Involvement of mammalian sirtuin 1 in the action of ethanol in the liver

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You M, Liang X, Ajmo JM, Ness GC. Involvement of mammalian sirtuin 1 in the action of ethanol in the liver. Am J Physiol Gastrointest Liver Physiol 294: G892–G898, 2008.—Chronic ethanol feeding causes liver steatosis in animal models by upregulating the sterol regulatory element-binding protein 1 (SREBP-1), which subsequently increases the synthesis of hepatic lipid. SREBP-1 activity is regulated by reversible acetylation at specific lysine residues. The present study tests the hypothesis that activation of SREBP-1 by ethanol may be mediated by mammalian sirtuin 1 (SIRT1), a NAD+-dependent class III protein deacetylase. The effects of ethanol on SIRT1 were determined in cultured rat hepatoma cells and in the livers of ethanol-fed mice. In rat H4IIEC3 cells, we observed that ethanol exposure induced SREBP-1c lysine acetylation and SREBP-1c transcriptional activity. The effect of ethanol was abolished by expression of wild-type SIRT1 or by treatment with resveratrol, a known potent SIRT1 agonist. Conversely, knocking down SIRT1 by the small silencing SIRT1 plasmid SIRT1shRNA or expression of a SIRT1 mutant, SIRT1(H363Y), did not negate the ethanol effect. These findings suggest that the effect of ethanol on SREBP-1c is mediated, at least in part, through SIRT1 inhibition. Consistent with the in vitro findings, chronic ethanol feeding substantially downregulated hepatic SIRT1 in mice. Inhibition of hepatic SIRT1 activity was associated with an increase in the acetylated active nuclear form of SREBP-1c in the livers of ethanol-fed mice. Our results indicate an essential role for SIRT1 in mediating the effects of ethanol on SREBP-1 and hepatic lipid metabolism, as well as the development of alcoholic fatty liver. Hence, SIRT1 may represent a novel therapeutic target for treatment of human alcoholic fatty liver disease.

alcoholic liver steatosis; lipid metabolism; acetylation; sterol regulatory element-binding protein-1c; peroxisome proliferator-activated receptor-γ coactivator-1α

STEROL REGULATORY ELEMENT-BINDING PROTEINS (SREBPs) ARE TRANSCRIPTION FACTORS THAT REGULATE CHOLESTEROL AND LIPID SYNTHESIS (13). PROCESSING OF SREBP TO GENERATE THE TRANSCRIPTIONALLY ACTIVE MATURE SREBP PROTEIN (~68 kDa) INVOLVES THE RELEASE OF THE N-TERMINAL DOMAIN OF THE INACTIVE PRECURSOR PROTEIN (~125 kDa) FROM THE ENDOPLASMIC RETICULUM (ER) AND NUCLEAR ENVELOPE BY A TWO-STEP PROTEOLYTIC CASCADE (13, 28). THE ACTIVE MATURE SREBP IS SUBSEQUENTLY TRANSPORTED TO THE NUCLEUS AND BINDS TO THE STEROL RESPONSE ELEMENTS (SRE) AT THE PROMOTERS OF SREBP TARGET GENES. THE MATURE NUCLEAR SREBP PROTEIN IS DEGRADATION BY A PROTEASOME-DEPENDENT PROCESS (13, 28). THERE ARE THREE MAJOR SREBP ISOFORMS, SREBP-1A, SREBP-1C, AND SREBP-2 (28). IN ANIMAL LIVER, THE SREBP-1C TRANSCRIPT PREDOMINATES AND MAINLY REGULATES LIPID SYNTHESIS BY INDUCING A PANEL OF GENES ENCODING LIPOGENIC ENZYMES INCLUDING FATTY ACID

SYNTHASE, STEAROYL-COOXYME A DESATURASE 1 (SCD1), MITOCHONDRIAL GLYCEROL-3-PHOSPHATE ACYLTRANSFERASE (GPAT1), ATP CITRATE LYASE, AND ACETYL-CoA CARBOXYLASE (13, 28).

THE INVOLVEMENT OF SREBP-1 IN THE ACTION OF ALCOHOLIC LIVER STÉTOSIS IN ANIMALS HAS BEEN DEMONSTRATED (5, 6, 14, 15, 30, 32, 37, 38, 39). WHILE BOTH ETHANOL-MEDIATED AMP-ACTIVATED KINASE (AMPK) INHIBITION AND ETHANOL-CAUSED ER STRESS HAVE ALTERNATIVELY BEEN PROPOSED TO CONTRIBUTE TO THE ACTIVATION OF SREBP-1, THE PRECISE MOLECULAR MECHANISMS BY WHICH ETHANOL STIMULATES SREBP-1 ACTIVITY REMAIN UNCLEAR (5, 15, 39).

SREBP PROTEIN STABILITY AND ACTIVITY ARE REGULATED BY REVERSIBLE ACETYLATION (10, 22, 31). HISTONE ACETYLTRANSFERASE (HAT) CAMP RESPONSE ELEMENT BINDING PROTEIN (CBP)/P300 ACETYLATES SREBP AT ITS LYSINE RESIDUES IN THE CORE DNA-BINDING DOMAIN AND INCREASES THE DNA-BINDING ACTIVITY OF SREBP (10). THESE ACETYLATED LYSINE RESIDUES ARE ALSO TARGETS OF UBIQUITINATION. THUS ACETYLATION INHIBITS PROTEASOME-MEDIATED DEGRADATION OF MATURE SREBP RESULTING IN SIGNIFICANT INCREASES IN THE PROTEIN LEVELS OF SREBP, WHEREAS DEACETYLATION BY HISTONE DEACETYLASES (HDACs) AND ULTIMATELY UBIQUITINATION LEAD TO DECREASED SREBP ACTIVITY (10).

MAMMALIAN SIRTUIN 1 (SIRT1) IS A NAD+-DEPENDENT CLASS III PROTEIN DEACETYLASE. IT USES NAD+ AS PART OF THE DEACETYLATION REACTION TO PRODUCE NICOTINAMIDE AND ACETYL-ADP-RIBOSE (11). SIRT1 IS LOCALIZED EXCLUSIVELY TO THE NUCLEUS AND DEACETYLATES A VARIETY OF IMPORTANT NUCLEAR HISTONE AND NONHISTONE PROTEINS (11).

RECENT STUDIES HAVE SUGGESTED A CRUCIAL ROLE FOR SIRT1 IN REGULATING LIPID METABOLISM. IN ADIPOSE TISSUE, SIRT1-MEDIATED REPRESSION OF PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR-γ ACTIVITY INHIBITS THE EXPRESSION OF GENES INVOLVED IN FAT STORAGE AND, THEREFORE, REDUCES triglyceride ACCUMULATION (24). IN THE LIVER, SIRT1-MEDIATED DEACETYLATION OF PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR-γ COACTIVATOR-1α (PGC-1α) LEADS TO A GENE EXPRESSION PATTERN THAT FAVORS OXIDATION AND ENERGY EXPENDITURE RATHER THAN FAT SYNTHESIS (2, 9, 18, 25, 26). THE POTENTIAL CONNECTION BETWEEN SIRT1 AND SREBP-1 HAS RECENTLY BEEN REPORTED (26). HEPATIC SIRT1 KNOCKDOWN IN MICE INDUCES THE EXPRESSION OF SREBP-1C AND ITS TARGET GENES ENCODING LIPOGENIC ENZYMES (26).

THERE IS INCREASING EVIDENCE THAT SIRT1 MAY BE INVOLVED IN ALCOHOLIC OR NONALCOHOLIC FATTY LIVER. EXPRESSION OF SIRT1 PROTEIN IS REDUCED SIGNIFICANTLY IN NONALCOHOLIC FATTY LIVER DISEASE INDUCED BY A HIGH-FAT DIET IN RATS (4). THE PROTECTIVE EFFECTS OF RESVERATROL, A KNOWN POTENT AGONIST OF SIRT1, AGAINST ALCOHOL-INDUCED LIVER TOXICITIES HAVE BEEN REPORTED (16, 17, 23). IN ADDITION TO ITS WELL-KNOWN ANTIOXIDANT PROPERTIES (23), UPREGULATION OF HEPATIC SIRT1 SIGNALING BY RESVERATROL MAY
contribute to its hepatic protective effects against alcoholic liver injuries.

In the present study, we tested the hypothesis that SIRT1 inhibition may represent an essential regulatory mechanism of altered lipid metabolism in response to ethanol exposure.

**MATERIALS AND METHODS**

Reagents and antibodies. Most chemicals and supplies were purchased from Sigma Chemical (St. Louis, MO), Schleicher and Schuell, GIBCO-BRL, and DuPont NEN Research Products. Resveratrol was obtained from Sigma. Ethanol was purchased from Aldrich (Milwaukee, WI). Splitomicin and N-acetyl-leucinal-leucinal-norleucinal (ALLN) were from Calbiochem (San Diego, CA). The rat hepatoma H4IIEC3 cell line was purchased from the American Type Culture Collection and was grown in MEM supplemented with 10% FBS, 100 μg/ml streptomycin, and 63 μg/ml penicillin G.

**Plasmids.** The pSyn SRE plasmid, containing a generic TATA and three SRE elements (representing those found at 325 to 225 bp of the hamster HMG-CoA synthase promoter fused into the luciferase pGL2 basic vector) as well as the expression vector for the active, processed nuclear form of SREBP1c (nSREBP-1c), were kind gifts from T. F. Osborne (University of California, Irvine, CA). The CBP plasmid was a kind gift from T.-P. Yao (Duke University, Durham, NC). Wild-type SIRT1 and deacetylase-defective SIRT1(H363Y) mutant expression plasmids were kind gifts from M. W. Mayo (University of Virginia, Charlottesville, VA) and W. Bai (University of South Florida, Tampa, FL). The p300 cDNA and small silencing SIRT1 plasmid (SIRT1shRNA) were purchased from Upstate Biotechnology (Lake Placid, NY).

**SREBP-1 acetylation and deacetylation assays.** Rat hepatoma H4IIEC3 cells were cotransfected with expression plasmids encoding nSREBP-1c, CBP/p300, wild-type SIRT1 (SIRT1wt), SIRT1 mutant [SIRT1(H363Y)], small silencing SIRT1 plasmid (SIRT1shRNA), or empty vector control. At 36 h posttransfection, the cells were harvested and soluble fraction were prepared. Cell extracts were immunoprecipitated with an antibody to SREBP-1 and immunoblotted with acetyl-lysine (Ac-Lys) or a SREBP-1 antibody. Western blots were quantified by a PhosphorImager and ImageQuant software analysis. Data are means ± SD (n = 3–4 replications). *P < 0.05, **P < 0.001 by one-way ANOVA.

**Fig. 1.** Mammalian sirtuin 1 (SIRT1) interacts with and deacetylates the active nuclear form of sterol regulatory element-binding protein 1c (nSREBP-1c) in rat hepatoma cells. A: Western blot of H4IIEC3 cells transfected with plasmids of wild-type SIRT1 (SIRT1wt) or SIRT1 mutant [SIRT1(H363Y)]. SIRT1 was detected with an anti-SIRT1 antibody. B: extracts of H4IIEC3 cells transfected with plasmids (5 μg each) of nSREBP-1c were immunoprecipitated with an antibody to SREBP-1 or SIRT1 and immunoblotted with an anti-SIRT1 or an anti-SREBP-1 antibody. C: cell extracts of H4IIEC3 cells transfected with plasmids (5 μg each) of p300, nSREBP-1c, SIRT1wt, SIRT1(H363Y), small silencing SIRT1 plasmid (SIRT1shRNA), and treated with resveratrol (RSV) were immunoprecipitated with an antibody to SREBP-1 and immunoblotted with acetyl-lysine (Ac-Lys) or a SREBP-1 antibody. Western blots were quantified by a PhosphorImager and ImageQuant software analysis. Data are means ± SD (n = 3–4 replications). *P < 0.05, **P < 0.001 by one-way ANOVA. aSignificant difference vs. control; bsignificant difference vs. p300 expression.
Fig. 3. Effect of ethanol on SIRT1-mediated inhibition of SREBP-1 transcriptional activity in rat H4IIEC3 cells. A: H4IIEC3 cells were transfected with a pSyn SRE-luciferase reporter (10 µg) and expression plasmids (5 µg/each) of p300, SIRT1wt, or SIRT1shRNA and β-galactosidase (2 µg; internal control). Ethanol or resveratrol (50 µM) was added for 24 h. Forty-eight hours after transfection, cell lysates were harvested, and luciferase and β-galactosidase activities were determined as described in MATERIALS AND METHODS. Normalized luciferase activities are means ± SD from at least 3 experiments performed in duplicate. B: representative Western blots of H4IIEC3 cells exposed to ethanol at the concentrations indicated for 24 h. *P < 0.05, **P < 0.001 by one-way ANOVA. *Significant difference vs. control; **significant difference vs. p300 expression.

posttransfection, cell lysates (25 µg/ml ALLN) were immunoprecipitated with an anti-SREBP-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and immunoblotted with an acetyl-lysine antibody (Cell Signaling).

Immunoprecipitation and Western blots. For immunoprecipitation, primary antibodies were mixed with precleared lysates and 20 µl of protein agarose A/G (Santa Cruz, CA), and reactions were tumbled overnight at 4°C. The agaroase beads were washed, and the protein was eluted and then subjected to Western blotting analysis. Levels of protein detected on Western blots were quantified by a Phosphor-Imager.

Transient transfection and luciferase assays. Hepatoma H4IIEC3 cells transfection assays were performed as described previously (38, 39). Transient transfections were performed using Lipofectamine reagent (Invitrogen) according to the manufacturer’s protocol. Luciferase assays were carried out using extracts from duplicate culture plates, and the results were averaged to represent a single data point for each transfaction. β-Galactosidase was used as an internal control to correct for transfection efficiency.

Animals and diets. The detailed animal feeding protocol was as described previously (38). Liquid diets were based on the Lieber-DeCarli formulation (Dyets; Bethlehem, PA). Six- to eight-week-old male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were divided into two dietary groups: 1) control low-fat diet (LF: fat comprising 10% of total calories and 72% of calories as carbohydrate) and 2) ethanol-containing low-fat diet (LF + E: identical to the control diet except with ethanol was added to account for 27.5% of total calories and the caloric equivalent of carbohydrate (maltose-dextrin) was removed). The animals were pair fed for 4 wk. The experimental protocols were approved by the respective Institutional Animal Care Use Committees of Indiana University School of Medicine and of University of South Florida.

Real-time quantitative RT-PCR. Liver or cell total RNA was prepared using the RNAeasy total RNA kit (Qiagen, Valencia, CA). Reverse transcription of 5 µg total RNA to cDNA was performed using the Stratascript QPCR cDNA synthesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s protocols. Real-time quantitative RT-PCR (qRT-PCR) amplification was performed in a MX4000 Spectrofluorometric thermal cycler (Stratagene) using a Brilliant SYBR Green QPCR master mix (Stratagene). Optimized primer sets tested for the targets for the SYBR Green based real-time PCR system were purchased from Superarray Bioscience (Frederick, MD). The following primer sets were purchased and used: SIRT1, GPAT1, SCD1, and GAPDH. The relative amount of target mRNA was calculated using the comparative threshold method by normalizing the target mRNA comparative threshold to those for GAPDH.

Liver nuclear extracts. Portions of fresh livers were homogenized in 10 mM HEPES (pH 7.9), 25 mM KCl, 10 mM NaF, 0.15 mM spermine, 1 mM EDTA, 2 M sucrose, 2% protease inhibitor (Roche Diagnostics, IN), 0.5 mM dithiothreitol, and 25 µg/ml ALLN, and nuclear proteins (50 µg/ml ALLN) were extracted as described previously (38, 39).

PGC-1α and nSREBP-1c acetylation assays. PGC-1α or nSREBP-1c protein was immunoprecipitated using liver nuclear extracts by an anti-PGC-1α or an anti-SREBP-1 antibody (Santa Cruz, CA). PGC-1α or SREBP-1c levels and acetylation were detected using specific antibodies for PGC-1α, SREBP-1, and acetyl-lysine (Cell Signaling, MA); 25–50 µg/ml ALLN were presented in all the procedures of nSREBP-1c analysis.

HDAC or HAT enzyme activity assays. The activity of HDAC was measured with the use of a nonisotopic assay that used a fluorescent derivative of ε-acetyl-lysine (AK-500). HDAC fluorescence activity assay kit, Biomol). HAT activity was determined with ELISA (HAT activity assay kit, Upstate Biotechnology).
Statistical analysis. In vivo experiments were performed on five to eight mice per group with values as means ± SD and were analyzed with Student’s t-test. In vitro assays were analyzed by one-way ANOVA analysis followed by post hoc testing with Fishers protected least squares difference test. P < 0.05 was considered statistically significant.

RESULTS

Ethanol blocks SIRT1-mediated-SREBP-1c deacetylation in rat hepatoma cells. Rat hepatoma H4IIEC3 cells metabolize ethanol through the activities of alcohol dehydrogenase and aldehyde dehydrogenase (38, 39). H4IIEC3 cells express modest levels of SIRT1 protein, which can be increased significantly by ectopic expression of a SIRT1wt or SIRT1(H363Y) (Fig. 1A). Thus this cell line provides an adequate system in which the effects of ethanol on SIRT1 can be studied. The dominant-negative SIRT1(H363Y) mutant construct specifies an amino acid substitution in which the histidine residue at position 363 of the SIRT1 protein catalytic site is substituted by tyrosine. This substitution abolishes SIRT1 deacetylase activity (35, 36). We focused on studying SREBP-1c because it was demonstrated that the ratio of SREBP-1c to SREBP-1a was nearly 9:1 in normal animal liver and that ethanol feeding induced hepatic triglyceride synthesis by activation of hepatic SREBP-1c in animals (13, 15, 28, 38, 39).

We first determined whether SIRT1 and SREBP-1c physically interact with each other. We transfected hepatoma H4IIEC3 cells with vector encoding active nSREBP1c or SIRT1 and performed coprecipitation experiments. As shown in Fig. 1B, an antibody to SIRT1 coprecipitated SREBP-1c and an antibody to SREBP-1 coprecipitated SIRT1 from cells expressing both SREBP-1c and SIRT1 proteins. Thus SIRT1 associates with SREBP-1c.

To determine whether SIRT1 deacetylates nSREBP-1c, we transfected the nSREBP-1c along with p300 into H4IIEC3 cells with empty vector, vector encoding SIRT1wt, SIRT1(H363Y), or SIRT1shRNA. SREBP-1c immunoprecipitates were prepared with an antibody to SREBP-1 and analyzed with an antibody to acetylated lysine on Western blots. As expected, expression of p300 effectively led to the acetylation of nSREBP-1c (Fig. 1C). A SIRT1 mutant that lacks enzymatic activity, SIRT1(H363Y), had a minor effect (Fig. 1C). However, expression of SIRT1wt or treatment with resveratrol, a known SIRT agonist, largely abolished p300-mediated acetylation of nSREBP-1c (Fig. 1C). Moreover, while knocking down SIRT1 by SIRT1shRNA coexpression slightly increased p300-mediated nSREBP-1c acetyaltion (data not shown), co-expression of SIRT1shRNA plasmid blocked the effect of resveratrol on nSREBP-1c acetylation (Fig. 1C). Collectively, these data show that SIRT1 deacetylates SREBP-1c in a manner that is dependent on its deacetylase activity.

To investigate the effect of ethanol on SIRT1-mediated SREBP-1c deacetylation, we transfected the nSREBP-1c plasmid into H4IIEC3 cells with either empty vector or vector encoding p300, SIRT1wt, or SIRT1H363Y and exposed the cells to ethanol for 24 h. Cell extracts were immunoprecipitated with an antibody to SREBP-1 and immunoblotted with an antibody to acetylated lysine or with SREBP-1 antibody to determine the nSREBP-1c amount; 25–50 μg/ml ALLN were included in the procedures. IgG antibodies were used as a negative control. All data are means ± SD (n = 5–8 animals). **P < 0.001, by paired t-test vs. pair-fed controls.
anti-acetyl-lysine antibody. As shown in Fig. 2A, ethanol exposure substantially augmented p300-mediated nSREBP-1c acetylation in a dose-dependent manner. Importantly, ectopic expression of SIRT1wt largely abolished ethanol-induced nSREBP-1c acetylation by p300, but a SIRT1 mutant [SIRT1(H363Y)] that lacks enzymatic activity had no effect (Fig. 2B). Similar results were produced by coexpression of a CBP plasmid (data not shown).

Ethanol abrogates SIRT1-mediated inhibition of SREBP-1c transcriptional activity in rat H4IIEC3 cells. Our previous work (38, 39) demonstrated that ethanol significantly induced SREBP-1 transcriptional activity in H4IIEC3 cells. To examine the role of SIRT1 in this activation, we evaluated SREBP-1 transcriptional activity in H4IIEC3 cells transfected with a SREBP-regulated promoter, pSyn SRE-luciferase, either alone or constructed together with p300, SIRT1wt, or SIRT1shRNA. As shown in Fig. 3A, coexpression of p300 activated pSyn SRE-luciferase in the absence of ethanol. However, ectopic expression of SIRT1wt or addition of resveratrol abolished the p300-mediated activation of the pSyn SRE-luciferase, whereas knocking down SIRT1 by SIRT1shRNA failed to block the p300 activation or resveratrol inhibition of the SRE reporter (Fig. 3A). These results demonstrate that SIRT1-mediated SREBP-1 deacetylation inhibits SREBP-1 transcriptional activity.

As expected, ethanol exposure significantly augmented the p300-mediated pSyn SRE-luciferase activity (Fig. 3A). However, coexpression of the SIRT1wt or addition of resveratrol diminished the ethanol-induced pSyn SRE-luciferase activity (Fig. 3A). Conversely, expression of SIRT1shRNA did not negate the ethanol effect (Fig. 3A). Furthermore, expression of SIRT1 shRNA abolished the inhibitory effect of resveratrol (Fig. 3A). Taken together, our data suggest that activation of SREBP-1 transcriptional activity by ethanol was mediated, at least in part, through SIRT1 inhibition.

We further examined the effect of ethanol on SIRT1 protein expression in H4IIEC3 cells. Western blot analysis showed that incubation of the H4IIEC3 cells for 24 h with ethanol significantly reduced nuclear SIRT1 protein levels to nearly 50% of controls (Fig. 3B).

SIRT1 regulates expression of SREBP-1-target genes encoding lipogenic enzymes in rat hepatoma cells. To verify the consequence of the SIRT1-mediated inhibition of SREBP-1 transcriptional activity, we transfected nSREBP-1c either alone or together with p300, SIRT1wt, SIRT1(H363Y), or SIRT1shRNA into H4IIEC3 cells and evaluated the mRNA expression levels of two SREBP-1c targeted lipogenic enzymes, SCD1 and GPAT1.

As shown in Fig. 4, p300 significantly increased mRNA expression of SCD1 or GPAT1 compared with controls. However, the p300-mediated increases in mRNA of SCD1 or GPAT1 were largely prevented by coexpression of SIRT1wt (Fig. 4). Conversely, knocking down SIRT1 by SIRT1shRNA or expression of SIRT1(H363Y) did not affect SCD1 or GPAT1 expression (Fig. 4). Our findings suggest that SIRT1 is partially required for repression of the SREBP-1c-regulated gene encoding lipogenic enzymes in H4IIEC3 cells.

Ethanol feeding downregulates hepatic SIRT1 in mice. We previously reported that feeding mice a low-fat ethanol-containing diet for 4 wk led to the development of liver steatosis (38). As we published, ethanol feeding markedly increased hepatic triglyceride content by nearly 3.5-fold compared with pair-fed control mice (38). Therefore, the current study assessed hepatic SIRT1 levels using liver samples from the same mice. SIRT1 expression was not changed at the messenger RNA level by ethanol feeding (Fig. 5A); however, SIRT1 protein levels were significantly reduced ~40% in the ethanol-fed mice (Fig. 5B).

Pgc-1α activity is positively regulated by SIRT1-mediated deacetylation (2, 9, 18, 25, 26). The acetylation status of PGC-1α is generally regarded as a reliable marker of in vivo SIRT1 activity (2, 18). To determine whether ethanol feeding alters hepatic SIRT1 deacetylase activity, PGC-1α was immunoprecipitated from liver samples using an anti-PGC-1α antibody. Western analysis was performed with an anti-acetyl-lysine antibody. Figure 5C shows that the acetylation level of PGC-1α in the ethanol-fed mice was markedly higher than in the controls. As a result, the ratio of acetylated PGC-1α to total PGC-1α protein was significantly increased by ethanol feeding, indicating that hepatic SIRT1 enzymatic activity was inhibited in the livers of ethanol-fed mice.

We previously demonstrated that ethanol feeding increased the expression of hepatic nSREBP-1c protein in mice (38). To study whether the increased nSREBP-1c protein is associated with its acetylation status, we analyzed nSREBP-1c acetylation using immunoprecipitation of SREBP-1c from liver nuclear extracts (Fig. 5D). As expected, ethanol exposure increased nSREBP-1c acetylation, and coexpression of SIRT1wt or addition of resveratrol diminished the ethanol-induced nSREBP-1c acetylation (Fig. 5D). Conversely, expression of SIRT1shRNA did not negate the ethanol effect (Fig. 5D).}

Fig. 6. Proposed role of SIRT1 in the pathogenesis of alcoholic fatty liver. Chronic ethanol feeding downregulates hepatic SIRT1. Ethanol inhibition of SIRT1 may promote SREBP-1 activity and inactivate PGC-1α by inducing acetylation, thereby increasing hepatic lipid synthesis and slowing mitochondrial fatty acid oxidation. CBP/p300, cAMP response element binding protein.
extracts with an anti-SREBP-1 antibody followed by western analysis using an anti-acetyl-lysine antibody. As shown in Fig. 5D, the acetylation levels of hepatic nSREBP-1c in the ethanol-fed mice were ~1.8-fold higher than the pair-fed controls.

Ethanol feeding had no significant effects on total hepatic HAT activity or overall hepatic HDAC activity (data not shown). The hepatic HDAC activity was measured in the absence of NAD\(^+\) cofactor. Thus our results suggest that ethanol inhibition of SIRT1 activity might cause site-specific hyperacetylation of PGC-1α or nSREBP-1c.

**DISCUSSION**

The present study investigated the molecular mechanisms of the ethanol-induced SREBP-1 activation by identifying SIRT1 as a novel target of ethanol. Using rat hepatoma H4IIEC3 cells, we showed that SIRT1 regulated SREBP-1c activity by deacetylating SREBP-1c and by inhibiting its transcriptional activity, resulting in transcription repression of the SREBP-1c-regulated lipogenic enzymes. As we expected, ethanol exposure increased SREBP-1c lysine acetylation and increased SREBP-1c translational activity. Importantly, ectopic expression of wild-type SIRT1 or treatment with resveratrol, a known potent SIRT1 agonist, abolished the ethanol-induced SREBP-1c hyperacetylation and blocked ethanol-mediated increases in SREBP-1 translational activity, whereas knocking down SIRT1 by SIRT1shRNA or expression of a SIRT1 mutant [SIRT1(H363Y)] failed to block the effects of ethanol on SREBP-1. Moreover, ethanol exposure significantly decreased SIRT1 protein expression levels in H4IIEC3 cells.

Consistent with these in vitro findings, chronic ethanol administration to mice decreased hepatic SIRT1 protein levels and significantly reduced its deacetylase activity as judged by a significantly increased ratio of acetylated PGC-1α to total PGC-1α protein in mouse livers. Ethanol-caused inhibition of SIRT1 activity was associated with hepatic nSREBP-1c hyperacetylation. Taken together, our findings for the first time suggest that SIRT1 may play a crucial role in regulating ethanol-mediated lipid accumulation in liver (Fig. 6).

The precise mechanism by which ethanol downregulates SIRT1 remains to be elucidated. Our data show that ethanol exposure decreased SIRT1 protein levels while mRNA levels remained unchanged, indicating that ethanol regulates SIRT1 at a posttranscriptional level. SIRT1 protein synthesis is known to be elevated by pyruvate both in hepatocytes and in animal livers (25). Ethanol is metabolized in the liver by alcohol dehydrogenase and aldehyde dehydrogenase (19). The resulting shift of the redox state results in an accumulation of NADH and reduction of NAD\(^+\) accompanied by a significant increase in the lactate to pyruvate ratio (19). We have recently observed that SIRT1 protein levels in cultured rat hepatocytes were reduced by treatment of cells with lactate (X. Liang and M. You, unpublished observations). Thus it is possible that the ethanol metabolism induced increase in lactate may reduce hepatic SIRT1 protein levels.

Our findings demonstrated that ethanol feeding suppressed hepatic SIRT1 deacetylase activity. SIRT1 enzymatic activity is regulated by multiple factors including NAD\(^+\), NADH, intracellular nicotinamide, and the cellular NAD\(^+\) concentration-to-NADH concentration ratio (3, 7, 20, 21). Therefore, it is logical to speculate that the ethanol metabolism-induced NAD\(^+\) depletion or overproduction of NADH could significantly suppress SIRT1 deacetylase activity. Nevertheless, the mechanism by which ethanol downregulates SIRT1 in vivo could be much more complicated. It is known that ethanol metabolism-induced acute shifts in redox state normalize after extended ethanol exposure in baboons but that fatty liver persists in those animals (27). Whether hepatic SIRT1 activity is continually inhibited and altered SIRT1 activity contributes to persistent liver steatosis and subsequent progression into further liver injury will be of great interest to determine.

Hepatic CYP2E1-mediated ethanol metabolism releases reactive oxygen species (19). Recent evidence suggests that oxidative stress downregulates SIRT1 (8, 33, 34). It is tempting to postulate that the ongoing reactive oxygen species generation by ethanol metabolism could prolong the suppression of SIRT1 activity in the liver. In addition, metabolites generated by ethanol metabolism including acetaldehyde or acetate may directly interfere with SIRT1 activity. Additional studies addressing these potential multiple mechanisms involved in ethanol regulation of SIRT1 are required and are currently under investigation in our laboratory.

Our previous work (38, 39) showed that ethanol exposure selectively increased mature SREBP-1 protein levels in both cultured hepatocytes and in mouse livers. Increased nSREBP-1c acetylation by ethanol via SIRT1 suppression would inhibit nSREBP-1c proteasominal degradation resulting in increased nSREBP-1c protein levels. Intriguingly, we previously found that ethanol-stimulated SREBP-1 activity occurs partially through downregulating AMPK (39). Recently, a possible association between SIRT1 and AMPK has been suggested (2, 33). Further investigations are needed to determine the mechanism of ethanol regulation in the potential SIRT1-AMPK axis. Furthermore, the development of alcoholic fatty liver in rats has also been associated with suppressed SREBP-1c activity, clearly suggesting that multiple molecular mechanisms may be involved in the alcoholic fatty liver (1, 12).

In addition to regulating the SIRT1-SREBP-1c axis, our data show that ethanol feeding increased the acetylation status of hepatic PGC-1α in mice. Considerable evidence suggests that the SIRT1-PGC-1α axis induces fatty acid oxidation through regulating the genes encoding the enzymes involved in fatty acid oxidation (2, 9, 18, 25, 26). It is possible that deregulation of SIRT1-PGC-1α axis by ethanol acts as an upstream trigger leading to impairment of fatty acid oxidation and contributes to lipid accumulation (Fig. 6).

In summary, our findings suggest a novel molecular mechanism by which ethanol activates SREBP-1 and define an essential role of SIRT1 in the development of alcoholic fatty liver. Our study suggests a possible therapeutic value for SIRT1-activating compounds such as resveratrol, a dietary polyphenol, in preventing and/or reversing human alcoholic fatty liver disease.

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