Insulin resistance of gluconeogenic pathways in neonatal rats after prenatal ethanol exposure

Li Chen, Tong Zhang, and B. L. G. Nyomba. Insulin resistance of gluconeogenic pathways in neonatal rats after prenatal ethanol exposure. Am J Physiol Regul Integr Comp Physiol 286: R554–R559, 2004. First published November 13, 2003; 10.1152/ajpregu.00076.2003.—Alcohol exposure during pregnancy is associated with fetal growth restriction and programs the offspring to insulin resistance later in life. The underlying mechanisms are still uncertain, but a dysregulation of gluconeogenesis and adipose hormones may be contributory. Newborn rats from dams that had been given ethanol (EtOH) or water (controls) during pregnancy were studied. Adiponectin mRNA was determined in subcutaneous fat by RT-PCR, and serum adiponectin was measured by RIA. Subsets of rats were killed before and after intraperitoneal administration of insulin, to determine, by RT-PCR, the hepatic expression of gluconeogenic enzymes and that of the transcription factor peroxisome proliferator-activated receptor-coactivator (PGC)-1, which promotes gluconeogenesis. EtOH offspring had delayed hypoglycemic response to insulin but normal adiponectin mRNA and serum levels compared with controls. The inhibitory response of the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK) and PGC-1 mRNAs to insulin was blunted in EtOH offspring compared with controls. The data suggest that intrauterine EtOH exposure causes insulin resistance of genes for PGC-1 and PEPCK early in life.

Glucose metabolism in the liver. Indeed, the suppression of hepatic glucose production by insulin is enhanced by leptin and adiponectin (5, 10, 18) but impaired by resistin (34). There are currently limited data on the association of adipocyte hormones with IUGR. Leptin levels are relatively low in humans born with IUGR, and it has been suggested that this may explain obesity and insulin resistance in these individuals (22). Offspring of rats undernourished during pregnancy have decreased leptin levels early in life but elevated levels in adulthood in association with obesity (45). In a recent report (8), we have shown that prenatal EtOH exposure results in IUGR with impaired glucose homeostasis and increased resistin expression in both neonatal and adult rat offspring. The neonatal offspring had decreased leptin levels, whereas adult offspring had normal leptin levels. Adiponectin expression was not investigated in neonatal offspring. However, its expression was normal in the adult offspring, even after chronic feeding with saturated fat (9).

The present study was performed in neonatal rats exposed to EtOH in utero to examine the insulin response of enzymes involved in hepatic glucose production and to determine the expression of adiponectin and its association with glucose homeostasis.

MATERIALS AND METHODS

Animals and experimental design. Virgin Sprague-Dawley rats from Charles River Canada (Saint Constant, PQ) were treated as previously described (8, 9). Briefly, the rats were randomly divided into two weight-matched groups (n = 6/group), and after pregnancy was timed by the vaginal plug method, one group was given EtOH, 2 g/kg (36%), by gavage twice daily at 9:00 AM and 4:00 PM throughout gestation; the second group (control) was given the same volume of water instead of EtOH. The rats were otherwise allowed free access to tap water and commercial rat chow (Aagway ProLab, Syracuse, NY). Body weight and food intake were recorded from day 14 of gestation to parturition. Daily food intake (23.5 ± 1.3 vs. 25.7 ± 0.9 g) and weight gain (140.0 ± 15.5 vs. 140.0 ± 11.2 g) during pregnancy were similar between the EtOH and control dams. The rats were allowed to deliver spontaneously, and the period of gestation was similar between EtOH (21.5 ± 0.3 days; range 20–22 days) and control dams (21.3 ± 0.3 days; range 21–22 days). Litter size was also similar between EtOH (14.5 ± 1.5) and controls (15.8 ± 0.9). On the first day of life, male pups from each litter were weighed, and their length was measured from the tail tip to the crown. The pups were subjected to an insulin tolerance test (ITT) and randomly assigned to be killed by decapitation at different time points (0, 10, 20, 30, 60, and 90 min) after an intraperitoneal injection of insulin (Humulin R, Eli Lilly, Indianapolis, IN) at a dose of 1.0 IU/kg body wt (1). A total of 48 pups per group was used. The pups were separated from their mothers for

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Fetal growth restriction; adiponectin; gluconeogenesis; peroxisome proliferator-activated receptor-γ coactivator-1

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2 h before the experiments. Trunk blood was collected in heparinized capillary tubes and used for the determination of glucose and insulin. Livers were rapidly dissected out, snap-frozen in liquid nitrogen, and stored at −70°C. In pups killed at time 0, subcutaneous fat and blood were also used to determine adiponectin mRNA and plasma concentration, respectively (8, 9). The protocol was approved by the Committee for Animal Use in Research and Teaching of the University of Manitoba.

PCR. Trizol, oligo(deoxythymidine) primers, SuperScript reverse transcriptase, Taq DNA polymerase, and cDNA primers were obtained from Invitrogen (Carlsbad, CA). RT-PCR assays were performed as previously described (8, 9). Total RNA was extracted from ~100 mg frozen tissue by the Trizol method, and the first-strand cDNAs were synthesized from 5 μg total RNA using SuperScript reverse transcriptase and oligo(deoxythymidine) primers. The reverse transcription products (5 μl) were amplified by PCR using Taq DNA polymerase and specific primers (Table 1). Another 5 μl of the reverse transcription product was amplified with GAPDH primers as an internal control. For each gene, the PCR program consisted of denaturation at 94°C for 3 min, followed by 30 cycles of 45 s at 94°C, 45 s at specific annealing temperatures (Table 1), 90 s at 72°C, and a final 7 min at 72°C. The expected RT-PCR products are shown in Table 1. RT-PCR products (10 μl) were electrophoresed in a 1.5% agarose gel stained with ethidium bromide, and densitometrically analyzed using NIH Image software.

Glycogen phosphorylase. Glycogen phosphorylase activity in the liver was assayed in the direction of glycogenolysis, based on the incorporation of [U-14C]glucose-1-phosphate (PerkinElmer Life Sciences, Boston, MA) into glycogen in the absence of AMP and the presence of caffeine (39). Briefly, 20 mg of tissue were homogenized in 0.2 ml of a buffer containing a final concentration of 50 mM potassium phosphate (pH 7.4), 20 mM sodium fluoride, 10 mM EDTA, 6% glycerol, 2 mM sodium vanadate, 10 mM disodium pyrophosphate, 1 μg/ml leupeptin, 10 μg/ml aprotinin, and 0.1 mM PMSF. All the chemicals were from Sigma (St. Louis, MO). The homogenate was centrifuged at 1,000 g at 4°C, and 10 μl of the supernatant were added to 50 μl of a reaction mixture consisting of a final concentration of 200 mM potassium fluoride, 100 mM [U-14C]glucose-1-phosphate (0.1 μCi/reaction), 10 mM caffeine, and 1% glycogen. The incubation was carried out for 15 min in a 30°C water bath, and the reaction was stopped by precipitating 50 μl of the reaction mixture onto Whatman paper immediately dropped in 66% cold ethanol. After several washes in 66% cold ethanol, the filters were washed briefly in aceton, allowed to dry, and placed in scintillation vials containing 10 ml scintillate for radioactivity counting. Glycogen phosphorylase activity is expressed as micromoles of glucose transferred per minute per gram protein.

Other assays. Serum adiponectin was measured with a mouse RIA kit (Linco Research, St. Charles, MO), as previously described (9). Serum insulin was measured with a sensitive rat RIA kit (Linco), which has 100% cross-reactivity with human insulin. Glucose was measured using a 2300 glucose analyzer (YSI, Yellow Springs, OH). Tissue protein was determined by the Bradford method using BSA as standard.

Statistics. Statistical analyses were conducted with SPSS software (version 11.0 for Windows; SPSS, Chicago, IL). To detect changes in glucose and insulin concentrations after insulin injection, we performed for each group a one-way ANOVA followed by post hoc pairwise comparisons by Tukey’s test after ANOVA established significant differences. Data were otherwise analyzed using a two-factor ANOVA for the main effects of group (EtOH vs. control) and insulin treatment, as well as for group × insulin interaction followed by unpaired t-test for post hoc comparisons between groups or treatments. Insulin concentrations were log-transformed before analysis. Values are expressed as means ± SE. P < 0.05 was considered significant.

RESULTS

Animal characteristics. Neonatal rats exposed to EtOH in utero were shorter (4.9 ± 0.08 vs. 5.3 ± 0.05 cm, P < 0.0001) and weighed significantly less than controls (5.5 ± 0.1 vs. 6.4 ± 0.1 g, n = 48/group, P < 0.0001). Baseline plasma glucose (4.7 ± 0.6 vs. 3.5 ± 0.3 mmol/l, n = 8/group, P = 0.07) and insulin (0.67 ± 0.09 vs. 0.51 ± 0.11 ng/ml, P = 0.3) levels were similar between EtOH offspring and controls.

Insulin sensitivity. To investigate differences in insulin sensitivity, we performed an ITT using littermates at different time points (Fig. 1). Insulin was given intraperitoneally before neonatal rats were decapitated and blood was collected for the measurement of glucose and insulin. In control pups, glucose concentrations declined rapidly after insulin administration, and the decrease became significant by 30 min. In pups exposed to EtOH in utero, however, a decline in glucose concentrations did not become significant until 90 min after insulin injection. In control pups, the insulin concentrations after intraperitoneal insulin administration reached a maximum at ~30 min and then declined. In EtOH pups, insulin concentrations continued to rise (Fig. 1). Overall the insulin levels were similar between the two groups, except at 90 min, when it was higher in EtOH vs. control rats (P < 0.05). By two-way ANOVA, there was a significant main effect of insulin treatment on both glucose (P < 0.0001) and insulin (P < 0.01), but the effect of group was significant on glucose only (P < 0.0001), and there was no group × time interaction on either glucose or insulin concentrations.

Table 1. Primer sequences

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Size, bp</th>
<th>AT, °C</th>
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<tr>
<td>Adiponectin</td>
<td>5'-GGGAGGAGAGAAAGAGCCAGTAA-3'</td>
<td>178</td>
<td>55</td>
</tr>
<tr>
<td>Adiponectin</td>
<td>5'-AGACACGCCTGCATGAA-3'</td>
<td>338</td>
<td>62</td>
</tr>
<tr>
<td>G6Pase antisense</td>
<td>5'-AAAGAGATGAGCGAGGGCCA-3'</td>
<td>194</td>
<td>50–62</td>
</tr>
<tr>
<td>GAPDH antisense</td>
<td>5'-CCATGGAGAAGGCTGGG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH antisense</td>
<td>5'-GAGAGTTGATCATGACGAGCC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G6Pase antisense</td>
<td>5'-CCATGGAGAAGGCTGGG-3'</td>
<td></td>
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</tr>
<tr>
<td>G6Pase antisense</td>
<td>5'-GAATTGGTCTCAGATGACGAG-3'</td>
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</tr>
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<td>PGC-1 sense</td>
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<td>57</td>
</tr>
<tr>
<td>PGC-1 antisense</td>
<td>5'-AGCATGAGGAGGATATAC-3'</td>
<td></td>
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</table>

AT, annealing temperature; bp, base pairs; G6Pase, glucose-6-phosphatase; GK, glucokinase; PEPCK, phosphoenolpyruvate carboxykinase; PGC-1, peroxisome proliferator-activated receptor-γ coactivator-1.
Liver enzymes and peroxisome proliferator-activated receptor-γ coactivator-1. To investigate whether liver enzymes could contribute to the hyperglycemia observed in EtOH-exposed pups, we measured phosphoenolpyruvate carboxykinase (PEPCK), glucose-6-phosphatase (G6Pase), and glucokinase (GK) mRNA as well as glycogen phosphorylase activity in liver extracts before and 30 min after insulin injection. The latter time point was chosen because 1) insulin levels had peaked in control pups and 2) insulin concentrations were comparable between groups, and, therefore, the groups were being submitted to a similar insulin stimulus. The expression of the gluconeogenic enzymes at baseline was not different between EtOH-exposed pups and controls. After insulin administration, PEPCK mRNA decreased by 39% in controls and 25% in the EtOH group (Fig. 2). G6Pase mRNA did not change in either group. GK mRNA was similar between the two groups at baseline and did not significantly change (P = 0.08) after insulin injection in either group taken individually. By two-factor ANOVA, the main effect of group was not significant on any of the mRNAs, consistent with the lack of baseline mRNA differences between groups. However, there was a significant main effect of insulin on PEPCK (P < 0.0001) and GK (P < 0.01). Most importantly, there was a significant group × insulin interaction on PEPCK (P < 0.05) but not on GK (P = NS). This was consistent with a significant group difference in the response of PEPCK mRNA to insulin and a lack of group difference in the response of GK mRNA to insulin. Glycogen phosphorylase activity was not different between the control and EtOH groups either before (47.4 ± 2.9 vs. 41.5 ± 4.7 µmol·g protein⁻¹·min⁻¹) or after (44.1 ± 6.0 vs. 41.2 ± 8.7 µmol·g protein⁻¹·min⁻¹) insulin injection.

We next determined peroxisome proliferator-activated receptor-γ coactivator-1 (PGC-1) expression to explain differences in PEPCK expression. Before insulin injection, PGC-1 mRNA was not different between the two groups. After insulin administration, however, PGC-1 mRNA decreased by ~25% in controls but did not decrease in EtOH-exposed rats (Fig. 2). By two-way ANOVA, there was a significant main effect of insulin (P < 0.05) and a significant group × insulin interaction (P < 0.01) on PGC-1 mRNA.

Adiponectin. Because of the role played by adiponectin in the regulation of glucose homeostasis, we determined its mRNA in subcutaneous adipose tissue and its peptide level in serum from newborn rats. Both adiponectin mRNA (0.70 ±
DISCUSSION

We have recently shown that EtOH ingestion by the mother during pregnancy results in IUGR with impaired glucose homeostasis in the offspring (8, 9). Adult offspring were glucose intolerant despite hyperinsulinemia, an indication that they were insulin resistant. We now show that insulin resistance is already present early in the life of these offspring, as documented by a delayed hypoglycemic response to insulin. We also investigated whether the gluconeogenic pathway in this IUGR model is insulin resistant at this early age.

Increased hepatic glucose production due to glycogenolysis and gluconeogenesis is an important component of insulin resistance in type 2 diabetes (28). Glycogenolysis is regulated by glycogen phosphorylase, but in this study, glycogen phosphorylase activity was not different between EtOH-exposed offspring and controls. A lack of difference in glycogen phosphorylase activity has been reported between insulin-resistant and insulin-sensitive neonatal animals (42). In gluconeogenesis, the regulation of the rate-limiting (PEPCK) and the last committed (G6Pase) enzymes occurs mainly at the transcription level (26). However, the insulin response sequences in these enzymes are functionally different and their response to insulin is not identical (30). In the current study, G6Pase mRNA levels were not different between groups. PEPCK mRNA levels were suppressed by 25% in the EtOH offspring and by ~40% in controls after insulin administration. This represents ~35% reduction in insulin action on PEPCK in the EtOH group. Alterations of gluconeogenic enzymes have been reported in adult rat offspring born with IUGR due to protein malnutrition, placental ischemia, or glucocorticoid exposure (14, 24, 29, 33). In the malnutrition model, the adult offspring had increased liver PEPCK activity with a concomitant decrease in GK activity (14, 33). In the placental ischemia IUGR model, where newborn rats were also studied, hepatic PEPCK and G6Pase mRNA were both increased and GK mRNA was decreased (24). These results suggested that IUGR programs both an increase of gluconeogenesis and a reduction of glycogenolysis, resulting in an increased hepatic glucose output. However, an increase of PEPCK only, without an alteration of G6Pase or GK expression, was reported in rat offspring with glucocorticoid-inducing IUGR (29). Although these results were intriguing, the authors suggested that the increase in PEPCK, the rate-limiting enzyme in the gluconeogenesis pathway, may promote hepatic glucose production, even though G6Pase and GK are unaltered. Similarly, Sun et al. (40) have reported in transgenic mice that a modest increase in PEPCK expression and gluconeogenesis in rats exposed to dexamethasone in utero (29). Furthermore, a recent report of elevated PGC-1 mRNA levels in newborn and adult rat offspring with IUGR due to placental ischemia lends support to the suggestion that programming of gluconeogenesis by IUGR may be mediated through PGC-1 (24).

The response of GK mRNA to insulin was normal in EtOH offspring. As mentioned above, studies of GK expression in IUGR models have yielded variable results, with reports of decreased (14, 24, 33) or normal (29) levels, depending on models and experimental conditions. GK expression would be expected to be decreased in this study because it is usually low in newborn rat liver (43) and because EtOH has an inhibitory effect on this enzyme (48). An increase of GK expression can be triggered by hyperglycemia (6), as a compensatory antihyperglycemic mechanism to promote glucose disposal. An increase in glucose transporter-1, which also promotes glucose disposal, has been found in the liver of newborn rats in the placental ischemia model in association with an upregulation of gluconeogenesis (23, 24). Similarly, parallel increases of G6Pase and GK activity have been reported after feeding rats a high-sucrose diet, which is known to cause insulin resistance.

PEPCK gene transcription is upregulated by glucagon, glucocorticoids, and epinephrine and downregulated by insulin, which exerts a dominant effect (26). It is unlikely that hypoglycemia or hypoinsulinemia in utero or an increase in epinephrine, corticosterone, or glucagon after delivery was the explanation for the difference in PEPCK mRNA in the current study, because this was not different at baseline. Our results suggest that PEPCK mRNA response to insulin exposure is impaired by ~35% in the EtOH rat offspring, but it is unclear whether these changes are biologically important, because we did not measure hepatic glucose production. However, a suppression of PEPCK of this magnitude has been reported by Rossetti and colleagues (3) to be associated with enhanced hepatic insulin sensitivity after removal of visceral fat in rats.

A recent study has shown that the transcription factor PGC-1 modulates hepatic gluconeogenesis through an increase of PEPCK transcription (49). PGC-1 is a coactivator-like protein that interacts with PPAR-γ, an orphan receptor found in the nucleus of fat cells that has been the target of diabetes treatments with thiazolidinediones. PGC-1 is not significantly expressed in the liver in the feeding state but is readily detectable after fasting and in states of diminished insulin action where gluconeogenesis is increased (49). PGC-1 overexpression increases glucose production and the transcription of genes encoding gluconeogenic enzymes, including PEPCK and G6Pase. PGC-1 is upregulated by cAMP-dependent mechanisms and glucocorticoids and downregulated by insulin. However, PGC-1 is not the sole regulator of these enzymes. PGC-1 interacts with the hepatic nuclear factor-4α and the glucocorticoid receptor for fully activating PEPCK expression. It is unclear, however, whether this explains the observed differences in the effects of insulin on PEPCK between the EtOH pups and controls. It is also unclear whether the 25% suppression of PGC-1 mRNA by insulin in the current study has any biological significance. However, mRNA changes of this magnitude have been reported to be important for hepatic glucose metabolism. For example, a 25% increase in hepatic glucocorticoid receptor expression has been reported to be a mechanism for increased PEPCK expression and gluconeogenesis in rats exposed to dexamethasone in utero (29). Furthermore, a recent report of elevated PGC-1 mRNA levels in newborn and adult rat offspring with IUGR due to placental ischemia lends support to the suggestion that programming of gluconeogenesis by IUGR may be mediated through PGC-1 (24).

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(12). Sun et al. (40) have suggested that liver insulin resistance may be selective to gluconeogenesis while sparing glycolysis. Adiponectin has been implicated as a factor that suppresses hepatic glucose production and plays a major role in the regulation of insulin action (19). Adiponectin expression is reduced in insulin-resistant states (21), increased with caloric restriction and thiazolidinedione treatment (11), and correlates with insulin sensitivity determined by euglycemic clamps (20, 47). Administration of this hormone increases insulin sensitivity, inhibits hepatic glucose production, and reduces glycemia in mice (5, 10). In the current study, however, we found no effect of prenatal EtOH exposure on adiponectin expression. In adult rat offspring exposed to EtOH in utero, adiponectin expression was also normal despite glucose intolerance and hyperinsulinemia (9). These results suggest that the insulin resistance associated with EtOH-induced IUGR is not explained by changes in adiponectin levels.

In summary, the gluconeogenic enzyme PEPCK and the transcription factor PGC-1, which regulates the expression of this enzyme, had an impaired response to insulin in rat newborns exposed to EtOH in utero. The data suggest that intrauterine EtOH exposure causes insulin resistance of genes for PGC-1 and PEPCK early in life. Further studies are required to investigate the presence of these abnormalities in the adult offspring exposed to EtOH during pregnancy to confirm the suspicion that insulin resistance of hepatic PGC-1 is an early defect programming the liver to enhanced gluconeogenesis later in life.

GRANTS

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48. Wimhurst JM and Harris EJ. The actions of avenacilolide and ethanol on gluconeogenesis and on related enzyme activities in the isolated perfused rat liver. Biochim Biophys Acta 437: 51–61, 1976.
