.

Activity of Succinate Dehydrogenase (Complex II)

Succinate dehydrogenase (SDH) activity (at 30°C) was measured spectrophotometrically at 600 nm in liver homogenates at pH 7.4 in 50 mM phosphate, 5 mM MgCl2, 1 mg/ml fatty acid-free BSA, 0.02% saponine, 5 µM rotenone, 4 µM antimycin A, 3 mM NaN3, 50 µM decylubiquinone, 50 µM 2, 6-dichlorophenol-indophenol, and 20 mM succinate (6). Liver homogenates were prepared in an all-glass Potter-Elvehjem homogenizer in 50 mM HEPES, pH 7.2, 0.25 M sucrose, 40 mM KCl, 2 mM EGTA, and 1 mg/ml BSA and centrifuged for 4 min at 2,000 g.

Protein Content and CS Activity

Protein concentration and CS activity in the tissue samples and in the isolated mitochondria were determined in 10% homogenates prepared in buffer (50 mM Tris-HCl, 0.6 mM MnCl2, 2 mM citrate, and 0.1% Triton X-100, pH 7.4; Ultra Turax homogenizer). Protein concentrations were determined using Lowry’s method (33). CS activity was assayed at 25°C (50).

Western Blotting

Ten percent tissue homogenates (wt/vol) were prepared by homogenization in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% NaDeoxycholate, 1% NP-40, 0.1% SDS, 1 µg/ml peptatin A, 1 mM Na3VO4, 1 mM NaF, Sigma PI no. 1, Sigma PI no. 2, Tablet Complete, Mini) using a TissueLyzer, followed by centrifugation for 30 min at 20,000 g. Twenty-five micrograms of protein of each sample was subjected to SDS-PAGE (10% Bis-Tris precast gels; NuPage) at 125 V for 90 min using 1× MOPS as running buffer. Proteins were electrophoresed onto polyvinylidene difluoride membranes at 30 V for 90 min and then washed in 0.1% Tween-20 in TBS (TBS-T) and blocked for 1 h in 5% powdered skimmed milk in TBS-T. Subsequently, membranes were probed with monoclonal rabbit anti-Akt (pan; no. 4691) and phosphorylated Akt (Ser473; no. 4060) from Cell Signaling Technology (Danvers, MA) and diluted 1:1,000 in 5% bovine serum albumin (fatty acid free) overnight at 4°C. Immunoblots were washed in TBS-T and incubated for 1 h at room temperature with horseradish peroxidase-conjugated anti-rabbit (DakoCytomation, Glostrup, Denmark) diluted 1:2,000 for Akt (pan) and phosphorylated Akt (p-Akt) in 5% powdered skimmed milk. After washing, specific binding was detected using ECL+ (Akt; GE Life Science).
Sciences, Pittsburgh, PA) or SuperSignal West Femto chemiluminescent substrate (p-Akt; Pierce Technology, Omaha, NE) visualization systems. Quantification was performed using the Image J gel analysis software (open source: http://rsbweb.nih.gov). All buffers and gels were purchased from Invitrogen (Carlsbad, CA).

Thiobarbituric Acid Reactive Substances Assay

This assay does not measure ROS production but provides a measure of lipid peroxidation (38). One-hundred microliters of the 10% (wt/vol) tissue homogenates from muscle and liver (preparation described under Western blot method) was analyzed for tissue homogenates’ capacity to react with thiobarbituric acid according to the consensus protocol published online by the Animal Models of Diabetic Complications Consortium at http://www.diacomp.org/shared/showProtocol.aspx?id=33.

Statistical Analysis

ANOVA was performed on data in the R programme (R 2.9.0; www.r-project.org). The level of significance was set at $P < 0.05$.

RESULTS

Test of the Progression of T2DM With Age in the GK Rat Model

Oral glucose tolerance tests. The result of the oral glucose tolerance test (OGTT) is shown in Fig. 1. Both GK groups have significant ($P < 0.05$) hyperglycemia and a markedly decreased ($P < 0.05$) glucose tolerance compared with the Wistar control ($P < 0.05$). Furthermore, there are significant differences ($P < 0.05$) between the OGTT of the GK6 and GK16 groups at 90 and 120 min. This difference between the two GK groups is supported by a significant increase ($P < 0.05$) in fasted plasma insulin from 64.6 ± 20 pM ($n = 7$) in the GK6 group to 144 ± 32 pM ($n = 7$) in the GK16 group.

$p$-Akt/Akt ratio. The $p$-Akt/Akt ratio is shown in Fig. 2. The ratio was similar in the three groups of animals in muscle, al-

![Fig. 1. Oral glucose tolerance test in Goto-Kakizaki (GK) rats of 6 (GK6) and 16 wk of age (GK16) and in Wistar control (W. cont). Glucose concentrations (mmol/l) are shown as time course at left and area under the curve (AUC) at right. *$P < 0.05$; (**$P < 0.06$. Data are means ± SE; $n = 7–8$.

![Fig. 2. Western blots of Akt and phosphor-ylated Akt (p-Akt) in muscle (left) and liver homogenates (right) from GK6 and GK16 rats and in W. cont. The histograms show the quantification of the blots in arbitrary units. *Significance of $P < 0.05$; (**) $P < 0.06$. Data are means ± SE; $n = 6–8$.](http://ajpendo.physiology.org/ Downloaded from http://ajpendo.physiology.org/ by 10.220.32.247 on June 28, 2017)
though there was a trend toward an increased ratio in the GK6 group. However, in liver we observed an ~40% decrease (P < 0.05) in the Akt phosphorylation during the progression of diabetes (from GK6 to GK16). However, it should be noted that our measurements were conducted under basal nonstimulated conditions, which may not reflect the in vivo status during insulin stimulation (58).

**Thiobarbituric acid-reactive substances.** In skeletal muscle the level of lipid peroxidation, as indicated by the amount of thiobarbituric acid-reactive substances (TBARS), was similar in the three groups, as shown in Fig. 3. However, in liver the TBARS levels were approximately threefold higher (P < 0.05) than in muscle in the control group, and there was a strong progression (2- to 3-fold, P < 0.05) from the GK6 to the GK16 group.

**Oxygen Consumption**

Typical traces of oxygen consumption in isolated liver and muscle mitochondria are given in Fig. 4. The detailed analyses of oxygen consumption are given in Table 1 for skeletal muscle and in Table 2 for liver mitochondria. The state 4, state 3, and VO2max respiration were recorded with six different substrate combinations, where VO2max is defined as the state 3 respiration of a given substrate, with the complex II substrate succinate added as well.

**Muscle.** The state 3 respiration as well as the VO2max was higher (P < 0.05) in the GK6 and GK16 groups under all conditions compared with the control (Table 1). Also, the increase in respiration followed by succinate addition was higher (P < 0.05) in GK6 animals compared with controls under all conditions. However, when considering the progression in diabetes, i.e., comparing the GK6 and GK16 animals, we observed an ~15% decrease (P < 0.05) in state 3 respiration and VO2max with palmitoyl carnitine (PC) or pyruvate plus PC as substrates. The increase in oxygen consumption when succinate was also added was similar with all substrates in both GK groups. The RCR was unchanged between groups, and the well-known RCR and P/O ratio decrease with PC as substrate was observed. There was no progression in the P/O ratio from the GK6 to the GK16 group.

**Liver.** Mitochondrial state 3 respiration with pyruvate was increased (P < 0.05) compared with Wistar control in both diabetic groups, but succinate addition increased oxygen consumption (P < 0.05) only in GK16 compared with control (Table 2). With PC as substrate, the increase in state 3 respiration with succinate addition was smaller (P < 0.05) in GK6 animals compared with control, and in the GK16 group there was no increase with succinate at all, suggesting a decreased complex II flux already in the early diabetic state of the GK6 animals and a profoundly affected complex II flux in the manifest diabetic animals represented by the GK16 group. RCR values were increased (P < 0.05) in GK16 compared with GK6 when pyruvate was added to the mitochondria. P/O ratios were increased (P < 0.05) in the manifest diabetic GK16 group compared with both control and GK6.

**Comparison of muscle and liver data.** Muscle mitochondria has a five- to sixfold higher respiration rate (nmol oxygen/mg mitochondrial protein) than liver mitochondria with pyruvate as substrate. Yet, with PC as the substrate, respiration in muscle mitochondria was only twofold higher than in liver mitochondria, confirming the well-known fact that liver mitochondria has a relatively higher capacity for metabolizing fatty acids than carbohydrates compared with muscle.

However, the effects of succinate addition on top of the other substrates were unexpected. The addition of succinate to muscle and liver mitochondria oxidizing pyruvate induced a similar increase in oxygen consumption in both diabetic groups compared with control (Tables 1 and 2). Yet, with PC as substrate, the response was very different in liver and muscle mitochondria and with significant differences also between the two GK groups. First, the liver displayed a much smaller succinate effect than muscle. Second, the GK6 group showed an ~50% smaller effect compared with controls, whereas in the GK16 group of manifest diabetic animals no increase at all was seen with succinate addition, indicating a profoundly changed SDH flux as a result of the progression of T2DM.

To test whether this decrease in SDH flux specifically in liver could be caused by decreased SDH activity in the GK groups, we measured this parameter, but as indicated in Table 3, there was no difference in SDH activity among groups.

**DISCUSSION**

The central findings in this study were as follows. 1) There are different mitochondrial phenotypes in the young and the adult GK rats, reflecting the effects of the progression of type 2 diabetes. 2) Liver and muscle mitochondria are affected differently; i.e., in liver, mitochondrial state 3 respiration increases in the GK rats from 6 to 16 wk, whereas in skeletal muscle mitochondria the reverse is true. 3) Liver mitochondria displayed a pronounced decrease in complex II flux in connection with fatty acid oxidation, which was not present in muscle mitochondria. 4) RCR and P/O ratios were unaffected by the progression of T2DM in muscle mitochondria, whereas there was a significant increase in both parameters in liver mitochondria during the progression of T2DM. 5) The two tissues had different activity of lipid peroxidation, with no change in muscle but an increase in the liver with age of the GK animals.

Thus, the mitochondria in the liver are clearly more affected (or affected earlier) by the progression of T2DM than in skeletal muscles.
muscle, and the flux in complex II, as well as the respiratory coupling, is involved.

**Test of the Progression of T2DM With Age of the GK Rat Model**

Insulin resistance and type 2 diabetes are known to develop spontaneously and progress with age in the GK rat (20). In the present study, we have demonstrated this progression from 6 to 16 wk of age by several independent parameters. At the whole body level a decreased glucose tolerance was observed (Fig. 1), and fasting plasma insulin was increased approximately twofold at 16 wk compared with 6 wk of age. At the organ level, lipid peroxidation, as estimated by the TBARS measurements (38), was increased very strongly in liver, whereas muscle tissue was not affected (Fig. 3). Simi-

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**Fig. 4. Respirometric measurements on isolated mitochondria from rat liver and skeletal muscle.** The graphs show typical time traces of PO2 and rate of oxygen consumption. A: muscle mitochondria from W. cont. B: liver mitochondria from W. cont. C: muscle mitochondria from GK6. D: liver mitochondria from GK6. E: muscle mitochondria from GK16. F: liver mitochondria from GK16. Final concentrations of added substrates were 2 mM malate, 1.5 mM pyruvate, 0.20 (for P/O measurements) and 3 mM ADP, and 6 mM succinate.
Table 1. Oxygen consumption in muscle mitochondria

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Wistar (16 wk)</th>
<th>GK6</th>
<th>GK16</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malate + pyruvate</td>
<td>9.0 ± 1.5a</td>
<td>10 ± 1.2a</td>
<td>9.0 ± 1.0a</td>
</tr>
<tr>
<td>Malate + PC</td>
<td>7.6 ± 1.5b</td>
<td>10 ± 0.69**</td>
<td>9.2 ± 1.0**</td>
</tr>
<tr>
<td>Malate + pyruvate + PC</td>
<td>10 ± 2.3a</td>
<td>12 ± 0.90b</td>
<td>10 ± 1.0b</td>
</tr>
<tr>
<td>Malate + pyruvate + succinate</td>
<td>172 ± 26a</td>
<td>215 ± 20a*</td>
<td>205 ± 17a**</td>
</tr>
<tr>
<td>Malate + PC</td>
<td>78 ± 13b</td>
<td>122 ± 5.7a*</td>
<td>104 ± 11a**</td>
</tr>
<tr>
<td>Malate + pyruvate + PC</td>
<td>184 ± 28a</td>
<td>256 ± 17a*</td>
<td>226 ± 24a**</td>
</tr>
<tr>
<td>V̇O₂max</td>
<td>9.0 ± 1.5a</td>
<td>10 ± 1.2a</td>
<td>9.0 ± 1.0a</td>
</tr>
<tr>
<td>Malate + pyruvate + succinate</td>
<td>220 ± 34a</td>
<td>281 ± 22a*</td>
<td>264 ± 20a*</td>
</tr>
<tr>
<td>Malate + PC + succinate</td>
<td>109 ± 20a</td>
<td>168 ± 8.4a*</td>
<td>143 ± 13a**</td>
</tr>
<tr>
<td>Malate + pyruvate + PC + succinate</td>
<td>232 ± 33a</td>
<td>310 ± 18a*</td>
<td>277 ± 26a**</td>
</tr>
</tbody>
</table>

Data are means ± SE and expressed in nmol·min⁻¹·mg⁻¹; n = 7–8. PC, palmitoyl carnitine; GK6, 6-wk-old Goto-Kakizaki rats; GK16, 16-wk-old Goto-Kakizaki rats; RCR, respiratory coupling ratio. *Significantly different from Wistar (16 wk), P < 0.05; †significantly different from GK6, P < 0.05; ‡P < 0.06. Different superscripted letters indicate significant differences within 1 group in 1 respiratory state.

Table 2. Oxygen consumption in liver mitochondria

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Wistar (16 wk)</th>
<th>GK6</th>
<th>GK16</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malate + pyruvate</td>
<td>7.2 ± 0.76a</td>
<td>6.7 ± 0.6a</td>
<td>6.7 ± 1.0a</td>
</tr>
<tr>
<td>Malate + PC</td>
<td>12.9 ± 2.8a</td>
<td>10.9 ± 1.0b</td>
<td>13.2 ± 1.2b</td>
</tr>
<tr>
<td>Malate + pyruvate + PC</td>
<td>13.6 ± 2.1b</td>
<td>13.2 ± 1.4c</td>
<td>14.1 ± 1.4c</td>
</tr>
<tr>
<td>Malate + pyruvate + succinate</td>
<td>25.9 ± 2.9a</td>
<td>30.9 ± 1.7**</td>
<td>41.9 ± 5.8**</td>
</tr>
<tr>
<td>Malate + PC</td>
<td>35.5 ± 12ab</td>
<td>59.8 ± 6.5a*</td>
<td>76.1 ± 8.8a*</td>
</tr>
<tr>
<td>Malate + pyruvate + PC</td>
<td>39.0 ± 7.3a</td>
<td>55.4 ± 2.7a*</td>
<td>72.0 ± 9.1a*</td>
</tr>
<tr>
<td>V̇O₂max</td>
<td>65.3 ± 7.9a</td>
<td>75.0 ± 7.5a</td>
<td>89.4 ± 9.6a**</td>
</tr>
<tr>
<td>Malate + pyruvate + succinate</td>
<td>60.1 ± 5.1ab</td>
<td>72.7 ± 6.1a</td>
<td>91.3 ± 14.7a†</td>
</tr>
<tr>
<td>Malate + PC + succinate</td>
<td>58.9 ± 9.0a</td>
<td>70.3 ± 10.6a</td>
<td>65.6 ± 10.7a</td>
</tr>
<tr>
<td>Malate + pyruvate + PC + succinate</td>
<td>65.3 ± 7.9a</td>
<td>75.0 ± 7.5a</td>
<td>89.4 ± 9.6a**</td>
</tr>
</tbody>
</table>

Data are means ± SE and expressed in nmol·min⁻¹·mg⁻¹; n = 7–8. *Significantly different from Wistar (16 wk), P < 0.05; †significantly different from GK6, P < 0.05. Different superscripted letters indicate significant differences within 1 group in 1 respiratory state.
Mitochondrial Respiration

The involvement of mitochondria in the pathogenesis of insulin resistance and T2DM has been implicated by many studies for more than a decade (26, 37, 44, 51). Most studies of type 2 diabetic patients find that mitochondrial respiratory capacity is either impaired (45, 35, 53) or unchanged (9, 12), and yet increased respiration of liver mitochondria has been reported in diabetic ob/ob mice (10, 13).

However, in the present study, we found an increased respiration in both GK groups compared with control (Tables 1 and 2), an observation also reported by Ferreira and colleagues (16, 17) in the GK rat model. These authors suggest that the increased respiration of liver mitochondria is likely to reflect an increased activity of SDH. We decided to elucidate this issue further by adding the complex II substrate succinate in addition to a carbohydrate (pyruvate) and/or a fatty acid substrate palmitoyl carnitine (PC). Our measurements revealed that in both liver and muscle mitochondria oxidizing pyruvate, addition of succinate induced a 20–30% increased respiration (Tables 1 and 2). However, when the mitochondria were oxidizing PC, the situation was very different in the two tissues. In muscle, succinate addition caused an ~40% state 3 respiration increase, whereas in liver mitochondria of the GK16 animals the succinate addition did not increase respiration at all. Thus, our data indicate an impaired and/or a saturated complex II pathway in the liver of type 2 diabetic rats and that this effect is clearly progressing from the early (GK6) to the manifest diabetic state (GK16). We tested whether this finding was related to a decreased activity of the complex II, but as shown in Table 3, the activity of complex II was equal in the three groups.

Thus, the observed reduction in SDH flux specifically in liver mitochondria from a diabetic animal must have another cause other than reduced complex II activity. One possibility is that in the diabetic animal the FADH2-dependent respiration is already running near maximum capacity, as supplied by the β-oxidation pathway, with PC as substrate. Therefore, under such conditions, further addition of a FAD-coupled substrate (succinate here) causes little or no increase in respiration. This interpretation finds support in the oxygen consumption data of Table 2, showing that oxygen consumption in liver mitochondria increases drastically with the progression of diabetes from 35.5 ± 12 nmol·min⁻¹·mg protein⁻¹ in the control group to 59.8 ± 6.5 and 76.1 ± 8.8 nmol·min⁻¹·mg protein⁻¹ in the 6- and 16-wk-old animals, respectively. In muscle, the effect of the progression of T2DM on PC-induced increase in oxygen consumption is also present but is smaller than in liver (Table 1).

All in all, our data support the notion that progression of T2DM in the GK animal involves an enhanced β-oxidation flux of a magnitude where eventually the reoxidation of SDH-generated FADH2 may be inhibited, thereby also compromising the TCA flux. However, these important findings need further experimental elucidations to clarify the underlying mechanisms and indeed why liver is affected earlier than muscle mitochondria. Also, it is important to establish whether the observed complex II involvement in the progression of type 2 diabetes is in fact a general phenomenon of T2DM or whether it is a specific manifestation of the GK rat. However, our findings are in line with recent observations of complex II involvement in human disease and aging (47).

**Table 3. Complex II activity in livers of Wistar, GK6, and GK16 rats**

<table>
<thead>
<tr>
<th></th>
<th>U/g wet wt</th>
<th>U/U CS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wistar</td>
<td>18.6 ± 2.45 (13)</td>
<td>1.18 ± 0.15 (7)</td>
</tr>
<tr>
<td>GK6</td>
<td>16.1 ± 1.65 (15)</td>
<td>1.01 ± 0.12 (8)</td>
</tr>
<tr>
<td>GK16</td>
<td>22.7 ± 3.71 (14)</td>
<td>1.71 ± 0.38 (6)</td>
</tr>
</tbody>
</table>

Data are means ± SD (no. of rats in parentheses). CS, citrate synthase. One unit corresponds to the amount of enzyme, which converts 1 μmol/min substrate succinate.

Conclusion

In this study, we observed a change in phenotype of liver and skeletal muscle mitochondria with the development of T2DM, as occurs with age in the GK rat model. In muscle, the changes were insignificant. However, in liver, the respiratory capacity increased with all substrate combinations tested. But most importantly, we showed that the increase in respiration, normally seen with the addition of the complex II substrate succinate, was decreased severely in the GK6 animals and completely lacking in the manifest diabetic state of the GK16 animals. The observations support the notion that the pathogenesis of T2DM of the GK rat is initiated in the liver and only later also affects muscle mitochondria (27), and it indicates that a decreased complex II flux, as well as an enhanced respiratory coupling, is involved.

**GRANTS**

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**DISCLOSURES**

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

**AUTHOR CONTRIBUTIONS**

W.J., N.G., and B.Q. researched data, analyzed and interpreted the results of experiments; W.J., P.J., K.A.R., N.G., and B.Q. performed the experiments; W.J., P.J., K.A.R., N.G., and B.Q. drafted the figures; W.J. and B.Q. drafted the manuscript; W.J., N.G., and B.Q. edited and revised the manuscript; W.J., P.J., K.A.R., N.G., and B.Q. approved the final version of the manuscript.
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