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Original Article

Asiatic acid promotes liver fatty acid metabolism in diabetic models

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Abstract: Asiatic acid (AA) has lipid-lowering and anti-diabetic properties. This study aimed to investigate the effects of AA on liver fatty acid metabolism. A mouse model of spontaneous type 2 diabetes was established by administering a high-fat diet to db/db mice. After treatment with AA for four weeks, liver morphology, fat synthesis, oxidation-related gene expression, and adenosine 5'-monophosphate-activated protein kinase (AMPK) signaling were assessed. The capacity of AA to protect against dysfunctional metabolism was also assessed in a free fatty acid (FFA)-induced hepatic cell line. Model mice weighed more and had heavier livers, higher ratios of liver to body weight, and higher levels of fasting serum glucose, cholesterol, and triglycerides, than control mice. AA treatment ameliorated these changes. Importantly, AA treatment reversed abnormal expression of SREBP1c, FAS, ACC1, SIRT1, PGC1α, PPARα, and CPT1α. *In vitro,* it was observed that AA reduced expression of genes associated with fatty acid synthesis and increased expression of genes associated with fatty acid oxidation. AA treatment induced phosphorylation of AMPK and acetyl CoA carboxylase. Taken together, results suggest that AA ameliorates liver fatty acid metabolism dysfunction in diabetic models, likely through inhibiting fatty acid synthesis and promoting fatty acid oxidation by activating AMPK signaling pathways.

Keywords: Asiatic acid, fatty acid metabolism, diabetics, AMPK

Introduction

Diabetes has reached an epidemic level, worldwide [1], severely affecting patient quality of life and causing an immeasurable social burden. Diabetes is caused by multiple factors. It is characterized by metabolic dysfunction, including impaired uptake and utilization of glucose, altered lipid metabolism, accumulation of various lipid species in the circulation and in tissues, and disruption of metabolic signaling pathways that regulate insulin secretion from pancreatic islet β-cells [2]. Fatty liver is one of the most common findings in type 2 diabetes, indicated by metabolic syndrome, liver cirrhosis, and cardiovascular disease. The liver is the primary organ responsible for lipid and glucose metabolism. As reported, this process is additionally regulated by insulin [3].

Fatty acids (FA) belong to a family of molecules within the lipid macronutrient class and, when completely oxidized by β-oxidation and the citric acid cycle, are the most effective source of ATP. However, unrestricted FA production without oxidation causes lipid accumulation, especially in the liver. Imbalanced regulation of FA synthesis and oxidation is a typical characteristic of metabolic disorders, including diabetes.

Centella asiatica is a Chinese medicine widely-used to treat varicose veins and chronic venous insufficiency. It is found in ointments to treat psoriasis and heal minor wounds [4]. Asiatic acid (AA) is an active component of *C. asiatica*. It possesses many biological activities, including anti-oxidant [5], liver-protecting [6], lipid-lowering [7], anti-cancer [8], and anti-diabetic [9] effects. AA has been reported to lower glu-

cose levels via an anti-inflammatory effect, regulating glucose metabolism enzymes, and reducing fibrosis in pancreatic islets [7, 10]. Tis study further investigated the mechanisms by which AA affects liver fatty acid metabolism in diabetic models.

Materials and methods

Mouse model and treatments

Twelve-week-old male db/db mice and age-matched male C57BL/6J mice were provided by the Model Animal Research Center of Nanjing University. Experimental protocols were approved by the Ethics Committee of Beijing University of Chinese Medicine. Mice were housed at Beijing Animal Experimental Center at 23 ± 2°C in 55 ± 10% humidity with a 12/12-h light/dark cycle. They were provided with food and water *ad libitum*. Db/db mice were fed a full-formula high-fat diet (composition: basal diet, cholesterol, egg-yolk powder, lard, bile salt), while C57BL/6J mice were fed a common basal diet. After one week of feeding, tail blood was collected. Non-fasting blood glucose concentration > 11.1 mmol/L was considered to indicate successful diabetic modeling.

Twelve modeled mice were randomly divided into the model and AA groups (six in each group). Six healthy male C57BL/6J mice were used as the normal group. AA (50 mg/kg, Chengdu Purui Biotech, China, 14053103) was administered orally once per day for 4 consecutive weeks. Saline served as negative control. The general condition of the mice (mental state, activity, hair color) was observed and body weight was recorded each week. After 4 weeks of treatment, the mice were fasted for 12 hours. Venous blood was obtained and fasting serum glucose (Glucose Detection Kit, YZB/Jing 0111-2013), cholesterol (Total cholesterol assay kit, YZB/Jing 0698-2010), and triglyceride (Serum Triglyceride Assay Kit, YZB/Jing 06997-2010) levels were measured using kits (Biosino Biotech, Beijing, China).

Hematoxylin and eosin

Liver tissue fixed in paraformaldehyde was used for hematoxylin and eosin (HE) staining. After fixation in 10% neutral formalin, the tissues were embedded in paraffin, sectioned (5-μm slice thickness), and stained with HE.

Cell culture

H4IIE cells (American Type Culture Collection, Bethesda, MD, USA) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, NY, USA), supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin-streptomycin (Sigma, USA) in 5% CO_2 at 37°C.

Neutral Red assay

Cells (5×10^4/ml) were seeded in 96-well culture plates and divided into normal, FFA, and FFA + AA (50 μM, 100 μM) groups. The main components of FFA included sodium oleate (Sigma, USA), which was dissolved in 0.1 M NaOH as a storing liquid for inducing deposition of triglycerides (TG). Concentration of FFA was 20 μM. AA was dissolved in DMSO (final concentration < 0.1 %) and concentration of AA was calculated based on the *in vivo* dose. A similar concentration of DMSO was applied in the FFA group. After incubation for 24 hours, 50 μg neutral red was added to each well. Three hours later, the cells were washed in PBS and neutral red was dissolved. Absorbance was measured using a microplate reader (Promega, USA) at a wavelength of 540 nm.

Oil Red O staining

Cells (5×10^4/ml) were seeded in 12-well culture plates and divided into normal, FFA, FFA + 50 μM AA, and FFA + 100 μM AA groups. Concentration of FFA was 20 μM. After 72 hours of incubation, the cells were washed in PBS and fixed in PFA for 10 minutes. Finally, the cells were stained with 60% Oil Red for 15 minutes. Images were taken by light microscope.

Real-time PCR

Total RNA was extracted from liver tissues and cells using TRIzol Reagent. RNA concentrations were determined spectrophotometrically and 1 μg RNA was reverse transcribed using an avian myeloblastosis virus reverse-transcriptase kit (Promega, Madison, WI, USA, 0000076581). PCR primers were as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH): 5'-TGAAGCAGGCATCTGAGGG-3' (Forward), 5'-CGAAGGTGGAAGAGTGGGAG-3' (Reverse); SIRT1: 5'-TCGTGGAGACATTTTTAATCAGG-3' (Forward), 5'-GCTTCATGATGGCAAGTGG-3' (Reverse); PGC-1α: 5'-TGCCATTGTTAAGACCGAG-3' (Forward),

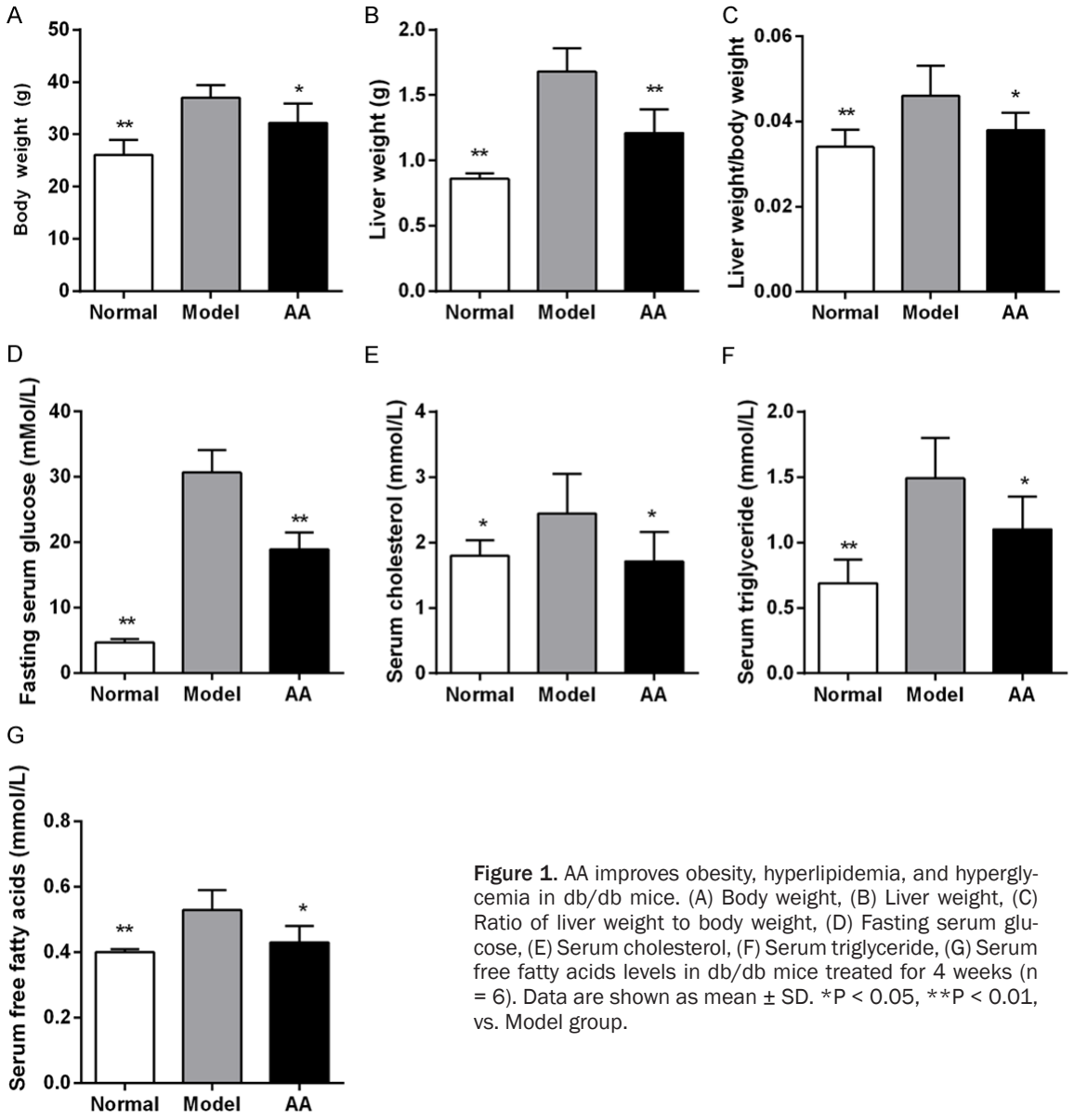

Figure 1. AA improves obesity, hyperlipidemia, and hyperglycemia in db/db mice. (A) Body weight, (B) Liver weight, (C) Ratio of liver weight to body weight, (D) Fasting serum glucose, (E) Serum cholesterol, (F) Serum triglyceride, (G) Serum free fatty acids levels in db/db mice treated for 4 weeks (n = 6). Data are shown as mean ± SD. *P < 0.05, **P < 0.01, vs. Model group.

5'-GGTCATTTGGTGACTCTGG-3' (Reverse); PPARα: 5'-CCTGCTTCCTGCCACTTG-3' (Forward), 5'-GTTCACCCTGATTCCTGATGTC-3' (Reverse); CPT-1α: 5'-AGGACCCTGAGGCATCTATT-3' (Forward), 5'-ATGACCTCCTGGCATTCTCC-3' (Reverse); SREBP1c: 5'-CCATCGACTACATCCGCTTCTT-3' (Forward), 5'-ACTTCGCAGGGTCAGGTTCTC-3' (Reverse); FAS: 5'-CTGCGGAAACTTCAGGAAATG-3' (Forward), 5'-GGTTCGGAATGCTATCCAGG-3' (Reverse); ACC: 5'-GGCCAGTGCTATGCTGAGAT-3' (Forward), 5'-AGGGTCAAGTGCTGCTCCA-3' (Reverse).

Amplification reactions were carried out using a 7500 real-time PCR system (Applied Biosystems) with an initial hold step (95°C for 10 minutes) and 40 cycles of a two-step PCR (95°C for 15 seconds and 60°C for 1 minute). Comparative computed tomography method was used to determine the amount of target, normalized to an endogenous reference (GAPDH) and relative to a calibrator ($2^{-\Delta\Delta Ct}$).

Western blotting

After treatment with the indicated drugs, cells were collected and proteins were extracted for Western blotting, as previously described [6]. Antibodies directed towards p-AMPKα1, t-AMPKα, p-ACC, ACC, and β-Actin (Cell Signaling

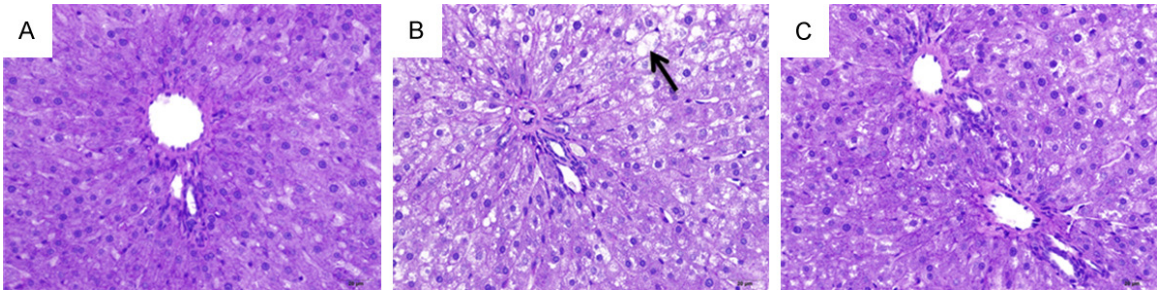

Figure 2. H&E staining of liver tissue in different groups. (A) Normal group, (B) Model group, (C) AA group (n = 6). (Magnification: 200×). Arrow indicated abnormal cell morphology.

Technology, Beverly, USA) were incubated overnight at 4°C at a dilution of 1:1000. An ECL reagent kit was applied to assist staining. Blots were scanned by ChemiDocTM XRS (Bio-Rad, USA). Grey density was analyzed by Image J 7.0 software.

Statistical analysis

Data are presented as mean ± standard deviation. One-way analysis of variance with *post-hoc* Bonferroni's tests for multiple comparisons were performed. Differences are considered significant at $p < 0.05$.

Results

Asiatic acid ameliorates obesity and diabetes in db/db mice

Initially, this study assessed measures of obesity and diabetes in db/db mice after AA treatment. Before intervention, body weights were significantly higher in the model group than the normal group (**Figure 1A**). However, AA treatment mitigated the increase in body weight. Livers of the model group mice were heavier than those of the normal group mice (**Figure 1B**), but AA treatment also attenuated liver weight. The ratio of liver weight to body weight was also compared (**Figure 1C**). The ratio was elevated in the model group, which was attenuated by AA treatment. In addition, fasting serum glucose (**Figure 1D**), cholesterol (**Figure 1E**), TG (**Figure 1F**), and free fatty acid (**Figure 1G**) levels were all higher in the model group. In contrast, AA treatment significantly decreased those parameters.

Asiatic acid affects fatty acid synthesis and fatty acid oxidation

Liver cell morphological changes were observed using H&E staining (**Figure 2**). Liver cells in the normal group were arranged in a radial distribution with clear morphology. The nucleus was located in the center of the cells. In contrast, cells in the model group were irregularly arranged with condensed nuclei. Hepatocyte steatosis was also observed. The nucleus was congested to one side of the cell. Importantly, AA treatment ameliorated these morphological changes in diabetic mice. Hepatocyte steatosis was rarely observed and cytosol lipid content was obviously reduced.

As indicated in **Figure 3**, this study also assessed fatty acid synthesis and fatty acid oxidation-related gene expression. SREBP1c, FAS, and ACC1 expression was significantly increased (**Figure 3A**), while expression of SIRT1, PGC1α, PPARα, and CPT1α were significantly lower in the model group than normal group (**Figure 3B**). After AA treatment, abnormal expression of SREBP1c, FAS, ACC1, SIRT1, PGC1α, PPARα, and CPT1α were ameliorated. These results suggest that AA regulates synthesis and oxidation of liver FFA.

Asiatic acid inhibits lipid accumulation in FFA-induced H4IIE cells

H4IIE hepatoma cell line is a physiologically relevant model used to investigate liver function [11]. H4IIE cells were incubated with different concentrations of AA (25, 50, 100 μM). Twenty-four hours later, the neutral red assay was applied to assess cell viability. As shown in **Figure 4A**, 50 to 100 μM AA significantly increased cell viability. Lipid deposition was analyzed by Oil Red O staining. In cells incubated with 20 μM FFA, increased fat deposition was observed, indicated by red stained lipid (**Figure 4C**). After AA treatment for 72 hours, lipid levels were significantly decreased in a dose-dependent manner (50-100 μM) (**Figure**

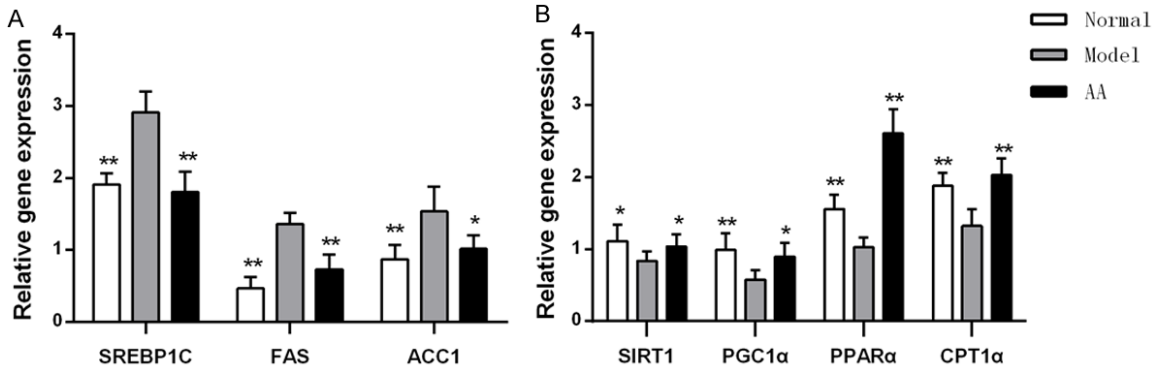

Figure 3. AA regulates hepatic fatty acid synthesis and oxidation-related gene expression in db/db mice. Expression of genes related to fatty acid synthesis (A) and fatty acid oxidation (B) in the liver of db/db mice (n = 6). Data are shown as mean ± SD. *P < 0.05, **P < 0.01, vs. Model group.

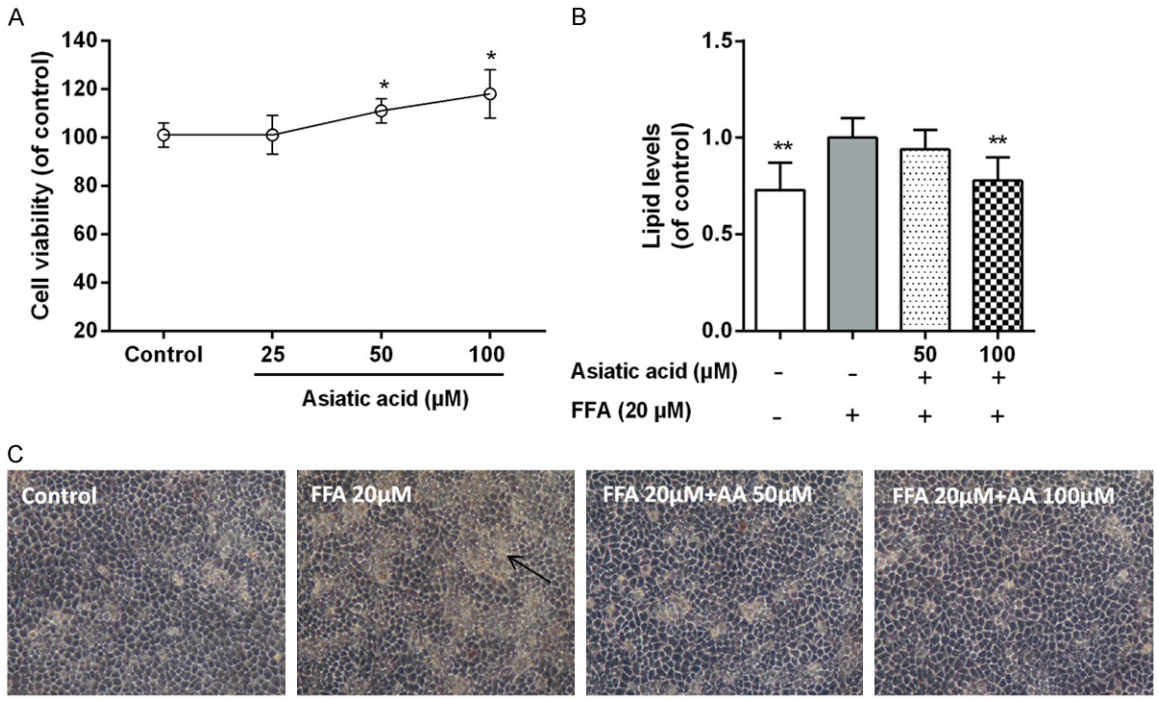

Figure 4. AA treatment ameliorates lipid accumulation in H4IIE cells induced by FFA. A. Cell viability was measured by Neutral Red test after H4IIE cells exposed to different concentration of AA for 24 hours. Data are expressed as the percentage of the control and shown as mean ± SD (n = 4). B and C. Cells were cultured in the absence or presence of AA (50, 100 μM) and FFA (20 μM) for 24 hours. B. Lipid accumulation was measured by optical density (OD) values at 540 nm after Oil Red O staining. Values are shown as mean ± SD (n = 6). *P < 0.05, **P < 0.01, vs. FFA 20 μM group. C. Oil Red O staining visualized intracellular oil droplets (Magnification: 200×). Arrow indicated oil droplets.

4B). These data suggest that AA significantly eliminated FFA-induced lipid deposition.

Fatty acid synthesis/oxidation-related gene expression was detected in FFA-induced H4IIE cells (**Figure 5**). FFA increased expression of SREBP1C and FAS, while decreasing SIRT1, PGC1α, CPT1α, and PPARα expression. Consistent with *in vivo* findings, AA treatment also reversed abnormal expression of those genes in FFA-induced H4IIE cells.

AMPK signaling pathways are very important. They have been reported to regulate fatty acid

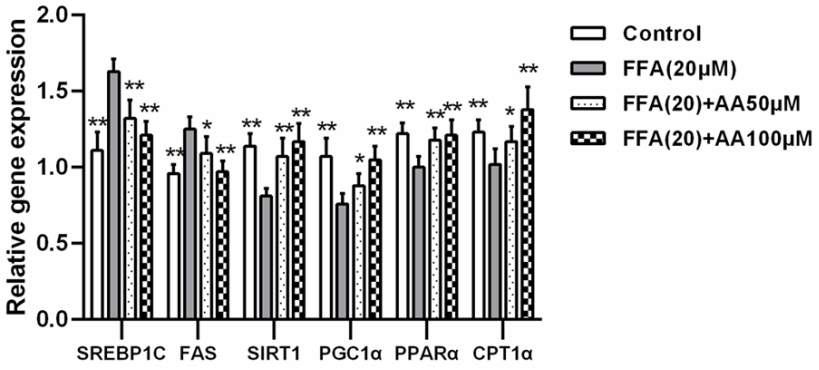

Figure 5. AA regulates hepatic fatty metabolism-related gene expression in FFA-induced H4IIE cells. Expression of genes related to fatty acid synthesis (SREBP1C, FAS) and fatty acid oxidation (SIRT1, PGC1α, PPARα, CPT1α) (n = 6). Data are shown as mean ± SD. *P < 0.05, **P < 0.01, vs. Control.

synthesis [12]. Phosphorylation of adenosine 5'-monophosphate-activated protein kinase (AMPK) and its downstream Acetyl CoA carboxylase (ACC) are important markers of AMPK activity. FFA treatment for 30 minutes significantly decreased the ratio of p-AMPKα to AMPKα and p-ACC to ACC. However, AA treatment (50 and 100 μM) elevated the ratio of p-AMPKα to AMPKα and p-ACC to ACC (**Figure 6**). These data suggest that AA treatment might regulate fatty acid synthesis and oxidation by activating AMPKα pathways.

Discussion

The present study demonstrated that AA treatment reduced liver FFA levels in a diabetic mouse model. Interestingly, AA treatment inhibited FFA synthesis-related gene expression and upregulated oxidation-related gene expression in diabetic mice. Moreover, these effects were confirmed by *in vitro* experiments. Finally, it was observed that AMPK signaling pathways are involved in the effects of AA.

Anti-diabetic effects of AA have been reported in both of genetic and streptozotocin (STZ)-induced models of diabetes. Liu et al. demonstrated that AA preserved beta cell mass and mitigated hyperglycemia in a diabetic rat model [13]. AA has also been reported to regulate carbohydrate metabolism in STZ-induced diabetic rats [10]. Antihyperlipidemic activity of AA has been reported in diabetic rats [7]. In addition, AA has been reported to reduce islet fibrosis in a diabetic model [9]. These results indicate the anti-diabetic effects of AA. The present study found that AA reduced symptoms of diabetes. More importantly, this study demonstrated a new function of AA in regulating liver metabolism.

The diabetic model used in this study was db/db transgenic mice treated with a high-fat diet. These mice exhibited typical characteristics of obesity, including increased body weight and liver weight along with high cholesterol, triglycerides, and FFA levels. The data implicated liver metabolism dysfunction in the modeled mice. Administration of AA reversed dysfunction. Consistent with previous reports, AA not only ameliorated diabetic symptoms, but also repaired the function of injured organs [14].

This study reported that expression of genes associated with fatty acid biosynthesis (SREBP1c, FAS, and ACC1) was elevated in diabetic mice on a high-fat diet, while expression of genes related to fatty acid oxidation (PGC1α, PPARα, CPT1A, and SIRT1) was depressed. SREBP1c is required for FAS promoter activation [15]. As reported, mice with liver-specific overexpression of mature Srebp1c expressed higher levels of Fas and Scd1 genes [16]. Here, it was demonstrated that AA treatment decreased SREBP1c, FAS, and ACC1 mRNA levels in diabetic mice. It was also found that AA treatment elevated PGC1α, PPARα, CPT1α, and SIRT1 expression in diabetic mice, as well as in the FFA-induced cell model. This study also found that AA treatment reduced serum cholesterol in diabetic mice. In future experiments, genes associated with cholesterol synthesis, such as SREBP2 and HMGCR, should also be measured [17].

In addition to the *in vivo* diabetic model, this study also treated a hepatic carcinoma cell line, H4IIE, with FFA. This cell line possesses the physiological characteristics of hepatic cells. In this study, 50-100 μM AA significantly enhanced the viability of H4IIE cells. FFA incubation remarkably increased lipid accumulation in H4IIE cells, but this was reversed by AA treat-

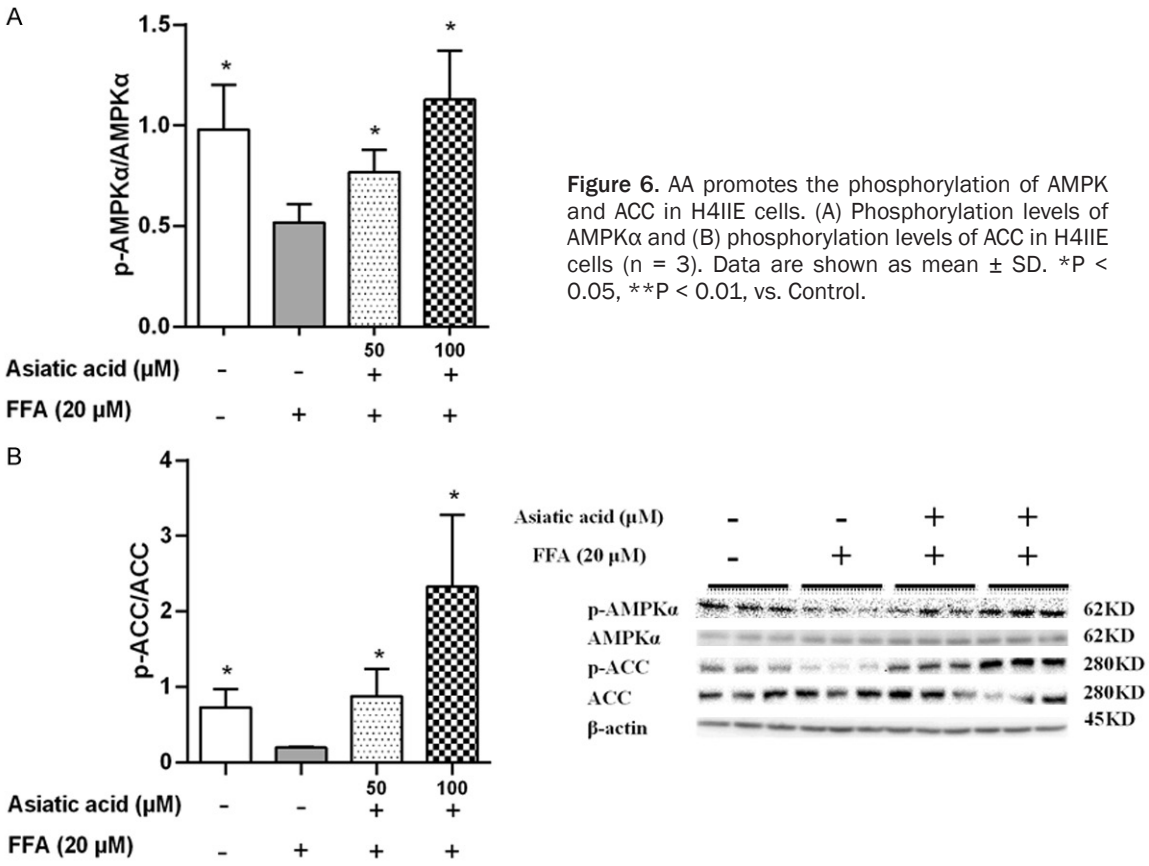

Figure 6. AA promotes the phosphorylation of AMPK and ACC in H4IIE cells. (A) Phosphorylation levels of AMPKα and (B) phosphorylation levels of ACC in H4IIE cells (n = 3). Data are shown as mean ± SD. *P < 0.05, **P < 0.01, vs. Control.

ment. *In vitro* experiments further supported the theory that AA could eliminate FFA-induced injury of hepatic cells. Consistent with *in vivo* data, it was observed that AA treatment reduced expression of genes associated with fatty acid synthesis and increased expression of oxidation-related genes.

AMPK, a key cellular energy sensor, plays a critical role in improving liver lipid metabolic homeostasis. Many reports have indicated that the extent of AMPKα (Thr172) phosphorylation reflects the degree of AMPK activation [17]. ACC is the first downstream enzymatic target of AMPK and is involved in promoting fatty acid synthesis and inhibiting fatty acid oxidation. Phosphorylation of AMPK suppresses ACC activation by promoting phosphorylation of ACC, followed by a decrease in fatty acid synthesis and an increase in fatty acid oxidation. Therefore, AMPK and its downstream signaler ACC play critical roles in modulating fatty acid production and lipid metabolism [18, 19]. AMPK likely activates the mTor signaling pathway to inhibit SREBP1 gene expression and fatty acid synthesis [20]. Obesity and type 2 diabetes are causally linked through their association with skeletal muscle insulin resistance [21]. Besides the effects on fatty acid synthesis, AMPK activation also increases fatty acid oxidation in skeletal muscle, likely by activating PPARα and PGC-1 [22]. In addition, AMPK activation also ameliorates fatty acid-induced increases in NF-κB transactivation in cultured human umbilical vein endothelial cells [23].

Conclusion

The present study demonstrated the therapeutic effects of AA on liver fatty acid metabolic dysfunction in a mouse model of diabetes. Based on changes in morphology and measurement of gene expression, it was concluded that AA could activate fatty oxidation and inhibit fatty acid synthesis, likely by activating AMPK signaling pathways.

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Disclosure of conflict of interest

None.

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