Chronic Ethanol Intake Impairs Insulin Signaling in Rats by Disrupting Akt Association with the Cell Membrane

ROLE OF TRB3 IN INHIBITION OF Akt/PROTEIN KINASE B ACTIVATION*

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Chronic and excessive alcohol consumption is an important and modifiable risk factor for type 2 diabetes. We previously reported elevations in hepatic Class I alcohol dehydrogenase (ADH) expression in ethanol-fed rats that corresponded with reduced levels of mature, nuclear sterol-regulatory element-binding protein-1 (SREBP-1), an insulin-induced transcriptional repressor of the ADH gene. In this report, we have studied the effects of insulin and ethanol on ADH gene expression in a highly differentiated rat hepatoma cell line (FGC-4), as well as the in vivo effects of chronic intake of an ethanol-containing diet on hepatic insulin signaling. Insulin inhibited ADH gene expression, and this was abolished by LY294002 (a phosphatidylinositol 3-kinase inhibitor) and small interfering RNA knockdown of SREBP-1. Chronic ethanol intake led to decreased phosphorylation of Akt (protein kinase B) at Thr308, increased phosphorylation of Akt at Ser473, and decreased phosphorylation of glycogen synthase kinase-3β (a downstream effector of Akt). Hepatic membrane-associated Akt content was decreased and cytosolic Akt content was increased in rats fed an ethanol-containing diet. Thus, disruptive effects of ethanol on insulin signaling occurred via impaired phosphorylation of Akt at Thr308. TRB3, a negative regulator of Akt, was induced in liver of ethanol-fed rats. In ethanol-treated FGC-4 cells, small interfering RNA knockdown of TRB3 increased membrane-associated Akt and the phosphorylation of Akt at Thr308. Our results suggest that ethanol induces TRB3, which, through binding to the pleckstrin homology domain of Akt, prevents its plasma membrane association, Akt-Thr308 phosphorylation, and subsequent Akt-mediated signaling. Ethanol inhibition of insulin signaling reduces nuclear SREBP accumulation and results in disinhibition of Class 1 ADH transcription.

Writings from as early as the 17th century, as well as modern epidemiological studies, suggest that chronic and excessive alcohol consumption is positively associated with the onset of type 2 diabetes (1–5). Ethanol intake has been reported to decrease glucose uptake and utilization consistent with the development of insulin resistance, a central component of diabetes (6). Results from these previous studies suggest that the ethanol impairment of insulin action is likely to be downstream from PI3 kinase; however, the molecular mechanisms underlying the effects of alcohol on insulin resistance and type-2 diabetes remain to be determined (7).

We previously reported that chronic intragastric infusion of an ethanol-containing diet to rats results in unique and predictably recurring cyclic fluctuations in plasma ethanol concentrations (8) as a consequence of cyclic expression of the major alcohol metabolizing enzyme, hepatic Class I alcohol dehydrogenase (ADH). Further studies from our laboratory demonstrated that alcohol induces hepatic ADH gene transcription via decreased levels of nuclear SREBP-1c protein, a negative regulator of the ADH gene (9). SREBP-1c is encoded by an insulin-responsive gene (10) and is an important early mediator in the pathway of insulin action in the liver (11). These observations led to the current hypothesis under study, namely that ethanol may suppress nuclear SREBP-1c via disruption of insulin signaling, which may be a potential link between alcohol consumption and insulin resistance. In this study, we elucidate the mechanism by which chronic ethanol intake inhibits insulin action and identify TRB3, a previously identified modulator of Akt signaling, as a primary ethanol-responsive modifier of insulin signaling (12).

EXPERIMENTAL PROCEDURES

Materials—All chemicals, unless otherwise specified, were purchased from Sigma. Antibodies were purchased from commercial suppliers: SREBP-1 was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); GSK3, and phosphatase and tensin homolog deleted on chromosome 10 (PTEN) were from Upstate Biotechnology (Lake Placid, NY); phospho-GSK3, Akt, phospho-Akt[ Thr308], phospho-Akt[ Ser473], phospho-PTEN, phosphoinositide-dependent kinase 1 (PKD1), p85, and p110α were from Cell Signaling, Inc. (Beverly, MA). LY294002 was purchased from Cell Signaling, Inc., and Lipofectamine 2000 was from Invitrogen. The TRB3 antibody was kindly provided by Dr. Marc Montminy. Rictor antibody was from Bethyl Laboratories, Inc. (Montgomery, TX).

Animal Experimental Protocols—Experiments conformed to ethical guidelines for animal research and received prior approval by the Institutional Animal Care and Use Committee of the University of Arkansas for Medical Sciences. Adult male Sprague-Dawley rats were purchased from Harlan Industries (Indianapolis, IN). An intragastric cannula was surgically implanted into each animal, and they were allowed to recover for total enteral nutrition. In this total enteral nutrition model, rats are infused with an ethanol-containing diet (13 g/kg/day)

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‡ The abbreviations used are: PI3, phosphatidylinositol 3; PI3K, phosphatidylinositol 3-kinase; SRE, sterol-responsive element; SREBP, SRE-binding protein-1; nSREBP, nuclear SREBP; ADH, alcohol dehydrogenase; siRNA, small interfering RNA; GSK3, glycogen synthase kinase; PH, pleckstrin homology; UEC, urine ethanol concentration; GFP, green fluorescent protein; ADU, arbitrary densitometric units; PIP2, phosphatidylinositol-3, 4, 5-trisphosphate; PDK, phosphoinositide-dependent kinase 1; PTEN, phosphatase and tensin homolog deleted on chromosome 10.
using a computer-driven programmable pump that supplies diet con-
tinuously, except for 1 h each day needed to refill the syringes, as
described previously (13). The control rats were infused the same diet
except that ethanol was isocalorically replaced with carbohydrate. We
used urine ethanol concentrations (UECs) to track blood ethanol con-
centrations since ethanol equilibrates with body water and we have
previously demonstrated that UECs accurately reflect blood ethanol
concentrations (36). Rats were sacrificed following 21–30 days of con-
tinuous diet infusion and when their UECs were greater than 300 mg/dl,
as described previously (8, 9, 14). The actual time of sacrifice occurred
while diets were being infused to avoid any changes in insulin levels
associated with stopping the diet infusions. Liver was collected and
stored at −70 °C. Type 2 diabetic rat livers were from a previously
reported study (15). Briefly, Sprague–Dawley rats (100 g) were fed a
erobustly high fat diet for 14 days and then treated with low dose (45 mg/kg) strepto-
zotocin. Type 2 diabetes was confirmed by hyperglycemia, normoinsu-
linemia, and oral glucose tolerance testing (15).

**Northern Blot Analysis**—Northern blot analysis was conducted as
described previously (8, 9). A 400-bp rat Class 1 ADH cDNA probe was
used for the detection of ADH mRNA. Rat TR3B2 probe was generated by
using the following primers: 5′-TTGGCTCGATTGTTCTTACG-3′
(5′-primer) and 5′-CTCCCTCAACCCGGATGTA-3′ (3′-primer).
18 S ribosomal RNA antisense oligonucleotide was synthesized (Bio
Synthesis, Inc., Lewisville, TX). All filters were probed with the 18 S
rRNA oligonucleotide as an internal control. Bands were quantitated by
densitometry of the autoradiogram, and the ratio of ADH message to 18
S rRNA in the same sample was determined and expressed as relative
RNA units or as percentage of that for the control.

**Preparation of Rat Liver Extracts, Crude Plasma Membrane Fra-
cions, Cytosolic Fractions, Nuclear Extracts, and Measurement of Akt
Activity**—Crude plasma membrane fractions and cytosolic fractions
were prepared from liver tissue homogenized with a Dounce homoge-
nizer using a previously described procedure (16). Nuclear extracts were
prepared as described previously (9). Akt activity was measured by using
an Akt kinase assay kit from Cell Signaling.

**Western Immunoblot Analysis**—Samples with equal amounts of pro-
tein determined by the Bradford protein assay were resolved on poly-
acrylamide gels. One was transferred to a Hybond-P membrane (Amer-
sham Biosciences), and the other was stained with Coomassie Blue to
confirm equal sample loading. Membranes were blocked overnight at
4 °C in Tris-buffered saline with Tween plus 5% (w/v) milk powder with
gentle shaking and were then incubated with different primary antibod-
ies diluted in Tris-buffered saline with Tween. Proteins were visualized
using the enhanced chemiluminescence plus system (ECL Plus; Amer-
sham Biosciences) following incubation with secondary antibodies.
Detection by autoradiography was as described previously (9).

**Plasmid Construction**—Mammalian expression vectors for pEGFP-
Akt2 and pEGFP-PH (Akt2) were generated by PCR using the following
primers: sense, 5′-CGTAGCTACCCATGAGGATCTCTGG-
CATCAA-3′ (5′-primer representing the Akt2 sense-strand sequence
flanked by an HindIII site (underlined) and containing an initiating
codon (bold)); antisense, 5′-GGGATCCCTCGCTCCAGATCC-
GCTGAGTAGGA-3′ for full-length Akt2, 5′-GGGTGATCCCGC-
ACATTTGGCCCTGCTGAC-3′ for pleckstrin homology
(PHP) domain (1–147 amino acids) (primer inversely complementary to
the 3′ Akt sequence flanked by a BamHI site (underlined))(17) followed
by subcloning into the pEGFP-N1 plasmid (Clontech). The plasmid
expression cassettes were sequenced to confirm identity.

**Cell Culture, Transfection, and Confocal Microscopy**—Rat hepatoma
FGC-4 cells (obtained from Mary C. Weiss, Pasteur Institute, Paris,
France) were seeded in medium (Dulbecco’s modified Eagle’s medium
containing 100 units/ml penicillin and 100 μg/ml streptomycin sulfate
and 5% fetal bovine serum). For transfection, FGC-4 cells at 60% con-
fluence were incubated for 6 h with plasmid/Lipofectamine 2000 mix-
ture following the manufacturer’s protocol. Subcellular localization of
GFP-tagged Akt was studied in cells cultured in Flaskette chamber slides
sealed with a gasket (Nunc International, Naperville, IL) after treatment
with insulin and/or ethanol and followed by fixation in 4% paraformal-
dehyde and examination with a Zeiss LSM410 laser scanning confocal
microscope. For experiments with ethanol treatment, cells were cul-
tured in glucose-free Dulbecco’s modified Eagle’s medium supplemen-
ted with 1 mM pyruvate and 5% fetal bovine serum; all caps were
rigidly closed for the remainder of the experiment to prevent the evap-
orative loss of ethanol.

**RNA Interference and Co-immunoprecipitation**—Double-stranded
stealth RNA duplexes (Invitrogen) corresponding to rat TR3B (165–189
bp, NM_144755) coding region (5′-ATCTCTGGCTTCTCTGCG-
ATGTGT-3′ were transfected into FGC-4 cells. SREBP-1 siRNA was
purchased from Dharmacon Inc. (Lafayette, CO). The effect of RNA
interference was measured after 48 h of incubation. The negative
control siRNA was used in control transfections (Ambion). For co-immu-
noprecipitation, cells were transfected with pEGFP-Akt expression vec-
tors or the pEGFP-N1 control vector by Lipofectamine 2000 for 24 h;
total cell lysates were obtained and incubated with antisense to GFP
and protein G slurry overnight at 4 °C. Immunoprecipitates were col-
lected, washed, and separated by SDS-PAGE (12%) followed by Western
blotting using TRB3 antiserum.

**Statistics**—The Student’s t test was used to determine whether group
means differed at a significance level of p < 0.05.

**RESULTS**

**Effects of Chronic Ethanol Intake on UECs**—UECs did not reach a
steady-state level, although the ethanol-containing diet was infused
continuously for more than 3 weeks (data not shown). Rather, UECs
fluctuated in the form of large regular “pulses” such that concentrations
varied between a nadir of near zero to peak levels of greater than 500
mg/dl over a 6-day period, similar to our previous observations (8). The
mean blood and urine alcohol concentrations were greater than 300
mg/dl at the time of sacrifice (data not shown); hepatic nSREBP-1 pro-
tein was reduced by more than 50%, and hepatic Class I ADH was
induced by greater than 2-fold, results nearly identical to those previ-
ously reported (9). Serum insulin concentrations did not differ signifi-
cantly between control and ethanol-fed rats (1.00 ± 0.11 versus 0.85 ±
0.10 ng/ml, respectively).

**Effects of Ethanol and Insulin on Class 1 ADH mRNA in Hepatoma
FGC4 Cells and Livers of Type 2 Diabetic Rats**—The effects of ethanol
and insulin on ADH mRNA expression were studied in highly differen-
tiated rat hepatoma FGC-4 cells. Fig. 1A presents a Northern blot of
FGC-4 cells treated with various doses of insulin (0.1–10 nM) in the
presence or absence of ethanol (50 mM). The data demonstrate that
ethanol induced and insulin reduced ADH mRNA abundance in a dose-
dependent manner (p < 0.05), an effect that was completely abolished
by 50 mM ethanol, confirming that insulin inhibits in vitro ADH expres-
sion. Insulin treatment increased nuclear SREBP-1c protein expression
(p < 0.05), and ethanol treatment not only prevented the insulin induc-
tion of nSREBP-1 but reduced (p < 0.05) it to levels below the control
(Fig. 1C). These data are consistent with the model that insulin regulates
ADH via changes in nSREBP-1c (9). The PI3K inhibitor LY294002 not
only blocked the insulin inhibition of ADH gene expression but also
induced ADH mRNA expression more than 2-fold over control (Fig. 2A).
FIGURE 1. Effects of insulin and ethanol on ADH mRNA and nuclear accumulation of SREBP-1c in FGC-4 cells. A, FGC-4 cells were incubated with the indicated concentrations of insulin and/or ethanol for 12 h. Aliquots of total RNA (20 μg) from FGC-4 cells were subjected to Northern blot hybridization with 32P-labeled ADH probe and 18S rRNA oligonucleotide. Each lane represents cells from an individual well. B, densitometric analysis. The means ± S.E. are shown for normalized ADH mRNA levels. *, p < 0.05 as compared with control (0 nM insulin). C, Western blot of SREBP-1c in nuclear extracts from FGC-4 cells treated with 1 nM insulin ± 50 mM ethanol for 12 h. Numbers at bottom of panel C are densitometric units (AU), means ± S.E., of nuclear SREBP-1c relative to a control value of 1. Values bearing * = p < 0.05 as compared with control, and values bearing ** = p < 0.05 as compared with insulin-treated group. P, SREBP-1c precursor; N, nuclear SREBP-1c.

FIGURE 2. ADH mRNA levels in treated FGC-4 cells and in type 2 diabetic rats. Aliquots of total RNA (20 μg) from rat liver or FGC-4 cells were subjected to Northern blot hybridization with 32P-labeled ADH probe and 18S rRNA oligonucleotide. A, FGC-4 cells were incubated with LY294002 (LY, 50 μM) and/or 5 nM insulin as indicated for 12 h, and LY294002 was added 1 h prior to the addition of insulin. CON, control; B, groups of cells were treated with 100 nM negative control siRNA (lanes 1–3); 100 nM negative control siRNA + 5 nM insulin (lanes 4–6); 100 nM SREBP-1 siRNA (lanes 7–9); 100 nM SREBP-1 siRNA + 5 nM insulin (lane 10–12). C, ADH mRNA in rat liver tissues. Each lane represents an individual rat. Values bearing * = p < 0.05 as compared with control, and values bearing ** = p < 0.05 as compared with insulin-treated group (for panels A and B). ND, non-diabetic rat; DB, type 2 diabetic rat.
These data suggest that insulin inhibits ADH gene expression through the PI3K pathway. When SREBP-1 was knocked down by specific siRNA, insulin-inhibition of ADH gene expression was negated, and ADH mRNA was significantly increased (Fig. 2B). This evidence confirmed our previous report that SREBP-1 is a repressor of ADH gene expression (9). The above data indicate that insulin inhibits ADH gene expression through PI3K and SREBP-1.

To substantiate the inhibition by insulin of ADH gene expression, we examined ADH mRNA levels in livers of type 2 diabetic rats and found that ADH mRNA was induced 4-fold as compared with liver from control rats (Fig. 2C). These results are consistent with the elevated ADH activity reported in diabetic male rats (18).

**Effects of Ethanol on the Phosphorylation of GSK3, Akt, and Proximal Insulin Signaling in Rat Liver**—We examined GSK3 because it inhibits SREBP-1c transcriptional activity (19) and stimulates ubiquitin-dependent degradation of SREBP-1c (20). Insulin has been reported to inhibit GSK3 as a result of phosphorylation. Western blot analyses were performed on liver whole cell lysates from rats fed an ethanol-containing diet and that were killed when blood ethanol concentrations were observed in total GSK3 levels, p-GSK3 phosphorylated at Thr308 (p-Akt-Thr308); and Akt phosphorylated at Ser473 (p-Akt-Ser473) and Akt are shown. δ, the corresponding densitometric analysis (means ± S.E.) of data in panel A. Asterisks indicate significant differences from the respective control at p < 0.05.

Western blot analysis was used to monitor the effects of ethanol on the subcellular localization of PDK1, p110α (the catalytic subunit of PI3K), p85, and Rictor (a critical component of the PDK2 complex) (Fig. 4A). In agreement with a previous report demonstrating that ethanol treatment increased PI3K activity in rat liver (6), we found significant recruitment of PDK1, p110α, p85, and Rictor to the hepatic plasma membrane of rats fed an ethanol-containing diet (Fig. 4A). Cellular distribution of Rictor abundance was assessed and found to be a membrane-enriched protein (Fig. 4B). There were no statistically significant differences in hepatic phospho-PTEN (1.00 ± 0.017 versus 0.99 ± 0.012 AU) or PTEN (1.00 ± 0.041 versus 1.095 ± 0.041 AU) between control and ethanol-fed rats, respectively.

**Subcellular Localization of Akt in Rat Liver**—Data in Fig. 3 demonstrate that ethanol impairs phosphorylation of Akt at Thr308. Translocation of Akt from the cytosol to the plasma membrane and binding to phosphatidylinositol-3,4,5-triphosphate (PIP₃) are crucial for the phosphorylation on Thr308 by membrane-located PDK1 (21, 22). We, therefore, examined the subcellular location of Akt in response to ethanol consumption. Ethanol reduced (p < 0.05) membrane-localized Akt (Fig. 5A), in agreement with the observed ethanol suppression of p-Akt-Thr308 (Fig. 5A), whereas ethanol increased (p < 0.05) the amount of Akt in the cytosolic fractions (Fig. 5A). These data suggest that ethanol prevents association of Akt with the plasma membrane-bound PIP₃, a prerequisite for PDK1 phosphorylation of Akt-Thr308.

To visualize the subcellular localization of Akt in response to insulin and ethanol, we transfected a GFP-Akt (full-length) expression plasmid into FGC-4 cells and monitored intracellular localization of GFP-Akt by confocal microscopy. Upon insulin stimulation, a significant portion of GFP-Akt was distributed to the plasma membrane, whereas ethanol abolished the insulin-stimulated GFP-Akt membrane localization (Fig. 5A).
To confirm these results, plasma membrane fractions were examined for GFP-Akt protein in Western blot analysis. As can be seen in Fig. 6, B and C, insulin-stimulated cells had five times greater levels of GFP-Akt \((p < 0.05)\), and this was blocked by ethanol treatment. It is noteworthy that there are 3 amino acid residues that differ between our cloned Akt2 cDNA from Sprague-Dawley rats (GenBank number DQ198085) and Akt2 previously cloned from Rattus norvegicus, but these 3 amino acid residues are identical to mouse Akt2 at the corresponding positions.

Role of TRB3 in Activation of Akt—TRB3 has been reported to inhibit insulin signaling by binding directly to Akt and preventing phosphorylation at Thr\(^{308}\) (12). Thus, induction of TRB3 is a potential mechanism by which ethanol could block Akt activation. We found that both protein (Fig. 5A) and mRNA (Fig. 7A) abundance of TRB3 were greater \((p < 0.05)\) in hepatic tissues from rats fed ethanol-containing diet. In whole cell lysates of FGC-4 cells, ethanol increased \((p < 0.05)\) the abundance of TRB3 protein and decreased \((p < 0.05)\) p-Akt-Thr\(^{308}\) and nuclear SREBP-1 (Fig. 8A), suggesting the possibility that ethanol blocks Akt activation by promoting the association between TRB3 and Akt. To confirm the inhibitory action of TRB3 on the activation process, RNA interference was used to suppress TRB3 levels in ethanol-treated FGC-4 cells. In these cells, siRNA decreased TRB3 \((p < 0.05)\) and increased p-Akt-Thr\(^{308}\) and nuclear SREBP-1 (Fig. 8B), supporting elevated translocation of Akt to the plasma membrane upon reduced TRB3. We confirmed this by demonstrating that siRNA knockdown of TRB3 in FGC-4 cells also prevented the ethanol-induced inhibition of insulin-stimulated membrane translocation of GFP-Akt (Fig. 8, C and D).

The above data raised the possibility that TRB3 binds to the PH domain of Akt, prevents this domain from binding PIP\(_3\), and thereby localizes Akt to the cytoplasm. To test this possibility, we constructed a truncated Akt (1–147 amino acids)-GFP fusion protein containing the PH domain of Akt2, which mediates interaction between myosin II and Akt (23). Full-length and truncated GFP-Akt constructs and control
GFP vector were transfected into FGC-4 cells, and cell extracts were prepared. Antibody against GFP was used to immunoprecipitate the GFP-Akt fusion proteins, and immunoprecipitates were probed with TRB3 antiserum in Western blots (Fig. 8E). Truncated GFP-Akt as well as full-length GFP-Akt were co-immunoprecipitated with TRB3, whereas the GFP control protein did not complex with TRB3. These data indicate that TRB3 associates with the PH domain of Akt2.

**DISCUSSION**

Chronic alcohol consumption can result in alcoholic liver disease, insulin resistance, and type 2 diabetes. Although there have been extensive studies on alcoholic liver disease, less research has been conducted on the mechanisms underlying ethanol impairment of insulin signaling with some inconsistencies in reported results apparent. For example, although both acute and chronic ethanol intake have been reported to cause insulin resistance, as evidenced by decreased glucose utilization and impaired insulin suppression of hepatic glucose production, some reports suggest that this is accompanied by enhanced phosphorylation of insulin receptor, IRS-1 and IRS-2, and by increased PI3K activity (6), whereas other reports using chronic ethanol exposure models have found reduced phospho-IRS-1 levels and lower activities of PI3K and Akt in liver (24). Thus, mechanisms underlying ethanol-induced insulin resistance remain to be clarified.

We previously reported that ethanol induces ADH in part by disinhibition of ADH gene expression. This is accomplished by reducing the abundance of mature nSREBP-1c, which is a negative regulator of the ADH gene and which functions in concert with the CCAAT/enhancer-binding proteins to mediate ethanol regulation of ADH (9, 14). Because SREBP-1c has been documented as an important early mediator in the pathway of insulin action in liver (11) and alcoholism is a known risk factor for diabetes and insulin resistance, we became interested in the potential linkage of alcohol, insulin, and regulation of ADH gene expression. In this report, we assessed insulin effects on ADH gene expression as well as ethanol effects on insulin signaling pathways. We found that insulin inhibits ADH gene expression. This inhibition occurs through the PI3K pathway and involves SREBP-1, as both LY294002 (a PI3K inhibitor) and knockdown of SREBP-1 abolished insulin effects and disinhibited ADH gene expression. These data establish a relationship between insulin-Pi3K-SREBP1-ADH gene expression and confirm that SREBP-1 is a negative regulator of the ADH gene, as suggested in our previous report (9).

SREBP-1c must translocate from the cytosol to the nucleus, where it...
FIGURE 7. TRB3 mRNA levels in rat liver. A shows TRB3 mRNA levels in rat liver. 20 μg of total RNA from rat hepatic tissues were subjected to Northern blot hybridization with 32P-labeled rat TRB3 probe and 18 S rRNA oligonucleotide. Ethanol-fed rats were sacrificed when the UECs were on the high UEC limb of the UEC pulse. Control rats were fed non-ethanol-containing diets and were sacrificed at the same time (n = 6 rats/group). Each lane represents an individual rat. B depicts the densitometric analysis of the above Northern blots (means ± S.E.). Asterisk indicates a significant difference from the control at p < 0.05. CON, control.

FIGURE 8. TRB3 affects Akt membrane translocation and phosphorylation in FGC-4 Cells. A, Western blot analyses of TRB3, p-Akt-Thr308, and total Akt in whole cell lysates and nuclear SREBP-1 in nuclear extracts from control and ethanol-treated (100 mM) FGC-4 cells for 12 h (medium was changed once after 6 h of incubation). B, TRB3, p-Akt-Thr308, total Akt, and nuclear SREBP-1 protein levels in FGC-4 cells transfected with negative control siRNA duplex or with TRB3 siRNA (siTRB3) oligonucleotides and treated with 100 mM ethanol. C, Western blot of GFP-Akt in the plasma membrane fractions and in whole cell lysates from FGC-4 cells transfected with GFP-Akt2 (full-length) expression plasmid, where groups of cells were treated with: negative control siRNA (lanes 1–3); 30 nM insulin negative control siRNA (lanes 4 – 6); 30 nM insulin + 100 mM ethanol (lanes 7–9); 30 nM stealth siRNA (lanes 10–12); 100 mM ethanol + 30 nM insulin (lanes 13–15). Also shown is the TRB3 level in whole cell lysates. D depicts the densitometric analysis of GFP-Akt levels in the plasma membrane fractions (in D, bars = means ± S.E.). Means with different superscripts differ at p < 0.05. The top section of E depicts a co-immunoprecipitation assay of endogenous TRB3 proteins from FGC-4 cells transfected with control GFP, GFP-tagged Akt2 (full-length, GFP-F), and GFP-tagged PH domain of Akt2 (GFP-PH) expression vectors, respectively. The middle section shows a Western blot of input of GFP, GFP-F, and GFP-PH. The bottom section shows the input levels of endogenous TRB3.
binds SREs on the promoter of ADH gene and prevents gene transcription. Ethanol consumption favors binding of enhancer-binding proteins β and δ (C/EBP β and δ) to the promoter and decreases SREBP-1 abundance and nuclear binding to dis inhibit ADH gene transcription (9). GSK3β negatively regulates SREBP-1c nuclear accumulation (19, 20). GSK3β phosphorylated at Ser9 (p-GSK3β) is inactive. We found that p-GSK3β was decreased in hepatic lysates of rats fed the ethanol-containing diet. This suggests that ethanol could be reducing nuclear accumulation of nSREBP-1 by inhibiting phosphorylation of GSK3β, and thus, activating this kinase to increase SREBP phosphorylation and decrease its stability.

Insulin stimulates SREBP-1c signaling by inhibiting GSK3β kinase activity, and this inhibition involves Akt-dependent phosphorylation of GSK3β (25). Full activation of Akt requires phosphorylation at both Thr308 and Ser473 amino acid residues (26). We found that ethanol-treated rats had lower Akt activity, and this was associated with a significantly altered phosphorylation pattern at Akt-Thr308 and Akt-Ser473. Ethanol reduced the abundance of hepatic p-Akt-Thr308, an effect probably responsible for the observed decrease in Akt activity in our rats.

Akt is phosphorylated at Thr308 by the membrane-bound protein, PDK1 (27). We examined the Akt protein level in whole cell lysates and membrane fractions in livers of ethanol-fed rats. Ethanol decreased membrane-associated Akt and blocked the Akt-membrane translocation and effectively prevented phosphorylation at the Thr308. To corroborate these findings, a fusion protein (GFP-Akt2) was constructed for visualizing its subcellular localization. Upon insulin stimulation, the GFP-Akt2 protein increased in the membrane fraction, and ethanol abolished this insulin action. It is worth noting that ethanol decreased membrane-localized GFP-Akt2 as compared with the control group because these data may explain why ethanol also induced ADH in FGC-4 cells in the absence of insulin.

TRB3 is a cytosolic protein that is reported to directly associate with Akt and to block phosphorylation at Akt-Thr308 (12). Increased TRB3-Akt binding has been associated with reduced Akt activity and with inhibition of insulin signal transduction. We found that ethanol increased cytosolic TRB3 protein levels. We surmise that TRB3 binds and prevents Akt from membrane translocalization. This is based on the following data from ethanol-fed rats showing: 1) decreased levels of membrane-associated Akt; 2) increased cytosolic Akt levels; and 3) elevated TRB3 in the cytosol. Indeed, when TRB3 expression was suppressed by RNA interference, the phosphorylation of Akt at Thr308 and the membrane-localized GFP-Akt2 were increased, and ~60% of insulin stimulation was restored in the presence of ethanol. This is consistent with previous data showing that overexpression of TRB3 in liver increases glucose production (12) and that knockdown of TRB3 by RNA interference improves glucose tolerance (28).

Thus, ethanol may be blocking the insulin-mediated SREBP-1 pathway by regulating TRB3. We next used procedures reported by Brazil et al. (29) to study lipid and protein binding to the PH domain of Akt. We showed that endogenous TRB3 co-immunoprecipitated with the PH domain of Akt, suggesting that there is a TRB3 binding site(s) at the PH domain.
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domain. Since TRB3 has been shown previously to bind Akt (lacking PH domain) near Thr308 to prevent its phosphorylation (12), our data suggest a second TRB3 binding site on Akt. We propose that TRB3 binds to the PH domain and blocks the lipid binding site, thereby preventing membrane association and providing a second site on Akt at which TRB3 can inhibit insulin signaling. Ethanol appears to be impairing this insulin signaling pathway by increasing TRB3 expression, which leads to subsequent inhibition of Akt translocation and decreased Akt phosphorylation. The increased PI3K activity may be due to the decreased “retrophosphorylation” of insulin receptor and insulin receptor substrates by downstream kinase cascades, and also, acetaldehyde (metabolite of ethanol) reduces protein tyrosine phosphatase activity (30).

Interestingly, p-Akt-Ser473 was elevated in liver of rats fed an ethanol-containing diet. PDK2 was recently identified as an Akt-Ser473 kinase (31, 32). We measured Rictor (a critical component of this PDK2 enzy-
matic complex) protein levels in whole cell extracts, cytosol, and cytoplasmic membrane extracts and found that Rictor is a membrane-associated protein. Ethanol increased the membrane association of Rictor, PDK1, p110α, or p85. However, ethanol-induced TRB3 binds to Akt and prevents its membrane association. Therefore, one might assume that phosphorylation at both Thr308 and Ser473 should be similarly affected by ethanol. Since this was not the case, we surmise that Akt phospho-status is determined by a balance between kinase and phosphatase activities. Phosphatases that specifically dephosphorylate Ser473, such as PH domain leucine-rich repeat protein phosphatase (PILPP) (33), might be inhibited by downstream effectors or by ethanol (or its metabolite, acetaldehyde), resulting in increased phosphorylation at Ser473. Moreover, since phosphorylation of Ser473 boosts the phosphorylation of Thr308 (31), the increased phosphorylation of Ser473 might be compensatory as TRB3 disrupts insulin signaling.

Based on the data presented herein and previously published data from our laboratory and well as others, we propose the following insulin-independent scheme by which chronic ethanol intake can induce expression of Class I ADH in the rat (Fig. 9). Insulin binding to its receptor results in tyrosine phosphorylation of IRS-1 and IRS-2, which provides docking sites for PI3 kinase and also activates this kinase to phospho-
rylate phosphatidylinositol-4, 5-biphosphate, generating PIP3 (34, 35). Akt binds PIP3 via its PH domain, triggering the phosphorylation of Akt-Thr308 by PDK1. To fully activate Akt, PDK2 (mammalian target of 
PDK2) was recently identified as an Akt-Ser473 kinase (19) and stimu-
lates as a negative modulator of Akt by binding directly to Akt and blocking Akt phosphorylation, resulting in the maintenance of GSK3α activation. GSK3α-induced phosphorylation of SREBP-1c reduces the abundance of nSREBP-1c, thereby disinhibiting ADH gene trans-
scription. Future studies in our laboratory will examine the mechanisms underlying ethanol effects on TRB3 mRNA and protein expression.