Adult rats prenatally exposed to ethanol have increased gluconeogenesis and impaired insulin response of hepatic gluconeogenic genes

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Yao, Xing-Hai, Li Chen, and B. L. Grégoire Nyomba. Adult rats prenatally exposed to ethanol have increased gluconeogenesis and impaired insulin response of hepatic gluconeogenic genes. J Appl Physiol 100: 642–648, 2006. First published October 20, 2005; doi:10.1152/japplphysiol.01115.2005.—Rat offspring exposed to ethanol (EtOH rats) during pregnancy are insulin resistant, but it is unknown whether they have increased gluconeogenesis. To address this issue, we determined blood glucose and liver gluconeogenic genes, proteins, and enzyme activities before and after insulin administration in juvenile and adult EtOH rats and submitted adult EtOH rats to a pyruvate challenge. In juvenile rats, basal glucose; peroxisome proliferator-activated receptor-coactivator-1α protein and mRNA; and phosphoenolpyruvate carboxykinase enzyme activity, protein, and mRNA were similar between groups. After insulin injection, these parameters failed to decrease in EtOH rats, but glucose decreased by 30% and gluconeogenic enzymes, proteins, and mRNAs decreased by 50–70% in control rats. In adult offspring, basal peroxisome proliferator-activated receptor-coactivator-1α protein and mRNA levels were 40–80% higher in EtOH rats than in controls. Similarly, basal phosphoenolpyruvate carboxykinase activity, protein, and mRNA were 1.8-fold greater in EtOH rats than in controls. These parameters decreased by 50% after insulin injection in control rats, but they remained unchanged in EtOH rats. After insulin injection in the adult rats, glucose decreased by 60% in controls but did not decrease significantly in EtOH rats. A subset of adult EtOH rats had fasting hyperglycemia and an exaggerated glycemic response to pyruvate compared with controls. The data indicate that, after prenatal EtOH exposure, the expression of gluconeogenic genes is exaggerated in adult rat offspring and is insulin resistant in both juvenile and adult rats, explaining increased gluconeogenesis. These alterations persist through adulthood and may contribute to the pathogenesis of Type 2 diabetes after exposure to EtOH in utero.

Prenatal ethanol; insulin resistance

ADVERSE EVENTS DURING PREGNANCY programs the fetus to later develop insulin resistance and Type 2 diabetes in adulthood, as suggested by epidemiological studies (2, 36, 40) and confirmed in animal models of intrauterine growth restriction (IUGR) employing malnutrition (20, 51), placental ischemia (45), glucocorticoid exposure (43), or diabetes (1) during pregnancy. Ethanol (EtOH) consumption during pregnancy can lead to abnormal fetal development, a spectrum of effects that include fetal alcohol syndrome (FAS) and less severe abnormalities known as fetal alcohol effects (46). Classic characteristics of FAS include IUGR, abnormal facial features, and central nervous system problems, but organ malformations may be absent in less severe forms of fetal alcohol exposure. After puberty, children with FAS are no longer underweight, although they remain shorter than age-matched controls (19, 46). This suggests that adult individuals with FAS are relatively obese and possibly insulin resistant. The prevalence of FAS is increased in populations with lower socioeconomic status (3), where Type 2 diabetes is also common (28). One study in humans (5) and several studies in rodents (7, 8, 17, 27, 52) have reported the presence of abnormal glucose tolerance after prenatal EtOH exposure. We have demonstrated, in addition, that EtOH-exposed rats have in vivo insulin resistance (9) and impaired insulin signaling in skeletal muscle (10).

Besides skeletal muscle, the liver is a major insulin-sensitive organ, which contributes to the pathogenesis of glucose intolerance through increased glucose production. Increased basal hepatic glucose production or its resistance to suppression by insulin contributes to both fasting and postprandial hyperglycemia observed in diabetes, impaired fasting glucose, and impaired glucose tolerance (13, 29, 49). Increased hepatic glucose production is due to increased glycogenolysis or gluconeogenesis or both. We have reported that EtOH-exposed neonatal rat offspring have normal hepatic expression of the glycolytic enzyme phosphorylase, suggesting that glycogenolysis was normal (11). The expression of the last committed gluconeogenic enzyme glucose-6-phosphatase and the glycolytic enzyme glucokinase was also normal (11). However, these neonatal rats had increased expression of phosphoenolpyruvate carboxykinase (PEPCK), the rate-limiting gluconeogenic enzyme, and of peroxisome proliferator-activated receptor-coactivator (PGC)-1α, which increases PEPCK transcription (57). These alterations in PEPCK and PGC-1α are not known to occur in adult rats in this model, and it is not known whether these rats have increased endogenous glucose production. We hypothesized that, besides insulin resistance in skeletal muscle (10, 17), EtOH-exposed rat offspring develop glucose intolerance due to exaggerated hepatic glucose production caused by increased PEPCK expression or its resistance to insulin. We studied gluconeogenesis and the insulin response of PEPCK and PGC-1α in juvenile and adult rat offspring exposed to EtOH in utero.

METHODS

Materials. EtOH was obtained from pharmaceutical services at the Health Sciences Centre (Winnipeg, MB, Canada). Protease inhibitor cocktail tablets were purchased from Roche Diagnostics (Penzberg, Germany). Electrophoresis and electroblotting consumables were from Bio-Rad (Hercules, CA). Antibodies were from Chemicon.
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International (Temecula, CA) and Santa Cruz Biotec technology (Santa Cruz, CA). Enhanced chemiluminescence kit was obtained from Amersham Pharmacia (Piscataway, NJ). Trizol, SuperScript reverse transcriptase, Tag DNA polymerase, and cDNA and oligo(deoxythymidine) primers were obtained from Life Technologies (Rockville, MD). Isopropyl alcohol and methanol were from Fisher Scientific (Nepean, ON, Canada). All other chemicals were purchased from Sigma-Aldrich (Oakville, ON, Canada).

Animals. All of the animal studies were approved by the Committee for Animal Use in Research and Teaching of the University of Manitoba. Rat offspring were exposed to EtOH in utero according to a protocol established in our laboratory (7–10). Briefly, Sprague-Dawley rats were purchased from Charles River Canada (Saint Constant, PQ), time-mated, and randomly divided into two weight-matched groups. One group was given EtOH [2 g/kg (36%)] by gavage twice daily for the duration of the gestation, whereas the other group was given the same volume of water instead of EtOH. This method results in alcoholemia of 115 mg/dl and 70 mg/dl at 2 and 4 h after ingestion, corresponding to human binge drinking (7, 58). Only male offspring were used because, in the vast majority of studies of IUGR rats, abnormalities of glucose metabolism were found only in males (15, 17, 47). At the time of this study, adult rat offspring were 12 and 14 wk old, i.e., within the age range previously reported to coincide with glucose intolerance in EtOH-exposed rats, and we were not expecting any major metabolic differences between them with such a small age difference (7–9). These rats were subsequently referred to as adult 1 and adult 2 (see RESULTS). Juvenile rats were used at 7 days of age. For this study, one or two offspring were randomly taken per litter per treatment group. Juvenile and adult offspring were fasted for 2 and 16 h, respectively, and they were given an intraperitoneal injection of regular insulin (Novolin Toronto, 2 U/kg, NovoNordisk, Mississauga, ON, Canada) or an equivalent volume of saline. Blood was drawn for glucose and insulin determination before and 2 h after insulin injection. The rats were killed at each of these time points, and the liver was rapidly removed, immediately frozen in liquid nitrogen, and stored at −70°C until used. Groups of adult rats (adult 2) were submitted to a pyruvate challenge as described below.

Pyruvate tolerance test. To estimate gluconeogenesis, a pyruvate load was administered as described (30, 37, 50). Briefly, rats were injected intraperitoneally with 2 g/kg pyruvate dissolved in saline. Control experiments were performed after oral administration of 3-mercaptopicolinic acid (3-MPA; 30 mg/kg), an inhibitor of gluconeogenesis (16, 42), 30 min before the pyruvate injection. Glucose was determined in tail blood every 30 min for 2 h.

Gene expression. PGC-1α and PEPCK mRNAs and proteins were determined by RT-PCR and Western blot as applicable, following protocols described before (7, 8, 10, 11).

PEPCK activity. PEPCK activity was determined by a modification (23) of Wimmer’s method (55) whereby the transformation of oxaloacetate to phosphoenolpyruvate and ATP is monitored. Briefly, liver homogenates were incubated with oxaloacetate and inosine triphosphate, which mediates the formation of phosphoenolpyruvate. Next, the mixtures were incubated with ADP and pyruvate kinase, resulting in the formation of ATP, which was determined via a reaction with luciferase reagent using the ATP bioluminescence assay kit CLS II (Roche Diagnostics, Indianapolis, IN).

Other assays. Blood glucose was determined with the Ascensia Elite XL blood glucose meter (Bayer HealthCare), and plasma insulin was measured with the ultrasensitive rat insulin ELISA kit (CrystalChem, Downers Grove, IL).

Statistics. Statistical analyses were performed with SPSS software (version 11.0 for Windows, SPSS, Chicago, IL). Differences between groups were analyzed by unpaired t-test or one-way ANOVA with Tukey’s honestly significant difference test for post hoc pairwise comparisons. A two-way ANOVA was used to evaluate the effects of EtOH exposure and insulin treatment. Changes in glucose concentra-

RESULTS

Baseline weight characteristics and glycemic response to insulin. The weight characteristics of rat offspring prenatally exposed to EtOH was significantly lower than that of controls at birth and remained lower at 7 days but was normal in adult rats (Table 1). Liver weights were similar among age-matched groups. In juvenile rat offspring, fasting plasma insulin levels were ~35% higher in the EtOH group than in controls, whereas basal blood glucose concentrations were similar between the two groups. Fasting insulin levels in 3-mo-old rat offspring were about twofold higher in EtOH-exposed rats compared with controls, whereas glucose concentrations were similar between groups (Table 1). After insulin injection, glucose concentrations in juvenile rat offspring decreased by 30% (P < 0.01) in controls, but no change could be shown in the rats exposed to EtOH (Fig. 1A). In adult offspring, glucose concentrations decreased by ~60% (P < 0.01) after insulin injection in controls, whereas no significant change was seen in those prenatally exposed to EtOH (Fig. 1C). In rats of both age groups, the changes in glucose concentrations paralleled changes in PEPCK enzymatic activity (Fig. 1, B and D). A subgroup of EtOH-exposed rats (Table 1) had fasting glucose concentrations in the diabetic range (range 7.5–11.9), significantly greater (P < 0.001) than in age-matched controls (range 4.7–6.8). Closer examination revealed that these rats (now referred to as adult 2) were 14 wk old, whereas the normoglycemic rats (adult 1) were 12 wk old, in addition to being from different litters. In adult 2 rats, fasting insulin levels tended to decrease compared with adult 1 rats (P = 0.09).

Table 1. Baseline characteristics

<table>
<thead>
<tr>
<th>Age group</th>
<th>Control</th>
<th>EtOH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Body weight, g</td>
<td></td>
</tr>
<tr>
<td>Neonate</td>
<td>6.1 ± 0.1</td>
<td>5.4 ± 0.1†</td>
</tr>
<tr>
<td>Juvenile</td>
<td>14.6 ± 0.3</td>
<td>12.9 ± 0.4‡</td>
</tr>
<tr>
<td>Adult 1</td>
<td>43.0 ± 17</td>
<td>447 ± 22</td>
</tr>
<tr>
<td>Adult 2</td>
<td>410 ± 28</td>
<td>427 ± 22</td>
</tr>
<tr>
<td></td>
<td>Liver weight, g</td>
<td></td>
</tr>
<tr>
<td>Juvenile</td>
<td>0.36 ± 0.01</td>
<td>0.35 ± 0.01</td>
</tr>
<tr>
<td>Adult 1</td>
<td>12.73 ± 0.55</td>
<td>13.27 ± 0.55</td>
</tr>
<tr>
<td>Adult 2</td>
<td>14.09 ± 0.40</td>
<td>13.68 ± 0.54</td>
</tr>
<tr>
<td></td>
<td>Insulin, ng/ml</td>
<td></td>
</tr>
<tr>
<td>Juvenile</td>
<td>0.61 ± 0.04</td>
<td>0.83 ± 0.06*</td>
</tr>
<tr>
<td>Adult 1</td>
<td>1.10 ± 0.45</td>
<td>2.75 ± 0.30*</td>
</tr>
<tr>
<td>Adult 2</td>
<td>1.26 ± 0.17</td>
<td>2.04 ± 0.30*</td>
</tr>
<tr>
<td></td>
<td>Glucose, mM</td>
<td></td>
</tr>
<tr>
<td>Juvenile</td>
<td>4.0 ± 0.2</td>
<td>4.7 ± 0.3</td>
</tr>
<tr>
<td>Adult 1</td>
<td>5.4 ± 0.2</td>
<td>5.8 ± 0.6</td>
</tr>
<tr>
<td>Adult 2</td>
<td>5.9 ± 0.3</td>
<td>9.6 ± 0.8‡</td>
</tr>
</tbody>
</table>

Values are means ± SE; no. of rats/group was n = 6, except for neonates (n = 24) and adult 2 (n = 12). Adult 1, 12-wk-old rats; adult 2, 14-wk-old rats. *P < 0.05, †P < 0.01, ‡P < 0.001 vs. age-matched controls.

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Effect of prenatal EtOH on PEPCK expression. To explain the changes in PEPCK activity, we determined PEPCK expression at the protein and mRNA levels. In juvenile rat offspring, basal PEPCK protein (Fig. 2A) and mRNA (Fig. 2B) levels were similar between EtOH-exposed rats and controls. Insulin injection to control rat offspring caused a 50% reduction ($P < 0.01$) in the levels of both PEPCK protein (Fig. 2A) and mRNA (Fig. 2B). In EtOH-exposed rats, however, no significant changes could be demonstrated after insulin injection. In adult rat offspring, unlike juvenile rats, basal levels of PEPCK protein (Fig. 2C) and mRNA (Fig. 2D) were 40–80% higher ($P < 0.01$) in the EtOH group compared with controls. Insulin caused a 30% reduction ($P < 0.01$) in the levels of PEPCK protein (Fig. 2C) and mRNA (Fig. 2D) in control rats but had no significant effect in EtOH-exposed rats.

Effect of prenatal EtOH on PGC-1α expression. Because hepatic PGC-1α regulates PEPCK expression (21, 25, 57), we measured PGC-1α expression in these animals. In juvenile rat offspring, basal PGC-1α protein (Fig. 3A) and mRNA (Fig. 3B) levels were similar between EtOH-exposed rats and controls. Insulin injection in control rat offspring caused a 60–70% reduction ($P < 0.01$) in PGC-1α protein and mRNA levels, but PGC-1α levels did not change significantly ($P = $ not significant) after insulin injection in EtOH-exposed rats. In adult rat offspring, unlike juvenile rats, basal PGC-1α protein (Fig. 3C) and mRNA (Fig. 3D) levels were 40–80% higher in the EtOH group compared with controls. Similar to juvenile rats, insulin caused an 50% reduction ($P < 0.01$) in the levels of PGC-1α protein and mRNA in control rats, but no significant reduction could be demonstrated in EtOH-exposed rats.

Effect of prenatal EtOH on pyruvate-induced gluconeogenesis. To account for the fasting hyperglycemia in adult 2 rats, we also investigated the effect of prenatal EtOH exposure on pyruvate-induced gluconeogenesis by measuring blood glu-
cose response to the administration of pyruvate, a gluconeogenesis substrate (Fig. 4). In control rats, blood glucose concentration increased 30 min after pyruvate administration ($P < 0.001$ vs. zero-time value), plateaued, and decreased after 90 min ($P < 0.05$ comparing 90 min vs. 120 min). In EtOH-exposed rats, however, blood glucose concentration plateaued by 60 min and did not decrease significantly after 90 min. As a result, the blood glucose concentration from 60–120 min was significantly greater in EtOH-exposed rat offspring compared with controls ($P < 0.05$). Prior injection of 3-MPA diminished the increase in blood glucose concentration in both groups of rats. Even in the presence of 3-MPA, however, the pyruvate-induced glycemic changes were still greater in rats prenatally exposed to EtOH compared with controls (Fig. 4).

**DISCUSSION**

In previous studies, we reported that prenatal EtOH exposure results in IUGR with impaired glucose homeostasis in the rat offspring (7–9, 11). Adult rat offspring were glucose intolerant despite hyperinsulinemia and had decreased insulin sensitivity, as determined by frequently sampled intravenous glucose tolerance test with minimal modeling (9). Newborn rat offspring exposed to EtOH in utero had a delayed hypoglycemic response to insulin in association with an impaired suppression of PEPCK and PGC-1α mRNAs by insulin, suggesting that insulin resistance and increased hepatic glucose production were already present early in the life of these offspring (11). In the present report, we extend these findings to older rats prenatally exposed to EtOH and we report that these animals have increased gluconeogenesis as an explanation for their glucose intolerance.

Increased gluconeogenesis is an important component of insulin resistance in Type 2 diabetes (13, 29, 49). PEPCK and glucose-6-phosphatase are two key enzymes that drive gluconeogenesis. PEPCK is the rate-limiting enzyme, whereas glucose-6-phosphatase catalyzes the final step of gluconeogenesis, the production of free glucose from glucose 6-phosphate. Both enzymes are suppressed by insulin at the transcription level. In states of insulin resistance and Type 2 diabetes, the effect of insulin to suppress these two enzymes is diminished, which enhances gluconeogenesis (33). Consistent with the rate-limiting role of PEPCK, its increased expression was shown to increase gluconeogenesis despite normal glucose-6-phosphatase expression (32, 48). We have previously shown that PEPCK gene expression is insulin resistant in neonatal EtOH-exposed rats (11), but it is not known whether this persists through adulthood. Because adult EtOH-exposed rat offspring are also insulin resistant, we hypothesized that PEPCK expression may be also affected in these animals. To address this question, we measured PEPCK mRNA before and...
after insulin administration. Insulin decreased PEPCK mRNA in control but not in EtOH-exposed rats regardless of age, suggesting that EtOH exposure during pregnancy causes insulin resistance of PEPCK gene expression in adult rat offspring. The action of insulin to suppress PEPCK mRNA correlated with its hypoglycemic effect in these animals. In addition, EtOH exposure was associated with elevated basal PEPCK mRNA in adult EtOH-exposed rats, despite normal fasting glucose concentrations. Elevated expression of PEPCK has been reported in models of IUGR induced by prenatal malnutrition (4, 14), glucocorticoid exposure (32), and placental insufficiency (53), where it was shown to precede the development of diabetes (53). In addition, failure of insulin to suppress hepatic glucose production has been reported in development of diabetes (53). In addition, failure of insulin to suppress hepatic glucose production has been reported in offspring of mothers fed a low-protein diet (34). Overall, these findings led to the conclusion that IUGR associated with an abnormal intrauterine milieu leads to permanent changes in hepatic glucose metabolism in offspring. We were surprised by the finding of elevated fasting glucose concentrations in the diabetic range in some EtOH-exposed adult rat offspring, clustering at ~14 wk of age. Reexamination of our previous data in 13- to 16-wk-old rats indicated that some rats had fasting glucose >7 mM (7–9). Thus, rather than indicating a fundamental difference in glucose metabolism between 12- and 14-wk-old rats, the data suggest that diabetes in these animals probably starts developing in this age group as preluded by the increased gluconeogenic gene expression. The question whether hyperglycemia starts at 12–16 wk of age and whether diabetes incidence in this model increases with age will be further investigated in future studies.

Fasting hyperglycemia in diabetes is mainly explained by increased hepatic glucose production due to gluconeogenesis (13). To investigate whether gluconeogenesis was increased in these animals, we administered the gluconeogenesis precursor pyruvate. Administration of pyruvate markedly increased blood glucose concentration in adult rats exposed to EtOH in utero, whereas blood glucose increase was lower in controls. This glucose increase was diminished by 3-MPA, a pharmacological inhibitor of PEPCK (16, 30, 37, 50), suggesting that the pyruvate effect was related to PEPCK-driven gluconeogenesis. Blood glucose concentration in the presence of 3-MPA was still higher in EtOH-exposed rats compared with controls, which is not surprising because the dosage of 3-MPA was similar between the two animal groups, even though the EtOH group had a much higher PEPCK level. Thus, as found in other IUGR models (4, 14, 32, 53), the increased PEPCK expression in EtOH-exposed rats is the likely explanation for the elevated gluconeogenesis and contributes to glucose intolerance and hyperglycemia.

As previously reported in newborn rats (11), prenatal exposure to EtOH was also associated with an impaired response of PGC-1α expression to insulin in both juvenile and adult rats, with increased basal expression of this protein in adult offspring. Elevated PGC-1 expression has also been reported in rat offspring with IUGR due to placental ischemia (26). PGC-1 is a coactivator initially identified as a protein that interacts with PPARγ, an orphan receptor found in the nucleus of fat cells that has been the target of diabetes treatment with thiazolidinediones (39). PGC-1 has several known isoforms, including PGC-1α, PGC-1β, and PGC-related coactivator. PGC-1α is highly expressed in tissues with high metabolic rates such as heart, muscle, and brown adipose tissue, where it promotes mitochondrial biogenesis and energy expenditure (38). PGC-1α is not significantly expressed in the liver in the fed state but is readily detectable after fasting and in states of diminished insulin action where gluconeogenesis is increased (21, 57). Hepatic PGC-1α overexpression increases glucose production and the transcription of genes encoding gluconeogenic enzymes. It is upregulated by cAMP-dependent mechanisms and glucocorticoids and downregulated by insulin. PGC-1α-deficient mice experience fasting hyperglycemia (25), whereas hepatic PGC-1α expression is elevated in mouse models of Type 2 diabetes (21, 57).

EtOH administration in our rat model corresponds to heavy drinking in humans (58), which is a risk factor for the development of Type 2 diabetes (22, 54). High EtOH exposure is associated with impaired glucose utilization, although moderate EtOH consumption may increase insulin sensitivity and protect against cardiovascular disease (12, 31, 54). EtOH consumption increases circulating glucose concentrations, glucose intolerance, and insulin resistance (18, 44, 56). Heavy EtOH exposure causes oxidative stress, which can impair insulin signaling (6, 24). However, our study does not reflect direct effects of EtOH because the results were obtained several days to weeks after exposure. Whether the observed abnormalities are due to IUGR per se, chronic effects of EtOH, or both is uncertain. We have previously reported that rats prenatally exposed to EtOH can be insulin resistant even with a normal birth weight (9). In fact, long-lasting oxidative stress (41) could be a common ground between prenatal EtOH exposure and other IUGR models where oxidative injury has been proposed to explain hepatic insulin resistance (35).

In summary, rats exposed to EtOH in utero had an increased gluconeogenesis as shown by the pyruvate tolerance test. In the liver of these rats, the expression of the gluconeogenic enzyme PEPCK and of the transcription factor PGC-1α, which regulates the expression of this enzyme, had an impaired response to insulin, an indication of hepatic insulin resistance. There was a difference in the basal expression of PEPCK and PGC-1α between juvenile and adult rats prenatally exposed to EtOH in that, in the basal state, these genes were increased in adult but not in juvenile rat offspring, which could be considered to have a lesser defect. This defect, however, worsens with age once it has been programmed by the EtOH insult. The data suggest that intrauterine EtOH exposure causes insulin resistance of the expression of PGC-1α and PEPCK genes starting early in life. These genetic and enzymatic alterations are long lasting, worsen with age, and have the potential to contribute to the pathogenesis of Type 2 diabetes after exposure to EtOH in utero. Future work will need to focus on the characteristics of diabetes in aging rats in this model.

GRANTS
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