Ethanol-induced hepatic steatosis is modulated by glycogen level in the liver

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Abstract Alcoholic liver disease (ALD) is a major health problem worldwide and hepatic steatosis is an early response to alcohol consumption. Fat and glycogen are two major forms of energy storage in the liver; however, whether glycogen metabolism in the liver impacts alcohol-induced steatosis has been elusive. In this study, we used a mouse model with overexpression of PPP1R3G in the liver to dissect the potential role of glycogen on alcohol-induced fatty liver formation. PPP1R3G is a regulatory subunit of protein phosphatase 1 and stimulates glycogenesis in the liver. Chronic and binge ethanol (EtOH) feeding reduced glycogen level in the mouse liver and such inhibitory effect of EtOH was reversed by PPP1R3G overexpression. In addition, PPP1R3G overexpression abrogated EtOH-induced elevation of serum levels of alanine aminotransferase and aspartate aminotransferase, increase in liver triglyceride concentration, and lipid deposition in the liver. EtOH-stimulated sterol regulatory element-binding protein (SREBP)-1c, a master regulator of lipogenesis, was also reduced by PPP1R3G overexpression in vivo. In AML-12 mouse hepatocytes, PPP1R3G overexpression could relieve EtOH-induced lipid accumulation and SREBP-1c stimulation.

In conclusion, our data indicate that glycogen metabolism is closely linked to EtOH-induced liver injury and fatty liver formation.—Gu, J., Y. Zhang, D. Xu, Z. Zhao, Y. Zhang, Y. Pan, P. Cao, Z. Wang, and Y. Chen. Ethanol-induced hepatic steatosis is modulated by glycogen level in the liver. J. Lipid Res. 2015. 56: 1329–1339.

Supplementary key words alcoholic liver disease • fatty liver • lipid metabolism • glycogen synthase • sterol regulatory element-binding protein

Alcoholic liver disease (ALD) is a major health problem worldwide, with an estimated 3.8% of all global deaths and 4.6% of global disability-adjusted life-years attributable to alcohol (1). ALD is manifested as a broad spectrum of disorders, ranging from simple fatty liver to more severe forms of liver injury, including alcoholic hepatitis, cirrhosis, and superimposed hepatocellular carcinoma (2, 3). Alcohol is a true hepatotoxin that causes hepatocellular damage and is not simply caused by malnutrition (4). Hepatic steatosis is an early response to alcohol consumption and it happens in more than 90% of heavy drinkers, with about 30% of heavy drinkers developing more severe forms of ALD, such as fibrosis and cirrhosis.

Hepatic steatosis is characterized by the accumulation of fat, such as triglycerides, phospholipids, and cholesterol esters, in hepatocytes. Earlier studies indicated that alcohol consumption increases the ratio of reduced nicotinamide adenine dinucleotide/oxidized nicotinamide adenine dinucleotide in hepatocytes, leading to disruption of mitochondrial β-oxidation of fatty acids and steatosis (5). However, recent studies have revealed that alcohol exposure directly or indirectly regulates transcription factors that control lipid metabolism, leading to stimulation of lipogenesis and inhibition of fatty acid oxidation. Alcohol can increase fatty acid synthesis in hepatocytes via up-regulation of sterol regulatory element-binding protein (SREBP)-1c, a master transcription factor that promotes fatty acid synthesis through upregulation of lipogenic genes (6, 7). It was reported that alcohol is able to directly increase transcription of SREBP-1c gene via its metabolite, acetaldehyde (6). On the other hand, alcohol inhibits fatty acid oxidation in hepatocytes mainly via inactivation of PPARα, a nuclear hormone receptor that controls...

Abbreviations: ALD, alcoholic liver disease; ALT, alanine aminotransferase; ASAT, aspartate aminotransferase; ChREBP, carbohydrate-responsive element-binding protein; CPT1, carnitine palmitoyltransferase; EtOH, ethanol; GP, green fluorescence protein; GP, glycogen phosphorylase; GS, glycogen synthase; HDL-c, HDL cholesterol; IL, interleukin; LDL-c, LDL cholesterol; L-PK, liver-pyruvate kinase; MCAD, acylCoA dehydrogenase; PAS, Periodic acid Schiff; PP1, protein phosphatase 1; PP1c, catalytic subunit of which the protein phosphatase 1 holoenzyme is composed; SRE, sterol regulatory element; SREBP, sterol regulatory element-binding protein; TC, total cholesterol.

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transcription of a range of genes involved in free fatty acid transport and oxidation. It was found that acetaldehyde converted from ethanol (EtOH) could directly inhibit the transactivation activity and DNA-binding ability of PPARα in hepatocytes (8).

In addition to fat, glycogen is another form of energy storage in the liver. In particular, the liver takes up approximately one-third of the oral glucose load in the animal and most of the glucose is stored in the hepatocytes in the form of glycogen (9). Glycogen metabolism in the liver is regulated in a complex manner and two critical enzymes are directly involved in the process, including glycogen synthase (GS) for glycogenesis and glycogen phosphorylase (GP) for glycogenolysis (10–12). The activities of GS and GP are regulated by phosphorylation/dephosphorylation events, but in opposing directions. GS is inhibited by phosphorylation at multiple sites mediated by protein kinases, such as protein kinase A and GS kinase 3, and activated by dephosphorylation via GS phosphatase. On the other hand, GP is activated by phosphorylation at a single residue near the N terminus by phosphorylase kinase and inhibited by dephosphorylation by protein phosphatase 1 (PP1). PP1 plays a critical role in glucose metabolism because of its regulatory effects on glycogen-metabolizing enzymes, including GS and GP. The PP1 holoenzyme is composed of a catalytic subunit (PP1c) and a regulatory subunit (13). In regulating glycogen metabolism, PP1c is anchored to the glycogen particles by a group of glycogen-targeting regulatory subunits (G subunits) that modulate the activities of the glycogen-metabolizing enzymes through PP1-mediated dephosphorylation. According to the GenBank database, there are seven genes encoding G subunits (PP1R3A to PP1R3G), all of which possess a PP1-binding domain and a glycogen-binding domain (14). Recent studies in our laboratory indicate that PP1R3G is changed along the fasting-feeding cycle and plays a critical role in postprandial glucose homeostasis (15). In addition, we found that PP1R3G overexpression in the liver can impact on liver triglyceride metabolism via its regulation on hepatic glycogenesis (16).

Inasmuch as alcohol induces fat deposition in the liver and that fat and glycogen are two major forms of energy storage in the liver, it is conceivable that glycogen metabolism in the liver may impact alcohol-induced steatosis. However, this issue has not been investigated before in the field. In this study, we used a mouse model with overexpression of PP1R3G in the liver to start dissecting the potential role of glycogen on alcohol-induced fatty liver formation.

MATERIALS AND METHODS

Animal studies

The Institutional Animal Care and Use Committee of the Institute for Nutritional Sciences, Chinese Academy of Sciences approved all animal procedures and protocols. Blood samples were taken and other tissues of interest were snap-frozen in liquid nitrogen immediately after resection and stored at −80°C until further analysis. Food intake and body weight measurement were described previously (17). The generation and characterization of the transgenic mice with liver-specific expression of PP1R3G was reported previously (16). The chronic and binge EtOH-fed mouse model was established as previously described with minor modifications (17, 18). In brief, the mice were exposed to chronic EtOH administration for 10 days ad libitum by oral feeding with Lieber-DeCarli EtOH liquid diet plus a single binge on day 11. The mice were fasted for 12 h before the binge. The binge was performed by gavage with 31.5% EtOH in water (v/v) at 7:00 AM. The gavage volume (in microliters) was body weight in grams times 20. The mice were euthanized at 4:00 PM. Ten- to twelve-week-old WT or PP1R3G liver-specific transgenic mice (female, weight 20–22 g) were randomly allocated into three groups: WT mice that were not administered with alcohol (control group, n = 6); WT mice exposed to alcohol treatment (WT-EtOH group, n = 6); and PP1R3G transgenic mice exposed to alcohol (R3G-EtOH group, n = 6). The mice were maintained on a regular 12 h dark/ light cycle with free access to water under a specific pathogen-free condition with temperature at 22 ± 1°C and humidity at 60 ± 10%. Hematoxylin and eosin staining as well, as Oil Red O staining, were performed as previously reported (17). Periodic acid Schiff (PAS) staining was performed as previous reported (16). The activities of liver GS and GP were measured using protocols as previously reported (16, 19).

Measurement of serum and liver variables

Hepatic lipids were extracted with a previously reported method (16). Serum and liver alanine aminotransferase (ALT), aspartate aminotransferase (AST), TG, total cholesterol (TC), HDL cholesterol (HDL-c), and LDL cholesterol (LDL-c) concentrations were determined by colorimetric methods according to the procedures as previously reported (16). The assay kits for ALT, AST, TG, TC, HDL-c, and LDL-c were from Shanghai, China. The blood alcohol level was measured by an EtOH assay kit from Sigma-Aldrich (St. Louis, MO). Briefly, the serum was diluted in the EtOH assay buffer to a final volume of 50 μl, and then mixed with EtOH probe and EtOH enzyme mix at 37°C for 30 min before colorimetric assay at 570 nm.

RNA isolation and real-time PCR analysis

Total RNA of liver tissues was isolated by using TRIzol reagent (Invitrogen, Carlsbad, CA). Oligo (dT) prime RNA (1 μg) was reverse-transcribed with the Super-Script First-Strand synthesis system (Tiangen, Shanghai, China) to obtain cDNA. Real-time quantitative PCR was performed with the SYBR Green PCR system (Applied Biosystems, Foster City, CA) by using β-actin as an internal control for normalization. The primers used in PCR were reported previously (16, 17). Applied Biosystems Prism 7900 HT was used for real-time PCR.

Cell culture and luciferase assay

The mouse AML-12 hepatocyte cells were cultured in DMEM/F-12 medium supplemented with 10% FBS, 1% penicillin-streptomycin, 0.1 mM dexamethasone, and insulin-transferrin-selenium (Invitrogen). Huh7 cells were cultured in DMEM (high-glucose concentration) supplemented with 10% FBS and 1% penicillin-streptomycin. The cells were grown at 37°C with 5% CO2. The construction of a SREBP-responsive luciferase reporter and the luciferase assay with Huh7 cells were reported previously (17). In brief, the luciferase reporter was driven by a sterol regulatory element (SRE)-containing promoter and a cell line stably expressing this reporter (named Huh7-SRE-Luc) (20). For EtOH treatment, 100 mM EtOH was added to the culture medium, followed by incubation for 36 h.
Measurement of intracellular triglyceride

Cells were harvested by using 0.25% trypsin-EDTA solution, and total intracellular lipids were extracted from cell lysates by using a chloroform/methanol mix (2:1, v/v). Intracellular triglyceride concentrations were measured by using a triglyceride determination kit (Sigma-Aldrich).

Staining of lipid droplets in hepatocytes

Cells were fixed for 10 min with 4% paraformaldehyde prepared in PBS, washed three times with PBS, and incubated for 20 min at room temperature in Nile Red to detect intracellular lipid droplets. After washing with PBS, the cells were stained with Hoechst 33342 (Molecular Probes, Eugene, OR) to detect the nuclei.

Western blot analysis

Immunoblotting was performed as previously described (16). The antibodies used in the assay were as follows: anti-tubulin and β-actin antibodies from Sigma-Aldrich; anti-green fluorescence protein (GFP) and anti-SREBP-1 antibodies from Santa Cruz Biotechnology (Santa Cruz, CA); anti-FAS antibody from BD Pharmingen (San Diego, CA). The anti-PPP1R3G antibody was generated in our laboratory as previous reported (15).

Statistical analysis

All results are expressed as mean ± SEM unless indicated otherwise. Significant differences were assessed by two-tailed Student’s t-test for pairwise comparison between two groups and two-way ANOVA was used for experiments with more than two groups. P < 0.05 was considered statistically significant.

RESULTS

Establishment of chronic-binge EtOH feeding in transgenic mice with liver-specific expression of PPP1R3G

To investigate the potential role of glycogen on alcohol-induced fatty liver formation, we applied chronic-binge EtOH feeding to transgenic mice with liver-specific expression of PPP1R3G (16). Compared with the WT mice, both the mRNA and protein levels of PPP1R3G were profoundly elevated in the transgenic mice (Fig. 1A, B), confirming that PPP1R3G was indeed overexpressed in these mice. As expected, the blood EtOH level was significantly raised by alcohol feeding (Fig. 1C). EtOH administration could increase liver weight in the WT mice (Fig. 1D). EtOH exposure had no effect on body weight in the WT mice (Fig. 1E), although PPP1R3G overexpression slightly reduced body weight at certain time points (Fig. 1E). On the other hand, food intake was not markedly altered among the three experiment groups (Fig. 1F).

Alcohol exposure reduces hepatic glycogen level that is increased by PPP1R3G overexpression

We analyzed the potential effect of EtOH administration on glycogen level in the liver. Intriguingly, alcohol exposure markedly reduced hepatic glycogen content (Fig. 2A). On the other hand, the glycogen level in the liver upon alcohol exposure was elevated in the transgenic mice (Fig. 2A), consistent with the function of PPP1R3G in stimulation of glycogen synthesis. PAS staining with the liver sections also indicated that EtOH exposure reduced liver glycogen level and this effect was relieved by PPP1R3G overexpression (Fig. 2B). As glycogen metabolism in the liver is mainly regulated by GS for glycogenesis and GP for glycogenolysis (10–12), we analyzed the effects of alcohol exposure on the activities of GS and GP. Interestingly, EtOH feeding significantly reduced the GS activity of the liver, but had no effect on the GP activity (Fig. 2C). On the other hand, overexpression of PPP1R3G could abrogate alcohol-induced reduction of GS activity (Fig. 2C). In summary, these data indicate the EtOH exposure inhibits liver glycogen synthesis mainly by suppression of GS activity, and overexpression of PPP1R3G reverses such an inhibitory effect of alcohol.

EtOH-induced hepatotoxicity is reduced by PPP1R3G overexpression

It has been reported that alcohol is a true hepatotoxin that causes hepatocellular damage (4). As expected, alcohol exposure could increase serum ALT and AST activities in the WT mice in comparison to the control group. EtOH administration induced liver damage, shown as a 59% increase in serum ALT concentration and a 58% increase in serum AST concentration, respectively (Fig. 3A). The elevated serum ALT and AST concentration in the R3G-EtOH group relative to the WT-EtOH group was significantly reduced by 48 and 49%, respectively (Fig. 3A). In the experiment, we found that EtOH exposure didn’t induce obvious dyslipidemia, as the serum levels of TG, TC, HDL-c, and LDL-c were not significantly different among the three groups (Fig. 3B). It is noteworthy that the blood lipid levels, as well as the body weight, were not significantly changed by EtOH feeding in our experiment, different from a previous report (17). This was likely due to the difference in the age of animals used in the studies. We used 10- to 12-week-old mice instead of the 6-week-old mice used in the other study (17). Based on the mouse protocol for chronic-binge EtOH feeding (18), younger mice are more susceptible to lose weight upon EtOH treatment.

PPPIR3G overexpression relieves EtOH-induced hepatic steatosis

We next analyzed the development of fatty liver upon alcohol administration. As expected, chronic and binge alcohol feeding significantly elevated triglyceride level in the liver (Fig. 4A), indicating that liver steatosis was successfully induced by EtOH in our mouse model. Interestingly, PPP1R3G overexpression significantly reduced EtOH-induced elevation of the liver triglyceride concentration by 20% (Fig. 4A). Hematoxylin and eosin staining revealed that EtOH profoundly caused lipid vacuoles in hepatocytes, and the EtOH-induced pathologic changes were reversed by overexpression of PPP1R3G (Fig. 4B). Consistently, Oil Red O staining revealed that EtOH markedly induced fat deposition in the liver and PPP1R3G overexpression could reduce this effect (Fig. 4C). Together, these data suggest that the liver steatosis induced by chronic alcohol administration can be alleviated by PPP1R3G overexpression.
EtOH itself is a macronutrient and 1 g of EtOH has seven calories, higher than carbohydrate and protein. To rule out the possibility that the effect of PPP1R3G on metabolism was dependent on the binge, which could produce extra energy to the animal, we performed an animal study with chronic EtOH feeding but without the binge. As shown in supplementary Fig. 1, chronic EtOH feeding itself was able to significantly reduce the glycogen level and elevate the triglyceride level in the liver. PPP1R3G overexpression could abrogate the chronic EtOH feeding-induced alterations of liver glycogen and triglyceride levels. These findings are consistent with the results using chronic-and-binge protocol. Therefore, the observed effect of PPP1R3G overexpression to improve hepatic steatosis is not likely caused by the overnutrition of the EtOH binge.

**EtOH-induced expression of genes involved in lipid synthesis and inflammation is relieved by PPP1R3G overexpression**

Previous studies have indicated that SREBP-1c, a key regulator of fatty acid synthesis, is implicated in the development of fatty liver upon EtOH exposure (6, 7). Consistently, we found that the mRNA levels of SREBP-1c, as well as its target gene FAS, were upregulated by EtOH treatment (Fig. 5A). However, the mRNA level of SREBP-2 was not altered by EtOH feeding (Fig. 5A). As a negative control, EtOH feeding had no effect on the mRNA level of \( \beta \)-actin (supplementary Fig. 2). EtOH significantly induced expression of SREBP-1c by 213% and FAS by 166%, respectively. On the other hand, PPP1R3G overexpression significantly abrogated EtOH-induced expression of these genes.
Glycogen is linked to hepatic steatosis in EtOH-induced hepatic steatosis, but also suggest that PPP1R3G overexpression may alleviate fatty liver formation through downregulation of the SREBP-1c pathway. We also analyzed hepatic expression of a few other genes involved in lipid metabolism. EtOH could reduce the mRNA levels of PPARα and its target genes, carnitine palmitoyltransferase (CPT1) and acyl-CoA dehydrogenase (MCAD), while PPP1R3G overexpression abrogated the EtOH effect (Fig. 5A). As PPARα plays an important role in β-oxidation of fatty acid (21), these results indicate that PPP1R3G transgenic mice had decreases in SREBP-1c by 55% and FAS by 45%, respectively (Fig. 5A). Such effects were confirmed by Western blotting in which the protein levels of both SREBP-1 precursor and FAS were elevated by EtOH exposure and abrogated by PPP1R3G overexpression (Fig. 5B). It is noteworthy that the antibody detected both SREBP-1c and SREBP-1a, but the expression level of SREBP-1c is much higher than SREBP-1a in the liver. These results not only indicate that the SREBP-1c pathway is involved in EtOH-induced hepatic steatosis, but also suggest that PPP1R3G overexpression may alleviate fatty liver formation through downregulation of the SREBP-1c pathway.

We also analyzed hepatic expression of a few other genes involved in lipid metabolism. EtOH could reduce the mRNA levels of PPARα and its target genes, carnitine palmitoyltransferase (CPT1) and acyl-CoA dehydrogenase (MCAD), while PPP1R3G overexpression abrogated the EtOH effect (Fig. 5A). As PPARα plays an important role in β-oxidation of fatty acid (21), these results indicate that

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**Fig. 2.** EtOH exposure reduces liver glycogen level and inhibits GS activity that is increased by PPP1R3G overexpression. Mice consumed a control diet or an EtOH-containing diet with or without liver-specific overexpression of PPP1R3G. A: Hepatic concentration of glycogen. B: Representative images (200×) of liver PAS staining. C: The activities of GS and GP. Values are mean ± SEM for (A) and mean ± SE for (C); n = 6 for each group. *P < 0.05, **P < 0.01 between the groups as indicated.
Protein was much higher in cells which were infected with Ad-PPP1R3G adenovirus (Fig. 6A). The glycogen level was also elevated by PPP1R3G overexpression in EtOH-induced AML-12 cells (Fig. 6B). PPP1R3G overexpression decreased EtOH-induced lipid accumulation in mouse hepatocytes (Fig. 5C), consistent with the in vivo results. EtOH treatment significantly elevated TG concentration by 83% in AML-12 cells, while PPP1R3G overexpression markedly prevented EtOH-induced increase of TG concentrations, showing as a 19% decrease (Fig. 6C). We also used Nile Red staining to directly analyze lipid droplets in these cells. EtOH exposure dramatically increased the intensity and quantity of lipid droplets in AML-12 cells, and this effect was apparently abrogated while PPP1R3G was overexpressed (Fig. 6D).

EtOH could activate the SREBP pathway at the cellular level, shown as stimulation of a SRE-responsive luciferase reporter by EtOH treatment (Fig. 6E). Interestingly, PPP1R3G overexpression could abrogate the stimulatory effect of EtOH on SREBP activity (Fig. 6E). Consistently, EtOH treatment also elevated the mRNA levels of SREBP-1c and FAS (Fig. 6F). PPP1R3G overexpression abrogated the effect of EtOH on the expression of these genes (Fig. 6F). The protein levels of SREBP-1 precursor and FAS were also elevated by EtOH exposure and the EtOH effect was abrogated by PPP1R3G overexpression (Fig. 6G). Collectively, these results indicate that PPP1R3G overexpression could reduce EtOH-induced lipid accumulation in hepatocytes, at least partly, by inhibiting the SREBP pathway.

PPP1R3G overexpression increases glycogen level and decreases EtOH-induced lipid accumulation in mouse hepatocytes

We next analyzed the effect of glycogen on alcohol-induced lipid deposition in mouse hepatocytes. AML-12 cells were infected with adenovirus Ad-GFP as a control or Ad-PPP1R3G that contained a PPP1R3G DNA with a Flag tag at the N terminus. Consistently, the expression level of PPP1R3G protein was much higher in cells which were infected with Ad-PPP1R3G adenovirus (Fig. 6A). The glycogen level was also elevated by PPP1R3G overexpression in EtOH-induced AML-12 cells (Fig. 6B). PPP1R3G overexpression decreased EtOH-induced lipid accumulation in mouse hepatocytes (Fig. 5C), consistent with the in vivo results. EtOH treatment significantly elevated TG concentration by 83% in AML-12 cells, while PPP1R3G overexpression markedly prevented EtOH-induced increase of TG concentrations, showing as a 19% decrease (Fig. 6C). We also used Nile Red staining to directly analyze lipid droplets in these cells. EtOH exposure dramatically increased the intensity and quantity of lipid droplets in AML-12 cells, and this effect was apparently abrogated while PPP1R3G was overexpressed (Fig. 6D).

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We also investigated whether PPP1R3G could affect incorporation of exogenous EtOH and glucose into glycogen or lipid. In AML12 cells, exogenous EtOH could elevate production of both glycogen and triglyceride, similar to the effect of exogenous glucose. PPP1R3G overexpression could elevate glycogen production from both exogenous EtOH and exogenous glucose (supplementary Fig. 3). However, PPP1R3G overexpression reduced triglyceride production from exogenous EtOH (supplementary Fig. 3). These data, therefore, further support our conclusion that elevation of glycogen synthesis can reduce EtOH-mediated lipid production in the liver.

DISCUSSION

Our studies reveal, for the first time, that liver glycogen plays an important role in the development of ALD, especially in alcohol-induced liver injury and fatty liver formation. Elevation of liver glycogen content by transgenic expression of PPP1R3G relieved alcohol-induced increases of serum levels of ALT and AST. PPP1R3G overexpression markedly reduced alcohol-induced hepatic steatosis, shown by a significant reduction in TG content and lipid accumulation in the liver. Interestingly, we found that the alcohol-induced fatty liver was accompanied by reduction of...
Fig. 5. EtOH-induced expression of genes involved in lipid metabolism and inflammation in the liver is reduced by PPP1R3G overexpression. A: Hepatic mRNA abundances of representative genes involved in lipid metabolism and inflammation in mice fed a control diet or an EtOH-containing diet with or without PPP1R3G overexpression. B: The mRNA levels were detected by quantitative real-time RT-PCR and shown as fold changes compared with the control group. Western blotting was used to detect the protein levels of SREBP-1c and FAS and the quantitation of the blots is shown in the right panels. Values are mean ± SEM; n = 6. *P < 0.05, **P < 0.01 between the groups as indicated.
Glycogen is linked to hepatic steatosis that controls transcription of a range of genes involved in free fatty acid transport and oxidation. Recently, it was discovered that the activity ChREBP, an important transcription factor for de novo fatty acid synthesis (22), is elevated by alcohol exposure (24, 25). In our experimental setting, we found that the mRNA level of ChREBP was not increased by EtOH treatment. However, we could not rule out the possibility that the activity of ChREBP was augmented by EtOH exposure. Nevertheless, our study indicates that glycogen contributes to the development of hepatic steatosis. We propose that while alcohol directly stimulates lipid deposition in the liver via SREBP-1c, PPARγ, and likely other factors (Fig. 7), alcohol also inhibits glycogenesis, consequently leading to release of free glucose which fuels the process of lipogenesis (Fig. 7). In addition, EtOH itself can produce acetyl-CoA via conversion from acetic acid by acetyl-CoA synthase. Therefore, an increase in glycogen accumulation, such as through overexpression of PPP1R3G, would reduce the level of free glucose available for fatty acid synthesis.
SREBP-1c and inhibition of PPAR overexpression could abrogate alcohol-mediated stimulation of lipogenesis upon alcohol exposure. In addition, PPP1R3G overexpression reduces glucose release and consequently reduces glycogenesis via inhibition of GS activity, resulting in reduced liver glycogen, such as through PPP1R3G overexpression could abrogate alcohol-mediated stimulation of SREBP-1c and inhibition of PPARα, contributing to reduction of hepatic steatosis.

It is noteworthy that alcohol exposure only reduces glycogenosis at the in vivo level (Fig. 4), but not at the cellular level (Fig. 6). Such a discrepancy is likely caused by the difference in EtOH metabolism between in vivo and in vitro. At the in vivo level, EtOH is metabolized to acetaldehyde via alcohol dehydrogenase and acetaldehyde is then converted to acetic acid via aldehyde dehydrogenase. It is known that acetaldehyde is a highly unstable compound and quickly forms free radical structures which are highly toxic if not quenched by antioxidants. We speculate that the acetaldehyde free radicals generated from EtOH are mainly responsible for their inhibitory effect on glycogenesis in the liver. In the cell culture, the free radical structure of acetaldehyde could be quickly quenched by antioxidants available in the culture medium. However, the acetaldehyde free radicals are slowly quenched by limiting the amount of antioxidants in vivo, leading to inhibition on glycogenesis.

In addition to the regulation on glycogenesis, EtOH is also able to modulate gluconeogenesis in the liver. It was recently reported that acute EtOH administration promotes fasting hypoglycemia by reduction of hepatic gluconeogenesis via inhibiting CREB-mediated activation of the gluconeogenic program in response to glucagon (26). Interestingly, both gluconeogenesis and glycogenesis are inhibited by EtOH feeding. The reduced glycogen storage in the liver may also contribute to hypoglycemia during fasting, as glycogen is the major source of blood glucose in the early phase of fasting. It is noteworthy that EtOH-induced hypoglycemia occurs in the fasting state, not in the fed state. We speculate that the liver glucose converted from the acetyl-CoA generated from EtOH during the fed state mainly goes to the lipid synthesis pathway; it will not go for glycogen synthesis, as this program is inhibited by EtOH.

Another unanswered question in this study is how reduced glycogen level is linked to stimulation of the SREBP-1c pathway under alcohol exposure. One possibility is that the reduced glycogenesis would lead to an increase of free glucose in the liver, leading to increased free fatty acid in the liver. It has been found that increased free fatty acids are associated with transcription of SREBP-1c (27), although the detailed molecular mechanism remains to be clarified. Nevertheless, the contribution of glycogen metabolism to alcohol-induced hepatic steatosis would be an interesting topic to explore in the future. Furthermore, alteration of glycogen metabolism might stand as a new approach to relieve ALD in clinical settings.

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REFERENCES

1. Rehm, J., C. Mathers, S. Popova, M. Thavorncharoensap, Y. Teerawattananon, and J. Patra. 2009. Global burden of disease and injury and economic cost attributable to alcohol use and alcohol disorders. Lancet. 373: 2223–2233.
2. Gao, B., and R. Bataller. 2011. Alcoholic liver disease: pathogenesis and new therapeutic targets. Gastroenterology. 141: 1572–1585.
3. Orman, E. S., G. Odena, and R. Bataller. 2013. Alcoholic liver disease: pathogenesis, management, and novel targets for therapy. J. Gastroenterol. Hepatol. 28(Suppl 1): S75–81.
4. Lieber, C. S., D. P. Jones, and L. M. Decarli. 1965. Effects of prolonged ethanol intake: production of fatty liver despite adequate diets. J. Clin. Invest. 44: 1009–1021.
5. Baraona, E., and C. S. Lieber. 1979. Effects of ethanol on lipid metabolism. J. Lipid Res. 20: 289–315.
6. You, M., M. Fischer, M. A. Deeg, and D. W. Crabb. 2002. Ethanol induces fatty acid synthesis pathways by activation of sterol regulatory element-binding protein (SREBP). J. Biol. Chem. 277: 29342–29347.
7. Ji, C., C. Chan, and N. Kaplowitz. 2006. Predominant role of sterol response element binding proteins (SREBP) lipogenic pathways in hepatic steatosis in the murine intragastric ethanol feeding model. J. Hepatol. 45: 717–724.
8. Galli, A., J. Pinaire, M. Fischer, R. Dorris, and D. W. Crabb. 2001. The transcriptional and DNA binding activity of peroxisome proliferator-activated receptor alpha is inhibited by ethanol metabolism. A novel mechanism for the development of ethanol-induced fatty liver. J. Biol. Chem. 276: 68–75.
9. Shoemaker, W. C., and D. H. Elwyn. 1969. Liver: functional interactions within the intact animal. Annu. Rev. Physiol. 31: 227–268.
10. Hers, H. G. 1976. The control of glycogen metabolism in the liver. Annu. Rev. Biochem. 45: 167–189.
11. Moore, M. C., A. D. Cherirington, and D. H. Wasserman. 2003. Regulation of hepatic and peripheral glucose disposal. Best Pract. Res. Clin. Diabetes. 17: 343–364.
12. Agius, L. 2008. Gluokakine and molecular aspects of liver glycogen metabolism. Biochem. J. 414: 1–18.
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13. Ceulemans, H., and M. Bollen. 2004. Functional diversity of protein phosphatase-1, a cellular economizer and reset button. Physiol. Rev. 84: 1–39.

14. Munro, S., H. Ceulemans, M. Bollen, J. Dipleixcto, and P. T. Cohen. 2005. A novel glycogen-targeting subunit of protein phosphatase 1 that is regulated by insulin and shows differential tissue distribution in humans and rodents. FEBS J. 272: 1478–1489.

15. Luo, X., Y. Zhang, X. Ruan, X. Jiang, L. Zhu, X. Wang, Q. Ding, W. Liu, Y. Pan, Z. Wang, et al. 2011. Fasting-induced protein phosphatase 1 regulatory subunit contributes to postprandial blood glucose homeostasis via regulation of hepatic glycogenesis. Diabetes. 60: 1435–1445.

16. Zhang, Y., D. Xu, H. Huang, S. Chen, L. Wang, L. Zhu, X. Jiang, X. Ruan, X. Luo, P. Cao, et al. 2014. Regulation of glucose homeostasis and lipid metabolism by PPP1R3G-mediated hepatic glycogenesis. Mol. Endocrinol. 28: 116–126.

17. Liu, G., Y. Zhang, C. Liu, D. Xu, R. Zhang, Y. Cheng, Y. Pan, C. Huang, and Y. Chen. 2014. Luteolin alleviates alcoholic liver disease induced by chronic and binge ethanol feeding in mice. J. Nutr. 144: 1009–1015.

18. Bertola, A., S. Mathews, S. H. Ki, H. Wang, and B. Gao. 2013. Mouse model of chronic and binge ethanol feeding (the NIAAA model). Nat. Protoc. 8: 627–637.

19. Frolow, J., and C. L. Milligan. 2004. Hormonal regulation of glycogen metabolism in white muscle slices from rainbow trout (Oncorhynchus mykiss Walbaum). Am. J. Physiol. Regul. Integr. Comp. Physiol. 287: R1344–R1353.

20. Tang, J. J., J. G. Li, W. Qi, W. W. Qu, P. S. Li, B. L. Li, and B. L. Song. 2011. Inhibition of SREBP by a small molecule, betulin, improves hyperlipidemia and insulin resistance and reduces atherosclerotic plaques. Cell Metab. 13: 44–56.

21. Fruchart, J.-C. 2009. Peroxisome proliferator-activated receptor-alpha (PPARalpha): at the crossroads of obesity, diabetes and cardiovascular disease. Atherosclerosis. 205: 1–8.

22. Filhoulaud, G., S. Guilmeneau, R. Dentin, J. Girard, and C. Postic. 2015. Novel insights into ChREBP regulation and function. Trends Endocrinol. Metab. 24: 257–268.

23. Petrasek, J., S. Bala, T. Csak, D. Lippai, K. Kody, V. Menasy, M. Barrieu, S. Y. Min, E. A. Kurt-Jones, and G. Szabo. 2012. IL-1 receptor antagonist ameliorates inflammasome-dependent alcoholic steatohepatitis in mice. J. Clin. Invest. 122: 3476–3489.

24. Liangpunsakul, S., R. A. Ross, and D. W. Crabb. 2013. Activation of carbohydrate response element-binding protein by ethanol. J. Investig. Med. 61: 270–277.

25. Marmier, S., R. Dentin, M. Daoua-Chavanieu, H. Guillou, J. Bertrand-Michel, S. Gerbal-Chaloin, J. Girard, S. Lotersztajn, and C. Postic. Novel role for carbohydrate responsive element binding protein in the control of ethanol metabolism and susceptibility to binge drinking. Hepatology. Epub ahead of print. March 11, 2015; doi:10.1002/hep.27778.

26. Tsai, W. W., S. Matsumura, W. Liu, N. G. Phillips, T. Sonntag, E. Hao, S. Lee, T. Hai, and M. Moutminn. 2015. ATF3 mediates inhibitory effects of ethanol on hepatic gluconeogenesis. Proc. Natl. Acad. Sci. USA. 112: 2699–2704.

27. Ou, J., H. Tu, B. Shan, A. Luk, R. A. DeBoe-Boyd, Y. Bashmakov, J. L. Goldstein, and M. S. Brown. 2001. Unsaturated fatty acids inhibit transcription of the sterol regulatory element-binding protein-1c (SREBP-1c) gene by antagonizing ligand-dependent activation of the LXR. Proc. Natl. Acad. Sci. USA. 98: 6027–6032.