

Hepatic NPC1L1 overexpression attenuates alcoholic autophagy in mice

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Abstract. Alcohol consumption causes liver steatosis in humans. Metabolic disorders of lipids are one of the factors that cause liver steatosis in hepatocytes. Hepatic Niemann-Pick C1-like 1 (NPC1L1) regulates lipid homeostasis in mammals. The relationship between NPC1L1 and autophagy in those with a history of alcohol abuse is unclear. The present study aimed to investigate the function of NPC1L1 in the activation of hepatic autophagy in a mouse model with a human (h)NPC1L1 transgene under alcohol feeding conditions. The mice expressing hNPC1L1 (Ad-L1) or controls (Ad-null) were created by retro-orbital adenovirus injection. The Ad-L1 and Ad-null mice were fed with alcohol or a non-alcoholic diet to mimic chronic alcohol consumption in humans. Hepatic autophagy was demonstrated in isolated primary hepatocytes by monitoring autophagic vacuoles under fluorescence microscopy, and by western blotting for autophagic makers. Isolated hepatocytes from the livers of Ad-L1 mice were treated with different doses of ezetimibe to study the restoration of autophagy. Chronic alcohol feeding caused liver injury and steatosis, shown by significantly higher levels of plasma alanine transaminase and aspartate transaminase activity, and by hematoxylin and eosin staining in Ad-L1 and Ad-null mice. Compared to Ad-null control mice, the microtubule-associated proteins 1A/1B light chain 3 (LC3) particles in the isolated hepatocytes of Ad-L1 mice were decreased, both under alcohol

and non-alcoholic feeding. The ratio of LC3II/LC3I was significantly decreased, and the level of p62/sequestosome-1 protein was significantly increased in Ad-L1 mice compared with Ad-null mice after alcohol feeding. Levels of LC3II protein were statistically increased in hepatocytes isolated from Ad-L1 mice with ezetimibe treatment. The increase in LC3II expression was dose dependent. Within the tested range, it reached its highest level at 40 μ M. The livers of Ad-L1 mice represent a more human-like state for the study of hepatic autophagy. Hepatic expression of human NPC1L1 resulted in an inhibition of autophagy; it may contribute to alcoholic fatty liver disease in humans.

Introduction

Those with a history of chronic alcohol abuse are at a higher risk of developing alcoholic fatty liver disease (AFLD), cirrhosis and, ultimately, liver failure (1). AFLD is represented by abnormal hepatic lipid accumulation in the early stages and steatohepatitis in the latter stages (1,2). Lipids storage in lipid droplets is influenced by synthesis and degradation in the liver (3). Therefore, an increase in lipid degradation in lipid droplets via autophagy or lipolysis could ameliorate AFLD (4,5).

Autophagy is a lysosome-dependent degradation process of intracellular organelles in mammalian cells. It is activated during stressful periods, such as energy deficiency (6), and is also influenced by cellular cholesterol levels and alcohol intake (6-8). The relationship between alcoholic beverages and autophagy is complex (9). Autophagy ameliorates liver steatosis *in vivo* (5,10,11).

Alterations in cholesterol levels in cells can regulate autophagy (8,12). By extension, the expression level of the cholesterol transporter Niemann-Pick C1-like 1 (NPC1L1) in the liver may also influence autophagy. NPC1L1 is a transmembrane cholesterol absorption transporter expressed in the liver and intestines in humans (13). The function of

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hepatic NPC1L1 is to prevent excessive cholesterol loss in humans (14,15). By contrast, mouse NPC1L1 is expressed only in the intestines (16). The expression of human NPC1L1 in the mouse liver results in an elevation of the total plasma cholesterol level, and hepatic triglyceride secretion (17,18). The hepatic cholesterol level is increased in the livers of apolipoprotein E-knockout mice with NPC1L1 overexpression (19). Furthermore, inhibition of NPC1L1 by ezetimibe can significantly activate autophagy in human hepatocytes (20). It is indicated that NPC1L1 may inhibit hepatic autophagy. In humans, NPC1L1 may be a potential factor contributing to alcoholic steatosis by reducing autophagy.

The present study assessed the function of NPC1L1 in the activation of hepatic autophagy in a mouse model expressing human NPC1L1 in the liver under conditions of alcohol consumption. It was concluded that NPC1L1 expression reduced hepatic autophagy.

Materials and methods

Generation of recombinant adenoviruses. cDNA of human NPC1L1 was cloned and NPC1L1 adenoviral vectors (Ad-L1) were constructed using the AdMax system (Hanbio Biotechnology Co., Ltd.) and purified through sucrose gradient ultracentrifugation (21). A control adenovirus (Ad-null) without encoded cDNA was also constructed. Adenoviral vectors were administered at a dose of 1×10^{11} particles (10.5×10^9 plaque-forming units) via the retro-orbital vein into the mice. The sequences of the primers used to amplify human (h)NPC1L1 from the mouse liver for reverse transcription-quantitative PCR (RT-qPCR) are listed in Table I.

Animals and feeding. A total of 24 male mice (C57BL/6J, weighting 20–22 g) at the age of 8 weeks (Jackson Laboratory; stock no. 002207) were housed in standard cages at 4 mice per cage. The conditions were 23°C, 50% humidity with a 12 h light/dark cycle and free access to water and a chow diet. A 10 day feeding plan was used to induce alcoholic liver steatosis during NPC1L1 expression (Fig. 1A). At the beginning, the mice were fed on a non-alcoholic liquid diet *ad libitum* (Bio-Serv; product no. F1259SP) for acclimatization. A total of 5 days later, 12 mice were changed to the Lieber-DeCarli alcoholic liquid diet (Bio-Serv; product no. F1258SP; 5 g/kg body weight). The other 12 mice continued to be administered the non-alcoholic diet. After 3 days, Ad-L1 and Ad-null adenoviruses were injected retro-orbitally into 6 mice on the alcohol diet and 6 mice on the non-alcoholic diet. At the end of feeding, mice on the alcoholic diet were euthanized at 9 h after a single binge gavage (20% alcohol). Instead of alcohol, the non-alcohol-fed mice were also euthanized at the same time after receiving a single dose of maltodextrin for energy balance. All mice were euthanized by 5% isoflurane (Hairui Chemical; cat. no. HR135327).

SDS-PAGE and western blot analysis. Liver tissues or primary hepatocytes were homogenized in the RIPA buffer (cat. no. 89900; Thermo Fisher Scientific, Inc.) followed by centrifugation at $12,000 \times g$ for 10 min at 4°C to isolate the proteins for western blotting. Then, the protein concentrations were quantified by BCA protein assay kit (cat. no. 23227; Thermo Fisher

Scientific, Inc.) via absorbance measurement using the LAS-4000 lumino-image analyzer (Fujifilm). Then, 30 μg protein was loaded to each lane of 10% SDS-PAGE gel. Western blotting was performed following protocols described previously (17). Data were normalized against β -actin. The antibodies used in the present study were: Anti-microtubule-associated proteins 1A/1B light chain 3 (LC3) A/B antibody (cat. no. ab128025; Abcam); anti- β -actin antibody (cat. no. ab8227; Abcam); anti-p62/sequestosome-1 (SQSTM1) antibody (cat. no. ab91526; Abcam), anti-hNPC1L1 antibody (cat. no. ab124801; Abcam); anti-pan-cadherin antibody (cat. no. ab6528; Abcam); goat anti-mouse IgG (cat. no. ab6788; Abcam); and goat anti-rabbit IgG (cat. no. ab6720; Abcam). Primary antibodies were diluted as 1:3,000 in 5% BSA for overnight incubation at 4°C. Secondary antibodies were diluted as 1:10,000 in 5% BSA for 1 h incubation at room temperature.

Duration of NPC1L1 expression. To determine how long the hNPC1L1 expression lasted in the liver, a total of 10 C57BL/6J male mice received Ad-L1 injection, 2 mice were sacrificed at each time point (0, 1, 3, 7 and 10 days) after injection and liver samples were harvested. Western blotting was performed using the aforementioned protocol and anti-hNPC1L1 antibody. The pan-cadherin antibody was used as a loading control.

Evaluation of liver injury by histopathology and biochemistry assay. Hematoxylin and eosin (H&E) and Sirius red staining were performed to evaluate pathological changes in the liver. All specimens were fixed overnight at room temperature by 10% formalin and were embedded in paraffin wax. Paraffin were cleared from 5 μm thick sections in three changes of xylene for 2 min per change at room temperature. The samples were hydrated at room temperature through three changes of 100% ethanol for 2 min per change followed by 95% ethanol and 70% ethanol for 2 min, respectively. The slides were rinsed in running water at room temperature for 5 min. The sections were stained in hematoxylin solution (cat. no. 143350, Shanghai Seebio Biotech, Inc.) for 3 min at room temperature, then rinsed under running water at room temperature for 10 min. The sections were stained working eosin Y solution (cat. no. C0850110535, Nanjing reagent) for 2 min before dehydration by transfer from 95% ethanol (20 min) to 100% ethanol (2 min each change), then three changes in xylene for 2 min per change. Slides were covered by glass coverslip with mounting medium (22). Picro-sirius red solution (cat. no. ab150681; Abcam) was using for 1 h staining at room temperature after de-waxing and hydration as described above. Histological analyses were evaluated at $\times 200$ magnification using a Leica DM 2700P microscope (Leica Microsystems GmbH). Aspartate transaminase (AST) and alanine transaminase (ALT) were measured using an AST activity colorimetric assay kit (cat. no. K753; Bio-Vision, Inc.) and an ALT activity colorimetric assay kit (cat. no. K752; Bio-Vision, Inc.), respectively.

Primary hepatocyte isolation and fluorescence microscopy. Primary hepatocytes were isolated from non-alcohol-fed and alcohol-fed mice, as described previously (23). The hepatocytes were seeded onto collagen-coated coverslips in six-well plates. Cells were collected and fixed with 4% paraformaldehyde for 20 min at room temperature. LC3 protein was detected by

Table I. Primer sequences.

Name	Forward (5'-3')	Reverse (3'-5')	Primer melting temperature (°C)
hNPC1L1	AGAGTGAGCCTTACACAACCA	GCAGGACACGTTGGAGAGT	60
mAtg5	TGTGCTTCGAGATGTGTGGTT	ACCAACGTCAAATAGCTGACTC	60
mAtg7	TCTGGGAAGCCATAAAGTCAGG	GCGAAGGTCAGGAGCAGAA	60
mAtg12	TGGCCTCGGAACAGTTGTTTA	GGGCAAAGGACTGATTCACAT	60
GAPDH	GAGCCAAACGGGTCATCATC	CATCACGCCACAGCTTTCCA	60

immunofluorescence antibody (cat. no. ab128025; Abcam) as previously described. Briefly, slides were incubated for 10 min with PBS containing 0.1% Triton X-100 then rinsed three times in PBS. The cells were incubated with 10% goat serum (cat. no. ab7481; Abcam) for 30 min at room temperature, then incubated cells in diluted primary antibody (1:100) in 1% BSA in PBST overnight at 4°C. Then, cells were incubated with goat anti-rabbit IgG (1:1,000, cat. no. ab150079; Abcam) at room temperature for 1 h. The cell nucleus was stained with DAPI in Prolong Diamond Antifade Mountant (cat. no. P36962; Thermo Fisher Scientific, Inc.) while the slides were sealed. LC3 protein (red dot) in the cells was observed under a Leica DM2500 fluorescence microscope (magnification x1,000; Leica Microsystems GmbH). Quantification was determined in 20 cells from captured images using Image J software (version 1.8.0, National Institutes of Health).

Ezetimibe treatment in cultured hepatocytes. To determine whether inhibition of NPC1L1 expression may restore the autophagy in alcohol-fed mice, primary hepatocytes were incubated in 5% CO₂ at 37°C in different concentrations of ezetimibe (0, 10, 20 and 40 μM; HR138776, Hairui Chemical, Co., Ltd.). After 1 h, hepatocytes were collected for the protein expression of LC3 by western blotting.

RT-qPCR. RNA extraction from hepatocytes of Ad-L1 mice and Ad-null mice (n=6) were performed as described using a RNeasy Mini kit 250 (cat. no. 74106; Qiagen, Inc.). Purified RNA was transcribed at 42°C by QuantiTectRev. Transcription kit (cat. no. 205313; Qiagen GmbH), and qPCR was performed using a SYBR® Green PCR Master Mix kit (cat. no. 4309155; Thermo Fisher Scientific, Inc.). Thermocycling conditions were 95°C for 2 min, then 40 cycles of 95°C for 15 sec, annealing for 15 sec, 72°C for 45 sec. After amplification, a melting curve was used to assess product purity. Annealing temperatures were optimized for each primer pair (Table I). GAPDH was used as an internal control and mRNA expression levels were calculated based on the 2^{-ΔΔC_q} method (24). mRNA levels for each gene represent the amount relative to that in control mice, which was arbitrarily standardized to 1. Sequences of the primers used for qPCR are given in Table I.

Statistical analysis. Data are presented as the mean ± SEM of at least four independent experiments. Densitometric analysis was performed on the immunoblots using ImageJ 1.8.0 (National Institutes of Health). Statistical differences were determined using the Student's t-test and one-way

ANOVA (post hoc test: Tukey's multiple comparison test) or two-way ANOVA (post hoc test: Bonferroni). All statistics were performed using GraphPad Prism 5 (GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Ethics statement. Experimental procedures and animal use and care protocols were in accordance with the guidelines of the Northwest A&F University Institutional Committee for the Care and Use of Laboratory Animals, and were approved by the Committee on Ethical Use of Animals of Northwest A&F University.

Results

Expression of human NPC1L1 (Ad-L1) in the mouse liver via adenovirus transfection. According to the experimental workflow (Fig. 1A), adenovirus-packaged human NPC1L1 cDNA was transfected retro-orbitally at day 3 after the commencement of the alcohol diet. NPC1L1 mRNA expression levels were evaluated in the mouse liver 3 days post-injection. The results revealed that mRNA expression in the Ad-L1 mice was ~200-fold higher than that in the Ad-null mice (Fig. 1B; P<0.001). Western blot analysis showed that NPC1L1 protein was markedly expressed in the mouse liver starting from day 3 until day 7 after adenoviral injection (Fig. 1C), and was decreased at about 10 days post-injection.

The Ad-L1 mice behaved normally under the experimental conditions. The alcohol consumption and adenoviral treatment did not result in significant changes in body weight, liver weight or the liver/body weight ratio in Ad-null and Ad-L1 mice (Fig. 1D and E).

Lipids in the blood and liver were measured. Plasma total cholesterol was significantly increased in Ad-L1 mice regardless of alcohol feeding (Fig. 1F; P<0.05). However, total hepatic cholesterol levels were similar under both non-alcoholic and alcohol feeding conditions (Fig. 1G). In contrast with the total cholesterol, total plasma triglyceride levels were similar between Ad-null and Ad-L1 mice (Fig. 1H) under both feeding states. Hepatic triglyceride levels were significantly higher in alcohol-fed mice in the Ad-L1 group (Fig. 1I; P<0.05).

Chronic-binge alcohol uptake causes mild liver injury and steatosis in mice. A chronic-binge alcohol feeding plan was followed in the present study to induce alcoholic liver injury. Plasma AST and ALT activity were measured to evaluate liver

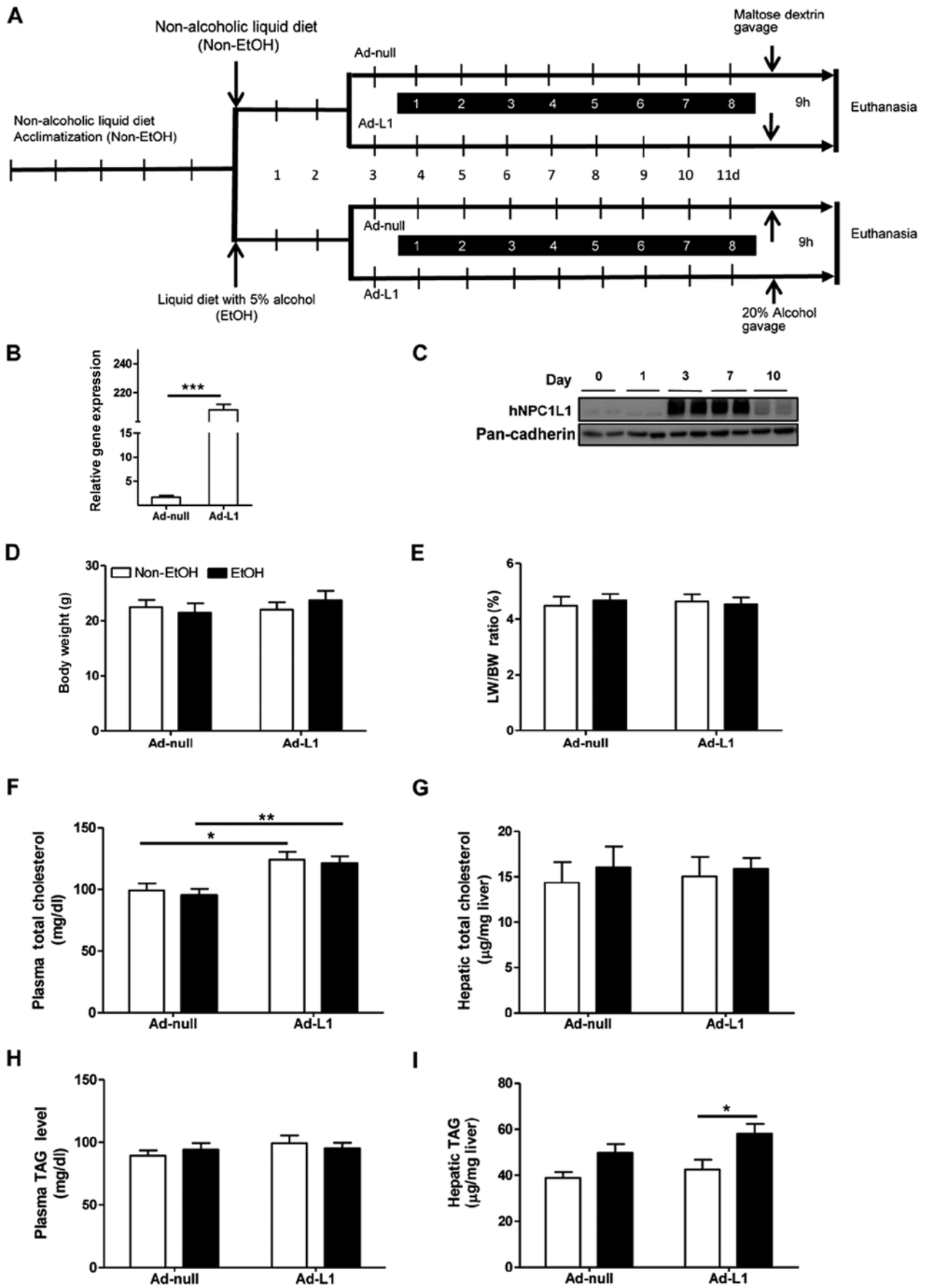


Figure 1. Creation and characterization of Ad-L1 and Ad-null mice. (A) Experimental workflow. (B) mRNA expression in the liver. (C) Protein expression in the livers of Ad-L1 mice. (D) Body weight comparison between EtOH and non-EtOH mice. (E) LW/BW ratio of EtOH mice compared with non-EtOH mice. (F) Plasma total cholesterol level. (G) Hepatic total cholesterol level. (H) Plasma TAG level. (I) Hepatic TAG level. Data in the graphs represent the mean \pm SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. TAG, triglyceride; EtOH, alcohol-fed; hNPC1L1, human Niemann-Pick C1-like 1; LW/BW, liver weight/body weight.

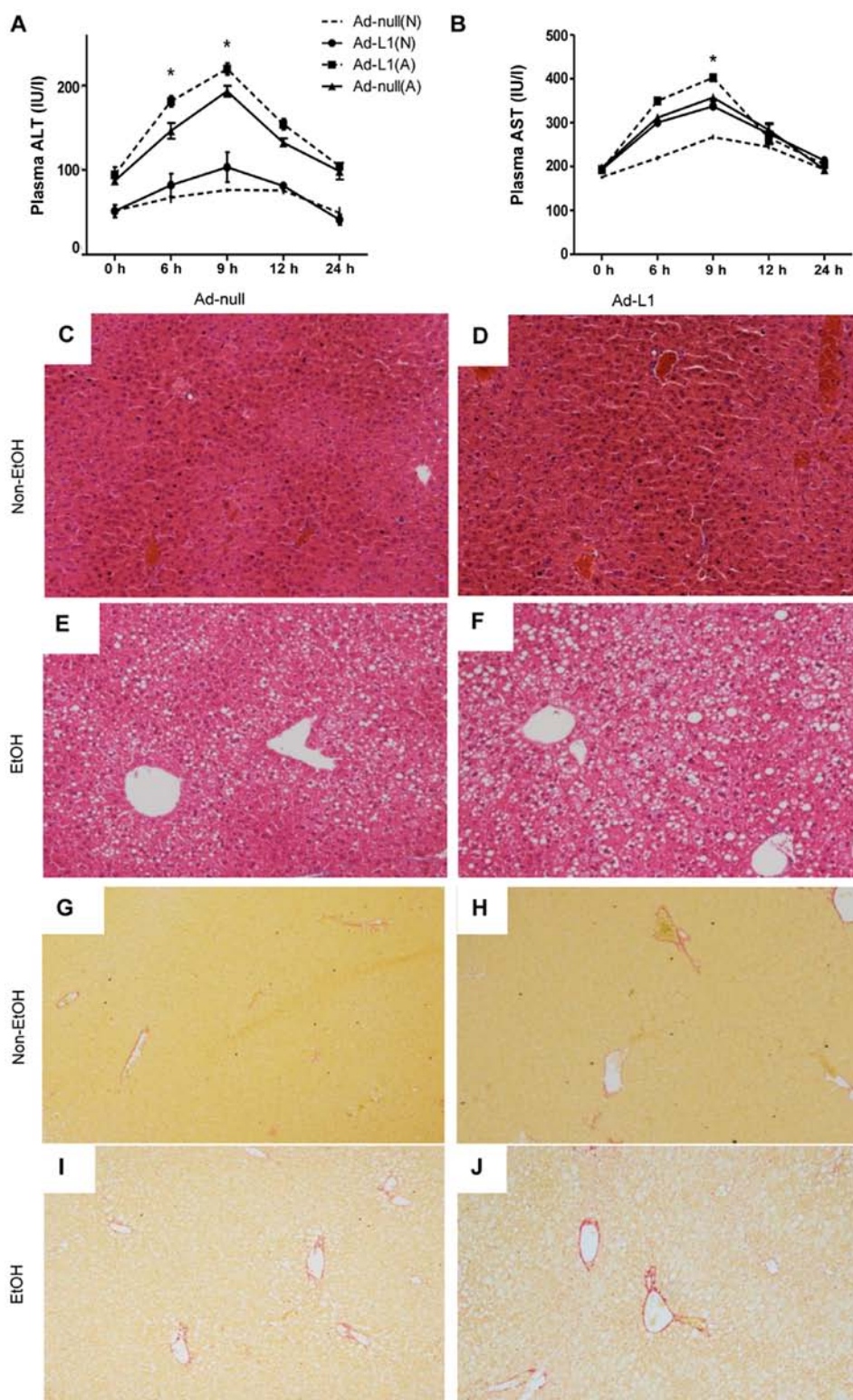


Figure 2. Alcohol feeding causes liver injury and steatosis. (A) Plasma ALT activity. (B) Plasma AST activity. Hematoxylin and eosin staining images of liver sections from (C) Ad-null and (D) Ad-L1 mice fed with non-EtOH diet, and (E) Ad-null and (F) Ad-L1 mice treated with EtOH diet. Sirius red staining images of liver sections from (G) Ad-null and (H) Ad-L1 mice fed with non-EtOH diet and (I) Ad-null and (J) Ad-L1 mice fed with EtOH diet. Magnification, x200. ALT, alanine transaminase; AST, aspartate transaminase; EtOH, alcohol-fed. * $P < 0.05$.

injury. The results revealed that alcohol feeding caused increases in AST and ALT activity in the blood of Ad-L1 and Ad-null mice. Compared with the Ad-null mice after alcohol feeding, the Ad-L1 mice had significantly higher ALT activity (Fig. 2A; $P < 0.05$). A small but significant difference was detected in

plasma AST activity between Ad-L1 and Ad-null mice at 9 h after gavage (Fig. 2B; $P < 0.05$). This suggested that 9 h after the gavage was the appropriate time to evaluate liver injury.

Histologically, H&E staining showed mild lipid accumulation in the livers of alcohol-fed mice (Fig. 2C-F). The lipid

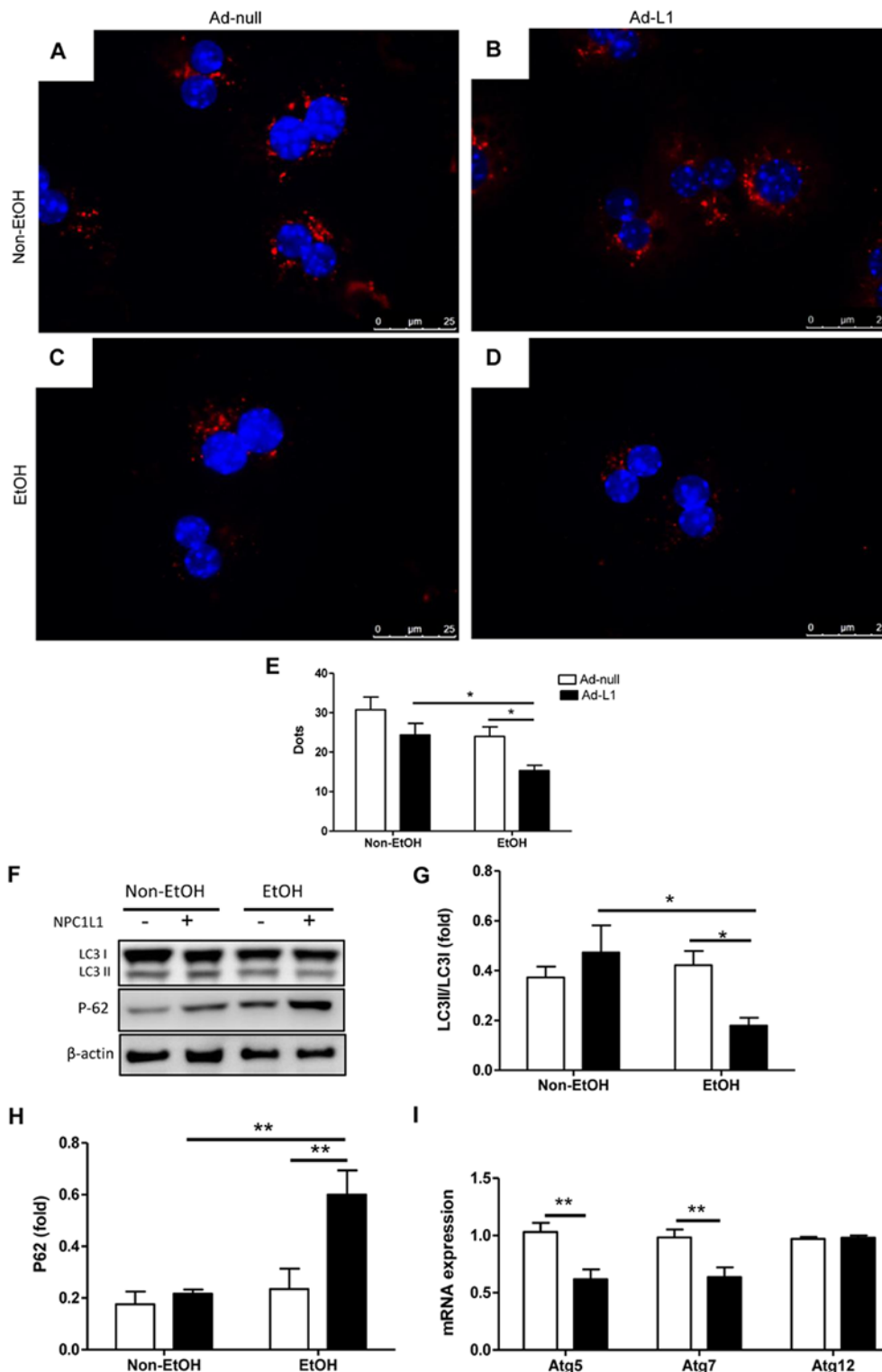


Figure 3. Autophagy is impaired in hepatocytes. Immunofluorescence images of isolated hepatocytes from (A) Ad-null and Ad-L1 (B) mice fed with non-EtOH diet, and (C) Ad-null and (D) mice fed with EtOH diet. (E) Quantification of LC3 particles (red dots) in the fluorescence images. (F) Western blot analysis of LC3 and p62 proteins. (G) Densitometry of LC3 protein expression. (H) Densitometry of p62 protein expression. (I) Reverse transcription-quantitative PCR analysis of the mRNA expression of autophagy-related genes after alcohol feeding. Data in the graphs represent the mean \pm SEM. * $P < 0.05$; ** $P < 0.01$. LC3, microtubule-associated proteins 1A/1B light chain 3; EtOH, alcohol-fed; NPC1L1, Niemann-Pick C1-like 1.

accumulation was more pronounced in the livers of AD-L1 mice than that in Ad-null mice. This demonstrated that alcohol uptake induced mild liver steatosis. Sirius red staining showed no detectable fibrosis in the livers of alcohol-fed mice (Fig. 2G-J).

Autophagy is reduced in the hepatocytes of Ad-null mice after alcohol feeding. Fluorescence microscopy was used to monitor the formation of autophagic particles in isolated primary hepatocytes (Fig. 3A-D). Red dots indicate the intracellular autophagy marker LC3II, and blue dots stained with DAPI

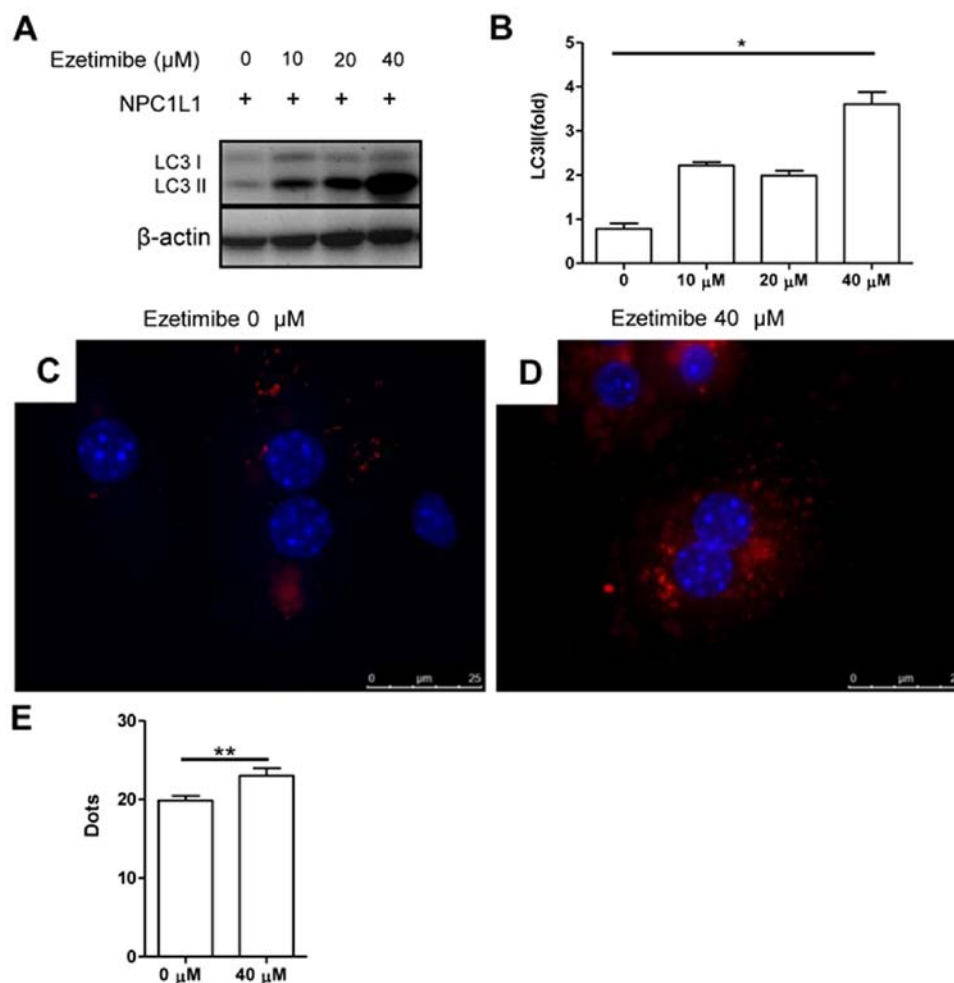


Figure 4. Ezetimibe activates autophagy in the hepatocytes of Ad-L1 mice after alcohol feeding. (A) Western blot analysis of LC3 expression in hepatocytes at different doses of ezetimibe. (B) Densitometry of LC3II expression. Immunofluorescence images with (C) no ezetimibe treatment and (D) treatment with 40 μM ezetimibe. (E) Quantification of LC3 (red dots) in the images. Data in the graphs represent the mean ± SEM. *P<0.05; **P<0.01. LC3, microtubule-associated proteins 1A/1B light chain 3; NPC1L1, Niemann-Pick C1-like 1.

indicate the nuclei of the hepatocytes. Quantification of 20 hepatocytes from captured images (Fig. 3A-D) showed fewer red dots in the alcohol-fed mice (Fig. 3D and E; P<0.05). The result was confirmed through analysis of the protein expression of the autophagy marker p62/SQSTM1 (Fig. 3F and H; P<0.01). The ratio of LC3II/LC3I in the liver was unchanged in the alcohol-fed mice compared with the non-alcohol-fed mice.

Autophagy is attenuated in the hepatocytes of Ad-L1 mice. According to the immunofluorescence images, Ad-L1 mice had significantly less LC3 expression (Fig. 3D and E) compared with Ad-null mice under both alcoholic and non-alcoholic feeding conditions (Fig. 3B and E). These results were confirmed by western blot analysis (Fig. 3F). The LC3II expression was reduced. By contrast, p62/SQSTM1 protein expression was increased in Ad-L1 mice compared with Ad-null mice. This indicated that autophagy was inhibited. Quantification by densitometry showed that the LC3II/LC3I expression ratio (Fig. 3G) was significantly decreased and p62/SQSTM1 protein content (Fig. 3H) was significantly increased in Ad-L1 mice compared with Ad-null mice after alcohol feeding. The mRNA expression of autophagy-related markers atg5, atg7 and atg12 was evaluated after alcohol

feeding in both Ad-L1 and Ad-null mice. The expression of atg5 and atg7, but not that of atg12, was significantly reduced in Ad-L1 mice compared with Ad-null mice after alcohol feeding (Fig. 3I; P<0.01). Together, these results indicated that NPC1L1 expression caused the inhibition of autophagy in the livers of mice after alcohol feeding.

Ezetimibe activates autophagy in alcohol-fed mouse hepatocytes. To further demonstrate that the reduction in autophagy was due to the NPC1L1 expression, primary hepatocytes from Ad-L1 mice were treated with different doses of ezetimibe, an inhibitor of NPC1L1 (Fig. 4A and B). The results indicated that LC3II protein expression was restored in Ad-L1 mice after ezetimibe treatment. The increase in the LC3II expression level was dose-dependent and reached its highest level at 40 μM (Fig. 4A and B; P<0.05). Quantification of the LC3 particles in fluorescence images indicated an increase at 40 μM of ezetimibe (Fig. 4C-E; P<0.01).

Discussion

Autophagy is the process of eliminating unnecessary or toxic intracellular components, including extra lipids, through

activation of the lysosomal degradation process. Increased understanding of autophagy in hepatocytes after alcohol abuse could contribute to the treatment of AFLD (6). The effects of alcohol on hepatic autophagy have been studied in mouse models (25,26). However, the findings from these studies are not entirely applicable to humans, because the expression of NPC1L1 is restricted to the intestines in rodents (27). The present study investigated hepatic autophagy in a more appropriate mouse model in which NPC1L1 was expressed in the liver, as it is in humans. It was also demonstrated that 10 day alcohol feeding plus a single binge gavage is an effective alcohol treatment procedure, because liver injury and steatosis were detected via elevation of blood AST and ALT activity, and by H&E staining. This result was consistent with a previously reported binge model for AFLD (28,29).

Alcohol treatment resulted in a reduction of hepatic autophagy in Ad-null mice, as supported by the results of the fluorescence microscopy in hepatocytes and liver p62 western blotting. The fluorescence microscopy images showed that there was a statistically significant reduction in LC3 in the hepatocytes of alcohol-fed mice compared with non-alcohol-fed mice. Moreover, p62 protein expression was also significantly increased in alcohol-fed mice. These results were in accordance with the consensus on alcoholic autophagy: That chronic alcohol treatment impairs autophagy (30), while acute alcohol treatment increases autophagy (5,11).

In comparison to that in Ad-null mice, the expression of NPC1L1 in the liver led to a more pronounced reduction in autophagy in both the non-alcohol and alcohol feeding states. These results were confirmed by the expression of LC3 using fluorescence microscopy and western blotting. Other autophagic markers, p62/SQSTM1 protein expression and the mRNA level of atg5 and atg7, also indicated that hepatic autophagy was inhibited in the livers of Ad-L1 mice. The reduction in autophagy in primary hepatocytes of Ad-L1 mice could be restored by ezetimibe treatment. It was concluded that the expression of NPC1L1 impaired hepatic autophagy in Ad-L1 mice treated with chronic alcohol feeding.

A reasonable explanation for the autophagy inhibition mediated by NPC1L1 may involve changes in cellular cholesterol homeostasis. It is known that autophagy is highly sensitive to variations in cellular cholesterol homeostasis, and cholesterol-lowering drugs such as statins, cholestyramine and ezetimibe increase autophagy in hepatocytes (9). Hepatic NPC1L1 prevents cholesterol loss from bile in humans (15,18). Overexpression of NPC1L1 in the liver results in free cholesterol absorption (30) and further impairs autolysosome clearance (9).

The experiments in human hepatocytes demonstrated that NPC1L1 was associated with the inhibition of autophagy, and ezetimibe treatment activated autophagy (15). To the best of our knowledge, this was the first *in vivo* study to show that overexpression of NPC1L1 in the liver impairs hepatic autophagy under alcohol feeding conditions. The findings of the present study suggested that activation of autophagy may be a potential therapy for AFLD via inhibition of NPC1L1. However, long-term investigation in other animal models, and clinical studies, are necessary before the treatment of AFLD patients with NPC1L1 inhibition.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

YW, PY and QL made substantial contributions to the conception and design of the study. YW, PY, BZ, YD and SL were involved in data analysis and interpretation. YH and XG performed histological examination. YW, PY and QL drafted the manuscript and revised it critically for important intellectual content. All authors approved the final version of the manuscript and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Experimental procedures and animal use and care protocols were in accordance with the guidelines of the Northwest A&F University Institutional Committee for the Care and Use of Laboratory Animals, and were approved by the Committee on Ethical Use of Animals of Northwest A&F University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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