Impaired oxidative phosphorylation in hepatic mitochondria in growth-retarded rats

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Peterside, Iyalla E., Mary A. Selak, and Rebecca A. Simmons. Impaired oxidative phosphorylation in hepatic mitochondria in growth-retarded rats. *Am J Physiol Endocrinol Metab* 285: E1258–E1266, 2003; 10.1152/ajpendo.00437.2002.—Intrauterine growth retardation (IUGR) has been linked to the development of type 2 diabetes in adulthood. We have developed an IUGR model in the rat whereby the animals develop diabetes between 3 and 6 mo of age that is associated with insulin resistance. Alterations in hepatic glucose metabolism are known to contribute to the hyperglycemia of diabetes; however, the mechanisms underlying this phenomenon have not been fully explained. To address this issue, intact liver mitochondria were isolated from IUGR and control offspring at different ages to examine the nature and time course of possible defects in oxidative metabolism. Phosphoenolpyruvate carboxykinase (PEPCK) expression was also measured in livers of IUGR and control offspring. Rates of ADP-stimulated (state 3) oxygen consumption were increased for succinate in the fetus and for α-ketoglutarate and glutamate at day 1, reflecting possible compensatory mitochondrial adaptations to acute hypoxia and acidosis in IUGR rats. By day 14, oxidation of glutamate and α-ketoglutarate had returned to normal, and by day 28, oxidation rates of pyruvate, glutamate, succinate, and α-ketoglutarate were significantly lower than those of controls. Rotenone-sensitive NADH-O_{2} oxidoreductase activity was similar in control and IUGR mitochondria at all ages, showing that the defect responsible for decreased pyruvate, glutamate, and α-ketoglutarate oxidation in IUGR liver precedes the electron transport chain and involves pyruvate and α-ketoglutarate dehydrogenases. Increased levels of manganese superoxide dismutase suggest that an antioxidant response has been mounted, and hydroxynonenal (HNE) modification of pyruvate dehydrogenase E2- (catalytic) and E3-binding protein subunits suggests that HNE-induced inactivation of this key enzyme may play a role in the mechanism of injury. The level of PEPCK mRNA was increased 250% in day 28 IUGR liver, indicating altered gene expression of the gluconeogenic enzyme that precedes overt hyperglycemia. These results indicate that uteroplacental insufficiency impairs mitochondrial oxidative phosphorylation in the liver and that this derangement predisposes the IUGR rat to increased hepatic glucose production by suppressing pyruvate oxidation and increasing gluconeogenesis. Liver; growth retardation; diabetes; gluconeogenesis

SEVERAL EPIDEMIOLOGICAL STUDIES show an increased incidence of type 2 diabetes in individuals who were growth retarded at birth (3, 4, 25, 43, 45, 54). The mechanisms by which an abnormal intrauterine milieu leads to the development of diabetes in adulthood are not known. To examine such mechanisms, we have developed a rat model of fetal growth retardation due to uteroplacental insufficiency that is induced by bilateral uterine artery ligation at 19 days of gestation (term is 22 days) (38, 39, 48, 53). 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. Intrauterine growth-retarded (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 (49) and impaired insulin suppression of hepatic glucose output (56). Basal hepatic glucose production is also increased (56).

In type 2 diabetes, excessive hepatic glucose production is a major contributor to both fasting hyperglycemia and exaggerated postprandial hyperglycemia (8, 12, 14, 59). A number of studies indicate that increased gluconeogenesis is responsible for the majority of excess hepatic glucose output in subjects with type 2 diabetes (13, 23, 42, 44). Elevated levels of phosphoenolpyruvate carboxykinase (PEPCK) in diabetes raise the possibility that increased PEPCK activity contributes to changes in the ability of insulin to regulate hepatic glucose production. Although PEPCK plays a crucial role in the control of gluconeogenesis, the interplay with other enzymes, such as pyruvate carboxylase and pyruvate dehydrogenase (PDH), is also critical (6, 21, 24, 27, 47). A decrease in the activity of PDH would be expected to favor gluconeogenesis by diverting pyruvate away from oxidation toward carboxylation with formation of oxaloacetate. Thus alterations in oxidation of key mitochondrial substrates, in particular pyruvate, may play a vital role in the control of gluconeogenic flux, although impaired mitochondrial function distal to PDH could compound realignment of hepatic metabolic function in the IUGR rat.

In previous studies (35), we found that hepatic redox state and expression of key mitochondrial enzymes were altered in IUGR animals. In light of these findings, and given the crucial role of mitochondria in...
Impaired oxidative phosphorylation in IUGR liver

Reagents. Reagent grade chemicals, porcine heart PDH, and mammalian protease inhibitor cocktail were obtained from Sigma Chemical (St. Louis, MO). Cell lysis buffer was purchased from Cell Signaling Technology (Beverly, MA). Rabbit anti-manganese superoxide dismutase (MnSOD) polyclonal antibody was obtained from StressGen (Victoria, BC, Canada), and mouse anti-pyruvate dehydrogenase E2/E3-binding protein subcomplex monoclonal antibody was purchased from Molecular Probes (Eugene, OR). Rabbit anti-hydroxyxynonalen (anti-HNE) polyclonal antibody was generously donated by Dr. Koji Uchida, of Nagoya University (Nagoya, Japan), and goat anti-rabbit conjugated to horseradish peroxidase was from Bio-Rad Laboratories (Hercules, CA). Sheep anti-mouse antibody conjugated to horseradish peroxidase, and Protein G Sepharose, were purchased from Amersham Pharmacia (Piscataway, NJ).

Animal model. Our surgical methods have previously been described (38, 39, 48, 49). Briefly, time-dated Sprague-Dawley pregnant rats were individually housed under standard conditions and allowed free access to standard rat chow and water. On day 19 of gestation (term is 22 days), the maternal rats were anesthetized with intraperitoneal xylazine (8 mg/kg), and ketamine (40 mg/kg), and both uterine arteries were ligated (IUGR). Rats recovered within a few hours and had ad libitum access to food and water. On day 21, fetuses were delivered by cesarean section after appropriate anesthesia. The other animals were allowed to deliver spontaneously, and litters were culled to eight at birth to ensure uniformity of litter size between IUGR and control litters. Subsequently, liver was harvested at days 1, 14, and 28 of life for experiments. These ages were chosen so that the evolution of the disease state could be examined during postnatal development. Previous studies in IUGR liver showed time-dependent changes in expression of key bioenergetic enzymes at these ages (35).

These studies were approved by the Animal Care Committee of the Children’s Hospital of Philadelphia and the University of Pennsylvania.

Isolation of liver mitochondria. The isolation buffer (H buffer) was composed (in mM) of 220 mannitol, 70 sucrose, 5 HEPES, pH 7.2, and 1 EGTA. All operations and buffers were at 2–4°C. Liver was surgically excised, placed in H buffer, rinsed, weighed, and minced. Minced liver was suspended in H buffer supplemented with 0.5% wt/vol fatty acid-free bovine serum albumin (BSA) and homogenized using a motor-driven Teflon pestle. The homogenate was centrifuged for 10 min at 1,500 g, and the supernatant was re-centrifuged for 10 min at 12,000 g. The mitochondrial pellet was washed one time with H buffer and collected by centrifugation at 10,000 g for 10 min. The final washed mitochondrial pellet was dispersed with a cold finger, resuspended in a small volume of H buffer, and used immediately for polarographic measurements.

Polarographic measurement of oxygen consumption. Oxygen consumption of liver mitochondria was measured polarographically using an Instech (Plymouth Meeting, PA) oxygen electrode in a magnetically stirred, thermostatically regulated chamber (30°C). Approximately 1 mg of mitochondrial protein was suspended in a total volume of 0.6 ml of air-saturated buffer composed in mM of 220 mannitol, 70 sucrose, 5 HEPES, pH 7.2, 1 EGTA, and 5 K$_2$HPO$_4$. After a basal rate of respiration with appropriate substrate(s) was recorded, two or three consecutive additions of 0.25 mM ADP were made. Oxygen consumption rate was measured for each of the following substrate(s): 10 mM pyruvate, 10 mM glucose, 10 mM α-ketoglutarate, each with 2 mM malate, 10 mM succinate + 5 μM rotenone, and 10 mM α-glycerophosphate. Oxidation of carnitine esters was measured in the presence of 1 mM malate with 2 mM acetylcyarnitine or 0.2 mM palmitoylcarnitine followed by addition of ADP. Cytochrome oxidase activity was measured using 6 mM ascorbate, 0.6 mM N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD), and 1 mM EDTA after recording and correcting for a basal rate of oxygen consumption in the presence of 50 μM horse heart cytochrome c. Rotenone-sensitive NADH-O$_2$ oxidoreductase activity was measured in the presence of 1 mM NADH and 50 μM horse heart cytochrome c after mitochondrial, diluted in 25 mM phosphate buffer, pH 7.2, were disrupted by three freeze-thaw cycles (5).

Rates of substrate oxidation with or without ADP were expressed as nanomoles of oxygen consumed per minute per milligram mitochondrial protein. Mean rates of oxygen consumption for each substrate were calculated for two or three consecutive additions of ADP. Respiratory control ratios (RCR) were calculated as the ratios of the rate of oxygen uptake in the presence of added ADP (state 3) to the rate observed when added ADP had been completely phosphorylated to ATP (state 4). ADP-to-O$_2$ ratios were calculated from the concentration of oxygen consumed during state 3 respiration and the concentration of ADP added (11, 20, 36).

PEPCK mRNA. Total RNA was extracted from liver using RNAzol B (Tel-Test, Friendswood, TX). RT-PCR with an internal standard was used to quantify levels of mRNA (34).

Immunoprecipitation. Porcine heart PDH and lysates of isolated liver mitochondria from day 28 control and IUGR rats were subjected to immunoprecipitation using PDH E2/E3-binding protein subcomplex antibody. Lysates were prepared using cell lysis buffer supplemented with 10% vol/vol glycerol and protease inhibitor cocktail. Immunocomplexes were collected with Protein G-Sepharose, washed, and eluted with Laemmli sample buffer, and aliquots were separately immunoblotted using antibodies directed against PDH E2/E3-binding protein subcomplex (1:1,100) and HNE (1:5,000).

Western blot analysis. All liver mitochondrial samples were subjected to reducing SDS-PAGE by use of 12% Tris-
significantly reduced in IUGR fetuses (38). However, at 28 days of life, body and liver weights did not differ from those of controls. Previous studies have reported that glucose, insulin, and glucagon levels are significantly reduced in IUGR fetuses (38). However, these values normalize within 24 h of the bilateral uterine artery ligation, and glucose, insulin, nonesterified fatty acid (NEFA), and glucagon levels were similar between IUGR and control pups at days 1, 14, and 28 (Table 1). Glycogen content did not vary between IUGR and control pups after birth (data not shown).

**Mitochondrial yield.** Despite a decrease in fetal liver weight, we found mitochondrial yield (mg mitochondrial protein/g liver wet weight) in the IUGR liver to be 200% of control. However, by day 28, mitochondrial yield was similar between IUGR animals and controls (Table 2).

### Table 1. Metabolic profile of control and IUGR rats

<table>
<thead>
<tr>
<th></th>
<th>Fetus</th>
<th>Day 1</th>
<th>Day 14</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body weight, g</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>IUGR</td>
<td>2.72 ± 0.21*</td>
<td>5.32 ± 0.8*</td>
<td>29.7 ± 2.7</td>
<td>89.2 ± 16.1</td>
</tr>
<tr>
<td>Control</td>
<td>3.58 ± 1.32</td>
<td>6.64 ± 1.5</td>
<td>32.3 ± 5.4</td>
<td>85.3 ± 12.2</td>
</tr>
<tr>
<td><strong>Liver weight, g</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IUGR</td>
<td>0.211 ± 0.002*</td>
<td>0.344 ± 0.002</td>
<td>1.17 ± 0.22</td>
<td>3.94 ± 0.14</td>
</tr>
<tr>
<td>Control</td>
<td>0.305 ± 0.002</td>
<td>0.342 ± 0.002</td>
<td>1.33 ± 0.16</td>
<td>4.12 ± 0.41</td>
</tr>
<tr>
<td><strong>Glucose, mg/dl</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>IUGR</td>
<td>33.1 ± 4.1*</td>
<td>99.2 ± 6.9</td>
<td>96.4 ± 9.8</td>
<td>102.2 ± 15.2</td>
</tr>
<tr>
<td>Control</td>
<td>89.2 ± 6.5</td>
<td>102.3 ± 12.5</td>
<td>97.2 ± 8.9</td>
<td>109.3 ± 13.2</td>
</tr>
<tr>
<td><strong>Insulin, µU/ml</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IUGR</td>
<td>95 ± 10*</td>
<td>79 ± 15</td>
<td>45 ± 6</td>
<td>42 ± 7</td>
</tr>
<tr>
<td>Control</td>
<td>245 ± 32</td>
<td>68 ± 19</td>
<td>52 ± 9</td>
<td>38 ± 6</td>
</tr>
<tr>
<td><strong>Glucagon, pg/ml</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IUGR</td>
<td>455 ± 78*</td>
<td>345 ± 35</td>
<td>410 ± 32</td>
<td>308 ± 54</td>
</tr>
<tr>
<td>Control</td>
<td>210 ± 21</td>
<td>390 ± 45</td>
<td>390 ± 67</td>
<td>359 ± 65</td>
</tr>
<tr>
<td><strong>NEFA, meq/l</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IUGR</td>
<td>ND</td>
<td>ND</td>
<td>0.22 ± 0.04</td>
<td>0.24 ± 0.04</td>
</tr>
<tr>
<td>Control</td>
<td>ND</td>
<td>ND</td>
<td>0.21 ± 0.05</td>
<td>0.30 ± 0.05</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 7. IUGR, intrauterine growth retarded; NEFA, nonesterified fatty acids; ND, not determined. *P < 0.05, IUGR vs. control.

**Mitochondrial substrate oxidation rates and phosphorylating capacities.** By measuring rates of ADP-stimulated oxygen consumption with various substrates that are oxidized by different dehydrogenases in different pathways and that donate reducing equivalents to different complexes of the electron transport chain, defects that may account for mitochondrial dysfunction can be dissected. Comparative rates of state 3 oxygen consumption of IUGR and control liver mitochondria with different substrates are presented in Fig. 1. State 3 refers to oxygen consumption stimulated by a limiting amount of ADP, and state 4 refers to oxygen consumption after phosphorylation of the added ADP to ATP. The rate of state 3 oxygen consumption in IUGR liver mitochondria was initially increased for succinate in the fetus and for α-ketoglutarate and glutamate at day 1. However, by day 14, oxidation of α-ketoglutarate and glutamate had returned to control levels, and oxidation of succinate was significantly less than control values (Fig. 1A). By day 28, oxidation rates for pyruvate, glutamate, α-ketoglutarate, and succinate were significantly lower than those of controls (Fig. 1A).

There was no significant difference observed for the oxidation of acetyl carnitine and palmitoyl carnitine between control and IUGR mitochondria at day 14 or day 28 (Fig. 1B). Oxidation of α-glycerophosphate was significantly higher in IUGR pups only at day 28.

### Table 2. Mitochondrial yield

<table>
<thead>
<tr>
<th>Age</th>
<th>Control</th>
<th>IUGR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetal (n = 6)</td>
<td>5.2 ± 0.6</td>
<td>9.7 ± 1.2*</td>
</tr>
<tr>
<td>Day 1 (n = 4)</td>
<td>9.2 ± 2.2</td>
<td>10.5 ± 2.0*</td>
</tr>
<tr>
<td>Day 14 (n = 7)</td>
<td>12.6 ± 1.5</td>
<td>17.0 ± 2.5*</td>
</tr>
<tr>
<td>Day 28 (n = 7)</td>
<td>6.0 ± 1.2</td>
<td>6.0 ± 1.5</td>
</tr>
</tbody>
</table>

*P < 0.05 vs. controls. Values are means ± SD; n = nos. of animals/treatment group. Mitochondrial yield is expressed as mg of mitochondrial protein/liver wet weight.
ever, by day 28, there was no difference between IUGR pups and controls. There was insufficient tissue to determine rates of oxidation of these substrates in mitochondria from fetus and day 1 pups.

To determine whether other factors might also contribute to decreased substrate oxidation in IUGR liver, additional parameters reflective of specific mitochondrial functions were also examined. These included proton permeability, efficiency of oxidative phosphorylation, respiratory control, adenine nucleotide transport, and electron transport chain activity. The rates of state 4 oxygen consumption (Table 3) and ADP/O ratios were similar in control and IUGR mitochondria with all substrates. The degree of coupling between substrate oxidation and ADP phosphorylation is best described by the RCR, the rate of oxygen consumption in the presence of ADP divided by the rate when ADP has been exhausted. RCR values were high for all substrates and did not differ between IUGR and controls (Table 3). Rotenone-sensitive NADH-O₂ oxidoreductase activity, measured in disrupted mitochondria in the presence of exogenous NADH, was comparable in control and IUGR mitochondria (Table 4). Cytochrome oxidase (complex IV) measured with TMPD/ascorbate was also similar in control and IUGR mitochondria (Table 4).

Assessment of oxidative stress in rat liver mitochondria. IUGR is associated with increased oxidative stress in the human fetus (30, 32). Both acute and chronic production of reactive oxygen species (ROS) are often associated with large increases in the level of

| Table 3. Rates of state 4 oxygen consumption and respiratory control ratios of control and IUGR mitochondria |
|-------------------------------|-----------------|-----------------|
| **Age** | **Substrate** | **Control** | **IUGR** |
| **Oxygen consumption** | | | |
| Day 1 (n = 4) | Pyruvate + malate | 10.0 ± 2.6 | 12.5 ± 1.5 |
| | Glutamate + malate | 20.0 ± 8.0 | 15.5 ± 0.5 |
| | α-Ketoglutarate + malate | 15.0 ± 1.0 | 19.7 ± 3.5 |
| | Succinate + rotenone | 37.0 ± 1.1 | 37.0 ± 1.0 |
| Day 14 (n = 7) | Pyruvate + malate | 13.5 ± 1.6 | 14.9 ± 1.5 |
| | Glutamate + malate | 15.4 ± 1.4 | 16.7 ± 2.2 |
| | α-Ketoglutarate + malate | 15.3 ± 1.9 | 18.1 ± 1.5 |
| | Succinate + rotenone | 31.8 ± 4.7 | 34.9 ± 3.3 |
| Day 28 (n = 7) | Pyruvate + malate | 20.9 ± 6.7 | 14.6 ± 4.9 |
| | Glutamate + malate | 14.7 ± 2.7 | 12.3 ± 3.4 |
| | α-Ketoglutarate + malate | 10.1 ± 0.9 | 10.1 ± 1.1 |
| | Succinate + rotenone | 31.7 ± 9.7 | 30.8 ± 11.9 |
| **RCR ratios** | | | |
| Day 1 (n = 4) | Pyruvate + malate | 2.7 | 2.1 |
| | Glutamate + malate | 3.0 | 4.0 |
| | α-Ketoglutarate + malate | 2.7 | 2.9 |
| | Succinate + rotenone | 2.1 | 2.2 |
| Day 14 (n = 7) | Pyruvate + malate | 3.6 | 3.0 |
| | Glutamate + malate | 4.7 | 4.4 |
| | α-Ketoglutarate + malate | 4.2 | 3.4 |
| | Succinate + rotenone | 3.6 | 2.8 |
| Day 28 (n = 7) | Pyruvate + malate | 3.0 | 3.0 |
| | Glutamate + malate | 5.0 | 4.8 |
| | α-Ketoglutarate + malate | 5.0 | 4.5 |
| | Succinate + rotenone | 4.0 | 3.9 |

Values of state 4 oxygen consumption are means ± SE expressed in nA of O₂· min⁻¹·mg mitochondrial protein⁻¹. Respiratory control ratios (RCR) were calculated from mean rates of state 3 and state 4 oxygen consumption; n, nos. of animals for each treatment group.
Table 4. Enzymatic activity in rat liver mitochondria of control and IUGR animals

<table>
<thead>
<tr>
<th>Age</th>
<th>Control</th>
<th>IUGR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rotenone-sensitive NADH-O2 oxidoreductase</td>
<td></td>
</tr>
<tr>
<td>Fetal (n = 6)</td>
<td>121 ± 123</td>
<td>113 ± 9</td>
</tr>
<tr>
<td>Day 1 (n = 4)</td>
<td>244 ± 35</td>
<td>191 ± 22</td>
</tr>
<tr>
<td>Day 14 (n = 7)</td>
<td>279 ± 43</td>
<td>244 ± 36</td>
</tr>
<tr>
<td>Day 28 (n = 7)</td>
<td>289 ± 51</td>
<td>278 ± 44</td>
</tr>
<tr>
<td></td>
<td>Cytochrome oxidase</td>
<td></td>
</tr>
<tr>
<td>Fetal (n = 6)</td>
<td>471 ± 48</td>
<td>377 ± 75</td>
</tr>
<tr>
<td>Day 1 (n = 4)</td>
<td>226 ± 25</td>
<td>244 ± 20</td>
</tr>
<tr>
<td>Day 14 (n = 7)</td>
<td>174 ± 21</td>
<td>170 ± 20</td>
</tr>
<tr>
<td>Day 28 (n = 7)</td>
<td>255 ± 6</td>
<td>214 ± 33</td>
</tr>
</tbody>
</table>

Values are means ± SE expressed in nA O2·min⁻¹·mg mitochondrial protein⁻¹. Activity of cytochrome oxidase was measured in intact mitochondria with TMPD/ascorbate; NADH oxidase was measured in hypotonically-swollen, freeze-thawed mitochondria in the presence of exogenous NADH.

MnSOD protein, suggesting that mitochondria have mounted antioxidant defenses (19). Western blot analysis of liver mitochondrial protein showed that the amount of MnSOD protein was significantly higher in IUGR than in control animals at all ages (Fig. 2A).

Oxidative stress can also lead to the formation of the highly reactive aldehyde trans-4-hydroxy-2-nonenal (HNE) that is produced when 6,6-polyunsaturated fatty acids undergo free radical-mediated peroxidation (16, 51, 52). HNE can react with the sulfhydryl group of cysteine, imidazole moiety of histidine, ε-amino group of lysine, and reduced lipoyl moieties of proteins, the latter including PDH and α-ketoglutarate dehydrogenase (α-KGDH), to form covalently modified proteins with altered function. Levels of a 35-kDa HNE-modified protein, determined by Western blot analysis, were significantly increased in IUGRs and ranged between 1.5- and 2-fold higher than in controls (Fig. 2B; P < 0.05).

Pyruvate oxidation was significantly decreased in IUGR liver at day 28, suggesting that PDH might have undergone HNE modification and inactivation due to oxidative damage. To determine whether this was true, PDH subunits were immunoprecipitated from mitochondrial lysates by using an anti-PDH E2/E3-binding protein antibody. The E2/E3-binding protein antibody immunoprecipitated three proteins that were visible when immunoblotted and probed with the same antibody: two strongly reacting proteins with molecular masses corresponding to PDH E3-(72 kDa) and E3-binding protein (55 kDa) subunits and an E2 subunit band (35 kDa). When the proteins immunoprecipitated with E2/E3-binding protein antibody were probed with an anti-HNE antibody, HNE modification was observed in the PDH E2- and E3-binding protein subunits. The E2- and E3-binding protein subunits of PDH showed a level of HNE modification that was twofold higher in day 28 IUGR mitochondria than in corresponding control mitochondria (HNE modification of E3-binding protein is shown in Fig. 3).

To determine whether decreased pyruvate oxidation was associated with concomitant changes in PEPCK expression, mRNA levels were measured by real-time PCR in day 28 IUGR liver (n = 7) and were 250% those of controls (150.1 ± 12.7 vs. 59.2 ± 4.9 arbitrary units, P < 0.05).

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. IUGR animals display insulin resistance early in life, before the onset of hyperglycemia. Whole body glucose disposal in response to insulin is markedly attenuated (49), and hepatic glucose production is significantly increased in IUGR animals (56). Our studies demonstrate that the mechanisms responsible for altered hepatic glucose metabolism in IUGR rats are due to impaired oxidation of pyruvate and a marked increase in PEPCK expression. These changes occurred early in life, before the onset of diabetes, suggesting that abnormal hepatic glucose metabolism represents an early
defect that contributes to the eventual onset of fasting hyperglycemia. It is plausible, therefore, to speculate that a defect that contributes to the eventual onset of fasting hyperglycemia occurs beyond a certain threshold, peripheral glucose disposal decreases, or any combination of the above.

The partitioning of pyruvate between decarboxylation to form acetyl-CoA by PDH and carboxylation to form oxaloacetate (OAA) by pyruvate carboxylase (PC) places pyruvate at the crucial branch point between two major metabolic fates: oxidation to generate ATP and conversion to substrates for biosynthesis. The exclusive intracellular location of both PDH and PC makes the mitochondrial matrix the primary location for pyruvate to cytosolic phosphoenolpyruvate for gluconeogenesis. The conversion of pyruvate to OAA by PC is allosterically activated by acetyl-CoA, which may be provided by fatty acid or pyruvate oxidation. Decreased oxidation of pyruvate by PDH would influence gluconeogenic flux by increasing substrate availability for PC, and increased levels of cytosolic PEPCK would promote conversion of OAA to phosphoenolpyruvate for production of glucose. Therefore, the combined effects of decreased pyruvate oxidation, increased pyruvate carboxylation, and increased PEPCK would favor increased gluconeogenesis in IUGR liver. Once the IUGR animal is no longer able to compensate, fasting hyperglycemia occurs.

Although recent studies have demonstrated that overexpression of PEPCK alone can increase hepatic glucose production (50, 55), other data suggest that gluconeogenesis is regulated by activities of multiple enzymes in combination with PEPCK (6, 24). Indeed, the regulation of flux between the multiple pathways involved in glucose metabolism in the liver plays a crucial role in maintaining metabolic homeostasis.

Oxidation of pyruvate, glutamate, α-ketoglutarate, and succinate was decreased in isolated day 28 IUGR liver mitochondria under state 3 conditions in the presence of saturating substrate, suggesting that there is an underlying defect in oxidative phosphorylation in this tissue. Therefore, to determine what mechanisms are responsible for impaired substrate oxidation, mitochondrial respiratory and phosphorylating activities were examined in detail. Rates of state 4 oxygen consumption were similar in control and IUGR mitochondria for all substrates, indicating that endogenous proton conductance of the inner mitochondrial membrane was not increased in IUGR liver. This conclusion is supported by the observation that ADP/O₂ ratios were not decreased, showing that the efficiency of energy coupling was not adversely affected in IUGR mitochondria. RCR values were high for all substrates, demonstrating that IUGR liver mitochondria were well coupled and showed high physical and functional integrity. High RCR values additionally denote that the adenine nucleotide and phosphate translocases were not impaired in IUGR liver mitochondria.

To determine whether the observed decreases in substrate oxidation in IUGR liver mitochondria result from a defect that precedes or resides within the electron transport chain, rotenone-sensitive NADH-O₂ oxidoreductase activity was measured in disrupted mitochondria in the presence of excess exogenous NADH to render the electron transport chain independent of substrate-generated NADH. NADH-O₂ oxidoreductase activity thus measures the maximal capacity of and electron flow through the electron transport chain (complexes I, III, and IV). NADH-O₂ oxidoreductase was similar in control and IUGR liver at all ages, indicating that substrate oxidation in day 28 liver mitochondria was restricted at a site proximal to the respiratory chain. Oxidation of α-glycerophosphate was normal, as was the activity of cytochrome oxidase, confirming that respiratory chain complexes III and IV were fully functional. Collectively, these data clearly demonstrate that state 3 oxygen consumption in isolated IUGR liver mitochondria is limited at the level of substrate dehydrogenases.

Impaired oxidation of substrates was not likely due to increased fatty acid oxidation or elevated levels of NEFAs. Palmitoyl carnitine oxidation in IUGR mitochondria was similar to that of controls, showing that the acylcarnitine/carnitine transporter carnitine palmitoyltransferase II and β-oxidation are unchanged. However, because fatty acid oxidation was measured in vitro, it cannot be completely ruled out that in vivo fatty acid oxidation is altered in IUGR liver. Normal oxidation rates of acetyl carnitine demonstrate further that the defect in IUGR mitochondria is not associated with enzymes of the first third of the tricarboxylic acid cycle, i.e., citrate synthase, aconitase, and isocitrate dehydrogenase. Increased serum free fatty acids have been implicated as causative factors in excess hepatic glucose production in type 2 diabetes. Oxidation of fatty acyl-CoAs may increase intramitochondrial acetyl-CoA/CoASH and NADH/NAD⁺ ratios and inhibit PDH by allosteric mechanisms and by promoting phosphorylation of the E1 subunit. However, although β-oxidation of fatty acids was not measured directly in vivo in IUGR animals, our data suggest that an alternate mechanism to the one involving fatty acids is responsible for decreasing pyruvate oxidation, with consequent increased glucose production.

**Fig. 3.** Western blot analyses of total and HNE-modified pyruvate dehydrogenase (PDH) immunoprecipitated from rat liver mitochondria isolated from IUGR (I) and control (C) rats. Mitochondria were immunoprecipitated with PDH E2/E3 antibody and then probed with anti-HNE antibody or with PDH E2/E3 antibody.
Levels of MnSOD are particularly elevated in IUGR fetuses and day 1 newborns. This is not surprising, as uterine artery ligation induces ischemia that would be associated with high levels of oxidative stress. As the IUGR animal recovers, oxidative stress decreases. However, our finding that MnSOD levels are still markedly higher in IUGR rats compared with controls at day 14 suggests that oxidative stress is ongoing. Furthermore, at day 29, MnSOD levels are higher than at day 14, suggesting that oxidative stress is slowly increasing. Increased MnSOD suggests that IUGR mitochondria have mounted an anti-oxidant response to increased reactive oxygen species (ROS). The severe oxidative stress that occurs in the fetus appears to be both transient and largely counterbalanced by upregulation of at least one ROS-scavenging enzyme (Mn-SOD), such that PDH is not subjected to oxidative modification during this interval, as judged by normal rates of pyruvate oxidation. However, this initial stress in the IUGR fetus destabilizes the mitochondrion and sets in motion a cycle of gradually escalating and sustained stress, which ultimately renders PDH susceptible to oxidative damage by day 28. This supports our hypothesis that oxidative stress is a self-perpetuating process whereby overproduction of ROS elicits mitochondrial dysfunction, inducing further production of ROS, and creating a vicious cycle.

Generation of superoxide anion, hydrogen peroxide, and hydroxyl radicals damages mitochondrial and cellular proteins, nucleic acids, and lipids, and so impairs energy metabolism. Specifically, the iron-sulfur centers in aconitase and electron transport chain complexes I, II, and III, and the activity of α-KGDH and PDH, have been reported to be sensitive to damage by ROS (22, 33, 57). IUGR caused by uteroplacental insufficiency results in decreased placental blood flow, fetal hypoxemia, and acidosis. All of these, singly or in combination, have the capacity to stimulate the production of oxygen-derived free radicals in various organs (1, 31). Thus our finding of oxidative stress, as demonstrated by increased levels of MnSOD and HNE-modified proteins, specifically PDH, in IUGR liver, indicates that this is the most likely mechanism responsible for decreased activity of PDH.

PDH and α-KGDH are structurally and catalytically similar. Both dehydrogenases are comprised of an α-keto dehydrogenase (E1), a dihydrolipoyl transacetylase (E2), and a dihydrolipoyl dehydrogenase (E3). PDH also has an E3-binding protein. Although we have examined only PDH and found that E2 catalytic and E3-binding protein subunits undergo HNE modification in day 28 IUGR liver mitochondria, it is likely that α-KGDH may also be susceptible to inactivation by HNE. HNE modification of lipoic acid moieties in PDH and α-KGDH, with concomitant loss of enzymatic activity, has been observed by other investigators (28, 29, 33). We speculate that lipoic acid and/or cysteine, lysine, and histidine residues in these key dehydrogenases undergo HNE modification in IUGR liver mitochondria, providing a molecular mechanism for the observed decreases in substrate oxidation.

The mechanisms underlying the increase in PEPCK in IUGR animals are less clear. PEPCK expression is not turned on until after birth at the time of the first feed. PEPCK gene transcription is increased by glucocorticoids and glucagon and is negatively and dominantly regulated by insulin. Levels of these hormones are altered in the IUGR fetus. Insulin concentrations are low, and corticosterone and glucagon levels are elevated (38). However, within a few hours of birth, levels of all three hormones normalize. Therefore, even though PEPCK transcription rates are exceedingly low in the fetus, it is possible that alterations in insulin, glucagon, and or corticosterone that occur during a critical window in fetal life result in a permanent increase in PEPCK gene transcription after birth.

Alternatively, PEPCK transcription may be altered by ROS. Of significance is the finding by a number of investigators (9, 31, 41) that HNE stimulates adenyl cyclase activity in a variety of cell types, including hepatocytes. cAMP is one of the most potent stimulators of PEPCK gene transcription. Because levels of HNE-modified proteins are elevated in liver of IUGR rats, it is conceivable that oxidative stress may be the underlying mechanism responsible for increased PEPCK expression.

PEPCK is also increased in nondiabetic adult offspring of a different model of IUGR (15). Protein deprivation during pregnancy in the rat induces a twofold increase in PEPCK mRNA levels in liver of adult offspring. This elevation in PEPCK is associated with increased basal hepatic production (7) and an impaired ability of insulin to suppress hepatic glucose output (15, 40). Although it is not known whether oxidative stress occurs in liver of adult offspring of protein-deprived dams, oxidative stress is present in the pancreas (37). Taurine, a ubiquitous sulfur-containing amino acid, is an antioxidant, and administration to the protein-deprived pregnant rat reduces oxidative stress in the pancreas of the offspring (37). Taurine administration also decreases oxidative stress in liver of aged rats (18). Thus these studies support our hypothesis that an abnormal intrauterine milieu associated with oxidative stress can permanently alter gene expression, leading to aberrant glucose homeostasis in adulthood.

Alterations in glucokinase (10, 46) and glycogen metabolism (17) have also been shown to increase hepatic glucose production in humans with type 2 diabetes and in animal models of type 2 diabetes. In contrast, we did not observe any changes in glucokinase activity or glycogen content in livers of 28-day-old IUGR animals, suggesting that changes in substrate oxidation/availability are likely to play a greater role in the eventual development of fasting hyperglycemia.

Interestingly, mitochondrial protein content, an index of mitochondrial number, was significantly higher in IUGR animals in the fetus and young pups (days 1–14). This likely represents a compensatory response to acute hypoxia and acidosis observed in other cell types and in our IUGR model (2, 26, 35). Our previous studies demonstrated increased expression of mito-
chondrial-encoded proteins and nuclear-encoded bioen-
ergetic enzymes (35).

In summary, our studies suggest that an aberrant
intraterane milieu permanently alters mitochondrial
oxidation of key substrates and function of vital he-
patic enzymes, such that gluconeogenesis is aug-
mented in the IUGR rat. These processes occur early in
life, before the onset of hyperglycemia, and indicate
that uteroplacental insufficiency causes a primary de-
fect in gene expression and mitochondrial metabolism
that leads to the eventual development of overt hyper-
glycemia.

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