Abnormal glucose homeostasis in adult female rat offspring after intrauterine ethanol exposure

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Ethanol (EtOH) is a common component of the human diet. About 7% of pregnant women drink EtOH, and in some communities nearly 50% of women of reproductive age are binge drinkers (30, 51). Some children whose mothers consume EtOH during pregnancy are born with multiple birth defects, mental retardation, and delayed growth collectively known as Fetal Alcohol Syndrome; however, the majority have less severe abnormalities known as fetal alcohol effects (3, 25). Abnormalities of glucose homeostasis have been reported in the offspring of humans (8) and animals in association with prenatal EtOH exposure (29, 47). More recently, we and others have demonstrated that EtOH consumption during pregnancy in amounts resulting in blood EtOH levels found in nonintoxicated EtOH users (46) is associated with insulin resistance, hyperlipidemia, and glucose intolerance in male rat offspring (10, 11, 13, 18, 33) without necessarily causing IUGR (12). These male rats may develop diabetes with fasting hyperglycemia during adulthood and have impaired muscle signaling in skeletal muscle (13, 18, 53). This was demonstrated by probing steps in the phosphatidylinositol 3 (PI3)-kinase insulin signaling pathway, which is necessary for insulin-stimulated glucose transporter 4 (glut4) docking to plasma membranes prior to glucose transport (9, 38). Insulin signaling downstream of PI3-kinase is mediated by the serine/threonine kinases Akt/protein kinase B (PKC) (19, 22, 26). Akt and PKCζ are activated by PI3-kinase and PKCζ phosphorylation is reduced. In addition, the expression of the protein tribbles-3 and the phosphatase enzyme activity of phosphatase and tensin homolog deleted on chromosome 10 (PTEN), which prevent Akt activation, were increased in muscle from EtOH-exposed rats. Female rat offspring exposed to EtOH in utero develop insulin-resistant diabetes in association with excessive PTEN and tribbles-3 signaling downstream of the phosphatidylinositol 3-kinase pathway in skeletal muscle, which may be a mechanism for the abnormal glucose tolerance.

Prenatal adverse factors have been implicated in the pathogenesis of chronic diseases in adulthood. The importance of these factors was first recognized by epidemiological studies describing associations between intrauterine growth restriction (IUGR) and insulin resistance, type 2 diabetes, and cardiovascular diseases later in life (2). Because the offspring exposed to such pre-natal factors were small at birth, these abnormalities are considered to be a consequence of IUGR. In animal models of IUGR employing malnutrition (32), placental ischemia (39), glucocorticoid exposure (4), or diabetes (1) during pregnancy, the offspring develop insulin resistance, glucose intolerance, and obesity with aging.

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pregnant, as a prelude to gestational studies. We report here that adult female rat offspring exposed to EtOH in utero develop abnormal glucose homeostasis at an earlier age than what is known in other models of prenatal adverse events (20, 31, 44).

METHODS

Animals. We exposed rat offspring to EtOH in utero as described previously (10, 11), with minor modifications. Briefly, virgin Sprague-Dawley rats were purchased from the University of Manitoba Central Animal Care Facility, randomly divided into three weight-matched groups and time-mated. One group was given EtOH, 2 g/kg (36%) by gavage twice daily from gestational day 1 to parturition, and the other 2 groups were given the same volume of water instead of EtOH. Among the latter, one group (pair-fed, PF) was fed the amount of chow consumed by the EtOH group, whereas the other group was given free access to chow (Control). With this method, we have obtained a peak alcoholemia of 115 mg/dl and 70 mg/dl at 2 and 4 h after ingestion, respectively (10), similar to levels found in clinically nonintoxicated EtOH users (46). The dams were housed individually, and body weight and food intake were measured daily as reported (10, 11). Offspring were culled to eight per lactating dam and kept with their own mothers until weaning on day 21. At 12–13 wk of age (3 mo), female offspring from each group were fasted for 15 h, and the procedures described below were performed. Each procedure included offspring from each treatment group and from each litter in a treatment group. All the studies were approved by the Committee for Animal Use in Research and Teaching of the University of Manitoba.

Glucose tolerance test. At 3 mo of age, offspring in the three rat groups were fasted overnight and underwent an intraperitoneal glucose tolerance test (IPGTT) by 9:00 AM the next morning. Glucose (30% wt/vol, 2 g/kg body wt) was injected intraperitoneally, and tail blood (40 μl) was sequentially collected for glucose and insulin determinations. The rats were killed by exsanguination, and the red gastrocnemius muscle was stored at −20°C until used. Aliquots of plasma were stored at −20°C until assayed.

Hyperinsulinemic euglycemic clamp. Rats were laid on a heating table to maintain normal body temperature. Indwelling catheters were placed in the left carotid artery and the right internal jugular vein, after a single intraperitoneal injection of ketamine (90 mg/kg) and xylazine (10 mg/kg). This allowed maintaining anesthesia throughout the experiment. The trachea was cannulated to allow for spontaneous breathing. The rats were allowed to stabilize for 60 min after surgery before infusions were started, during which time no changes in glycemia were observed (37). Insulin, 0.8 U·kg−1·h−1 and variable amounts of 20% glucose were infused for 3 h through the intravenous catheter using a CMA/100 microinjection pump (CMA Microdialysis AB, Solna, Sweden) and an IVAC 710 pump (IVAC, San Diego, CA), respectively. Blood was sampled every 5–10 min through the arterial line. Glycemia was clamped at ~5 mmol/l. Glucose infusion rate during the last 30 min of insulin infusion was considered to be an estimate of whole body glucose utilization.

PCR analysis. TRIZol, oligo(deoxythymidyidine) primers, SuperScript reverse transcriptase, Taq DNA polymerase, and cDNA primers were obtained from Invitrogen (Carlsbad, CA). RT-PCR assays were performed as previously described (10, 11). 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(deoxythymidyidine) primers. The reverse transcription product (5 μl) was amplified by PCR using specific primers for PTEN (sense 5′-GGAAAGAGGCAGGTCTCTGTA-3′, antisense 5′-TGCCAATCTGACCGTATCCA-3′) and TRB3 (sense 5′-ACCAACCCCCAGCTACACCTC-3′, antisense 5′-CCCCACCTCCCTTTCCCT-3′) with Taq DNA polymerase. Another 5 μl of the reverse transcription product was amplified with β-actin primers as an internal control (sense 5′-GCCAAACTG-
Tukey's HSD. Data are expressed as the means ± SE. *P < 0.05 was considered significant.

RESULTS

Body weight and food intake. Figure 1 shows body weight and food intake of dams during pregnancy. The average daily chow intake of EtOH and PF rats during pregnancy was ~30% lower than in controls. With the calories from EtOH (4 g·kg⁻¹·day⁻¹ = 28 kcal·kg⁻¹·day⁻¹), however, the EtOH dams had ~11 kcal/day in excess vs. PF and control dams, respectively. The weight of EtOH dams was not significantly different from that of PF and control rats throughout pregnancy. The duration of pregnancy was 22 days in control rats, whereas it spanned from 21 to 23 days in EtOH and PF rats (Table 1). Litter size was comparable between groups, but 2 out of 7 pups from one PF dam and 5 out of 11 pups from one EtOH dam died during the first 24 h after birth.

On day 1 of life, the variance of offspring body weight was slightly different between groups [F(2,78) = 3.59, P < 0.05], and Tukey's intergroup comparison showed a slightly increased weight in PF pups, but no weight difference between the EtOH and control groups (Table 1). Subsequently, growth curves diverged (Fig. 2A), so that at 3 mo of age, the variance of body weight was highly different between groups [F(2,33) = 6.59, P < 0.005], and offspring of EtOH, and PF dams were significantly heavier than controls (Table 1).

Glucose tolerance. We measured glucose and insulin levels during an IPGTT after an overnight fast in 3-mo-old rats. Glucose and insulin levels were significantly higher in EtOH offspring than in both PF and control rats and fasting blood glucose concentrations were >7.0 mmol/l in all EtOH animals examined (Fig. 2, B and C). These results indicate that EtOH offspring had insulin-resistant diabetes.

Euglycemic hyperinsulinemic clamp. During insulin infusion, the insulin levels progressively increased in all three groups of rats and were similar during the last hour of the infusion (Fig. 3). In EtOH rats, blood glucose concentrations (which were elevated during fasting) decreased toward control values, and glucose was clamped at ~5.0 mmol/l in all three groups. The glucose infusion rate at euglycemia was similar between PF and controls but significantly lower in EtOH offspring, confirming the presence of insulin resistance in these rats.

Insulin signaling to Glut4. Glut4 translocation to the plasma membrane is a requirement for insulin-stimulated glucose transport and requires intact signaling through PI3-kinase, which has Akt and PKCζ as downstream targets. To explain the whole body insulin resistance determined during IPGTT and clamp procedures, we examined the effect of an insulin bolus on membrane glut4, and on PDK1, Akt, and PKCζ phosphorylation, which is an indication of their activation (Figs. 4 and 5). We found no group difference in basal glut4 level or in phospho-PDK1, phospho-Akt, or phospho-PKCζ phosphorylation, which is an indication of their activation. During insulin infusion, insulin significantly increased the phosphorylation of Akt (Fig. 4, C and D), PDK1 (Fig. 5B), and PKCζ (Fig. 5C) in PF and control rats but failed to increase their phosphorylation in EtOH rats. Similarly, insulin increased the membrane content of Akt (Fig. 4B) and PKCζ (Fig. 5D) in PF and controls, but not in EtOH rats.

Akt inhibiting proteins. To further explain at the molecular level the lack of activation of Akt, we determined the expression of PTEN and TRB3, two known inhibitors of Akt (Fig. 6). Muscle PTEN enzymatic activity was similar between PF and control offspring but was significantly increased in EtOH rats [F(2,15) = 8.31, P < 0.005]. PTEN level was determined by Western blot and was also significantly elevated in EtOH offspring compared with both PF and control offspring [F(2,15) = 9.44, P < 0.05], but there was no difference between the latter two groups. Similarly, PTEN mRNA level was increased in EtOH rats [F(2,15) = 16.92, P < 0.0001], but not in PF rats, compared with controls. The expression of TRB3 determined by both Western blot [F(2,15) = 21.50, P < 0.0001] and RT-PCR [F(2,15) = 10.70, P < 0.001] was also significantly increased in EtOH rats vs. PF and control rats.

Table 1. Offspring characteristics

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>PF</th>
<th>EtOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestation, days</td>
<td>22.0±0.0</td>
<td>22.3±0.3</td>
<td>21.6±0.2</td>
</tr>
<tr>
<td>Litter size</td>
<td>12.5±2.3</td>
<td>12.8±2.1</td>
<td>13.4±0.7</td>
</tr>
<tr>
<td>Birth weight, g</td>
<td>5.8±0.1</td>
<td>6.0±0.1*</td>
<td>5.7±0.1</td>
</tr>
<tr>
<td>Adult weight, g</td>
<td>247±5</td>
<td>288±8*</td>
<td>276±10*</td>
</tr>
</tbody>
</table>

Values are presented as means ± SE. Female offspring: n = 25–30/group. *P < 0.05 vs. control, by Tukey’s honestly significant difference test. PF, pair-fed; EtOH, ethanol.
DISCUSSION

We have previously reported that prenatal EtOH exposure results in impaired glucose homeostasis in male rat offspring (10–12, 14). Adult male rat offspring were glucose intolerant in spite of hyperinsulinemia and had decreased insulin sensitivity, as determined by frequently sampled intravenous glucose tolerance test with minimal modeling (12). The glucose intolerance was associated with increased hepatic gluconeogenesis and impaired skeletal muscle insulin signaling (13). A subset of the adult EtOH rats was diabetic with fasting hyperglycemia (53). In the present report, we extend these findings to female rats, and we report that adult female rats prenatally exposed to EtOH develop glucose intolerance with fasting hyperglycemia. In addition, the rats have whole body insulin resistance, as suggested by IPGTT and confirmed by euglycemic clamp as an explanation for their glucose intolerance. Furthermore, the rats have impaired skeletal muscle insulin signaling as an explanation of total body insulin resistance.

Clamp studies were carried out while the rats were sedated with only one injection of ketamine-xylazine. At a high dose, these chemicals have been reported to suppress plasma insulin, while increasing counterregulatory hormones, resulting in hyperglycemia in fed, but not in fasting rats (37). These drugs were given to all our rats while fasting, and the abnormal glucose homeostasis in offspring of EtOH rats was demonstrated using several other techniques, including fasting glucose, IPGTT, insulin signaling proteins, and gene expression. Therefore, differences in glucose homeostasis between groups cannot be attributed to anesthesia. In addition, these effects are clearly related to prenatal EtOH exposure and not to malnutrition, because offspring of PF dams had a normal glucose
homeostasis, even though their mothers were fed less total calories than EtOH dams.

There have been several reports of altered glucose homeostasis in male adult rat offspring as a consequence of maternal EtOH ingestion during pregnancy. Most studies reported normal glucose concentrations with increased insulin levels as a manifestation of insulin resistance (reviewed in Ref. 45). Only three previous studies formally assessed insulin sensitivity in these rats. Elton et al. (18) reported that male rats exposed to EtOH in utero had a reduced glucose uptake in soleus muscle, but whole body insulin sensitivity measured during euglycemic clamp was not affected. The discrepancy between in vivo and in vitro insulin sensitivity was attributed to a predominance of white muscle over red muscle in rats.

Fig. 4. Membrane-associated glut4 (A), Akt (B), and phospho-Akt (C, D) in control (CF, white bars), EtOH (EF, black bars), and pair-fed (PF, gray bars) female rat offspring before (−, nonhatched bars) and after (+, hatched bars) intravenous insulin administration. Actin was used as a control for protein loading. Protein levels are expressed in arbitrary units relative to controls. Representative blots are shown. The results are shown as the means ± SE, n = 6/group. *P < 0.05, **P < 0.01, CF(+) vs. CF(−); #P < 0.05, ##P < 0.01, PF(+) vs. PF(−), by paired t-test.

Fig. 5. Membrane-associated PDK1 (A), phospho-PDK1 (B), PKCζ (C), and phospho-PKCζ (D) in control (CF, open bars), EtOH (EF, solid bars), and pair-fed (PF, gray bars) female rat offspring before (−, nonhatched bars) and after (+, hatched bars) intravenous insulin administration. Actin was used as a control for protein loading. Protein levels are expressed in arbitrary units relative to controls. Representative blots are shown. The results are shown as the means ± SE, n = 6/group. **P < 0.01, CF(+) vs. CF(−); #P < 0.05, ##P < 0.01, PF(+) vs. PF(−), by paired t-test.
Of note, this study assessed insulin responsiveness expressed as glucose clearance per gram of body weight, as opposed to insulin sensitivity, which takes into account prevailing insulin levels (27, 35). We have shown a reduction of insulin sensitivity and glucose intolerance in male rats exposed to EtOH in utero using the minimal model approach (12). Sadri et al. (36), using a technique called rapid insulin sensitivity test, showed increased insulin resistance in female rats prenatally exposed to EtOH, which was attributed to the release of a putative hepatic insulin-sensitizing substance. We have previously shown that male rats exposed to EtOH in utero have a reduced glut4 content in gastrocnemius muscle (10, 11, 13), which is in agreement with findings of reduced glucose uptake in isolated soleus muscle of rats exposed to EtOH in utero (18). In addition, EtOH-exposed rats had increased resistin expression, which may have contributed to insulin resistance (11). We now show that 3-mo-old female rats are insulin resistant and diabetic as a result of EtOH exposure early in life. As fasting hyperglycemia was inconsistently found in male offspring, these results are consistent with the well-known sensitivity of females to EtOH and complement other models in which female offspring did not develop glucose intolerance or developed insulin resistance only during senescence (20, 31, 44). It is also interesting that EtOH-exposed female rats had abnormal glucose homeostasis despite having normal birthweight. This may seem to contradict our previous report in male rat offspring that glucose intolerance occurs with decreased birthweight after intrauterine EtOH exposure (10). In fact, male rat offspring had a bimodal distribution of birthweight, which was low or normal, and glucose intolerance was prevalent regardless of birthweight (13). The findings of abnormal glucose homeostasis despite normal birthweight and the fact that offspring of PF dams in this study had normal glucose tolerance suggest that EtOH, not decreased food intake, is the cause of the observed anomalies.

Whole body insulin resistance is primarily explained by a reduction of insulin-stimulated glucose transport in skeletal muscle (16). Insulin-stimulated glucose uptake requires translocation of glut4 from the intracellular compartment to the plasma membrane (15, 40). Elton et al. (18) have demonstrated a reduction of insulin-stimulated glucose uptake in isolated soleus muscle from adult male rats exposed to EtOH in utero, and we have reported a reduction of gastrocnemius muscle membrane glut4 after an oral glucose load or insulin administration in these animals (10, 11, 13). We show here that the association of glut4 with muscle cell membrane in response to insulin is reduced in female EtOH-exposed rats compared with PF and controls. In skeletal muscle, the effects of insulin on glucose uptake are mediated by Akt and PKCi in the PI3-kinase arm of the insulin-signaling pathway (38, 41). Insulin binding to the α-subunit of the insulin receptor activates the intrinsic receptor tyrosine kinase (IRTK), which phosphorylates the receptor β-subunit and causes insulin receptor substrate-1 (IRS-1) tyrosine phosphorylation. The IRS-1 molecule binds the p85 subunit of PI3-kinase, activating the PI3-kinase arm of the insulin-signaling pathway (38, 41). Insulin binding to the α-subunit of the insulin receptor activates the intrinsic receptor tyrosine kinase (IRTK), which phosphorylates the receptor β-subunit and causes insulin receptor substrate-1 (IRS-1) tyrosine phosphorylation. The IRS-1 molecule binds the p85 subunit of PI3-kinase, causing activation of the p110 catalytic subunit of the kinase. This, in turn, generates the lipid product PIP₃, which via PDK1 leads to activation of enzymes such as Akt and PKCi, resulting in glut4 translocation. We have previously shown that EtOH-exposed rats had decreased tyrosine phosphorylation of the insulin receptor β-subunit and of IRS-1, as well as reduced IRS-1-associated PI3-kinase in the gastrocnemius muscle (13). We show here that insulin-stimulated PDK1 and Akt phosphorylation, and thus activity, is decreased in EtOH-exposed rats, which could account for the decreased glut4 translocation and insulin resistance.
We also examined the atypical PKC\(\varepsilon\) activation by insulin because, besides Akt, PI3-kinase can also activate PKC\(\varepsilon\), which can contribute to GLUT4 translocation to the plasma membrane (19, 26). Upon insulin stimulation, PKC\(\varepsilon\) moves to the cell membranes and fuses with GLUT4 vesicles before translocating to plasma membranes, providing a mechanism for insulin-stimulated glucose transport (23, 43). We found reduced PKC\(\varepsilon\) phosphorylation in response to insulin in rats exposed to EtOH in utero, in agreement with reports in rodents, primates, and humans with glucose intolerance or type 2 diabetes (19, 48). We also found a reduced amount of PKC\(\varepsilon\) associated with muscle membranes in response to insulin in these rats. To verify that membrane-associated PKC\(\varepsilon\) represented the active enzyme, we probed membranes with phospho-PKC\(\varepsilon\) antibody. As expected, we found a reduction of membrane-associated phospho-PKC\(\varepsilon\) after insulin treatment in EtOH-exposed rats compared with controls. These results strongly suggest that a defective Akt and PKC\(\varepsilon\) activation by insulin is an explanation for the whole body insulin resistance found in rats exposed to EtOH in utero.

Because of the recent identification of PTEN and TRB3 as Akt inhibitors, we sought to determine their expression in EtOH-exposed rats. PTEN is a lipid phosphatase that dephosphorylates PIP3 and prevents PI3-kinase induced PDK1 activation and subsequent activation and recruitment of Akt and PKC\(\varepsilon\) to the cell membrane. Because PI3-kinase catalyzes the formation of PIP3, PTEN antagonizes this PI3-kinase function and inhibits Akt and PKC\(\varepsilon\) activation. Studies using inhibition of PTEN production in the liver and adipocytes have shown an improvement of insulin sensitivity in diabetic db/db and ob/ob mice (7), whereas elevated levels of PTEN were reported in skeletal muscle of diabetic obese Zucker (fa/fa) rats (28). In line with these studies, we found increased PTEN expression in skeletal muscle from insulin-resistant EtOH rats, as shown by studies of mRNA, Western blot analysis, and enzyme activity. The reason for increased PTEN expression is currently unclear. However, resistin, which is increased in EtOH-exposed rats (11), is known to upregulate PTEN (42). This association suggests a role for resistin to reduce insulin sensitivity in these animals, as previously proposed in male rats (11).

TRB3 is an adaptor protein that binds to Akt and prevents its phosphorylation, resulting in insulin resistance. TRB3 is overexpressed in liver during fasting and in insulin-resistant mice, and its inhibition improves insulin sensitivity (17). TRB3 is also expressed in skeletal muscle, where it is induced by glucose deprivation (52). It has been reported that TRB3 is induced in liver of EtOH-fed rats and could explain insulin resistance in these animals (21). Our observation of increased expression of TRB3 in muscle of rats prenatally exposed to EtOH is consistent with the observed insulin resistance in these animals.

In conclusion, this study suggests that female rat offspring exposed to EtOH in utero develop insulin-resistant diabetes in association with excessive PTEN and TRB3 signaling downstream of the PI3-kinase pathway in skeletal muscle, which may be a mechanism for the abnormal glucose tolerance.

**GRANTS**

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**REFERENCES**


