Alpha-Lipoic Acid Decreases Hepatic Lipogenesis Through Adenosine Monophosphate-Activated Protein Kinase (AMPK)-Dependent and AMPK-Independent Pathways

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Fatty liver is common in obese subjects with insulin resistance. Hepatic expression of sterol regulatory element binding protein-1c (SREBP-1c), which plays a major role in hepatic steatosis, is regulated by multiple factors, including insulin, adenosine monophosphate–activated protein kinase (AMPK), liver X receptors (LXRs), and specificity protein 1. Alpha-lipoic acid (ALA), a naturally occurring antioxidant, has been shown to decrease lipid accumulation in skeletal muscle by activating AMPK. Here we show that ALA decreases hepatic steatosis and SREBP-1c expression in rats on a high fat diet or given an LXR agonist. ALA increased AMPK phosphorylation in the liver and in cultured liver cells, and dominant-negative AMPK partially prevented ALA-induced suppression of insulin-stimulated SREBP-1c expression. ALA also inhibited DNA-binding activity and transcriptional activity of both specificity protein 1 and LXR. *Conclusion:* These results show that ALA prevents fatty liver disease through multiple mechanisms, and suggest that ALA can be used to prevent the development and progression of nonalcoholic fatty liver disease in patients with insulin resistance. (HEPATOLOGY 2008;48:1477-1486.)

Nonalcoholic fatty liver disease (NAFLD) is common in obese individuals with insulin resistance and is considered an important hepatic manifestation of metabolic syndrome.[1,2] The onset of NAFLD is characterized by increased intracellular triglyceride accumulation. Sterol regulatory binding protein-1c (SREBP-1c) is one of the major regulators of the expression of genes involved in hepatic triglyceride synthesis.[3] Its role in the development of fatty liver disease is now well established.[4,5]

The regulation of hepatic SREBP-1c *in vivo* is largely dependent on nutritional status. Under fasting conditions, activation of adenosine monophosphate–activated protein kinase (AMPK) reduces lipogenesis in the liver by suppressing SREBP-1c activity. AMPK activators, including metformin and thiazolidinediones, have been shown to inhibit the expression of the SREPB-1c gene and to prevent the development of hepatic steatosis.[6,7] Conversely, activation of liver X receptor (LXR) and specificity protein 1 (Sp1) increases SREBP-1c expression under insulin-stimulated conditions and leads to hepatic lipogenesis.[8,9] Thus, identifying pharmacologic agents that inhibit the activity of LXR and Sp1, but stimulate AMPK activity in hepatocytes, may provide more effective treatment options for fatty liver disease.

Abbreviations: ALA, alpha-lipoic acid; AMPK, adenosine monophosphate–activated protein kinase; DN-AMPK, dominant negative form of AMPK; HFD, high fat diet; LXR, liver X receptor; NAFLD, nonalcoholic fatty liver disease; Sp1, specificity protein 1; SREBP-1c, sterol regulatory element binding protein-1c.

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We previously reported that alpha-lipoic acid (ALA), a naturally occurring thiol antioxidant, activates AMPK and reduces lipid accumulation in skeletal muscle of obese rats.[10] Recent studies from other laboratories also demonstrated that ALA activates AMPK in various tissues, including liver, heart, and pancreatic beta cells.[11,12] Here we report that ALA decreases hepatic lipogenesis in animals by inhibiting SREBP-1c expression through both AMPK-dependent and AMPK-independent pathways.

Materials and Methods

Materials. See Supporting Materials and Methods.

Animal Experiments. All procedures were performed in accordance with the institutional guidelines for animal research. In studies of ALA effects on hepatic steatosis in high fat diet (HFD)-fed rats, 8-week-old male Sprague-Dawley rats (Orient, Sungnam, Korea) weighing 250 to 300 g were given normal rat chow or placed on an HFD that provided 60% of calories as fat (D12492; Research Diets, New Brunswick, NJ). After 3 weeks, rats in the HFD group were further divided into three groups and maintained on the following dietary regimens for 3 days: (1) control HFD group; (2) ALA group given 0.5% (wt/wt) ALA mixed in food; and (3) pair-fed group. The pair-fed group was given the same amount of food as that consumed by the ALA group on the previous day. After being fasted for 5 hours on the morning of the last day, rats were sacrificed and their livers were rapidly removed and kept frozen at −70°C for subsequent measurement of SREBP-1c expression and AMPK phosphorylation. The effects of ALA on hepatic and peripheral insulin sensitivity were determined using the hyperinsulinemic-euglycemic clamp method, as described.[13,14] (See Supporting Materials and Methods for detailed protocols.)

In a separate experiment, HFD-fed rats received an intraperitoneal injection of 100 mg/kg ALA in saline. At 30 minutes, 60 minutes, and 24 hours after the ALA injection, rats were sacrificed and their livers were removed for measurement of AMPK phosphorylation.

To explore the effects of ALA on LXR-induced hepatic steatosis, 8-week-old male C57BL/6 mice (SLC, Japan) weighing 20 to 25 g were fed rat chow ad libitum. The LXR agonist, T0901317 (50 mg/kg body weight), was administered by oral gavage (0.2 mL/mouse) once daily for 7 days. The T0901317 treatment group was fed with or without 0.5% (wt/wt) ALA mixed in food as described above.

Analysis of Blood Samples. See Supplementary Materials and Methods.

Liver Triglyceride Content. Triglyceride content in liver was determined in duplicate using the Triglyceride (GPO-Trinder) kit as described by the manufacturer (Sigma, St. Louis, MO).

Histological Analysis of Liver. Liver tissue was fixed with 4% paraformaldehyde, embedded in paraffin, and stained with hematoxylin and eosin. Alternatively, intrahepatic lipids were stained by the oil red O method. Images were captured using an Olympus BX60 camera (Tokyo, Japan) and processed in Adobe PhotoShop (Adobe Systems).

Cell Culture. HepG2 (human hepatoma cell line) cells were cultured in MEM (Gibco-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and antibiotics at 37°C in a humidified, 5% CO_2/95% air atmosphere. After reaching ~80% confluence, cells were serum-starved for 24 hours in medium containing 0.5% fetal bovine serum, and then treated as indicated in the text.

Isolation and Culture of Primary Rat Hepatocytes. Primary rat hepatocytes were isolated from the livers of 7-week-old male Sprague-Dawley rats. (See Supplementary Materials and Methods for detailed protocols and treatments.)

Recombinant Adenovirus. Plasmids encoding c-Myc-tagged forms of dominant-negative α1 and α2 AMPK (DN-AMPK) and a constitutively active form of AMPK (CA-AMPK)[15-17] were kind gifts from Dr J. Ha (Department of Molecular Biology, Kyung Hee University College of Medicine, Seoul, Korea). For details of preparation of recombinant adenovirus, refer to the Supplementary Materials and Methods.

Northern Blot Analysis. Messenger RNA (mRNA) expression was measured by northern blot analysis. For details, refer to Supplementary Materials and Methods.

Western Blot Analysis. Protein expression in cells and tissues was measured by western blot analysis. For details of western blotting procedures, refer to the Supplementary Materials and Methods.

In Vitro *Transient Transfection and Reporter Assays.* Promoter activity was measured using in vitro transfection and luciferase reporter assays. For details, refer to the Supplementary Materials and Methods.

Electrophoretic Mobility Shift Assay. DNA-binding activity was assessed using an electrophoretic mobility shift assay. For details, refer to the Supplementary Materials and Methods.

Statistical Analyses. Data were evaluated using analysis of variance followed by a post hoc least significant difference test, and expressed as means ± standard error of the mean. Values of $P < 0.05$ were considered statistically significant. All experiments were performed at least three times.

Table 1. Metabolic Parameters of Rats Fed a High-Fat Diet (HFD) with or without ALA

	Control	HFD	HFD + ALA	HFD + pair-fed
Caloric intake (kcal/day)	88.6 ± 3.6	117.1 ± 4.5*	81.9 ± 8.3*,†	89.5 ± 4.6†
Body weight (g)	441.7 ± 2.1	456.5 ± 15.4	440.7 ± 18.2†	449.0 ± 9.6
Liver weight (g)	13.6 ± 0.9	18.6 ± 0.6*	14.0 ± 0.9†	15.1 ± 0.4
Liver/body weight ratio (%)	3.21 ± 0.21	4.06 ± 0.13*	3.39 ± 0.15†	3.67 ± 0.23
Plasma glucose (mg/dL)	184.3 ± 2.4	190.2 ± 5.15	179.7 ± 4.9	173.3 ± 2.6
Plasma insulin (μU/mL)	7.1 ± 0.6	11.2 ± 0.7*	8.9 ± 0.5†	10.3 ± 0.7
Glucose/insulin ratio	25.9 ± 1.8	16.6 ± 1.3*	21.1 ± 0.8†	16.8 ± 1.6*
Triglycerides (mmol/L)	86.7 ± 6.4	131.4 ± 14.7*	66.8 ± 7.2†	89.3 ± 4.4
Free fatty acid (mmol/L)	128.8 ± 43.3	258.9 ± 20.5*	168.4 ± 36.6†	217.9 ± 23.1

Values are mean ± SEM; n = 5.
*$P < 0.05$ compared with control rats.
†$P < 0.05$ compared with HFD-fed untreated rats.

Results

ALA Reduces Lipid Accumulation and SREBP-1c Expression in HFD-Fed Rats. ALA has been shown to reduce body weight in rodents.[17] To demonstrate direct effects of ALA on hepatic steatosis and SREBP-1c gene expression independent of its effects on body weight, we compared the short-term effects of ALA on hepatic lipid accumulation and SREBP-1c expression in HFD-fed rats with those of untreated and pair-fed rats. As expected, ALA reduced food intake (Table 1) and tended to decrease body weight. Interestingly, the liver weight of ALA-treated rats was significantly lower than that of HFD-fed rats, resulting in a liver/body weight ratio that was significantly lower than that of the control HFD group (Table 1). Plasma glucose levels were not significantly different between the groups, but plasma insulin levels were lower and insulin sensitivity (glucose/insulin ratio) was higher in the ALA-treated group than in the control HFD group. Plasma triglyceride and free fatty acid levels were also lower in ALA-treated group. In the pair-fed group, all values for these parameters were intermediate between those of ALA and control HFD groups (Table 1).

Histological examination showed that hepatocytes of HFD-fed rats were distended by large cytoplasmic lipid droplets. This change in cellular morphology was nearly completely prevented by ALA treatment (Fig. 1A). Triglyceride concentration and SREBP-1c expression in the liver were significantly higher in HFD-fed rats than in chow-fed rats (Fig. 1B,C). Administration of ALA significantly decreased both liver triglyceride levels and SREBP-1c expression compared to untreated and pair-fed rats (Fig. 1B,C). Pair-feeding also attenuated fatty changes, and reduced liver triglyceride levels and SREBP-1c expression compared to HFD-feeding. However, these changes were significantly smaller than those observed in ALA-treated rats (Fig. 1).

ALA Inhibits Insulin-Stimulated SREBP-1c Expression in Cultured Hepatocytes. Insulin is one of the most important hormones involved in regulating hepatic lipogenesis. In cultured hepatocytes, insulin has been shown to increase lipogenesis primarily through induction of SREBP-1c.[18] Pretreatment with ALA for 24 hours dose-dependently attenuated insulin-stimulated SREBP-1c mRNA expression in primary rat hepatocytes and HepG2 cells (Supporting Fig. 1A,B). Moreover, ALA inhibited the insulin-stimulated expression of the SREBP-1c target genes, acetyl coenzyme A carboxylase (ACC) and fatty acid synthetase (FAS) (Supporting Fig. 1B).

ALA Inhibition of SREBP-1c Expression Is Partially Mediated by AMPK Activation. ALA has been shown to activate AMPK in skeletal muscle.[10] Because AMPK activators, including metformin and thiazolidinediones, inhibit SREBP-1c transcription and act to prevent the development of hepatic steatosis,[6,7] we examined the effect of ALA on AMPK phosphorylation in the liver of HFD-fed rats. As expected, intraperitoneal administration of ALA increased hepatic AMPK phosphorylation for up to 1 day after injection (Fig. 2A). However, this effect was not sustained and AMPK phosphorylation levels in rats receiving ALA in their diet returned to baseline within 3 days (Fig. 2A,B). In cultured hepatocytes, ALA also increased AMPK phosphorylation. This effect was time-dependent, with AMPK phosphorylation levels increasing within 1 hour and peaking at 24 hours (Fig. 2C). In HepG2 cells, blocking AMPK activity with adenovirus-carrying dominant-negative AMPKs (a mixture of α1 and α2 dominant-negative AMPKs; see Supporting Materials and Methods) restored approximately 50% of the insulin-stimulated increase in SREBP-1c mRNA that was inhibited by ALA at 24 hours (Fig. 2D). Similarly, transient transfection studies showed that blocking AMPK activity with DN-AMPKs partially restored ALA-induced inhibition of SREBP-1c promoter activity at 12

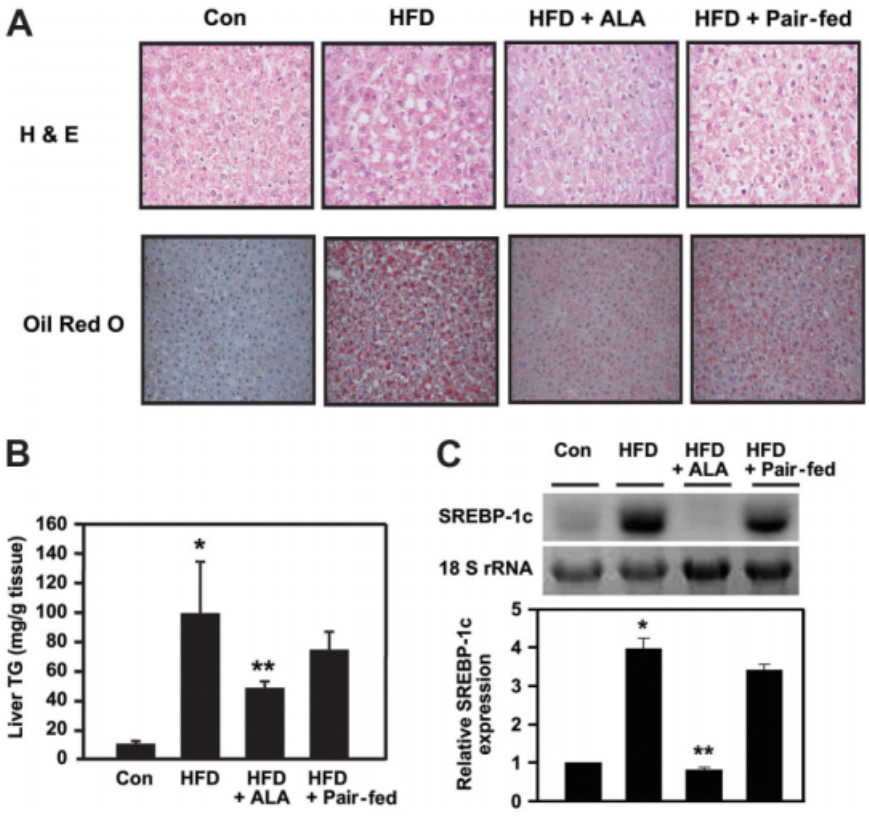

Fig. 1. ALA reduces triglyceride accumulation and SREBP-1c expression in the liver of HFD-fed rats. (A) Histological analysis of liver sections of HFD-fed rats treated with or without ALA, or pair-fed rats (food restricted without ALA treatment). Hematoxylin and eosin (H&E) staining (upper panel) and Oil red O staining (lower panel). Lipid droplets in hepatocytes appear as darkly stained cytoplasm containing varying amounts of brownish red lipofuscin. Original magnification $\times 400$ ($n = 5$). (B) Triglyceride concentration in the liver. Data are presented as the mean $\pm$ standard error of the mean (SEM). $^{*}P < 0.05$ compared with untreated rats and $^{**}P < 0.05$ compared with HFD-fed rats. (C) Northern blot analysis of the effect of ALA on SREBP1-c expression in the liver. Data are presented as the mean $\pm$ SEM. $^{*}P < 0.001$ compared with control, $^{**}P < 0.001$ compared with HFD or HFD+pair-fed.

and 24 hours (Fig. 2E, lower panel). However, inhibition of the SREBP-1c promoter activity induced by short-term (3 and 6 hours) ALA treatment was not influenced by DN-AMPK (Fig. 2E, upper panel), indicating that AMPK did not act at these earlier time points to decrease SREBP-1c promoter activity. Collectively, these data suggest that ALA suppression of insulin-stimulated SREBP-1c expression is partly mediated by AMPK activation, but also point to the involvement of additional, AMPK-independent mechanisms.

ALA Inhibits LXR-Stimulated Hepatic Steatosis and SREBP-1c Expression. LXR plays a major role in regulating SREBP-1c expression, especially under insulin-stimulated conditions.[19] Thus, we examined whether ALA can inhibit LXR-stimulated hepatic steatosis. Consistent with previous reports,[20] fatty liver was induced by 7-day administration of the LXR ligand, T0901317 (Fig. 3A), and was accompanied by an increase in the expression of SREBP-1c, ACC, and FAS (Fig. 3B). Coadministration of ALA profoundly attenuated fatty changes, reduced plasma and liver triglyceride levels, and decreased expression of lipogenic genes compared with T0901317-treated control and pair-fed mice (Fig. 3).

We next examined the mechanism by which ALA inhibits LXR ligand-stimulated SREBP-1c expression. ALA inhibited T0901317-stimulated SREBP-1 gene expression in primary rat hepatocytes in a dose-dependent manner (Fig. 4A). Transient transfection with a promoter-reporter construct showed that T0901317 induced a marked increase in SREBP-1c promoter activity that was dose-dependently inhibited by ALA (Fig. 4B). Moreover, T0901317 induced a dose-dependent increase in the DNA-binding activity of LXR, which was inhibited by ALA in a dose-dependent manner (Fig. 4C). Interestingly, the inhibitory effect of ALA on LXR activity was AMPK-independent (Supporting Results; Supporting Fig. 2).

ALA Inhibits Insulin-Stimulated Transcriptional Activity of Sp1. In addition to LXR, other transcription factors have been proposed to be involved in insulin-stimulated SREBP-1c expression.[9] We found that insulin stimulated the luciferase activity of an LXR-mut-SREBP-1c promoter-reporter construct in which two LXR binding sites were mutated (Fig. 5A), although the activation by insulin was decreased compared to that of the wild type. These results support the notion that LXR is not the only factor responsible for insulin-stimulated SREBP-1c expression. Interestingly, ALA inhibited insulin-stimulated LXR-mut-SREBP-1c promoter activity, reinforcing the idea that ALA inhibits transcription factors other than LXR (Fig. 5A).

A recent study[8] showed that Sp1 plays an important role in insulin-stimulated SREBP-1c transcription. Accordingly, we examined whether ALA inhibits insulin-

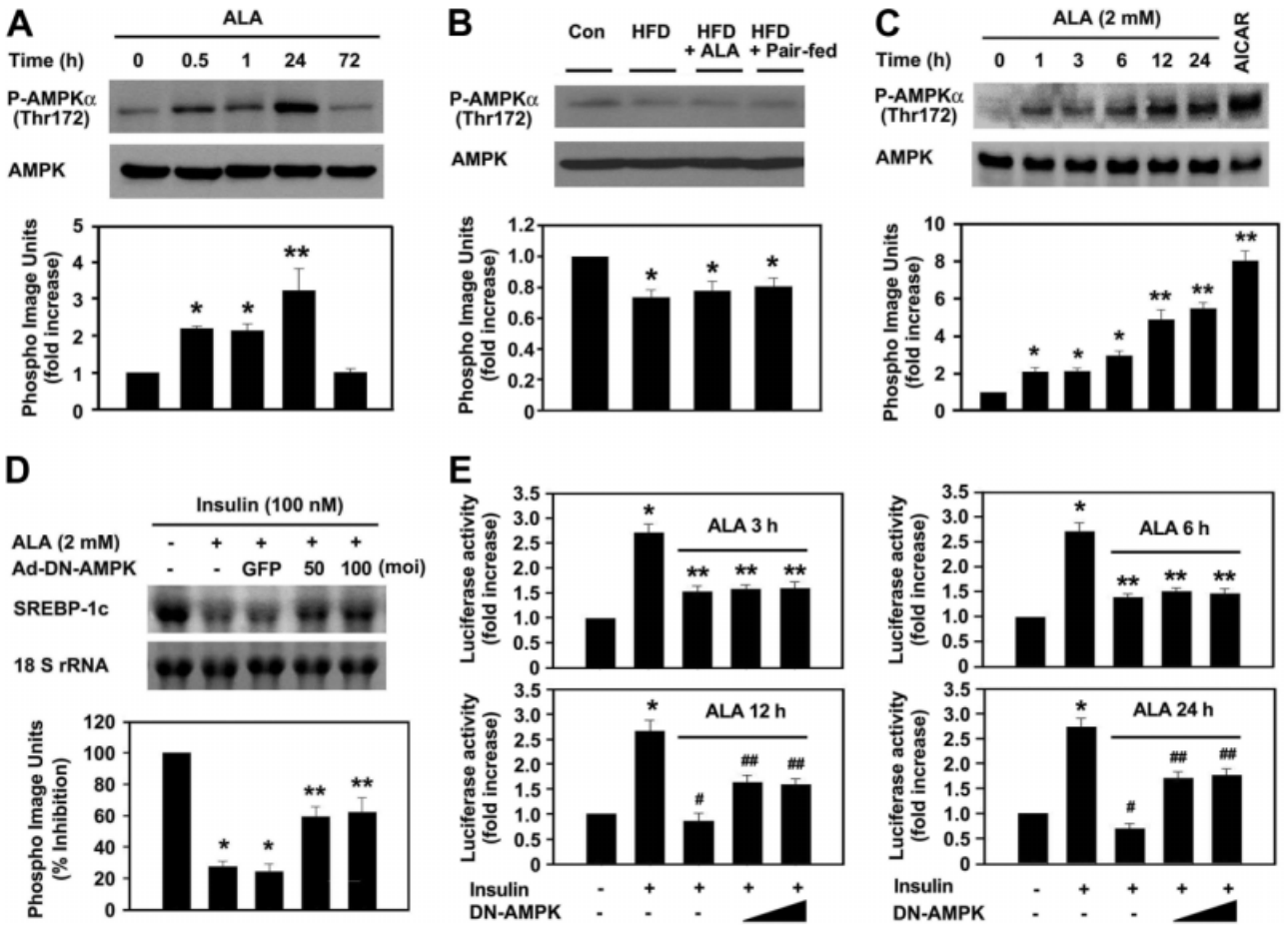

Fig. 2. ALA inhibition of insulin-stimulated SREBP-1c expression is partly mediated by AMPK activation. (A) Time-dependent changes in AMPK phosphorylation in the liver of rats given ALA. HFD-fed rats were given an intraperitoneal injection of 100 mg/kg ALA in saline or 0.5% (wt/wt) ALA mixed in food for 3 days. The rats were sacrificed at the indicated time points for measurement of AMPK phosphorylation. AMPK phosphorylation levels were normalized to AMPKα protein levels. Data are presented as the mean $\pm$ SEM of three separate measurements. *$P < 0.05$ and **$P < 0.01$ compared with control. (B) The effect of ALA on AMPK phosphorylation in the liver of HFD-fed rats. HFD-fed rats were divided into three groups: with ALA (0.5% wt/wt mixed in food), without ALA, and pair-fed; groups were maintained on their respective diets for 3 days. *$P < 0.05$ compared with control. (C) Western blot analysis showing the effect of ALA on AMPK phosphorylation. HepG2 cells were treated with ALA (2 mmol/L) for the indicated times. adenosine analog 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) (500 μmol/L for 1 hour) was used as a positive control. *$P < 0.01$ and **$P < 0.001$ compared with control. (D) Northern blot analysis showing the effect of adenovirus-carrying dominant-negative AMPKs (Ad-DN-AMPKs) on SREBP-1c mRNA expression. HepG2 cells were infected with the indicated dose (multiplicity of infection, MOI) of Ad-DN-AMPKs or adenovirus-carrying GFP (Ad-GFP) (100 MOI) for 24 hours. Cells were treated with ALA and/or insulin. RNA levels were normalized to 18S ribosomal RNA (rRNA) levels. Data are presented as the mean $\pm$ SEM of three separate measurements. *$P < 0.001$ compared with insulin alone; **$P < 0.01$ compared with insulin plus ALA with or without Ad-GFP. (E) Time course of the effects of ALA and DN-AMPKs on insulin-stimulated SREBP-1c promoter activity. HepG2 cells were cotransfected with a pSREBP-1c ($-1516/+40$)-luciferase construct (300 ng/well) and the DN-AMPKs expression vectors or control empty vector (100 or 200 ng/well each) and then stimulated with 100 nmol/L insulin for 6 hours, with or without ALA (2 mmol/L) pretreatment for the indicated time. *$P < 0.01$ compared with control, **$P < 0.05$ and $^{\#}P < 0.01$ compared with insulin alone, and $^{\#\#}P < 0.05$ compared with insulin and ALA.

stimulated Sp1 transcriptional activity. Using a reporter construct composed of four synthetic Sp1-response elements ([Sp1]×4 luciferase), we examined the effect of ectopic expression of Sp1, insulin and/or ALA. As expected, the combination of Sp1 and insulin led to approximately a five-fold increase in [Sp1]×4 luciferase activity (Fig. 5B). Addition of ALA induced a dose-dependent decrease in [Sp1]×4 luciferase activity. Interestingly, electrophoretic mobility shift assay revealed that, while insulin did not enhance the binding of Sp1 to the SREBP-1c promoter,[8] ALA profoundly decreased it (Fig. 5C).

Discussion

In the present study, we found that ALA decreased hepatic lipogenesis in animal models of hepatic steatosis through inhibition of SREBP-1c expression. Part of this effect was via AMPK activation. ALA activated AMPK in the liver and in cultured hepatocytes, and DN-AMPK

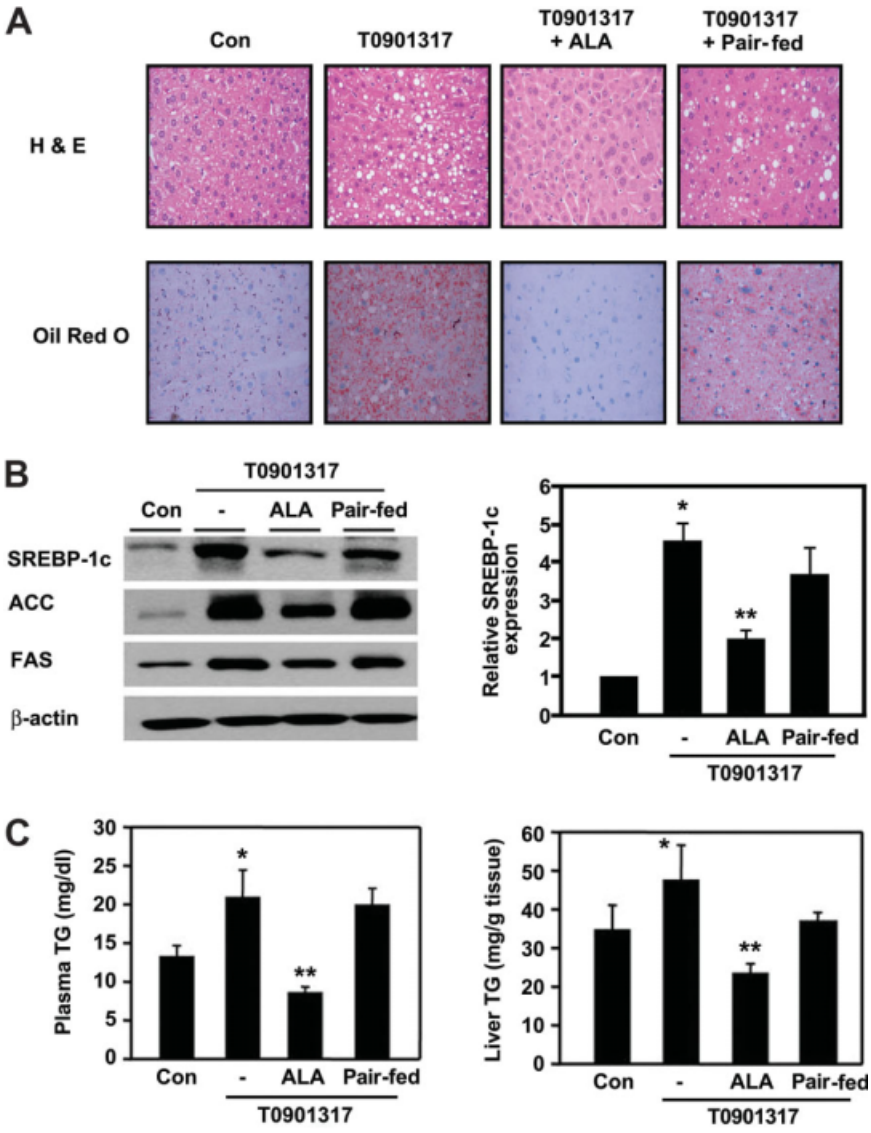

Fig. 3. ALA inhibits hepatic steatosis and SREBP-1c expression in mice given an LXR agonist. (A) Histological analysis of liver sections from T0901317-treated mice treated with or without ALA, or pair-fed. Upper panel: H&E staining. Lower panel: Oil red O staining. (B) Representative western blot showing the effect of ALA on LXR-induced SREBP-1c, ACC, and FAS expression ($n = 5$). Data are presented as the mean ± SEM of three separate measurements. $*P < 0.001$ compared with untreated mice and $**P < 0.001$ compared with T0901317-treated mice without ALA treatment. (C) Plasma triglyceride (TG) concentration (left) and hepatic triglyceride content (right). Fatty liver was induced in C57BL/6 mice by treatment with T0901317 for 7 days. These mice were treated with or without ALA, or pair-fed ($n = 5$). $*P < 0.05$ compared with untreated mice and $**P < 0.05$ compared with T0901317-treated mice without treatment of ALA.

significantly blocked ALA-induced suppression of SREBP-1c expression induced by insulin. In addition, ALA inhibited the activities of LXR and Sp1, known mediators of insulin-dependent SREBP-1c expression.[9] To our knowledge, ALA is the first drug shown to stimulate AMPK activity and inhibit LXR and Sp1 activities in hepatocytes (Fig. 6).

ALA is an essential cofactor for mitochondrial respiratory enzymes.[21] We previously showed that ALA activates AMPK activity in vascular endothelial cells and skeletal muscle.[10,22] It has also been shown that AMPK activators, including metformin and thiazolidinediones, inhibit the expression of the SREBP-1c gene and prevent the development of hepatic steatosis.[6,7] Thus, we hypothesized that ALA inhibits hepatic lipogenesis by acting through AMPK to inhibit SREBP-1c expression. As expected,[11] ALA activated AMPK in HFD-induced insulin-resistant rats and in insulin-stimulated, cultured hepatocytes. In addition, adenovirus-carrying dominant-negative AMPKs significantly, although not completely, reduced the effect of ALA on SREBP1c transcription at 12 and 24 hours. When activated, AMPK increases fatty acid oxidation and decreases lipid accumulation in tissues.[6,7,21,22] Thus, the preventive effect of ALA on hepatic steatosis may be partly explained by its effect on AMPK. However, ALA's effect on hepatic steatosis may not be wholly explained by this action. First, ALA activation of AMPK in the liver was not sustained: ALA increased hepatic AMPK phosphorylation for up to 1 day, but AMPK phosphorylation levels returned to baseline within 3 days. Second, inhibition of SREBP-1c promoter activity with short-term (3 and 6 hours) ALA treatment was unaffected by DN-AMPKs. Taken together, these data suggest that, although AMPK is an important mediator of ALA's effect at certain time points, an alternative,

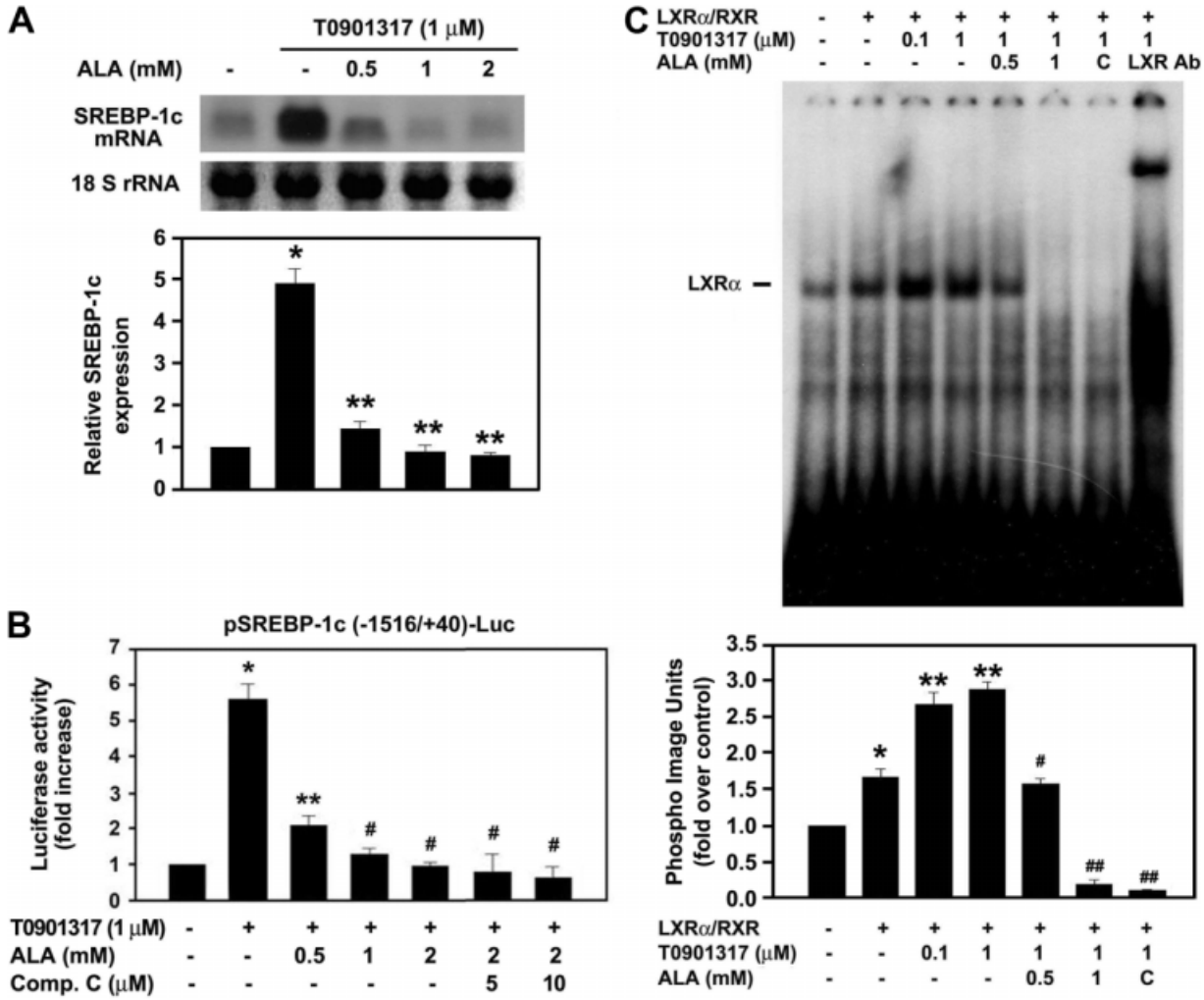

Fig. 4. ALA inhibits LXR activity in cultured cells. (A) Representative northern blot showing the effect of ALA on T0901317-stimulated SREBP-1c mRNA expression. Primary rat hepatocytes were incubated with 1 μmol/L T0901317 for 24 hours, with or without pretreatment with the indicated concentrations of ALA for 24 hours. Data are presented as the mean $\pm$ SEM of three separate measurements. $^{*}P < 0.001$ compared with control, $^{**}P < 0.001$ compared with T0901317 alone. (B) The effect of ALA on T0901317-stimulated SREBP-1c promoter activity. HepG2 cells were transfected with a pSREBP-1c ($-1516/+40$)-luciferase construct and then stimulated with 1 μmol/L T0901317, with or without the indicated concentrations of ALA or compound C. Data are presented as the mean $\pm$ SEM of three separate measurements. $^{*}P < 0.001$ compared with control, $^{**}P < 0.01$ and $^{\#}P < 0.001$ compared with T0901317 alone. (C) The effect of ALA on T0901317-stimulated LXR-DNA binding activity. HepG2 cells were cotransfected with pCMV7-LXRα (100 ng/well) and pCDNA3-retinoid X receptor α (RXRα) (100 ng/well) and then incubated with 1 μmol/l T0901317, with or without the indicated concentrations of ALA for 24 hours. Data are presented as the mean $\pm$ SEM of three separate measurements. $^{*}P < 0.01$ and $^{**}P < 0.001$ compared with control, $^{\#}P < 0.01$ and $^{\#\#}P < 0.001$ compared with LXRα/RXR and T0901317.

AMPK-independent pathway(s) mediates ALA's effect on hepatic steatosis during other periods.

In this context, it was recently shown that the expression of SREBP-1c in response to insulin is mediated by increased Sp1-mediated transactivation of the SREBP-1c promoter.[8] Our results support such a mechanism, showing that insulin enhanced and ALA decreased SREBP-1c transcription by modulating the ability of Sp1 to transactivate the SREBP-1c promoter. It should be noted that, although insulin did not change Sp1-DNA binding activity,[8] ALA dose-dependently decreased it. Taken together, these data suggest that suppression of insulin-stimulated SREBP-1c expression by ALA is mediated by inhibition of Sp1 activity as well as by stimulation of AMPK activity.

LXR is a member of a nuclear receptor superfamily that regulates the expression of key proteins involved in lipid metabolism and inflammation, serving as a kind of nuclear cholesterol sensor.[23] Because of its ability to increase adenosine triphosphate-binding cassette transporter A1 (ABCA1)-dependent reverse cholesterol transport, LXR is considered an attractive therapeutic target in the treatment of atherosclerosis. However, LXR also increases the expression of SREBP-1c, which leads to increased hepatic triglyceride synthesis. In our study, ALA inhibited hepatic

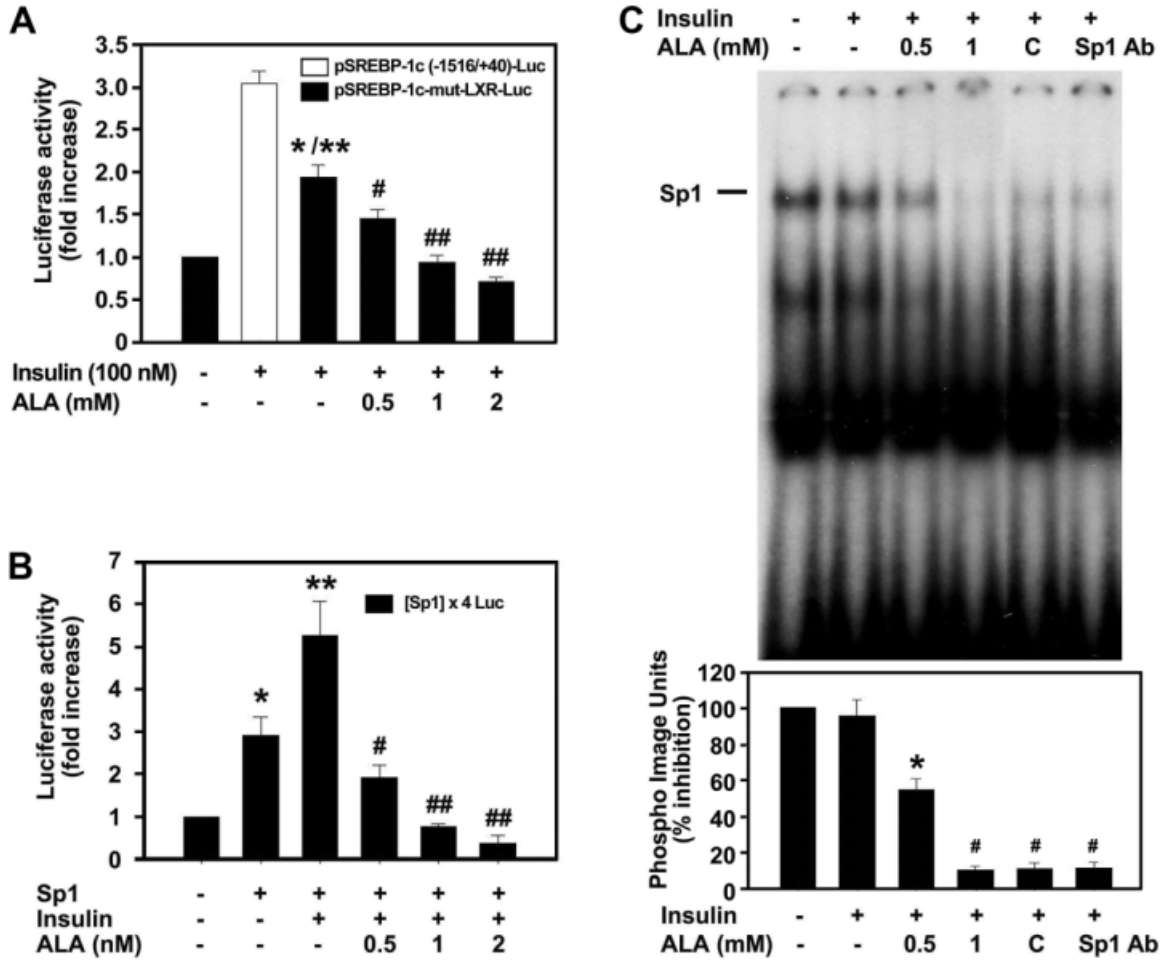

Fig. 5. ALA inhibits Sp1 activity. (A) The effect of ALA on the insulin-stimulated reporter activity of an LXR-binding-site-mutant SREBP-1c promoter (mut-LXR). HepG2 cells were transfected with pSREBP-1c (−1516/+40)-luciferase (300 ng/well) or pSREBP (−1516/+40) mutLXR1/mutLXR2-luciferase (300 ng/well) and then stimulated with 100 nmol/L insulin for 6 hours, with or without pretreatment with the indicated concentrations of ALA for 24 hours. Data are presented as the mean ± SEM of three separate measurements. $^{*}P < 0.01$ compared with control, $^{**}P < 0.01$ compared with insulin-stimulated SREBP-1c-promoter activity, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with insulin-stimulated LXR-mut-SREBP-1c promoter activity. (B) The effect of ALA on insulin-stimulated Sp1 transactivating activity. HepG2 cells were cotransfected with pCMV-Sp1 (Sp1) and the pGL3-Sp1 reporter (300 ng/well, [Sp1]×4 luciferase) and then stimulated with 100 nmol/L insulin for 6 hours, with or without pretreatment with the indicated concentrations of ALA for 24 hours. Data are presented as the mean ± SEM of three separate measurements. $^{*}P < 0.01$ and $^{**}P < 0.001$ compared with control, $^{\#}P < 0.001$ and $^{\#\#}P < 0.001$ compared with Sp1 and insulin-stimulated [Sp1]×4 luciferase. (C) The effect of ALA on insulin-stimulated Sp1 DNA binding activity. HepG2 cells were incubated with 100 nmol/L insulin for 6 hours, with or without pretreatment with the indicated concentrations of ALA for 24 hours. Data are presented as the mean ± SEM of three separate measurements. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with insulin alone.

steatosis in LXR ligand-treated mice and inhibited LXR ligand-stimulated expression of SREBP-1c and SREBP-1c target genes, both *in vivo* and *in vitro*. Gel shift assays and *in vitro* transient transfection studies employing promoter-reporter constructs showed that inhibition of LXR ligand-stimulated SREBP-1c expression by ALA is mediated by inhibition of LXR-DNA binding activity at the SREBP-1c promoter. Thus, it is likely that ALA's effect on hepatic steatosis and SREBP-1c expression is multifactorial: ALA inhibited insulin-stimulated SREBP-1c expression through activation of AMPK and inhibition of Sp1, and also inhibited LXR-mediated SREBP-1c transcription.

It is now well known that hepatic steatosis is closely associated with insulin resistance.[24] Previous reports that ALA increases insulin sensitivity in humans and animals[10,25] are supported by the present study, which showed that ALA improved insulin sensitivity in the liver as well as in peripheral tissues. Improved insulin sensitivity in ALA-treated rats was accompanied by a significant reduction in plasma insulin levels. This may be a physiological, adaptive mechanism to prevent hypoglycemia, but Targonsky et al.[12] recently demonstrated a direct inhibitory action of ALA on pancreatic beta cells. Insulin is one of the most important hormones in the regulation of hepatic lipogenesis. In cultured hepatocytes, insulin in-

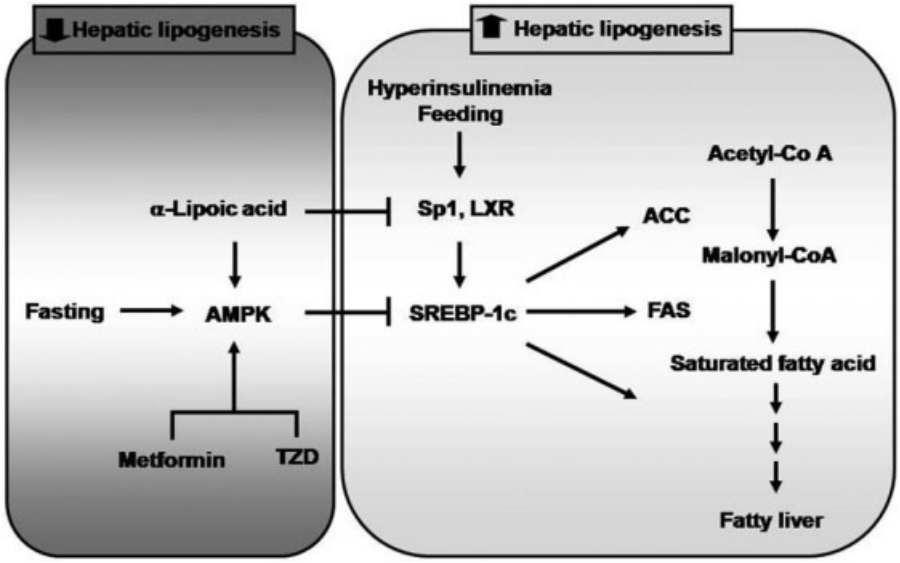

Fig. 6. Schematic model depicting ALA effects on hepatic lipogenesis. ALA decreases hepatic lipogenesis in animals through AMPK-dependent and AMPK-independent pathways. ALA inhibits SREBP-1c expression by activating AMPK and inhibiting LXR and Sp1 activity.

creases lipogenesis through induction of SREBP-1c.[18] Thus ALA's effect on hepatic steatosis may be partly due to a lowering of plasma insulin levels and consequent inhibition of Sp1 and/or LXR pathways, as described above.

Hepatic steatosis is emerging as the most important cause of chronic liver disease associated with the increasing incidence of obesity. It can progress to nonalcoholic steatohepatitis in 10%-20% of patients,[26] and even to advanced cirrhosis and hepatocellular carcinoma.[27,28] Although AMPK activators, including metformin and thiazolidinedione, have been shown to prevent hepatic steatosis in insulin-resistant animals, it is not yet known whether these drugs are also effective in human subjects with NAFLD. In the present study, we showed that ALA, which is currently used in clinical practice to treat patients with diabetic neuropathy,[29] prevents hepatic steatosis in animals through both AMPK-dependent and AMPK-independent pathways. This raises the possibility that ALA can be used to prevent the development and progression of NAFLD in patients with insulin resistance.

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