Ethanol administration exacerbates the abnormalities in hepatic lipid oxidation in genetically obese mice

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Everitt H, Hu M, Ajmo JM, Rogers CQ, Liang X, Zhang R, Yin H, Choi A, Bennett ES, You M. Ethanol administration exacerbates the abnormalities in hepatic lipid oxidation in genetically obese mice. Am J Physiol Gastrointest Liver Physiol 304: G38–G47, 2013. First published November 8, 2012; doi:10.1152/ajpgi.00309.2012.—Alcohol consumption synergistically increases the risk and severity of liver damage in obese patients. To gain insight into cellular or molecular mechanisms underlying the development of fatty liver caused by ethanol-obesity synergism, we have carried out animal experiments that examine the effects of ethanol administration in genetically obese mice. Lean wild-type (WT) and obese (ob/ob) mice were subjected to ethanol feeding for 4 wk using a modified Lieber-DeCarli diet. After ethanol feeding, the ob/ob mice displayed much more pronounced changes in terms of liver steatosis and elevated plasma levels of alanine aminotransferase and aspartate aminotransferase, indicators of liver injury, compared with control mice. Mechanistic studies showed that ethanol feeding augmented the impairment of hepatic sirtuin 1 (SIRT1)-AMP-activated kinase (AMPK) signaling in the ob/ob mice. Moreover, the impairment of SIRT1-AMPK signaling was closely associated with altered hepatic functional activity of peroxisome proliferator-activated receptor γ coactivator-α and lipin-1, two vital downstream lipid regulators, which ultimately contributed to aggravated fatty liver observed in ethanol-fed ob/ob mice. Taken together, our novel findings suggest that ethanol administration to obese mice exacerbates fatty liver via impairment of the hepatic lipid metabolism pathways mediated largely by a central signaling system, the SIRT1-AMPK axis.

The liver is one of the major organs in which sirtuin 1 (SIRT1), an NAD+-dependent protein deacetylase, plays a crucial role in the regulation of lipid metabolism by regulating the acetylation/deacetylation status of a wide range of molecules including histones and transcriptional regulators (29, 34–36). AMP-activated kinase (AMPK) is another master lipid regulator controlling the pathways of hepatic lipid metabolism largely through direct phosphorylation and subsequent inhibition of acetyl-CoA carboxylase (ACC), a rate-limiting enzyme in hepatic fatty acid biosynthesis that exerts its effects through the inhibition of malonyl-CoA, a basic substrate for fatty acids (33).

SIRT1 and AMPK reciprocally regulate each other’s activity and share several common target molecules (4, 17, 19). Whereas SIRT1 stimulates AMPK activity through LKB1, AMPK enhances SIRT1 activity by increasing cellular NAD+ levels (4, 17, 19). In liver, activation of the SIRT1-AMPK axis protects against the development of steatosis by turning on fatty acid oxidation pathways and inhibiting lipid synthesis through interaction with multiple transcriptional factors and cofactors such as sterol regulatory element-binding protein 1 (SREBP-1), peroxisome proliferator-activated receptor α (PPARα), and PPARγ coactivator-α (PGC-1α) (9, 24).

Lipin-1 has recently emerged as a vital lipid regulator by displaying two distinct functions in regulating lipid metabolism dependent upon its subcellular localization. In the cytosol, lipin-1 functions as a Mg2+-dependent phosphatidate phosphatase, catalyzing a key step in the synthesis of glycerolipids (10). The nuclear-localized lipin-1 acts as a cotranscriptional regulator to increase the capacity of the liver for fatty acid oxidation and reduce the lipogenesis through interacting with transcriptional regulators including PGC-1α, PPARα, and SREBP-1 (10, 24).

The development of AFLD and NAFLD have been attributed to a combination of increased fatty acid uptake, de novo lipogenesis, decreased fatty acid oxidation, and impaired fatty acid export from the liver (3, 7, 16). Considerable evidence from human and rodent studies demonstrates that both AFLD and NAFLD are associated with impairment of the hepatic the SIRT1-AMPK signaling system and subsequent derangement of PGC-1α or lipin-1 (3, 4, 7, 16, 17, 19). Nevertheless, the role of hepatic SIRT1-AMPK signaling in the development of fatty liver disease that is manifest by the synergism of ethanol and obesity is largely unknown.

In the present study, we used a murine model of obesity (ob/ob mouse model) to examine the effects of chronic ethanol administration to obese ob/ob mice and investigated the hepatic lipid metabolism pathways affected by this combination, which results in a synergistic enhancement of fatty liver.
Materials and methods

Animals and ethanol feeding. Twelve-week-old male ob/ob mice (C57BL/6-J-Rj-ob) and their lean (+/-) wild-type (WT) littermates were purchased from Jackson Laboratory (Bar Harbor, ME). Animals were housed individually in stainless steel wire-bottom cages in a temperature- and humidity-controlled room (23°C and 50% relative humidity) with a 12:12-h light-dark cycle.

Liquid diets were based on the modified Lieber-DeCarli formula and provided 1 kcal/ml (prepared by Dyets, Bethlehem, Philadelphia, PA) (1, 30). Male obese ob/ob mice and their age-matched WT control mice were divided into four dietary groups: 1) control: WT mice fed a polysaturated fat control diet (PUFA; 40% of calories from fat, primarily from corn oil); 2) WT mice fed a PUFA diet plus ethanol identical to the control PUFA diet but with ethanol added to account for 27.5% of total calories and the caloric equivalent of carbohydrate (maltose-dextrin) removed; 3) ob/ob mice fed the PUFA control diet; 4) ob/ob mice fed the PUFA diet supplemented with ethanol. All diets were freshly prepared each day, and the dietary and nutritional intake of control WT or ob/ob mice were matched to those of the ethanol-fed mice by pair feeding the same volume of isocaloric liquid diet for 4 wk (1, 30, 40). Once the mice were accommodated to the liquid diet, ethanol was gradually introduced up to 27.5% of total calories for 2 days, then 18% for 2 days, and finally 27.5%.

For animals on an ethanol-containing diet, animal cages were placed on heating pads to maintain body temperature since ethanol consumption can induce hypothermia. Food intake of each mouse in all four groups was recorded daily. After a 4-wk feeding, the animals were euthanized, at which time blood, liver, and adipose tissues were collected. The studies were approved by the Institutional Animal Care Use Committees of University of South Florida.

Histology and biochemical assays. Sections of the liver were stored in 10% formalin on excision and then stained with hematoxylin and eosin as reported (1, 30). Total hepatic triglyceride and cholesterol levels were measured with the Serum Triglyceride Determination Kit (Sigma-Aldrich, St. Louis, MO). Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), cholesterol, and triglyceride were determined by using kits from Point Scientific (Canton, MI). Plasma β-hydroxybutyrate (β-OHB) was measured by a β-OHB LiquiColor procedure (SanBio Laboratory, Boerne, TX). Liver samples were deproteinized and the concentrations of lactate and pyruvate were determined by using commercial kits from BioVision (Mountain View, CA). Serum levels of adiponectin were determined with a colorimetric assay kit from BioAssay Systems (Hayward, CA).

Lipid peroxidation. The levels of lipid peroxidation product in liver were determined based on the formation of the thiobarbituric reactive substances and expressed as the extent of malondialdehyde (MDA) production (1, 30).

Total RNA isolation and qRT-PCR. Total RNA was prepared from liver and adipose tissue with use of TRIZol reagent (Sigma-Aldrich) (1, 30). Reverse transcription of total RNA to cDNA was performed with the High Capacity RNA-to-cDNA Master Mix from Applied Biosystems (Carlsbad, CA). Real-time quantitative polymerase chain reaction (qRT-PCR) amplification was performed in an iCycler Spectrofluorometric thermal cycler (Bio-Rad Laboratories, Hercules, CA) by using RT2 SYBR Green qPCR Master Mix and primer sets designed in-house and provided by Integrated DNA Technologies (Coralville, IA) (Table 1). The relative amount of target mRNA was calculated by the comparative cycle threshold (Ct) method by normalizing target mRNA Ct to those for GAPDH.

Immunoblot analysis. Liver nuclear and cytosolic proteins were extracted and separated by using the nuclear extract kit (Active Motif, Carlsbad, CA) according to the manufacturer’s protocol (1, 30, 40). Immunoblot analyses were performed by using 25–40 μg liver protein extracts separated by electrophoresis in a SDS-polyacrylamide gel and transferred to nitrocellulose filters. SIRT1 and lipin-1 were visualized with antibodies from Santa Cruz. AMPKα, phospho-AMPKα, and phospho-AMPKα were detected by a chemiluminescent substrate kit (Cell Signaling Technology, Danvers, MA). The blots were visualized with an Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE). The optical density of each band was quantified using Odyssey software (Li-Cor Biosciences), and the ratio of target proteins to β-actin was calculated. Target protein expression was normalized to control (1, 30).

Table 1. Primer sequences and annealing temperatures used in this study

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<th>Gene</th>
<th>Primer 5'–3'</th>
<th>Tm (°C)</th>
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<tr>
<td>Acox1</td>
<td>F-TGCTATGGTGGTGAATGAC</td>
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</tr>
<tr>
<td></td>
<td>R-AAATTCACACTCCATTG</td>
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<tr>
<td>Fg21</td>
<td>F-GGAGATCGAACGTCCCTGGA</td>
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<td>R-GGAGCTTACACATCGCTGG</td>
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<td>Gapdh</td>
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<td>R-TCATGTCATCGGCGAAGTG</td>
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<td>Tfα</td>
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<td></td>
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Table 2. Selected parameters in obese ob/ob mice or lean WT mice fed ethanol

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Ethanol</th>
<th>Ob/ob</th>
<th>Ob/ob + Ethanol</th>
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<tbody>
<tr>
<td>Starting body weight, g</td>
<td>24.5 ± 3.9b</td>
<td>25.8 ± 3.0b</td>
<td>60.2 ± 6.1a</td>
<td>54.7 ± 4.2a</td>
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<tr>
<td>Final body weight, g</td>
<td>27.5 ± 3.9b</td>
<td>27.2 ± 1.6b</td>
<td>53.6 ± 1.6a</td>
<td>54.9 ± 3.5a</td>
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<tr>
<td>Liver weight, g</td>
<td>1.12 ± 0.1b</td>
<td>1.27 ± 0.1b</td>
<td>2.33 ± 0.2a</td>
<td>2.88 ± 0.2a</td>
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<tr>
<td>Liver weight/body weight</td>
<td>0.042 ± 0.002b</td>
<td>0.046 ± 0.002b</td>
<td>0.045 ± 0.002b</td>
<td>0.053 ± 0.002b</td>
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<tr>
<td>Serum FGF21, μg/ml</td>
<td>0.78 ± 0.2</td>
<td>1.53 ± 0.2</td>
<td>2.62 ± 0.1b</td>
<td>6.14 ± 0.7b</td>
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<tr>
<td>Plasma β-OHB, mg/dl</td>
<td>10.3 ± 1.3b</td>
<td>4.6 ± 1.2b</td>
<td>5.9 ± 1.2b</td>
<td>17.6 ± 2.1b</td>
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<tr>
<td>Liver MDA, mmol/mg protein</td>
<td>315 ± 71a</td>
<td>781 ± 203a</td>
<td>218 ± 13b</td>
<td>239 ± 31b</td>
</tr>
<tr>
<td>Plasma FFA, μM/dl</td>
<td>414 ± 40a</td>
<td>557 ± 39a</td>
<td>505 ± 141a</td>
<td>388 ± 38b</td>
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<tr>
<td>Plasma TG, mg/dl</td>
<td>43 ± 2a</td>
<td>58 ± 8a</td>
<td>26 ± 8a</td>
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</table>

Lean wild-type or obese ob/ob mice were divided into 4 groups as follows: 1) pair-fed control diet (Control); 2) ethanol containing diet (27.5% of total calories) (Ethanol); 3) control diet-fed ob/ob (ob/ob); and 4) ob/ob mice fed with the ethanol containing diet (ob/ob + Ethanol). The animals were euthanized after 4-wks. FGF21, fibroblast growth factor 21; MDA, malondialdehyde; β-OHB, β-hydroxybutyrate; MDA, malondialdehyde; FFA, free fatty acid; TG, triacylglycerol. Results are expressed as means ±SE of 4–11 mice. Means without a common letter differ, P < 0.05.
AMPKα, AMPKβ, phospho-AMPKβ, and phospho-ACC, mammalian target of rapamycin (mTOR), phospho-mTOR, PGC-1α, and PPARγ were visualized with monoclonal antibodies (Cell Signaling Technology, Danvers, MA). A polyclonal rabbit anti-actin antibody or Lamin A/C antibody (Sigma) was used to normalize the signal obtained for total or nuclear liver protein extracts.

Liver nuclei isolation. Liver nuclei were isolated as described (8, 18). Briefly, livers were homogenized in buffer (0.25 M sucrose; 50

![Liver TG/Protein (mg/g)](image1)

![Liver Chol/Protein (mg/g)](image2)

Fig. 1. Chronic ethanol feeding exacerbates the development of fatty liver in obese ob/ob mice. Lean wild-type or obese ob/ob mice were fed either a control diet (C; ob/ob) or ethanol-containing diets (E; ob/ob+E). A: hematoxylin and eosin staining (original magnification ×40) of liver sections. B: immunohistochemical staining for collagen (original magnification ×40) of liver sections. C: hepatic triglyceride (TG) levels. D: hepatic cholesterol (Chol) levels. Results are expressed as means ± SE of 4–11 mice. Means without a common letter differ, P < 0.05.

![Serum ALT (U/dL)](image3)

![Serum AST (U/L)](image4)

Fig. 2. Chronic ethanol feeding exacerbates the development of fatty liver in obese ob/ob mice. Mice were fed as described in Fig. 1. A: serum alanine aminotransferase (ALT) levels. B: serum aspartate aminotransferase (AST) levels. Results are expressed as means ± SE of 4–11 mice. Means without a common letter differ, P < 0.05.
mM Tris·HCl, pH 7.4; 25 mM KCl; 5 mM MgCl₂ and 1 mM PMSF). Homogenate was centrifuged at 800 g for 10 min. The pellet was then subjected to two resuspension/centrifugation cycles as described in the accompanying literature, and the resultant supernatant containing purified nuclei was stored at −80°C until required.

Immunofluorescence imaging. Immunofluorescence imaging using isolated mouse liver nuclei was performed as described (8, 39). Briefly, isolated liver nuclei were pipetted onto fibronectin-plated coverslips and left to adhere for 10 min before being fixed for 30 min in 4% paraformaldehyde. The samples were permeabilized with 0.1% Triton X-100 in PBS for 15 min, washed three times with PBS, and incubated for 1 h at room temperature in blocking buffer (PBS plus 2% BSA). Coverslips were incubated with the primary anti-lipin-1 antibody (Abnova, Walnut, CA) (1:100 in blocking buffer) at 4°C overnight, rinsed, and incubated in secondary antibody (anti-rabbit Cy3; Jackson ImmunoResearch Laboratories) for 1 h at room temperature. Images were obtained with a Leica DM4000 upright microscope and Leica DFC350X camera, and processed with Leica LAS software.

Statistical analysis. Data are presented as means ± SE. All data were analyzed by two-way ANOVA followed by Tukey’s multiple comparison procedure, with P < 0.05 being considered significant.

RESULTS

Ethanol feeding exacerbates the fatty liver injury in obese ob/ob mice. Male lean WT and obese ob/ob mice were pair fed modified Lieber-DeCarli liquid diets with or without ethanol (27.5% of the total calories) for 4 wk (1, 30, 40). During the 4-wk feeding period, food intake was similar in all groups, and ethanol feeding had no apparent effect on the health status of the lean WT or obese ob/ob mice (data not shown). The body weights of ob/ob mice were more than double their lean WT counterparts when the feeding regime began and remained heavier throughout the feeding period (Table 2).

Consistent with our previous findings (1, 30, 40), ethanol induced mild liver steatosis in lean WT mice compared with pair-fed WT controls (Fig. 1). The ob/ob mice fed with a control diet displayed excessive hepatic fat accumulation (Fig. 1). However, chronic ethanol administration substantially aggravated the development of fatty liver in ob/ob mice compared with all other groups, as determined by increased liver weight-to-body weight ratio, significantly elevated

![Fig. 3](http://ajpgi.physiology.org/)

Fig. 3. Ethanol feeding augments the impairment of hepatic SIRT1-AMPK signaling system in obese ob/ob mice. Mice were fed as described in Fig. 1. A: hepatic relative SIRT1 mRNA expression levels. B: Western blots were performed by using anti-SIRT1, anti-phosphorylated-AMPKα (anti-p-AMPKα), anti-AMPKα, anti-phosphorylated acetyl CoA carboxylase (p-ACC), anti-ACC, anti-phosphorylated mTOR (anti-p-mTOR) and anti-mTOR antibodies, against liver nuclear or cytosolic extracts from mice. C: hepatic relative protein levels of SIRT1, p-AMPKα, AMPKα, p-ACC, p-mTOR, and mTOR. All data are expressed as means ± SE (n = 5–8 animals). Means without a common letter differ, P < 0.05. AMPK, AMP-activated kinase; mTOR, mammalian target of rapamycin; SIRT1, sirtuin 1; PPARγ, peroxisome proliferator-activated receptor γ; PGC-1α, PPARγ coactivator-1α.
Fig. 4. Ethanol feeding disrupts hepatic SIRT1 signaling system in obese ob/ob mice. Mice were fed as described in Fig. 1. Immunohistochemistry of hepatic SIRT1 in lean wild-type or obese ob/ob mice fed with or without ethanol. Arrows indicate SIRT1-positive cells. Original magnification: ×400.

Fig. 5. Ethanol and obesity synergistically augment the SIRT1-target genes PGC-1α and PPARγ. Mice were fed as described in Fig. 1. A: hepatic relative mRNA levels of PGC-1α, and acyl-CoA oxidase (AOX). B: hepatic relative mRNA levels of PPARγ, FGF-21, and fat-specific protein 27 (FSP-27). C: Western blots were performed by using an anti-PGC-1α or anti-PPARγ antibody against liver nuclear or cytosolic extracts from mice. D: hepatic relative protein levels of PGC-1α and PPARγ. All data are expressed as means ± SE (n = 5–8 animals). Means without a common letter differ, P < 0.05.
hepatic triglyceride and cholesterol contents, and synergistically enhanced ALT and AST levels, all indicators of liver injury (Figs. 1 and 2; Table 2). It is important to note that during the 4-wk ethanol feeding period there were no significant differences in the average food intake among in all groups (data not shown), discounting the possibility that the aggravated fatty liver in the ethanol-fed ob/ob mice could be due to higher ethanol consumption by the ob/ob mice compared with lean littermates. Moreover, blood ethanol levels in ethanol-fed WT mice (46.2 ± 14 mg/dl) were similar to the blood ethanol levels in ethanol-fed ob/ob mice (42.3 ± 5 mg/dl). Despite a significant induction of fat accumulation by ethanol administration to ob/ob mice, augmented hepatic fibrosis judged by hepatic collagen deposition and mRNA expression of early markers of hepatic fibrosis such as α-smooth muscle actin and collagen I was not found in our ethanol-fed ob/ob mice (Fig. 1B and data not shown). These findings suggest that for ethanol-fed ob/ob mice to progress from fatty liver to fibrotic liver may require a prolonged period of alcohol intake (e.g., 8-wk ethanol feeding) (11).

Taken together, our data clearly demonstrate that ethanol administration for 4 wk exacerbates the development of fatty liver in ob/ob mice.

Ethanol administration augments the impairment of hepatic SIRT1-AMPK signaling system in ob/ob mice. To elucidate the cellular and molecular mechanisms by which ethanol and obesity are synergistically causing enhanced hepatic fat accumulation, the effects of chronic alcohol feeding on SIRT1-AMPK axis were examined in the livers of ob/ob and WT mice. Ethanol feeding modestly but significantly decreased total mRNA and protein levels of hepatic SIRT1 in WT mice compared with pair-fed WT controls whereas SIRT1 mRNA levels were even lower in ob/ob mice fed with or without ethanol (Figs. 3A and 4). However, the decrease in the abundance of nuclear SIRT1 protein was more pronounced in the livers of ethanol-fed ob/ob mice compared with other groups, suggesting that the SIRT1 expression may be largely regulated at posttranscriptional levels in ethanol-fed obese mice (Fig. 3, B and C; and Fig. 4) (1).

Livers from ethanol-fed ob/ob mice displayed marked decreases in both phosphorylated and total protein levels of AMPKα as well as phosphorylated levels of ACC, a known AMPK-regulated downstream molecule (Fig. 3, B and C). Conversely, total and phosphorylated levels of mTOR, a molecule negatively regulated by SIRT1, were increased in ob/ob mice with a further increase in the ethanol-fed ob/ob mice (Fig. 3, B and C) (12).

SIRT1-AMPK signaling pathways are reciprocally modulated through LKB1 (17). However, altered acetylation or phosphorylation of LKB1 in ethanol-fed WT or ob/ob mice was not detected (data not shown).
Taken together, these results clearly demonstrate that ethanol and obesity synergistically impaired the hepatic SIRT1-AMPK signaling system in ob/ob mice.

Ethanol and obesity synergistically inhibit hepatic PGC-1α signaling in ob/ob mice. The SIRT1-AMPK signaling pathway controls the activity of PGC-1α, a key regulator in hepatic lipid metabolism (9). When ob/ob mice were fed with ethanol, there was a significant decrease in mRNA levels of PGC-1α (Fig. 5A). Strikingly, the nuclear PGC-1α levels were largely depleted in the livers of ethanol-fed ob/ob mice (Fig. 5). Interestingly, the mRNA levels for acyl-CoA oxidase were significantly decreased in ethanol-fed WT and ob/ob mice compared with controls, suggesting involvement of both PGC-1α-dependent and -independent mechanisms (Fig. 5A).

We further examined whether the impaired hepatic PGC-1α signaling led to reduced hepatic fatty acid oxidation capacity. Ethanol feeding to WT mice increased the plasma β-OHB levels by nearly 3.6-fold compared with WT controls (Table 2) (1, 30). However, ethanol feeding to ob/ob mice only increased the plasma β-OHB levels by ~2-fold compared with WT controls and ~1.2-fold compared with ob/ob control mice, suggesting that ethanol in combination with obesity was capable of perturbing hepatic fatty acid oxidation, reducing generation of ketone bodies, and inducing accumulation of hepatic fat in mice. It is worthwhile to point out that ethanol feeding did not significantly alter expression of hepatic PPARα, a partner of PGC-1α, in WT or ob/ob mice compared with controls (data not shown).

PPARγ is another target of SIRT1 (21, 25). Both mRNA and protein levels of PPARγ were markedly increased in the livers of ethanol-fed ob/ob mice compared with other groups (Fig. 5, B–D). In accordance, mRNA expression of fat-specific protein 27 (FSP27), a known PPARγ target that heavily regulates lipodroplet formation, was also significantly increased in the ethanol-fed ob/ob mice (Fig. 5B). Furthermore, there was a significant increase in both mRNA and circulating levels of fibroblast growth factor 21 (FGF21) in ethanol-fed ob/ob mice compared with other groups (Fig. 5B and Table 2) (38).

Ethanol feeding to ob/ob mice aggravated the alteration of the levels of hepatic lactate and pyruvate, which represent the ratio of NAD⁺ and NADH concentrations compared with all other groups (Fig. 6, A and B). Whereas the mRNA levels of hepatic tumor necrosis factor-α (TNF-α) were depressed in WT mice receiving ethanol, the administration of ethanol to ob/ob mice had no effect on TNF-α mRNA levels (Fig. 6C). Meanwhile, in the adipose tissue, there was a significant increase in TNF-α mRNA in the ethanol-fed ob/ob mice compared with other groups (Fig. 6C) (5). However, we were unable to detect the changes in serum levels of TNF-α in all the groups, implying ethanol-obesity synergy may be mediated via TNF-α independent pathway. Cytochrome P450 2E1 (CYP2E1) plays a prominent role in the pathogenesis of alcoholic liver injury

![Graph](https://via.placeholder.com/150)

**Fig. 7.** Ethanol and obesity synergistically block hepatic lipin-1 nuclear entry in mice. Mice were fed as described in Fig. 1. A: Western blots were performed by using of an anti-lipin-1 antibody against liver cytosolic (Cyto) or nuclear (Nuc) extracts from mice. B: immunofluorescence for lipin-1 (green) and DAPI (blue) from isolated mouse liver nuclei. Original magnification ×1,000. C: relative levels of hepatic cytosolic or nuclear lipin-1. D: hepatic Lpin1/βα ratio. All data are expressed as means ± SE (n = 5–8 animals). Means without a common letter differ, P < 0.05.
However, ethanol feeding significantly induced the mRNA and protein expression levels of CYP2E1 to the same extent in WT or ob/ob mice compared with their respective controls (Fig. 6D and data not shown). Interestingly, whereas the serum levels of free fatty acids in ethanol-fed WT mice and ob/ob mice were increased, ethanol administration to ob/ob mice entirely prevented the elevation of free fatty acids, suggesting that the rates of very low-density lipoproteins (VLDL) secretion may be impaired in those ob/ob mice (Table 2).

Ethanol and obesity synergistically block the lipin-1 nuclear entry in the livers of ob/ob mice. Lipin-1 has recently emerged as a pivotal player in the pathogenesis of AFLD (10, 14). Hence we examined the role of lipin-1 signaling in the development of fatty liver induced by ethanol-obesity synergism. Consistent with our recent findings concerning subcellular localization of lipin-1 (18, 39), the livers of ob/ob mice fed an ethanol-containing diet displayed significantly higher cytosolic levels and lower nuclear levels compared with other groups (Fig. 7, A–C).

Alternative mRNA splicing generates two major lipin-1 isoforms, lipin-1α and lipin-1β, which exhibit different subcellular localizations (13, 23, 26). Ethanol feeding modestly but significantly increased the ratio of hepatic Lipin1 β/α in WT mice compared with the WT control mice (Fig. 7D). However, ethanol administration to ob/ob mice resulted in the highest ratio of Lipin1 β/α compared with all other groups (Fig. 7D).

Lipin-1 mRNA alternative splicing is known to be regulated by the splicing factor, SFRS10. (26). Ethanol or obesity each significantly reduced the mRNA and protein levels of SFRS10 whereas ethanol administration to ob/ob mice significantly exacerbated the reduction of SFRS10 protein (Fig. 8).

Collectively, our results suggest that ethanol-induced alteration of lipin-1 mRNA splicing may be mediated, at least in part, by inhibition of SFRS10. This alteration may be the underlying mechanism that hinders the entry of lipin-1 into the nuclei of hepatocytes in ethanol-fed ob/ob mice.

DISCUSSION

In the present study, we utilized lean WT and genetically obese mice to investigate the cellular and molecular mechanisms underlying the synergistic effect of ethanol and obesity on the development of fatty liver disease. We demonstrated that hepatic SIRT1-AMPK signaling was severely impaired in the livers of chronically ethanol-fed ob/ob mice. The down-regulation of the SIRT1-AMPK signaling system correlated closely with diminished hepatic PGC-1α activity and altered lipin-1 signaling, which, in turn, putatively led to the impairment of fatty acid oxidation observed in the mouse livers. Taken together, our novel findings suggest that ethanol administration exacerbates the development of fatty liver in obese mice by aggravating the abnormalities in hepatic lipid metabolism pathways largely controlled by the hepatic SIRT1-AMPK signaling system (Fig. 9).

Our 4-wk ethanol feeding model has not recapitulated the full spectrum of liver injuries such as hepatitis, fibrosis, or cirrhosis, which have been observed in obese animals or alcoholics (7, 11, 15, 20, 28, 31, 37). Nevertheless, our findings suggest that the severity of aggravated liver injuries

![Fig. 8](http://ajpgi.org)

**Fig. 8.** Ethanol and obesity impair expression levels of SFRS10, a splicing factor, in mice. Mice were fed as described in Fig. 1. A: relative hepatic mRNA levels of SFRS10. B: Western blots were performed by using an anti-SFRS10 antibody against liver total protein extracts from mice. C: relative hepatic protein levels of SFRS10. All data are expressed as means ± SE (n = 5–8 animals). Means without a common letter differ, P < 0.05.

![Fig. 9](http://ajpgi.org)

**Fig. 9.** Proposed underlying mechanism by which ethanol-obesity synergy leads to aggravated fatty liver in mice.
induced by the interaction of ethanol and obesity are likely dependent on the duration and dose of ethanol intake in ob/ob mice. Indeed, it was demonstrated that chronic modest ethanol consumption alleviated liver steatosis in ob/ob mice (11). The underlying mechanisms whereby heavy or moderate alcohol drinking exerts influences on the severity and progress of liver diseases in obese mice or individuals warrant future investigation.

Our present study clearly suggests that dysregulation of the hepatic SIRT1-AMPK signaling system represents a central mechanism for ethanol-obesity synergism in the development of liver steatosis. However, the exact mechanism by which ethanol exacerbates the obesity-induced dysregulation of hepatic SIRT1-AMPK signaling remains to be determined.

Oxidative stress has been implicated in regulation of SIRT1 and AMPK (29, 33), and several in vivo studies have reported that ethanol and obesity can induce reactive oxygen species (ROS) formation and trigger lipid peroxidation (2, 3, 7, 11, 15, 20, 28, 31, 37). Paradoxically, despite the pronounced increase in hepatic fat accumulation, levels of hepatic MDA, a biomarker for oxidative stress, were not augmented by ethanol administration to ob/ob mice, suggesting involvement of ROS-independent mechanisms (Table 2). The functionality and activity of SIRT1 and AMPK are influenced by the absolute or relative concentrations of NAD⁺ and NADH (29). Indeed, the levels of NAD⁺ and NADH as suggested by the levels of hepatic lactate and pyruvate were significantly altered by ethanol administration to ob/ob mice. Therefore, it is likely that ethanol and obesity may conspire to disrupt SIRT1-AMPK signaling through an augmented shift in the ratio of hepatic NAD⁺ to NADH concentration, rather than ROS formation.

A novel finding of this present study is that the exacerbated fatty liver observed in chronically ethanol-fed ob/ob mice may be mediated through alterations in PGC-1α and lipin-1 as a downstream effect of impaired SIRT1-AMPK signaling. Accumulating evidence suggests that PGC-1α and lipin-1 are two crucial regulators of hepatic lipid metabolism through their interactions with various transcriptional regulators (10, 24). More importantly, nuclear-localized lipin-1 forms a complex with PGC-1α/PPARα, leading to induction of fatty acid oxidation genes (10). Indeed, our data have shown that the livers of ethanol-fed ob/ob mice handled fatty acid load much less effectively than livers from lean WT mice fed ethanol or ob/ob mice fed with a control diet, leading to increased fat accumulation. In addition to regulating fatty acid oxidation, PGC-1α and lipin-1 each can also play a role in hepatic triacylglycerol (TG) synthesis and VLDL-TG secretion in mice (6, 9). Therefore, it is logical to speculate that pronounced decrease in PGC-1α or lipin-1 in ethanol-fed ob/ob mice may further induce TG synthesis and suppress VLDL-TG secretion, ultimately leading to massive steatosis.

The mechanism by which SIRT1-AMPK signaling pathway regulates lipin-1 nuclear localization and its function is unknown. However, our group has recently demonstrated that chronic ethanol exposure alters acetylation/SUMOylation modifications of lipin-1 and sequesters lipin-1α to the cytosol, disturbing lipin-1 nuclear functioning in both AML-12 hepatocytes and mouse livers (18, 39). Interestingly, coexpression of SIRT1 alleviated ethanol-mediated impairment of lipin-1 in hepatocytes, suggesting the involvement of SIRT1 (39). A recent study suggests that phosphorylation of lipin-1 regulated by a known SIRT1 target, mTOR, is one of the crucial determinants of lipin-1 nuclear retention in hepatocytes (24). We found that mTOR activity was indeed significantly augmented by ethanol administration to ob/ob mice. Therefore, it is tempting to postulate that ethanol and obesity may synergistically block the lipin-1 nuclear entry in the livers of ob/ob mice by targeting the SIRT1-mTOR axis. Whether and how ethanol-mediated impairment of the SIRT1-AMPK-mTOR axis in ob/ob mice is associated with lipin-1 cellular localization in a phosphorylation-, acetylation-, or SUMOylation-dependent manner is currently under investigation in our laboratory.

Further studies establishing a causal relationship among the signaling pathways regulated by SIRT1, AMPK, PGC-1α, or lipin-1 in ethanol-fed ob/ob mice would be made possible through use of various genetically modified cell culture or animal models. We are also currently identifying the missing links between these pathways and their mechanisms of regulation by ethanol and obesity. Moreover, it will be of great interest to verify our present findings observed in genetically obese mice in diet-induced obese mouse models or in obese alcoholics.

In summary, our findings suggest that the synergistic effect of ethanol and obesity on the development of fatty liver is largely mediated by impairment of a central SIRT1-AMPK signaling system in the liver. Our novel findings shed light on the mechanism by which obesity and ethanol synergistically interact to cause excessive hepatic lipid accumulation.

**GRAMS**

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**


**REFERENCES**


