Impaired oxidative phosphorylation in skeletal muscle of intrauterine growth-retarded rats

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Selak, Mary A., Bayard T. Storey, Iyalla Peterside, and Rebecca A. Simmons. Impaired oxidative phosphorylation in skeletal muscle of intrauterine growth-retarded rats. Am J Physiol Endocrinol Metab 285: E130–E137, 2003.—Intrauterine growth retardation (IUGR) has been linked to the development of type 2 diabetes in later life. We have developed a model of uteroplacental insufficiency, a common cause of intrauterine growth retardation, in the rat. Early in life, the animals are insulin resistant and by 6 mo of age they develop diabetes. Glycogen content and insulin-stimulated 2-deoxyglucose uptake were significantly decreased in muscle from IUGR rats. IUGR muscle mitochondria exhibited significantly decreased rates of state 3 oxygen consumption with pyruvate, glutamate, α-ketoglutarate, and succinate. Decreased pyruvate oxidation in IUGR mitochondria was associated with decreased ATP production, decreased pyruvate dehydrogenase activity, and increased expression of pyruvate dehydrogenase kinase 4. Such a defect in IUGR mitochondria leads to a chronic reduction in the supply of ATP available from oxidative phosphorylation. Impaired ATP synthesis in muscle compromises energy-dependent GLUT4 recruitment to the cell surface, glucose transport, and glycogen synthesis, which contribute to insulin resistance and hyperglycemia of type 2 diabetes.

diabetes; intrauterine growth retardation; pyruvate dehydrogenase

UTEROPLACENTAL INSUFFICIENCY resulting in fetal growth retardation is a common complication of pregnancy and a significant cause of perinatal morbidity and mortality. Evidence is mounting that uteroplacental insufficiency may also have permanent consequences later in life. Several epidemiological studies show an increased incidence of type 2 diabetes in individuals who were growth retarded at birth (1, 2, 20, 37, 38, 49). The mechanisms by which an abnormal uterine milieu leads to the development of diabetes in adulthood are not known. To determine such mechanisms, we have developed a model of intrauterine growth retardation (IUGR) in the rat, induced by bilateral uterine artery ligation at 19 days of gestation (term is 21.5 days) (34, 44, 48). Fetuses have decreased levels of glucose, branched-chain amino acids, insulin, IGF-I, and oxygen. All of these metabolic parameters are normal at birth, yet birthweight is reduced in IUGR newborn pups. The unique feature of this model is its ability to induce diabetes in adult animals with the salient features of most forms of type 2 diabetes in the human: defects in insulin action and insulin secretion. IUGR animals exhibit marked insulin resistance early in life (before the onset of hyperglycemia), characterized by blunted whole body glucose disposal in response to insulin (45). Before the onset of diabetes, IUGR animals are hyperinsulinemic and obese and have elevated levels of fatty acids. Once the animal develops diabetes, insulin levels fall, presumably due to a marked decline in β-cell mass. Fat pad mass also decreases, and nonesterified fatty acid (NEFA) levels normalize, yet insulin resistance worsens. The underlying mechanism(s) responsible for the reduced capacity of peripheral tissue, e.g., skeletal muscle, to respond to the metabolic effects of insulin in IUGR animals was the focus of this study.

Control of hyperglycemia requires transport of glucose from the blood into storage tissues, which is mediated by insulin (3). Normally, in skeletal muscle, insulin recruits GLUT4 to increase glucose flux across the plasma membrane and stimulate flux through the glycogen synthase pathway (29, 39, 43). Decreased insulin-stimulated glucose uptake resulting in impaired glycogen synthesis in skeletal muscle has been shown to be a major contributing factor to insulin resistance in type 2 diabetes (7, 11, 36, 40, 41, 43, 55).

The pathway from glucose transport into the cell to glycogen deposition is highly energy dependent. Glycogen synthesis utilizes two molecules of ATP per mole glucose incorporated into glycogen: 1 mol is used to form glucose 6-phosphate (G-6-P) and the second to form UDP-glucose (18). ATP is additionally required by cAMP-, calmodulin-, and diacylglycerol-stimulated kinases, which reciprocally control glycogen synthase and glycogen phosphorylase activities (18). The intracellular ATP pool available for these noncontractile signaling and other metabolic reactions is expected to have profound effects on the ability of muscle to accumulate glucose and store it as glycogen. Impairment of ATP production by oxidative phosphorylation, resulting in diminution of this energy pool, would compro...
mise GLUT4 recruitment, glucose uptake, and glyco-
genesis in response to insulin and promote insulin resistance.

A unique advantage of our IUGR model is the ability to examine glucose homeostasis and the effects of mito-
chondrial dysfunction on insulin resistance during the evolution of the diabetic disease state. In previous
studies (27), we found that mitochondrial NADH/
NAD+ ratios were increased in IUGR rats at day 21 of life, suggesting an underlying mitochondrial cytopathy
in skeletal muscle. Therefore, the focus of this study was to determine whether mitochondrial function is
impaired in IUGR skeletal muscle, the major depot for glucose storage as glycogen.

MATERIALS AND METHODS

Reagents

Reagent-grade chemicals, luciferase assay reagents, and diagnostic kits for lactate and glucose were obtained from Sigma Chemical (St. Louis, MO). A NEFA C diagnostic kit for NEFA was obtained from Wako Chemicals (Neuss, Germany). Rabbit antiserum to rat pyruvate dehydrogenase kinase-4 (PDK4) was the generous gift of Dr. Robert A. Harris, Indiana University. Rabbit antiserum to GLUT1 and GLUT4 was obtained from East Acres Biologicals (Southbridge, MA). Goat anti-rabbit IgG conjugated to horseradish peroxidase was from Bio-Rad Laboratories (Hercules, CA).

Animals

Time-dated, pregnant Sprague-Dawley rats were obtained from Charles River Laboratories, housed under standard conditions, and allowed free access to standard rat chow and water. To induce IUGR, bilateral uterine artery ligation was performed in rats on day 19 of a 21.5-day gestation period, as previously described (45). Sham-operated animals served as controls. Pregnant rats were allowed to deliver spontaneously, litters were culled to eight animals, and the pups remained with their mothers until they were weaned. These studies were approved by the Animal Care Committees of Children’s Hospital of Philadelphia and the University of Pennsylvania.

Isolation of Skeletal Muscle Mitochondria

The following three buffers were used in the isolation protocol: 100 mM KCl, 50 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 1 mM EDTA, and 1 mM ATP (buffer A); 100 mM KCl, 50 mM Tris·HCl, pH 7.2, 1 mM MgCl2, 0.2 mM EDTA, and 0.2 mM ATP (buffer B); and 220 mM mannitol, 70 mM sucrose, and 15 mM Tris·HCl, pH 7.2 (MST buffer). MST buffer was used for final resuspension of isolated mitochondria. All operations were carried out at 2–4°C. Predomi-
nantly red muscle, collected from the front and hind legs of 3- to 6-mo-old aged-matched control and IUGR rats, was cleaned to remove fat and connective tissue, weighed and

In this study, we found that mitochondrial function is impaired in skeletal muscle of IUGR rats, as indicated by increased NADH/NAD+ ratios and decreased oxygen consumption rates. These findings suggest mitochondrial dysfunction in IUGR muscle, which may contribute to insulin resistance and altered glucose homeostasis.

**Oxygen Consumption Measurement**

Oxygen consumption of isolated skeletal muscle mitochondria was measured using a Clark-type oxygen electrode. The isolated mitochondria were resuspended in buffer A and incubated at 37°C for 10 min. Then, a basal rate of oxygen consumption was measured, followed by the addition of ADP to achieve state 3 respiration. The respiratory control ratio (OCR) was calculated as the ratio of state 3 to state 2 oxygen consumption rates.

**Mitochondrial Enzyme Activities**

Mitochondrial enzyme activities were measured using standard assay protocols. 

**Results**

- Mitochondrial NADH/NAD+ ratios were increased in IUGR muscle compared to controls.
- Oxygen consumption rates were significantly decreased in IUGR mitochondria.
- Respiratory control ratios were lower in IUGR mitochondria.

**Discussion**

The results of this study suggest that mitochondrial dysfunction is an important factor in the development of insulin resistance in IUGR muscle. Further studies are needed to elucidate the mechanisms underlying this dysfunction and to develop strategies for preventing or treating insulin resistance in this model.
Spectrophotometric Quantitation of Respiratory Chain Components

Substrate-reduced minus oxidized spectra at anaerobiosis were recorded at room temperature in an Amino DW2 split-beam spectrophotometer (Silver Spring, MD) using mitochondria suspended in MSTPE buffer at 1 mg/ml protein (9, 28). The reference cuvette contained 0.25 mM ADP, and the sample cuvette contained 10 mM glutamate, 2 mM malate, 10 mM succinate, and 0.25 mM ADP. Dithionite was subsequently added to the sample cuvette, and a dithionite-reduced minus oxidized spectrum was recorded. Nanomoles of substrate-reducible electron transport chain carrier per milligram of mitochondrial protein were calculated from the absorbance changes in the anaerobic reduced minus oxidized difference spectra at the following wavelength pairs in accord with the Beer-Lambert relationship by using the following millimolar extinction coefficients: cytochrome $a_3$ (445–455 nm; 80 cm$^{-1}$ m$^{-1}$); cytochrome $a$ (605–630 nm; 24 cm$^{-1}$ m$^{-1}$); cytochrome $c$ ($c_1$) (550–540 nm; 19.1 cm$^{-1}$ m$^{-1}$); cytochrome $b$ (562–575 nm; 20 cm$^{-1}$ m$^{-1}$); the redox state of flavoproteins was qualitatively assessed using the wavelength pair of 465–510 nm (11 cm$^{-1}$ m$^{-1}$) (32, 33).

Measurement of Aconitase, Citrate Synthase, and Pyruvate Dehydrogenase

The specific activities of aconitase, citrate synthase, and pyruvate dehydrogenase (PDH) were measured spectrophotometrically at 25°C using assay methods described by Robinson et al. (39). PDH was measured in the presence of dichloroacetate (10 mM) and oxamic acid (25 mM) to inhibit pyruvate dehydrogenase kinase (PDK) and lactate dehydrogenase (LDH), respectively. Freeze-thawed, isolated muscle mitochondria were assayed in the presence of N-dodecyl-$\beta$-maltoside (0.2 mM) to ensure complete enzyme release from the matrix. Recovery of mitochondria from skeletal muscle was monitored by measuring the specific activity of citrate synthase throughout the course of organelle isolation and was determined from the total units of citrate synthase present in the final mitochondrial preparations relative to the total units of citrate synthase present in the initial muscle homogenates.

Measurement of Muscle Glycogen and Serum Lactate, Glucose, and NEFA

Lactic acid, glucose, and NEFA concentrations were determined using Sigma Diagnostic and Wako assay kits, respectively, in flash-frozen serum samples collected from animals at the time animals were killed. Skeletal muscle glycogen content was determined in perchloric acid extracts prepared from flash-frozen, pulverized skeletal muscle by the method of Keppler and Decker (25). Glycogen released following hydrolysis of glycogen with amyloglucosidase (glucoamylase) from Aspergillus niger was corrected for endogenous glucose present in muscle. Glucose was quantitated spectrophotometrically by monitoring NADP$^+$ reduction in a coupled enzyme reaction employing hexokinase and glucose-6-phosphate dehydrogenase.

Measurement of Glucose Uptake

Glucose uptake was measured in epimysial muscle of IUGR and control animals by use of the glucose analog 2-deoxyglucose (2-DG) and the procedure of Young et al. (54). Glucose uptake is expressed as micromoles 2-DG per milliliter of intracellular water per 20 min.

Western Blots

Isolated muscle mitochondria and tissue extracts prepared from flash-frozen skeletal muscle were subjected to reducing SDS-PAGE using 12% Tris-glycine gels. Proteins were electrophoresed from the gels onto polyvinylidene difluoride membranes and probed with GLUT1 (1:10,000), GLUT4 (1:5,000), or PDK4 antisera (1:500), followed by goat anti-rabbit horseradish peroxidase-conjugated secondary antibody. Enzyme-catalyzed chemiluminescence (ECL) mediated by peroxidase was developed with an ECL kit from Amersham Pharmacia Biotech (Piscataway, NJ) and detected with Super RXX-ray film from Fuji Medical Systems (Stamford, CT). Densitometric analyses of GLUTs and PDK4 Western blots were performed using MacBAS, Fuji PhotoFilm, and Kohshin Graphics.

Electron Microscopy of Muscle Samples

Longitudinal and cross sections of skeletal muscle preserved in 4% paraformaldehyde were examined by electron microscopy by the Biomedical Imaging Core Facility of the University of Pennsylvania. Samples were fixed at 4°C for 1 h with glutaraldehyde in phosphate-buffered saline containing 1 mM CaCl$_2$ and postfixed in 2% OsO$_4$ in 0.1 M sodium cacodylate for 30 min at 4°C. Muscles were dehydrated with a graded ethanol (75–100%) series and embedded in Epon-812. Thin (700 Å) sections were cut and collected on nickel grids.

Protein Determination

Mitochondrial and muscle homogenate protein concentrations were determined with the bicinchoninic acid reagent from Pierce (Rockford, IL) using bovine serum albumin (BSA) as protein standard.

Statistical Analyses

Analyses were performed using, as required, paired and unpaired two-tailed Student’s $t$-tests by StatView from SAS Institute (Cary, NC) and Statistics for Macintosh from Blackwell Scientific (Oxford, UK) statistical programs. Differences were taken as significant for $P < 0.05$.

RESULTS

IUGR Animals: Metabolic Profile

Prediabetes. Before developing diabetes (3–4 mo), IUGR rats had normal glucose but elevated insulin levels (Table 1). Serum concentrations of NEFA were also elevated in IUGR rats compared with controls (Table 1). IUGR rats had higher body weights (402.5 ± 26.2 g, $P < 0.05$, IUGR vs. controls, respectively) and total fat pad mass (25.5 ± 2.6 vs. 17.9 ± 1.5 g, $P < 0.05$, IUGR vs. controls, respectively). Glycogen content of skeletal muscle was decreased by 27% ($P < 0.05$) in IUGR animals compared with corresponding controls.

Insulin-stimulated 2-DG uptake was significantly blunted in isolated muscle from IUGR animals compared with controls, whereas there was no difference between the two groups with respect to basal 2-DG uptake (Fig. 1). GLUT1 and GLUT4 protein levels, as
determined by Western blot analyses and quantitated by densitometry, were similar in IUGR and control muscle (data not shown).

Diabetes. IUGR rats developed diabetes at ~6 mo of age. At the time of this study, IUGR animals were no longer obese (fat pad mass: 16.4 ± 1.2 vs. 18.1 ± 2.5, IUGR vs. control, respectively), and body weights were less than in controls (305.2 ± 63 vs. 387.6 ± 31 g, IUGR vs. control, respectively). Levels of serum insulin and NEFAs were similar in the two groups; however, glucose and lactate concentrations were both markedly elevated (Table 1). Glycogen content of skeletal muscle was decreased by 43% (*P < 0.05) in IUGR animals compared with corresponding controls.

Basal 2-DG uptake in isolated muscle was similar to that of controls; however, maximal 2-DG uptake in response to insulin (2 mU/ml) increased only 55% in IUGR animals compared with a more than fourfold increase in controls (Fig. 1). Similar to prediabetic IUGR animals, diabetic IUGR animals had GLUT1 and GLUT4 protein levels that were similar to control levels (data not shown).

Mitochondrial Morphology and Cytochrome Levels

No obvious pathology was evident in prediabetic or diabetic IUGR skeletal muscle compared with controls as documented by electron microscopy (data not shown). Mitochondrial morphology, numbers, and distribution appeared normal in both groups of animals. Recovery of mitochondria was also similar for control, prediabetic, and diabetic IUGR muscle. Concentrations of electron transport carriers (cytochrome a3, cytochrome a, cytochrome c1, cytochrome b, and flavoproteins), normalized to mitochondrial protein, also did not differ between groups. The content of respiratory chain proteins in these rat skeletal muscle preparations is similar to the content reported for mouse skeletal muscle (28). Subsequent addition of dithionite did not further increase reduction levels, showing that all cytochromes present were functionally associated with the electron transport chain (data not shown).

Respiratory Chain-Linked Electron Transfer and Energy Coupling In Muscle

Analysis of the respiratory and phosphorylating activities of freshly isolated, intact, coupled mitochondria is the most powerful in vitro method currently available for determining the site and severity of putative mitochondrial energetic defects. Hence, this approach was used to analyze skeletal muscle mitochondria from control and adult prediabetic and diabetic IUGR rats to ascertain whether mitochondrial function was compromised in IUGR animals. Comparison of the rates of oxidative phosphorylation for different substrates in skeletal muscle is presented in Table 2. ADP-stimulated respiration with palmitoyl carnitine did not differ between groups, demonstrating that fatty acid oxidation was not impaired in IUGR rat muscle. Similar rates of acetyl carnitine oxidation in control and IUGR mitochondria indicated that there was no defect associated with the enzymes in the first third of the tricarboxylic acid cycle.

Table 1. Serum glucose, insulin, lactate, and NEFA concentrations in control (age 3 and 6 mo) and IUGR rats in the prediabetic (age 3 mo) and diabetic states (age 6 mo)

<table>
<thead>
<tr>
<th></th>
<th>Glucose, mg/dl</th>
<th>Insulin, ng/ml</th>
<th>Lactate, mg/dl</th>
<th>NEFA, meq/l</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>IUGR</td>
<td>Control</td>
<td>IUGR</td>
</tr>
<tr>
<td>3 mo</td>
<td>92 ± 13.2</td>
<td>99 ± 20.3</td>
<td>35.3 ± 5.1</td>
<td>59.3 ± 8.9*</td>
</tr>
<tr>
<td></td>
<td>3.53 ± 0.4</td>
<td>6.8 ± 0.9</td>
<td>0.34 ± 0.05</td>
<td>1.21 ± 0.13*</td>
</tr>
<tr>
<td>6 mo</td>
<td>130 ± 18.9</td>
<td>280 ± 19.4*</td>
<td>42.6 ± 4.8</td>
<td>39.1 ± 5.6</td>
</tr>
<tr>
<td></td>
<td>0.29 ± 0.06</td>
<td>0.28 ± 0.11</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values shown are means ± SD; n = 5 determined using flash-frozen serum collected at the time animals were killed. *P < 0.05 IUGR vs. control. NEFA, nonesterified fatty acids; IUGR, intrauterine growth retarded.
ATP production was decreased by 21% in mitochondria from prediabetic and diabetic IUGR rats. Pyruvate-supported ATP production was decreased by 21 ± 4% (n = 3, P < 0.05), and the specific activity of pyruvate dehydrogenase (PDH) was decreased by 20 ± 6% (n = 3, P < 0.05) in IUGR mitochondria. Because the activity state of PDH can vary widely depending on conditions of sample manipulation as well as the metabolic state, measurements of PDH activity may be subject to error and can conceivably give misleading results. Thus the fully activated enzyme probably provides the most reliable estimate of total enzyme activity. Maximal activity of PDH was induced by pretreatment of muscle mitochondria with the activator dichloroacetate; the decrease of 20% observed in IUGR mitochondria may actually underestimate the extent to which the enzyme complex may be inhibited in vivo by short-term and long-term effects mediated by acetyl-CoA and NADH.

Table 2. Rates of state 3 and state 4 oxygen consumption of skeletal muscle mitochondria from control and IUGR rats

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Control State 3</th>
<th>Control State 4</th>
<th>Control RCR</th>
<th>IUGR State 3</th>
<th>IUGR State 4</th>
<th>IUGR RCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate + malate</td>
<td>196 ± 11</td>
<td>38 ± 4</td>
<td>5.2</td>
<td>167 ± 12*</td>
<td>41 ± 6</td>
<td>4.1</td>
</tr>
<tr>
<td>3 mo</td>
<td>195 ± 12</td>
<td>44 ± 12</td>
<td>4.4</td>
<td>145 ± 7*</td>
<td>43 ± 12</td>
<td>3.4</td>
</tr>
<tr>
<td>Glutamate + malate</td>
<td>190 ± 16</td>
<td>33 ± 2</td>
<td>5.8</td>
<td>174 ± 12*</td>
<td>35 ± 4</td>
<td>5.0</td>
</tr>
<tr>
<td>3 mo</td>
<td>193 ± 11</td>
<td>30 ± 2</td>
<td>6.4</td>
<td>153 ± 11*</td>
<td>42 ± 7</td>
<td>3.6</td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>181 ± 21</td>
<td>32 ± 14</td>
<td>5.7</td>
<td>156 ± 11*</td>
<td>37 ± 8</td>
<td>4.8</td>
</tr>
<tr>
<td>3 mo</td>
<td>197 ± 17</td>
<td>31 ± 11</td>
<td>6.4</td>
<td>136 ± 13*</td>
<td>41 ± 7</td>
<td>3.3</td>
</tr>
<tr>
<td>Succinate</td>
<td>231 ± 28</td>
<td>80 ± 6</td>
<td>2.9</td>
<td>192 ± 10*</td>
<td>81 ± 8</td>
<td>2.4</td>
</tr>
<tr>
<td>3 mo</td>
<td>248 ± 21</td>
<td>98 ± 13</td>
<td>2.5</td>
<td>161 ± 3*</td>
<td>75 ± 1</td>
<td>2.2</td>
</tr>
<tr>
<td>C2-carnitine + malate</td>
<td>120 ± 29</td>
<td>23 ± 6</td>
<td>5.2</td>
<td>96 ± 19</td>
<td>31 ± 4</td>
<td>3.1</td>
</tr>
<tr>
<td>6 mo</td>
<td>150 ± 30</td>
<td>47 ± 11</td>
<td>3.2</td>
<td>157 ± 42</td>
<td>55 ± 6</td>
<td>2.8</td>
</tr>
<tr>
<td>C16-carnitine + malate</td>
<td>11 ± 3</td>
<td>32 ± 14</td>
<td>5.7</td>
<td>156 ± 11*</td>
<td>37 ± 8</td>
<td>4.8</td>
</tr>
</tbody>
</table>

Rates of oxygen consumption were determined in the active ADP-stimulated state of oxidative phosphorylation (state 3) and in the resting ADP-depleted state on completion of phosphorylation (state 4) with calculated respiratory control ratios (RCR) for various substrates. [Values are means ± SD n = 4.] RCRs were calculated as the ratio of the mean state 3 rate to the mean state 4 rate. *P < 0.05, IUGR vs. control.

In contrast to normal fatty acid oxidation, rates of state 3 oxygen consumption with pyruvate, glutamate, α-ketoglutarate (each plus malate), and succinate (plus rotenone) were decreased in mitochondria from prediabetic and diabetic IUGR rats. Pyruvate-supported ATP production was decreased by 21 ± 4% (n = 3, P < 0.05), and the specific activity of pyruvate dehydrogenase (PDH) was decreased by 20 ± 6% (n = 3, P < 0.05) in IUGR mitochondria. Because the activity state of PDH can vary widely depending on conditions of sample manipulation as well as the metabolic state, measurements of PDH activity may be subject to error and can conceivably give misleading results. Thus the fully activated enzyme probably provides the most reliable estimate of total enzyme activity. Maximal activity of PDH was induced by pretreatment of muscle mitochondria with the activator dichloroacetate; the decrease of 20% observed in IUGR mitochondria may actually underestimate the extent to which the enzyme complex may be inhibited in vivo by short-term and long-term effects mediated by acetyl-CoA and NADH.

Regulation of activity of the PDH complex is controlled primarily by interconversion of its PDH component (E1) between an active, nonphosphorylated form and an inactive, phosphorylated form. In skeletal muscle, phosphorylation of E1 is primarily catalyzed by the isoenzyme PDK4 (8, 53). Therefore, to determine whether an elevated level of PDK4 might be responsible for the impaired activity of PDH in IUGR muscle, we performed Western blot analyses. These analyses showed that expression of PDK4 protein was increased between four and ninefold (n = 12; P < 0.01) in prediabetic and diabetic IUGR skeletal muscle mitochondria.

To determine whether other factors might also contribute to decreased substrate oxidation in IUGR muscle, additional parameters reflective of specific mitochondrial functions were examined. These included proton permeability, respiratory control, adenine nucleotide transport, efficiency of oxidative phosphorylation, ATP synthase activity, electron transport chain activity, and calcium uptake. The rates of state 4 oxygen consumption were similar in control and IUGR mitochondria with all substrates (Table 2), indicating that the endogenous proton conductance of the inner mitochondrial membrane was not increased in IUGR muscle. RCRs were high for all substrates in control mitochondria and, albeit lower for IUGR mitochondria, were still robust (Table 2). The RCR reflects the degree of coupling between substrate oxidation and ADP phosphorylation; by this criterion, IUGR mitochondria were well coupled and showed high physical and functional integrity. The RCRs additionally denote that the adenine nucleotide and phosphate translocases were not impaired in diabetic muscle. Similar ADP/O ratios indicate similar efficiencies of oxidative phosphorylation. The ratio of oxygen consumption rates in state 3 to those in the presence of the uncoupler CCCP was similar in IUGR and control mitochondria (data not shown), showing that proton translocation to the ATP synthase did not limit state 3 oxygen consumption in IUGR mitochondria. By increasing proton conductance, uncouplers disrupt coupling between respiration and ATP synthesis and stimulate maximal oxygen consumption (21, 47).

Rotenone-sensitive NADH:O2 oxidoreductase (NADH oxidase) activity measured in disrupted mitochondria in the presence of exogenous NADH was comparable in control and IUGR mitochondria (data not shown), demonstrating that overall capacity of and electron flow through the electron transport chain were not impaired in IUGR mitochondria. Control and IUGR mitochondria had similar Ca2+/O ratios (data not shown), suggesting that a failure of mitochondria to accumulate calcium to stimulate PDH and α-ketoglutarate dehydrogenase (α-KGDH) (12) was unlikely to account for the observed decreases in state 3 pyruvate, gluta-
mate, and α-ketoglutarate oxidation by IUGR mitochondria. Activities of other key mitochondrial enzymes, such as citrate synthase, aconitase, and cytochrome oxidase, also did not differ between IUGR and controls (data not shown).

DISCUSSION

Uteroplacental insufficiency limits availability of substrates to the fetus and retards growth during gestation. These studies demonstrate that alterations in the intrauterine milieu have a profound impact on glucose homeostasis in the offspring, culminating in the development of type 2 diabetes in adulthood. The distinct advantage of our animal model of diabetes in IUGR rats is the ability to examine time-dependent changes in glucose homeostasis and their contribution to the induction of diabetes.

The etiology of insulin resistance within skeletal muscle in the human with type 2 diabetes is multifactorial, involving impairments in hormonal signaling, enzyme and transporter activity, and substrate availability. Recent studies have also implicated decreased oxidative capacities of skeletal muscle of human diabetics as contributory to insulin resistance. Simoneau and Kelley found decreased activities of citrate synthase and cytochrome-c oxidase, suggesting that the oxidative capacity of muscle in individuals with type 2 diabetes might be diminished. Our studies have extended these observations to demonstrate that oxidative phosphorylation of key substrates is reduced, leading to decreased ATP production in both prediabetic and diabetic IUGR animals. We suggest that diminished ATP production may be the underlying molecular mechanism responsible for impaired insulin signaling in muscle of IUGR rats.

Muscle ATP is functionally and physically compartmentalized in the myocyte, with by far the largest pool being used for contractile work in the myofibrils. ATP in this pool is strongly buffered by the creatine phosphate-creatine kinase system. Therefore, in vivo measurements of ATP content in muscle cannot accurately reflect the amount of ATP that is available for metabolic processes other than contraction. Deficits in mitochondrial oxidative phosphorylation are therefore reflected in reduced glycogen synthesis due largely to impaired glucose transport and conversion of glucose to G-6-P, the initial substrate for glycogen synthesis; both processes consume ATP.

The observed reduction in oxidative phosphorylation capacity, although significant, amounts to 20–35%, raising the question whether this reduction would be sufficient to compromise glycogen synthesis and insulin-signaling pathways in muscle. A similar reduction in ATP production in 3T3-L1 adipocytes results in an increase in basal glucose transport (which is mediated by GLUT1 and not dependent on the energy-requiring process of translocation) but a significant decrease in insulin-stimulated glucose transport, translocation of GLUT4 to the plasma membrane, and insulin-induced phosphorylation and/or activation of insulin receptor Akt-1, p70 S6 kinase, and extracellular signal-related kinase-1/2 (22, 25). Inhibition of cellular energy production also significantly impairs insulin action in skeletal muscle (31). Furthermore, patients with a mutation in the mitochondria tRNALEU(UUR) gene develop diabetes characterized by defects in both insulin secretion and insulin action (17, 51). The percentage reduction in oxidative phosphorylation in skeletal muscle would thus be expected to be more than adequate to decrease glycogen production to the extent that insulin resistance would be manifest.

State 4 oxygen consumption rates were similar in control and IUGR mitochondria with all substrates, indicating that proton conductance of the inner membrane is not increased in IUGR muscle. ADP/O ratios were not decreased in IUGR mitochondria, which shows that the efficiency of energy coupling was not adversely affected in diabetic animals. Although RCRs in IUGR mitochondria were lower than in controls, they were still robust, further indicating that oxidative phosphorylation was well coupled in IUGR mitochondria. Activity of the electron transport chain, measured as rotenone-sensitive NADH:O2 oxidoreductase, was normal in IUGR mitochondria, demonstrating that substrate oxidation was restricted at a site proximal to the respiratory chain. The decrease in oxidative phosphorylation rates in IUGR mitochondria cannot be attributed to impairment of electron transport or to partial uncoupling of mitochondria but can rather be attributed to decreased substrate oxidation in state 3 at the dehydrogenase level.

Decreased oxidation of pyruvate, α-ketoglutarate, glutamate, and succinate would result from a primary defect at the level of PDH activity, which would subsequently lead to secondary decreases in activities of both succinate dehydrogenase (SDH) and α-KGDH. SDH is inhibited by oxaloacetate, which accumulates because the acetyl-CoA produced from pyruvate oxidation is inadequate to facilitate its rapid removal by citrate synthase (52). Inhibition of SDH would, in turn, lead to an elevated level of succinyl-CoA, thereby inhibiting α-KGDH. Because glutamate is exclusively oxidized by α-KGDH following transamination in skeletal muscle mitochondria, the decrease in glutamate oxidation rate is most likely attributable to this inhibition of α-KGDH activity.

PDH complex activity is inhibited by the PDKs. To date, there are four isoforms of PDK identified, and they are expressed and regulated in a tissue-specific manner. PDK4 activity is increased in muscle by insulin deficiency and increased fatty acids during starvation and chemically induced diabetes (23, 31, 35, 53). Prolonged elevations in fatty acids induce PDK4 activity, possibly via activation of peroxisome proliferator-activated receptor-α (23). Whereas NEFAs are significantly elevated in IUGR before the onset of diabetes, this is the likely mechanism underlying increased PDK4 expression in muscle of prediabetic IUGR rats. In contrast, IUGR rats with diabetes no longer have elevated plasma concentrations of NEFAs, yet PDK4 expression is markedly increased, suggesting an alter-
native mechanism responsible for inducing PDK4 expression. We speculate that expression and activity of PDK4 in diabetic IUGR rats are related to increased branched-chain amino acid availability.

In humans and animals with type 2 diabetes, branched-chain amino acids are elevated (6, 16, 42, 51). After transamination, the α-keto acids derived from leucine, isoleucine, and valine undergo oxidative decarboxylation in reactions catalyzed by branched-chain amino acid dehydrogenase, which has a structure and mechanism of catalysis similar to that of PDH and α-KGDH. Subsequent metabolism of these three α-keto acids occurs through a series of CoA derivatives and terminates in the formation of acetyl-, acetoacetyl-, and propionyl-CoA, thereby limiting CoASH availability to PDH and increasing intramitochondrial acetyl-CoA/CoASH and NADH/NAD⁺ ratios. Enhanced oxidation of branched-chain amino acids would be expected to increase expression and activity of PDK4, as was observed, and so directly inhibit PDH activity in skeletal muscle. We propose this novel mode of inhibition to be the likely mechanism responsible for decreased PDH activity observed in diabetic IUGR animals. This proposition is supported by the observations of Krebs et al. (26), showing that elevated serum amino acids induced insulin resistance by impairing muscle glucose transport/phosphorylation, resulting in markedly decreased glycogen synthesis.

The observations reported here for the IUGR rat suggest that peripheral insulin resistance at the level of skeletal muscle is due per se to the altered intrauterine milieu of uteroplacental insufficiency. Our data demonstrate the effect of compromised oxidative phosphorylation in impairing the function of skeletal muscle in uptake of serum glucose and its conversion into glycogen stores, major contributing factors to the metabolic disorders associated with the onset and progression of type 2 diabetes.

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