

Oroxin A from *Oroxylum indicum* improves disordered lipid metabolism by inhibiting SREBPs in oleic acid-induced HepG2 cells and high-fat diet-fed noninsulin-resistant rats

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Abstract

Background

Lipid metabolism disorders have become a major global public health issue. Due to the complexity of these diseases, much more research and many more drugs are needed to address them. Oroxin A, the major component of *Oroxylum indicum* (L.) Kurz (Bignoniaceae), can improve the lipid profiles of diabetic and insulin-resistant (IR) rats. Since insulin resistance is highly correlated with lipid metabolism, improving insulin resistance may also be an effective way to improve lipid metabolism. Thus, more research on the efficacy and mechanism of oroxin A under non-IR conditions is needed.

Method

In this research, we established lipid metabolism disorder rats by high-fat diet feeding and fatty HepG2 cell lines by oleic acid induction and evaluated the therapeutic effect and mechanism of oroxin A *in vitro* and *in vivo* by biochemical indicators, pathological staining, immunoblotting, and immunofluorescence staining.

Results

Oroxin A improved disordered lipid metabolism under non-IR conditions, improved plasma and hepatic lipid profiles, and enhanced the lipid-lowering action of atorvastatin. Additionally, oroxin A reduced the total triglyceride (TG) level by inhibiting SREBP1 expression and reducing the expression of ACC and FASN *in vivo* and *in vitro*. Oroxin A also reduced the total cholesterol (TC) level by inhibiting SREBP2 expression and reducing HMGCR expression *in vivo* and *in vitro*. In addition, oroxin A bound LDLR and increased AMPK phosphorylation.

Conclusion

Our results suggested that oroxin A may modulate the nuclear transcriptional activity of SREBPs by binding to LDLR proteins and increasing AMPK phosphorylation, thereby reducing lipid synthesis for lipid metabolism disorder treatment and prevention.

Introduction

Lipid metabolism disorders, which mainly include hyperlipidaemia and nonalcoholic fatty liver disease (NAFLD), have become major global health security issues associated with changes in people's living standards and diet structure (Cariou et al., 2021; Ferrer et al., 2021). More than 20% of people suffer from these diseases worldwide. Lipid metabolism disorders in turn affect the development and progression of many other diseases, such as type 2 diabetes and atherosclerosis, and thus reduce quality of life

(Guillamat-Prats et al., 2019; Taroni and Gellera, 2020). Therefore, an effective approach for retarding the development of lipid metabolism disorders is urgently needed. Currently, there are various drugs for treating disorders of lipid metabolism, especially hyperlipidaemia. For example, statins are 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, which are the most efficient drugs for treating hyperlipidaemia. However, these drugs also show some disadvantages; in severe patients, the administration of high-dose statins does not increase their hypolipidaemic effects and can damage human health in certain ways through effects such as kidney function damage, liver injury, and gastrointestinal dysfunction (Sultan et al., 2019). Additionally, statins do not reduce hepatic fat accumulation effectively, and no Food and Drug Administration (FDA)-approved drugs are available for the treatment of NAFLD (Wegermann et al., 2018). Thus, further research on and drugs for lipid metabolism disorders are needed.

The positive effects of natural products on lipid metabolism disorders have recently attracted increasing attention. Flavonoids are a group of polyphenols that are currently receiving attention because of their ability to improve lipid metabolism disorders. For example, hesperetin and baicalein can reduce high-fat diet (HFD)-induced hepatic and plasma lipid profiles in rats (Li et al., 2021; Sun et al., 2020). *Oroxylum indicum* (L.) Kurz (Bignoniaceae) is a traditional Chinese medicine and was first recorded in "Ben Cao Gang Mu Shi Yi". It has the effects of moistening the lung, improving the throat, draining the liver and harmonizing the stomach and is mainly used for lung fever, cough, mute, sore throat, liver and stomach gas pain, and sores (Dinda et al., 2015; National Commission of Chinese Pharmacopoeia, 2010). Modern research has found that *Oroxylum indicum* (L.) Kurz has antibacterial, anti-inflammatory, antiviral, antioxidant, anticancer, hypoglycaemic and other pharmacological effects and can be used clinically for respiratory and digestive system diseases. Oroxin A or baicalein-7-O-glucoside (PubChem CID: 5320313), a typical flavonoid, is the major component of *Oroxylum indicum* (L.) Kurz (Bignoniaceae). Our previous research in mice showed that oroxin A can decrease blood glucose, increase insulin sensitivity and improve lipid metabolism during the development from prediabetes to diabetes, suggesting that oroxin A might have a therapeutic effect on lipid metabolism disorders under conditions of insulin resistance (Sun et al., 2018, 2021). However, insulin resistance might induce lipid metabolism, and improving insulin resistance may be an effective way to improve lipid metabolism. Therefore, it is essential to evaluate the effect of oroxin A on lipid metabolism disorders under noninsulin-resistant (IR) conditions. To address this issue, the mechanism by which oroxin A improves lipid metabolism disorders could be explored under non-IR conditions. Furthermore, based on the lipid-lowering mechanism of oroxin A, it will also be important to search for a solution to the damage caused by high-dose statins through the combined use of statins and oroxin A.

In this study, we evaluated the role of oroxin A in disordered lipid metabolism and its potential mechanisms in HFD-fed non-IR rats and oleic acid-induced fatty HepG2 cells. Furthermore, we evaluated the synergistic effect of the combination of oroxin A and statins on the hypolipidaemic effect.

Materials and Methods

Materials

A protein assay kit, protein extraction kit, glucose assay kit (glucose oxidase method), and total cholesterol (TC), total triglyceride (TG), low-density lipoprotein cholesterol (LDLC), high-density lipoprotein cholesterol (HDL), aspartate transaminase (AST), and alanine transaminase (ALT) kits were purchased from the Jiancheng Institute of Biotechnology (Nanjing, China). An insulin kit was purchased from Cusabio Technology Co. Ltd. (Wuhan, China). A Western Bright ECL kit, Dulbecco's modified Eagle's medium (DMEM), antibiotics, Lipofectamine 3000, foetal bovine serum, and trypsin were purchased from Solarbio Co., Ltd. (Beijing, China). Polyvinylidene fluoride (PVDF) membranes were purchased from Millipore Co., Ltd. (Massachusetts, USA). A luciferase assay kit and luciferase reporter vector were purchased from Promega Co., Ltd. (Shanghai, China). HepG2 (human hepatocellular carcinoma) cells were obtained from the American Type Culture Collection (Maryland, USA). HFD chow (D16492) containing 36.4% carbohydrates, 25.6% fat, 20% protein, 1% cholesterol and 0.1% bile acid was purchased from SYSE Co., Ltd. (Changzhou China). Recombinant mouse low-density lipoprotein receptor (LDLR) protein (ab206024) was purchased from Abcam (Cambridge, UK). Primary antibodies against precursor sterol regulatory element-binding protein 1 (pSREBP1; 1:1000, PA005992) and phospho-SREBP1 (1:1000, PA050140) were purchased from Cusabio Technology Co. Ltd. (Wuhan, China). A primary antibody against precursor sterol regulatory element-binding protein 2 (pSREBP2; 1:1000, ab30682) was purchased from Abcam (Cambridge, UK). A primary antibody against phospho-SREBP2 (Ser455) (pSREBP2; 1:1000, PA5-106042) was purchased from Thermo Fisher Scientific (Massachusetts, USA). Primary antibodies against acetyl coenzyme A carboxylase (ACC; 1:1,000, bs2745R), fatty acid synthase (FASN; 1:1000, bs1498R), 3-hydroxy-3-methylglutaryl CoA reductase (HMGCR, 1:100 bsm-52822R), and mature SREBP1 (mSREBP1; 1:1000, bs1402R) were purchased from Bioss Technology Co. Ltd. (Beijing, China). Primary antibodies against β -actin (1:1,000, K200058M) and secondary antibodies were purchased from Solarbio Co., Ltd. Primary antibodies against sterol regulatory element-binding protein 2 (SREBP2) (1:1000, ab30682) and LDLR (ab30532) were purchased from Abcam (Cambridge, UK). A primary antibody against phospho-SREBP2 (Ser455) (1:500, PA5-64716) was purchased from Thermo Fisher Scientific Inc. (Massachusetts, USA).

Preparation of oroxin A

Oroxin A (more than 96% pure; Fig. S1, Table S1, and Table S2) was isolated from *O. indicum* following the method of our previous research (Sun et al., 2017; Sun et al., 2018). In short, the seed extract of *O. indicum* was dissolved in ethanol, applied to a reversed-phase column (ODS-C18) and eluted with 20–80% acetonitrile to obtain four fractions. Fraction 2 was concentrated and injected into the prepacked ODS column again to obtain pure oroxin A.

ELISA and docking experiments

Protein binding assay

Based on previous studies, we used an enzyme-linked immunosorbent assay (ELISA) to measure the binding efficiency of LDLR-oxoxin A (Li et al., 2017). Oxoxin A at a range of concentrations was coated onto a polystyrene plate, which was then incubated at 4°C overnight and blocked with 1% gelatine for 2 h. After these steps, recombinant LDLR protein (50 µL/well, 20 µg/mL) was added, and incubation was performed for 1 h. Then, the HRP-labelled goat anti-mouse IgG antibody was added for incubation to detect LDLR. It is worth noting that the plate needed to be washed three times with PBS after each step. Finally, the samples were visualized. After adding H₂O₂ and o-phenylenediamine for colour development, the reaction was stopped by adding 2 mM sulfuric acid, and the absorbance at 492 nm was measured within the specified time.

Docking experiment

Docking was carried out according to a previous research method (Sun et al., 2018). In brief, the ligand-binding domain structure of the LDLR protein was prepared according to a previous method (Sun et al., 2018), which was derived from the Protein Data Bank (PDB ID: 3SO6, www.rcsb.org). The Schrodinger Maestro 9.4 package was then used to predict the binding effect of the LDLR protein ligand binding domain on oxoxin A. In particular, we generated a docking grid (36 × 36 × 36 Å) and selected five optimal conformations according to empirical glide G scores (kcal/mol).

In vitro experiments

Cell culture

HepG2 cells were cultured in tissue culture flasks by adding DMEM containing 10% heat-inactivated foetal bovine serum (FBS) and antibiotics and were incubated at 5% humidified CO₂ and 37°C. The culture media was routinely changed every 2 days, and the cells were passaged by trypsinization before reaching confluence. The cell model was established by inducing cells to increase excess fat accumulation with 1 mM oleic acid as described in our previous method.

In vitro Oil red O staining

HepG2 cells were inoculated in 10-cm culture dishes and cultured to 70%-80% confluence for the experiment. After starvation for 12 h, HepG2 cells were treated with either DMSO or oxoxin A (5 µM or 50 µM) in combination with 1 mM oleic acid and incubated for 24 h, while the control cells were not treated with any treatment. HepG2 cells were subsequently fixed with 1 ml of 2% formaldehyde stained with Oil red O according to a previous description (Li et al., 2021). Finally, the stained samples were observed at an appropriate magnification of 40 x.

Cellular TC and TG assays

The cytotoxicity of oxoxin A was determined in our previous research (Sun et al., 2018). We determined whether the adipose HepG2 cell model was successfully established by measuring the cellular TC and TG levels. After successful model establishment, the cells in the treatment group were given different concentrations (5 µM and 50 µM) of oxoxin A, while the negative control cells were treated with DMSO.

After treatment for 24 h, we determined the cellular TC and TG levels with a commercial kit. Each treatment was repeated 6 times.

SREBP transcriptional activity assay

SREBP transcriptional activity in the cell model was assessed as described in our previously reported methods (Sun et al., 2020). Furthermore, we cotreated cells with different concentrations (0 μ M, 5 μ M, 50 μ M) of oroxin A and 1 mM oleic acid for 24 h and then assessed the action of oroxin A on the transcriptional activity of SREBP.

In vitro immunoblot analysis

After treating fatty HepG2 cells with DMSO or oroxin A (5 μ M or 50 μ M) for 24 h (cell treatments with pAMPK, phospho-SREBP1, and phospho-SREBP2 were performed for 1 h, 1 h, and 6 h, respectively), we collected the cells and extracted the proteins using a protein extraction kit according to the instructions. We then separated protein samples by 12% SDS-polyacrylamide gel electrophoresis (PAGE), which was used to detect mSREBP1, mSREBP2, AMPK, pAMPK and β -actin levels. We separated pSREBP1, phospho-SREBP, pSREBP2, phospho-SREBP2, ACC, FASN and HMGCR by 8% SDS-PAGE. Then, immunoblot analysis of the protein samples was performed as per our previously described method. Each treatment was repeated 3 times.

AMPK phosphorylation inhibition assay

Experiments were performed after starvation treatment of the fatty HepG2 cells for 12 h. The cells were given DMSO or 10 μ M compound C (dorsomorphin). After 1 h of treatment, the cells were then given DMSO or oroxin A. Then, the cellular TC and TG levels and SREBP transcriptional activity were determined as described for previous experiments.

In vitro immunofluorescence staining

An *in vitro* immunofluorescence staining experiment was performed with minor modifications according to a previously reported method (Donaldson, 2015). The cells were treated with different concentrations (0 μ M, 5 μ M, and 50 μ M) of oroxin A for 24 h for subsequent immunofluorescence staining *in vitro*. The fixed HepG2 cells were cultured for 10 minutes with BSA/FBS to block nonspecific sites of antibodies and then with PBS, pH 7.4, containing a 1% concentration of the SREBP1 or SREBP2 antibody. Then, the cells were washed with 1 ml PBS/FBS three times to remove unbound antibodies and incubated for 5 min each time. Then, the fixed HepG2 cells were incubated with 1% fluorophore-conjugated secondary antibodies. After adding the secondary antibody, the fixed HepG2 cells were covered with culture dishes and cultured away from light for 60 min at room temperature. Then, the cells were washed with 1 ml PBS/FBS to remove unbound antibodies three times and incubated for 5 min each time. Finally, we stained the nuclei with 4',6-diaminophenylindole (DAPI) and blocked the cells with neutral gel to observe the fluorescent signal using an Olympus IX81 microscope. When SREBP1 or SREBP2 underwent nuclear translocation, the FITC fluorescence signal could be observed in DAPI-stained nuclei to quantify SREBP1 or SREBP2.

***In vivo* experiments**

Animal experiments

Male Sprague–Dawley rats (180–220 g) were purchased from the Shandong Laboratory Animal Center (Jinan, China) (permission number SCXK 2014-0007). The current study protocol followed international ethical guidelines, and all animal handling procedures were performed in a standard laboratory and approved by the Institutional Animal Care and Use Committee of Shandong University of Technology (YLX20210801).

Experiment 1

Eight rats fed a standard diet served as the negative control group (NC). A model of lipid metabolism disorder was established in rats induced by HFD feeding. The remaining 32 rats fed a high-fat diet were randomized into four groups (n = 8): (I) high-fat diet group (HFD); (II) Essentiale Forte N treatment group (EN, positive control, 196.3 mg/kg); (III) low-dose oroxin A treatment group (LA, 50 mg/kg); and (IV) high-dose oroxin A treatment group (HA, 200 mg/kg). In addition, both the CON and HFD groups were randomly administered vehicle (0.5% CMC-Na) by gavage every day for 3 months.

Experiment 2

Eight rats fed a standard diet served as the negative control group (NC2). Disordered lipid metabolism was induced in the rats by HFD feeding. The remaining 48 rats fed a high-fat diet were randomized into six groups (n = 8): (I) high-fat diet group (HFD2); (II) high-dose oroxin A treatment group (HA2, 200 mg/kg); (III) low-dose atorvastatin treatment group (AT(10), 10 mg/kg/d); (IV) high-dose atorvastatin treatment group (AT(30), 30 mg/kg/d); (V) low-dose atorvastatin and oroxin A combination treatment group (AT (10) + HA); and (VI) high-dose atorvastatin and oroxin A combination treatment group (AT (30) + HA). Furthermore, both the CON and HFD groups were randomly administered vehicle (0.5% CMC-Na) by gavage every day for 3 months.

Physiological and biochemical properties

In the last week of the animal experiments, food intake was assayed. All rats were euthanized by isoflurane inhalation. In addition, we recorded the premortem weight of each group of rats and collected blood samples and liver samples for processing and preservation for further analysis. The plasma levels of fasting plasma glucose (FPG), 2-h postprandial glucose (2h-PG), fasting insulin (FINS), TC, TG, LDLC, HDLC, ALT, and AST were assayed according to the commercial kit instructions. Additionally, TC, TG, SOD, MDA, and GPx levels in the hepatic tissues were also analysed using commercial kits according to the instructions.

In vivo immunoblot analysis

For *in vivo* immunoblot analyses, protein samples were excised from the hepatic tissues of all groups of rats in Experiment 1, and the abundance of phospho-SREBP1, pSREBP1, mSREBP1, phospho-SREBP2,

SREBP2, AMPK, pAMPK, ACC, FASN, HMGCR, and β -actin was analysed as mentioned above.

Histological analysis

Some liver tissue samples of rats in each group in Experiment 1 were excised and fixed in formalin solution and embedded in paraffin to make 3 μ m paraffin sections. The sections were then stained with haematoxylin-eosin or Oil red O and observed at an appropriate magnification of 40 $\times$.

In vivo immunofluorescence staining

In vivo immunofluorescence staining was performed with minor modifications as described previously. (Liu et al., 2021). The washes mentioned in the following steps were performed using PBS for three minutes at a time. The steps were as follows: 1) dewaxing and hydration: after baking at 65°C for 1 h, the antigens were dewaxed twice using xylene immersion for 10 minutes each time, followed by hydration in 100%, 100%, 95%, 85% and 75% ethanol for 2 minutes, washing in distilled water 2 times for 3 minutes each time, and washing in PBS for 3 minutes; 2) repair of antigens: high-pressure heat treatment with EDTA repair solution for 2.5 minutes, and after natural cooling, washed 3 times; 3) closure of endogenous peroxidase: adding 3% hydrogen peroxide and cultured for 10 minutes at room temperature, and then washed 3 times; 4) closure: animal serum was added and sealed at 37°C for 10 minutes; 5) primary antibody incubation: after removal of the blocking solution, anti-SREBP1/anti-SREBP2 antibodies (1:100) were added and cultured overnight at 4°C, then washed 3 times; 6) secondary antibody incubation: after incubating with FITC-labelled secondary antibody (30 minutes at 37°C) at 4°C for 1 h, washed 3 times; 7) dehydration: dehydrated in 75%, 85%, 95% and 100% ethanol for 2 minutes, followed by two immersions in xylene for 2 minutes each; 8) staining and sealing: the nuclei were stained with DAPI, sealed with neutral gel and observed under the microscope (Olympus IX81). When SREBP1 or SREBP2 underwent nuclear translocation, the FITC fluorescence signal could be observed in DAPI-stained nuclei to quantify SREBP1 or SREBP2.

Statistical analyses

SPSS 17.0 was used for statistical analysis, and both *in vitro* and *in vivo* experimental results were expressed as the mean $\pm$ standard deviation. The level of significant differences (* $P < 0.05$) was analysed by one-way ANOVA, and the least significant difference (LSD) test was used for multiple comparisons. In addition, different letters (a, b, c) in the corresponding graphs or tables indicate statistically significant differences.

Results

Oroxin A reduced lipid accumulation in oleic acid-induced fatty HepG2 cells

The structure of oroxin A is shown in Fig. 1A. The levels of TC and TG and lipid accumulation were significantly increased in the fatty HepG2 cells induced by 1 mM oleic acid. (Fig. 1B-D). In contrast, oroxin

A decreased cellular TG and TC levels in a dose-dependent manner (Fig. 1B and 1C). These data indicated that oroxin A might reduce fatty acid synthesis and cholesterol synthesis. Furthermore, oroxin A reduced the area of Oil Red O staining (Fig. 1D). Taken together, these data indicate that oroxin A could inhibit oleic acid-induced cellular lipid accumulation.

Oroxin A inhibited the transcriptional activity of SREBPs

The transcriptional activity of SREBPs in HepG2 cells was determined by a double-luciferase system and found to be significantly increased by oleic acid. Oroxin A significantly inhibited increases in SREBP transcription levels in a dose-dependent manner, and 50 μ M oroxin A exhibited the strongest effect (Fig. 1E).

Oroxin A inhibited the SREBP1-related signalling pathway *in vitro*

We administered high concentrations of oleic acid to induce a fatty HepG2 cell model. To determine the effect of oroxin A on the SREBP1-related signalling pathway, the cells were given oroxin A for 24 h or phospho-SREBP1 for 6 h. The results showed that oroxin A elevated the phosphorylation level of SREBP1 and the expression level of pSREBP1 and reduced the expression level of mSREBP1 (Fig. 1F-I). Similarly, oroxin A reduced the expression levels of ACC and FASN after oroxin A treatment for 24 h (Fig. 1F, J and K). All of the above results showed a dose-dependent effect. In addition, immunofluorescence analysis showed that oroxin A treatment increased the amount of SREBP1 in the cytoplasm and decreased the amount in the nucleus (Fig. 2A).

Oroxin A inhibited the SREBP2-related signalling pathway *in vitro*

We treated fatty HepG2 cells with oroxin A for 24 h or phospho-SREBP2 for 6 h. The results indicated that oroxin A elevated the cellular phosphorylation level of SREBP2 and the expression level of pSREBP2 and reduced the expression level of mSREBP2 (Fig. 1L-O). Moreover, oroxin A reduced the expression level of HMGCR after oroxin A treatment for 24 h (Fig. 2L and P). In addition, immunofluorescence analysis indicated that oroxin A increased the amount of SREBP2 in the cytoplasm and reduced the amount in the nucleus (Fig. 2B).

Oroxin A inhibited the SREBP-related signalling pathway by phosphorylating AMPK

To further confirm the mechanism by which oroxin A inhibits SREBP transcriptional activity, we determined the phosphorylation levels of several signalling proteins. We found that oroxin A increased the phosphorylation level of AMPK (Fig. 3A). Furthermore, to determine the regulatory mechanism of oroxin A, the cells were treated with DMSO (control), oroxin A (50 μ M), compound C (dorsomorphin, an

AMPK phosphorylation inhibitor, 10 μ M) or a combination of compound C and oroxin A (Fig. 3B). The results indicated that compound C treatment significantly increased the transcriptional activity of SREBP compared with oroxin A treatment. Moreover, compared with oroxin A treatment, compound C treatment elevated the cellular TC and TG levels (Fig. 3C and D). These data indicate that oroxin A can inhibit the SREBP-related signalling pathway by phosphorylating AMPK.

Oroxin A might regulate the phosphorylation of AMPK by binding the LDLR protein

We used ELISA to study binding activity between the receptor protein and oroxin A. We found tight binding between the LDLR protein and oroxin A. The binding of LDLR to oroxin A was significantly increased by increasing oroxin A concentrations (Fig. 3E). Furthermore, we used Schrodinger Maestro 9.4 to predict the site in LDLR that binds oroxin A. Oroxin A was observed to bind to residues Phe168 and Ile115 of LDLR in the form of two hydrogen bonds, and these strong interactions formed the foundation of oroxin A-LDLR binding (Fig. 3F).

Oroxin A improved the plasma lipid profile in HFD-fed non-IR rats

Only the weights of the HFD and NC groups differed, and the HFD group was significantly increased compared with the NC group (Fig. 4A). In contrast, there were no differences in body weight and food intake among the HFD, LA and HA groups (Fig. 4B). In addition, there was no difference in FPG and 2h-PG between the HFD and NC groups, which indicated that HFD feeding did not induce an increase in blood glucose or insulin resistance, and there was no difference in FPG or 2h-PG among the HFD, LA, and HA groups (Fig. 4C). TC, TG and LDLC levels were significantly increased, and the HDLC level was significantly reduced after HFD feeding for 3 months (Fig. 4D-G). However, there was a significant improvement in the plasma lipid profile after treatment with oroxin A or EF. Although the HDLC level was unchanged, oroxin A significantly decreased the TC, TG, and LDLC levels (Fig. 4D-G).

Oroxin A improved the function, hepatic lipid profile, and morphology of HFD-fed non-IR rats

We observed that ALT and AST levels elevated significantly after administration of an HFD for 3 months, while oroxin A or EF-treated groups significantly reduced ALT and AST levels (Fig. 4H and 4I), suggesting that oroxin A can improve hepatic function. In addition, the hepatic TC and TG levels were also significantly increased after HFD feeding for 3 months, and oroxin A or EF significantly reduced TC and TG levels, indicating that oroxin A can reduce hepatic lipid profile (Fig. 4J and 4K). Regarding morphology, cellular swelling and a significant increase in the number of large and/or small hollow spaces were observed after HFD administration for 3 months, but oroxin A or EF reversed this damage, suggesting that oroxin A can largely restore the structure and morphology of the liver (Fig. 4L). Additionally, the area of Oil red O staining increased after HFD administration for 3 months, but oroxin A

or EF reversed this damage, which indicates that oroxin A can reduce lipid accumulation in the liver (Fig. 4M).

Oroxin A inhibited the SREBP-related signalling pathway *in vivo*

The results indicated that oroxin A increased AMPK phosphorylation after 3 months of treatment (Fig. 5A and 5B). Furthermore, oroxin A elevated the phosphorylation level of SREBP1 and the expression level of pSREBP1 and reduced the expression levels of mSREBP1, ACC and FASN (Fig. 5C-H). Similarly, oroxin A also elevated the phosphorylation level of SREBP2 and the expression level of pSREBP2 and decreased the expression levels of mSREBP2 and HMGCR (Fig. 5I-M). Furthermore, immunofluorescence analysis indicated that oroxin A increased the amounts of SREBP1 and SREBP2 in the cytoplasm and reduced the amounts in the nucleus (Fig. 6A and 6B).

Oroxin A enhanced the lipid-lowering effect of atorvastatin

After 3 months of combined treatment with oroxin A and atorvastatin, body weight, food intake, and blood glucose levels were unchanged compared with those observed upon treatment with oroxin A or atorvastatin alone (Fig. 7A-C). Furthermore, when the plasma lipid profiles were examined, TC and TG levels were significantly lower under combined oroxin A and atorvastatin treatment than under treatment with oroxin A or atorvastatin alone (Fig. 7D and E). Additionally, when the hepatic lipid profiles were examined, TC and TG levels were also lower under combined oroxin A and atorvastatin treatment than under treatment with oroxin A or atorvastatin alone, although the differences were not significant (Fig. 7F and G).

Discussion

Lipid metabolism disorders have become a major global public health issue, and more effective approaches for adjusting to different patient conditions are needed. In particular, there is an urgent need to address the challenge imposed by the possibility that high doses of statins may not significantly increase their hypolipidaemic effects but may cause serious side effects (Sultan et al., 2019). Oroxin A, the main component of *O. indicum*, accounts for nearly 30% of the *O. indicum* seed extract, which means that oroxin A is easy to obtain from *O. indicum* (Sun et al., 2017) and may be used to develop high-value products. Our previous results indicated that oroxin A is well tolerated and can improve the lipid profile of IR mice (Sun et al., 2018). However, the mechanism of action of oroxin A on lipid metabolism disorders has not been clearly elucidated, and more basic research is needed under non-IR conditions. Clearly defining the mechanism of oroxin A in lipid metabolism disorders would be beneficial for the application and development of treatments involving *O. indicum* or oroxin A. In preliminary experiments, we performed toxicity tests to determine the experimental dose of oroxin A and found that 0–2 g/kg oroxin A for 2 weeks failed to cause mortality in rats and that 3 g/kg oroxin A caused slight behavioural changes in rats (data not shown). Therefore, we considered an oroxin A dose of 2 g/kg as a safe dose for rats and

selected 200 mg/kg/d oroxin A (a dose equal to 1/10 of this safe dose) as the experimental dose. Furthermore, in our previous studies, many flavonoids, such as hesperetin and baicalin, had hypolipidaemic effects when given at high doses (200 mg/kg/d), while low doses (50 or 100 mg/kg/d) also tended to act (Li et al., 2021; Sun et al., 2018, 2020). Therefore, doses of 50 mg/kg/d and 200 mg/kg/d were used in subsequent animal experiments. In this study, our results showed that oroxin A could ameliorate lipid metabolism disorders under non-IR conditions similarly to EF, as it could reduce plasma and hepatic lipid profiles, ameliorate NAFLD, and enhance the lipid-lowering effect of atorvastatin. Our results also indicated that oroxin A could inhibit the transcriptional activity of SREBP1 and SREBP2. Furthermore, oroxin A reduced TG levels by inhibiting SREBP1 and TC levels by inhibiting SREBP2. Further results indicated that oroxin A could bind LDLR and increase AMPK phosphorylation, which might be the mechanism by which oroxin A regulates SREBPs.

SREBPs, which are located in the endoplasmic reticulum, are intracellular cholesterol sensors that regulate the transcription of genes encoding lipid-related enzymes. SREBPs are also typical nuclear transcription receptors (Jeon and Osborne, 2012). In a resting state, the SREBP precursor protein is located in the cytoplasm. When SREBP is activated, SREBP in the endoplasmic reticulum is further cleaved to form mSREBP and is translocated to the nucleus, where it regulates the transcription of corresponding genes (Horton et al., 2002). SREBPs are encoded by the SREBP-1 and SREBP-2 genes. SREBP1-c, which is mainly expressed in the liver, and SREBP1-a are translated from the SREBP1 gene and regulate the expression of genes that synthesize fatty acids and triglycerides, such as those encoding FASN and ACC (Hu et al., 2020). ACC is the rate-limiting enzyme of fatty acid synthesis, and its overexpression can promote fat synthesis. Studies have shown that the ACC content in the adipose tissue and livers of obese people is significantly higher than that in the corresponding tissues of people of normal weight. ACC catalyses the first step of fatty acid synthesis (i.e., acetyl coenzyme A carboxylation to synthesize malonyl-coenzyme A), after which malonyl-coenzyme A further synthesizes growing fatty acid chains under the action of an enzyme system responsible for fatty acid carbon chain extension (Chen et al., 2019). FASN is a multifunctional enzyme that plays an important role in fatty acid synthesis, catalysing the de novo biosynthesis of long-chain saturated fatty acids starting from acetyl-CoA and malonyl-CoA (Smith, 1994). Our results indicated that oroxin A decreased the nuclear transcription level of SREBP1 and decreased the expression levels of ACC and FASN *in vivo* and *in vitro*, which may be the main mechanism by which oroxin A reduces hepatic TG levels. Additionally, due to the circulation of lipids between the liver and blood, decreased hepatic TG levels lead to a decrease in plasma TG. SREBP2 is translated from the SREBP2 gene and regulates the expression of cholesterol synthesis genes, such as HMGCR (Karpaleetal., 2021). Cholesterol in the human body comes mainly from an endogenous pathway, and HMGCR, the rate-limiting enzyme in cholesterol synthesis, directly affects the speed of cholesterol synthesis and the amount of cholesterol in the body. Previous research indicated that HMGCR-related gene mRNA expression is upregulated after hepatocyte steatosis. Increases in HMGCR and cholesterol synthesis in the liver can increase systemic cholesterol in circulation (Ference et al., 2016). Our results indicated that oroxin A decreased the nuclear transcription level of SREBP2 and decreased the expression level of HMGCR *in vivo* and *in vitro*, which is the main mechanism by which oroxin A can reduce hepatic

TC. Similarly, due to the circulation of lipids between the liver and blood, decreased hepatic TC levels result in decreased plasma TC levels. In fact, HMGCR is the main target of statins, which inhibit HMGCR activity. However, when hyperlipidaemia occurs under severe conditions, high-dose statins do not exert a better lipid-lowering effect than low-dose statins (Frishman, 2015). To address this issue, a suitable drug for combination treatment with statins is needed. Oroxin A, which can reduce the abundance of HMGCR, might show potential for use in combined treatment with statins. Our results suggest that the lipid-lowering effect of oroxine A in combination with atorvastatin is superior to that of oroxine A or atorvastatin alone. Hence, oroxin A might be a good choice to enhance the therapeutic effects of statins.

To study the mechanism by which oroxin A regulates SREBPs, we explored the role of oroxin A on a series of protein kinases. Our results indicated that oroxin A elevated the phosphorylation level of AMPK and that an AMPK phosphorylation inhibitor (compound C) reduced the inhibitory effect of oroxin A on SREBPs. The above findings suggest that AMPK is a key factor in the regulatory effect of oroxin A on SREBPs. Many other researchers have also reported the relationship between AMPK and SREBPs, supporting our results (Ma et al., 2017). Furthermore, we measured the binding efficacy between oroxin A and many receptors related to lipid metabolism, and our results indicated tight binding between oroxin A and the LDLR protein. LDLR is a transmembrane glycoprotein that plays an important role in maintaining constant cholesterol levels (Meoli et al., 2019). Previous research demonstrated that when the LDLR protein was inhibited, the phosphorylation of AMPK increased (Wang et al., 2012; Zhong et al., 2019). This might be the mechanism by which oroxin A regulates AMPK/SREBPs. However, to our knowledge, the regulation of LDLR is very complicated, and LDLR can be regulated by many proteins, such as PPAR γ and other protein kinases (Guo et al., 2012; Bogachev et al., 2011). Thus, further determination of whether the binding between oroxin A and LDLR directly results in changes in the AMPK/SREBP pathway would be quite difficult. Taking these results into consideration, oroxin A might regulate the AMPK/SREBP pathway by binding the LDLR protein; however, more research is needed.

In addition to its lipid-lowering effect, oroxin A also has many other biological activities that might be beneficial for the treatment of lipid metabolism disorders. Our previous research indicated that oroxin A has antioxidant properties (Sun et al., 2017). Because oxidative stress injury causes the development of NAFLD and its progression, the antioxidant activity of oroxin A might also be beneficial for NAFLD prevention and treatment (Sumida et al., 2013). Additionally, inflammatory and mitochondrial apoptosis are also involved in the development of NAFLD and hyperlipidaemia (Di Naso et al., 2015). However, much more research needs to be done.

Conclusion

In conclusion, oroxin A significantly improved disordered lipid metabolism and could improve the plasma lipid profile for the treatment of hyperlipidaemia under normal conditions, enhance the lipid-lowering effect of statins for the treatment of severe hyperlipidaemia, improve liver function and reduce hepatic lipid accumulation for NAFLD treatment and prevention. Further mechanistic analysis indicated that oroxin A can inhibit the nuclear transcriptional activity of SREBPs to reduce lipid synthesis and that this

effect might be regulated by the binding of oroxin A to the LDLR protein and increased phosphorylation of AMPK. Our research expands the range of *O. indicum* applications and supports the use of oroxin A as a natural lead compound for improving disordered lipid metabolism. However, more detailed mechanistic and clinical studies are required to establish its appropriate application.

Abbreviations

insulin-resistant (IR)

high-fat diet (HFD)

non-alcoholic fatty liver disease (NAFLD)

Food and Drug Administration (FDA)

total triglyceride (TG)

total cholesterol (TC)

high-density lipoprotein cholesterol (HDL)

low-density lipoprotein cholesterol (LDL)

aspartate transaminase (AST)

alanine transaminase (ALT)

polyvinylidene fluoride (PVDF)

Dulbecco's modified Eagle's medium (DMEM)

HepG2 (human hepatocellular carcinoma cells)

low-density lipoprotein receptor (LDLR)

precursor sterol regulatory element-binding protein 1 (pSREBP1)

precursor sterol regulatory element-binding protein 2 (pSREBP2)

acetyl coenzyme A carboxylase (ACC)

fatty acid synthase (FASN)

3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR)

mature SREBP1 (mSREBP1)

sterol regulatory element-binding protein 2 (SREBP2)

enzyme-linked immunosorbent assay (ELISA)

fasting plasma glucose (FPG)

2-h postprandial glucose (2h-PG)

fasting insulin (FINS)

least significant difference (LSD)

Declarations

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Author contributions

Wenlong Sun, Jingda Li, and Lingru Li conceived and designed the research. Tianqi Cai, Xiaoxue Xu, Ling Dong, Shufei Liang, and Meiling Xin performed the experiments and the statistical analyses. Tianqi Cai, Chao Wang, Zhengbao Xu, Meng Wang, and Xinhua Song analysed the data and wrote and revised the manuscript. Tianxing Li helped with the animal experiments. Xudong Wang and Weilong Zheng helped with the WB experiments. Tianqi Wang helped with the cell experiments. All data were generated in-house, and no paper mill was used. All authors agree to be accountable for all aspects of work and for ensuring the integrity and accuracy of the data.

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Availability of data and materials

The data used to support the findings of this study are available from the corresponding author upon request.

Ethics approval and consent to participate

All animal handling procedures were performed in a standard laboratory and approved by the Institutional Animal Care and Use Committee of Shandong University of Technology (Y LX20210801).

Consent for publication

All authors agree to publish this article.

Competing interests

The authors declare that there are no conflicts of interest.

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References

1. Bogachev, O., Majdalawieh, A., Pan, X., Zhang, L., Ro, H.S., 2011. Adipocyte enhancer-binding protein 1 (AEBP1) (a novel macrophage proinflammatory mediator) overexpression promotes and ablation attenuates atherosclerosis in ApoE (-/-) and LDLR (-/-) mice. *Mol. Med.* 17, 1056–1064.
2. Cariou, B., Byrne, C.D., Loomba, R., Sanyal, A.J., 2021. Nonalcoholic fatty liver disease as a metabolic disease in humans: A literature review. *Diabetes Obes. Metab.* 23, 1069–1083.
3. Chen, L., Duan, Y., Wei, H., Ning, H., Bi, C., Zhao, Y., Qin, Y., Li, Y., 2019. Acetyl-CoA carboxylase (ACC) as a therapeutic target for metabolic syndrome and recent developments in ACC1/2 inhibitors. *Expert Opin. Investig. Drugs* 28, 917–930.
4. Dinda, B., SilSarma, I., Dinda, M., Rudrapaul, P., 2015. *Oroxylum indicum* (L.) Kurz, an important Asian traditional medicine: from traditional uses to scientific data for its commercial exploitation. *J Ethnopharmacol.* Doi: 10.1016/j.jep.2014.12.027.
5. Di Naso, F.C., Porto, R.R., Fillmann, H.S., Maggioni, L., Padoin, A.V., Ramos, R.J., Mottin, C.C., Bittencourt, A., Marroni, N.A., de Bittencourt, P.I., 2015. Obesity depresses the anti-inflammatory HSP70 pathway, contributing to NAFLD progression. *Obesity* 23, 120–129.
6. Donaldson, J.G., 2015. Immunofluorescence staining. *Curr. Protoc. Cell Biol.* 69, 4.3.1–4.3.7.

7. Ference, B.A., Robinson, J.G., Brook, R.D., Catapano, A.L., Chapman, M.J., Neff, D.R., Voros, S., Giugliano, R.P., Smith, G.D., Fazio, S., Sabatine, M.S., 2016. Variation in PCSK9 and HMGCR and risk of cardiovascular disease and diabetes. *N. Engl. J. Med.* 375, 2144–2153.
8. Ferrer, M.J., Silva, A.F., Abruzzese, G.A., Velázquez, M.E., Motta, A.B., 2021. Lipid metabolism and relevant disorders to female reproductive health. *Curr. Med. Chem.* Doi: 10.2174/0929867328666210106142912.
9. Frishman, W.H., 2015. Can clinicians improve on the effectiveness of statins with additional lipid lowering therapies? *Am. J. Med.* 128, 1160–1161.
10. Guillamat-Prats, R., Rami, M., Herzig, S., Steffens, S., 2019. Endocannabinoid signalling in atherosclerosis and related metabolic complications. *Thromb. Haemost.* 119, 567–575.
11. Guo, D., Reinitz, F., Youssef, M., Hong, C., Nathanson, D., Akhavan, D., Kuga, D., Amzajerdi, A. N., Soto, H., Zhu, S., Babic, I., Tanaka, K., Dang, J., Iwanami, A., Gini, B., Dejesus, J., Lisiero, D. D., Huang, T. T., Prins, R. M., Wen, P. Y., Robins, H. I., Prados, M. D., Deangelis, L. M., Mellinghoff, I. K., Mehta, M. P., James, C. D., Chakravarti, A., Cloughesy, T. F., Tontonoz, P., Mischel P. S., 2012. Correction: An LXR agonist promotes glioblastoma cell death through inhibition of an EGFR/AKT/SREBP-1/LDLR-dependent pathway. *Cancer Discov.* 2, 190–191.
12. Horton, J.D., Goldstein, J.L., Brown, M.S., 2002. SREBPs: Activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J. Clin. Invest.* 109, 1125–1131.
13. Hu, Q., Mao, Y., Liu, M., Luo, R., Jiang, R., Guo, F., 2020. The active nuclear form of SREBP1 amplifies ER stress and autophagy via regulation of PERK. *FEBS J.* 287, 2348–2366.
14. Jeon, T.I., Osborne, T.F., 2012. SREBPs: Metabolic integrators in physiology and metabolism. *Trends Endocrinol. Metab.* 23, 65–72.
15. Karpale, M., Käräjämäki, A. J., Kumm, O., Gylling, H., Hyötyläinen, T., Orešič, M., Tolonen, A., Hautajärvi, H., Savolainen M. J., Ala-Korpela, M., Hukkanen, J., Hakkola. J., 2021. Activation of nuclear receptor PXR induces atherogenic lipids and PCSK9 through SREBP2 mediated mechanism. *British Journal of Pharmacology*. In press.
16. Li, J., Wang, T., Liu, P., Yang, F., Wang, X., Zheng, W., Sun, W., 2021. Hesperetin ameliorates hepatic oxidative stress and inflammation via the PI3K/AKT-Nrf2-ARE pathway in oleic acid-induced HepG2 cells and a rat model of high-fat diet-induced NAFLD. *Food Funct.* Doi: 10.1039/D0FO02736G.
17. Li, J., Xiu, Z., Wang, R., Yu, C., Chi, Y., Qin, J., Fu, C., Matsuura, E., Liu, Q., 2017. The lipid moiety 7-ketocholesteryl-9-carboxynonanoate mediates binding interaction of oxLDL to LOX-1 and upregulates ABCA1 expression through PPAR γ . *Life Sci.* 177, 27–40.
18. Liu, P., Wu, P., Yang, B., Wang, T., Li, J., Song, X., Sun, W., 2021. Kaempferol prevents the progression from simple steatosis to non-alcoholic steatohepatitis by inhibiting the NF- κ B pathway in oleic acid-induced HepG2 cells and high-fat diet-induced rats. *J. Funct. Foods* 85, 104655.
19. Ma, L., Qiu, H., Li, L., Gou, D., 2017. MicroRNAs regulate lipid metabolism through PPAR and AMPK/SREBPs signaling pathways. *Chem. Life* 37, 1017–1029.

20. Meoli, L., Ben-Zvi, D., Panciotti, C., Kvas, S., Pizarro, P., Munoz, R., Stylopoulos, N., 2019. Intestine-specific overexpression of *Idlr* enhances cholesterol excretion and induces metabolic changes in male mice. *Endocrinology* 160, 744–758.
21. National Commission of Chinese Pharmacopoeia, 2010. *Pharmacopoeia of Peoples Republic of China*. China Chemical Industry Press, Beijing p. 60.
22. Smith, S., 1994. The animal fatty acid synthase: One gene, one polypeptide, seven enzymes. *FASEB J.* 8, 1248–1259.
23. Sultan, S., D'Souza, A., Zabetakis, I., Lordan, R., Tsoupras, A., Kavanagh, E.P., Hynes, N., 2019. Statins: Rationale, mode of action, and side effects, in: Zabetakis, I., Lordan, R., Tsoupras, A. (Eds.), *The Impact of Nutrition and Statins on Cardiovascular Diseases*. Academic Press, Cambridge, MA, pp. 171–200.
24. Sumida, Y., Niki, E., Naito, Y., Yoshikawa, T., 2013. Involvement of free radicals and oxidative stress in NAFLD/NASH. *Free Radic. Res.* 47, 869–880.
25. Sun, W., Liu, P., Wang, T., Wang, X., Zheng, W., Li, J., 2020. Baicalein reduces hepatic fat accumulation by activating AMPK in oleic acid-induced HepG2 cells and high-fat diet-induced non-insulin-resistant mice. *Food Funct.* 11, 711–721.
26. Sun, W., Liu, P., Yang, B., Wang, M., Wang, T., Sun, W., Wang, X., Zheng, W., Song, X., Li, J., 2021. A network pharmacology approach: Inhibition of the NF- κ B signaling pathway contributes to the NASH preventative effect of an *Oroxylum indicum* seed extract in oleic acid-stimulated HepG2 cells and high-fat diet-fed rats. *Phytomedicine*, 153498. Doi: 10.1016/j.phymed.2021.153498.
27. Sun, W., Sang, Y., Zhang, B., Yu, X., Xu, Q., Xiu, Z., Dong, Y., 2017. Synergistic effects of acarbose and an *Oroxylum indicum* seed extract in streptozotocin and high-fat-diet induced prediabetic mice. *Biomed. Pharmacother.* 87, 160–170.
28. Sun, W., Zhang, B., Yu, X., Zhuang, C., Li, X., Sun, J., Xing, Y., Xiu, Z., Dong, Y., 2018. Oroxin A from *Oroxylum indicum* prevents the progression from prediabetes to diabetes in streptozotocin and high-fat diet induced mice. *Phytomedicine* 38, 24–34.
29. Taroni, F., Gellera, C., 2020. Disorders of lipid metabolism, in: Rosenberg, R.N., Pascual, J.M. (Eds.), *Rosenberg's Molecular and Genetic Basis of Neurological and Psychiatric Disease*. Elsevier, Amsterdam, NL, pp. 731–753.
30. Wang, Y.X., Kong, W.J., Li, Y.H., Tang, S., Li, Z., Li, Y.-B., Shan, Y.Q., Bi, C.W., Jiang, J.D., Song, D.Q., 2012. Synthesis and structure–activity relationship of berberine analogues in LDLR up-regulation and AMPK activation. *Bioorg. Med. Chem.* 20, 6552–6558.
31. Wegermann, K., Diehl, A.M., Moylan, C.A., 2018. Disease pathways and molecular mechanisms of nonalcoholic steatohepatitis. *Clin. Liver Dis.* 11, 87–91.
32. Zhong, S., Li, L., Zhang, Y.L., Zhang, L., Lu, J., Guo, S., Liang, N., Ge, J., Zhu, M., Tao, Y., Wu, Y.C., Yin, H., 2019. Acetaldehyde dehydrogenase 2 interactions with LDLR and AMPK regulate foam cell formation. *J. Clin. Invest.* 129, 252–267.

Supplementary Material

Supplementary Material is not available with this version

Figures

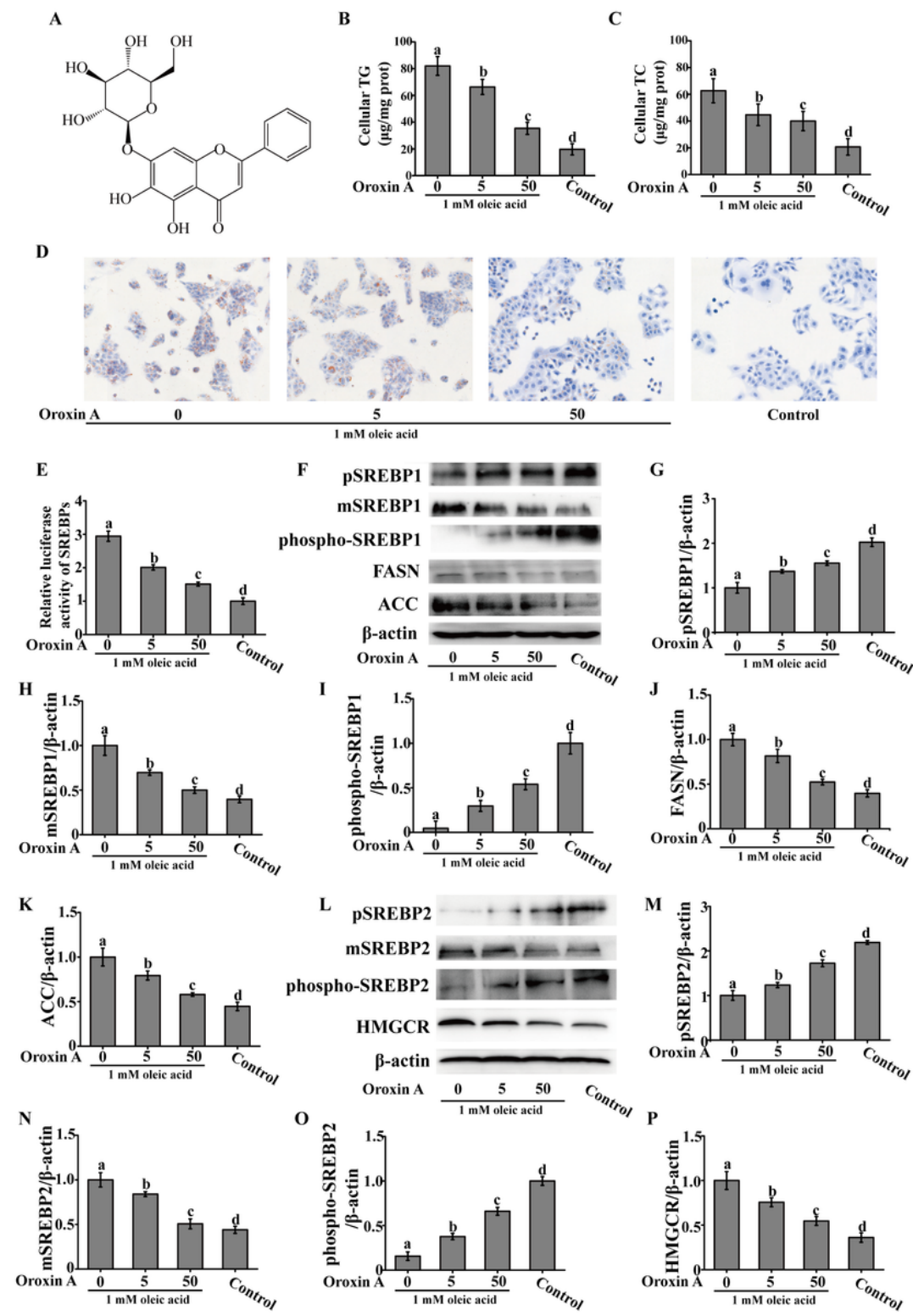


Figure 1

Effects of oroxin A on lipid accumulation, SREBPs, the SREBP1 pathway, and the SREBP2 pathway in oleic acid-induced fatty HepG2 cells. (A) Structure of oroxin A; (B) Cellular TG levels; (C) Cellular TC levels; (D) Oil red O staining; (E) Transcriptional activity of SREBPs; (F) Immunoblot analysis of pSREBP1, mSREBP1, phospho-SREBP1, FASN and ACC; (G) Densitometry analysis of pSREBP1; (H) Densitometry analysis of mSREBP1; (I) Densitometry analysis of phospho-SREBP1; (J) Densitometry analysis of FASN; (K) Densitometry analysis of ACC; (L) Immunoblot analysis of pSREBP2, mSREBP2, phospho-SREBP2 and HMGCR; (M) Densitometry analysis of pSREBP2; (N) Densitometry analysis of mSREBP2; (O) Densitometry analysis of phospho-SREBP2; (P) Densitometry analysis of HMGCR. The values are means $\pm$ standard deviations (n = 3 for each group). Significant differences ($p < 0.05$) were analysed using one-way ANOVA followed by the LSD test for multiple comparisons. The values sharing a common letter (a, b, c, and d) were not significantly different.

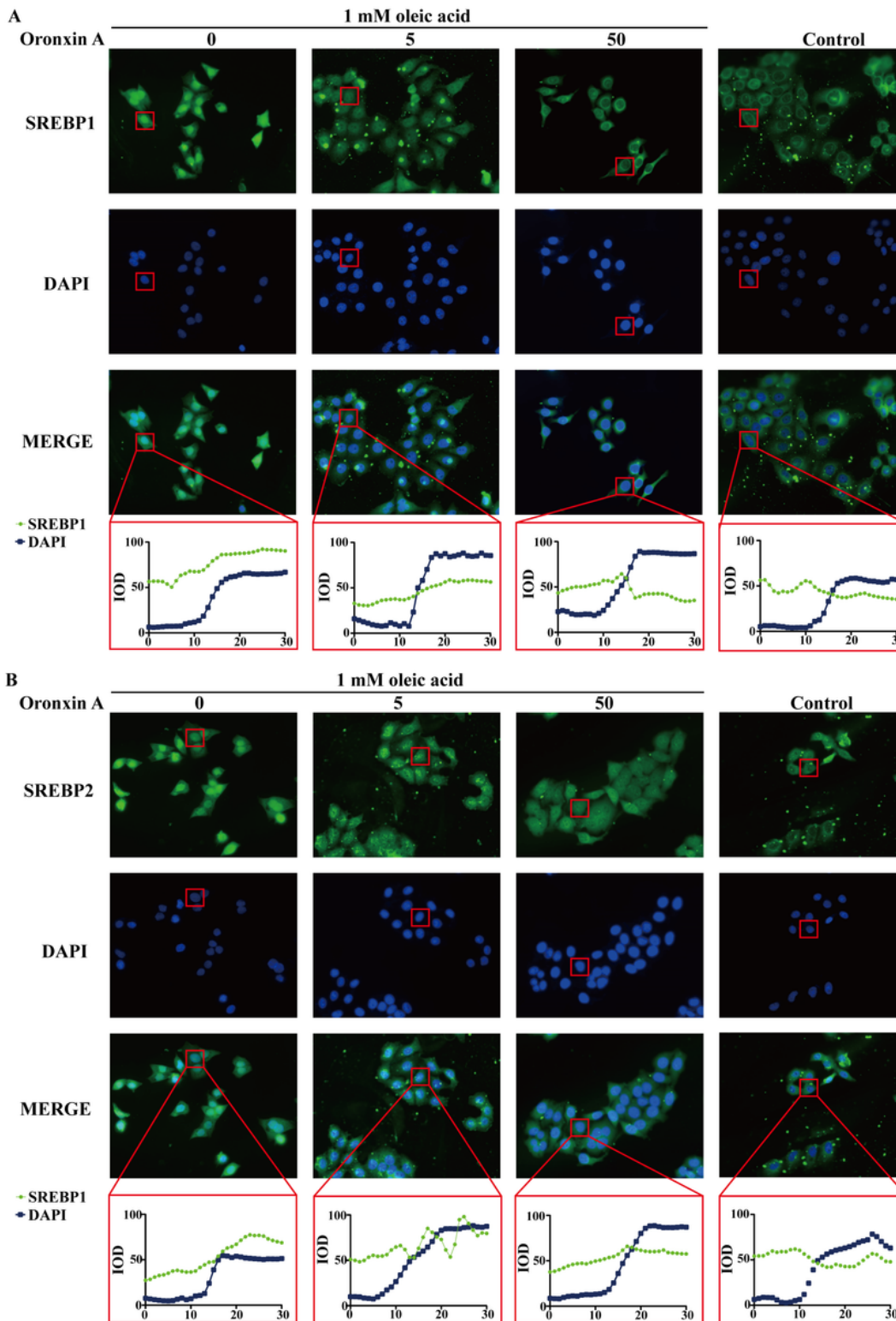


Figure 2

Effects of oroxin A on SREBP1 and SREBP2 in oleic acid-induced fatty HepG2 cells evaluated using immunofluorescence. (A) Immunofluorescence analysis of DAPI and SREBP1, merged image of DAPI and SREBP1 and relative luminance curves of DAPI and SREBP1 in a representative area (from the edge to the centre of the marked yellow frame); (B) Immunofluorescence analysis of DAPI and SREBP2, merged image of DAPI and SREBP2 and relative luminance curves of DAPI and SREBP2 in a representative area

(from the edge to the centre of the marked yellow frame). The values are means $\pm$ standard deviations ($n = 3$ for each group). Significant differences ($p < 0.05$) were analysed using one-way ANOVA followed by the LSD test for multiple comparisons. The values sharing a common letter (a, b, c, and d) were not significantly different.

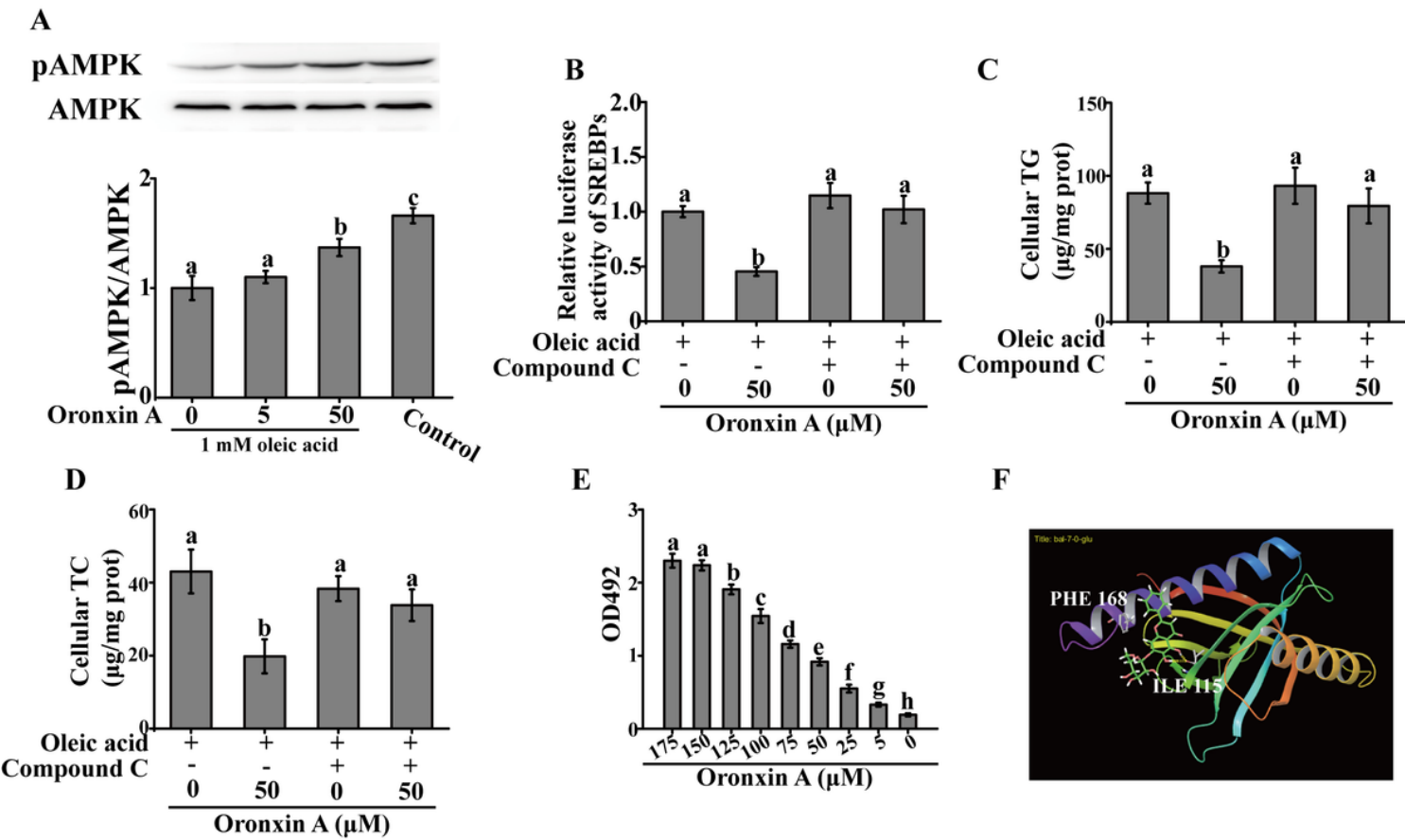


Figure 3

Effect of oroxin A on AMPK and LDLR levels in oleic acid-induced fatty HepG2 cells. (A) Immunoblot and densitometry analysis of pAMPK and AMPK; (B) Transcriptional activity of SREBPs; (C) Cellular TG levels; (D) Cellular TC levels; (E) The binding of oroxin A and LDLR; (F) Docking of LDLR and oroxin A. The values are means $\pm$ standard deviations ($n = 3$ for each group). Significant differences ($p < 0.05$) were analysed using one-way ANOVA followed by the LSD test for multiple comparisons. The values sharing a common letter (a, b, c, and d) were not significantly different.

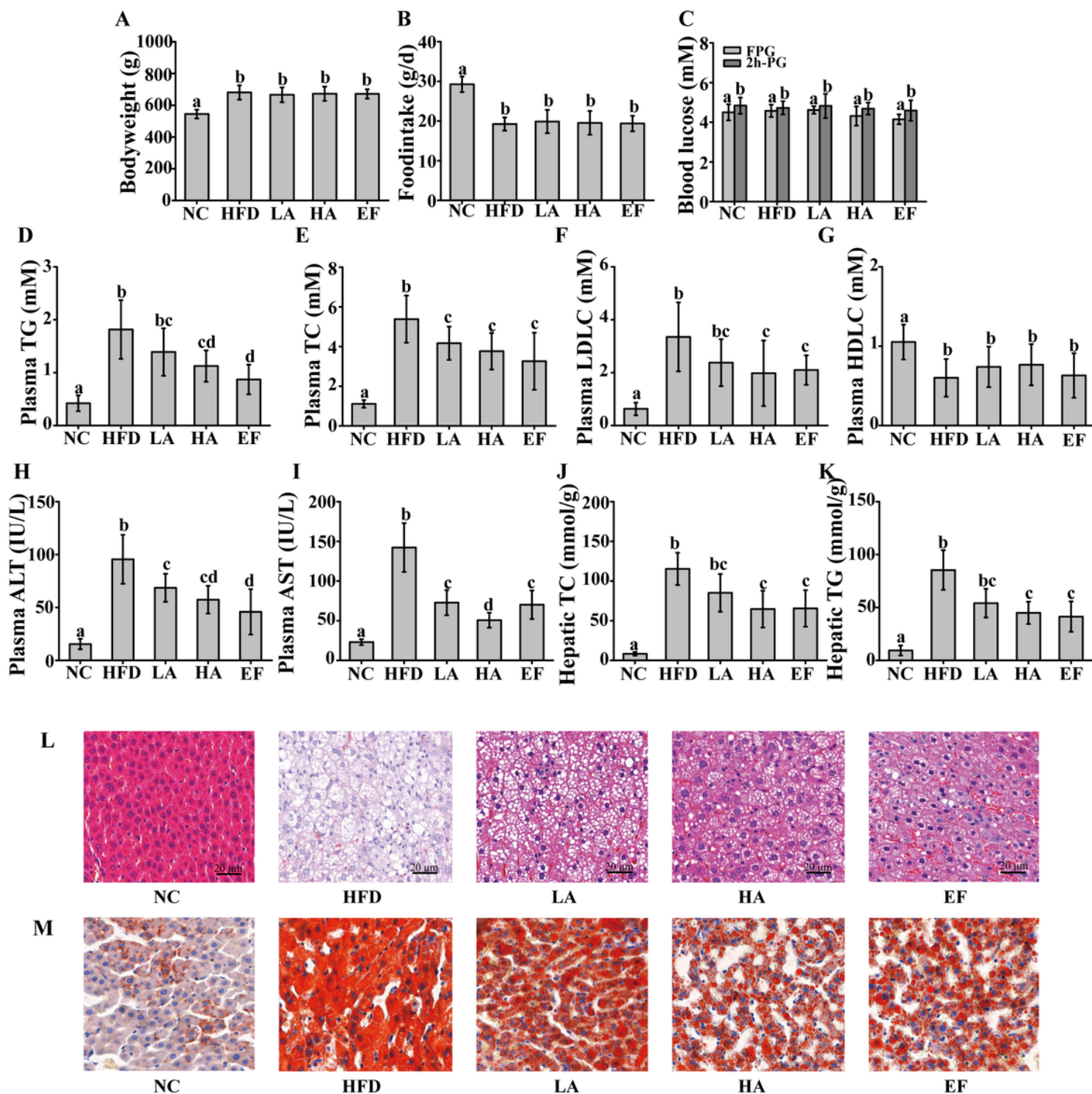


Figure 4

Effects of oroxin A on the plasma lipid profile, hepatic lipid profile, and liver function, architecture, and morphology in HFD-fed rats. (A) Body weight; (B) Food intake; (C) Blood glucose; (D) Plasma TG; (E) Plasma TC; (F) Plasma LDLC; (G) Plasma HDLC; (H) Plasma ALT; (I) Plasma AST; (J) Hepatic TC; (K) Hepatic TG; (L) HE staining of liver; (M) Oil red O staining of liver. NC: rats fed standard chow were administered 0.5% CMC-Na; HFD: rats fed HFD chow were administered 0.5% CMC-Na; LA: rats fed HFD

chow were administered 50 mg/kg/d oroxin A in 0.5% CMC-Na; HA: rats fed HFD chow were administered 200 mg/kg/d oroxin A in 0.5% CMC-Na; EF: rats fed HFD chow were administered 196.3 mg/kg Essentiale Forte N in 0.5% CMC-Na. The values are means $\pm$ standard deviations ($n = 8$ for each group). Significant differences ($p < 0.05$) were analysed using one-way ANOVA followed by the LSD test for multiple comparisons. The values sharing a common letter (a, b, c, and d) were not significantly different.

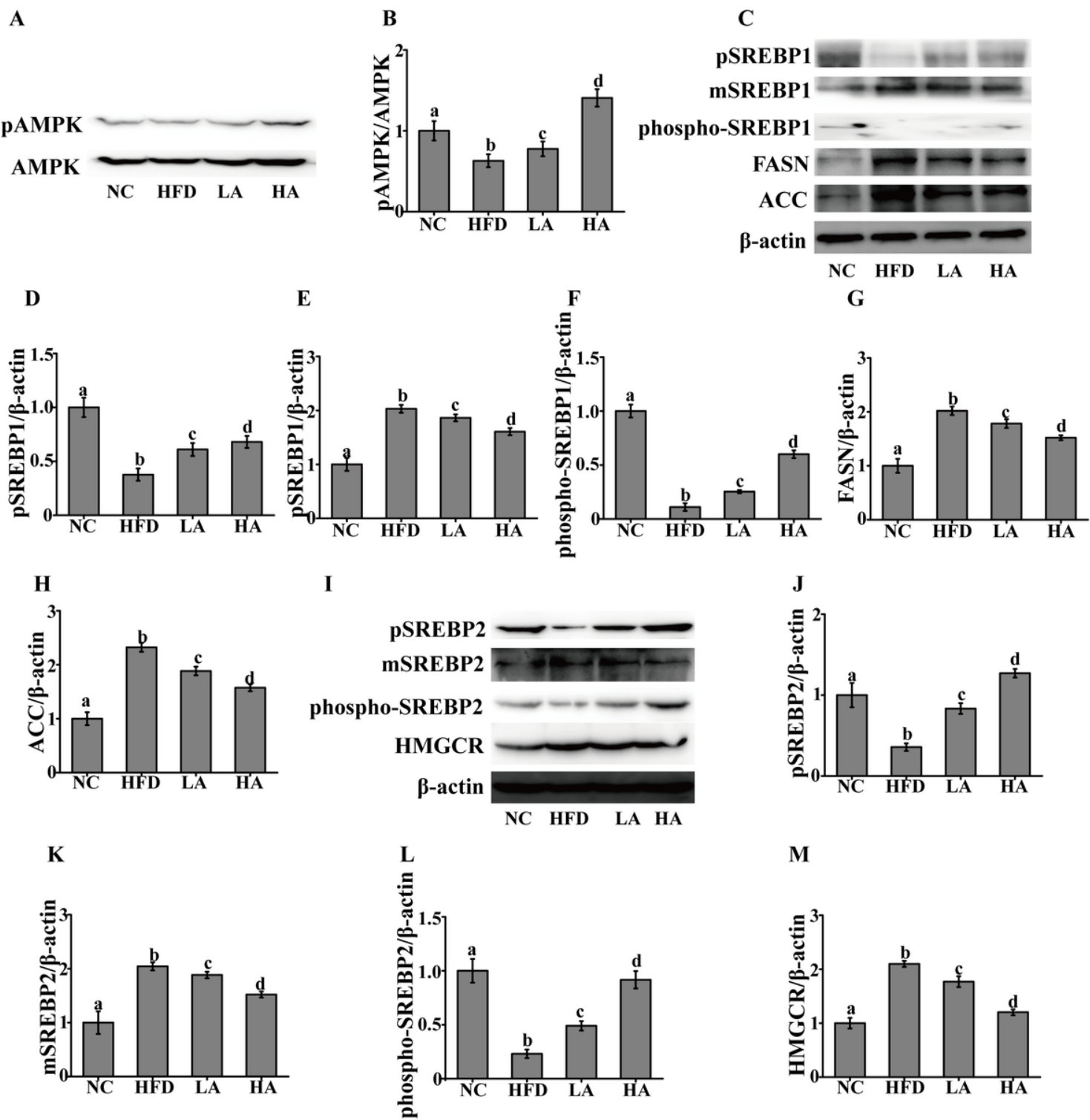


Figure 5

Effects of oroxin A on the AMPK/SREBP pathway in HFD-fed rats. (A) Immunoblot analysis of pAMPK and AMPK; (B) Densitometry analysis of pAMPK and AMPK; (C) Immunoblot analysis of pSREBP1, mSREBP1, phospho-SREBP1, FASN and ACC; (D) Densitometry analysis of pSREBP1; (E) Densitometry analysis of mSREBP1; (F) Densitometry analysis of phospho-SREBP1; (G) Densitometry analysis of FASN; (H) Densitometry analysis of ACC; (I) Immunoblot analysis of pSREBP2, mSREBP2, phospho-SREBP2 and HMGCR; (J) Densitometry analysis of pSREBP2; (K) Densitometry analysis of mSREBP2; (L) Densitometry analysis of phospho-SREBP2; (M) Densitometry analysis of HMGCR. NC: rats fed standard chow were administered 0.5% CMC-Na; HFD: rats fed HFD chow were administered 0.5% CMC-Na; LA: rats fed HFD chow were administered 50 mg/kg/d oroxin A in 0.5% CMC-Na; HA: rats fed HFD chow were administered 200 mg/kg/d oroxin A in 0.5% CMC-Na. The values are means $\pm$ standard deviations (n = 8 for each group). Significant differences ($p < 0.05$) were analysed using one-way ANOVA followed by the LSD test for multiple comparisons. The values sharing a common letter (a, b, c, and d) were not significantly different.

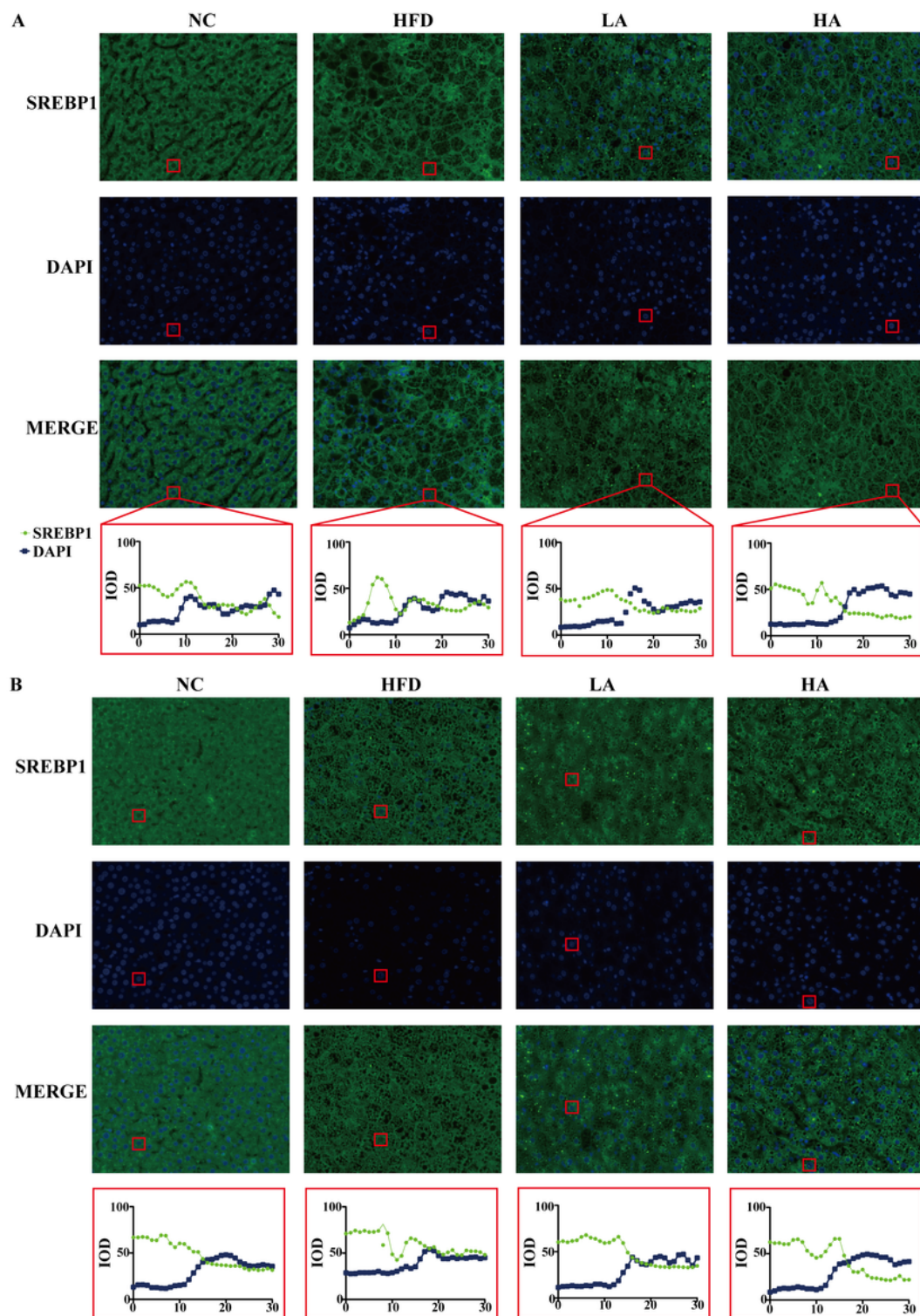


Figure 6

Effect of oroxin A on SREBP1 and SREBP2 in the livers of rats fed a high-fat diet evaluated using immunofluorescence. (A) Immunofluorescence analysis of DAPI and SREBP1, merged image of DAPI and SREBP1 and relative luminance curves of DAPI and SREBP1 in a representative area (from the edge to the centre of the marked yellow frame); (B) Immunofluorescence analysis of DAPI and SREBP2, merged image of DAPI and SREBP2 and relative luminance curves of DAPI and SREBP2 in a representative area

(from the edge to the centre of the marked yellow frame). NC: Rats fed standard chow were administered 0.5% CMC-Na; HFD: Rats fed HFD chow were administered 0.5% CMC-Na; LA: Rats fed HFD chow were administered 50 mg/kg/d oroxin A in 0.5% CMC-Na; HA: Rats fed HFD chow were administered 200 mg/kg/d oroxin A in 0.5% CMC-Na. The values are means $\pm$ standard deviations ($n = 8$ for each group). Significant differences ($p < 0.05$) were analysed using one-way ANOVA followed by the LSD test for multiple comparisons. The values sharing a common letter (a, b, c, and d) were not significantly different.

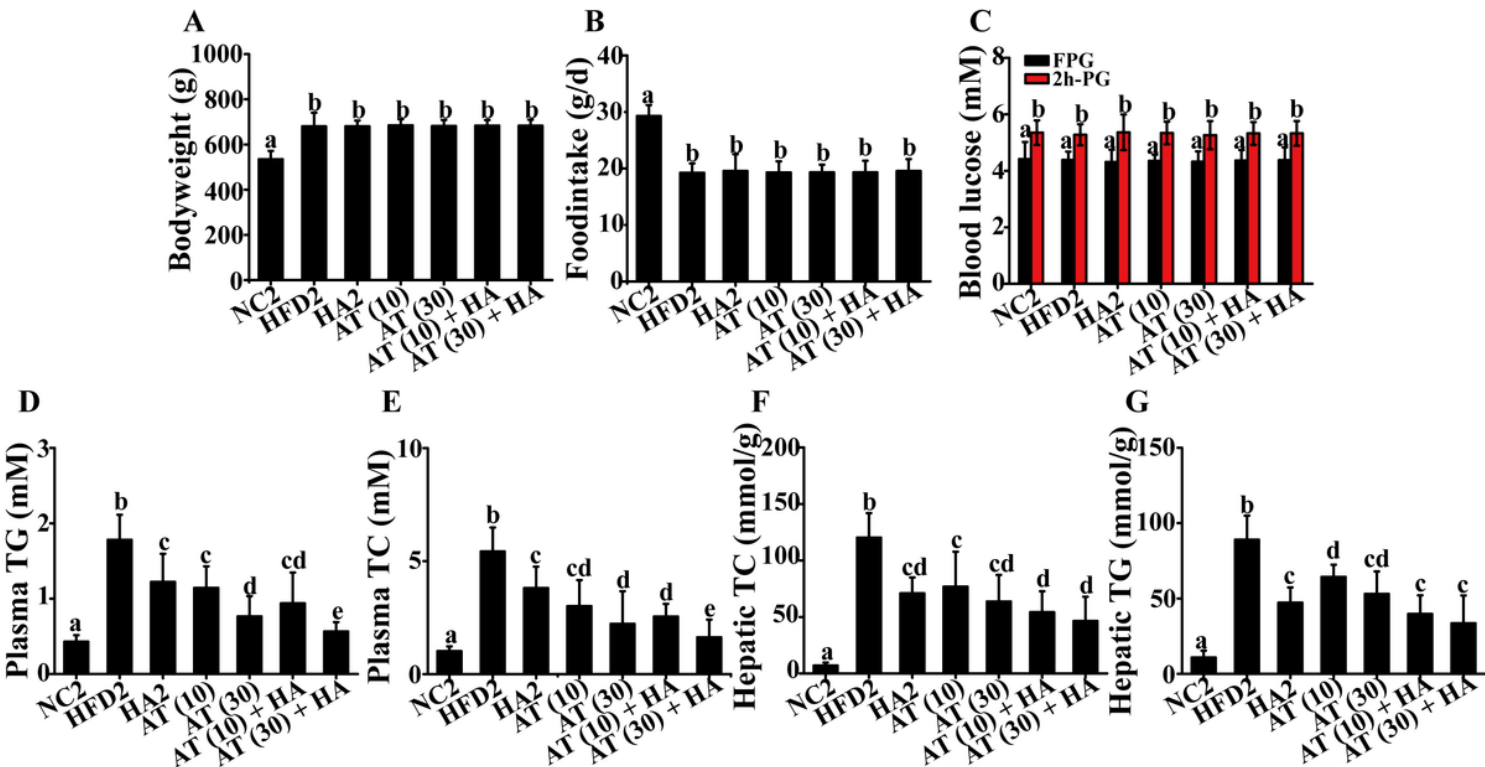


Figure 7

Effects of oroxin A combined with atorvastatin on the plasma lipid profile of HFD-fed rats. (A) Body weight; (B) Food intake; (C) Blood glucose; (D) Plasma TG; (E) Plasma TC; (F) Hepatic TC; (G) Hepatic TG. NC2: Rats fed standard chow were administered 0.5% CMC-Na; HFD2: Rats fed HFD chow were administered 0.5% CMC-Na; HA2: Rats fed HFD chow were administered 200 mg/kg/d oroxin A in 0.5% CMC-Na; AT (10): Rats fed HFD chow were administered 10 mg/kg/d atorvastatin in 0.5% CMC-Na; AT (30): Rats fed HFD chow were administered 30 mg/kg/d atorvastatin in 0.5% CMC-Na; AT (10) + HA: Rats fed HFD chow were administered 10 mg/kg/d atorvastatin and 200 mg/kg/d oroxin A in 0.5% CMC-Na; AT (30) + HA: Rats fed HFD chow were administered 30 mg/kg/d atorvastatin and 200 mg/kg/d oroxin A in 0.5% CMC-Na; The values are means $\pm$ standard deviations ($n = 8$ for each group). Significant differences ($p < 0.05$) were analysed using one-way ANOVA followed by the LSD test for multiple comparisons. The values sharing a common letter (a, b, c, and d) were not significantly different.

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