Ethanol and its nonoxidative metabolites promote acute liver injury by inducing ER stress, adipocyte death and lipolysis

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Binge alcohol drinking

- High concentrations of ethanol
  - Adipocyte death
  - Tissue inflammation
  - ER stress
  - Free fatty acids
  - Lipolysis
  - Bcl2 (anti-apoptotic)

- Alcohol-Associated Liver Disease
  - ER stress
  - Cell death
  - FAEEs
  - DNA damage

- AcH
  - ADH
  - Ethanol

[White Adipose Tissue]
adipocyte death and lipolysis

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disease; ALDH2: aldehyde dehydrogenase 2; ALT: alanine aminotransferase; AST: aspartate aminotransferase; ATGL: adipose triglyceride lipase; Bcl2: B-cell lymphoma 2; Bcl-xL: B-cell lymphoma extra-large; CAT: catalase; CYP2E1: cytochrome p450 2E1; EtOH: ethanol; FFAs: free fatty acids; FAEEs: fatty acid ethyl esters; GTT: glucose tolerance test; HFD: high fat diet; HSL: hormone sensitive lipase; IL: interleukin; IPA: ingenuity pathway analysis; ITT: insulin tolerance test; JNK: c-Jun N-terminal kinase; MAPK: mitogen-activated protein kinase; MEOS: microsomal ethanol oxidizing system; PBA: 4-phenylbutyric acid; PKA: protein kinase A; ROS: reactive oxygen species; SA: salicylic acid; SVF: stromal vascular fraction; TG: triglyceride; TUDCA: tauroursodeoxycholic acid; TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling; WAT: white adipose tissue; WT: wild-type; 3-AT: 3-Amino-1,2,4-triazole; 4-MP: 4-Methylpyrazole

SYNOPSIS:

Binge drinking induces acute liver injury and is an important risk factor for alcohol-associated liver disease. Binge alcohol and its non-oxidative metabolites promote acute liver injury by inducing ER stress, adipocyte death, and lipolysis.
Background & Aims: Binge drinking in patients with metabolic syndrome accelerates the development of alcohol-associated liver disease (ALD). However, the underlying mechanisms remain elusive. We investigated if oxidative and non-oxidative alcohol metabolism pathways, diet-induced obesity, and adipose tissues influence the development of acute liver injury in a single ethanol binge model.

Methods & Results: A single ethanol binge was administered to chow-fed or high-fat diet (HFD)-fed wild-type and genetically modified mice. Oral administration of a single dose of ethanol induced acute liver injury and hepatic ER stress in chow- or HFD-fed mice. Disruption of the alcohol dehydrogenase 1 (Adh1) gene elevated blood ethanol concentration and exacerbated acute ethanol-induced ER stress and liver injury in both chow-fed and HFD-fed mice, while disruption of the aldehyde dehydrogenase 2 (Aldh2) gene did not affect such hepatic injury despite high blood acetaldehyde levels. Mechanistic studies revealed that alcohol, not acetaldehyde, promoted hepatic ER stress, fatty acid synthesis, increased adipocyte death and lipolysis, contributing to acute liver injury. Elevated serum fatty acid ethyl esters (FAEEs), which are formed by an enzyme-mediated esterification of ethanol with fatty acids, were detected in mice post ethanol gavage with higher levels in Adh1 knockout mice than that in wild-type mice. Deletion of the carboxylesterase 1d (Ces1d) gene in mice markedly reduced acute ethanol-induced elevation of blood FAEE levels with slight but significant reduction of serum aminotransferase levels.

Conclusion: Ethanol and its non-oxidative metabolites, FAEEs, not acetaldehyde, promoted acute alcohol-induced liver injury by inducing ER stress, adipocyte death, and lipolysis.

Keywords: binge, ADH, ALDH, FAEE, carboxylesterase 1d
Alcohol-associated liver disease (ALD) is a major cause of chronic liver diseases.\textsuperscript{1} It represents a spectrum of histopathological changes from steatosis, steatohepatitis, cirrhosis, and hepatocellular carcinoma. The risk of ALD is associated with the quantity of alcohol consumed. Patients with alcohol-induced steatosis who continue to consume alcohol more than 400 grams per week significantly increase the risk for cirrhosis.\textsuperscript{2} In another large study of more than 13,000 patients, a risk for ALD is considerably increased with drinking more than 7 to 13 beverages per week in women and 14 to 27 beverages per week in men.\textsuperscript{3} In addition to the quantity of alcohol consumed, the pattern of drinking is also associated with the development of ALD. Binge drinking, a heavy episodic alcohol intake, is defined as consuming 5 or more drinks on an occasion for men or 4 or more drinks on an occasion for women.\textsuperscript{4} It is a drinking pattern associated with an increase in blood alcohol concentration above the legal limit within 2 hours.\textsuperscript{4} Binge drinking is thought to be an important risk factor for ALD observed in the millennials\textsuperscript{5, 6} and patients with metabolic syndrome.\textsuperscript{7, 8} However, the underlying mechanisms of binge drinking-associated ALD remain elusive.

Once ingested, alcohol is metabolized, primarily in the liver, by oxidative and non-oxidative pathways.\textsuperscript{9} More than 90\% of ingested alcohol is converted into acetaldehyde by oxidative enzymes, alcohol dehydrogenase (ADH), cytochrome P450 2E1 (CYP2E1), and catalase.\textsuperscript{9} Many adverse effects of ethanol among chronic drinkers are mediated by its byproduct, acetaldehyde.\textsuperscript{10} Acetaldehyde is further metabolized into acetate by mitochondrial aldehyde dehydrogenase 2 (ALDH2) in the liver. Interestingly, our recent studies suggest that the liver ALDH2 is responsible for less than 50\% blood acetaldehyde clearance.\textsuperscript{11} In addition, a non-oxidative pathway to metabolize alcohol is mediated by the generation of lipophilic fatty acid ethyl esters (FAEEs), a combination of alcohol with free fatty acids.\textsuperscript{12} The generation of FAEEs is mediated by tissue-specific FAEE synthase enzymes, FAEE carboxylesterase (encoded by Ces1d in murine and CES1 in humans) in the liver, and carboxylester lipase (Cel) in the pancreas.\textsuperscript{13-15} FAEEs accumulate in tissues after acute alcohol intoxication causing organ inflammation and injury.\textsuperscript{12} The implications of binge drinking on oxidative and non-oxidative pathways in association with liver injury remain obscure.

The pathogenesis of ALD is complex and the inter-organ crosstalk between the liver and other tissues contributes to the progression of ALD.\textsuperscript{16} Inter-organ crosstalk leads to inflammation, metabolic alternations, and cell death in ALD.\textsuperscript{16} Adipose tissue is an important organ in regulating lipid homeostasis.\textsuperscript{17} Chronic alcohol consumption markedly increases adipocyte death\textsuperscript{18} and lipolytic activity in murine adipose tissue.\textsuperscript{19, 20} An increase in adipose tissue lipolysis causes the release of non-esterified free fatty acids, providing an extrahepatic source of free fatty acids for accumulation in hepatocytes
binge alcohol consumption and liver injury remains elusive.

The development of the chronic-plus-binge ethanol model has led to the discovery of multiple pathways in ALD pathogenesis. However, the molecular mechanisms underlying binge ethanol-induced liver injury have not been carefully investigated. To address these mechanisms, we utilized a single ethanol binge model in several genetically modified mouse models to identify the pathways linking binge drinking, obesity, and acute liver injury. There is a challenge in developing a preclinical model for binge alcohol consumption in rodent. The amount of alcohol consumed in humans during a binge episode, which may lead to liver injury, is approximately 0.8-1 g/kg in a 70-kilogram subject. In mice, ethanol metabolism and clearance are approximately 5.5 fold faster than that in humans. We, therefore, carefully selected the quantity of alcohol being administered in our mouse model at ~5-7 g/kg. By using this model, here we investigated the role of a single ethanol binge and acute liver injury by focusing on the following objectives. First, we examined if oxidative and non-oxidative alcohol metabolism pathways influence the development of acute liver injury. Second, we explored the implications of a single ethanol binge and diet-induced obesity on acute liver injury. Third, we determined the role of the liver and adipose tissue crosstalk in the pathogenesis of liver injury after a single ethanol binge.
RESULTS

A single ethanol binge induces acute liver injury in mice via the induction of ER stress

A single ethanol binge via oral gavage to C57BL/6N mice induced liver injury in a dose-dependent manner, from 5 to 7 g/kg of ethanol (Fig. 1A). The highest peak of serum ALT (~220 IU/L) and AST (~400 IU/L) was observed in mice receiving 7 g/kg of ethanol approximately 9 hours after the administration (Fig. 1A). We found a positive correlation between body weight and serum ALT levels at 9 hours after a single ethanol binge (7 g/kg) in both C57BL/6N and C57BL/6J mice (Fig. 1B). However, the elevation of serum ALT levels was higher in C57BL/6N mice than those in C57BL/6J mice after a single binge (7 g/kg), suggesting that C57BL/6N mice were more sensitive to acute alcoholic injury than C57BL/6J mice (Fig. 1B). In addition, our data revealed that male mice were more susceptible to acute alcohol-induced liver injury than female mice (serum ALT: ~220 IU/L in male mice versus ~160 IU/L in female mice). Alcohol-induced liver injury was also observed in BALB/c mice, again in a dose dependent manner with higher serum AST and ALT levels in 7 g/kg ethanol-treated than in 5 g/kg ethanol-treated BALB/c mice (ALT: ~250 IU/L, AST: ~340 IU/L in the 7g/kg binge group versus ALT: ~125 IU/L, AST: ~180 IU/L in the 5g/kg binge group).

Activation of the ER stress pathway has been implicated in ethanol-induced liver injury after chronic ethanol feeding or chronic-plus-binge ethanol feeding. We next asked if the pathway related to liver injury after a single ethanol binge is also attributed to ER stress. As illustrated in Fig. 1C, a single ethanol binge (7 g/kg) rapidly upregulated the expression of several ER stress-related proteins, notably p-PERK, p-eIF2α, IRE1α, p-JNK, CHOP, and BiP with the peak expression approximately 3 to 6 hours post binge. Along with the induction of ER stress-related proteins, hepatic expressions of pro-apoptotic protein such as BAD (Fig. 1C), and mRNA expressions of Gadd34, Bak, Bim, Ero1b, and Dr5 were markedly increased after a single ethanol binge (Fig. 1D).

The CHOP is induced by ER stress and mediates cellular apoptosis. To confirm that ER stress pathway is crucial in the pathogenesis of liver injury in a single ethanol binge model, we employed, (i) a loss of function approach using Chop−/− mice, and (ii) ER stress inhibitors, PBA, and TUDCA. The serum ALT levels in Chop−/− mice receiving a single ethanol binge at 7 g/kg were markedly lower than those in wild-type controls (Fig. 1E). Additionally, the administration of ER stress inhibitors (PBA and TUDCA) but not JNK inhibitor significantly attenuated serum ALT levels in mice receiving a single ethanol binge (Fig. 1F). Taken together, these data suggest that a single ethanol binge induces hepatocyte injury via the induction of ER stress.
Disruption of the oxidative metabolizing enzyme, alcohol dehydrogenase 1 (Adh1), exacerbates acute ethanol-induced liver injury in chow- and HFD-fed mice

To examine whether ethanol or its oxidative metabolites play an important role in alcohol-induced liver injury in our single ethanol binge model, we performed the experiments in mice lacking Adh1, a key oxidizing enzyme converting ethanol to acetaldehyde. As expected, blood ethanol concentrations were much higher in Adh1−/− mice than in WT mice, notably at 6 and 9 hours after an ethanol binge, whereas the level of serum acetaldehyde was comparable in Adh1−/− and WT mice (Fig. 2A). Interestingly, a single ethanol binge induced higher serum ALT levels in Adh1−/− mice than those in WT mice (Fig. 2A). In agreement with this finding, hepatocyte death, hepatic ER stress-related proteins and genes were higher in Adh1−/− mice than those in WT mice after a single ethanol binge (Fig. 2B-D).

In addition to ADH, ethanol can be oxidized by a microsomal ethanol oxidizing system (MEOS), cytochrome P450 2E1, and peroxisomal catalase. We, therefore, carried out the experiments to determine the potential role of CYP2E1 and catalase in ALD after a single ethanol binge. There was no difference in the level of serum ALT in WT and Cyp2e1−/− mice receiving a single ethanol binge. Treatment with the catalase inhibitor 3-Amino-1,2,4-triazole (3-AT) or salicylic acid (SA) slightly, but not significantly increased serum ALT levels in ethanol-treated mice (data not shown). Moreover, binge alcohol intake elevated hepatic MDA levels, however, significant differences between WT and Adh1−/− mouse were not observed in MDA, Sod1, Sod2, Catalase, or several inflammatory markers (data not shown). Collectively, these data suggest that among alcohol metabolizing enzymes, ADH1, but not CYP2E1 and catalase, plays an important role in acute liver injury in a single ethanol binge model via the inhibition of ER stress but not oxidative stress.

As previously stated, binge drinking, in combination with metabolic syndrome or obesity, cause a synergistic effect for ALD. Next, we further explored whether the deficiency of Adh1 is also more susceptible to acute alcoholic liver injury under HFD-mediated obese condition. WT and Adh1−/− mice fed with HFD for 3 months without alcohol gavage, had similar body weight gain and glucose homeostasis, as measured by the GTT and ITT (Fig. 3A). Because oral administration of 6 or 7 g/kg ethanol caused high mortality in HFD-fed mice, we only used 5 g/kg ethanol gavage in HFD-fed mice in the following experiments. As illustrated in Fig. 3B, HFD-fed Adh1−/− mice receiving a single ethanol binge had higher levels of blood ethanol concentrations and serum ALT compared to HFD-fed WT mice. However, blood acetaldehyde levels were comparable between WT and Adh1−/− mice after a single ethanol binge (Fig. 3B). Furthermore, HFD-plus-binge ethanol-fed Adh1−/− mice had greater liver fibrogenic response and hepatocyte death as demonstrated by H&E, Sirius Red, and TUNEL staining,
compared to that of WT mice (Fig. 3C), and higher levels of ER stress and fibrotic genes as demonstrated by RT-qPCR analyses (Fig. 3D). Finally, we also performed in vitro experiments in primary hepatocytes isolated from WT and Adh1−/− mice treated with palmitic acid (200 μM) in the presence and absence of ethanol (100 mM). We found that mRNA expression of Chop and Bim was significantly higher in Adh1 deficient hepatocytes treated with both palmitic acid and ethanol when compared to those treated with either palmitic acid or ethanol (Fig. 3E).

Depletion of the oxidative metalizing enzyme Aldh2 does not exaggerate acute alcohol-induced liver injury in chow-fed and HFD-fed mice

Once generated from the metabolism of alcohol by oxidative enzymes, acetaldehyde is subsequently converted to acetate by aldehyde dehydrogenase (ALDH2). To evaluate the role of acetaldehyde in a single binge ethanol model, we performed the experiments using Aldh2−/− mice. An administration of ethanol at 7 g/kg caused significantly high mortality in Aldh2−/− mice; we, therefore, used ethanol at 6 g/kg in Aldh2−/− mice and their controls, in the subsequent experiments. After ethanol gavage, blood ethanol concentrations were slightly but significantly higher in Aldh2−/− than those in WT mice, whereas much higher blood acetaldehyde levels were observed in Aldh2−/− mice than in WT mice post ethanol gavage (Fig. 4A). Despite higher levels of acetaldehyde, the serum ALT levels in Aldh2−/− and WT mice were comparable, suggesting that high blood acetaldehyde does not play a significant role in alcohol-induced liver injury in our model (Fig. 4A). Hepatic expression of several ER stress-related genes except for Gadd34 (DNA damage-inducible gene) was also similar between both groups after ethanol gavage (Fig. 4B). Protein expressions of CYP2E1, ER stress-related proteins, CHOP and BIM, were also similar between both groups after a single ethanol gavage (Fig. 4C).

We also explored the involvement of ALDH2 on liver injury induced by ethanol binge in a combination with the HFD feeding. Aldh2−/− mice had similar body weight gains and glucose homeostasis, as measured by the GTT and ITT when compared to corresponding WT groups after 3 months HFD feeding without ethanol gavage (Fig. 5A). After ethanol gavage (5 g/kg), HFD-fed Aldh2−/− mice had much higher levels of blood acetaldehyde levels compared to WT mice, but both groups had similar serum ALT levels (Fig. 5B), hepatocyte death, hepatic collagen deposition and hepatic expression of ER stress and fibrotic genes (Fig. 5C).

Administration of ethanol, but not acetaldehyde, induces liver injury by promoting ER stress and hepatocyte death
To further determine whether ethanol or acetaldehyde induces acute liver injury in a single binge ethanol model, ethanol or acetaldehyde was administered via an intraperitoneal route to WT mice. As illustrated in Fig. 6A, the intraperitoneal administration of ethanol (6 g/kg) rapidly elevated blood ethanol levels (left panel) and serum ALT levels (right panel). Hepatic expression of ER stress-related genes (Chop and Xbp-1), cell death-associated gene (Bim, Bax, and Bak), and neutrophil infiltration related genes (Ly6g, Lcn2, and Cxcl1) were upregulated with the peak induction at 9 hours post administration (Fig. 6B). Next, we also administered ethanol intraperitoneally to WT and Adh1−/− mice and euthanized them 9 hours post administration. As illustrated in Fig. 6C, serum ALT and ethanol levels, not acetaldehyde, were higher in Adh1−/− mice compared to corresponding WT mice. Concomitantly, hepatic expressions of Chop, Xbp-1, and Bim were greater in Adh1−/− mice than in WT mice (Fig. 6C).

Next, we also evaluated the involvement of acetaldehyde in acute liver injury by intraperitoneal injection of acetaldehyde (50 mg/kg). As illustrated in Fig. 6D, injection of acetaldehyde rapidly elevated blood acetaldehyde levels with the peak at 15 minutes post injection; its level gradually declined to very low levels at 3 hours post injection, which is probably because acetaldehyde is short-lived and rapidly metabolized in the liver. Administration of acetaldehyde slightly increased serum ALT levels up to 1 hour, but it did not reach a statistical difference, suggesting that acetaldehyde does not attribute to acute liver injury (Fig. 6D).

Acute alcohol gavage increases hepatic expression of fatty acid synthesis-related genes, elevates serum FFA, and increases adipose tissue lipolysis

To understand molecular mechanisms of acute liver injury after a single ethanol binge, we subjected liver tissues for microarray analyses and analyzed lipid metabolism-associated gene expression profiles. A large number of dysregulated genes was observed in the livers of mice receiving 5 g/kg or 7 g/kg ethanol gavage compared to those from control mice, with the most alternations from the 7 g/kg ethanol gavage group (Fig. 7A). The genes related to fatty acid metabolism were the most altered in mice receiving 7 g/kg ethanol gavage compared to controls and those with 5 g/kg ethanol gavage (Fig. 7B, C). In more details, oral administration of 7 g/kg ethanol markedly upregulated hepatic expression of de novo lipogenesis genes (acetyl-CoA carboxylase [Acaca], fatty acid synthase [Fasn] and cytochrome P450 sterol 14α-demethylase [Cyp51]), but attenuated hepatic expression of the fatty acid oxidation pathway (peroxisome proliferator-activated receptor [Ppara], fibroblast growth factor 21 [Fgf21] and cytochrome P450 4A [Cyp4a10]) with limited impacts on hepatic triglyceride (TG) synthesis (diacylglycerol O-acyltransferase 2 [Dgat2] and low density lipoprotein receptor [Ldlr]) (Fig. 8A). Interestingly, lipid droplet dynamics related genes (apoprotein E [Apoe] and apolipoprotein B
was highly associated with pro-inflammatory responses and inversely associated with fatty acid oxidation (Fig. 8A).

To further explore how 7 g/kg ethanol gavage induces acute liver injury and how fatty acid metabolism is involved, we measured serum and hepatic free fatty acids (FFAs) and hepatic triglyceride levels. As illustrated in Fig. 8B, oral administration of 7 g/kg ethanol markedly elevated serum and hepatic FFAs as well hepatic triglyceride levels. In addition, hepatic levels of several FFAs including palmitic acid, oleic acid, and linoleic acid were drastically elevated after oral alcohol administration (Fig. 8C). Hepatic expressions of FASN and SCD1 proteins, which play an important role in the fatty acid synthesis, were highly upregulated after acute alcohol gavage (Fig. 8C).

Because serum levels of FFAs were highly elevated after a single ethanol binge, and adipose lipolysis has been shown to promote steatosis,20 we wondered whether adipose tissue is the source of circulating FFAs through the lipolytic process in the adipose tissues. To test this hypothesis, we performed western blot analyses of lipolysis-related proteins. Our data show that oral ethanol administration elevated the expression of lipolysis proteins such as p-PKA, p-HSL, and ATGL in adipose tissues, suggesting acute alcohol gavage-induced adipose tissue lipolysis (Fig. 8D).

**Acute alcohol gavage causes adipocytes death and ER stress in adipose tissues, which is enhanced in Adh1−/− but not in Aldh2−/− mice**

We have previously demonstrated that adipocyte death triggers lipolysis.30 Thus, we speculated whether acute ethanol-induced lipolysis was due to the induction of adipocyte death. To answer this question, we measured ethanol and acetaldehyde concentrations in adipose and liver tissues post ethanol gavage. As illustrated in Fig. 9A, total ethanol concentrations were comparable between the liver and adipose tissues post ethanol gavage except for 1-hour time point in which ethanol concentrations were higher in the liver than in adipose tissues, where the levels of acetaldehyde were much lower in adipose tissues than in liver tissues after a single binge (Fig. 9A).

Next, we examined adipose tissue histology after a single ethanol gavage. Our preliminary data show that the number of crown-like structure (CLS) was higher in epididymal fat than that in subcutaneous fat after alcohol gavage (both “single binge” and “3M HFD-plus-binge”). In addition, we did not observe significant morphological changes/gene expression of brown adipose tissues in single binge model and HFD-plus-binge ethanol feeding model compared to their corresponding controls (data not shown). Thus, we focused on epididymal fat in this study and found that epididymal adipose tissues had an increase in CLS after a single ethanol binge compared to the control group, and the number of
a typical pattern of adipocyte death formed by the accumulation of macrophages around the dead/injured adipocytes. Caspase 3/7 activity was elevated in the binge alcohol gavage group compared to the control group (Fig. 9C). Furthermore, RT-qPCR data showed that the expression of several ER stress-related genes in adipose tissues was upregulated after acute alcohol gavage (Fig. 9C).

We further explored acute alcohol-induced adipocyte death and lipolysis in Adhl\(^{-}\) mice. Fig. 9D shows that adipose tissues from Adhl\(^{-}\) mice had a greater number of the CLS and TUNEL\(^{+}\) cells. As expected, Adhl\(^{-}\) mice had higher levels of ethanol and caspases 3/7 activity, but lower levels of acetaldehyde than WT mice after a single ethanol gavage (Fig. 9E). The levels of serum FFA, the expression of ER stress-, cell death- and lipolysis-related genes in adipose tissues were greater in Adhl\(^{-}\) mice compared to WT mice after ethanol gavage (Fig. 9E, F).

In addition, binge alcohol gavage (6 g/kg) was given to Aldh2\(^{-}\) and WT mice. After the binge alcohol gavage, acetaldehyde concentration in white adipose tissues was much higher in Aldh2\(^{-}\) mice than that in WT mice, as we expected (Fig. 10A). Interestingly, ethanol levels in adipose tissues were also slightly higher in Aldh2\(^{-}\) mice than in that WT mice (Fig. 10A). Despite higher levels of acetaldehyde, Aldh2\(^{-}\) mice showed similar CLS formation, TUNEL\(^{+}\) cells, and caspase 3/7 activity (Fig. 10B, C). RT-qPCR analyses revealed expression of several ER stress-related genes in adipose tissues was higher in Aldh2\(^{-}\) mice compared to WT controls, but the expression of cell death- and lipolysis-related genes tended to increase but was not statistically significant (Fig. 10D).

**Ethanol directly induces adipocytes death and ER stress in cultured adipocytes**

The above data revealed that a high concentration of ethanol but not acetaldehyde correlates with greater adipocyte death in vivo, suggesting ethanol but not acetaldehyde induces adipocyte death. To investigate the effects of ethanol on adipocyte injury in the in vitro setting, we analyzed adipocyte death by annexin-V and propidium iodide (PI) staining. As shown in Fig. 11A, ethanol exposure to differentiated adipocyte cell line (3T3-L1) markedly increased annexin-V and PI staining. Furthermore, ethanol treatment induced adipocyte death as evidenced by an elevation of Cyto c ox gene copy, DNA fragmentation, and expression of several apoptosis-related genes (Fig. 11B). To understand the mechanisms by which ethanol induces adipocyte death and lipolysis, we treated the differentiated pre-adipocytes from white adipose tissues with ethanol, and then performed immunoblotting and RT-qPCR analyses for ER stress- and lipolysis-related proteins. As illustrated in Fig. 11C, D, ethanol treatment markedly increased expression of several ER stress- or lipolysis-related proteins and genes in differentiated adipocytes.
Inhibition of adipocyte death ameliorates acute alcohol or acute-on-chronic ethanol-induced liver ER stress and injury

To examine whether adipocyte death induced by binge alcohol gavage contributes to alcohol-induced lipolysis and liver injury, we developed adipocyte-specific Bcl2 transgenic mice (Bcl2AdipoTg) in which anti-apoptotic BCL2 protein was specifically over-expressed in adipose tissue. Bcl2AdipoTg mice had less adipocyte death after ethanol gavage, as evidenced by reduction of CLS formation and TUNEL+ cells, and lower caspase 3/7 activity compared to WT mice (Fig. 12A, B). Less adipocytes death in Bcl2AdipoTg mice correlated with reduced adipocyte lipolysis in these mice, as indicated by downregulated expression of lipolysis-related genes (Fig. 12B) and decreased serum FFA levels (Fig. 12C). Blood ethanol and acetaldehyde levels were comparable between Bcl2AdipoTg and WT mice after a single ethanol gavage (Fig. 12C). We further examined acute alcoholic liver injury in Bcl2AdipoTg and WT mice. Serum ALT levels, hepatic TG levels and hepatic mRNA expression of several lipid metabolism-, ER stress-, and cell death-related genes were lower in Bcl2AdipoTg mice compared to WT mice after ethanol binge (Fig. 12C, D).

Resistance to ethanol-induced adipocytes death in Bcl2AdipoTg mice was also found in an acute-on-chronic ethanol feeding model as demonstrated by the results that Bcl2AdipoTg mice had lower levels of caspase 3/7 activity in adipose tissues than WT mice after ethanol gavage (Fig. 13A). Bcl2AdipoTg mice had lower levels of serum FFAs and reduced expression of several lipolysis-related genes in adipose tissues (Fig. 13B, C). Furthermore, serum ALT levels and hepatic TG levels were lower in Bcl2AdipoTg mice than in WT mice after chronic-plus-binge ethanol feeding (Fig. 13D, E). Hepatic expressions of several ER stress- and DNA damage-related mRNAs/proteins were lower in ethanol-fed Bcl2AdipoTg mice compared to WT mice after chronic-plus-binge ethanol feeding (Fig. 13F, G).

To further explore the effect of adipocyte death on alcohol-induced liver injury, we also used BclxL adipocyte-specific KO mice. After acute-on-chronic ethanol feeding, BclxLAdipoKO mice exhibited increased adipocytes death (higher levels of CLS formation and caspase 3/7 activity in adipose tissue) (Fig. 14A, B) and increased lipolysis (higher expression of lipolysis-related genes in adipose tissue) (Fig. 14C) compared to WT mice. Serum FFA levels were also higher in BclxLAdipoKO mice compared to control (Fig. 14D). Furthermore, BclxLAdipoKO mice had more steatosis, elevated caspase3/7 activity and greater expression of several ER stress-related genes in the liver than WT mice after chronic-plus-binge ethanol feeding (Fig. 14E, F). There were no statistical differences in serum ALT levels between BclxLAdipoKO and WT mice after chronic-plus-binge ethanol feeding (Fig. 14G).
In addition to the oxidative process converting alcohol to acetaldehyde by alcohol metabolizing enzymes, a non-oxidative alcohol metabolism has also been implicated in the pathogenesis of alcohol-induced organ damage via the production of fatty acid ethyl esters (FAEEs) (Fig. 1A). Our data suggest that acetaldehyde does not play a key role in acute liver injury after a single ethanol binge, so we wondered whether non-oxidative ethanol metabolites, FAEEs, contribute to acute ethanol-induced liver injury. We evaluated serum FAEE levels using gas chromatography with a flame ionization detector (GC-FID), as previously described. After acute alcohol administration, a significant increase in serum FAEEs was observed compared to those in controls (Fig. 1B). Intriguingly, serum FAEE levels were almost 3 times higher in Adh1/− mice than those in WT mice. The major FAEEs were composed of palmitic acid ethyl ester (16:0 ethyl ester), stearic acid ethyl ester (18:0 ethyl ester), and oleic acid ethyl ester (18:1n9 ethyl ester) (Fig. 1C). Similarly, Adh1/− mice fed with HFD for 3 months and a single ethanol binge showed higher levels of FAEEs compared to those in WT mice (Fig. 1C). Taken together, acute alcohol administration leads to an increase in serum FAEEs, and the inhibition of oxidative alcohol metabolism in Adh1/− mice results in an increase in the production of non-oxidative ethanol metabolites in both chow- and HFD-fed mice.

Next, we investigated the hepatic expression of FAEE synthesis associated gene, Ces1d. As illustrated in Fig. 16A, the highest expression of Ces1d was detected in the liver compared to other organs. In the liver, Ces1d was mainly detected in hepatocytes, while its expression was detected at very low levels in other non-hepatic parenchymal cells. To examine the role of Ces1d in acute alcohol-induced liver injury, Ces1d/− and WT mice were subjected to a single ethanol binge. Serum levels of ethanol and acetaldehyde were comparable between Ces1d/− and WT mice, while the levels of serum FAEEs were much lower in Ces1d/− mice than in WT mice after acute ethanol gavage (Fig. 16B). A small reduction in serum ALT levels was observed in ethanol-fed Ces1d/− mice compared to ethanol-fed WT mice, which reached a statistical difference when a large number of mice were used (Fig. 16C). In addition, hepatic expression of several ER stress-mediated proteins was lower in Ces1d/− mice than in WT mice (Fig. 16D). Carboxylester lipase (Cel) is another important FAEE synthesis enzyme to catalyze FAEE synthesis, however, Cel mRNA was mainly expressed in the pancreas and hardly detected in the liver. Here we also found the CEL protein expression was detected at high levels in the pancreas but not in the liver tissues (Fig. 16E). Deletion of the Cel gene did not attenuate acute alcohol-induced liver injury (Fig. 16F). Taken together, Ces1d is highly expressed in hepatocytes and partially contributes to acute alcohol-induced liver injury via FAEE production, while carboxyl ester lipase CEL does not significantly contribute to acute alcohol-induced liver injury.
In the current study, we extensively studied the mechanisms of ethanol binge-induced liver injury by using various lines of genetically modified mice. Several interesting findings are observed. First, ethanol, not acetaldehyde, directly contributes to acute alcohol-induced liver injury by inducing ER stress and fatty acid synthesis in the liver. Second, ethanol induces adipocyte death and lipolysis, resulting in an elevation in free fatty acids and liver injury. Third, FAEEs, non-oxidative ethanol metabolites, contribute to acute liver injury in our ethanol binge model. Our working model of acute alcoholic liver injury is depicted in the graphical abstract.

In humans, the development of ALD is associated with the quantity and duration of alcohol consumed. Liver is the major organ which metabolizes more than 90% of ingested alcohol into acetaldehyde through several enzymatic mechanisms, although our recent studies suggest that the liver ALDH2 is responsible for less than 50% blood acetaldehyde clearance. Several studies have shown that acetaldehyde is one of the principal culprits mediating chronic alcoholic liver injury and fibrogenic effects. An elevated level of acetaldehyde during chronic alcohol consumption impairs cellular function by forming adducts with DNA and proteins. However, the role of acetaldehyde in mediating liver injury induced by binge ethanol is not known. Binge drinking leads to a transient increase and a rapid decline in the levels of acetaldehyde due to its very short half-life, approximately 18-31 minutes.

To explore the role of ethanol and acetaldehyde in acute alcoholic liver injury, we employed Adh1−/− and Aldh2−/− mouse models in which ethanol metabolism is markedly inhibited. Interestingly, we found that acute alcoholic liver injury was markedly exacerbated in mice lacking the key ethanol metabolism enzyme ADH1, as indicated by an increase in serum alcohol concentration, serum transaminases, hepatic ER stress, and hepatocyte apoptosis after acute ethanol gavage. These results suggest that liver injury in an ethanol binge model is likely acetaldehyde independent. To further test this hypothesis, we conducted experiments in mice lacking Aldh2, an oxidative enzyme converting acetaldehyde to acetate. Despite an increase in blood acetaldehyde after ethanol binge, Aldh2−/− mice had a similar liver injury (serum ALT levels) as WT mice. Given the combination of binge drinking and the presence of metabolic syndrome as a major risk of ALD, We also determined the implications of ethanol and acetaldehyde in liver injury using the HFD+binge model in Adh1−/− and Aldh2−/− mice. We again observed enhanced liver injury and activation of hepatic ER stress pathway in Adh1−/− mice challenged with HFD+binge compared to WT mice, whereas the lack of Aldh2 did not affect liver injury in this model. Taken together, our results suggest that the mechanism underlying liver injury induced by binge ethanol intake is acetaldehyde independent and directly related to ethanol. This notion was further confirmed in our experiments when we found that intra-peritoneal administration of ethanol, but not acetaldehyde, induces liver injury, ER stress, and hepatocyte death.
analyzed our transcriptomic data in the liver of mice receiving ethanol binge at 2 doses (5 g/kg and 7 g/kg). Our analyses revealed that a single ethanol binge significantly upregulated hepatic expression of genes related to fatty acid synthesis and inflammatory response but downregulated the genes associated with fatty acid oxidation. In addition to de novo lipogenesis within the hepatocytes, adipose tissue lipolysis is another important source of hepatic fatty acids.\textsuperscript{17,33} Alcohol disturbs adipose tissue functions by mediating adipocyte death, altering secretion of pro-inflammatory mediators, and release of free fatty acids.\textsuperscript{34} We have previously demonstrated that adipocyte death of epididymal fat promotes liver injury by stimulating adipose tissue lipolysis, inflammation, and free fatty acid release.\textsuperscript{30} To conclusively address the role of adipocyte death in inducing liver injury in our ethanol binge model, we performed experiments in adipocyte specific Bcl-2 transgenic mice (\textit{Bcl2}\textsuperscript{AdipoTg}), a model using the anti-apoptotic BCL2 protein to inhibit adipocyte death. We found that the inhibition of adipocyte death ameliorates acute liver injury and hepatic ER stress in our model, suggesting that an increase in lipolysis secondary to adipocyte death is a driver of acute liver injury in a single ethanol binge model. Furthermore, we provided several lines of evidence suggesting that ethanol, not acetaldehyde, contributes to adipocyte death after acute ethanol gavage. First, we found a high concentration of alcohol in white adipose tissues, which is in the comparable amount of that in the liver after ethanol gavage. The levels of acetaldehyde, however, were much lower in adipose tissues likely reflecting the lower amount of ADH1 when compared to that in the liver.\textsuperscript{35} Second, in our model, a single ethanol binge induced ER stress and adipocyte death, and activated adipose tissue lipolysis, together likely caused an increase in circulating free fatty acids. These observations were augmented in \textit{Adh1}\textsuperscript{−/−} mice, in which we found high levels of ethanol in adipose tissues, but not in \textit{Aldh2}\textsuperscript{−/−} mice despite high levels of acetaldehyde in these mice. Finally, incubation with ethanol directly induced adipocyte apoptosis \textit{in vitro}.

In addition to the oxidative metabolism, the liver uses a non-oxidative pathway to metabolize ethanol by combining ethanol with free fatty acids to produce lipophilic fatty acid ethyl esters (FAEEs).\textsuperscript{12} The generation of FAEEs occurs via diverse FAEE synthase enzymes, such as FAEE carboxylesterase (encoded by \textit{Ces1d} in murine and CES1 in humans) and carboxylester lipase (\textit{Cel}).\textsuperscript{13-15} The distribution of these carboxylases is tissue-specific. The \textit{Ces1d} gene is highly expressed in the liver, while \textit{Cel} is primarily located in the pancreas.\textsuperscript{12,36} FAEEs are important mediators of ethanol-induced organ damage, such as alcohol-induced cardiomyopathy and alcohol-induced pancreatitis.\textsuperscript{31,37,38} We found a significant increase in the level of circulating FAEEs, such as palmitic ethyl ester, stearic ethyl ester, and oleic ethyl ester, in mice receiving a single ethanol binge. Their levels increased further in \textit{Adh1}\textsuperscript{−/−} mice when ethanol oxidative metabolism is inhibited. Furthermore, deletion of \textit{Ces1d} markedly reduced serum levels of FAEEs and hepatic ER stress and a reduction of serum ALT levels after a single ethanol binge. On the other hand, deletion of the \textit{Cel}, a gene primarily expressed in the
an important role of hepatic FAEEs in mediating liver injury via activation of ER stress in a single ethanol binge model.

Clinical and mechanistic implications of this study: While it is believed that binge drinking exacerbates ALD, the human and animal data supporting this notion is limited. An increase in the levels of serum transaminases is reported among patients with alcohol intoxication, suggesting the possibility of acute liver injury in these patients. In a large cohort of more than 6,000 patients without baseline liver disease, binge drinking frequency is directly associated with ALD and its association is more pronounced in patients with metabolic syndrome. The mechanism underlying these findings in human studies remains obscured. Our findings that ethanol, not acetaldehyde, is the key driver of acute liver injury in our model deserve further discussion, especially in the context of allelic variants of ADH and ALDH and their enzymatic activities to metabolize ethanol. It was reported that serum ALT levels were much lower in alcohol-intoxicated patients with ALDH2*1/2 (low activity) than those with ALDH2*1/1 (full activity). Whether patients with a genetic variant leading to low ADH enzymatic activity are more susceptible for acute liver injury from binge drinking should be further explored. In summary, in a single ethanol binge model, we found that ethanol and its non-oxidative metabolites (FAEEs), not acetaldehyde, promote acute alcohol-induced liver injury by inducing ER stress, adipocyte death, and lipolysis.
**Animal experiments**

The following lines of genetically modified mice were used in this study: Adh1\(^{+/−}\), Aldh2\(^{+/−}\), Chop\(^{+/−}\), Cyp2e1\(^{+/−}\), Bcl2\(^{AdipoTG}\), BclxL\(^{AdipoKO}\) and Ces1d\(^{+/−}\) mice. In detail, Adh1\(^{+/−}\) and Aldh2\(^{+/−}\) mice and their wild-type controls were previously described.\(^{41,42}\) Chop\(^{+/−}\) mice were purchased from the Jax Laboratory (Bar Harbor, Maine, USA). Cyp2e1\(^{+/−}\) mice were kindly provided by Dr. BJ Song (NIAAA) and backcrossed to a C57B/6N background for more than 10 generations. Cre-inducible Bcl2 transgenic mice were previously described\(^{43}\) and were used to generate adipocyte-specific Bcl2 transgenic mice (Bcl2\(^{AdipoTG}\)) with Bcl2 overexpression by crossing with AdipoCre mice (Jax laboratory). Bcl-xL floxed mice were kindly provided by Dr. You-Wen He (University of North Carolina) and were used to generate adipocyte-specific Bcl-xL KO mice by crossing with AdipoCre mice (Jax laboratory). Ces1d\(^{+/−}\) mice on a C57BL/6 background were previously described\(^{44}\), heterozygous Ces1d\(^{+/−}\) mice were crossed to generate Ces1d\(^{+/−}\) mice and their littermate wild-type controls. Unless noted, male mice were used for all experiments. Mice were housed in polycarbonate cages (maximum 4 mice per cage) and maintained in a temperature and light controlled facility (12h:12h light-dark cycle) under standard food and water *ad libitum*. For the induction of acute alcoholic liver injury, ethanol (5 to 7 g/kg) was administered orally or intra-peritoneally and euthanized at different time points. After the administration of ethanol, heating pad (38°C) was placed under the cage to provide thermal support and minimize body heat loss. In some experiments, mice were subjected to “acute-on-chronic ethanol feeding” and “HFD-plus-binge ethanol feeding” (denotes as E10d+1B and 3M HFD+binge, respectively) using the models, as described in previous studies\(^{45,46}\). Male mice (6-7 weeks of age) were also fed on high-fat diet (HFD) (60% kcal fat; D12492; Research Diets, New Brunswick, NJ) or a chow diet (10% kcal fat) for 3 months. In some experiments, ER stress inhibitors (PBA, cat.8209860025, 1 mg/kg; TUDCA, cat.580221, 500 mg/kg) (Merck-Millipore, Billerica, MA), JNK inhibitor II (cat.420119, 20 mg/kg) (Merck-Millipore, Billerica, MA), ethanol (6 g/kg) or acetaldehyde (50 mg/kg) was administered intra-peritoneally. All animals were received humane care in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. All animal experiments were approved by the NIAAA Animal Care and Use Committee.

**Histological analysis**

The liver and perigonadal adipose tissues were fixed with 10% neutral buffered formalin. After deparaffinization and rehydration, paraffin sections with 4 μm thickness were stained with hematoxylin and eosin (H&E) and Sirius Red. Terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining was performed with an ApopTag® Peroxidase *In Situ* Apoptosis Detection Kit (S7101, Merck-Millipore, Billerica, MA). Paraffin-embedded liver and adipose
3% H$_2$O$_2$ for 30 minutes and non-specific binding was further blocked with 3% normal goat serum for 1 hour. Tissues were incubated with primary antibody overnight at 4°C and incubated with ABC staining kit (PK-4001, rabbit IgG, Vector Laboratories, Inc., Burlingame, CA). Reactions were developed with DAB peroxidase substrate (SK-4105, Vector Laboratories, Inc., Burlingame, CA), according to the manufacturer’s instruction and then slides were counterstained with hematoxylin.

**RT-qPCR**

For RT-qPCR assays, total RNA was isolated from tissues or cells with TRIzol reagent (Thermo Fisher Scientific). Total RNA was obtained and reverse-transcribed into cDNA using High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific), according to manufacturer’s instructions. RT-qPCR was performed by SYBR Green Real time PCR master mix. The mRNA expression levels of the target genes were normalized to β-actin mRNA expression. The mouse primer sequences are shown in Table 1 and were obtained from Thermo Fisher Scientific.

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**Western blot analysis**
Tissues or cell lysates were homogenized in RIPA lysis buffer at 4°C and centrifuged at 10,000 g for 10 minutes. After tissue homogenization, the supernatants were mixed with loading buffer and subjected to SDS-PAGE and the lysates were subjected to 4-12% Tri-glycine gradient gels (Criterion XT, BioRad). The extracted proteins were transferred onto nitrocellulose membranes. Membranes were blocked in 5% milk, incubated with primary antibodies at 1:1,000 in PBST. Protein bands were visualized by SuperSignal West Femto Maximum Sensitivity Substrate (#34096; Thermo Fisher Scientific). Comparative amounts were normalized with β-actin.

**Antibodies**

Primary antibodies specific for following proteins were used. For western blot analysis, antibodies against ADH1 (#5295s; CST), ALDH2 (ab194587; Abcam), CYP2E1 (AB1252; Sigma), p-SAPK/JNK (Thr183/Tyr185) (#4668s; CST), SAPK/JNK (#9258; CST), p-PERK (Thr982) (#3179s; CST), PERK (C33E10) (#3192; CST), p-elf2α (Ser51) (#3398; CST), elf2α (#5324; CST), p-IRE1α (phospho-S724) (ab48187; Abcam), IRE1α (14C10) (#3294; CST), XBP-1s (E9V3E) (#40435; CST), CHOP (#5554s; CST), BAD (D24A9) (#9239; CST), BIM (C34C5) (#2933; CST), BiP (#3177s; CST), p-histone H2A.X (Ser139) (#9718; CST), HIF-1α (D2U3T) (#14179; CST), ATF6 (D4Z8V) (#65880; CST), FASN (#3189; CST), SCD1 (C12H5) (#2794; CST), ATGL (#2138; CST), Ero1-Lα (#3264; CST), p-PKA c (Thr197) (#4781; CST), PKA c (#4782; CST), p-HSL (Ser660) (#4126; CST), HSL (#4107; CST), p-STAT3 (Tyr705) (#9145; CST), STAT3 (#30835; CST) were used. Pre-diluted rabbit anti-F4/80 (CST) antibody was used for immunohistochemistry. Secondary antibodies for western blot analyses were HRP-linked anti-rabbit IgG (#7074; CST) and HRP-linked anti-mouse IgG (#7076; CST) at 1:5,000 dilutions.

**Serum biochemical analyses**

Serum was collected for the evaluation of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) using VetTest Chemistry analyzer (IDEXX Laboratories), according to the manufacturer’s instructions. Levels of serum ethanol, acetaldehyde, and hepatic free fatty acids were evaluated using gas chromatography-mass spectrometry (GC-MS). Serum free fatty acids (FFAs) levels were measured with a commercial kit from the BioVision (#K612; Milpitas, CA), according to the manufacturer’s instruction. The results were obtained using Infinite M200 Pro microplate reader (Tecan Group Ltd.), equipped at Ewha Drug Development Research Core Center.

**FAEE identification and quantification**

A quantitative internal standard of ethyl heptadecanoate (E17:0) was added to the collected serum samples, and the lipids were extracted with acetone/hexane. Then, the FAEEs were isolated by solid phase extraction using aminopropyl column (1 ml/100 mg, Thermo Fisher Scientific), as described
chromatography/flame ionization detector (GC/FID).

**Glucose tolerance test (GTT) and insulin tolerance test (ITT)**

Animals were fasted overnight for GTT and 6 hours for ITT. Blood was collected through tail vein at 0, 15, 30, 45, 60, and 120 minutes after intraperitoneal injection of glucose (0.75 g/kg) or insulin (1.5 IU/kg). The levels of glucose were measured by a digital glucometer.

**Immunofluorescence staining**

The cultured cells were stained with Annexin V/PI double staining kit (#101-100, Biovision) to detect cell death, according to manufacturer’s instruction. The images were obtained by using LSM710 confocal microscope (Zeiss, Thornwood, NY).

**Cell death assay**

DNA fragmentation was measured with a cell death detection kit (#11544675001; Sigma-Aldrich). Samples were prepared and measured according to the manufacturer’s instruction. Caspase-3/7 activities in the adipose tissue samples were measured with a commercial kit (#G7791, Promega, Madison, WI, USA), according to the manufacturer’s instruction.

**Isolation of stromal vascular fraction (SVF) cells**

Perigonadal adipose tissues were harvested from mice and digested with collagenase A (#10103578001, Roche). Adipocytes and preadipocytes were separated by centrifuge (1500 rpm, 5 min) and preadipocytes was resuspended for adipocyte differentiation.

**Cell culture**

Cells were cultured in the DMEM supplemented with 10% FBS and penicillin/streptomycin solution (#15140122, Gibco) and maintained in a humidified incubator at 37°C and 5% CO₂. After reaching confluency, 3T3-L1 or SVF cell were cultured with 10% FBS, 0.125 mM Indomethacin, 1 μM Dexamethasone, 0.5 mM IBMX, 0.02 μM insulin, 1 μM Rosiglitazone to induce the differentiation for 2 days. The media were then replaced with 10% FBS, insulin, and Rosiglitazone. The media were renewed every 2 to 3 days during cell culture.

**Microarray Analyses of Mouse Liver Samples.** Liver samples from mice with ethanol (5g/kg or 7g/kg) gavage or maltose (control) were subjected to microarray analysis. Dye-coupled cDNAs were isolated by using a MiniElute PCR purification kit (Qiagen) and hybridized to an Agilent 44K mouse 60-mer oligo microarray (Agilent Technologies, Santa Clara, CA). The Genespring GX software package
control and 5g/kg ethanol groups were previously published and deposited in NCBI’s Gene Expression Omnibus (GEO accession number: GSE98153). The liver tissue microarray data from 7g/kg ethanol gavaged mice are deposed in NCBI’s Gene Expression Omnibus (GSE214778). Differential expression analysis was performed via the R package DESeq2 (v1.30.1). Genes with fold change >1.5 and padj <0.05 were put into gene set enrichment analysis via the R package cluster Profiler (v3.18.1). The R package heatmap (v1.0.12) was used to generate the heatmap plots. Interactive Venn diagrams and gene function analyses were processed by Ingenuity Pathway Analysis (IPA).

**Statistical analysis**

Data are presented as means ± SEM (n=6-15 per group). Statistical analysis was performed using the 2-tailed Student t-test or one-way analysis of variance. All P<0.05 values were considered statistically significant.
Figure 1. Binge alcohol induces acute liver injury by activating ER stress. C57BL/6N mice (8-10 weeks; male) were acutely gavaged with different doses of ethanol (5-7 g/kg). After ethanol gavage, mice were euthanized at different time points. (A) Serum ALT and AST levels were assessed. (B) Serum ALT levels in C57BL/6N and C57BL/6J mice were measured following an acute ethanol gavage (7 g/kg ethanol). (C, D) Liver tissues were subjected to Western blot (panel C) and RT-qPCR analysis (panel D). (E) Serum ALT levels were determined after a single alcohol gavage (7 g/kg) was administered to WT and Chop−/− mice (9 hours post alcohol gavage). (F) C57BL/6N mice were administered via intraperitoneal injection of JNK inhibitor II or ER stress inhibitors, PBA, and TUDCA, 15 minutes before 7 g/kg ethanol gavage. Serum ALT levels were measured. Data are expressed as the mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 versus the corresponding controls.

Figure 2. Deletion of the Adh1 gene exacerbates acute alcoholic liver injury and ER stress-mediated hepatocyte death. Chow-fed WT and Adh1−/− mice were given a single ethanol binge. Mice were euthanized at different time points post-gavage. (A) Serum ethanol, acetaldehyde, and ALT levels were evaluated. (B) Liver tissue sections were subjected to TUNEL staining. Representative images are shown. (C) Western blot analyses were performed on liver tissue lysates, and the density of bands was quantified. (D) Liver tissues were subjected to RT-qPCR analysis. Data are expressed as the mean ± SEM. *P < 0.05, **P < 0.01 versus the corresponding controls.

Figure 3. HFD-fed Adh1−/− mice are more susceptible to acute alcoholic liver injury and ER stress. (A) Adh1−/− and WT mice were fed an HFD for three months without ethanol binge. Body weight change was evaluated. Glucose tolerance test (GTT; 0.75 g/kg glucose i.p. injection) and insulin tolerance test (ITT; 1.5 IU/kg insulin i.p. injection) were performed. (B-D) Adh1−/− and WT mice were fed an HFD for three months followed by receiving a single alcohol gavage (5 g/kg) or isocaloric maltose. Mice were euthanized 9 hours after acute alcohol administration. Serum ethanol, acetaldehyde, and ALT levels were measured (panel B). H&E, Sirius Red, and TUNEL staining were performed on liver tissue sections (panel C). Liver tissue lysates were subjected to RT-qPCR analysis (panel D). (E) Freshly isolated hepatocytes (WT and Adh1 deficient hepatocytes) were treated with palmitic acid (200 μM) and ethanol (100 mM) for 12 hours, followed by performing RT-qPCR. Data are expressed as the mean ± SEM. *P < 0.05, **P < 0.01 versus the corresponding controls.
WT mice were subjected to ethanol gavage (4g/kg of ethanol) and euthanized 3 hours post gavage. Blood ethanol and acetaldehyde levels were measured. In addition, Aldh2\(^{-/-}\) and WT mice were subjected to ethanol gavage (various doses of ethanol) and euthanized 9 hours post gavage, followed by measuring serum ALT levels. (B, C) Aldh2\(^{-/-}\) and WT mice were subjected to ethanol gavage (6g/kg of ethanol) and euthanized 9 hours post gavage. RT-qPCR and western blot analyses were performed on liver tissue lysates. Data are expressed as the mean ± SEM. **\(P < 0.01\) versus the corresponding controls.

Figure 5. Deletion of the Aldh2 gene does not exacerbate the acute alcoholic liver injury, obesity, and insulin resistance. (A) Aldh2\(^{-/-}\) and WT mice were fed an HFD for three months without ethanol gavage. Body weight change, glucose tolerance test (GTT; 0.75 g/kg glucose i.p injection) and insulin tolerance test (ITT; 1.5 IU/kg insulin i.p. injection) were evaluated. (B, C) Aldh2\(^{-/-}\) and WT mice were fed HFD for three months followed by giving a single dose of ethanol (5 g/kg) or isocaloric maltose, and the mice were euthanized nine hours later. Serum acetaldehyde and ALT levels were assessed (panel B). Liver tissue lysates were subjected to RT-qPCR analysis (panel C). Data are expressed as the mean ± SEM. **\(P < 0.01\) versus the corresponding controls.

Figure 6. Intraperitoneal administration of ethanol, not acetaldehyde, induces acute liver injury and hepatic ER stress. (A-C) C57BL/6N mice were intraperitoneally administrated with 6 g/kg ethanol and were euthanized 9 hours post-injection. Serum samples ethanol and ALT levels were measured (panel A). Liver tissue lysates at different time points were subjected to RT-qPCR analyses (panel B). Serum and liver tissues from WT and Adh1\(^{-/-}\) mice were tested for ALT, ethanol, and acetaldehyde levels, as well as hepatic mRNA expression (panel C). (D) C57BL/6N mice were injected intraperitoneally with acetaldehyde (50 mg/kg) and euthanized 9 hours later. Serum acetaldehyde and ALT levels were determined. Data are expressed as the mean ± SEM. *\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\) versus the corresponding controls.

Figure 7. Binge ethanol causes an increased lipid synthesis and a decrease in beta-oxidation. (A) Z-score hierarchical clustering heat map visualization changes were shown. The colors reflect scaled expression levels, with blue representing low expression and red representing high expression. (B) Predicted upregulation/downregulation of top lipid metabolism functions using bio-functional analysis (control vs. 1B7g). (C) IPA analysis was used to show the top 20 predicted pathways significantly different from ethanol binge 5g/kg vs. ethanol binge 7g/kg.
Figure 8. Binge ethanol elevates hepatic free fatty acids and increases adipose tissue lipolysis. Mice were subjected to a single ethanol binge (5 or 7 g/kg) or isocaloric maltose and were euthanized at 9 hours after alcohol gavage. (A) Liver tissues were subjected to microarray analyses. The heat-map and IPA analyses for the liver mRNA expression are shown. (B) Sera were collected for FFA measurement. Liver FFA and TG levels were measured. (C) Gas chromatography-mass spectrometry was used to perform specific FFA studies on the liver tissues. Hepatic FASN and SCD1 proteins were analyzed using Western blots. (D) Collected white adipose tissues were analyzed using Western blot. Data are expressed as the mean ± SEM. *P < 0.05, **P < 0.01 versus the corresponding controls.

Figure 9. Binge ethanol induces adipocyte death and ER stress in adipose tissues with greater induction in Adh1⁻/⁻ mice than in WT mice. (A-C) C57BL/6N mice were treated with a single ethanol binge (7 g/kg) or isocaloric maltose and euthanized at various time points post alcohol gavage. Liver and white adipose tissues were collected for the following analyses. Ethanol and acetaldehyde concentrations in liver and white adipose tissues (epididymal fat and subcutaneous fat) were determined (panel A). Representative staining images of H&E, TUNEL, and F4/80 staining of epididymal fat tissues 9 hours post ethanol gavage (panel B). Caspase 3/7 activity assays, and RT-qPCR analysis of ER stress and lipolysis-associated genes in epididymal fat tissues 9 hours post ethanol gavage were performed (panel C). (D-F) Adh1⁻/⁻ and WT mice were subjected to 7 g/kg alcohol gavage and euthanized 9 hours post alcohol administration. H&E and TUNEL staining were performed on sections of white adipose tissue (panel D). Ethanol, acetaldehyde, and Caspase3/7 activity were measured in white adipose tissues (panel E). Serum FFA levels were assessed (panel E). Adipose tissues were subjected to RT-qPCR analyses (panel F). Data are expressed as the mean ± SEM. *P < 0.05, **P < 0.01 versus the corresponding controls.

Figure 10. Ablation of the Aldh2 gene does not affect acute alcohol-induced adipocyte death. Aldh2⁻⁻ and WT mice were subjected to 6 g/kg single alcohol gavage and euthanized 9 hours post alcohol administration. (A) White adipose tissue was subjected to evaluate ethanol and acetaldehyde levels. (B) Representative staining images of H&E, and TUNEL staining of white adipose tissue. (C) Caspase 3/7 activity in epididymal fat was assessed. (D) White adipose tissues were subjected to RT-qPCR analysis. Data are expressed as the mean ± SEM. *P < 0.05 versus the corresponding controls.
Differentiated 3T3-L1 cells were treated with 100 mM ethanol. Representative images for Annexin-V and PI staining to evaluate cell death (Panel A). The copy number of mitochondrial cyto c ox levels from total DNA extraction and DNA fragmentation were evaluated, and the mRNA levels of cell-death-associated genes were determined by RT-qPCR analysis (panel B). (C-D) Isolated stromal vascular fractions (SVF) from mouse white adipose tissue were differentiated and treated with ethanol (100 mM) for 24 hours. Cultured cells were collected and analyzed by using Western blotting (panel C), and mRNA expression was evaluated using RT-qPCR (panel D). Data are expressed as the mean ± SEM. *P < 0.05, **P < 0.001 versus the corresponding controls.

**Figure 12.** Bcl2AdipoTG mice are resistant to acute ethanol-induced adipocyte death and liver injury. Bcl2AdipoTG mice and littermate control mice were subjected to binge alcohol gavage (7 g/kg) and euthanized 9 hours post alcohol administration. (A) Representative staining images of H&E, and TUNEL staining on white adipose tissues. (B) Caspase3/7 activity and RT-qPCR analysis were conducted on white adipose tissues. (C) Serum ethanol, acetaldehyde, FFA, and hepatic TG levels were assessed. (D) RT-qPCR study of liver tissue lysates. Data are expressed as the mean ± SEM. *P < 0.05, **P < 0.01 versus the corresponding controls.

**Figure 13.** Bcl2AdipoTG mice are resistant to chronic-plus-binge ethanol-induced adipocyte death, lipolysis and hepatic ER stress. Bcl2AdipoTG mice and their littermate control mice were subjected to E10d+1B ethanol feeding. Adipose and liver tissues were collected 9 hours post binge. (A-C) White adipose tissues were collected for the measurement of caspase 3/7 activity (panel A), serum FFA levels (panel B), RT-qPCR analyses (panel C). (D) Sera were collected for the measurement of ALT levels. (E-G) Liver tissues were collected for the measurement of liver TG levels (panel E), RT-qPCR analyses of ER stress-related genes (panel F), and western blot analysis (panel G). Data are expressed as the mean ± SEM. *P < 0.05, **P < 0.01 versus the corresponding controls.

**Figure 14.** Deletion of the BclxL gene in adipocytes enhances lipolysis and liver TG synthesis after chronic-plus-binge ethanol feeding. BclxLA adipokO mice and their littermate control mice were subjected to E10d+1B ethanol feeding. Mice were euthanized nine hours post the gavage for analyses. (A-C) White adipose tissues were subjected to H&E staining (panel A), the measurement of caspase 3/7 activity (panel B), and RT-qPCR analysis (panel C). (D) Sera were collected for the measurement of FFA levels (panel D). (E-F) Liver tissues were collected for H&E staining (panel E), the measurement
collected for the measurement of ALT levels. Data are expressed as the mean ± SEM. *P < 0.05, **P < 0.001 versus the corresponding controls.

**Figure 15. Binge ethanol promotes the production of FAEEs in WT and Adh1−/− mice.** (A) A schematic overview of oxidative and non-oxidative alcohol metabolism. (B) Serum FAEEs were extracted using an aminopropyl column in solid-phase extraction, and FAEEs were detected using gas chromatography/flame ionization detection methods. The GLC-462 EE reference standards were used. (C) Levels of serum FAEEs were evaluated using serum samples from acute alcohol binge (7 g/kg) and 3M HFD-plus-binge alcohol (5 g/kg). Data are expressed as the mean ± SEM. *P < 0.05, **P < 0.001 versus the corresponding controls.

**Figure 16. Deletion of the Ces1d gene reduces acute ethanol-induced serum FAEE and ALT elevation.** (A) RT-qPCR analysis of Ces1d mRNA in multiple organs and cells in the liver from C57BL/6 mice. (B-D) Ces1d−/− mice and littermate control mice were administrated by alcohol gavage (7 g/kg) and euthanized 9 hours post alcohol administration. Serum ethanol, acetaldehyde and FAEE levels were evaluated (panel B). Serum ALT levels were evaluated (panel C). Protein expression was assessed by Western blotting on liver tissue lysates (panel D). (E-F) Cel−/− mice and littermate control mice were administrated by alcohol gavage (7 g/kg) and euthanized 9 hours post alcohol administration. CEL protein expression in the liver and pancreas was evaluated (panel E). Serum ALT levels were measured (panel F). Data are expressed as the mean ± SEM. *P < 0.05, **P < 0.001 versus the corresponding controls.

2. Teli MR, Day CP, Burt AD, Bennett MK, James OF. Determinants of progression to cirrhosis or fibrosis in pure alcoholic fatty liver. Lancet 1995;346:987-990.


4. Fillmore MT, Jude R. Defining “binge” drinking as five drinks per occasion or drinking to a .08% BAC: which is more sensitive to risk? Am J Addict 2011;20:468-75.


phenotypes of severe alcoholic hepatitis suggest different mechanisms driving injury and failure. J Clin Invest 2022;132.


Schematic overview ethanol metabolism

Oxidative alcohol metabolism

Acetaldehyde

Alcohol dehydrogenase

MEOS

Non-oxidative alcohol metabolism

Ethanol

Fatty acids

Fatty acid ethyl esters

FAEE synthase

CH₃CH₂OH

B

Gas chromatography FID peak height (pA)

Retention time (min)

Control serum (PBS)

WT serum (1B7g: 9h)

Adh1⁻⁻ serum (1B7g: 9h)

GLC-462 EE standard

C

Single alcohol gavage (1B7g: 9h)

Non-Tx WT

1B7g WT

1B7g Adh1⁻⁻

Serum FAEEs (µg/ml)

HFD plus binge ethanol (3M HFD+1B5g)

3M HFD only

3M HFD+1B (5 g/kg) WT

3M HFD+1B (5 g/kg) Adh1⁻⁻

FAEE 16:0 EE

Palmityl ethyl ester

FAEE 18:0 EE

Stearic ethyl ester

FAEE 18:1n9 EE

Oleic ethyl ester

Total FAEEs