

Inhibition of aldose reductase ameliorates alcoholic liver disease by activating AMPK and modulating oxidative stress and inflammatory cytokines

CHANGXUAN SHI^{1*}, YUANFANG WANG^{1*}, JING GAO^{1*}, SI CHEN¹, XIANGQIAN ZHAO¹,
CHENGCHAO CAI¹, CHANG GUO^{1,2} and LONGXIN QIU¹⁻³

¹Department of Biological Science and Technology, School of Life Sciences; ²Key Laboratory of Preventive Veterinary Medicine and Biotechnology (Longyan University), Fujian Province University;

³Fujian Provincial Key Laboratory for the Prevention and Control of Animal Infectious Diseases and Biotechnology, Longyan, Fujian 364012, P.R. China

Received May 30, 2016; Accepted April 13, 2017

DOI: 10.3892/mmr.2017.6895

Abstract. Aldose reductase (AR) expression is elevated in the livers of patients with alcoholic liver diseases. However, the role of AR in the development of alcoholic liver diseases remains unclear. The aim of the present study was to determine the effect of AR inhibition on ethanol-induced hepatosteatosis *in vivo* and *in vitro*, and to identify possible underlying molecular mechanisms. Alcoholic fatty livers were induced in C57BL/6 mice by feeding the mice with Lieber-DeCarli liquid diets. The expression of AR protein was elevated in the liver tissue of C57BL/6 mice fed with an ethanol diet and in mouse AML12 liver cells exposed to ethanol. In addition to the elevation in AR, hepatic steatosis was observed in ethanol diet-fed mice, and this ethanol-induced steatosis was significantly attenuated by inhibiting AR activity with a specific inhibitor, zopolrestat. The suppressive effect of AR inhibition was associated with decreased levels of hepatic lipoperoxides, decreased protein expression of hepatic cytochrome P450 2E1 (CYP2E1), increased phosphorylation of 5'-AMP-activated protein kinase (AMPK) and decreased mRNA expression of tumor necrosis factor- α (TNF- α). Treatment with the AR inhibitor attenuated the level of lipid accumulation and oxidative stress, activated AMPK, and suppressed the mRNA expression of TNF- α , interleukin-6 and transforming growth

factor- β 1 in ethanol-treated AML12 cells. The results of the present study demonstrated that inhibition of AR ameliorated alcoholic liver disease *in vivo* and *in vitro*, in part by activating AMPK, decreasing CYP2E1-mediated oxidative stress and ameliorating the expression of pro-inflammatory cytokines.

Introduction

Alcohol is a widely-consumed hepatic toxicant, and excessive alcohol consumption may lead to acute and chronic fatty liver diseases, including steatosis, steatohepatitis, liver fibrosis and liver cirrhosis. Although the pathogenesis of alcoholic liver disease (ALD) remains to be fully elucidated, oxidative stress is considered to be one of the principal factors responsible for alcoholic liver damage (1). Previous studies have demonstrated that ethanol-induced oxidative stress may lead to the production of cytokines and chemokines, including tumor necrosis factor (TNF)- α , transforming growth factor (TGF)- β 1 and reactive oxygen species (ROS), which are hypothesized to serve a role in the development of ALD (1,2).

Aldose reductase (AR; additionally termed AKR1B or EC1.1.1.21) catalyzes the reduction of glucose to sorbitol with the aid of the co-factor reduced nicotinamide adenine dinucleotide phosphate, in the polyol pathway (3). The role of AR in the development of complications in diabetes is well-established (4). Additionally, hepatic AR expression has been observed to be induced in a number of liver disease conditions, including alcoholic liver disease, non-alcoholic fatty liver disease, hepatitis, cirrhosis and hepatocellular carcinoma (5-8). It was previously reported that overexpression of AR induced the production of ROS and TNF- α in liver cells, and that administration of the AR inhibitor zopolrestat attenuated methionine-choline-deficient diet-induced hepatosteatosis and liver inflammation (8,9). However, the role of AR in the development of ALD remains unknown. Therefore, the aim of the present study was to investigate the effect of AR inhibition on ethanol-induced hepatic steatosis and injury in mice and AML12 mouse liver cells, and to investigate the mechanism through which AR affects the development of ALD.

Correspondence to: Professor Longxin Qiu, Department of Biological Science and Technology, School of Life Sciences, Longyan University, 1 North Dongxiao Road, Longyan, Fujian 364012, P.R. China
E-mail: qlongxin@tom.com

*Contributed equally

Key words: aldose reductase, alcoholic liver disease, 5'-AMP-activated protein kinase, oxidative stress, inflammatory cytokines

Materials and methods

Animal experiments. Male Kunming mice (age, 6 weeks; weight, 22–25 g; total $n=40$) were obtained from the Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China) and 5 mice/cage were maintained under a 12-h light/dark schedule, with a temperature range of 20 to 24°C. Experiments were conducted according to protocols and guidelines approved by the Longyan University Institutional Animal Care and Use Committee (Longyan, China). Liquid diets were based on the modified Lieber-DeCarli formulation and provided 1 kcal/ml (prepared by Trophic Animal Feed High-tech Co., Ltd., Nantong, China). The ethanol diet consisted of 18% of the total energy as protein, 35% as fat, 19% as carbohydrate and 28% as ethanol. In the control diet, ethanol was replaced isocalorically with carbohydrate. Following a 1-week period of adaptation to the environment with free access to the liquid diet, mice were randomly divided into four experimental groups ($n=10$ mice/group): Control diet-fed mice; control diet-fed mice + zopolrestat (zopol); ethanol diet-fed mice and ethanol diet-fed mice + zopol. Mice were administered 50 mg/kg/day zopol as a single daily intraperitoneal injection for 4 weeks. Equivalent volumes of saline were administered to the control groups of mice. On the final day, mice were sacrificed under anesthesia. Tissues were snap-frozen in liquid nitrogen immediately following resection and stored at -80°C until further analysis.

Histological examination. Liver tissues were fixed in 10% formalin for 24 h at room temperature and were routinely embedded in paraffin. Briefly, tissues were rinsed in tap water for 5 min at room temperature prior to dehydration with a series of alcohol (70, 80 and 90%, respectively, for 5 min each, followed by 3 incubations with 100% alcohol for 5 min each at room temperature). The tissues were then treated twice with xylene for 5 min each at room temperature, prior to paraffin embedding. Sections (5- μm) were deparaffinized in xylene twice for 5 min each and treated with 100% alcohol twice for 3 min, then 95, 70 and 50% alcohol, respectively, for 3 min each at room temperature. Sections were stained with hematoxylin and eosin (H&E) for histological analysis. Hepatic steatosis was graded as previously described (10), according to the percentage of lipid-laden hepatocytes: 0, 0%; 1, 0–25%; 2, 26–50%; 3, 51–75% and 4, 76–100%.

Cell culture. AML12 mouse hepatocyte cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium/Ham's F12 containing 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin and 10% fetal bovine serum (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA), in a 37°C incubator with 5% CO_2 .

Detection of ROS generation in AML12 cells. AML12 cells were plated on 24-well plates at a density of $\sim 1 \times 10^5$ cells/well. Following 24 h of incubation, cells were exposed to 100 mM ethanol and co-treated with 50 μM zopol. A total of 36 h subsequent to ethanol and zopol treatment, ROS generation in cells was determined using ROS assay kit (cat no S0033; Beyotime Institute of Biotechnology, Haimen, China), according to the manufacturer's instructions.

Tissue and cell lipid peroxidation assays. Total lipoperoxides were measured as thiobarbituric acid reactive substances (TBARS) in liver homogenates or cell lysates, using a lipid peroxidation malondialdehyde (MDA) assay kit (cat no S0131; Beyotime Institute of Biotechnology), according to the manufacturer's instructions. TBARS were quantified using MDA as a standard and Microsoft Excel 2013 (Microsoft Corporation, Redmond, WA, USA).

Quantitative analysis of mRNA expression using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from tissues and cells using TRIpure reagent (Roche Applied Science, Mannheim, Germany), according to the manufacturer's protocol. cDNA was synthesized from mRNA using RevertAid First Strand cDNA Synthesis kits (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Expression levels of hepatic TNF- α , TGF- β 1, interleukin (IL)-6 and sterol regulatory element binding protein (SREBP)-1c were analyzed. The specific primers used were: Forward, 5'-CGTGCTCCTCACCCACAC-3' and reverse, 5'-GGGTTTCATACCAGGGTTTGA-3' for TNF- α ; forward, 5'-ACAACCACGGCCTTCCCTACTT-3' and reverse, 5'-GTGTAATTAAGCCTCCGACT-3' for IL-6; forward, 5'-CAACTTCTGTCTGGGACCCT-3' and reverse, 5'-TAGTAGACGATGGGCAGTGG-3' for TGF- β 1; forward, 5'-ATCGGCGCGGAAGCTGTCTGGGGTAGCGTC-3' and reverse, 5'-ACTGTC TTGGTTGTTGATGAGCTGGAGCAT-3' for SREBP-1c and forward, 5'-CTATTGGCAACGAGCGGTTCC-3' and reverse, 5'-GCACTGTGTTGGCATAGAGGTC-3' for β -actin. qPCR was performed using the FastStart Universal SYBR-Green Master Mix (Roche Applied Science). The reaction was performed with the following thermocycling conditions: 95°C for 10 min, followed by 35 cycles of 95°C for 15 sec, 53–59°C for 30 sec and 72°C for 30 sec, then a final extension of 72°C for 10 min. The $2^{-\Delta\Delta\text{C}_q}$ method (11) was used for quantification and the data were normalized to β -actin.

Western blot analysis. Tissues and cells were homogenized in ice-cold radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology). Protein concentrations of the extracts were measured using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology), according to the manufacturer's protocol. A total of 40 μg of each protein sample was loaded and separated using 10–12% SDS-PAGE and transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). Membranes were blotted with 5% non-fat milk in TBS containing 0.1% Tween-20 for 1 h at room temperature. Membranes were then incubated with anti-AR (cat no. sc-17735; 1:500; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-cytochrome P450 2E1 (cat no. ab28146; 1:1,000; CYP2E1; Abcam, Cambridge, UK), anti-phosphorylated AMPK (cat no. 2535; 1:1,000), anti-AMPK (cat no. 2532; 1:1,000) (both from Cell Signaling Technology), anti-GAPDH (cat no. G8795; 1:2,000) or anti- β -actin (cat no. A2228; 1:2,000) (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 4°C overnight, and subsequently with horseradish peroxidase-conjugated anti-goat or anti-rabbit immunoglobulin G (cat nos. AP307P and AB324P, respectively; 1:2,000; Sigma-Aldrich; Merck KGaA) for 2 h at room temperature. Detection was achieved using the

SuperSignal Chemiluminescent Substrate kit (Beyotime Institute of Biotechnology).

Statistical analysis. All data were processed and analyzed using GraphPad Prism software, version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA) and were expressed as the mean \pm standard error of the mean. One-way analysis of variance and the post hoc Newman-Keuls multiple comparison test was used for multi-group analyses. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

AR is induced in the livers of ethanol diet-fed mice and in ethanol-treated mouse AML12 liver cells, and inhibition of AR activity attenuates ethanol-induced hepatic steatosis. Previous studies demonstrated that feeding C57BL/6 mice with Lieber-DeCarli ethanol diets induced fatty liver within 4–8 weeks (12). In order to investigate whether AR was involved in the development of ethanol diet-induced hepatosteatosis, the protein expression levels of hepatic AR were analyzed in mice fed with the ethanol diet. As presented in Fig. 1A, hepatic AR protein expression in C57BL/6 mice fed with the ethanol diet for 4 weeks was increased, compared with mice fed with the control diet. In addition to the induction of AR expression in mouse liver tissue, an increase in AR protein expression was observed in hepatocytes exposed to 100 mM ethanol for 36 h (Fig. 1B).

In order to further determine the role of AR in the development of ethanol-induced hepatosteatosis, AR activity was inhibited by treating mice with an AR-specific inhibitor, zopol. As presented in Fig. 2A and B, examination of H&E-stained sections demonstrated marked hepatic steatosis in mice fed with the ethanol diet for 4 weeks, while mice fed with the control diet exhibited low-grade steatosis. Following inhibition of AR activity, hepatic steatosis in mice fed with the ethanol diet was significantly attenuated ($P < 0.05$).

AR inhibition decreases ethanol-induced hepatic oxidative stress in vivo and in vitro. In order to clarify the mechanism through which AR is associated with the development of ethanol-induced steatosis, the effect of AR inhibition on hepatic lipoperoxide production was investigated. As presented in Fig. 3A, consumption of the ethanol diet resulted in an increase in hepatic TBARS levels, compared with the control diet, and the increase was attenuated significantly by treating the ethanol diet-fed mice with zopol ($P < 0.001$). The protein expression of CYP2E1, a principal mediator of lipid peroxidation (13,14), was induced by ethanol, and AR inhibition significantly prevented the induction of CYP2E1 (Fig. 3B). In addition, the protective effect of AR inhibition on ethanol-induced oxidative stress was analyzed *in vitro*. As presented in Fig. 3C and D, inhibition of AR resulted in a 33.0% decrease in MDA levels ($P < 0.001$) and a 33.9% decrease in ROS levels ($P < 0.05$) in ethanol-treated AML12 cells. However, CYP2E1 protein expression was not detected in AML12 cells. The results of the present study demonstrated that the induction of AR by ethanol may promote hepatic oxidative stress in mice with alcoholic liver disease, and that the increased hepatic oxidative stress is due in part to the AR-mediated induction of CYP2E1.

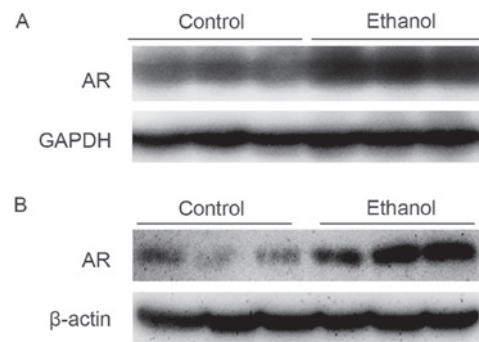


Figure 1. Hepatic AR is induced by ethanol *in vivo* and *in vitro*. Representative western blot analyses demonstrate the induction of hepatic AR protein expression in (A) ethanol-fed C57BL/6 mice and (B) ethanol-treated AML12 cells. AR, aldose reductase.

AR inhibition activates AMPK and suppresses SREBP-1c mRNA expression. Previous studies demonstrated that AMPK inactivation was associated with the development of ethanol-induced hepatic steatosis (15,16). In order to clarify the mechanisms whereby AR affects lipid accumulation in hepatocytes, the effect of AR inhibition on AMPK activity was investigated. As presented in Fig. 4A, phosphorylated AMPK protein expression levels in the livers of ethanol-fed mice treated with zopol were increased compared with ethanol-fed mice, indicating that AR inhibition activated hepatic AMPK in ethanol-fed mice. The activation on AMPK by the AR inhibitor was additionally confirmed in ethanol-treated AML12 cells (Fig. 4B). The effect of AR inhibition on the mRNA expression of SREBP-1c, a transcriptional factor regulated by AMPK which controls the synthesis of fatty acids, was assessed in the livers of ethanol-fed mice. As presented in Fig. 4C, AR inhibition did not significantly decrease the mRNA expression levels of SREBP-1c in ethanol-fed mice when compared with the ethanol-fed group. However, AR inhibition significantly decreased the mRNA expression levels of SREBP-1c in ethanol-treated AML12 cells (Fig. 4D).

AR inhibition suppresses the mRNA expression of certain inflammatory mediators in the livers of ethanol diet-fed mice and in hepatocytes. In order to evaluate the effect of AR inhibition on the development of ethanol-induced liver injuries, the expression levels of certain proinflammatory cytokines were assessed, including TNF- α , IL-6 and TGF- β 1. Mice fed on the ethanol diet exhibited a marked elevation of hepatic mRNA expression of TNF- α compared with mice fed on the control diet (Fig. 5A). However, the ethanol-induced TNF- α mRNA elevation was significantly attenuated by 51.4% ($P < 0.01$) in mice treated with zopol. Compared with TNF- α , the effect of AR inhibition on IL-6 and TGF- β 1 was less significant. Consistent with the *in vivo* results, AR inhibition in ethanol-treated AML12 cells resulted in a significant decrease in the mRNA expression levels of TNF- α ($P < 0.01$; Fig. 5B). AR inhibition in ethanol-treated AML12 cells resulted in a significant decrease in the mRNA expression levels of IL-6 ($P < 0.05$) and TGF- β 1 ($P < 0.05$). The results of the present study demonstrated that hepatic AR elevation in mice fed on the ethanol diet may directly affect the production of pro-inflammatory cytokines, particularly TNF- α , in the liver.

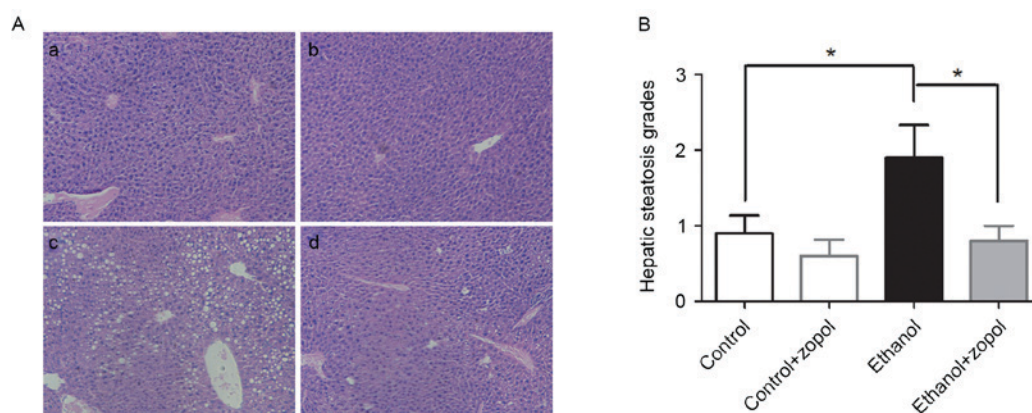


Figure 2. Treatment with an AR inhibitor improves ethanol-induced steatosis in C57BL/6 mice. (A) Hematoxylin and eosin-stained liver sections from mice fed with: (a) Control diet; (b) control diet + zopol; (c) ethanol diet; and (d) ethanol diet + zopol. Slides are representative of eight separate experiments (magnification, $\times 100$). (B) Effect of AR inhibition on scores for hepatic steatosis ($n=8$). Values are expressed as the mean \pm standard error of the mean. $^*P<0.05$. AR, aldose reductase; zopol, zopolrestat.

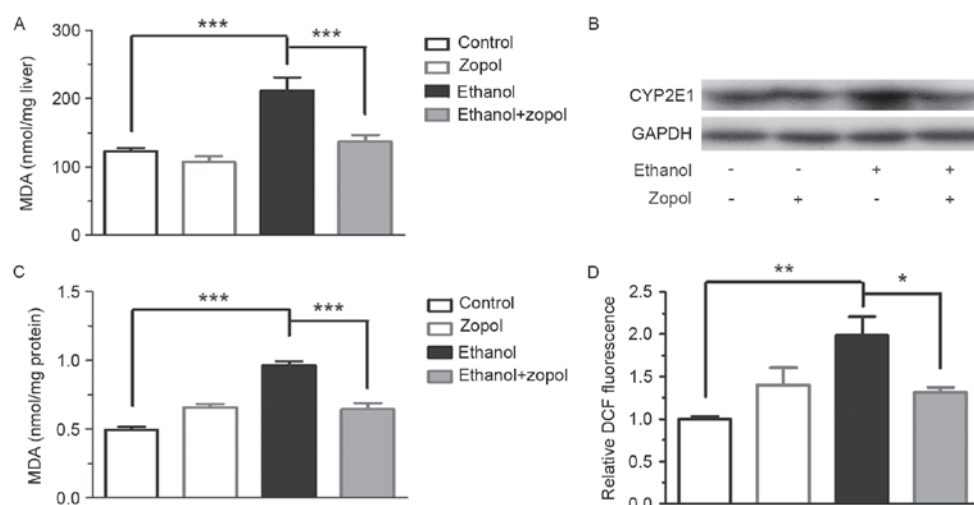


Figure 3. Inhibition of AR attenuates hepatic lipoperoxide content and CYP2E1 expression in ethanol-fed C57BL/6 mice, and attenuates lipoperoxide content and ROS levels in AML12 cells. (A) Hepatic lipoperoxide content and (B) CYP2E1 protein expression were determined in mice fed with the control diet, ethanol diet or ethanol diet treated with zopol for 4 weeks ($n=4$). (C) Lipoperoxide content and (D) ROS levels were assayed in ethanol-treated AML12 cells treated with zopol for 36 h ($n=4$). Values are expressed as the mean \pm standard error of the mean. $^{***}P<0.001$; $^{**}P<0.01$; $^{*}P<0.05$. AR, aldose reductase; CYP2E1, cytochrome P450 2E1; ROS, reactive oxygen species; zopol, zopolrestat; DCF, dichlorofluorescein; MDA, malondialdehyde.

Discussion

The induction of AR expression has been observed in certain liver disease conditions, including alcoholic liver disease and non-alcoholic fatty liver disease (6,8). Inhibition of AR improves the development of hepatic steatosis and liver inflammation in mice with non-alcoholic steatohepatitis (7-9). However, the role of AR in the development of ALD remains unclear. The results of the present study demonstrated that hepatic AR protein was induced in ethanol diet-induced hepatosteatosis in C57BL/6 mice, and in ethanol-treated mouse AML12 liver cells. Pharmacological inhibition of AR was conducted in the ethanol diet-fed C57BL/6 mice, to examine the role of AR in the development of ethanol-induced hepatosteatosis. It was demonstrated that AR inhibition improved the development of hepatosteatosis. The results of the present study indicated that AR may be involved in the development of ALD.

The alcohol metabolite acetaldehyde has previously been demonstrated to be one of the important causes of hepatic triglyceride accumulation and hepatocellular injury in alcoholic fatty livers (17). AR is able to catalyze the reduction of a variety of aldehydes and carbonyls (18,19). Therefore, AR has been postulated to serve a cytoprotective function by rapidly detoxifying aldehydes. AR inhibitors have been reported to prevent injuries in a variety of rodent models, including ischemia/reperfusion-induced liver (20), arterial balloon (21) and ischemic myocardial injuries (22). In the present study, it was demonstrated that AR exacerbated hepatocyte injury. As AR possesses the capacity to rapidly detoxify aldehydes, it is notable that AR inhibition prevented hepatocyte injury. The present study demonstrated that AR inhibition prevented the induction of CYP2E1 in ethanol diet-fed C57BL/6 mice. CYP2E1 serves an important role in the pathogenesis of ALD (13,14,23). CYP2E1 is able to catalyze the reduction of alcohol to acetaldehyde in the liver, resulting in the generation

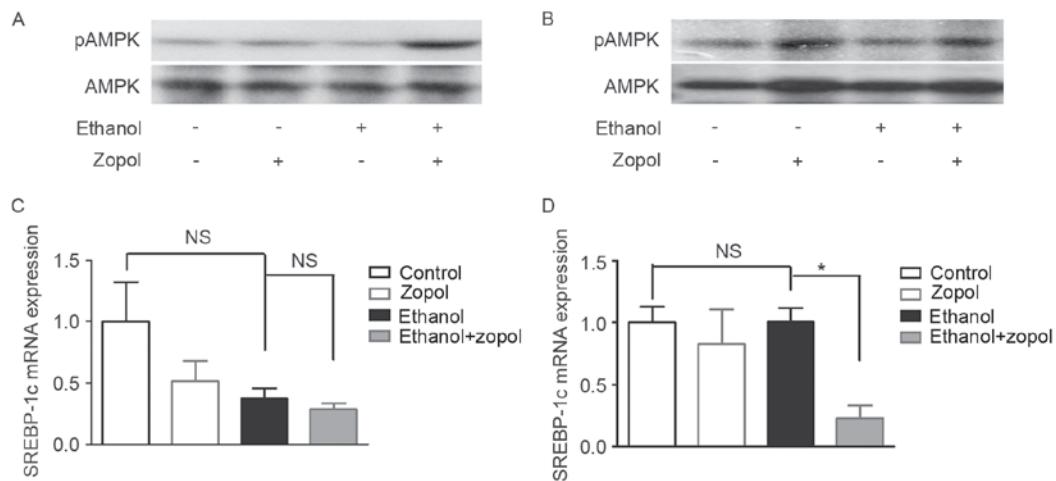


Figure 4. Effect of AR inhibition on the activation of AMPK and the mRNA expression of SREBP-1c. Activation of AMPK in (A) zopol-treated mice fed with ethanol diet and (B) zopol-treated AML12 liver cells exposed to ethanol was determined using western blotting. mRNA expression of SREBP-1c in (C) zopol-treated mice fed with ethanol diet and (D) zopol-treated AML12 liver cells exposed to ethanol was analyzed using reverse transcription-quantitative polymerase chain reaction, standardized against an internal control (β -actin). Data are expressed as fold differences compared with values obtained in control cells (n=4). Values are expressed as the mean \pm standard error of the mean. *P<0.05. NS, not significant; SREBP-1c, sterol regulatory element binding protein-1c; AR, aldose reductase; AMPK, 5'-AMP-activated protein kinase; zopol, zopolrestat; p, phosphorylated.

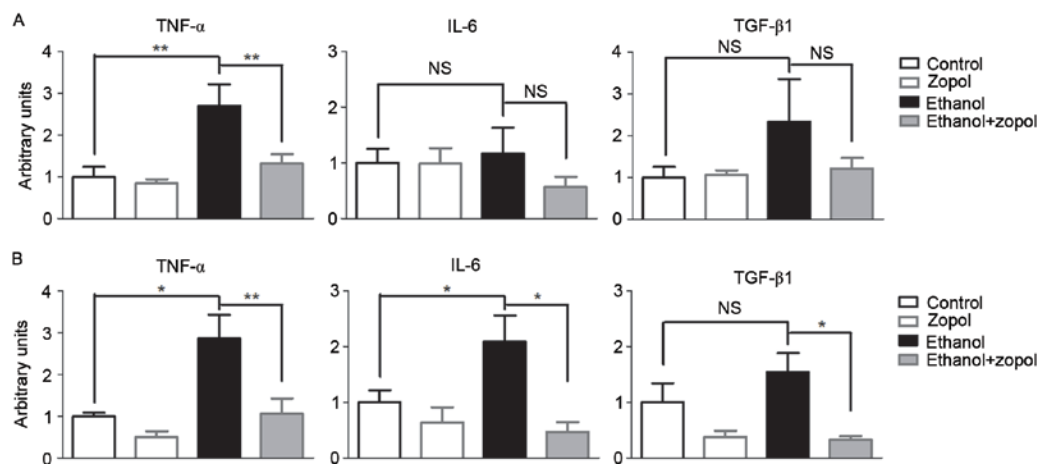


Figure 5. AR inhibition attenuates the ethanol-induced TNF- α mRNA expression in C57BL/6 mice and AML12 cells. mRNA expression levels of TNF- α , IL-6 and TGF- β 1 were analyzed in (A) C57BL/6 mice and (B) AML12 cells using reverse transcription-quantitative polymerase chain reaction, standardized against an internal control (β -actin). Data are expressed as fold differences compared with values obtained in control cells (n=4). Values are expressed as the mean \pm standard error of the mean. **P<0.01; *P<0.05. NS, not significant; AR, aldose reductase; TNF, tumor necrosis factor; IL, interleukin; TGF, transforming growth factor; zopol, zopolrestat.

of ROS, including the superoxide anion, hydrogen peroxide and the hydroxyl radical. The generation of ROS may initiate lipid peroxidation in the liver (13). The results of the present study suggested that the elevation of AR resulted in the induction of hepatic CYP2E1 expression in mice with ALD and, consequently, exacerbated lipid peroxidation and oxidative stress, and affected the development of ALD. Therefore, the prevention of the induction of hepatic CYP2E1 in mice with ALD may be part of the mechanism via which AR inhibition attenuates lipid peroxidation and ameliorates ALD.

AMPK is an enzyme that serves a role in cellular energy homeostasis. The effect of AMPK inhibition is suppression of fatty acid oxidation and stimulation of lipogenesis (24). Ethanol has been reported to suppress hepatic AMPK activity, and the inhibition of AMPK by ethanol serves an important role in the development of hepatosteatosis induced by chronic alcohol

consumption (15,16). In the present study, it was demonstrated that AR inhibition activated AMPK in ethanol-treated AML12 liver cells and in the livers of mice fed with ethanol. Therefore, activation of hepatic AMPK may be part of the mechanism via which AR inhibition ameliorates ethanol-induced hepatosteatosis.

It is well-known that chronic alcohol consumption increases TNF- α production and leads to hepatosteatosis and liver injury (2). Studies have demonstrated that the expression of TNF- α is elevated in patients with alcoholic liver diseases (25,26). In addition, TNF- α has been observed to induce lipid accumulation, and result in apoptosis and inflammation in liver cells (27). Therefore, the improvement of steatosis and liver injury in alcoholic livers by treatment with an AR inhibitor may be partially attributed to an inhibitory effect on ethanol-induced TNF- α mRNA overexpression.

In conclusion, the present study demonstrated that AR inhibition significantly ameliorated alcohol-induced hepatic steatosis in mouse AML12 liver cells and in Kunming mice, potentially by activating AMPK, decreasing CYP2E1-mediated oxidative stress and suppressing the expression of pro-inflammatory cytokines. These results indicate that AR inhibitors may have potential as alternative therapeutic strategies for the treatment of alcoholic fatty liver diseases.

Acknowledgements

The present study was supported in part by the Training Program of Fujian Excellent Talents in University (grant no. MJR201558) and the Science Planning Program of Longyan University (grant no. LG2014012).

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