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Alpha-lipoic acid improves high-fat diet-induced hepatic steatosis by modulating the transcription factors SREBP-1, FoxO1 and Nrf2 via the SIRT1/LKB1/AMPK pathway☆

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Abstract

Understanding the mechanism by which alpha-lipoic acid supplementation has a protective effect upon nonalcoholic fatty liver disease *in vivo* and *in vitro* may lead to targets for preventing hepatic steatosis. Male C57BL/6J mice were fed a normal diet, high-fat diet or high-fat diet supplemented with alpha-lipoic acid for 24 weeks. HepG2 cells were incubated with normal medium, palmitate or alpha-lipoic acid. The lipid-lowering effects were measured. The protein expression and distribution were analyzed by Western blot, immunoprecipitation and immunofluorescence, respectively. We found that alpha-lipoic acid enhanced sirtuin 1 deacetylase activity through liver kinase B1 and stimulated AMP-activated protein kinase. By activating the sirtuin 1/liver kinase B1/AMP-activated protein kinase pathway, the translocation of sterol regulatory element-binding protein-1 into the nucleus and forkhead box O1 into the cytoplasm was prevented. Alpha-lipoic acid increased adipose triacylglycerol lipase expression and decreased fatty acid synthase abundance. In *in vivo* and *in vitro* studies, alpha-lipoic acid also increased nuclear NF-E2-related factor 2 levels and downstream target amounts via the sirtuin 1 pathway. Alpha-lipoic acid eventually reduced intrahepatic and serum triglyceride content. The protective effects of alpha-lipoic acid on hepatic steatosis appear to be associated with the transcription factors sterol regulatory element-binding protein-1, forkhead box O1 and NF-E2-related factor 2.

Keywords: Alpha-lipoic acid; FoxO1; Nonalcoholic fatty liver disease; Nrf2; SIRT1; SREBP-1

1. Introduction

Nonalcoholic fatty liver disease (NAFLD), characterized by excessive triglyceride (TG) accumulation in the liver, is becoming increasingly prevalent in Asian-Pacific regions due to Westernization [1]. The central role of lipid accumulation in the pathogenesis of NAFLD has been confirmed in many clinical correlation studies and animal models [2]. Therapeutic options targeting hepatic lipid metabolism are therefore crucial to the management of NAFLD.

Sirtuin 1 (SIRT1) and AMP-activated protein kinase (AMPK) are known fuel-sensing molecules that modulate lipid metabolism [3]. AMPK serves as a cellular energy sensor and is activated by increased AMP/ATP ratio or by the upstream kinases liver kinase B1 (LKB1), Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK) and transforming growth

Abbreviations: ACC, acetyl-CoA carboxylase; ALA, alpha-lipoic acid; ALT, alanine aminotransferase; AICAR, 5-aminoimidazole-4-carboxamide riboside; AMPK, AMP-activated protein kinase; AST, aspartate aminostransferase; ATGL, adipose triacylglycerol lipase; CaMKK, Ca^{2+}/calmodulin-dependent protein kinase kinase; CAT, catalase; CC, compound CC; DAPI, 4′,6-diamidino-2-phenylindole; DBC1, deleted in breast cancer-1; DHE, dihydroethidium; FAS, fatty acid synthase; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; FoxO1, forkhead box O1; HDL, high-density lipoprotein; H&E, hematoxylin and eosin; HFD, high-fat diet; HMG-CoA synthase, 3-hydroxy-3-methylglutaryl-coenzyme A synthase; HO-1, hemeoxygenase-1; IP, immunoprecipitation; LDL, low-density lipoprotein; LKB1, liver kinase B1; NA, nicotinamide; NAFLD, nonalcoholic fatty liver disease; ND, normal diet; NEFA, nonesterified fatty acids; NF-κB, nuclear factor-κB; Nrf2, NF-E2-related factor 2; PGC-α, peroxisome proliferator-activated receptorγcoactivator1α; PVDF, polyvinylidene difluoride; ROS, reactive oxygen species; SIRT1, sirtuin 1; SOD, superoxide dismutase; SRE-1, sterol regulatory element 1; SREBP-1, sterol regulatory element-binding protein-1; TAK1, transforming growth factor-β-activated kinase-1; TC, total cholesterol; TG, triglyceride.

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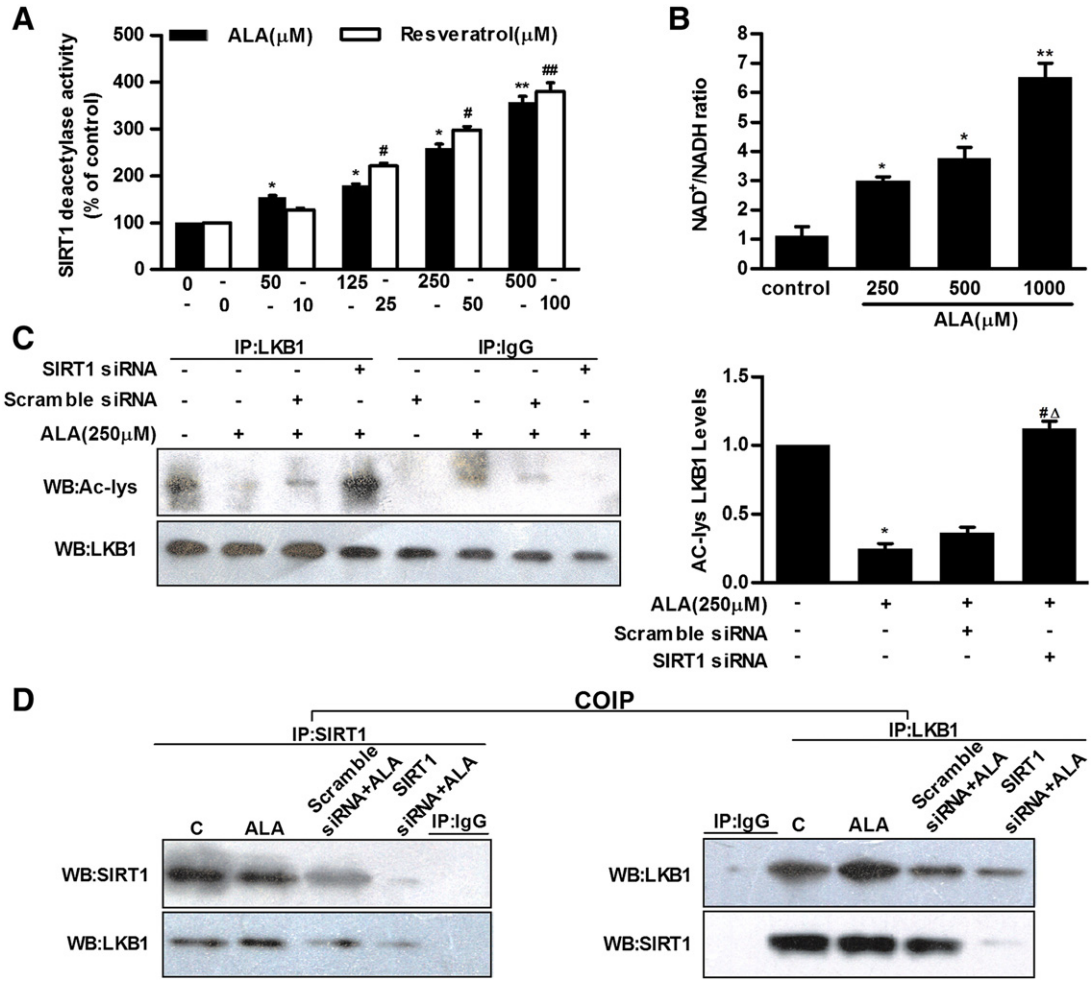


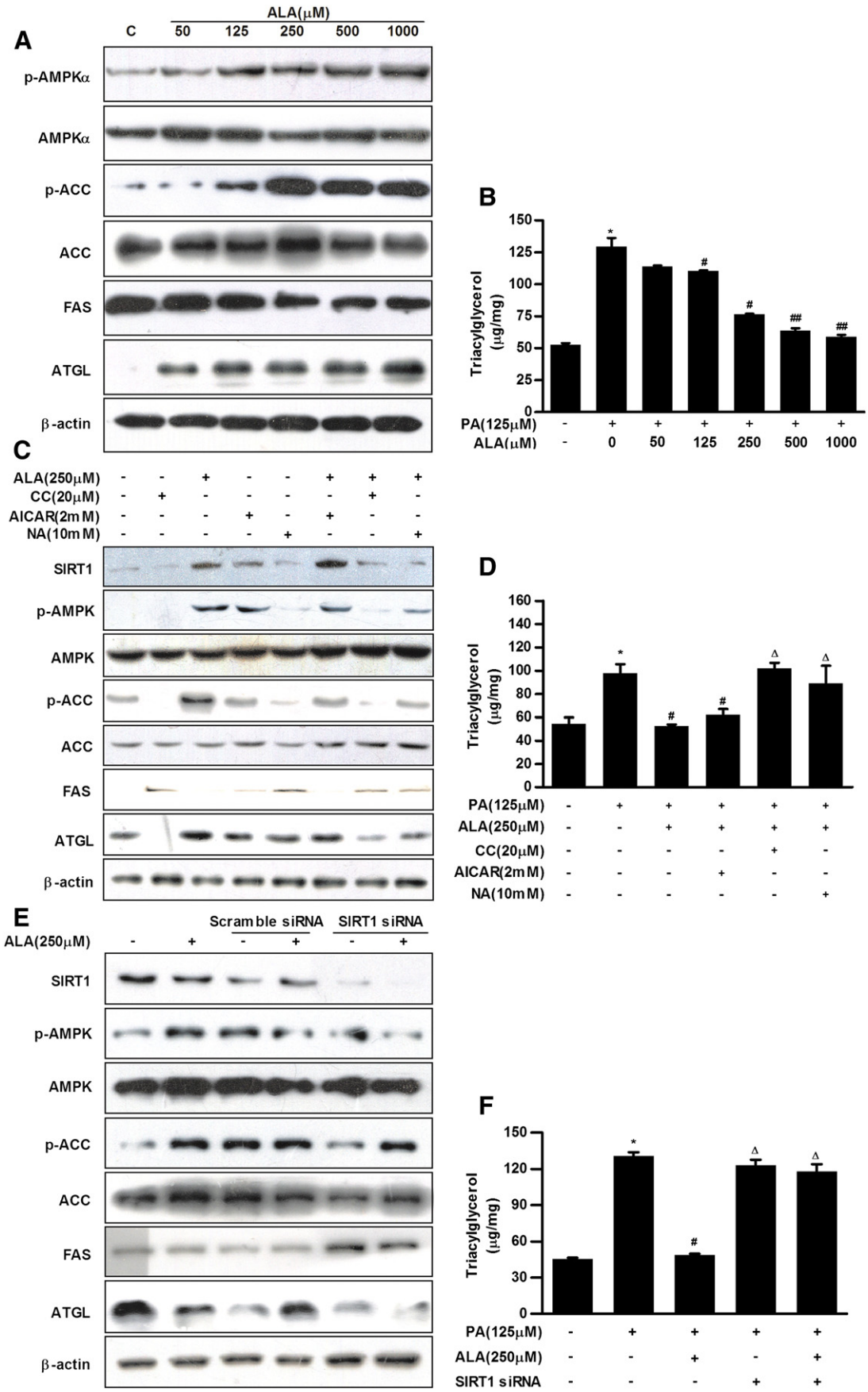
Fig. 1. ALA activates SIRT1/LKB1 pathway in HepG2 cells. (A) The dose–response effect of ALA on SIRT1 deacetylase activity. HepG2 cells were treated with 0, 50, 125, 250 and 500 μM ALA, or 0, 10, 25, 50 and 100 μM resveratrol used as a positive control for 24 h. Data are presented as mean±S.E.M. (n=5). *P<.05, **P<.01 vs. control (0 μM ALA); #P<.05, ##P<.01 vs. control (0 μM resveratrol). (B) Intracellular NAD^+/NADH ratio. HepG2 cells were treated with 0 (control), 250, 500 and 1000 μM ALA for 24 h. Data are presented as mean±S.E.M. (n=6). *P<.05, **P<.01 vs. control (0 μmol ALA). HepG2 cells were transfected with SIRT1siRNA or scramble siRNA for 24 h after incubation with ALA (250 μM, 6 h). (C) IP of acetylated liver kinase B1 (LKB1). *P<.05 vs. control (untreated HepG2 cells); #P<.05 vs. ALA group; ΔP<.05 vs. ALA+scramble siRNA group. (D) COIP of SIRT1 and LKB1. Nonspecific IgG was used as control.

factor-β-activated kinase-1 (TAK1) [4]. SIRT1 activation by polyphenols represents an upstream regulator in the LKB1/AMPK signaling axis [3]. Activation of SIRT1/AMPK signaling plays a central role in regulating hepatic fatty acid metabolism [5]. It abrogates ectopic fat accumulation by facilitating fatty acid oxidation and curbing the *de novo* fatty acid synthesis largely through deacetylation and phosphorylation, respectively, of transcription factors or coactivators, such as p53, forkhead box O (FoxO), nuclear factor-κB (NF-κB), peroxisome proliferator-activated receptorγcoactivator1α (PGC-α) and sterol regulatory element-binding protein 1 (SREBP-1) [6,7].

The transcription factor FoxO1 is regulated by dephosphorylation or deacetylation that causes its nuclear translocation to induce transcription of the rate-limiting enzymes of lipolysis, such as adipose triglyceride lipase (ATGL) [8]. In lipid metabolism, SREBP-1 up-regulates the expression of *de novo* lipogenesis via fatty acid synthase (FAS) [9]. SIRT1/AMPK signaling is intimately associated with the activation or inactivation of FoxO1 and SREBP-1 and therefore represents attractive targets in the development of therapies to repress hepatic steatosis.

Several lines of evidence suggest that chronic oxidative stress plays an important role in the progression of fatty liver due to a close link between dysregulated lipid homeostasis and oxidative stress [10]. The transcription factor NF-E2-related factors 2 (Nrf2) plays a central role in the defense against oxidative stress. Upon oxidative insults, Nrf2 translocates to the nucleus where it interacts with antioxidant response element (ARE) to mediate the transcription of its target genes, such as hemeoxygenase-1 (HO-1), superoxide dismutase (SOD), glutathione peroxidase and catalase [11]. The transcription of these genes enhances cellular resistance against oxidative stress. Previous studies have shown that Nrf2 plays an important role in the protection of hepatocytes from NAFLD [12]. SIRT1 is also known to markedly protect cells from oxidative

Fig. 2. ALA suppresses intracellular accumulation of lipids through the SIRT1/LKB1/AMPK signaling pathway in HepG2 cells. (A) HepG2 cells were treated with 0 (control, C), 50, 125, 250, 500 and 1000 μM ALA for 6 h. Protein expression of AMPK, p-AMPK, ACC, p-ACC, FAS and ATGL was determined by Western blot. (B) Measurement of intracellular triacylglycerol contents. HepG2 cells were treated with 125 μM PA and different concentrations of ALA for 12 h. (C) Effect of ALA (250 μM, 6 h) on protein expression levels in the presence or absence of AMPK inhibitor (CC, 20 μM, 0.5 h), SIRT1 inhibitor (NA, 10 mM, 12 h) and AMPK activator (AICAR, 2 mM, 1 h), respectively. (D) Measurement of intracellular triacylglycerol contents in the presence or absence of different activators and inhibitors. (E) HepG2 cells were transfected with SIRT1siRNA or scramble siRNA for 24 h after incubation with ALA (250 μM, 6 h). (F) Measurement of intracellular triacylglycerol in the presence or absence of SIRT1 siRNA. All data are presented as mean±S.E.M. (n=6). *P<.05 vs. control (untreated cells); #P<.05, ##P<.01 vs. PA group; ΔP<.05 vs. PA+ALA group.


A
ALA(μM)
C
50
125
250
500
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p-AMPKα
AMPKα
p-ACC
ACC
FAS
ATGL
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B
Triacylglycerol (μg/mg)
PA(125μM)
ALA(μM)
C
ALA(250μM)
CC(20μM)
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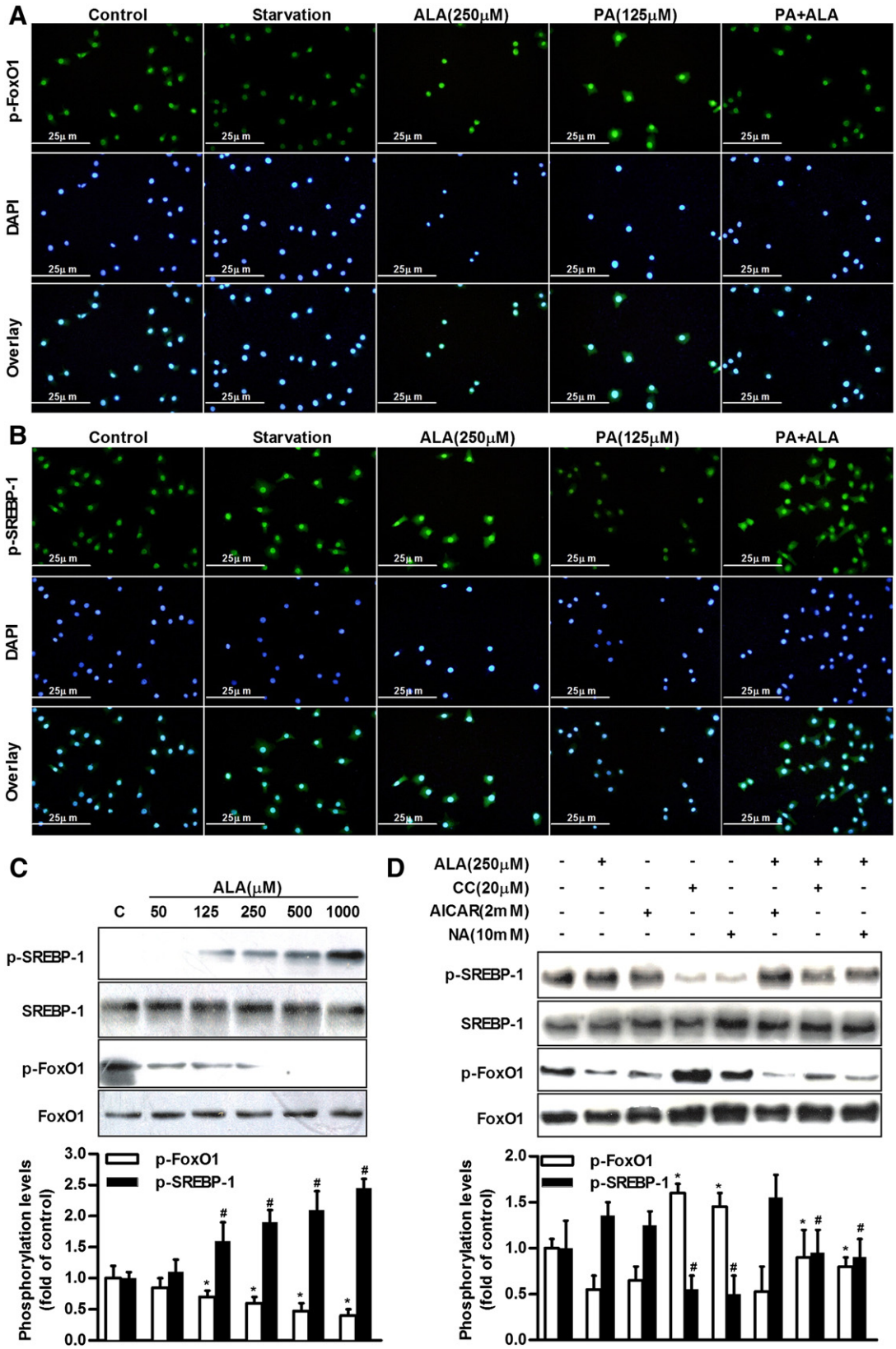


Fig. 3. ALA causes redistribution of transcription factors FoxO1 and SREBP-1 via the SIRT1/LKB1/AMPK signaling pathway. (A and B) HepG2 cells were treated with 125 μM PA and 250 μM ALA for 12 h. Starvation: HepG2 was incubated with serum-free medium. FoxO1 and SREBP-1 protein expression and distribution were determined by immunofluorescence staining (magnification, 400×). Green: FITC; blue: DAPI. (C) HepG2 cells were treated with 0 (control, C), 50, 125, 250, 500 and 1000 μM ALA for 12 h. $^{*}P<.05$, $^{\#}P<.05$ vs. control (untreated cells). (D) Effect of ALA (250 μM, 6 h) on FoxO1 and SREBP-1 phosphorylation levels in the presence or absence of AMPK inhibitor (CC, 20 μM, 0.5 h), SIRT1 inhibitor (NA, 10 mM, 24 h) and AMPK activator (AICAR, 2 mM, 1 h), respectively. $^{*}P<.05$, $^{\#}P<.05$ vs. ALA group.

stress injury [13]. Due to the critical role of SIRT1 and Nrf2 in oxidative stress [14], it might be interesting to explore the synergistic effect of SIRT1/LKB1/AMPK signaling and Nrf2 in NAFLD.

Alpha-Lipoic acid (ALA), a naturally occurring dithiol compound, plays an essential role in mitochondrial bioenergetics. It has gained considerable attention as an antioxidant in managing diabetic complications and NAFLD [15,16]. Multiple studies have described ALA's functions in lipid metabolism. For instance, it was shown that ALA activates both SIRT1 and AMPK and leads to lipid-lowering effects in a skeletal disease model [17], and ALA inhibits NF-κB and activates AMPK in skeletal muscles, which leads to several metabolic consequences [18,19]. Liver damage induced by n-6 polyunsaturated fatty acids including development of a fatty liver, fibrosis, inflammation and apoptosis in the liver of young rats was reduced by ALA injection [20]. In addition, ALA dephosphorylated FoxO1 and reversed the nuclear exclusion of FoxO liver cells [21]. The complex molecular network involved in NAFLD may represent a potential therapeutic target for the treatment and prevention of hepatic steatosis and its progression to steatohepatitis [22]. This study aimed to assess the dual effect of ALA in regulating both the energy metabolism and redox system. We then assess whether ALA has an effect on Nrf2, FoxO1 and SREBP-1 activities via the SIRT1/LKB1/AMPK pathway and controls lipogenesis protein expression. In addition, we investigated whether ALA is potentially effective against NAFLD and can improve hepatic steatosis by eliminating dyslipidemia in the liver. Thus, we hope to reveal for the first time the close connections between oxidative stress and lipid metabolism of liver cells through the interactions between the oxidative stress-related molecule Nrf2 and the energy metabolism-related molecule SIRT1.

2. Materials and methods

2.1. Animals and grouping

Male C57BL/6J mice (6-week-old; body weight: 22–24 g) were purchased from Beijing Vital River Biological Co., Ltd. (China) and housed in standard cage conditions at a constant temperature (22±1°C) and a 13:11-h light/dark cycle. All mice were allowed *ad libitum* access to normal diet and water for 2 weeks before dividing into four groups (n=8): normal diet (ND) (10% energy from fat; D12450B; Research Diets, New Brunswick, NJ, USA), high-fat diet (HFD) (60% energy from fat; D12492; Research Diets, USA) and HFD plus ALA (100 mg/kg or 200 mg/kg). These doses of ALA were selected to be similar to previous studies [17]. After 24 weeks of treatment, blood samples were collected after the eyeballs of the mice were extracted for serum preparation by centrifugation at 2000×g for 10 min at 4°C. The liver tissues were harvested in liquid nitrogen and stored at −80°C. All animal studies were approved by the Animal Research Committee of Ningxia Medical University, Yinchuan, China.

2.2. Biochemical analyses

Levels of serum biochemistry markers, including TG, total cholesterol (TC), aspartate aminotransferase (AST), alanine aminotransferase (ALT), high-density lipoprotein (HDL), low-density lipoprotein (LDL) and nonesterified fatty acids (NEFAs) were determined using a Biochem-Immuno autoanalyser (Brea, CA, USA). Serum glutathione (GSH)/glutathione disulfide (GSSG) was measured using Enzymatic Colorimetric Assay (BIOXYTECH, Portland, OR, USA).

2.3. Detection of intracellular reactive oxygen species (ROS) generation

For the detection of hepatic superoxide production, an oxidative fluorescent dye dihydroethidium (DHE) was used to evaluate the *in situ* production of superoxides. The 10 μm-thick liver frozen sections were incubated for 1 h with 50 μM DHE (Beyotime, Shanghai, China). Fluorescent signals were scanned using a fluorescence microscopy (Olympus IX71, Tokyo, Japan).

2.4. Cell culture and treatment

The human hepatocellular carcinoma (HepG2) cell line was obtained from the Chinese Academy of Medical Sciences tumor cell libraries (Beijing, China) and cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (GIBCO, Gran Island, NY, USA) at 37°C and 5% CO_2. HepG2 cells were treated with AMPK inhibitor (CC, 20 μM, 0.5 h), SIRT1 inhibitor (NA, 10 mM, 12 or 24 h), and AMPK activator (AICAR, 2 mM, 1 h) purchased from (Calbiochem-Novabiochem, San Diego, CA, USA), and palmitate (PA, 125 μM, 12 h) and ALA (250 μM, 6 or 12 h) provided by Sigma-Aldrich (St. Louis, MO, USA). The dose of ALA was selected to be similar to that used in previous studies [21].

2.5. Western blot

Total protein was extracted with Total Protein Extraction Kit (Applygen Technologies Inc, Beijing, China), and nuclear and cytosolic fractions were collected using a nuclear protein extraction kit (Pierce, Nashville, TN, USA) [23], according to the instructions of the manufacturers. HepG2 cell lysates were prepared using a lysis buffer containing 50 mM Tris–HCl (pH 7.5), 1 mM EDTA, 1%Triton, protease and phosphatase inhibitors, 0.1 mM PMSF, 1 μg/ml leupeptin. Lysates were centrifuged at 10,000×g for 10 min at 4°C. Protein concentration was measured using BCA kit (Pierce). Fifty micrograms of protein was loaded onto 8% or 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Pall Corporation, Port Washington, NY, USA). The membranes were incubated with 5% skimmed milk for 1 h at room temperature. Immunoblots were obtained using antibodies against the following proteins: AMPK (pThr172), AMPK, acetyl-CoA carboxylase (ACC) (pSer79), ACC, LKB1, HO-1, and acetyl-lysine (Cell Signaling Technology, Danvers, MA, USA); FAS and Lamin B (Abcam, Cambridge, MA, USA); SREBP-1 (pSer372), SREBP-1, SIRT1, ATGL, FoxO1 (pSer256), FoxO1, Nrf2, SOD-2, catalase and beta-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Western blots were exposed using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Waltham, MA, USA). Film images were scanned by Epson Perfection V33 (China), and signals were quantified with the Image Pro-Plus 6.0 software (Media Cybernetics, Rockville, MD, USA).

2.6. Immunoprecipitation

For each immunoprecipitation (IP) or co-immunoprecipitation (COIP), 1 mg or 100–500 μg protein was used. Samples were incubated with 20 μl Protein A-G (Santa Cruz Biotechnology) and 1–2 μg primary antibodies [anti-rabbit-SIRT1 antibody (Santa Cruz Biotechnology) and anti-rabbit-LKB1 antibody (Cell Signaling Technology)] for 1–2 h at 4°C under constant shaking. Nonspecific IgG (Santa Cruz Biotechnology) was used as a control. The immunoprecipitates were washed 3–4 times with cold phosphate-buffered saline (PBS), and the mixture boiled with SDS sample buffer. Immunoprecipitates were separated on a 10% SDS-PAGE followed by immunoblotting against antibody.

2.7. SIRT1 small interfering RNA and transfection

Predesigned human SIRT1 siRNA (sc-40986) and control scramble siRNA (sc-37007) were purchased from Santa Cruz Biotechnology. HepG2 (2×10^5 cells/well) cells were seeded in 6-well plates and allowed to adhere overnight. Then, 1 μg SIRT1 siRNA and 1 μg scramble siRNA were transfected into cells using Vigofect (Vigorous Biotechnology, Beijing, China) according to the manufacturer's instruction. HepG2 cells were transfected for 24 h after incubation with ALA (250 μM, 6 h).

2.8. NAD^+/NADH assay

The NAD^+/NADH ratio of HepG2 cells or liver tissues was measured using the NAD^+/NADH Quantification Kit (BioVision Inc, Milpitas, CA, USA) according to the manufacturer's instruction.

2.9. Measurement of SIRT1 deacetylase activity

SIRT1 deacetylase activity was determined with the SIRT1 Fluorometric Drug Discovery Kit (Enzo Life Sciences, AG, Lausen, Switzerland) according to the manufacturer's instruction. Resveratrol (Sigma-Aldrich) was used as a SIRT1 activator.

2.10. Measurement of HepG2 intracellular triacylglycerol

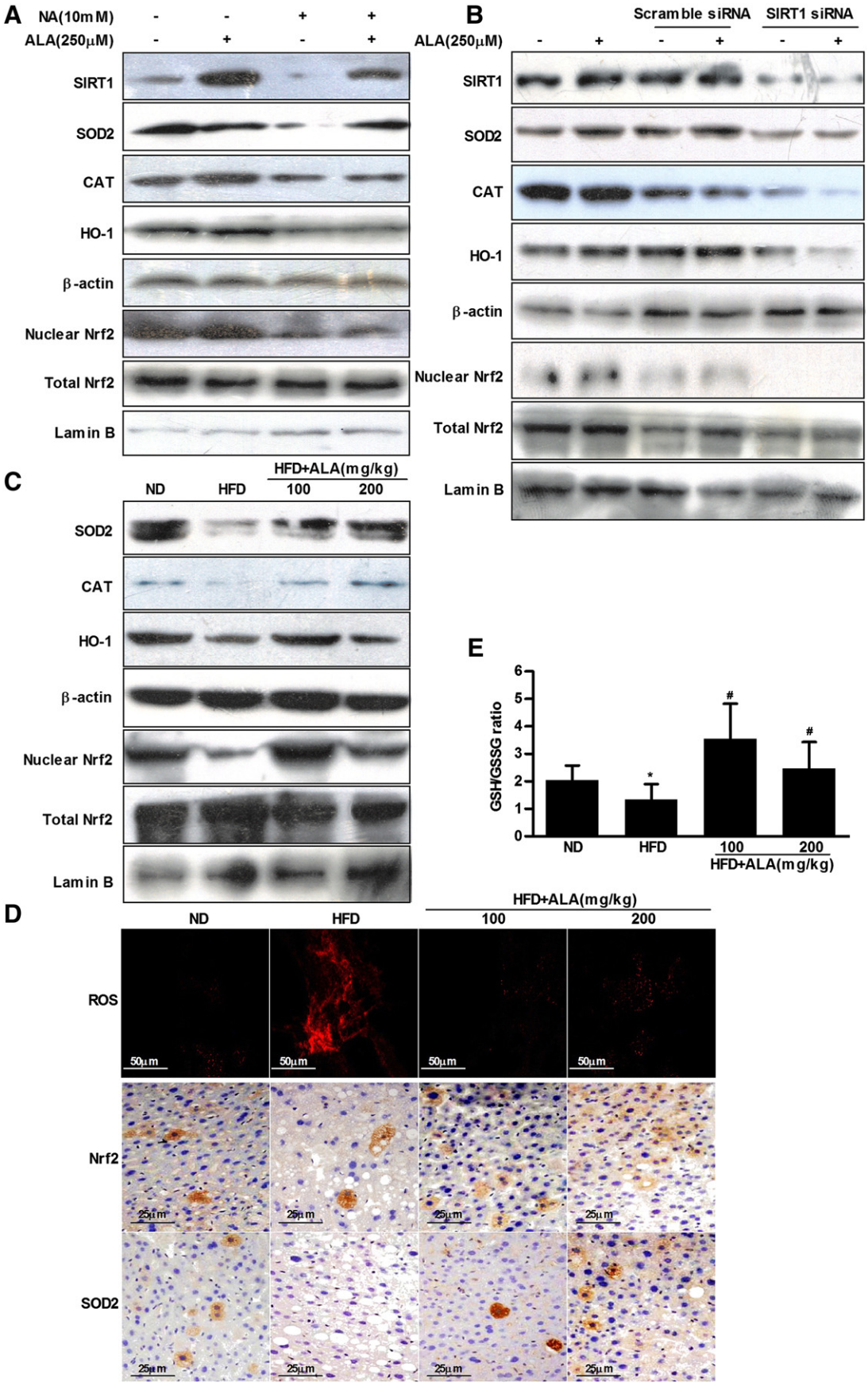
Intracellular triacylglycerol was measured with the Triacylglyceride Assay Kit (Applygen Technologies Inc), according to the manufacturer's instruction.

2.11. Histological analysis and immunohistochemical staining

Liver tissues were fixed in 4% paraformaldehyde for 24 h, embedded in paraffin, cut into 5–7 μm-thick sections, and stained with hematoxylin and eosin (H&E) or Oil Red to observe the lipid droplets in liver tissues. Steatosis and inflammation were used to assess pathological standard. The severity of steatosis was graded from 1 to 3 according to the percentage of cells with lipid droplets (1, 10%–33%; 2, 33%–66%; and 3, >66%). The intensity of inflammation was ranked as 1 (mild), 2 (moderate), and 3 (severe) [24]. For immunohistochemistry, liver sections were stained with Nrf2 or SOD-2 antibodies [12].

2.12. Immunofluorescence staining

HepG2 cells (5×10^3 cells/well) were seeded on glass coverslips and incubated for 12 h. The cells were fixed in 4% paraformaldehyde for 10 min at room temperature and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NA(10mM) - - + +
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SIRT1
SOD2
CAT
HO-1
β-actin
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Lamin B
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Scramble siRNA
SIRT1 siRNA
ALA(250μM) - + - + - +
SIRT1
SOD2
CAT
HO-1
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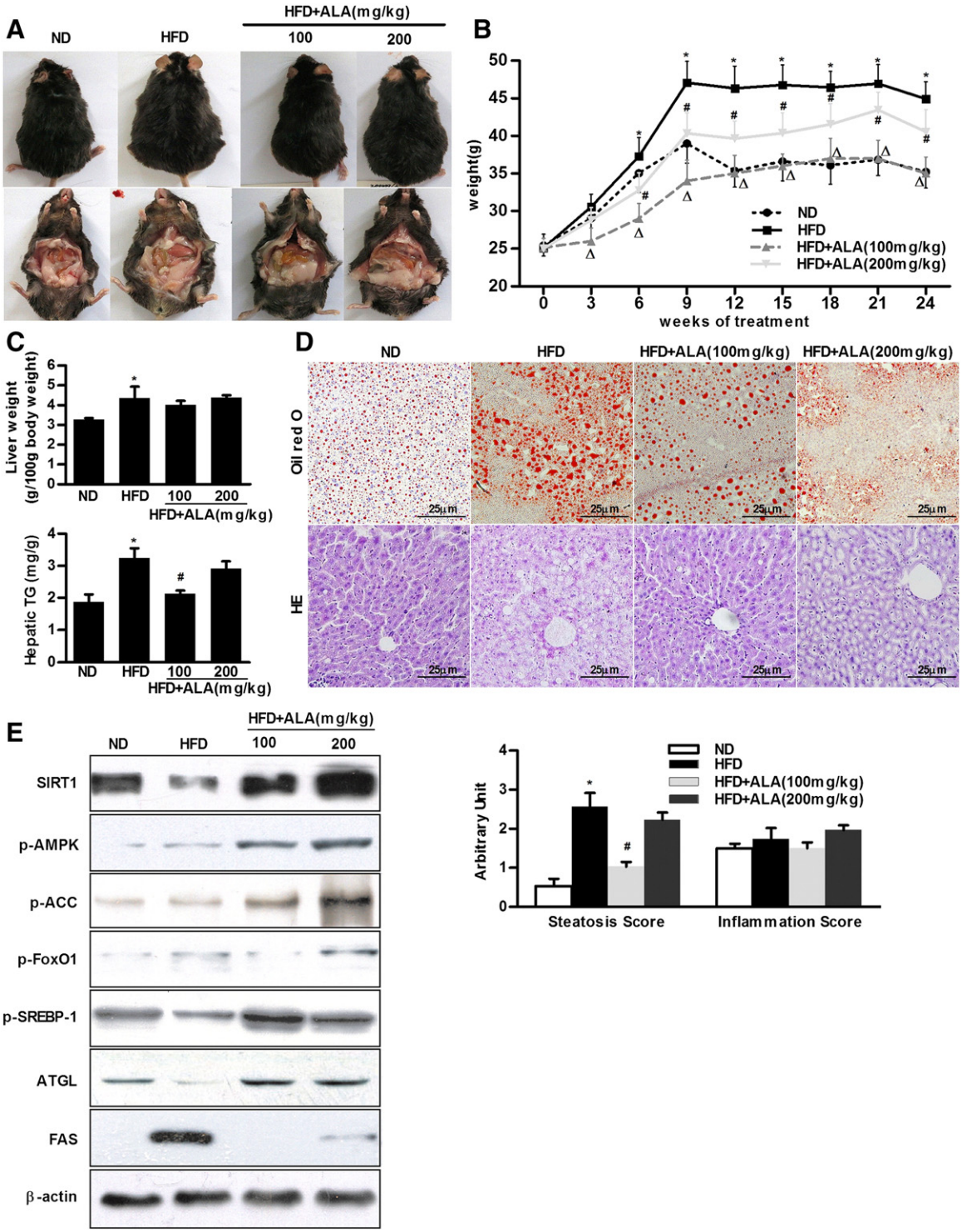

Fig. 5. ALA ameliorates hepatic lipid metabolism in HFD-induced hepatic steatosis mouse model. (A) Effect of ALA (100 mg/kg or 200 mg/kg) on gross contour of the whole body and visceral fat. (B) Effects of ALA (100 mg/kg or 200 mg/kg) on body weight changes. (C) Effect of ALA (100 mg/kg or 200 mg/kg) on fresh liver weight and hepatic triacylglycerol (TG) content. (D) Representative photomicrographs of hepatic steatosis and inflammation after H&E staining. Steatosis and inflammation scores were used to assess pathology. *P<.05 vs. ND-fed group. Lipid droplets in liver tissues were determined by Oil Red O staining. (E) Effects of ALA (100 mg/kg or 200 mg/kg) on SIRT1, p-AMPK, p-ACC, p-Foxo1, p-SREBP1, ATGL and FAS protein expressions. All data are presented as mean±S.E.M. (n=8). *P<.05 vs. ND-fed group, #P<.05 vs. HFD-fed group.

Fig. 4. ALA activates transcription factor Nrf2 depending on the SIRT1/LKB1/AMPK signaling pathway in *vitro* and in *vivo*. (A) Effect of SIRT1 inhibitor (NA, 10 mM, 12 h) and ALA (250 μM, 6 h) on SOD2, CAT, HO-1 and Nrf2 expressions in HepG2 cells. (B) Effect of SIRT1 siRNA (24 h) and ALA (250 μM, 6 h) on antioxidative protein and Nrf2 protein expression in HepG2 cells. (C) Effect of ALA (100 mg/kg or 200 mg/kg) on antioxidative protein and Nrf2 protein expression in HFD-induced hepatic steatosis mouse model. (D) Effect of ALA (100 mg/kg or 200 mg/kg) on the distribution of Nrf2, SOD2 and ROS *in vivo*. Nrf2 and SOD2 distribution was determined by immunohistochemistry. (E) Effect of ALA (100 mg/kg or 200 mg/kg) on serum GSH/GSSG ratio *in vivo*. Data are presented as mean±S.E.M. (n=8). *P<.05 vs. ND-fed group, #P<.05 vs. HFD-fed group.

washed once with cold PBS. We used rabbit anti-FoxO1 or SREBP-1 as primary antibodies and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG as secondary antibody. The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (5 mg/ml) (Beyotime) for 5 min in the dark. Finally, the coverslips were observed under a fluorescence microscope (Olympus IX71).

2.13. Statistical analysis

Data are presented as mean±standard error of the mean (S.E.M.). The SPSS 13.0 software (SPSS Inc, Chicago, IL, USA) was used to perform one-way analysis of variance with Student–Newman–Keuls test for post hoc analysis for multiple groups or unpaired *t* test for two groups. *P* value <.05 was considered statistically significant.

3. Results

3.1. ALA regulates hepatocyte lipid metabolism and decreases palmitate-induced hepatic cellular triacylglycerol accumulation via the SIRT1/LKB1/AMPK signaling pathway

To test whether ALA increases SIRT1 deacetylase activity in the liver, we first determined ALA-stimulated deacetylation of a peptide comprising amino acids Arg-His-Lys-Lys(Ac) 379–382 of human p53 [25]. The results showed that incubation with ALA (50 μM) significantly increased SIRT1 activity (Fig. 1A, $P<.05$ or $P<.01$). Since SIRT1 is an NAD^+-dependent enzyme that acts as a metabolic sensor for NAD^+ [25], we next measured the NAD^+/NADH ratio in HepG2 cells. Treatment of cells with ALA significantly increased the NAD^+/NADH ratio in HepG2 cells (Fig. 1B, $P<.05$ or $P<.01$). Treatment with ALA increased SIRT1 activity in HepG2 cells (Fig. 1A); the increase in the NAD^+/NADH ratio may have resulted in increased SIRT1 activity.

The exact mechanism underlying ALA-stimulated AMPK activation remains to be clarified. To date, two upstream AMPK kinases (AMPKK) have been identified: LKB1 and CaMKK [26]. LKB1 likely controls most of the AMPK activation in a majority of tissues. To determine whether ALA-mediated SIRT1 activation regulated LKB1 deacetylation, the acetylation level of LKB1 was measured by IP. As shown in Fig. 1C, ALA significantly reduced LKB1 acetylation ($P<.05$), when the levels of acetylation were normalized to protein input. We also found that SIRT1-LKB1 was coimmunoprecipitated from cells (Fig. 1D). The findings confirmed the interaction between SIRT1 and LKB1 following ALA treatment of HepG2 cells.

These data supported the notion that ALA activated SIRT1 and regulated the AMPK signaling pathway through LKB1 deacetylation in the liver. The SIRT1/LKB1/AMPK signaling pathway plays a key role in the ALA regulation of hepatocyte lipid metabolism [27].

To investigate whether ALA improved AMPK signaling pathway, we assessed the effects of ALA on phosphorylation of AMPK and ACC. We found that ALA increased phosphorylation of AMPK and ACC in HepG2 cells in a dose-dependent fashion (Fig. 2A).

With increased ALA concentration, triacylglycerol accumulation in palmitate environment significantly decreased (Fig. 2B, $P<.05$ or $P<.01$). We examined the effects of SIRT1 inhibitor (nicotinamide; NA), AMPK inhibitor (compound c; CC) and AMPK activator (5-aminoimidazole-4-carboxamide riboside; AICAR) on phosphorylation of AMPK and ACC. As shown in Fig. 2C, ALA increased expression of p-AMPK and p-ACC in the AIACR group, but a decrease was observed in the CC group. In addition, p-AMPK and p-ACC levels were reduced by NA, confirming that SIRT1 acts upstream of AMPK. The ALA and ALA +AICAR groups showed decreased triacylglycerol accumulation in the presence of palmitate (Fig. 2D, $P<.05$). Specifically the CC and NA groups increased triacylglycerol accumulation in HepG2 cells. To further determine the role of SIRT1 in regulating AMPK activation, SIRT1 siRNA was transfected to test whether p-AMPK production was reduced. Our data indicated that SIRT1 knockdown significantly decreased phosphorylation of AMPK and ACC (Fig. 2E). Accordingly, SIRT1 siRNA altered FAS and ATGL production (Fig. 2E). However, SIRT1 knockdown failed to attenuate palmitate-induced triacylglycerol accumulation in cells (Fig. 2F, $P<.05$). These results support the notion that ALA mediates lipid metabolism through the SIRT1/LKB1/AMPK signaling pathway [28].

3.2. ALA causes distribution of FoxO1 and SREBP-1 through SIRT1/LKB1/AMPK signaling

ALA decreases p-FoxO1 expression levels and reinforces FoxO1 activities, and mediates the downstream molecular targets of ATGL activity [29]. As demonstrated by immunofluorescence (Fig. 3A), FoxO1 was found mainly in the nuclei of untreated cells. ALA treated cells showed apparently exclusive nuclear expression of p-FoxO1. Upon treatment with palmitate, FoxO1 mostly translocated into the cytoplasm. As shown in Fig. 3A, nuclear translocation of p-FoxO1 was very obvious in the ALA alone and ALA+palmitic acid groups compared to the palmitic acid alone stimulation group. At increasing concentrations of ALA, p-FoxO1 expression decreased also suggesting migration of FoxO1 into the nucleus (Fig. 3C, $P<.05$).

The ALA induced nuclear translocation of FoxO1 was reversed by pretreatment with NA (Fig. 3D), confirming the ALA-mediated nuclear localization of FoxO1 occurred via SIRT1/LKB1/AMPK signaling. The phosphorylation of FoxO1 was increased by SIRT1 and AMPK inhibitors (Fig. 3D) compared to the ALA-treated cells ($P<.05$), suggesting that the ALA-mediated signal transduction pathway is activated via SIRT1/AMPK down to FoxO1, leading to increased expression of ATGL but treatment with inhibitors and ALA together reduced the increase.

SREBP-1 is a membrane-binding protein located on the surface of the endoplasmic reticulum membrane that controls lipid metabolism-related enzymes (FAS) [30]. To determine the influence of ALA on the subcellular localization of SREBP-1, cells were treated with ALA in the presence or absence of CC, NA or AICAR. The SREBP-1 protein was mainly found in the cytoplasm of ALA-treated cells (Fig. 3B). As shown in Fig. 3C, ALA caused significant increases in p-SREBP-1 levels ($P<.05$). Interestingly, the phosphorylation of SREBP-1 caused by ALA was reversed by CC or NA (Fig. 3D, $P<.05$).These findings suggested that ALA might repress the transcription of genes involved in lipid synthesis by preventing the entry of SREBP-1 into the nucleus through SIRT1/LKB1/AMPK signaling.

3.3. ALA significantly activated Nrf2/ARE signaling

Nrf2 is an important endogenous antioxidant mediator. When exposed to oxidative stress, cells exhibit adaptive activation of the Nrf2/ARE pathway to relieve cell damage [31]. When cells were pretreated with NA, and then treated with ALA, SIRT1, nuclear Nrf2, SOD, catalase and HO-1 expressions were decreased. By contrast, the total Nrf2 levels displayed no overt changes (Fig. 4A). SIRT1 knockdown also resulted in decreased protein expression (Fig. 4B). ALA (100 mg/kg or 200 mg/kg)-treated group also showed increased expression of Nrf2 signaling molecules compared with the HFD group (Fig. 4C). Immunohistochemical staining results also revealed elevated expression of Nrf2 and SOD2, in agreement with Western blot data (Fig. 4D).

Compared with the HFD group, ALA (100 mg/kg or 200 mg/kg) significantly increased the level of GSH/GSSG (Fig. 4E, $P<.05$).

3.4. ALA ameliorates lipid metabolic function and lipid accumulation in HFD-induced NAFLD mice

C57BL/6J mice, divided into four groups, were fed an HFD for 24 weeks to induce NAFLD followed by daily administration of ALA. Then, the effects of ALA on hepatic lipid accumulation in long-term HFD-fed mice were assessed. Administration of ALA markedly reduced visceral fat mass in mice (Fig. 5A). In addition, ALA treatment inhibited the appetite and caused a dramatic weight loss (Fig. 5B, all

Table 1
Variables associated with lipid metabolism after 24-week HFD treatment.

Variables	ND	HFD	HFD+ALA (100 mg/kg)	HFD+ALA (200 mg/kg)
Body weight (g)	36.65±2.00	48.76±2.83*	39.40±3.89#	42.45±2.84△
Perirenal fat (g)	0.55±0.12	1.17±0.19*	0.77±0.31#	0.98±0.16
AST (U/l)	262.66±30.56	312.00±30.80*	243.00±31.50#	218.66±22.19△
ALT (U/l)	74.96±30.43	161.80±13.50**	56.40±14.93##	101.16±30.39△
Triacylglycerol (mmol/l)	0.87±0.10	1.74±0.22*	0.86±0.11#	1.48±0.17△
Cholesterol (mmol/l)	3.57±0.20	5.35±0.93*	4.10±0.38#	5.53±1.61
HDL (mmol/l)	6.91±0.06	3.15±0.29*	4.26±0.76#	3.16±0.24
LDL (mmol/l)	0.23±0.10	0.54±0.10*	0.13±0.11#	0.31±0.06△
NEFA (μmol/l)	144.48±40.34	235.98±78.43**	158.50±43.50##	175.54±59.28△
Plasma glucose (mmol/l)	5.06±0.56	8.97±0.90*	5.79±0.35#	8.21±0.55

Note: Data are shown as mean±S.E.M. (n=8).
* P<.05 vs. ND-fed group.
** P<.01 vs. ND-fed group.
# P<.05 vs. HFD-fed group.
P<.01 vs. HFD-fed group.
△ P<.05 vs. HFD-fed group.

P<.05). Although liver weights in ALA-treated mice were not altered (Fig. 5C), the hepatic triacylglycerol content showed a significant difference (Fig. 5C, P<.05). Interestingly, lipid droplets as detected by Oil Red O staining were markedly reduced in the liver after ALA treatment (Fig. 5D).

Importantly, HFD-induced hepatic steatosis was significantly reduced in the ALA-treated group, as illustrated by the decrease in the steatosis and inflammation scores (Fig. 5D). As expected, ALA decreased perirenal fat weight (Table 1). All the serum variables obtained for ALA-fed mice were significantly lower compared with the HFD-fed group (Table 1, P<.05). Plasma glucose was significantly different in these groups (Table 1, P<.05).

These data showed that the weight of ALA-controlled mice was reflected by improved hepatic lipid metabolism and decreased triacylglycerol accumulation *in vivo*.

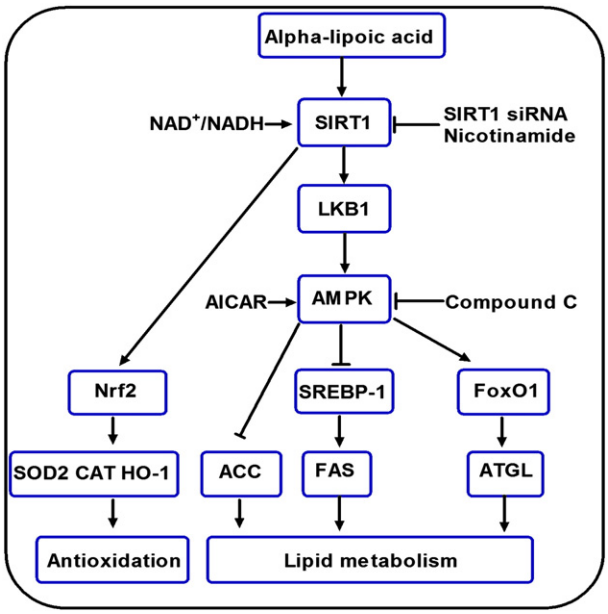

Fig. 6. Proposed scheme illustrating the role of ALA in the regulation of hepatocyte lipid metabolism and antioxidation. ALA reverses the HFD-induced changes in AMPK expression, which is involved in SIRT1/LKB1/AMPK-mediated signaling, and transcription factors FoxO1, SREBP-1 and nuclear Nrf2 protein expression, along with changes in the lipid metabolism and antioxidative enzyme expression.

ALA-fed mice showed increased SIRT1, p-AMPK, p-ACC, p-SREBP-1and ATGL production and decreased p-FoxO1 and FAS expression (Fig. 5E).

4. Discussion

Hepatic steatosis is characterized by obesity and dysregulated lipid metabolism, including increased *de novo* lipogenesis or decreased lipolysis [32]. The present study aimed to determine the mechanism underlying the ALA-mediated activation of the transcription factors SREBP-1, FoxO1 and Nrf2 for hepatic steatosis prevention, *in vivo* or *in vitro*.

Mounting evidence suggests that SIRT1 and AMPK are two critical signaling targets controlling the pathways of hepatic lipid metabolism. In the present study, we showed that ALA increases SIRT1 deacetylase activity, LKB1 deacetylation and AMPK activity in HepG2 cells (Fig. 6). ALA substantially prevents the impairment of lipid accumulation due to elevated phosphorylation of AMPK and ACC when HepG2 cells were exposed to palmitate. The effects of ALA were largely abolished by pharmacological and genetic inhibition of SIRT1, suggesting that the stimulation of AMPK and lipid-lowering effect of ALA depends on SIRT1 activity.

Furthermore, expression of SIRT1 and p-AMPK is stimulated by ALA in the mouse liver. AMPK activation by SIRT1 also protects against FAS production and lipid accumulation in HFD-fed mice. Our data suggest that SIRT1 is a crucial target of ALA in hepatic metabolism. ALA alleviates hepatic steatosis by coordinating multiple lipid metabolism signaling pathways, which in turn boost the oxidation of hepatic fatty acids and reduce hepatic lipogenesis in HFD-fed mice. As far as we know, this is the first study to demonstrate the synergy of ALA in regulating oxidative stress and improving lipid metabolism in liver cells.

The role of SIRT1 in lipid metabolism is being established in connection with FoxO1-mediated ATGL expression [29]. According to previous studies, transgenic mice overexpressing SIRT1 displayed significant decreases in total blood cholesterol and white adipose tissue, and it was proposed that deleted in breast cancer-1 (DBC1) induced SIRT1 regulation and contributed to HFD-induced liver steatosis in mice [33,34]. In cultured adipocytes, knockdown of SIRT1 decreased TG hydrolysis by reducing ATGL expression, following elevated levels of acetylated and phosphorylated FoxO1 [29]. Taken together, these studies agree with our results and indicate that the lipid-lowering effects of ALA are mediated by the SIRT1/LKB1/AMPK pathway to activate FoxO1/ATGL signaling. In addition to the effects on lipid catabolism, ALA may alternatively modulate lipid anabolism via SREBP-1/FAS (Fig. 6). We showed

that ALA decreased the expression of FAS in association with reduced nuclear translocation of SREBP-1. Activated AMPK reportedly suppressed the proteolytic cleavage and nuclear translocation of SREBP-1 via direct phosphorylation in hepatocytes, ultimately decreasing the plasma levels of TC and TG. SREBP-1 is known as the sterol regulatory element 1 (SRE-1) and contains regulatory elements in the promoter of low density lipoprotein receptor and 3-hydroxy-3-methylglutaryl-coenzyme A synthase (HMG-CoA synthase) genes, similar to the FAS gene [35]. SREBP-1 induces the basic transcription of these genes in the hamster liver [36]. Therefore, the decrease of the active SREBP-1 form probably reduces the production of cholesterol and fatty acids. This evidence supports our findings that ALA exerts lipid-lowering effects by activating SIRT1/LKB1/AMPK/SREBP-1 signaling.

One of the most important antioxidant mechanisms is mediated by the transcription factor Nrf2. Briefly, Keap1 binds to actin cytoskeleton and Nrf2 to retain Nrf2 in the cytoplasm under basal conditions. Upon exposure of cells to oxidative stress or chemopreventive compounds, Nrf2 dissociates from Keap1, translocates to the nucleus, forms a heterodimer with its obligatory partner Maf, and ultimately activates ARE-dependent gene expression. Nrf2/ARE signaling is also known to be primarily responsible for the up-regulation of antioxidant gene expression and hence constitutes a crucial cellular response to environmental stresses [37]. As expected, ALA increased both Nrf2 and HO-1/SOD2 expression regardless of stress [38]. Reports have recently demonstrated a regulatory role for SIRT1 in the Nrf2/ARE antioxidant pathway [14,39]. Indeed, it was suggested that indole-3-carbinol's protective action against hepatic steatosis is at least partially mediated through the up-regulation of SIRT1-AMPK signaling in the livers of HFD-fed mice [24]. In this study, we also found that ALA significantly activated Nrf2/ARE signaling. Enhancing the SIRT1 function by resveratrol and SIRT1 overexpression significantly promoted the nuclear accumulation, DNA binding and transcriptional activities of Nrf2, evidently up-regulated HO-1 and SOD levels and eventually diminished AGE-induced ROS production. Increasing SIRT1 levels in *in vivo* and *in vitro* models promoted resistance to diabetic renal fibrosis by activating Nrf2, similar to known Nrf2 activators, such as sulforaphane and cinnamic aldehyde [40]. It is also known that HO-1 induction mediates the antiapoptotic effect of AMPK, providing an important adaptive response to preserve endothelial cell viability during metabolic stress [41]. Interestingly, it was shown that ALA may alleviate lipopolysaccharide-induced acute lung injury through HO-1 induction, which in turn suppresses NF-κB-mediated inflammatory responses [42]. Our data suggested that ALA increases the interaction between SIRT1 and Nrf2 (Fig. 6). The impact of the interplay between these proteins on the activation of the Nrf2/ARE pathway requires further investigation. Encouragingly, we have conducted a study to evaluate the role of ALA in improving the fatty liver in HFD-induced obese mice. Interestingly, ALA at 100 mg/kg/day significantly reduced body weight and fat mass, and increased the fatty acid oxidation in the liver. Thus, both the *in vitro* and *in vivo* studies suggest a potential benefit for ALA as an anti-NAFLD agent.

Taken together, our results demonstrated that ALA reduces lipid accumulation in the liver by regulating the transcriptional factors SREBP-1, FoxO1, and Nrf2, and their downstream lipogenic targets via the activation of the SIRT1/LKB1/AMPK pathway. The present study reveals the promising role of ALA in the prevention and treatment of hepatic steatosis, *in vivo* and *in vitro*.

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