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

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**Short title:** Glycogen is linked to hepatic steatosis

**List of abbreviations:**

ALT, alanine aminotransferase; AST, aspartate aminotransferase; ChREBP, carbohydrate-responsive element-binding protein; CPT1, carnitine palmitoyltransferase; GP, glycogen phosphorylase; GS, glycogen synthase; IL-1β, interleukin-1 β; IL-6, interleukin-6; L-PK, liver-pyruvate kinase; MCAD, acyl-CoA dehydrogenase; PPARα, peroxisome proliferator-activated receptor alpha; RT-PCR, reverse transcription PCR; SREBP-1c, sterol regulatory element binding transcription factor 1C; TNF-α, tumor necrosis factor α.
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 on 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 feeding reduced glycogen level in the mouse liver and such inhibitory effect of ethanol was reversed by PPP1R3G overexpression. In addition, PPP1R3G overexpression abrogated ethanol-induced elevation of serum levels of alanine aminotransferase and aspartate aminotransferase, increase in liver triglyceride concentration, and lipid deposition in the liver. Ethanol-stimulated SREBP-1c, a master regulation of lipogenesis, was also reduced by PPP1R3G overexpression in vivo. In AML-12 mouse hepatocytes, PPP1R3G overexpression could relieve ethanol-induced lipid accumulation and SREBP-1c stimulation. In conclusion, our data indicate that glycogen metabolism is closely linked to ethanol-induced liver injury and fatty liver formation.
Introduction

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% heavy drinkers, with about 30% of heavy drinkers develop 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 di-nucleotide 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 upregulation of sterol regulatory element-binding protein 1c (SREBP-1c), a master transcription factor that promotes fatty acid synthesis through up-regulation of lipogenic genes (6, 7).
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 the peroxisome proliferator-activated receptor α (PPARα), a nuclear hormone receptor that controls transcription of a range of genes involved in free fatty acid transport and oxidation. It was found that acetaldehyde converted from ethanol 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 glycogen synthase kinase 3 (GSK3), and activated by dephosphorylation via glycogen synthase phosphatase (GSP). 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 (PPP1R3A to PPP1R3G), all of which possess a PP1-binding domain and a glycogen-binding domain (14). Recent studies in our laboratory indicate that PPP1R3G is changed along the fasting-feeding cycle and plays a critical role in postprandial glucose homeostasis (15). In addition, we found that PPP1R3G 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 on alcohol-induced steatosis. However, such issue has not been investigated before in the field. In this study, we used a mouse model with overexpression of PPP1R3G 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 PPP1R3G was reported previously (16). The chronic and binge ethanol-fed mouse model was established as previously described with minor modifications (17, 18). In brief, the mice were exposed to chronic ethanol administration for 10 days \textit{ad libitum} by oral feeding with Lieber-DeCarli ethanol 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% ethanol in water (vol/vol) at 7am. The gavage volume (in μl) was body weight in grams times 20. The mice were sacrificed at 4pm. Ten to twelve weeks old wild type or PPP1R3G liver-specific transgenic mice (female, weight 20–22 g) were randomly allocated into 3 groups: wild type (WT) mice that were not administered with alcohol (control group, n = 6); WT mice exposed to alcohol treatment (WT-EtOH group, n = 6); and PPP1R3G transgenic mice exposed to alcohol (TG-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 60±10%. Hematoxylin and eosin staining as well as Oil-O-Red staining were performed as previously reported (17). PAS staining was performed as previous reported (16). The activities of liver glycogen synthase (GS) and glycogen phosphorylase (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 ALT, AST, TG, TC, HDL and LDL cholesterol concentrations were determined by colorimetric methods according to the procedures as previously reported (16). The assay kits for alanine aminotransferase (ALT), aspartate aminotransferase (AST), total triglyceride (TG), total cholesterol (TC), HDL cholesterol (HDL-c), and LDL cholesterol (LDL-c) were from ShenSuoYouFu (Shanghai, China). The blood alcohol level was measured by an ethanol assay kit from Sigma-Aldrich (St. Louis, MO, USA). Briefly, the serum was diluted in the ethanol assay buffer to a final volume of 50 µl, and then mixed with ethanol probe and ethanol 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, USA). 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, USA) 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 realtime 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 SRE-containing promoter and a cell line stably expressing this reporter (named Huh-7/SRE-Luc) (20). For ethanol treatment, 100 mM ethanol 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 3 times with PBS, and incubated for 20 minutes 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, USA) 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-GFP and anti-SREBP-1 antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, USA), 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 means ± SEM unless indicated otherwise. Significant differences were assessed by two-tailed Student’s t test for pairwise comparison between two groups and 2-way ANOVA was used for experiments with more than two groups. $P < 0.05$ was considered statistically significant.
Results

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

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

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

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

**PPP1R3G overexpression relieves ethanol-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 (Figure 4A), indicating that liver steatosis was successfully induced by ethanol in our mouse model. Interestingly, PPP1R3G overexpression significantly reduced ethanol-induced elevation of the liver triglyceride concentration by 20% (Figure 4A). Hematoxylin and eosin staining revealed that ethanol profoundly caused lipid vacuoles in hepatocytes, and the ethanol-induced pathologic changes were reversed by overexpression of PPP1R3G (Figure 4B). Consistently, Oil-O-Red staining revealed that ethanol markedly induced fat deposition in the liver and PPP1R3G overexpression could
reduce such effect (Figure 4C). Together, these data suggest that the liver steatosis induced by chronic alcohol administration can be alleviated by PPP1R3G overexpression.

Ethanol itself is a macronutrient and 1 gram of ethanol has 7 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 ethanol feeding but without the binge. As shown in Supplemental Figure 1, chronic ethanol feeding itself was able to significantly reduce the glycogen level and elevate the triglyceride level in the liver. PPP1R3G overexpression could abrogate the chronic ethanol 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 overnutrition of the ethanol binge.

**Ethanol-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 ethanol exposure (6, 7). Consistently, we found that the mRNA levels of SREBP-1c as well as its target gene FAS (fatty acid synthase) were upregulated by ethanol treatment (Figure 5A). However, the mRNA level of SREBP-2 was not altered by ethanol feeding (Figure 5A). As a negative control, ethanol
feeding had no effect on the mRNA level of β-actin (Supplemental Figure 2). Ethanol significantly induced expression of SREBP-1c by 213% and FAS by 166% respectively. On the other hand, PPP1R3G overexpression significantly abrogated ethanol-induced expression of these two lipogenic genes. Compared with the EtOH group, PPP1R3G transgenic mice had decreases in SREBP-1c by 55% and FAS by 45% respectively (Figure 5A). Such effects were confirmed by Western blotting in which the protein levels of both SREBP-1 precursor and FAS were elevated by ethanol exposure and abrogated by PPP1R3G overexpression (Figure 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 SREBP1c pathway is involved in ethanol-induced hepatic steatosis, but also suggest that PPP1R3G overexpression may alleviate fatty liver formation through down-regulation of SREBP-1c pathway.

We also analyzed hepatic expression of a few other genes involved in lipid metabolism. Ethanol could reduce the mRNA levels of peroxisome proliferator-activated receptor alpha (PPARα) and its target genes carnitine palmitoyltransferase (CPT1) and acyl-CoA dehydrogenase (MCAD), while PPP1R3G overexpression abrogated the ethanol effect (Figure 5A). As PPARα plays an important role in β-oxidation of fatty acid (21), these results indicate that elevated glycogen level could decrease hepatic lipid level via elevation of fatty acid oxidation. We also analyzed carbohydrate-responsive element-binding protein (ChREBP), an important transcription factor for de novo fatty
acid synthesis (22). The mRNA levels of ChREBP and its target gene liver-pyruvate kinase (L-PK) were reduced by ethanol feeding and PPP1R3G overexpression could partially abrogate such reduction (Figure 5A).

We also analyzed markers involved in inflammation and lipid peroxidation, including interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor α (TNF-α) (Figure 5A). IL-1β signaling is required for the development of alcohol-induced liver steatosis, inflammation and injury (23). We found that the expression level of IL-1β was significantly elevated by ethanol exposure and abrogated by PPP1R3G expression (Figure 5A). However, the mRNA levels of IL-6 and TNF-α were not altered by PPP1R3G overexpression (Figure 5A), indicating that the protective role of PPP1R3G on the liver is only associated with changes of a few limited inflammatory markers.

**PPP1R3G overexpression increases glycogen level and decreases ethanol-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 (Figure 6A). The glycogen level was also
PPP1R3G overexpression decreased ethanol-induced lipid accumulation in mouse hepatocytes (Figure 5C), consistent with the *in vivo* results. Ethanol treatment significantly elevated TG concentration by 83% in AML-12 cells, while PP1R3G overexpression markedly prevented ethanol-induced increase of TG concentrations, showing as a 19% decrease (Figure 6C). We also used Nile Red staining to directly analyze lipid droplets in these cells. Ethanol exposure dramatically increased the intensity and quantity of lipid droplets in AML-12 cells, and such effect was apparently abrogated while PPP1R3G was overexpressed (Figure 6D).

Ethanol could activate SREBP pathway at the cellular level, shown as stimulation of a SRE-responsive luciferase reporter by ethanol treatment (Figure 6E). Interestingly, PPP1R3G overexpression could abrogate the stimulatory effect of ethanol on SREBP activity (Figure 6E). Consistently, ethanol treatment also elevated the mRNA levels of SREBP-1c and FAS (Figure 6F). PPP1R3G overexpression abrogated the effect of ethanol on the expression of these genes (Figure 6F). The protein levels of SREBP-1 precursor and FAS were also elevated by ethanol exposure and the ethanol effect was abrogated by PPP1R3G overexpression (Figure 6G). Collectively, these results indicate that PPP1R3G overexpression could reduce ethanol-induced lipid accumulation in hepatocytes at least partly be inhibiting SREBP pathway.
We also investigated whether PPP1R3G could affect incorporation of exogenous ethanol and glucose into glycogen or lipid. In AML12 cells, exogenous ethanol could elevate production of both glycogen and triglyceride, similar to the effect of exogenous glucose. PPP1R3G overexpression could elevate glycogen production from both exogenous ethanol and exogenous glucose (Supplemental Figure 3). However, PPP1R3G overexpression reduced triglyceride production from exogenous ethanol (Supplemental Figure 3). These data, therefore, further support our conclusion that elevation of glycogen synthesis can reduce ethanol-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 as 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 glycogen level in the liver and PPP1R3G overexpression abrogated such negative effect of alcohol. The reduced glycogen level in the liver appeared to be caused by inhibition of glycogen synthase activity. In addition, PPP1R3G overexpression also abrogated the stimulatory effect of alcohol exposure on SREBP-1c, the master regulator of lipogenesis in the liver. At the cellular level, we found that PPP1R3G overexpression could also abrogate alcohol-induced lipid accumulation and SREBP-1c stimulation in hepatocytes. Collectively, our studies pinpoint that increased liver glycogen level is associated with alleviation of alcohol-induced hepatic steatosis.

Interestingly, although it has been known for a long time that fat and glycogen are two major forms of energy storage in the liver, the role of glycogen in alcoholic fatty liver formation has been elusive. Currently, it is believed that alcohol-induced fatty liver is mainly caused by alterations of 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 upregulation SREBP-1c, a master transcription factor that promotes fatty acid synthesis through up-regulation of lipogenic genes (6, 7). Alcohol also inhibits fatty acid oxidation in hepatocytes mainly via inactivation of the peroxisome PPAR-α (8), a nuclear hormone receptor 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 ethanol treatment. However, we could not rule out the possibility that the activity of ChREBP was augmented by ethanol exposure. Nevertheless, our study indicates that glycogen contributes to the development of hepatic steatosis. We propose that while alcohol directly stimulates lipid deposit in the liver via SREBP-1c, PPARα and likely other factors (Figure 7). Alcohol also inhibits glycogenesis, consequently leading to release of free glucose which fuels the process of lipogenesis (Figure 7). The increased amount of free glucose released from the glycogen would favor formation of acetyl-CoA, the building block of fatty acid. Acetyl-CoA is converted from pyruvate via pyruvate dehydrogenase and pyruvate is formed from glucose via glycolysis. In addition, ethanol 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, contributing to relief of alcohol-induced fatty liver formation (Figure 7).

It is noteworthy that alcohol exposure only reduces glycogenesis at the in vivo level (Figure 4), but not at the cellular level (Figure 6). Such discrepancy is likely caused by the difference in ethanol metabolism between in vivo and in vitro. At the in vivo level, ethanol is metabolized to acetaldehyde via alcohol dehydrogenase (ADH) and acetaldehyde is then converted to acetic acid via aldehyde dehydrogenase (ALDH). 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 ethanol 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 amount of antioxidants in vivo, leading to inhibition on glycogenesis.

In addition to the regulation on glycogenesis, ethanol is also able to modulate gluconeogenesis in the liver. It was recently reported that acute ethanol 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 ethanol 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 ethanol-induced hypoglycemia occurs in fasting state, not in fed state. We speculate that the liver glucose converted from acetyl-CoA generated from ethanol during fed state mainly goes to the lipid synthesis pathway; and it won’t go for glycogen synthesis as this program is inhibited by ethanol.

Another unanswered question in this study is how reduced glycogen level is linked to stimulation of SREBP-1c pathway under alcohol exposure. One possibility is the reduced glycogenesis would lead to 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.

Acknowledgements

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References


Inhibition of SREBP by a small molecule, betulin, improves hyperlipidemia and insulin resistance and reduces atherosclerotic plaques. Cell Metab 13: 44-56.


Figure legends

Figure 1. Characterization of PPP1R3G transgenic mice with a chronic and binge EtOH feeding protocol

Mice consumed a control diet or an EtOH-containing diet with or without liver-specific overexpression of PPP1R3G. The mRNA level of PPP1R3G in the liver was determined by RT-PCR (A). The protein level of PPP1R3G in the liver was determined by Western blotting (B) and the quantitation of the blots was shown in the lower panel. The blood concentration of ethanol was determined in these mice (C). The ratio of liver weight vs body weight (D). The body weight and food intake of the mice (E and F). Values are means ± SEM; n = 6 for each group. * for P < 0.05 and ** for P < 0.01 between the groups as indicated. “ns” stands for non-significant. ^ for P < 0.05 between the WT-EtOH and the TG-EtOH groups. ## for P < 0.01 between the control and the WT-EtOH groups. Mouse groups: Control, no alcohol administration; WT-EtOH, wild type mice exposed to alcohol using a chronic and binge EtOH feeding protocol; TG-EtOH, PPP1R3G transgenic mice exposed to alcohol.

Figure 2. Ethanol exposure reduces liver glycogen level and inhibits glycogen synthase 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. Hepatic concentration of glycogen (A). Representative
images (200X) of liver PAS staining (B). The activities of glycogen synthase (GS) and for glycogen phosphorylase (GP) (C). Values are means ± SEM for (A) and means ± SE for (C); n = 6 for each group. * for P < 0.05 and ** for P < 0.01 between the groups as indicated. GS, glycogen synthase; GP, glycogen phosphorylase.

Figure 3. Ethanol-induced liver injury is relieved by PPP1R3G overexpression

Serum ALT (A), AST (B), TG (C), TC(D), HDL-C (E), and LDL-C (F) in mice fed a control diet with or without liver-specific overexpression of PPP1R3G. Values are means ± SEM; n = 6 for each group. * for P < 0.05 and ** for P < 0.01 between the groups as indicated. ALT, alanine aminotransferase; AST, aspartate aminotransferase; TG, total glyceride; TC, total cholesterol; HDL-c, HDL cholesterol; LDL-c, LDL cholesterol.

Figure 4. PPP1R3G overexpression relieves ethanol-induced hepatic steatosis

Mice consumed a control diet or an EtOH-containing diet with or without liver-specific overexpression of PPP1R3G. Hepatic concentrations of TG (A). Representative images (200X) of liver H&E staining and Oil-Red-O staining (B and C). Values are means ± SEM; n = 6 for each group. * for P < 0.05 and ** for P < 0.01 between the groups as indicated. TG, total glyceride.

Figure 5. Ethanol-induced expression of genes involved in lipid metabolism and
inflammation in the liver is reduced by PPP1R3G overexpression

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 (A). The mRNA levels were detected by quantitative realtime RT-PCR and shown as fold changes compared to the control group. Western blotting was used to detect the protein levels of SREBP-1c and FAS (B) and the quantitation of the blots was shown in the right panels. Values are means ± SEM; n = 6. * for P < 0.05 and ** for P < 0.01 between the groups as indicated. ChREBP, carbohydrate-responsive element-binding protein; CPT1, carnitine palmitoyltransferase; IL-1β, interleukin-1 β; IL-6, interleukin-6; L-PK, liver-pyruvate kinase; MCAD, acyl-CoA dehydrogenase; PPARα, peroxisome proliferator-activated receptor alpha; SREBP, sterol regulatory element binding transcription factor; TNF-α, tumor necrosis factor α.

Figure 6. Ethanol-induced lipid deposition and SREBP-1c stimulation in hepatocytes is abrogated by PPP1R3G overexpression

AML-12 cells were transfected with GFP vector or PPP1R3G (R3G) without or without ethanol (EtOH) treatment. Western blotting was used to detect PPP1R3G protein (A). Glycogen and TG concentrations of the cells were determined (B and C). Representative images of Nile Red staining for lipid droplets (D). Results of a SREBP-responsive luciferase reporter (E). Results of mRNA abundances of representative genes involved in
lipogenesis (F). Western blotting to detect the protein levels of SREBP-1 and FAS (G). Values are means ± SEM. * for P < 0.05 and ** for P < 0.01 between the groups as indicated. FAS, fatty acid synthase; R3G, PPP1R3G overexpression; SREBP-1, sterol regulatory element-binding protein 1; TG, total glyceride.

Figure 7. A model to depict the contribution of glycogen metabolism to alcohol-induced hepatic steatosis.

Chronic alcohol exposure induces fatty liver formation through increased lipogenesis via stimulation of SREBP-1c and reduced fatty acid oxidation via inhibition of PPARα. On the other hand, alcohol exposure reduces glycogenesis via inhibition of glycogen synthase activity, resulting in reduction of liver glycogen and release of free glucose that fuels lipogenesis. Increase of liver glycogen such as through PPP1R3G overexpression reduces glucose release and consequently reduces lipogenesis upon alcohol exposure. In addition, PPP1R3G overexpression could abrogate alcohol-mediated stimulation of SREBP-1c and inhibition of PPARα, contributing to reduction of hepatic steatosis.
Figure 2

A

![Bar graph showing liver glycogen levels](image)

B

![Histological images comparing control, WT-EtOH, and TG-EtOH](image)

C

![Graphs showing GS and GP activity](image)
Figure 4

A

Liver TG (mg/g protein)

Control  WT-EtOH  TG-EtOH

**  *  

B

Control  WT-EtOH  TG-EtOH

C

Control  WT-EtOH  TG-EtOH
Figure 5

A

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Figure 7