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AMPK-Regulated Autophagy Contributes to Ursolic Acid Supplementation-Alleviated Hepatic Steatosis and Liver Injury in Chronic Alcohol-Fed Mice

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Cite This: *ACS Omega* 2023, 8, 907−914

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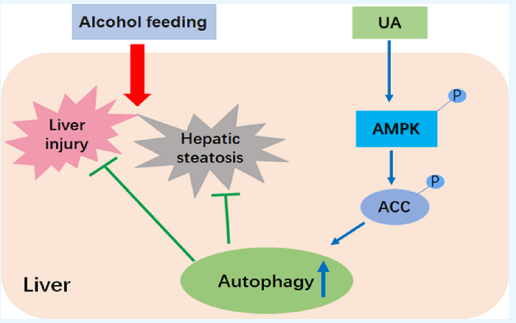
ABSTRACT: Alcoholic liver disease (ALD) is a chronic liver disease caused by long-term heavy consumption of alcohol. The pathogenesis of ALD is complex, and there is no effective clinical treatment at present. Ursolic acid (UA), a general triterpenoid with multiple biological roles, is widely distributed in plants. This study aims to explore the therapeutic effect and potential mechanisms of UA that protect against liver injury and hepatic steatosis in an ALD mouse model. In this study, we analyzed the lipid accumulation and the effect of UA treatment in a mouse model of ALD; AML12 and HepG2 cells were used to study the biological effect and potential mechanisms of UA on ethanol-induced hepatotoxicity. The morphologic and histological detections showed that UA significantly reduced alcohol-induced liver injury and hepatic steatosis. In addition, UA dramatically ameliorated alcohol-induced metabolic disorders, oxidative stress, and inflammation. Furthermore, UA treatment activated autophagy via the AMPK−ACC pathway to protect hepatocytes from lipotoxicity. Thus, these findings demonstrate that UA treatment alleviates alcoholic-induced liver injury by activating autophagy through the AMPK−ACC pathway. Therefore, UA may represent a promising candidate for the treatment of ALD.

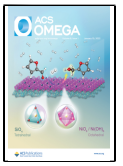


1. INTRODUCTION

Long-term excessive alcohol intake causes alcoholic liver disease, a type of chronic liver disease. Every year, millions of patients worldwide suffer from this disease, which is one of the most common causes of liver disease incidence and mortality.[1] The pathogenesis of alcoholic liver disease (ALD) is complex and obviously multifactorial.[2,3] Hepatic steatosis is the most common and earliest consequence of chronic alcohol consumption. In the presence of intracellular triglycerides, hepatocytes are more sensitive to the consequences of the "second hit", such as oxidative stress and proinflammatory cytokines, leading to the progression of steatohepatitis.[4,5] This disease encompasses a spectrum of clinical features, including simple fatty liver, to more advanced hepatitis, and fibrosis, which may progress to cirrhosis and even hepatocellular carcinoma.[6] Despite great efforts that have been made in understanding the disease pathogenesis, there are currently no effective therapies available for the treatment of ALD. Therefore, safe compounds with strong preventive and therapeutic effects are urgent needed. Ursolic acid (UA) is a pentacyclic triterpenoid compound that is widely distributed in a variety of plants.[7] The multiple biological efficacies of UA, including anti-oxidative, anti-inflammatory, anti-hyperlipidemic, anti-atherosclerotic, anti-carcinogenic, anti-mutagenic, and anti-microbial effects, have been demonstrated by numerous studies.[8,9] UA stimulates triglyceride (TG) hydrolysis by activating adipose TG lipase and hormone-sensitive lipase in primary-cultured adipocytes.[10] UA reduces metabolic syndrome and reduces diet-induced fatty liver disease in a mouse model.[11,12] In addition, UA can improve non-alcoholic fatty liver disease (NAFLD) through regulating lipid metabolism, ameliorating hepatic steatosis, and improving metabolic disorders in a rat model.[13] However, the effects of UA on ALD are rarely reported.

Therefore, the aim of the present study was to evaluate the protective effect of UA against alcoholic fatty liver and liver injury, inflammation, and oxidative stress in an alcohol-induced ALD mouse model and further explore its potential mechanisms in hepatocytes. UA is a potential candidate for improving hepatic steatosis in response to alcohol.

Received: September 27, 2022
Accepted: December 8, 2022
Published: December 16, 2022



2. MATERIALS AND METHODS

2.1. Animal Model and Experimental Protocol. Wild-type (WT) C57BL/6 mice were kept in ventilated cages and under specific pathogen-free conditions in the animal center of Zhejiang Chinese Medical University. The C57BL/6 mice were divided into three groups (n = 10 per group): pair-fed (PF) group, alcohol-fed (AF) group, and alcohol-fed/UA (0.25 g/100 mL liquid diet, Sigma-Aldrich, St. Louis, Missouri) (AF + UA) group. The AF mice were fed ad libitum on an ethanol-containing Lieber−DeCarli liquid diet (ethanol-derived calories were increased from 22 to 32% during the 4 weeks, with a 5% increase each week) for 4 weeks, whereas control mice in the PF group were fed an isocaloric control lipid diet with the amount consumed by the alcohol group the previous day. The food intake of mice was recorded daily, and body weight was recorded weekly. Mice were sacrificed under anesthesia with avertin (250 mg/kg body weight) after 4 h of fasting. Plasma, liver, and epididymal fat pad samples were collected for further analysis.

2.2. Biochemical Analysis. Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were measured using Nanjing Jiancheng Bioengineering Institute kits (Nanjing, China) according to the manufacturer's instructions. The levels of glycerin and free fatty acids (FFA) were measured in plasma with a glycerin assay kit and an FFA assay kit, respectively. The levels of total cholesterol (TC), TG, FFA, reduced glutathione/oxidized glutathione (T-GSH/GSSG), total superoxide dismutase (T-SOD), and malondialdehyde (MDA) in the liver were measured with TC, TG, FFA, T-GSH/GSSG, T-SOD, and MDA assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions, respectively.

2.3. Cell Lines and Culture Conditions. The non-hepatoma hepatocyte cell line alpha mouse liver (AML)-12 and the human hepatoma cell line HepG2 were both obtained from the American Type Culture Collection (ATCC, Manassas, Virginia, United States). The AML12 hepatocyte cell line was cultured in Dulbecco's modified Eagle medium/Ham's Nutrient Mixture F-12, 1:1 (DMEM/F-12, Sigma-Aldrich, 051 M8322), supplemented with 10% (v/v) fetal bovine serum (Life Technologies, 10099-141), 5 mg/mL insulin (Sigma-Aldrich, I9278), 5 mg/mL transferrin (Sigma-Aldrich, T8158), 5 ng/mL selenium (Sigma-Aldrich, 229865), 40 ng/mL dexamethasone (Sigma-Aldrich, D4902), 100 U/mL penicillin, and 100 mg/mL streptomycin (Life Technologies, 15140−122) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. HepG2 was cultured in DMEM containing 10% (v/v) fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

2.4. RNA Interference. The methods for RNA interference have been described previously.[14] Briefly, cultured cells were transfected with human AMPK siRNA (GenePharma, Shanghai, China) using ExFect2000 (Vazyme, Nanjing, China) according to the manufacturer's instructions. In the control group, cells were transfected with scrambled siRNA (GenePharma, Shanghai, China).

2.5. Histological Examination. Liver tissue samples were fixed with 4% paraformaldehyde, tissues were fixed with paraffin and sectioned, and paraffin sections were stained with hematoxylin and eosin (H&E). For Oil Red O staining, freshly isolated liver tissues were fixed in 4% paraformaldehyde and then frozen with Tissue-Tek O.C.T. compound. Frozen tissues were sectioned on a micron cryostat at −19 °C for 8 mm and then air dried. Rehydrated liver sections were stained with Oil Red O for 20 min and counterstained with Mayer's Hematoxylin.[14]

2.6. Quantitative Real-Time Reverse Transcription (RT)-PCR. The methods for RNA isolation have been described previously.[14] Quantitative PCR was performed using the SYBR Green PCR Master Mix (Qiagen). Samples were normalized to the general housekeeping gene β-actin and calculated using the $2^{-\Delta\Delta Ct}$ method. The primers are as follows: *IL-1β* forward, 5′-CTGGTACATCAGCACCTCTCAA-3′; *IL-1β* reverse, 5′-GAGACTGCCCATTCTCGACAA-3′; *TNF-α* forward, 5′-TGTCTCAGCCTCTTCTCATT-3′; *TNF-α* reverse, 5′-AGATGATCTGAGTGTGAGGG-3′; *18S* forward, 5′-ATA-CATGCCGACGGGCGCTG-3′; and *18S* reverse, 5′-CGGCTCGGGCCTGCTTTGAA-3′.

2.7. Western Blotting Analysis. Western blot analysis was performed as previously described,[14] and the following antibodies were used: anti-Caspase3 (Rabbit mAb, #9664), anti-Atg5 (Rabbit mAb, #9980), anti-Beclin1 (Rabbit mAb, #3495), anti-AMP-activated protein kinase (AMPK)/pAMPK (Rabbit mAb, #5831; #2535), *anti*-acetyl-CoA carboxylase (ACC)/pACC (Rabbit mAb, #3676; #3661) (Cell Signaling Technology, Danvers, Massachusetts, United States), anti-LC3B (Rabbit, L7543) (Sigma-Aldrich, St. Louis, Missouri, United States), and beta-actin antibody and GAPDH antibody (Boster, Wuhan, China).

2.8. Analysis of Autophagic Flux. Briefly, AML-12 cells were transfected with recombinant adenovirus GFP-LC3 (Hanbio Biotechnology Co., Ltd., Shanghai, China). The transfected cells were pretreated with the autophagy inhibitor chloroquine (CQ) before performing the indicated interventions. GFP-LC3 puncta were detected by laser scanning confocal microscopy (Nikon A1R, Japan) and analyzed for mean fluorescence intensity (MFI); in addition, LC3-II expression was detected by Western blot and analyzed for autophagic flux.

2.9. Statistical Analysis. Statistical analysis was performed using a one-way analysis of variance (ANOVA) by the Newman−Keuls test for the statistical difference using GraphPad software. Differences between treatments were considered to be statistically significant at $p < 0.05$. All data were expressed as mean ± SD.

3. RESULTS

3.1. Chronic Alcohol Consumption Induces Metabolic Disorders in Mice and the Effects of UA Treatment on the Characterization of ALD Mice. Male C57BL/6 mice were fed the Lieber−DeCarli alcohol-containing liquid diet for 4 weeks. We observed a significant reduction in body weight in the alcohol group (AF) compared to the control group (PF) during alcohol exposure, whereas the weight was regained in the UA-treated group (Figure 1A). Compared with the PF group, liver weight and liver index (liver weight/body weight) were significantly increased in the AF group, whereas liver weight and liver index were obviously decreased in the UA-treated group (Figure 1B,C). Moreover, the epididymal fat index (epididymal fat/body weight) was similar to the liver index, with a significant increase in the AF group compared with the PF group and a significant decrease in the UA-treated group (Figure 1D). We also measured glycerol and FFA contents (Figure 1E,F). After another 5 weeks of UA treatment, UA supplementation obviously reversed alcohol-induced metabolic disorders.

https://doi.org/10.1021/acsomega.2c06252
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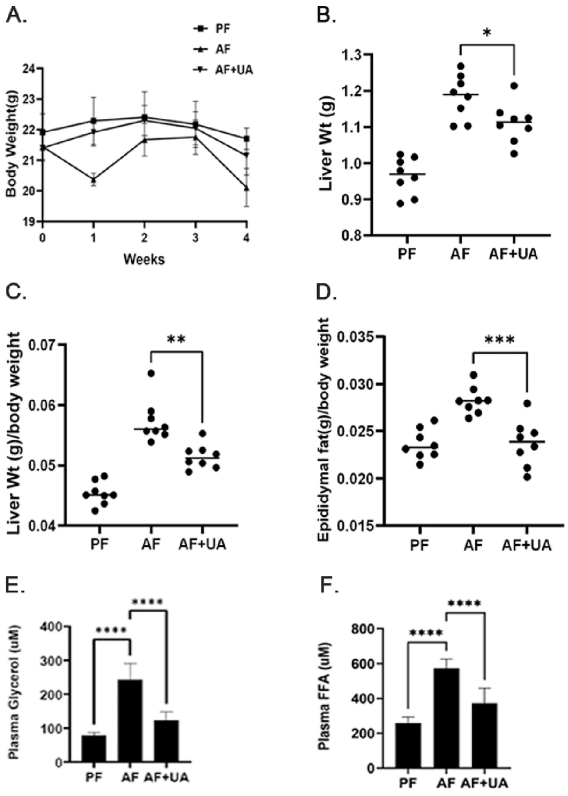

Figure 1. Effects of UA treatment on body weight, liver weight, plasma glycerol, and plasma FFA of the ALD mice. (A) Body weight. (B) Liver weight. (C) The ratio of liver weight to body weight. (D) The ratio of epididymal fat to body weight. (E) Plasma glycerol and (F) plasma FFA. Data are expressed as the mean ± SD (n = 8 mice per group). Bars with asterisks differ significantly (P < 0.05). PF: pair feeding; AF: alcohol feeding; UA: ursolic acid.

3.2. UA Supplementation Reverses Alcohol-Induced Liver Injury. Chronic alcohol exposure significantly increased plasma ALT and AST levels in mice (Figure 2A,B). Compared with the control group, the histological examination of mice showed visible liver steatosis and vacuolar degeneration in alcohol-fed mice (Figure 2C), which were further confirmed by Western blot analysis showing that apoptosis protein cleaved-caspase3 expression significantly increased in alcohol-fed mice (Figure 2D). After another 5 weeks of UA treatment, UA supplementation obviously reversed alcohol-induced liver injury (Figure 2).

3.3. UA Supplementation Alleviates Alcohol-Induced Fatty Liver. Moreover, Oil Red showed massive hepatic fat accumulation in alcohol-fed mice (Figure 3A). Compared with the PF group, AF mice illustrated a significant increase in hepatic TG contents (Figure 3B) and liver TC contents (Figure 3C). As shown in Figure 3, in comparison with the AF group, the mice with UA treatment ameliorated these parameters in the setting of chronic alcohol exposure. UA supplementation manifested alleviated alcohol-induced lipid accumulation in the liver.

3.4. UA Reduces Alcohol-Induced Oxidative Stress Markers. Chronic exposure to ethanol enhances levels of reactive oxygen species, reduces antioxidant activity in cells, and contributes to oxidative stress in the liver. In comparison to alcohol-fed animals, UA supplementation mice showed significantly lower GSSG levels and higher GSH levels, leading to a remarkable increase in the GSH/GSSG ratio (Figure 4A), which indicated recovery of hepatic glutathione homeostasis.

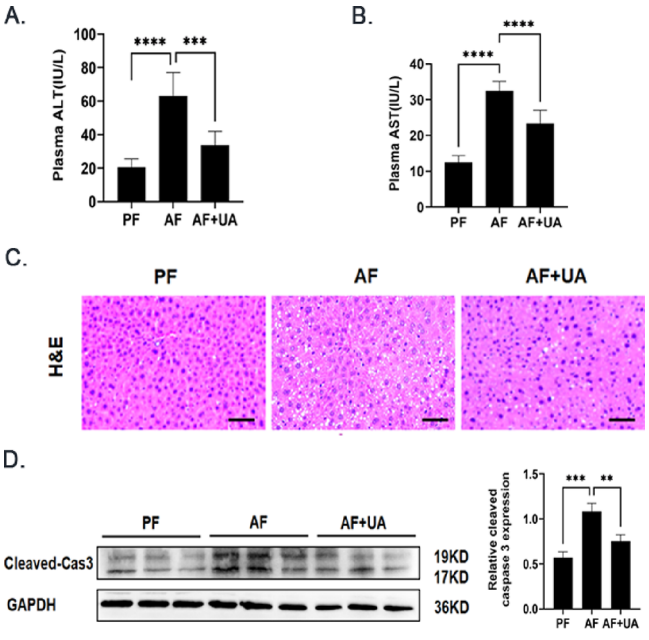

Figure 2. UA supplementation reverses alcohol-induced liver injury. (A) Plasma ALT levels. (B) Plasma AST levels. (C) H&E staining and (D) protein expression of Cleaved-Cas3 were detected by Western blotting. Data are expressed as the mean ± SD (n = 8 mice per group). Bars mean 100 μm; bars with asterisks differ significantly (P < 0.05). PF: pair feeding; AF: alcohol feeding; UA: ursolic acid.

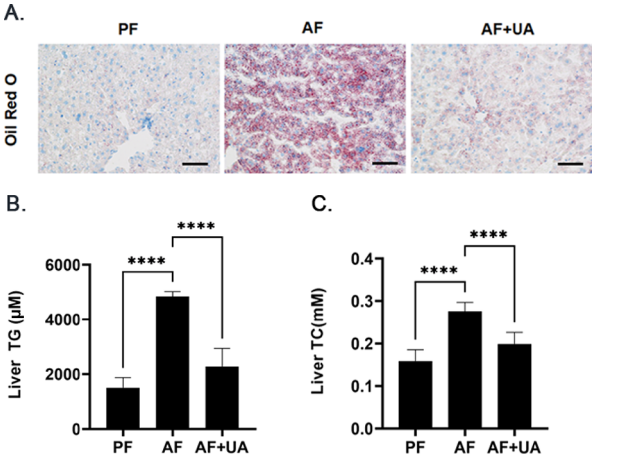

Figure 3. UA supplementation alleviates alcohol-induced fatty liver. (A) Liver Oil Red O staining. (B) Liver TG and (C) Liver TC contents. Data are expressed as the mean ± SD (n = 8 mice per group). Bars mean 100 μm; bars with asterisks differ significantly (P < 0.05). PF: pair feeding; AF: alcohol feeding; UA: ursolic acid.

Following the elevated hepatic GSH/GSSG ratio in UA supplementation mice, UA supplementation markedly increased T-SOD formation and decreased MDA formation (Figure 4B,C), suggestive of repressed lipid peroxidation in the liver.

3.5. UA Ameliorates Alcohol-Induced Hepatic Inflammation in Mice. Alcohol-induced liver injury is associated with hepatic inflammation. Compared with those of pair-fed mice, alcohol-fed mouse hepatic pathological alterations were associated with significantly increased IL-1β and TNF-α gene expression in mouse liver tissues. After another 4 weeks of UA treatment, UA supplementation obviously ameliorated alcohol-induced hepatic inflammation (Figure 5).

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ACS Omega 2023, 8, 907−914

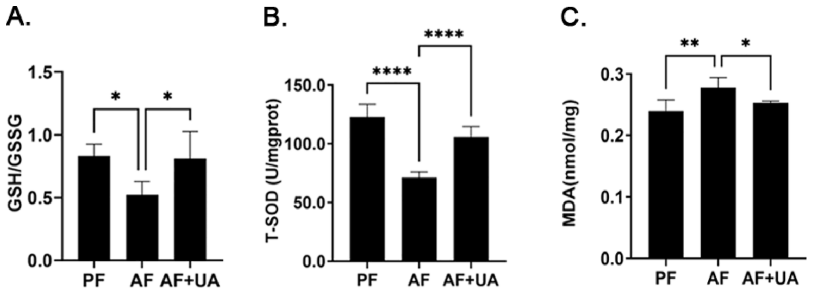

Figure 4. Effects of UA treatment on alcohol-induced oxidative stress markers. (A) GSH/GSSG. (B) T-SOD and (C) MDA. Data are expressed as the mean ± SD (n = 8 mice per group). Bars with asterisks differ significantly (P < 0.05). PF: pair feeding; AF: alcohol feeding; UA: ursolic acid.

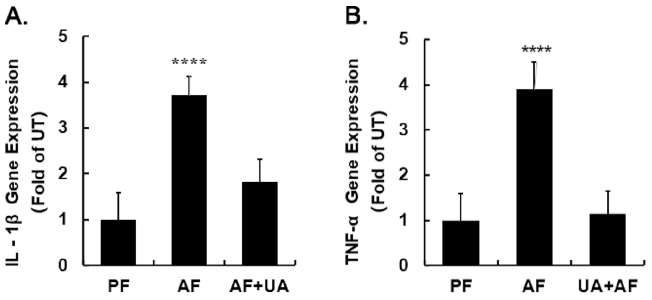

Figure 5. Effects of UA treatment on ethanol-induced inflammation in mouse liver. (A) IL-1β gene expression in mouse liver by qPCR and (B) TNF-α gene expression in mouse liver by qPCR. Data are expressed as the mean ± SD (n = 8 mice per group). Bars with asterisks differ significantly (P < 0.05). PF: pair feeding; AF: alcohol feeding; UA: ursolic acid.

3.6. UA Treatment Activates Autophagy in Hepatocytes. In our previous study, we found that autophagy activation further alleviated alcoholic-induced liver injury.[14] To investigate the effect of UA supplementation on the induction of hepatocyte autophagy in response to chronic alcohol exposure, we examined activation markers of hepatic autophagy like LC3-II conversion and Atg5 and Beclin1 expression in the livers of pair-fed, alcohol-fed, and alcohol-fed/UA treatment mice. As shown in Figure 6A, in comparison with alcohol-fed control animals, UA treatment mice manifested markedly increased hepatic LC3-II conversion and Atg5 and Beclin1 expression. Similarly, in AML12 cells, UA supplementation led to increased LC3-II conversion and Atg5 and Beclin1 expression (Figure 6B) and increased autophagic flux (Figure 6C).

3.7. UA Activates the AMPK−ACC Pathway to Induce Autophagy. Ethanol can induce energy metabolic disorders and liver injury. Autophagy may be the main protective mechanism limiting the toxicity of ethanol. To explore the effect of UA-activated autophagy in hepatocytes, we found that in comparison to pair-fed animals, alcohol-fed mice inhibited AMPK and ACC activation (pAMPK/AMPK, pACC/ACC) and UA treatment significantly reversed AMPK and ACC activation (Figure 7A). Similarly, in HepG2 cells, UA supplementation led to AMPK and ACC activation (Figure 7B), whereas AMPK siRNA knockdown inhibited autophagy activation (Figure 7C), suggesting that UA can activate the AMPK−ACC pathway to regulate autophagy activation.

4. DISCUSSION

We previously reported that UA treatment ameliorates hepatic steatosis and improves metabolic disorders in non-alcoholic fatty liver disease rats induced with high-fat diets.[13] This study aimed to examine the therapeutic effect of UA against alcoholic fatty liver and liver injury, inflammation, and oxidative stress in an ALD mouse model and further address its potential mechanisms.

Using a well-established Lieber−DeCarli mouse model of ALD, we reported the therapeutic role of UA on alleviating alcoholic fatty liver and liver injury and further improving alcohol-induced metabolic disorders, including serum lipid disorders, oxidative stress, and inflammation. Importantly, our data showed that UA supplementation ameliorates alcoholic-induced liver injury, which was associated with increased autophagy activation by regulating the AMPK−ACC pathway.

The liver is the main tissue for alcohol metabolism. Chronic alcohol consumption is associated with elevated oxidative stress and subsequent lipid peroxidation in the liver. Long-term alcohol exposure increases the production of reactive oxygen species, reduces the level of cellular antioxidation, and results in oxidative stress in the liver. Alcohol-induced liver injury is associated with increased formation of lipid radicals, lipid peroxidation, protein carbonyl formation, and decreased antioxidant defenses of liver.[15−17] Polyunsaturated fat (required for lipid peroxidation) is replaced with saturated fat or medium-chain TGs to reduce or prevent lipid peroxidation and alcohol-induced liver injury.[18−20] In the present study, chronic alcohol consumption induces metabolic disorders in mice and UA supplementation reverses the liver index (liver weight/body weight) and epididymal fat index (epididymal fat weight/body weight) (Figure 1); UA supplementation reverses alcohol-induced liver injury and alleviates alcohol-induced fatty liver (Figures 2 and 3). UA significantly alleviated oxidative stress in the liver, increased antioxidant enzyme T-SOD formation, and decreased lipid oxidative product (MDA) formation (Figure 4), suggestive of repressed lipid peroxidation in the liver. Over-production of tumor necrosis factor-α (TNF-α) is involved in the pathogenesis of ALD. Patients with alcoholic hepatitis have elevated systemic TNF levels, which is associated with disease severity and mortality.[21−23] Many inflammatory cytokines, such as IL-6 and TNF-α, have been demonstrated to promote insulin resistance and liver inflammation.[24,25] In this work, UA significantly reduced hepatic inflammatory evidenced by the reduction of chronic alcohol consumption that induced an increase in TNF-α and IL-1β gene expression in the liver, showing that the anti-inflammatory effect was mediated by UA.

Although there has been great progress in the past few years, our understanding of the pathogenesis of ALD at the molecular and cellular levels is still incomplete. More and more evidence shows that autophagy plays a crucial role in regulating basic homeostatic functions of the liver and disordered autophagy activation is related to the pathogenesis of various liver

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ACS Omega 2023, 8, 907−914

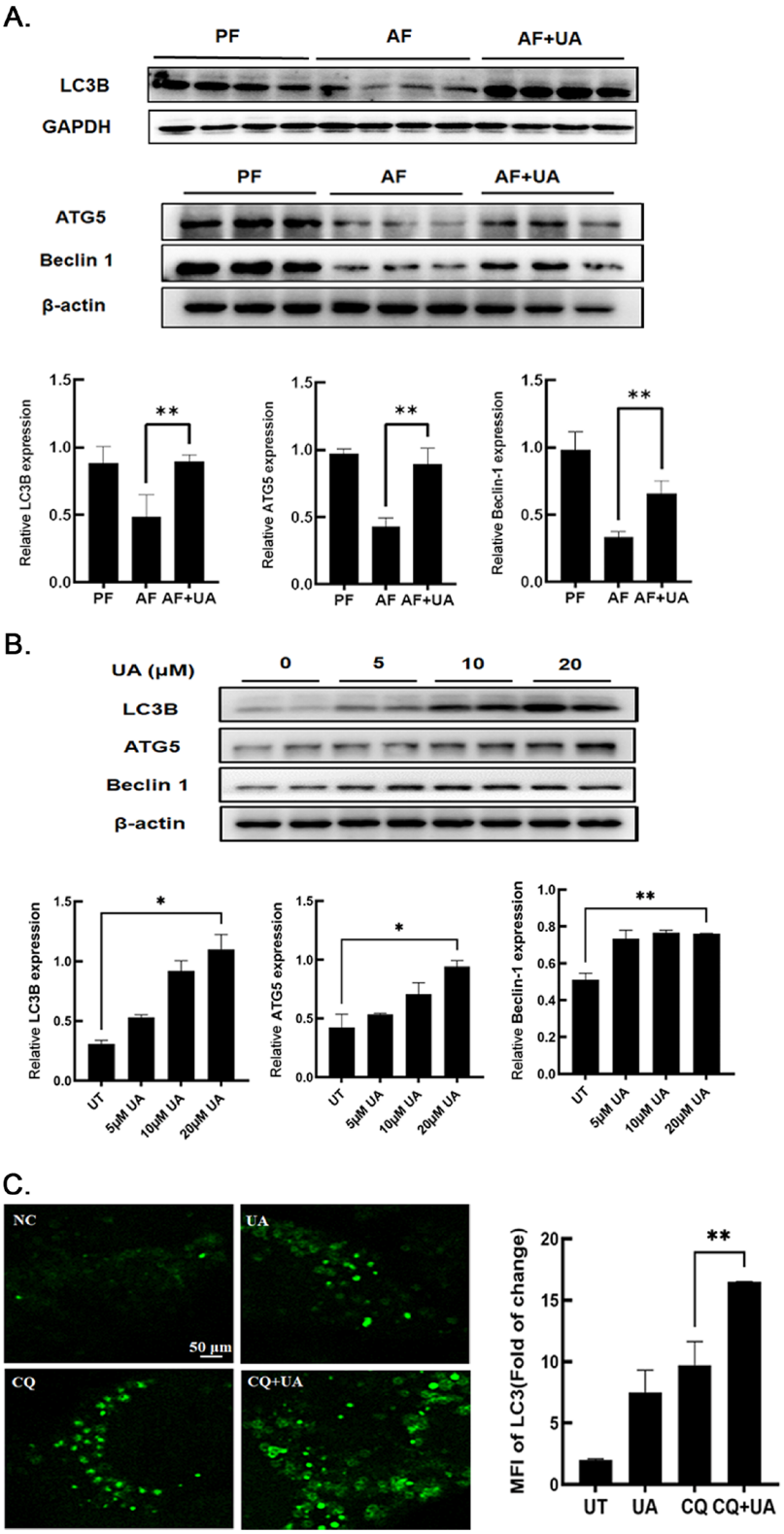

Figure 6. UA treatment activates autophagy in hepatocytes. (A) Protein expression of LC3B, Atg5, and Beclin-1 in various groups of mice as indicated by Western blotting. (B) Protein expression of LC3B, Atg5, and Beclin-1 in AML12 cells treated with different doses (0, 5, 10, 20 μM) of UA. (C) Recombinant adenovirus GFP-LC3 was transfected into HepG2 cells followed by CQ treatment, as indicated. Green puncta were counted. Bars with asterisks differ significantly ($P < 0.05$). PF: pair feeding; AF: alcohol feeding; UA: ursolic acid.

diseases.[26,27] The effects of alcohol on autophagy activation in liver remain unclear and appear to be dependent on dose and drinking patterns. Interestingly, while acute ethanol intake activated autophagy in the liver, chronic exposure to alcohol liquid diet inhibited hepatic autophagy activation. However, in both models, enhanced autophagy activation through chemical activators can prevent alcohol-induced liver injury, indicating that autophagy plays a pathological role in ALD.[28,29] UA is a

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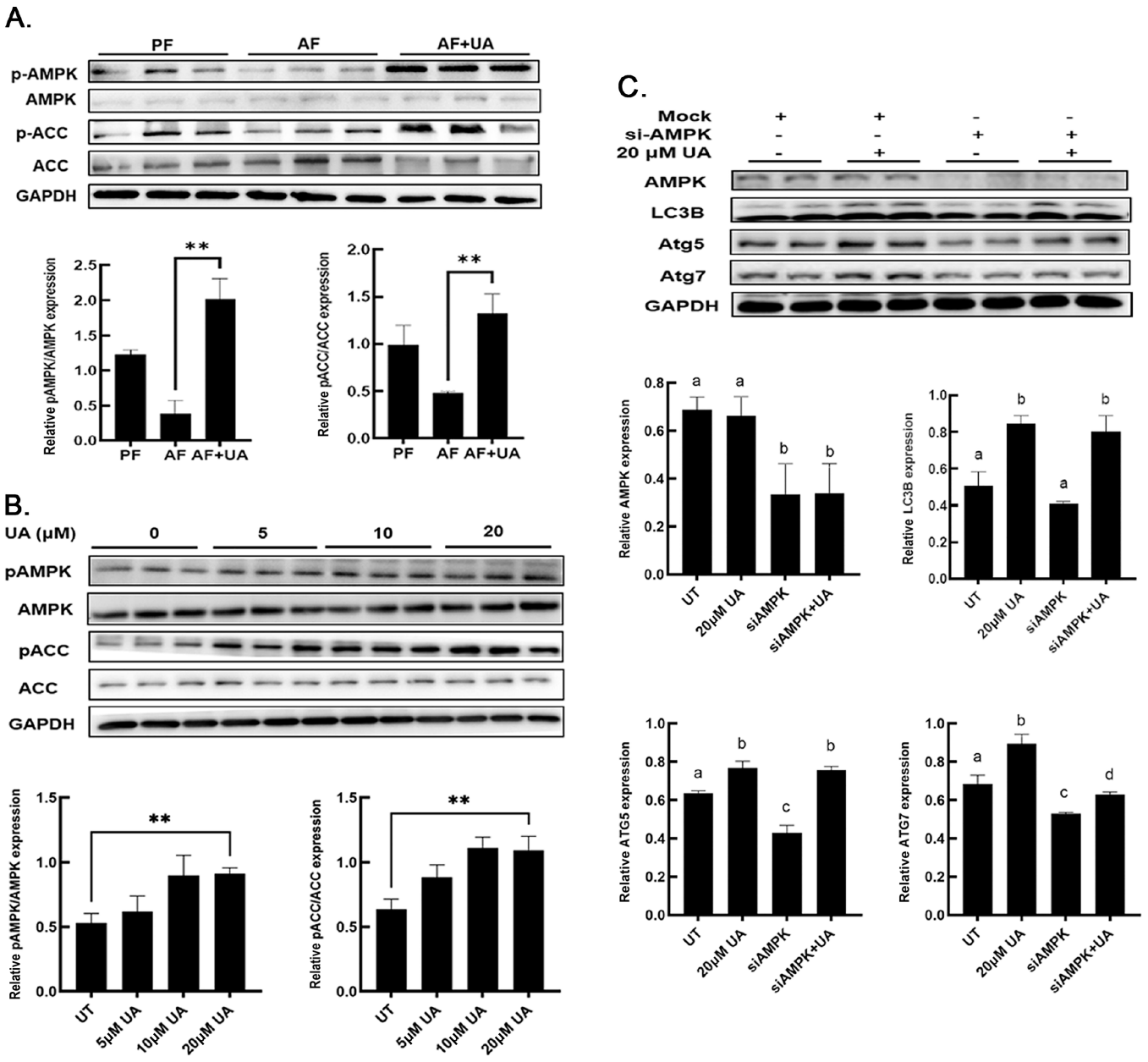

Figure 7. UA activates the AMPK−ACC pathway to induce autophagy. (A) pAMPK/AMPK and pACC/ACC expressions were detected in various groups of mice as indicated by Western blotting, respectively (n = 8). (B) Protein expression of pAMPK/AMPK and pACC/ACC in HepG2 cells treated with UA (5, 10, 20 μM). (C) HepG2 cells were transfected with siAMPK or scramble siRNA and treated with or without UA (20 μM) for 12 h. Immunoblotting assay for AMPK and LC3B, Atg5, and Atg7. Bars with different letters differ significantly ($P < 0.05$). PF: pair feeding; AF: alcohol feeding; UA: ursolic acid.

natural lipophilic pentacyclic triterpenoid found in many plants and has a wide range of biological functions, such as anti-oxidation, anti-inflammation, and anti-fibrosis.[30−32] Increasing evidence suggests that UA promoted cancer cell macrophage autophagy.[33−36] Evidence of UA-induced autophagy includes the formation of acidic vesicular organelles, increase of autophagolysosomes, and the accumulation of LC3-II.[37] UA treatment induced autophagy, increased SIRT1 expression, and reduced acetylation of lysine residues on Atg5.[38] UA treatment induced autophagy, but the downstream signaling pathway was blocked. In the present study, UA induced both primary macroautophagy from experimental mice and AML12 cell line autophagy occurrence by increasing LC3B as well as ATG5 and Beclin-1 expression (Figure 6). In addition, the changes in cell lines were significantly reversed by autophagy inhibitor CQ. More importantly, AF group mice blocked the AMPK−ACC pathway and reduced autophagy induction, hinting that autophagy induced by UA suppressed autophagy activation in the liver by regulating the AMPK−ACC pathway (Figure 7), whereas AMPK siRNA knockdown inhibited autophagy activation (Figure 7), suggesting that UA can activate the AMPK−ACC pathway to regulate autophagy activation.

In summary, the present study provides original evidence that UA administration protects alcohol-induced liver injury in ALD. Our data demonstrate that UA supplementation ameliorates chronic alcohol consumption-induced steatosis and liver injury, inflammation, and oxidative stress. Mechanistically, UA reversed alcohol-decreased autophagy activation via the AMPK−ACC

https://doi.org/10.1021/acsomega.2c06252
ACS Omega 2023, 8, 907−914

pathway in hepatocytes. Based on these findings, UA may serve as a potential therapeutic choice for the treatment of ALD.

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Author Contributions

#Y.M. and Q.D. contributed equally to this paper.

Notes

The authors declare no competing financial interest.

This study was carried out in strict accordance with the recommendations from the Guide for the Care and Use of Laboratory Animals of the Chinese Association for Laboratory Animal Science. All animal care and protocols were approved by the Animal Care and Use Committee of the Zhejiang Chinese Medical University. All killings were performed under sodium pentobarbital anesthesia, and efforts were taken to minimize animal suffering.

ACKNOWLEDGMENTS

This study was supported by grants from the National Natural Science Foundation of China (No. 81973041), Medical Science and Technology Project of Zhejiang Province (No. 2022RC127), Science Research Project of Zhejiang Province Education Department (Y202249223), Natural Science Foundation of Zhejiang Province (LR20H260001, LZ21H030001), and Special Project for the Research Institutions of Zhejiang Province (YS2021011).

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https://doi.org/10.1021/acsomega.2c06252
ACS Omega 2023, 8, 907−914