Hepatic Insulin Resistance Precedes the Development of Diabetes in a Model of Intrauterine Growth Retardation

Patricia Vuguin, Elisabeth Raab, Bing Liu, Nir Barzilai, and Rebecca Simmons

Intrauterine growth retardation (IUGR) has been linked to the development of type 2 diabetes in adulthood. We developed an IUGR model in rats whereby at age 3–6 months the animals develop a diabetes that is associated with insulin resistance. Hyperinsulinemic-euglycemic clamp studies were performed at age 8 weeks, before the onset of obesity and diabetes. Basal hepatic glucose production (HGP) was significantly higher in IUGR than in control rats (14.6 ± 0.4 vs. 12.3 ± 0.3 mg · kg⁻¹ · min⁻¹; P < 0.05). Insulin suppression of HGP was blunted in IUGR versus control rats (10.4 ± 0.6 vs. 6.5 ± 1.0 mg · kg⁻¹ · min⁻¹; P < 0.01); however, rates of glucose uptake and glycogenolysis were similar between the two groups. Insulin-stimulated insulin receptor substrate 2 and Akt-2 phosphorylation were significantly blunted in IUGR rats. PEPCK and glucose-6-phosphatase mRNA levels were increased at least threefold in liver of IUGR compared with control rats. These studies suggest that an aberrant intrauterine milieu permanently impairs insulin signaling in the liver so that gluconeogenesis is augmented in the IUGR rat. These processes occur early in life, before the onset of hyperglycemia, and indicate that uteroplacental insufficiency causes a primary defect in gene expression and hepatic metabolism that leads to the eventual development of overt hyperglycemia. Diabetes 53:2617–2622, 2004

U teroplacental insufficiency limits the availability of substrates to the fetus and retards growth during gestation (1,2). We have previously shown that this abnormal metabolic intrauterine milieu affects the development of the fetus by modifying the gene expression and function of susceptible cells in the pancreas, muscle, and liver (3–5). The end result is the development of type 2 diabetes in adulthood (4). The unique feature of our animal model of intrauterine growth retardation (IUGR) is its ability to induce diabetes in adult rats with the salient features of most forms of type 2 diabetes in humans, that is, defects in insulin action and insulin secretion. IUGR animals exhibit insulin resistance early in life (before the onset of hyperglycemia) that is characterized by blunted whole-body glucose disposal in response to insulin (4).

The liver plays an important role in maintaining blood glucose homeostasis by controlling hepatic glucose production (HGP) (6,7). In type 2 diabetes, the high levels of HGP and the inability of insulin to adequately suppress hepatic glucose output are major contributors to both fasting hyperglycemia and exaggerated postprandial hyperglycemia (8–14).

Insulin resistance is associated with a postreceptor defect(s) in the intracellular insulin-signaling cascade, leading to the failure of insulin to suppress HGP (15). Insulin binds to its receptor, which leads to activation of the insulin receptor, insulin receptor substrates (IRs) such as IRS-1 and -2, phosphatidylinositol 3-kinase, and Akt. Akt activation leads to decreased transcription of PEPCK and glucose-6-phosphatase (G6Pase) (16), thus reducing glucose production in the liver (17–21).

It has not yet been determined whether increased HGP is a contributing factor to the onset of type 2 diabetes or whether altered hepatic glucose metabolism is secondary to the diabetic disease state. A distinct advantage of our IUGR model is that it gives us the ability to examine glucose homeostasis before the onset of hyperglycemia and hyperlipidemia. The aims of the present study were to determine whether in vivo hepatic glucose metabolism is altered and identify the mechanisms underlying hepatic insulin resistance in the young pre-diabetic IUGR rat.

RESEARCH DESIGN AND METHODS

Our surgical methods have been described previously (4). In brief, time-dated Sprague-Dawley (SD) pregnant rats were individually housed under standard conditions and allowed free access to standard rat diet 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. 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.

Male SD rats (Charles River Laboratories, Wilmington, MA) were studied during young adulthood (weight 250–300 g, n = 12) before developing obesity and diabetes. IUGR animals generally develop increased fat mass at age 7–9 weeks (2). Animals were housed in individual cages and subjected to a standard light (6:00 A.M. to 6:00 P.M.)/dark (6:00 P.M. to 6:00 A.M.) cycle. All rats were fed ad libitum using regular rat diet that consisted of 64% carbohydrate, 30% protein, and 6% fat, with a physiological fuel value of 3.3 kcal/g food. At 1 week before the in vivo study, rats were anesthetized by inhalation of methoxyflurane, and indwelling catheters were inserted in the right internal jugular vein and left carotid artery (22–26). This method of anesthesia allows fast recovery and normal food consumption after 1 day. The venous catheter extended to the level of the right atrium, and the arterial catheter was
advanced to the level of the aortic arch. These chronically catheterized rats were not studied until their body weight was within 3% of their preoperative weight (~4–6 days). All studies were performed after animals were fasted for ~6 h; at the time of the study, animals were awake and unstressed. Animals (IUGR and control) were matched by body weight and fat mass and studied at approximately age 7 weeks.

Body composition was assessed as described earlier (22–26). Briefly, rats received an intra-arterial bolus injection of 20 μCi of tritiated-labeled water (3H2O, Du Pont-NEN, Boston, MA), and plasma samples were obtained at 30-min intervals for 3 h. Steady-state conditions for plasma 3H2O-specific activity were achieved within 45 min in all studies. Plasma samples (n = 5) obtained between 1 and 3 h were used in the calculation of whole-body distribution space of water.

The study protocol was reviewed and approved by the Animal Care and Use Committee of the Albert Einstein College of Medicine and the Children’s Hospital of Philadelphia, University of Pennsylvania.

**Hyperinsulinemic-euglycemic clamp studies.** To mimic components of in vivo physiological postmeal conditions, the following protocols were designed: 1) a saline study, in which saline was infused into rats for 3 h, and 2) a hyperinsulinemic-euglycemic clamp study. Rats received a primed continuous insulin infusion (3 mU · kg⁻¹ · min⁻¹) to obtain physiological, postmeal insulin levels and a variable infusion of dextrose (25%), periodically adjusted to clamp the plasma glucose concentration at the basal level for the 2 h of the clamp (22–26). A primed, continuous infusion of high-performance liquid chromatography-purified [3H]-glucose (Du Pont-NEN; 15–45 μCi bolus, followed by 0.4 μCi/min infusion) was initiated at t = 0 and maintained for 4 h. At the end of the clamp study, rats were killed using 60 mg pentobarbital sodium/kg body wt. The abdomen was quickly opened, and adipose fat depots and liver samples were freeze clamped in situ with aluminum tongs precooled in liquid nitrogen (27).

**Immunoprecipitations and Western blotting.** In a separate group of IUGR (n = 5) and control (n = 5) animals, after ketamine (40 mg/kg) and xylazine anesthesia (40 mg/kg), 2 units/kg of insulin were injected into the portal vein. After 5 min, 0.5% EGTA, 200 μmol/l NaVO3, 1 mmol/l microcystin, and 10% glycerol containing protease inhibitors (10 μg/ml leupeptin, 10 μg/ml aprotinin, and 34.4 μg/ml 4-[2-aminoethyl][benzenesulfonyl] fluorophore) was added to 50 μg of frozen liver. Tissue was disrupted by sonication, mixed by inversion at 4°C, and pelleted at 14,000 rpm. Liver homogenates (500 mg) were incubated with either anti–IRS-2 or anti–Akt-2 overnight with protein A agarose beads. Immunoprecipitates were then collected and washed and subjected to reducing SDS-PAGE using 12% Tris-glycine gels. Proteins were electroblotted from the gels onto nitrocellulose (PVDF) membranes. The blots were then incubated with tyrosine-phosphorylated IRS-2 antibody (Upstate Biotechnol- ogy, Lake Placid, NY). The membranes were stripped and then reprobed for IRS-2 or Akt-2 antibody (Cell Signaling Technology, Beverly, MA) overnight with protein A agarose beads. After being washed, immune complexes were resolved on 10% SDS-PAGE and electroblotted onto PVDF membranes. Akt-2 phosphorylation was determined on Ser474, as described above. The membranes were stripped and reprobed with anti–Akt-2 antibody. Protein bands were detected using enzyme-catalyzed chemiluminescence mediated by horseradish peroxidase (Amersham Pharmacia Biotech, Piscataway, NJ). Images were analyzed and bands were quantified using MacBas version 2.4 software (FujiFilm Film, Tokyo, Japan). Protein phosphorylation was calculated as the ratio of phosphorylated to total protein expression.

**G6Pase and glucokinase activity.** G6Pase activity was assayed in intact microsomes prepared from liver 4 h after the insulin clamps (n = 5 for each group). Frozen liver was pulverized under liquid nitrogen and homogenized in 10 mmol/l HEPES and 0.25 mol/l sucrose (pH 7.4). Activity was assayed at glucose-6-phosphate concentrations of 1, 2.5, and 10 mmol/l.

G6Pase was determined by the method described by Bontempo et al. (28) with 100 mg of liver tissue. Liver was homogenized in 50 mmol/l triethanol- amine, 5 mmol/l MgCl2, 1 mmol/dithiothreitol, and 5 mmol/l EDTA (pH adjusted to 7.5). The homogenate was centrifuged at 10,000g at 4°C, and the supernatant was retained for the activity assay. The spectrophotometric assay of glucose-phosphorylating activity was performed at two glucose concentra- tions: 100 mmol/l (measures all hexokinases including glucokinase) and 0.5 mmol/l (measures only the low-Km hexokinases). The difference between the two assays gives glucokinase activity. 1 unit is the amount of enzyme that catalyzes the formation of 1 μmol of substrate per minute in the conditions of the assay.

**TABLE 1**

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<th>Body composition of IUGR and control SD rats</th>
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<td><strong>Body weight (g)</strong></td>
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<td><strong>Lean body mass (g)</strong></td>
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<td><strong>Visceral fat (g)</strong></td>
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<td><strong>Epididymal fat (g)</strong></td>
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<td><strong>Perinephric fat (g)</strong></td>
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<td><strong>Mesenteric fat (g)</strong></td>
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Data are means ± SE.

**PEPCK and G6Pase mRNA.** Total RNA was isolated from liver (n = 5 per group) using RNAzol B (Tel-Test, Friendswood, TX). Quantitative PCRs were carried out using equivalent dilutions of each cDNA sample, the fluorescent indicator SYBR green, the emipirically determined concentration of each primer, and the Applied Biosystems model 7700 sequence detector PCR machine (PerkinElmer Life Sciences, Boston, MA). To verify that only a single PCR product was generated for each amplified transcript, the multicomponent data for each sample was subsequently analyzed using the Dissociation Curves 1.0 program (PerkinElmer Life Sciences). To account for differences in starting material, quantitative PCR was also carried out for each cDNA sample using the Applied Biosystems human glyceraldehyde-3-phosphate dehydroge- nase (GAPDH) 20× primer and probe reagent (PerkinElmer Life Sciences). The relative abundance of the target was divided by the relative abundance of GAPDH in each sample to generate a normalized abundance. Each reaction was carried out in triplicate. Standard PCR conditions were used.

**Analytic procedures.** Plasma glucose was measured by the glucose oxidase method (Glucose Analyzer II; Beckman Instruments, Palo Alto, CA). Insulin, corticosterone, glucagon, and free fatty acid levels were obtained in the fasted and clamped state. Plasma insulin concentrations were measured in duplicate by radioimmunoassays using rat insulin and porcine as the standards (Linco, St. Louis, MO). Plasma glucagon and corticosterone concentrations were measured in duplicate by radioimmunoassay (Penn Diabetes Core at the University of Pennsylvania). Plasma nonesterified fatty acid (NEFA) concentra- tions were determined by an enzymatic method with an automated kit, according to the manufacturer’s specifications (Waco Pure Chemical Indus- tries, Osaka, Japan). Liver glycogen content was determined in perchloric acid extracts prepared from flash-frozen, pulverized liver by the method of Keppler and Decker (29).

Plasma [1H]glucose radioactivity was measured in duplicates in the super- natants of Ba(OH)2 (2) and ZnSO4 precipitates (Somogyi procedure) of plasma samples (20 μl) after they were evaporated to dryness to eliminate tritiated water. The HGP was calculated as the difference between the tracer-derived rate of appearance and the infusion rate of glucose. Regression analysis of the slopes of 3H2O rates of appearance (used in the calculation of the rates of glycolysis) was performed at 60-min intervals. The rate of glycolysis was estimated from the rate of conversion of [3H]glucose to 3H2O, as previously described (22,30–33). Because tritium on the C-3 position of glucose is lost to water during glycolysis, it can be assumed that plasma tritium is present in either [3H]water or plasma. Plasma-tritiated water—specific activity was determined by liquid scintillation counting of the protein-free supernatant (Somogyi filtrate) before and after samples were evaporated to dryness.

**Statistical analysis.** The significance of group differences was evaluated by the two-sample Student’s t test. Pearson’s correlation coefficients were calculated to estimate the linear relation between variables. All data were presented as means ± SE. P < 0.05 was considered significant. All statistical analyses were performed using SPSS software (version 9).

**TABLE 2**

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<th>Basal metabolic characteristics of IUGR and control SD rats</th>
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<td><strong>Glucose (mg/dl)</strong></td>
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<td><strong>Insulin (μU/ml)</strong></td>
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<td><strong>Glucagon (pg/ml)</strong></td>
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<td><strong>Corticosterone (ng/ml)</strong></td>
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<td><strong>NEFA (mEq/l)</strong></td>
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<td><strong>Glycogen (mg/g liver)</strong></td>
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Data are means ± SE. *P < 0.001 vs. control rats.
RESULTS

Metabolic profile. As previously reported (4), the birth weight of IUGR animals was significantly lower than that of controls until approximately age 7–9 weeks, when IUGR rats caught up to controls (Table 1). At the time of the study, body weight, lean body mass, total fat mass, and epididymal, perinephric, and mesenteric fat depots were similar between IUGR and control animals (Table 1). The week before the study and during the study week, IUGR and control animals had an equal daily caloric consumption (20 g · kg⁻¹ · day⁻¹). There were no significant differences in basal concentrations of glucose, corticosterone, NEFAs, or glucagon between IUGR and control rats (Table 2). Liver glycogen content also did not differ between IUGR and control rats (Table 2). However, as previously reported (4), fasting plasma concentrations of insulin were nearly twofold higher in IUGR than in control rats (P < 0.01).

HGP. The basal HGP was significantly higher in IUGR compared with control rats (14.6 ± 0.4 vs. 12.3 ± 0.3 mg · kg⁻¹ · min⁻¹; P < 0.05). During the insulin clamp studies, the steady-state plasma insulin levels were similarly increased to physiological postprandial levels in both groups (65 ± 8 vs. 64 ± 6 μU/ml in IUGR and control rats, respectively). Steady-state plasma glucose levels were also increased to physiological postprandial levels in both groups (148 ± 3.7 vs. 146 ± 3.0 mg/dl in IUGR and control rats, respectively). Steady-state plasma insulin levels were similarly increased in both groups. During physiological hyperinsulinemia, HGP was suppressed by 30% in IUGR compared with 50% in control animals (10.4 ± 0.6 vs. 6.5 ± 1.0 mg · kg⁻¹ · min⁻¹, P < 0.01). Furthermore, the glucose infusion rate (Fig. 2) required to maintain normoglycemia was 20% lower in IUGR than in control rats (11.1 ± 0.9 vs. 14.2 ± 1.3 mg · kg⁻¹ · min⁻¹, P < 0.05). This decrease in the rate of glucose infusion was largely accounted for by a lack of suppression of HGP.

Insulin-mediated glucose uptake, glycolysis, and glycogen synthesis. During physiological hyperinsulinemia, the rate of glucose uptake in IUGR rats was similar to that of control animals (20 ± 0.4 vs. 20 ± 1.0 mg · kg⁻¹ · min⁻¹). Furthermore, glycogen synthesis and glycolysis did not differ between the groups (data not shown).

Insulin-signaling proteins in liver. Basal levels of IRS-2 protein did not differ between IUGR and control rats. However, IRS-2 tyrosine phosphorylation was significantly decreased in IUGR liver (Fig. 3). Furthermore, after the administration of insulin, no increase in phosphorylated IRS-2 was seen in IUGR liver, whereas a two- to threefold increase in phosphorylated IRS-2 was observed in control liver (Fig. 3). Both Akt-2 and phosphorylated Ser⁴⁷⁴·Akt-2 protein levels were significantly decreased in liver of IUGR animals compared with controls (Fig. 3). After administration of insulin into the portal vein, phosphorylation at Ser⁴⁷⁴ of Akt-2 significantly increased in control liver; however, phosphorylation of Akt-2 in response to insulin was markedly blunted in IUGR rats compared with controls (Fig. 3).

PEPCK and G6Pase mRNA. Previous studies showed a marked increase in PEPCK expression at age 28 days (5). To extend these observations, we measured PEPCK expression at age 7–9 weeks by real-time PCR. PEPCK mRNA levels were increased 3.5 ± 0.2-fold in IUGR compared with control liver (P < 0.05; n = 5). Similarly, mRNA expression of G6Pase was increased in IUGR rats, although not significantly compared with control rats (Table 3).

FIG. 1. Basal HGP (■) and suppression of HGP (□) during insulin infusion (3 mU · kg⁻¹ · min⁻¹) in IUGR (n = 12) and control (n = 8) rats. Data are means ± SE. *P < 0.05 for IUGR vs. control; **P < 0.05 for vehicle vs. insulin infusion.

FIG. 2. Glucose infusion rate during hyperinsulinemic clamp in IUGR (n = 12) and control (n = 8) rats. Data are means ± SE. *P < 0.05 for IUGR vs. control.
Statistical significance of insulin (Table 4). G6Pase and glucokinase activity did not differ in IUGR liver (40.5 ± 0.78 vs. 3.7 ± 0.29 mU/mg protein; P < 0.05). Similarly, insulin suppression of G6Pase mRNA levels was blunted in IUGR versus control rats (40.5 ± 3.9 vs. 69 ± 4.8%; P < 0.05; n = 5).

Effect of IUGR on G6Pase and glucokinase activity. Despite a significant increase in G6Pase mRNA levels, basal activity was only mildly elevated and did not reach statistical significance in IUGR compared with control rats (Table 4). G6Pase and glucokinase activity did not differ between IUGR and control animals 3 h after administration of insulin (Table 4).

DISCUSSION
Our studies demonstrated that alterations in the intrauterine milieu permanently alter hepatic glucose metabolism in offspring. These changes occurred early in life before the onset of obesity and diabetes, suggesting that abnormal hepatic glucose metabolism represents an early defect that contributes to the eventual onset of fasting hyperglycemia.

Basal HGP was mildly increased in IUGR animals. Inappropriate HGP could be due to increased flux through G6Pase and/or decreased flux through glucokinase. Because glucokinase activity, glycolysis, and glycogen content were all normal in IUGR animals, it is likely that increased gluconeogenesis was responsible for the observed increase in HGP in IUGR rats. A key step in gluconeogenesis is the formation of phosphoenolpyruvate from oxaloacetate, which is catalyzed by PEPCK. Recent studies have demonstrated that overexpression of PEPCK alone can increase HGP (19,34). A twofold elevation, similar to the magnitude of change observed in IUGR animals, results in a 30% increase in basal HGP, but normal plasma glucose levels (19).

Despite an elevation of G6Pase mRNA levels in IUGR rats, there was no increase in basal activity of this enzyme in those animals. Therefore, the elevation in basal HGP observed in IUGR animals was likely due to increased PEPCK expression. It is plausible, therefore, to speculate that in those destined to become diabetic, increased flux through the PEPCK pathway precedes the development of overt hyperglycemia. Fasting hyperglycemia develops in the IUGR animal once either HGP increases beyond a certain threshold, β-cell compensation fails, peripheral glucose disposal decreases, or any combination of the above occurs (4).

Data from animal models of IUGR induced by malnutrition (35–39) or glucocorticoid exposure in late gestation stages (40) further support the concept that poor fetal growth has permanent consequences in the regulation of HGP. Nyirenda et al. (40) have shown that in the adult offspring of rats exposed to dexamethasone in late pregnancy, the expression and activity of PEPCK are increased, thereby predisposing adult offspring to glucose intolerance. Protein restriction during pregnancy also retards fetal growth and offspring develop glucose intolerance later in life (35–38,41). PEPCK expression is increased in offspring of protein-restricted dams compared with controls (37). Furthermore, insulin not only fails to suppress HGP, it actually increases HGP in offspring of mothers fed a low-protein diet (39). These findings suggest that limited nutrient availability, such as occurs in uteroplacental insufficiency (42) or with protein restriction or glucocorticoid administration during pregnancy leads to permanent changes in hepatic glucose metabolism in offspring.

Of critical importance was our finding that the excess HGP was not due to increased fatty acids. Increased levels

**TABLE 4**

Hepatic G6Pase and glucokinase activities in control and IUGR SD rats

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<th>Control (n = 5)</th>
<th>IUGR (n = 5)</th>
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<tr>
<td></td>
<td>Basal</td>
<td>Insulin</td>
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<tr>
<td><strong>G6Pase (mU/mg protein)</strong></td>
<td><strong>1.95 ± 0.29</strong></td>
<td><strong>0.85 ± 0.02</strong></td>
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<tr>
<td><strong>Glucokinase (mU/mg protein)</strong></td>
<td><strong>3.42 ± 0.62</strong></td>
<td><strong>4.95 ± 0.78</strong></td>
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Data are means ± SE. Intact microsomes were prepared and activity of enzymes were assayed as described in RESEARCH DESIGN AND METHODS.
of serum free fatty acids have been implicated as a causative factor in excess HGP in type 2 diabetes (43–46). IUGR animals used in the present study did not yet exhibit increased serum NEFA levels, suggesting that an alternate mechanism other than one involving fatty acids is responsible for increased glucose production by the liver.

A key finding in our study was that the suppression of HGP by insulin was impaired in IUGR animals. Insulin normally inhibits HGP by suppressing both glycogenolysis and gluconeogenesis (6). Insulin also regulates the rate of gluconeogenesis through its inhibitory effects on lipolysis (46,47) and through reduction of plasma glucagon levels (48). It is generally believed that hepatic insulin resistance is a secondary defect in type 2 diabetes, caused by the abnormal metabolic milieu of associated obesity. Indeed, hepatic insulin resistance is directly correlated with visceral fat content, and a reduction in this fat depot by surgical removal or caloric restriction dramatically improves insulin action in liver (32,33). However, our results demonstrated that in this animal model of type 2 diabetes, insulin resistance in the liver is not due to increased fatty acids, but rather is a primary defect caused by programming of the hepatocyte by an abnormal intrauterine milieu.

We have previously shown that uteroplacental insufficiency induces oxidative stress in the fetal liver that creates a self-perpetuating process in which overproduction of reactive oxygen species elicits mitochondrial dysfunction, inducing further production of reactive oxygen species and thereby creating a vicious cycle (5). This process sets in motion a cycle of gradually escalating and sustained stress, leading to impaired hepatic insulin signaling. Prolonged exposure to reactive oxygen species has been shown to downregulate IRS-2 and Akt phosphorylation in vitro (49,50). Thus it is possible that IUGR-induced oxidative stress inhibits insulin signaling in liver. Phosphorylation of Akt-2 triggers insulin effects on the liver, such as glycogen synthesis and the suppression of HGP. Activation of Akt-2 contributes to insulin-mediated suppression of glycogenolysis by driving glycogen synthase through the activation of glycogen synthase (51). However, because there were no changes in glycogen content in IUGR rat liver, it seems likely that IUGR-induced hepatic insulin resistance is secondary to the inability of Akt signaling to drive insulin-negative regulation of PEPCK gene expression.

In summary, our studies suggest that an aberrant intrauterine milieu permanently impairs insulin signaling in the liver so that gluconeogenesis is augmented in the IUGR rat. These processes occur early in life, before the onset of hyperglycemia, and indicate that uteroplacental insufficiency causes a primary defect in gene expression and hepatic metabolism that leads to the eventual development of overt hyperglycemia.

ACKNOWLEDGMENTS

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