In vivo insulin signaling through PI3-kinase is impaired in skeletal muscle of adult rat offspring exposed to ethanol in utero

Li Chen,1,3 Xing-Hai Yao,4 and B. L. G. Nyomba1,2

Departments of 1Internal Medicine and 2Physiology, University of Manitoba, Winnipeg, Manitoba, Canada; and 3Department of Pharmacology, Jilin University, Changchun, China

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First published March 24, 2005; doi:10.1152/japplphysiol.01098.2004.—It is now known that prenatal ethanol (EtOH) exposure is associated with impaired glucose tolerance and insulin resistance in rat offspring, but the underlying mechanism(s) is not known. To test the hypothesis that in vivo insulin signaling through phosphatidylinositol 3 (P13)-kinase is reduced in skeletal muscle of adult rat offspring exposed to EtOH in utero, we gave insulin intravenously to these rats and probed steps in the PI3-kinase insulin signaling pathway. After insulin treatment, EtOH-exposed rats had decreased tyrosine phosphorylation of the insulin receptor β-subunit and of insulin receptor substrate-1 (IRS-1), as well as reduced IRS-1-associated PI3-kinase in the gastrocnemius muscle compared with control rats. There was no significant difference in basal or insulin-stimulated Akt activity between EtOH-exposed rats and controls. Insulin-stimulated PKC ζ isoform phosphorylation and membrane association were reduced in EtOH-exposed rats compared with controls. Muscle insulin binding and peptide contents of insulin receptor, IRS-1, p85 subunit of PI3-kinase, Akt/PKB, and atypical PKC ζ isoform ζ were not different between EtOH-exposed rats and controls. Thus insulin resistance in rat offspring exposed to EtOH in utero may be explained, at least in part, by impaired insulin signaling through the PI3-kinase pathway in skeletal muscle.

Akt; fetal growth restriction; insulin resistance; protein kinase C; intrauterine growth restriction

Epidemiologic studies have shown that adverse events during pregnancy may program the fetus to later develop insulin resistance and Type 2 diabetes in adulthood (4, 45, 46). Animal models of intrauterine growth restriction (IUGR) employing malnutrition (26, 60), placental ischemia (53), glucocorticoid exposure (6), or diabetes (59) during pregnancy have confirmed these data. Ethanol (EtOH) consumption during pregnancy can lead to abnormal fetal development, a spectrum of effects that include fetal alcohol syndrome and less severe abnormalities known as fetal alcohol effects (17). Fetal alcohol syndrome is characterized by IUGR, abnormal facial features, and central nervous system problems. Remarkably, the prevalence of fetal alcohol syndrome is elevated in populations with lower socioeconomic status (7), where Type 2 diabetes is also common (39). In a recent study in mice, the frequency of IUGR in the offspring was synergistically increased by maternal diabetes and EtOH exposure (43). We and others have shown that EtOH ingestion during pregnancy in amounts corresponding to human chronic drinking (69) leads to IUGR and is associated with glucose intolerance in the adult offspring (9, 14, 15, 23, 37). These studies have suggested that EtOH-induced IUGR is associated with insulin resistance in later life. Recently, we have demonstrated a reduction of whole body insulin sensitivity in association with tissue accumulation of triglycerides (13) and an ~35% reduction of the insulin-sensitive glucose transporter 4 (GLUT4) in skeletal muscle membranes of rats exposed to EtOH in utero (14, 15). Elton et al. (23) have reported a reduction of glucose uptake in isolated muscle from EtOH-treated rat offspring with, however, a normal insulin receptor kinase activity.

We hypothesized that in vivo insulin signaling downstream of the insulin receptor is impaired in skeletal muscle of rats exposed to EtOH in utero. This hypothesis was tested by probing steps in the phosphatidylinositol 3 (P13)-kinase insulin signaling pathway in adult rats with IUGR due to prenatal EtOH exposure. Insulin binding to its receptor activates the insulin receptor kinase, which leads to a tyrosine phosphorylation of several insulin receptor substrates (IRS) (31, 48). In skeletal muscle, IRS-1 is the most important of these substrates (12), and IRS-1 tyrosine phosphorylation promotes its association with the p85 regulatory subunit of PI3-kinase and activation of the p110 catalytic subunit of the kinase (2, 3). PI3-kinase activation is a necessary step for insulin-stimulated glucose transport (11, 52). Insulin signaling downstream of PI3-kinase is mediated by the serine/threonine kinases Akt1/2, the phosphoinositide 3-kinase (PI3K) and the atypical protein kinase C ζ (51). Both Akt and PKCζ play a role in glucose transport. One or several defective steps in this pathway could explain the in vivo insulin resistance found in rats exposed to EtOH in utero.

Methods

Materials. EtOH was obtained from pharmaceutical services of the Health Sciences Centre (Winnipeg, MN, Canada). Electrophoresis and electroblotting consumables were from Bio-Rad (Hercules, CA). Rabbit polyclonal phospho-Akt antibody (catalog no. 9275) and the Akt kinase assay kit (catalog no. 9840) were purchased from Cell Signaling Technology (Beverly, MA). Receptor grade porcine [125I]Tyr114 insulin (catalog no. NEX196) was purchased from Perkin-Elmer Life Science (Boston, MA). Anti-insulin receptor β-subunit (catalog no. 06–492), -phosphotyrosine (pY) (catalog no. 06–427), and -p85 (catalog no. 06–195) antibodies were from Upstate (Charlottesville, VA). Polyclonal anti-GLUT4 (catalog no. sc-7938), -IRS-1 (catalog no. sc-559), -Akt1/2 (catalog no. sc-8312), -PKCζ (catalog no. sc-216), -phospho-PKCζ (Thr410) (catalog no. sc-12894-R), and agarose-conjugated anti-IRS-1 (catalog no. sc-559AC) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Polyvinylidene difluoride membranes were from Bio-Rad (Hercules, CA). Nitrocellulose membranes were from Bio-Rad (Hercules, CA). The electroblots were from Bio-Rad (Hercules, CA). 3,3′-Diaminobenzidine was from Sigma-Aldrich (St. Louis, MO).

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Santa Cruz Biotechnology (Santa Cruz, CA). Protease inhibitor cocktail tablets were purchased from Roche Diagnostics (Penzberg, Germany). Enhanced chemiluminescence kit was obtained from Amersham Pharmacia (Piscataway, NJ). Isopropyl alcohol and methanol were from Fisher Scientific (Nepean, ON, Canada). All other chemicals were purchased from Sigma-Aldrich (Oakville, ON, Canada).

Animals. Adult rat offspring exposed to EtOH in utero were generated as described before (13–15). Briefly, time-mated Sprague-Dawley rats were randomly divided into two weight-matched groups. Throughout pregnancy, one group was given EtOH, 2 g/kg (36%), by gavage twice daily, and the other group was given the same volume of water instead of EtOH. The offspring exposed to EtOH were smaller than controls (14, 15). Male offspring were culled to four to five per lactating dam and kept with their mothers until weaning on day 21. At 16 wk of age, offspring from each group were fasted overnight, and by 9:00 AM the next morning they were anesthetized with an intraperitoneal injection of ketamine (90 mg/kg) and xylazine (10 mg/kg). The hindlimb muscles were exposed by carefully cutting free the exterior skin. Regular insulin (Humulin R, 2 U/kg, Eli Lilly, Indianapolis, IN) and the equivalent volume of saline, was injected into the left femoral vein followed by a rapid dissection of the lateral head of the gastrocnemius muscle 5 min later. This time frame has been demonstrated to be sufficient for in vivo signaling activation by insulin in rats (38, 67). The muscle was rapidly trimmed of any visible fat, immediately frozen in liquid nitrogen, and stored at −70°C until used. All animal studies were approved by the Committee for Animal Use in Research and Teaching of the University of Manitoba.

Preparation of muscle homogenate. Gastrocnemius muscle tissue was homogenized in an ice-cold 1-ml buffer containing 20 mM Tris, pH 7.4, 140 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, 1% Triton X-100, 10 mM sodium pyrophosphate, 10 mM NaF, 2 mM Na3VO4, 2 mg/ml benzamidine, 1 mM PMSF, and a protease inhibitors cocktail (1 tablet/10 ml). Muscle lysates were centrifuged for 10 min at 14,000 g. The supernatant was used for insulin signaling studies described below. Protein was measured using Bio-Rad protein assay method.

Preparation of cell membranes. Gastrocnemius muscle tissue was homogenized for 5 s in ice-cold TES buffer (20 mM Tris·HCl, pH 7.4, containing 250 mM sucrose, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.01 mM leupeptin, and 5 μg/ml aprotinin). After centrifugation at 3,000 g for 10 min at 4°C, the supernatant was subjected to centrifugation at 100,000 g for 90 min. The new supernatant was collected as cytosol, and the precipitate (membranes) was resuspended in ice-cold TES by shearing through 22-, 25-, and 30-gauge needles using at least 10 passes per needle until the precipitate was well resuspended.

Insulin binding. Insulin binding was performed as described by Saucier et al. (49). Membranes (200 μg/tube) were incubated for 20 h at 4°C in Tris·HCl buffer containing 125I-labeled insulin, 5% insulin free bovine serum albumin, and increasing concentrations of unlabeled porcine insulin. After the incubation, the samples were centrifuged on a sucrose layer for 20 min at 15,000 g. The pellet was obtained by cutting the lower end of the tube with a sharp razor blade and counted in a gamma counter.

Western blotting. Membranes or homogenate (50 μg protein/lane) were separated by SDS-PAGE and electroblotted onto nitrocellulose membranes. The blots were blocked with 5% dry milk and incubated overnight at 4°C with the following antibodies at 1:1,000 dilution, except when indicated otherwise: anti-IRS-1 (1 μg/ml), -p85 (1 μg/ml), -Akt, -phospho-Akt, -PKCζ, and -phospho-PKCζ. The blots were then washed in TBS-Tween for 15 min, incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody at 1:3,000 for 1 h at room temperature, and washed in TBS-Tween for 15 min. Immune complexes were detected using the enhanced chemiluminescence detection kit after the blots were exposed to a Kodak X-Omat AR (XAR-5) film. Protein contents were quantified by densitometry using NIH Image software, and the reading was corrected for that of the positive control used as standard (14).

Immunoprecipitation. Muscle homogenates (500 μg of protein) were immunoprecipitated overnight at 4°C with 4 μg of anti-insulin receptor antibody and protein A-Sepharose beads or with 5 μg of agarose-conjugated anti-IRS-1 antibody. The immunoprecipitates were washed twice with PBS, redisolved in 20 μl of buffer, subjected to SDS-PAGE, and transferred to nitrocellulose membranes. The blots were incubated with insulin receptor (4 μg) or IRS-1 (1 μg) antibodies to estimate the amounts of these proteins, or with phosphotyrosine (1 μg) or p85 (1 μg) antibodies to detect tyrosine phosphorylation and p85 association with IRS-1. After being washed, the blots were incubated with goat anti-rabbit antibody and immunoreactive bands were visualized and quantified as described. Tyrosine phosphorylation of the insulin receptor and IRS-1, and IRS-1-associated p85 in individual samples were corrected for the amounts of insulin receptor and IRS-1, respectively. To further ensure equal protein loading, the blots were stripped in a Tris buffer, pH 6.7, containing 2% SDS and 100 mM mercaptoethanol, and reprobed with the appropriate primary antibody.

Akt activity. Akt activity was determined using a kit from Cell Signaling Technology, according to the manufacturer’s instructions. Briefly, tissue lysates (200 μg of protein) were immunoprecipitated for 4 h at 4°C with a monoclonal anti-Akt antibody coupled to agarose beads. The immunoprecipitates were incubated with paramyosin-GSK-3α/β crosstide fusion protein in the presence of ATP, allowing GSK-3 phosphorylation by active Akt. After electrophoresis, phospho-GSK-3 was detected by Western blotting with anti-phospho-GSK-3α/β (Ser21/9) antibody. In parallel experiments, Akt immune pellets were separated on SDS-PAGE and blotted with the anti-Akt antibody. The bands were used for the correction of Akt activity.

Statistics. The data were compared by unpaired Student’s t-test or ANOVA with Duncan’s test as appropriate. Values are expressed as means ± SE. P < 0.05 was considered significant.

RESULTS

GLUT4 translocation to the plasma membrane is a requirement for insulin-stimulated glucose transport and requires intact signaling. This classically involves signaling through the PI3-kinase pathway. We have previously shown an ~35% reduction in GLUT4 content in muscle membranes after oral glucose challenge in rats with EtOH-induced IUGR (14, 15). We now show that muscle membrane GLUT4 content similarly decreases after insulin administration in these rats (Fig. 1). To examine whether EtOH-associated insulin resistance in these animals is due to alterations of the PI3-kinase signaling pathway, we examined the in vivo insulin activation of its intrinsic receptor tyrosine kinase (IRTK) and postreceptor steps in the PI3-kinase pathway. Insulin treatment increased by ~35% the tyrosine phosphorylation of the insulin receptor tyrosine kinase (Fig. 2). In EtOH-exposed rats, an indication that PI3-kinase activity was decreased after prenatal EtOH exposure (Fig. 3A), the insulin-stimulated p85 association of p85 with IRS-1 increased in control but not in EtOH-exposed rats, an indication that PI3-kinase activity was decreased after prenatal EtOH exposure (Fig. 3B).

In separate experiments, we examined the effect of insulin on Akt and PKCζ, which have been implicated in the regulation of GLUT4 translocation. Akt activity was slightly elevated.
in the basal state in the EtOH-exposed rats compared with controls, but the difference was not significant and the level of Akt activity after insulin treatment was similar between the two groups (Fig. 5A). Akt phosphorylation showed similar changes (Fig. 5B). We found no group difference in basal PKCζ phosphorylation in muscle homogenate, but PKCζ phosphorylation was decreased in EtOH rats after insulin treatment. Because PKCζ associates with the cell membranes on insulin stimulation (56), we measured its content and that of phospho-PKCζ in muscle membranes (Fig. 6A). There was no intergroup difference in membrane-associated PKCζ under basal conditions. However, insulin stimulated PKCζ association with membranes by ∼35% in controls but did not stimulate PKCζ association with membranes in the EtOH group. To investigate whether membrane associated PKCζ represents the active enzyme, which is threonine phosphorylated, we probed the membranes with anti-phospho-PKCζ antibody (Fig. 6B). Whereas PKCζ phosphorylation was similar between the two groups under basal conditions, it was increased twofold by insulin in controls but did not significantly change in the EtOH-exposed rats. PKCζ changes in response to insulin paralleled those of IRS-1-associated PI3-kinase. Tissue contents of either Akt or PKCζ proteins were comparable between the two groups of rats (Table 1).

**DISCUSSION**

Previous studies by our laboratory (13–15) and others (23, 37) have shown that rats with EtOH-induced IUGR have impaired glucose tolerance, but the underlying mechanism(s) remain(s) unclear. These rats have increased serum insulin (13–15) and triglycerides (13, 44), which are markers of insulin resistance. In addition, we have recently shown that these animals have increased intramuscular and intrahepatic triglycerides and a decreased whole body insulin sensitivity (13). The animal model we have been using involves EtOH administration by gavage throughout gestation at a dose resulting in alcoholemia of ∼30 mM (14), corresponding to human heavy drinking (69). Feeds intake of EtOH-treated dams is <10% that of controls, but weight gain during pregnancy, litter size, and perinatal mortality are similar to controls. Alcoholemia in our model is similar to that reported by Pennington and colleagues (23, 44). These investigators administered EtOH in a liquid diet, and pups were surrogate fostered to nontreated dams. Cross-fostering is used to prevent a delay in pup weight gain while they are suckling from their own undernourished mothers or to study the effect of chronic alcoholism during lactation or gestation separately (62). We did not use cross-fostering because dams in our model show no signs of malnutrition compared with normal (13–15) or pair-fed controls (unpublished observations). In addition, it has been suggested that cross-fostering probably does not influence offspring development after in utero EtOH exposure (62). We have also shown that insulin resistance develops in offspring even after EtOH exposure through lactation following an otherwise normal pregnancy (13). Thus either prenatal or postnatal EtOH exposure can cause insulin resistance in the rat offspring.

Whole body insulin resistance is primarily explained by a reduction of insulin-stimulated glucose transport in skeletal muscle (20). Insulin-stimulated glucose uptake requires translocation of GLUT4 from the intracellular compartment to the plasma membrane and their subsequent activation (19, 54). Elton et al. (23) have demonstrated a reduction of insulin-stimulated glucose uptake in isolated red muscle from adult rats exposed to EtOH in utero, and we have reported a reduction of muscle GLUT4 after an oral glucose load in these animals (14, 15). We show here that the association of GLUT4 with cell membrane in response to insulin was reduced in EtOH-exposed rats compared with controls. In skeletal muscle, the effects of insulin on glucose uptake are primarily mediated...
by the IRS-1/PI3-kinase arm of the insulin-signaling pathway (52, 55). Insulin binding to the α-subunit of the insulin receptor activates the IRTK, which phosphorylates the receptor β-subunit and causes 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 activates Akt, leading to GLUT4 translocation. We here show that IRTK has a reduced response to activation by insulin in EtOH-exposed rat offspring compared with controls, despite similar insulin binding among the two groups. As a consequence, the ability of insulin to stimulate IRS-1 phosphorylation and its association with PI3-kinase was also reduced in these animals. Basal Akt activity was also slightly increased in EtOH-exposed rats, but the level of enzyme activity after insulin treatment was normal, although it could be argued that the extent of insulin-induced changes was reduced. Because the insulin receptor and Akt proteins were similar between groups, their activities were not explained by differences in protein contents.

We also examined the atypical PKCζ activation by insulin because recent studies have shown that, besides Akt, PI3-kinase can also activate PKCα/ζ and that PKCα/ζ can contribute to GLUT4 translocation to the plasma membrane (24, 33). On insulin stimulation, PKCα/ζ moves to the cell membranes and fuses with GLUT4 vesicles before translocating to plasma membranes, providing a mechanism for insulin-stimulated glucose transport (29, 56). We found reduced PKCζ phosphorylation in response to insulin in rats exposed to EtOH in utero, despite normal PKCζ peptide content in the homogenate, in agreement with reports in rodents, primates, and humans with glucose intolerance or Type 2 diabetes (24, 61). We also found a reduced amount of PKCζ associated with muscle membranes in response to insulin in these rats. To verify that membrane-associated PKCζ represented the active enzyme, we probed membranes with phospho-PKCζ antibody. As expected, we found a reduction of membrane-associated phospho-PKCζ after insulin treatment in EtOH-exposed rats compared with controls. These results strongly suggest that a defective PKCα/ζ activation by insulin is an explanation for the whole body insulin resistance found in rats exposed to EtOH in utero.

There appears to be different regulations of Akt and PKCζ activities in EtOH-exposed rats for which a clear explanation is not readily available. Changes in PKCζ and IRS-1-associated PI3-kinase were parallel, whereas Akt activity was dissociated from the kinase in these animals. It is possible that Akt receiving signaling from insulin receptor-bound PI3-kinase, bypassing IRS-1 (22). This mechanism has been proposed in insulin-resistant mice with gestational diabetes as explaining increased Akt activity despite decreased IRS-1-associated PI3-kinase (50). A similar dissociation has been reported in humans, where fatty acid infusion inhibited PI3-kinase but spared Akt (34). It has been suggested that the paradoxical response of Akt and PKC-ζ is due to differential response to PI3,4,5-triphosphate, the lipid product of PI3-kinase (5).

A small number of studies have examined the PI3-kinase signaling pathway in skeletal muscle in offspring born with IUGR. PKCζ activation in these conditions has not been

### Table 1. Insulin signaling peptides in skeletal muscle

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<thead>
<tr>
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<th>Controls (n = 4)</th>
<th>EtOH (n = 4)</th>
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<tbody>
<tr>
<td>Insulin receptor, U</td>
<td>158 ± 12</td>
<td>151 ± 11</td>
</tr>
<tr>
<td>IRS-1, U</td>
<td>134 ± 10</td>
<td>132 ± 8</td>
</tr>
<tr>
<td>p85, U</td>
<td>108 ± 3.4</td>
<td>118 ± 7.5</td>
</tr>
<tr>
<td>Akt, U</td>
<td>148 ± 5.0</td>
<td>155 ± 4.6</td>
</tr>
<tr>
<td>PKCζ, U</td>
<td>101 ± 9.2</td>
<td>107 ± 7.2</td>
</tr>
</tbody>
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Values are means ± SE. EtOH, ethanol; U, arbitrary units; IRS, insulin receptor substrate.
reported before. A human study of genes involved in the PI3-kinase pathway found decreased insulin-stimulated GLUT4 expression but normal insulin receptor, IRS-1, and PI3-kinase expression in skeletal muscle from individuals with low birth weight (30). In rats exposed to global malnutrition in utero, muscle insulin receptor was increased, IRS-1 was normal, but PI3-kinase activation and GLUT4 were reduced (1). Rats exposed to a low-protein diet in utero had a normal insulin receptor and reduced expression of PKC\^{\text{\textregistered}} but PKC\^{\text{\textregistered}} phosphorylation or activity was not reported (42). In a study of EtOH-exposed offspring, muscle IRTK responded normally to insulin, and glucose uptake was reduced, but steps below the IRTK were not studied (23). In this study did not have measurable whole body insulin resistance (23), although they had decreased muscle glucose uptake (23) and increased serum triglycerides (44).

To explain the insulin resistance associated with IUGR, it has been proposed that adverse events during pregnancy cause hyperactivity of the hypothalamic-pituitary-adrenal axis in the offspring, downregulating hippocampal glucocorticoid receptors and thereby blunting feedback control on glucocorticoid secretion and upregulating hypothalamic-pituitary-adrenal activity throughout life, with the resulting hypercortisolism then programming the fetus to metabolic diseases in later life (6, 6, 35). More recently, it has been suggested that programming occurs through control of gene expression mediated by stress-induced alterations in DNA methylation or modifications of chromatin packaging, possibly via changes in histone acetylation. These epigenetic changes in gene expression would explain non-Mendelian transmission of certain features from mother to offspring and might account for programming of insulin resistance (21). Prenatal EtOH exposure can cause hypothalamic-pituitary-adrenal axis hyperactivity (65) and has been reported to alter DNA methylation in some tissues (58), but it is unclear how these changes would affect insulin signaling.

It is well known that chronic heavy EtOH consumption is a risk factor for the development of Type 2 diabetes (27, 57, 64). High EtOH exposure is associated with impaired glucose utilization, although moderate EtOH consumption may increase insulin sensitivity and protect against cardiovascular disease (18, 41, 64). EtOH consumption increases circulating glucose concentrations (25), glucose intolerance, and insulin resistance (51, 68). Heavy EtOH exposure causes oxidative stress (32), which can impair insulin signaling (10), and both Akt and PKC\^{\text{\textregistered}} activities may be sensitive to cellular redox status (16, 36, 40). Our study does not reflect direct effects of EtOH, because the results were obtained in 16-wk-old rat offspring, i.e., 4 mo after intrauterine exposure to EtOH. However, rat dams fed EtOH during pregnancy still showed stigmata of oxidative stress 6 wk after parturition (47), and it is
conceivable that oxidative damage caused by EtOH early in life becomes permanent, resulting in insulin resistance later in life.

In summary, our results indicate that EtOH-induced UGR causes insulin resistance in adult rat offspring by impairing insulin signaling through PI3-kinase and the atypical PKCζ. These results are in line with Barker’s hypothesis that an abnormal intrauterine milieu programs the offspring to insulin resistance later in life. The mechanistic link between EtOH exposure and defective insulin signaling requires further studies.

GRANTS
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REFERENCES


