Hepatic insulin resistance induced by prenatal alcohol exposure is associated with reduced PTEN and TRB3 acetylation in adult rat offspring

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Yao X-H, Nyomba BL. Hepatic insulin resistance induced by prenatal alcohol exposure is associated with reduced PTEN and TRB3 acetylation in adult rat offspring. Am J Physiol Regul Integr Comp Physiol 294: R1797–R1806, 2008. First published April 2, 2008; doi:10.1152/ajpregu.00804.2007.—Prenatal alcohol exposure (EtOH) results in insulin resistance in rats of both sexes with increased expression of hepatic gluconeogenic genes and glucose production. To investigate whether hepatic insulin signaling is defective, we studied 3-mo-old female offspring of dams that were given EtOH during pregnancy compared with those from pair-fed and control dams. We performed an intraperitoneal pyruvate tolerance test, determined the phosphorylation status of hepatic phosphoinositide-dependent protein kinase-1 (PDK1), Akt, and PKCα before and after intravenous insulin bolus, and measured mRNA and in vivo acetylation of TRB3 (tribles 3) and PTEN (phosphatase and tensin homolog deleted on chromosome ten) as well as the expression of the histone acetylase (HAT) PCAF (p300/CREB-binding protein-associated factor), histone deacetylase-1 (HDAC1), and HAT and HDAC activities. In EtOH compared with pair-fed and control offspring, basal and pyruvate-induced blood glucose was increased, insulin-induced PDK1, Akt, and PKCα phosphorylation was reduced, and expression of PTEN and TRB3 was increased while their acetylation status was decreased in association with increased HDAC and decreased HAT activities. Thus female adult rats prenatally exposed to EtOH have increased gluconeogenesis, reduced insulin signaling, and increased PTEN and TRB3 expression in the liver. In addition, PTEN and TRB3 are hypacetylated, which can contribute to Akt-inhibiting activity. These results suggest that hepatic insulin resistance in rats prenatally exposed to EtOH is explained, at least in part, by increased PTEN and TRB3 activity due to both increased gene expression and reduced acetylation.

intrauterine environment; gluconeogenesis; Akt

Prenatal adverse factors program the fetus to later develop insulin resistance and type 2 diabetes later in life, as suggested by epidemiological studies (2) and confirmed in animal models employing malnutrition (30), placental ischemia (33), glucocorticoid exposure (4), or diabetes (39) during pregnancy. We and others have demonstrated that moderate alcohol consumption during pregnancy is associated with low birth weight, insulin resistance, hyperlipidemia, tissue steatosis, and glucose intolerance in rat offspring (8–11, 15, 31). Contrary to controls, β-cell mass in alcohol-exposed offspring fails to increase in response to a nutrition challenge with a high-fat diet, which is an indication of β-cell dysfunction (9). The rat offspring of both sexes have impaired insulin signaling in skeletal muscle, as demonstrated by probing steps in the phosphatidylinositol 3 (PI3)-kinase insulin signaling pathway (11, 15, 44). Male rats also had increased gluconeogenesis and fasting hyperglycemia during adulthood (44).

Insulin signaling downstream of PI3-kinase is mediated by the serine/threonine kinases Akt/PKB (6, 40, 42) and PKCζ (16, 20, 25), which are activated by PI3-kinase via phosphoinositide-dependent protein kinase-1 (PDK1). Both Akt and PKCζ are under inhibitory control by the lipid phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome ten) (7, 26), whereas Akt is also negatively regulated by TRB3 (tribles 3) (14). Although we have demonstrated that insulin stimulation of Akt and PKCζ is impaired along with increased expression of PTEN and TRB3 in skeletal muscle of rat offspring prenatally exposed to alcohol (11, 45), mechanisms leading to hepatic insulin resistance and increased gluconeogenesis in these animals are only partially understood. Liver steatosis and increased expression of gluconeogenic genes may play a role, as previously reported (12, 44). Besides gene expression, however, recent studies suggest that post-translational protein modifications play an important role in the regulation of gluconeogenesis and liver steatosis. For example, the transcriptional coactivator peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), which regulates gluconeogenic enzymes, is controlled by the histone deacetylase (HDAC) SIRT1, which is posttranslationally induced by a nutrient signaling response mediated by pyruvate (32), whereas mice overexpressing HDAC1 exhibit a high incidence of hepatic steatosis (41). We have previously shown that pyruvate injection results in a dramatic blood glucose rise in alcohol-exposed rat offspring (44, 45), implying that PGC-1α deacetylation by SIRT1 is likely to be active in these animals. SIRT1 deacetylates PGC-1α, and this, in addition to PGC-1α gene expression, enhances the induction of gluconeogenic genes and hepatic glucose output (32). Recent evidence also indicates that PGC-1α promotes insulin resistance in liver via induction of TRB3 (24), whereas acetylation of PTEN under the control of the histone acetylase (HAT) PCAF (p300/CREB-binding protein-associated factor) has been shown to reduce its enzymatic activity (29). Because PTEN has been reported to enhance gluconeogenesis (18), its inhibition by acetylation suggests that it may be deacetylated in states of increased gluconeogenesis. It is not known whether TRB3 is also acetylated. It is also interesting that HDAC1 is associated with liver steatosis (41). The purpose of the current study was to investigate the acetylation status of PTEN and TRB3 as well as the balance between HAT and HDAC activities in alcohol-exposed rats as an additional explanation for their insulin resistance and increased gluconeogenesis.

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METHODS

Animals. We exposed rat offspring to alcohol in utero as described previously (8, 9), with minor modifications. Briefly, virgin Sprague-Dawley rats were randomly divided into three weight-matched groups and time mated. One group (EtOH) was given ethanol (2 g/kg, 36%) by gavage twice daily from gestational day 1 to parturition, and the other two groups were given the same volume of water instead of EtOH. Among the latter, one group (PF) was pair fed the amount of chow consumed by the EtOH group, whereas the other group (control) was given free access to chow. This EtOH dose is moderate (21, 23) and results in alcoholemia of 115 mg/dl 2 h after ingestion, decreasing to 70 mg/dl 4 h later (8, 9), which is considered safe for driving in humans. 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. The general characteristics of these animals, including duration of gestation, litter size, body weight, and feed intake, were previously reported (45).

Pyruvate tolerance test. To estimate gluconeogenesis, we administered a pyruvate load as described previously (44). Briefly, rats were injected intraperitoneally with pyruvate (2 g/kg) dissolved in saline. Control experiments were performed after oral administration of 3-mercaptopicolinic acid (3-MPA; 30 mg/kg), an inhibitor of gluconeogenesis, 30 min before the pyruvate injection. Glucose was determined in tail blood every 30 min for 2 h using the Ascensia Elite XL blood glucose meter (Bayer HealthCare).

Insulin treatment. Groups of rats were administered regular insulin (Novolin Toronto, 10 U/kg; NovoNordisk, Mississauga, ON, Canada) or an equivalent volume of saline through the jugular vein, and the liver was rapidly removed 10 min later, immediately frozen in liquid nitrogen, and stored at −80°C until used.

Polymerase chain reactions. Trizol, oligo(deoxynucleotides) 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 of frozen tissue by the Trizol method, and the first-strand cDNAs were synthesized from 5 μg of total RNA using SuperScript reverse transcriptase and oligo(deoxynucleotides) primers. The reverse transcription product (5 μl) was amplified by PCR using specific primers for PTEN (sense, 5'-GGAAAGGGAGCCTGTTGTA-3'; antisense, 5'-TGCCACCTGGTCTGTAATCCA-3') and TRB3 (sense, 5'-ACCAACCCCGACTCCTCCT-3'; antisense, 5'-CCCCACCTCCCTTCTC-3') with Taq DNA polymerase. Another 5 μl of the reverse transcription product were amplified with β-actin primers as an internal control (sense, 5'-GCCAACTGTGGAGGACC-3'; antisense, 5'-TTGCGCTGAAATTATCCG-3'). For each gene, the PCR program consisted of denaturation at 94°C for 10 min, followed by 35 cycles of 60 s at 94°C, 45 s at 60°C, and 90 s at 72°C, with a final 7 min at 72°C. The RT-PCR products (10 μl) were electrophoresed in a 1.5% agarose gel, stained with ethidium bromide, and densitometrically analyzed using NIH Image software.

Preparation of liver tissue extracts. Liver extracts were prepared as described previously (11) with minor modifications. Briefly, liver tissue was homogenized in ice-cold buffer containing 20 mM Tris·HCl, pH 7.4, 250 mM sucrose, 1 mM EDTA, 10 mM NaF, 2 mM Na3VO4, 2 mg/ml benzamidine, and a protease inhibitor cocktail (Sigma Aldrich, Mississauga, ON, Canada). After centrifugation at 3,000 g for 10 min at 4°C, the supernatant was centrifuged at 100,000 g for 90 min. The precipitate was resuspended in ice-cold buffer by shearing through 22-, 25-, and 30-gauge needles, using 10 passes per needle until the precipitate was well resuspended. For cytosolic protein acetylation assay, liver tissue was homogenized in RIPA buffer (1% phosphate-buffered saline, 1% Igepal, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) containing the above-mentioned protease and phosphatase inhibitors and deacetylase inhibitors (100 nM trichostatin A and 10 mM sodium butyrate).

The nuclear fraction was prepared as described previously (5). Briefly, liver tissue was homogenized in two volumes of ice-cold 0.25 M sucrose in TKM (0.05 M Tris·HCl, pH 7.5, 0.025 M KCl, and 0.005M MgCl2). For nuclear protein acetylation assay, the buffer contained deacetylase inhibitors. The homogenate was filtered through four layers of gauze, and 1.0 ml of filtrate was pipetted into a centrifuge tube and mixed with 2.0 ml of 2.3 M sucrose in TKM. The mixture was then underlaid by 1.0 ml of 2.5 M sucrose in TKM with a syringe and 13-gauge needle, forcing the lighter homogenate upward. After centrifugation at 124,000 g at 4°C for 30 min, the supernatant was poured off and the nuclear pellet was taken up in TKM buffer.

Western blotting. Proteins (40 μg/lane) were separated by SDS-PAGE and electroblotted onto nitrocellulose membranes. Protein amount was chosen because it was in the linear range of immunodetection for all proteins tested. The blots were blocked with 5% dry milk and incubated overnight at 4°C with the primary antibody at 1:1,000 dilution. Antibodies against acetyl-lysine, β-actin, Akt1/2, phospho-Akt (Ser473), HDAC1, IFN-γ, IL-6, PCAF, PKCζ, phospho-PKCζ, PTEN, and TRB3 were obtained from Santa Cruz Biotechnology, whereas antibodies against acetyl-lysine, phospho-Akt (Thr308), PDK1, phospho-PDK1, and TNF-α were obtained from New England Biolabs (Pickering, ON, Canada). The blots were then washed three times for 10 min each in Tris-buffered saline (TBS)-Tween, incubated with goat anti-rabbit or donkey anti-goat horseradish peroxidase-conjugated secondary antibody at 1:5,000 for 1 h at room temperature, and washed three times for 10 min in TBS-Tween. Immune complexes were detected using the ECL chemiluminescent detection kit after exposing the blots 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.

PTEN and TRB3 protein acetylation assay. Aliquots of 500 μg of protein diluted to 1 mg/ml in RIPA buffer were incubated overnight at 4°C with anti-PTEN, anti-TRB3, or anti-acetyl-lysine antibodies using the cytosolic protein acetylation assay, liver tissue was homogenized in RIPA buffer (1% phosphate-buffered saline, 1% Igepal, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) containing the above-mentioned protease and phosphatase inhibitors and deacetylase inhibitors (100 nM trichostatin A and 10 mM sodium butyrate).

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PTEN and TRB3 protein acetylation assay. Aliquots of 500 μg of protein diluted to 1 mg/ml in RIPA buffer were incubated overnight at 4°C with anti-PTEN, anti-TRB3, or anti-acetyl-lysine antibodies us-
ing constant rotation. Protein A agarose was added, and the incubation
was continued for 3 h at 4°C. The immune complexes were washed
three times with RIPA buffer, and after electrophoresis, they were
immunoblotted with anti-PTEN, anti-TRB3, or anti-acetyl-lysine
antibodies as applicable. The proteins were revealed by ECL after
incubation with horseradish peroxidase-conjugated secondary anti-
body.

PTEN enzymatic assay. PTEN activity was measured on tissue
immunoprecipitates using the PTEN Biomol Green assay (Biomol
Research Laboratories, Plymouth Meeting, PA) following the manu-

Fig. 2. Levels of phosphoinositol-depen-
dent protein kinase-1 (PDK1; A), phospho-
PDK1 (p-PDK1; B), Akt (C), phospho-Akt
(Ser122) [p-Akt(Ser122); D], phospho-Akt
(Thr308) [p-Akt(Thr308); E], PKCζ (F), and
phospho-PKCζ (p-PKCζ; G) in liver of CF,
EF, and PF female rat offspring before (-)
and after (+) intravenous insulin administra-
tion. Actin was used as a control for protein
loading. Protein levels are expressed in arbi-
trary units relative to controls. Representative
blots are shown. Data are means ± SE of n =
6 rats/group. *P < 0.05; **P < 0.01, CF(-)
vs. CF(+). #P < 0.05; ##P < 0.01, PF(-)
vs. PF(+) (t-test).
manufacturer’s instructions, as reported previously (46). Briefly, liver tissue was homogenized in RIPA buffer containing protease and phosphatase inhibitors. Aliquots of 500 μg of protein were diluted to 1.0 mg/ml in the buffer and incubated overnight at 4°C with 1 μg/ml of anti-PTEN antibody. Protein A agarose was then added, and the samples were further incubated for 3 h at 4°C with constant rotation. The immune complexes were washed three times in the buffer without phosphatase inhibitors before PTEN phosphatase activity assay. Samples were incubated with phosphatidylinositol 3,4,5-trisphosphate (PIP3) as substrate, from which PTEN causes the release of phosphate. Biomol Green reagent was added to stop the reaction and begin color development. After 20 min of incubation at room temperature, the absorbance was read at 620 nm and the amount of phosphate was calculated using a phosphate standard curve.

**HAT and HDAC activity assays.** HAT assay was carried out using the HAT activity colorimetric assay kit (BioVision, Mountain View, CA) following the manufacturer’s instructions. Briefly, 50 μg of nuclear proteins were incubated with the reagents provided with the kit in a 96-well plate, and the NADH produced during the incubation was detected spectrophotometrically at 440 nm. Cell nuclear extract provided with the kit was used as positive control. HAT activity was expressed as optical density (OD) per microgram of protein.

**HDAC assay was carried out using the HDAC activity colorimetric assay kit (BioVision) following the manufacturer’s instructions.** Briefly, 100 μg of nuclear proteins were added to each well of a 96-well plate. A standard curve was prepared using duplicate known amounts of the deacetylated standard included in the kit, whereas HeLa nuclear extract and the HDAC inhibitor trichostatin A were used as positive and negative controls, respectively.

**Statistics.** Statistical analyses were performed with SPSS software. Differences between groups were analyzed using t-test or one-way ANOVA with Tukey’s honestly significant difference test for post hoc comparisons as indicated. Glucose production during the pyruvate tolerance test was analyzed with ANOVA, using glucose concentrations at each time point and the area under the curve computed by the trapezoidal rule. Data are means ± SE. P < 0.05 was considered significant.

**RESULTS**

**Pyruvate tolerance.** We investigated the effect of prenatal alcohol exposure on pyruvate-induced gluconeogenesis by measuring blood glucose response to the administration of pyruvate, a gluconeogenesis substrate (Fig. 1). Basal and
pyruvate-induced blood glucose concentrations were higher in EtOH rats than in PF and control rats. In control and PF rats, blood glucose concentration increased 30 min after pyruvate administration and decreased after 60–90 min. In EtOH-exposed rats, however, blood glucose concentration plateaued by 90 min and did not significantly decrease after 120 min. The glucose area was significantly greater \[ F(2, 15) = 13.65, P < 0.001 \] in EtOH rats (1,634 ± 119 mM/min) than in PF rats (1,140 ± 54 mM/min) and controls (1,113 ± 57 mM/min). Prior injection of 3-MPA diminished the increase in blood glucose concentration in all three rat groups, although the pyruvate-induced glycemic rise tended to be greater \[ F(2, 15) = 3.20, P = 0.07 \] in EtOH rats (846 ± 91 mM/min) compared with control (607 ± 82 mM/min) and PF rats (631 ± 31 mM/min) (Fig. 1).

Distal PI3-kinase insulin signaling. We examined the effect of an insulin bolus on PDK1 and Akt phosphorylation, which is an indicator of their activation, to explain the increased gluconeogenesis in EtOH rats, since insulin inhibition of gluconeogenesis requires intact signaling through PI3-kinase with Akt as a downstream effector. We found no group difference in basal phospho-PDK1 or phospho-Akt in liver extracts (Fig. 2, A–E), which already points to impaired insulin signaling in EtOH rats, since they are known to have elevated insulin levels (45). Total PDK1 and Akt levels were not different among groups. However, insulin significantly increased the phosphorylation of PDK1 and Akt in PF and control rats but failed to increase their phosphorylation in EtOH rats (Fig. 2, B, D, and E).

We also examined the effect of insulin on the phosphorylation of PKCζ, another PDK1 downstream target, which in the liver is primarily involved in fatty acid synthesis (28). Whereas total PKCζ and basal phospho-PKCζ levels were similar among groups, insulin-stimulated phospho-PKCζ was decreased in EtOH rats compared with PF and control rats (Fig. 2, F and G).

Akt-inhibiting protein expression. 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. PTEN enzymatic activity was similar between PF and control offspring but was significantly increased in EtOH rats.

![Fig. 4. Acetylation of Akt-inhibiting proteins. Acetyl-lysine protein content immuno-precipitated (IF) with anti-PTEN antibody (A), PTEN protein immunoprecipitated with anti-acetyl-lysine antibody (B), acetyl-lysine protein content immunoprecipitated with anti-TRB3 antibody (C), TRB3 protein immunoprecipitated with anti-acetyl-lysine antibody (D), and expression of p300/CREB-binding protein-associated factor (PCAF) protein (E) in CF, EF, and PF female rat offspring before (−) and after (+) intravenous insulin administration. Protein levels are expressed in arbitrary units relative to controls. Representative blots are shown. Data are means ± SE of n = 6 rats/group. *P < 0.05; **P < 0.01 vs. CF. ##P < 0.01 vs. PF (Tukey’s test).](http://ajpregu.physiology.org/)}
PTEN and TRB3 acetylation. Because PTEN protein was recently shown to be regulated through acetylation (29), we determined PTEN acetylation status in EtOH offspring using anti-acetyl-lysine antibodies. The acetyl-lysine content in liver extracts immunoprecipitated with anti-PTEN antibody was reduced by ∼40% in EtOH rats compared with PF and control rats \([F(2, 15) = 14.15, P < 0.001]\) (Fig. 4A). Similar results \([F(2, 15) = 45.45, P < 0.0001]\) were obtained when anti-PTEN antibodies were used to immunoblot extracts immunoprecipitated with anti-acetyl-lysine antibodies (Fig. 4B). To investigate whether TRB3 is acetylated, we immunoprecipitated liver extracts with anti-TRB3 antibodies followed by immunoblotting with anti-PTEN and anti-acetyl-lysine antibodies. Conversely, extracts were immunoprecipitated with anti-acetyl-lysine antibodies and blotted with anti-TRB3 antibodies. Figure 4, C and D, shows that TRB3 is acetylated and that its acetylation status is reduced by ∼60% in EtOH rat offspring \([F(2, 15) = 18.47–38.08, P < 0.001–0.0001]\).

We also determined the protein expression of the histone acetylase PCAF, which has been shown to acetylate PTEN (29). As shown in Fig. 4E, PCAF expression was reduced by ∼45% in EtOH rats compared with PF and control rats \([F(5, 30) = 12.91, P < 0.001]\), and there was no insulin effect on PCAF expression at this time. To ascertain whether PCAF physically interacts with PTEN and TRB3, we immunoprecipitated liver extracts with anti-PTEN or anti-TRB3 antibodies followed by immunoblotting with anti-PCAF antibodies. As shown in Fig. 5, A and B, PTEN and TRB3 coimmunoprecipitated with PCAF, but there was ∼35–40% less PCAF in the PTEN and TRB3 immunoprecipitates in EtOH rats than in PF and control rats. Extract immunoprecipitation with anti-PCAF antibodies followed by immunoblotting with anti-PTEN and anti-TRB3 antibodies showed more PTEN \([F(2, 15) = 34.04, P < 0.0001]\) and TRB3 \([F(2, 15) = 10.70, P < 0.001]\) in the EtOH rats, consistent with the increased expression of these two proteins (Fig. 5, C and D).

Global tissue protein acetylation. To investigate global hepatic protein acetylation, we submitted cytoplasmic and nuclear extracts to Western analysis using anti-acetyl-lysine antibodies. We found a reduction in the intensity of protein acetylation in EtOH rats compared with PF and control rats (Fig. 6). This hypoacetylation was observed in both the cytoplasmic and nuclear fractions and involved proteins with molecular masses of ∼90, ∼60, ∼55, and ∼45 kDa, corresponding to PCAF, HDAC1, PTEN, and TRB3, respectively.

HDAC and HAT activities. To further investigate the effects of prenatal EtOH on protein acetylation, we determined HDAC and HAT activities in nuclear extracts. HDAC activity increased approximately twofold \([F(2, 15) = 30.72, P < 0.0001]\), whereas HAT activity was decreased by ∼35% \([F(2,
Liver tissue cytokines. We examined the levels of TNFα, IL-6, and IFN-γ by Western analysis in liver extracts, since these cytokines are associated with insulin resistance (38). There were no differences among EtOH, PF, and control rats for any of these cytokines (Fig. 8).

DISCUSSION

In this study, we report new findings that could explain hepatic insulin resistance previously reported in rat offspring exposed to alcohol in utero (12, 44). We found that PTEN and TRB3 proteins are hypoaecetylated in rats prenatally exposed to alcohol in association with reduced HAT and increased HDAC activities. Accordingly, EtOH rats had reduced expression of PCAF and increased expression of HDAC1. PTEN enzymatic activity increased in an inverse proportion with its acetylation status, in agreement with a recent report in 293T human embryonic kidney cells that PCAF modulates PTEN activity through acetylation (29).

PTEN is a lipid phosphatase that dephosphorylates PIP3 and prevents PI3-kinase-induced PDK1 activation and subsequent activation and recruitment of Akt and PKCζ to the cell membrane. Since PI3-kinase catalyzes the formation of PIP3, PTEN antagonizes this PI3-kinase function and inhibits Akt and PKCζ activation. Elevated levels of PTEN were reported in skeletal muscle of diabetic obese Zucker (fa/fa) rats (26), whereas inhibition of PTEN production in the liver and adipocytes improved insulin sensitivity in diabetic db/db and ob/ob mice (7). We have previously reported increased expression of PTEN mRNA and protein in skeletal muscle from insulin-resistant alcohol-exposed rat offspring (45). In line with these studies, we found increased PTEN expression in the liver of these animals as shown by studies of mRNA, Western blotting, and enzyme activity. In addition, PTEN acetylation was reduced in close association with increased enzymatic activity.

Fig. 6. Protein acetylation in nuclear (A) and cytoplasmic liver extracts (B) as revealed by Western blotting with anti-acetyl-lysine antibodies in CF, EF, and PF female rat offspring. Representative blots are shown. Bands of 90, 60, 55, and 45 kDa correspond to PCAF, histone deacetylase-1 (HDAC1), PTEN, and TRB3, respectively.

Fig. 7. HDAC (A) and histone acetylase (HAT) activities (B) and HDAC1 protein level (C) in nuclear liver extracts in CF, EF, and PF female rat offspring. Data are means ± SE of n = 6 rats/group. **P < 0.01 vs. CF. #P < 0.01 vs. PF (Tukey’s test).
The reasons for these translational and posttranslational alterations of PTEN are currently unclear but could be related to oxidative stress, which is known to regulate the acetylation of nonhistone proteins (3). PTEN has been proposed to amplify oxidative stress in neuronal and pancreatic cells, and we have previously documented a persistent oxidative stress status induced by prenatal alcohol exposure in the rat hypothalamus (13). PTEN is also upregulated by resistin (34), another protein associated with inflammation and oxidative stress, which we have shown to be increased in alcohol-exposed rat offspring (9). Resistin induces gluconeogenesis through a mechanism involving inhibition of adenosine monophosphate-activated protein kinase (1), whereas PTEN enhances gluconeogenesis by stimulating Foxo3a and, ultimately, phosphoenol pyruvate carboxykinase (PEPCK) (18). It is not known whether these effects of PTEN and resistin on gluconeogenesis are interconnected.

Besides alterations of gene expression, posttranslational protein modifications also have been reported in other key regulators of gluconeogenesis. Foxo transcriptional activity is inhibited by Akt induced phosphorylation, whereas another level of Foxo control is provided by the opposing actions of the acetylases p300, CREB-binding protein, and PCAF, which prevent transcription, and the protein deacetylase SIRT1, which promotes transcription (18). SIRT1 also deacetylates the transcriptional coactivator PGC-1α, contributing to the upregulation of gluconeogenic genes PEPCK and glucose-6-phosphatase. SIRT1 is posttranslationally induced by a nutrient signaling response mediated by pyruvate (32). The fact that pyruvate injection results in a dramatic blood glucose rise in alcohol-exposed rat offspring as shown in the current and previous studies (44, 45) suggests that PGC-1α deacetylation by SIRT1 is likely to be active in these animals.

As a novel finding, we report decreased acetylation in addition to increased expression of TRB3 in alcohol-exposed rats. 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 (14). Recent evidence indicates that the gluconeogenic regulator PGC-1α promotes insulin resistance in liver via induction of TRB3 (24). It has been reported that TRB3 is induced in liver of adult rats chronically fed an alcohol-containing diet (19). TRB3 is also expressed in skeletal muscle, where it is induced by glucose deprivation (43). We have previously demonstrated increased expression of TRB3 in muscle of rats prenatally exposed to alcohol as an explanation for the observed insulin resistance in these animals (45). Our current results in the liver are in agreement with this previous report. In addition to its increased expression, TRB3 acetylation status was reduced in rats exposed to alcohol in utero in close relationship with that of PTEN and an imbalance of HAT and HDAC activities favoring protein deacetylation. TRB3 acetylation has not been reported before, and the functional implication of its acetylation is currently unknown. We found in the current study a coimmunoprecipitation of TRB3 and PCAF that may be an indication of their interaction in vivo. Whether PCAF is a TRB3 acetylase, however, remains to be shown.

Hepatic insulin resistance is primarily explained by a reduction of insulin-induced inhibition of glucose production through gluconeogenesis with some contribution from glycogenolysis. Insulin inhibition of gluconeogenesis requires Akt-induced phosphorylation and the resulting suppression of gluconeogenic genes. We have previously reported a reduction of the suppressive effect of insulin on hepatic PGC-1α and PEPCK expression in rat offspring exposed to alcohol in utero, with as

**Fig. 8.** TNF-α (A), IL-6 (B), and IFN-γ levels (C) in CF, EF, and PF female rat offspring. Protein levels are expressed in arbitrary units relative to controls. Representative blots are shown. Data are means ± SE of n = 6 rats/group.
a result fasting hyperglycemia and a dramatic increase in pyruvate-induced gluconeogenesis (12, 44). We have shown presently that two upstream molecules that inhibit Akt and therefore promote gluconeogenesis are upregulated in alcohol-exposed rats compared with PF and control rats and that this upregulation occurs both translationally and posttranslationally. In the liver, the inhibitory effects of insulin on glucose production are mediated by Akt in the PI3-kinase arm of the insulin-signaling pathway. The PI3-kinase p110 catalytic subunit generates the lipid product PIP3, which via PDK1 leads to Akt activation. We have shown presently that insulin-stimulated PDK1 and Akt phosphorylation, and thus activity, is decreased in EtOH-exposed rats, consistent with increased PTEN and TRB3 expression, which could account for the reduced inhibition of hepatic glucose production and insulin resistance.

Although both PTEN and TRB3 inhibit Akt activation by insulin, PTEN also inhibits PKCζ activation. Consistently, we found decreased activation of PKCζ in the liver of alcohol exposed rats, as previously reported in skeletal muscle (11, 45). Whereas in muscle, both PKCζ and Akt promote glucose transport, their roles diverge in liver, where Akt inhibits gluconeogenesis while PKCζ, although having a role in insulin internalization and insulin action on glucose transport, primarily plays a role in fatty acid synthesis (17, 36). The diminished PKCζ activation in alcohol-exposed rats may reduce glucose transport across the cell membrane as well as lipid synthesis. This differs from insulin-resistant models, where the expression or activation of PKCζ is increased alongside an impairment of Akt activation (35). It was recently shown, however, that PKCζ expression may be reduced in insulin-resistant rats in association with decreased fatty acid synthesis and increased triglyceride storage (37). Our previous finding of increased hepatic triglyceride deposition (10) and current finding of reduced PKCζ activation in rats exposed to alcohol in utero agree with this study and suggest that other factors may be involved in hepatic steatosis in this rat model. One such factor is HDAC1, whose increased expression has been reported to cause steatosis in mice (41).

Perspectives and Significance

Overall, our results indicate an involvement of translational and posttranslational protein modifications in the pathogenesis of insulin resistance in rats prenatally exposed to alcohol. The expression of Akt inhibitors PTEN and TRB3 is upregulated whereas their acetylation status is reduced in these animals due to an imbalance between HATs and HDACs, which also seems to affect other proteins in both the cytoplasm and the nucleus. Abnormal acetylation of proteins has been reported in fetal and juvenile rats in association with altered DNA methylation in a model of uteroplacental insufficiency (22, 27). Given the association of histone acetylation with chromatin stability and gene transcription, our results suggest that prenatal alcohol exposure could have implications in epigenetic transmission of the abnormal glucose tolerance phenotype.

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