

RESEARCH ARTICLE

Ursolic acid improves lipid and glucose metabolism in high-fat-fed C57BL/6J mice by activating peroxisome proliferator-activated receptor alpha and hepatic autophagy

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Scope: This study investigated metabolic effects of ursolic acid (UA), a peroxisome proliferation-activated receptor (PPAR)- α activator, in vivo.

Methods and results: High-fat diet (HFD)-fed C57BL/6J mice were orally administered UA (50 or 200 mg/kg body weight) for 8 wk. UA reduced liver and adipose tissue mass, adipocyte size, and plasma leptin concentrations, plasma triglyceride and low-density-lipoprotein cholesterol concentrations, while it elevated the high-density-lipoprotein cholesterol and adiponectin concentrations significantly compared with controls. UA induced the expression of PPAR α and its responsive genes involved in fatty acid uptake and β -oxidation in the livers, whereas genes involved in lipogenesis, including sterol regulatory element-binding proteins-1c, were down-regulated. UA administration improved glucose tolerance and insulin sensitivity significantly compared with the HFD-fed control livers. UA administration also activated hepatic autophagy as assessed by the expression of microtubule-associated protein 1A/1B-light chain 3 (LC3)-II and other key proteins in the autophagy pathway.

Conclusion: Our findings suggest that UA ameliorates lipid and glucose metabolism in HFD-fed mice primarily by the activation of PPAR α and induction of the hepatic autophagy pathway. Thus, intake of UA in the diet or in an isolated form may ameliorate lipid and glucose metabolism.

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Abbreviations: ACOX1, peroxisomal acyl-coenzyme A oxidase 1; ACS1, acetyl-CoA synthetase 1; Akt, protein kinase B; CPT1, carnitine palmitoyltransferase 1; FAS, fatty acid synthase; FATP4, fatty acid transport protein 4; FPLC, fast protein liquid chromatography; HFD, high-fat diet; IR, insulin resistance; ITT, insulin tolerance test; LC3, microtubule-associated protein 1A/1B-light chain

1 Introduction

Disorders of lipid and glucose metabolism are major symptoms in several pathological phenomena, and considering the moderate net effects of natural medicine herbs combined with their minimal side effects, studies conducted over the last decade have focused on the biological effects

3; mTOR, mammalian target of rapamycin; OGTT, oral glucose tolerance test; PPAR, peroxisome proliferation-activated protein; SCD1, stearoyl-CoA desaturase 1; SREBP1c, sterol regulatory element binding protein 1c; UA, ursolic acid

of natural compounds from medicinal herbs. Ursolic acid (UA), a natural pentacyclitriterpenoid, is a major bioactive compound in several traditional medicinal herbs, including *Cornus officinalis*, and has been suggested to have multiple biological activities, including the regulation of lipid [1], and glucose metabolisms [2] and anticancer, anti-oxidative, anti-inflammatory, anti-atherosclerotic, hepato- and gastroprotective, and immune-regulatory effects [3]. The effects of UA on the regulation of lipids and glucose metabolism have been investigated in cultured cells and animal studies. UA significantly reduces body weight, visceral adiposity, and plasma triglyceride levels and improves the lipid profile in rodent models [4–6]. UA is suggested to act as a hepatoprotective component [7, 8] due to its effects on the reduction of hepatic lipid accumulation and improvement of glucose tolerance [9]. The hypoglycemic effects of UA have been investigated by several researchers. UA administration significantly decreases the plasma glucose concentration [4] and improves glucose and insulin tolerance in rodent models [2]. Although the hypolipidemic, hypoglycemic, and hepatoprotective effects of UA have been described previously, the molecular target and mechanism of actions of UA have not been clearly elucidated. Our previous research suggested that UA activated the nuclear receptor of peroxisome proliferator-activated receptor- α (PPAR α) and thereby reduced lipid accumulations in hepatocytes via the regulation of PPAR α -responsive genes in hepatic lipid metabolism [10].

As one of the nuclear receptors belonging to the steroid/thyroid hormone receptor superfamily [11], PPAR α is an essential regulator of genes involved in fatty acid transport and utilization and mitochondrial and peroxisomal fatty acid β -oxidation in the liver [12, 13], thus, the activation of PPAR α by either synthetic or natural compounds elevates the rate of cellular fatty acid uptake and subsequent oxidation [14]. Thus, the administration of PPAR α agonists ameliorates lipid and glucose metabolism simultaneously, reducing both plasma and hepatic triglyceride accumulation, improving glucose tolerance, and elevating high-density-lipoprotein (HDL) cholesterol concentrations [15, 16]. The critical role of PPAR α in lipid and glucose metabolism was confirmed in genetic studies, which demonstrated that PPAR α -deficient mice developed massive hepatic lipid accumulations, hyperglycemia, severe hypoketonemia, and increased plasma free fatty acid concentrations [17] and showed reduced capacity of mitochondrial fatty acid oxidation compared to wild-type mice [12], thus revealing a susceptibility to hepatic steatosis under overloaded dietary fat [18]. Moreover, PPAR α is suggested to regulate lipid homeostasis by providing energy to cells via increased fatty acid β -oxidation [19]. Therefore, the activation of PPAR α may regulate lipid accumulation in the whole body and the liver to reduce the plasma lipid concentrations and hepatic lipid accumulation.

Alternatively, hypotriglyceridemic and hypoglycemic regulation by UA is carried out by activation of the autophagy pathway. Autophagy acts under stress as a survival mechanism, thus maintaining the healthy state of cells through

the clearance of subcellular debris and the regeneration of metabolic precursors. The process of autophagy contributes to cellular and tissue homeostasis and affects pathogenesis [20]. Autophagy participates in the turnover of mitochondria and other organelles, such as the endoplasmic reticulum (ER) and peroxisomes [21]. The process of autophagy is initiated by enclosing a portion of the cytoplasm that contains nonfunctional proteins and damaged organelles by a phagophore or isolation membrane to form an autophagosome [22, 23]. The outer membrane of the autophagosome subsequently fuses with the endosome and then fuses with the lysosome [23]. Finally, the contents of the autophagosome are degraded by lysosomal acid hydrolases within the autophagosome inner membrane, and the resulting metabolites are released into the cytoplasm for metabolic recycling [20, 22, 23]. The induction of the autophagy pathway reduces lipid contents via the breakdown of undesirable lipid droplets [24] and regulation of proteins involved in lipid metabolism [25]. Autophagy is reported to protect against insulin resistance (IR) [26, 27] and against high-fat diet (HFD)-induced glucose intolerance [28]. Thus, the induction of autophagy contributes to the control of lipid and glucose metabolisms, especially in HFD-induced obesity or disorder of glucose metabolism. In this study, we investigated the metabolic effects and modes of action of UA in HFD-fed mice associated with PPAR α activation.

2 Materials and methods

2.1 Reagents and materials

Cell culture media and supplies were purchased from Hyclone (Logan, UT, USA), and penicillin/streptomycin was purchased from Welgene Inc. (Seoul, Korea). UA (456.71 g/mol) was purchased from Maya Reagents (Zhejiang, China), and fenofibrate (FF, 360.83 g/mol) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The ELISA kits for leptin, adiponectin, and insulin were purchased from Millipore (MA, USA), Abcam (Seoul, Korea), and Alpcos (NH, USA), respectively. RNAsiso Plus for the extraction of total RNA and SYBR[®] Premix Ex Taq[™] for real-time PCR were purchased from Takara (Otsu, Japan). Monoclonal anti-PPAR α , antifatty acid transport protein 4 (FATP4), anti-acetyl-CoA synthetase 1 (ACS1), anti-carnitine palmitoyltransferase 1 (CPT1), anti-peroxisomal acyl-coenzyme A oxidase 1 (ACOX1), anti-sterol regulatory element binding protein 1 (SREBP1), anti-fatty acid synthase (FAS), anti-stearoyl-CoA desaturase (SCD1), anti- α -tubulin antibodies, and the secondary antibodies (anti-mouse and anti-rabbit immunoglobulin G) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against proteins involved in the autophagy pathway, including anti-microtubule-associated protein 1A/1B-light chain 3 (LC3) and anti-Beclin1, were obtained from Novus (CO, USA).

2.2 Cell culture

HEK293 cells obtained from the Korean Cell Line Bank (Seoul, Korea) were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM) medium with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin at 37°C with a humidified atmosphere of 5% CO₂.

2.3 Mouse care and feeding

C57BL/6J male mice purchased from Samtako Co. (Kyung-gido, Korea) were maintained under a 12-h light/12-h dark cycle at a temperature of 21–25°C and a relative humidity of 50–60% and were fed 45% HFD (Central Lab. Animal Inc., Seoul, Korea) for 4 wk. The mice were then randomly assigned to 4 groups and fed only HFD and orally administered UA (50 and 200 mg/kg body weight; UA50 and UA200, respectively) or fenofibrate (50 mg/kg body weight, FF50) as the positive control for another 8 wk. The body weight and food intake were assessed weekly, and blood samples were collected every 4 wk. At the end of the experimental period, the mice were fasted for 12 h and killed according to a protocol approved by the Animal Experiment Committee of Korea University (Protocol No. KUIACUC-2013-139). Blood was collected in EDTA tubes (BD Vacutainer) retroorbitally or by cardiac puncture, centrifuged for 15 min at 12 000 rpm at 4°C to collect plasma samples, and stored at –80°C. The organs, including the liver, white adipose tissues (epididymal, visceral, perirenal), and leg skeletal muscle, were collected and snap-frozen in liquid nitrogen until analysis.

2.4 Histological analysis

Livers and epididymal adipose tissues were fixed with 4% paraformaldehyde and then stained by hematoxylin and eosin at the Histopathology Department of Anam Korea University Hospital (Seoul, Korea). Adipocyte sizes were assessed using an Axio Imager M1 microscope (Carl-Zeiss, Oberkochen, Germany).

2.5 Quantification of plasma lipids, glucose, and hormones

Triglyceride, total-, HDL- and LDL-cholesterol, and fasting glucose concentrations were quantified using an automated clinical chemistry analyzer (Cobas111, Roche, Basel, Switzerland) with enzymatic methods. The plasma leptin, adiponectin, and insulin levels were measured by ELISA assays according to the manufacturer's instructions. The homeostatic model assessment (HOMA) of IR and insulin-sensitivity index were calculated by the concentrations of plasma glucose and insulin.

2.6 Fast protein liquid chromatography (FPLC)

Plasma lipoprotein profiling was assessed by a FPLC system as described previously [29]. The fractions of HDL- and LDL-cholesterol were confirmed using Experion Protein Analysis Kits (Bio-Rad, PA, USA), and the cholesterol concentrations were measured using an Amplex Red Cholesterol Assay Kit (Invitrogen, Carlsbad, CA, USA).

2.7 Oral glucose tolerance test (OGTT) and insulin tolerance test (ITT)

After 7 wk of UA or FF administration, the mice were fasted overnight and orally administered glucose (1.5 g/kg body weight) for OGTT or fasted for 4 h and intraperitoneally injected insulin (0.35 unit/kg body weight) for ITT. The blood glucose concentrations were measured at 0, 15, 30, 60, 90, and 120 min after feeding or injection using a portable glucose meter (Accu-Check Go, Roche).

2.8 Quantitative (q) PCR analysis

Total RNA from the livers was extracted, and the cDNA was synthesized as described previously [30]. The livers were homogenized in RNAiso Plus reagent, and the total RNA samples were extracted according to the manufacturer's instructions. The synthesis of cDNA was performed by mixing 2 µg of total RNA with the M-MLV reverse transcriptase, oligo-dT, and dNTPs (Mbiotech, Korea). The real-time qPCR was performed with the Bio-Rad iQ5 Cycler System according to the manufacturer's instructions using glyceraldehyde 3-phosphate dehydrogenase as a reference for normalization. Primers were shown in Supporting Information Table 1.

2.9 Immunoblotting analysis

Proteins from the livers were extracted, and immunoblotting was performed as described previously [30]. Briefly, the livers were homogenized in RIPA buffer and centrifuged at 14 000 rpm for 10 min at 4°C to collect the supernatant. Protein concentrations were assessed by a Bio-Rad reagent (Bio-Rad). The denatured proteins were run on a SDS-PAGE gel, and immunoblotting was performed as described previously [31]. Proteins separated by SDS-PAGE were transferred to a membrane and incubated with primary and secondary antibodies to obtain the immunoblotting image with a ChemiDoc™ XRS+ imaging system (Bio-Rad).

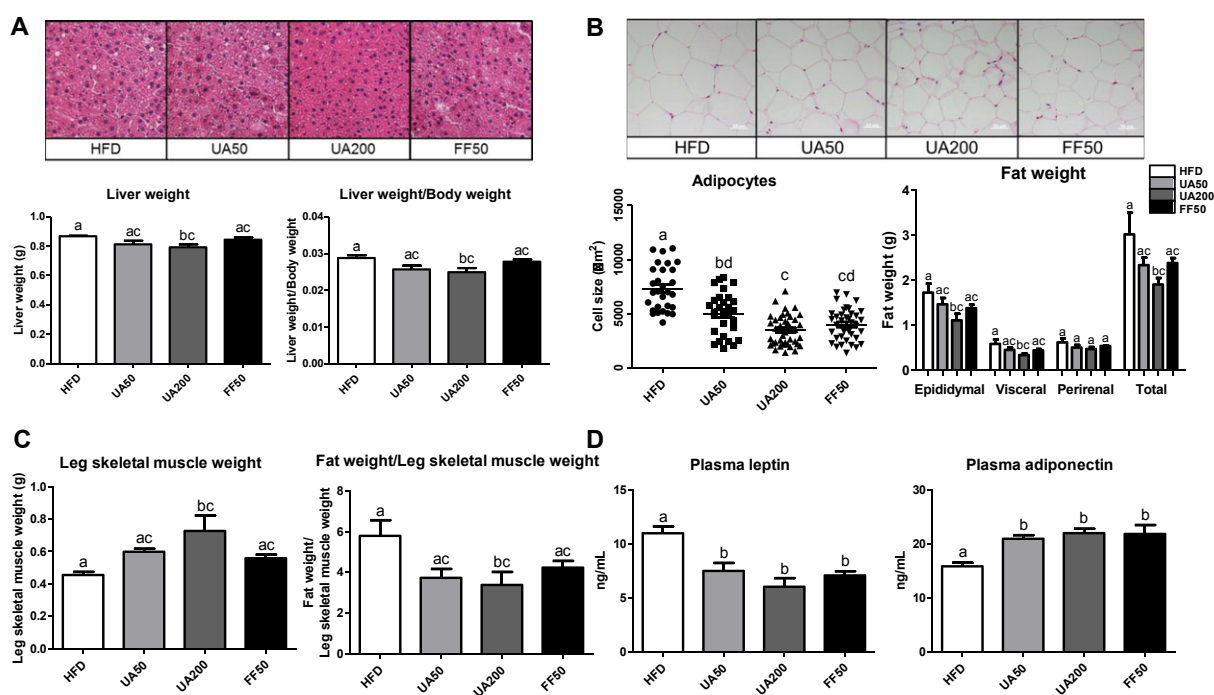


Figure 1. UA reduces lipid accumulation in the liver and adipose tissue and alters adiponectin and leptin concentrations. (A) Histological analysis of the liver based on hematoxylin and eosin staining and liver mass. (B) Histological analysis of white adipose tissue based on hematoxylin and eosin staining and fat mass. (C) Quantification of muscle mass and ratio of fat-to-muscle mass. (D) Plasma leptin and adiponectin concentrations. After oral administration of UA (UA50, 50 mg/kg body weight; UA200, 200 mg/kg body weight) or fenofibrate (FF50, 50 mg/kg body weight) for 8 wk, the mice were killed to collect blood and tissues for further analysis. White bar, high-fat diet (HFD)-fed mice; grey bar, HFD-fed mice administrated with 50 mg/kg body weight of UA; dark-grey bar, HFD-fed mice administrated with 200 mg/kg body weight of UA; black bar, HFD-fed mice administrated with 50 mg/kg body weight of FF. Data are presented as the means \pm SEM. Group ($n = 5-6$) comparisons were performed with a Tukey's test of one-way ANOVA. The different letters indicate significant differences among the groups.

2.10 Fluorescent microscopic analysis of hepatic autophagy process

HEK293 cells were cultured in a 6-well plate at 1.5×10^5 cells/mL for 24 h and were transfected with a tandem fluorescent mRFP-GFP-LC3 vector (Addgene, Cambridge, MA, USA) using FuGENE HD (Promega, Madison, WI, USA) according to the manufacturer's instructions. The transfected cells were selected with G418 antibiotics (Sigma) for several days. The selected cells' coverslips were fixed with 4% paraformaldehyde and mounted with anti-fade reagent after the nuclei were stained with 4',6-diamidino-2-phenylindole (Invitrogen). Images were taken using a Zeiss LSM 5 Exciter confocal microscope ($100 \times / 1.30$ oil DIC) and processed using the Zeiss LSM510 v.3.2 software (Carl Zeiss). The formation of autophagosomes and autolysosomes was traced with yellow and red puncta, respectively.

2.11 Statistical analysis

All of the data are shown as the means \pm SEM. Difference among group means were estimated using one-way ANOVA

followed by Tukey's post hoc test by GraphPad Prism 5.0 software. Significance was set up at $p < 0.05$.

3 Results

3.1 UA reduced lipid accumulation in liver and adipose tissues while increasing muscle weights

The purity of UA (90.2%) was analyzed by an ultra-performance liquid chromatography system (Supporting Information Fig. 1). To investigate the metabolic effects of UA in HFD-fed mice, the liver and adipocyte histology was assessed. Food intake was similar among groups (Supporting Information Table 2). In line with the reported anti-obesity and hepatoprotective effects, the results from this study revealed that the lipid accumulation in the liver and adipose tissues was significantly decreased in HFD-fed C57BL/6J mice (Figs. 1A and B). Although the body weight was unaltered (Supporting Information Fig. 2), the fat mass was reduced and the muscle weight was increased in UA mice compared with the HFD-fed controls (Fig. 1C). The levels of adiponectin were elevated and the concentration of leptin was reduced (Fig. 1D).

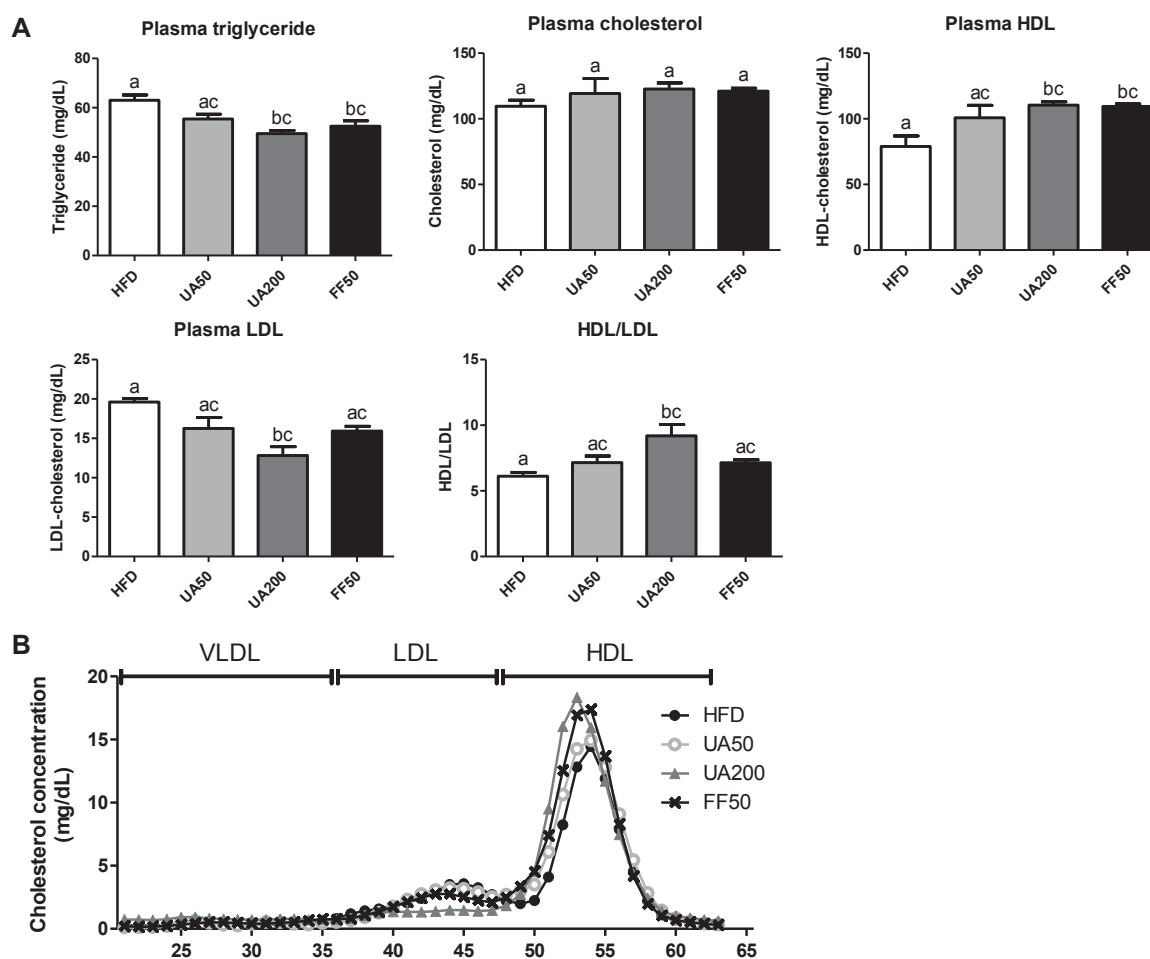


Figure 2. UA reduces plasma triglyceride concentrations and improves plasma cholesterol profiling. (A) Plasma triglyceride and total-, HDL-, LDL-, and VLDL-cholesterol concentrations. (B) Lipoprotein profiling of plasma cholesterol quantified by FPLC system. Data are presented as the means \pm SEM. The group ($n = 5-6$) comparisons were performed with a Tukey's test of one-way ANOVA. The different letters indicate significant differences among the groups.

These hormonal changes are known to be associated with improvement with obesity and IR.

3.2 UA reduces plasma triglyceride and LDL cholesterol and elevates HDL cholesterol concentrations in HFD-fed mice

Next, a plasma lipid analysis was performed. The plasma triglyceride concentrations were reduced in UA mice. This reduction was significant in UA200 mice compared with HFD-fed mice (Fig. 2A). The LDL cholesterol concentrations but not the total plasma cholesterol concentrations were reduced and the plasma HDL cholesterol concentration was increased significantly in the UA200 group compared with the HFD controls (Figs. 2A and B). The ratio of HDL-to-LDL cholesterol was significantly improved accordingly (Fig. 2A).

These results confirmed the hypolipidemic effects of UA on HFD-fed obese mice.

3.3 UA shows hypoglycemic effects with the improvement of glucose and insulin tolerance

Because UA is suggested as a hypoglycemic component in herbal medicines, we investigated the effects of UA on glucose metabolism. Mice in HFD for 4 wk did not dramatically induce fasting glucose levels; however, the results showed that the plasma glucose and insulin concentrations were significantly reduced in the UA200 group compared with those in the HFD-fed mice (Figs. 3A and B). The glucose and insulin tolerances were significantly improved in the UA200 group compared with the controls (Figs. 3A and B). UA improved insulin sensitivity as well (Figs. 3C and D). These

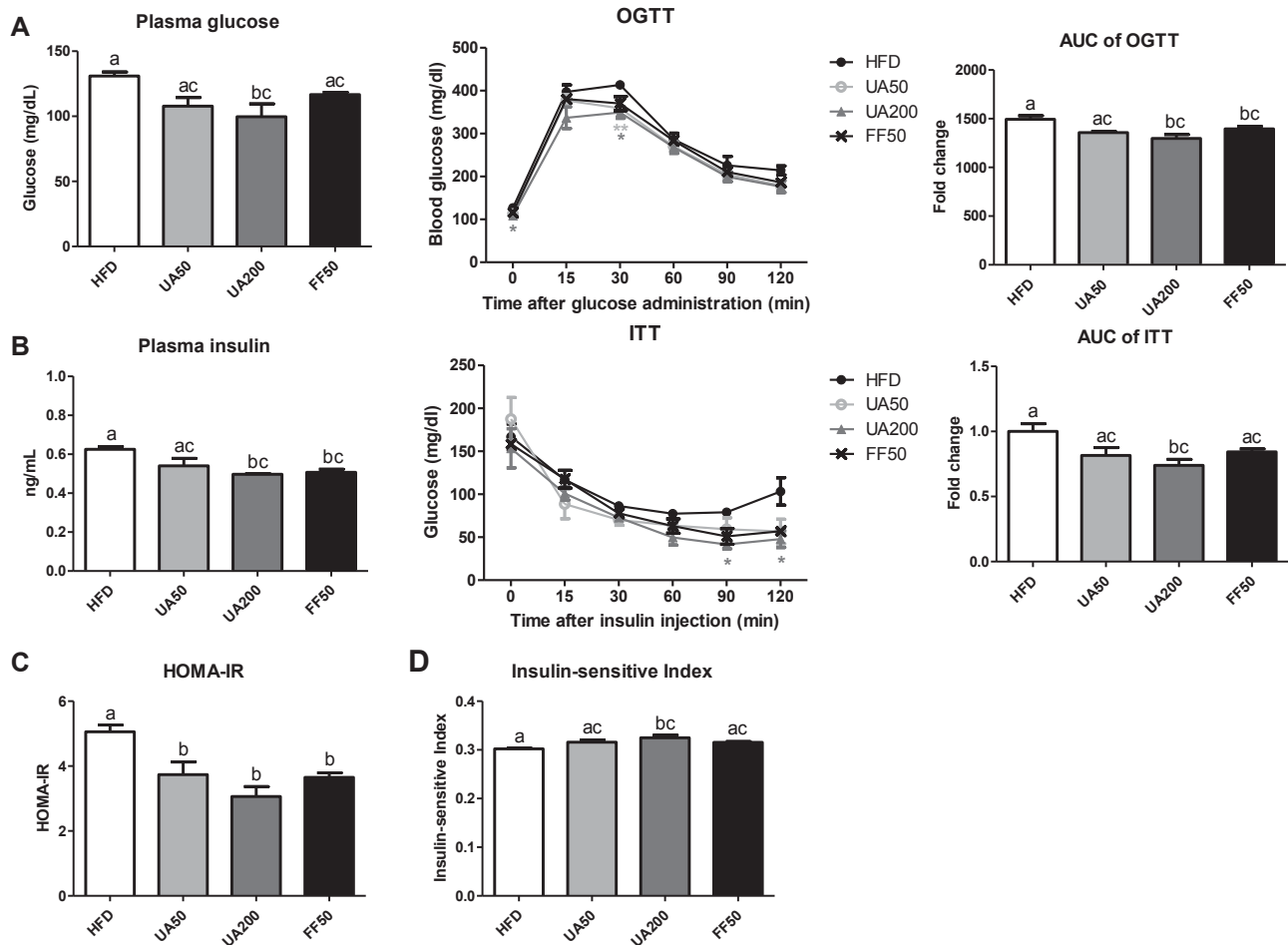


Figure 3. UA reduces fasting glucose and insulin concentrations and improves glucose tolerance and insulin sensitivity. (A) The concentrations of fasting glucose by OGTT analysis. (B) The concentrations of fasting insulin by ITT analysis. (C) The homeostatic model assessment (HOMA) of insulin resistance (IR). (D) The insulin-sensitivity index. After 7 wk of the oral administration of UA, the OGTT, and ITT were performed. The plasma glucose and insulin concentrations were measured after 12 h of fasting. AUC refers to the area under the curve. Data are presented as the means \pm SEM. The group ($n = 5-6$) comparisons were performed with a Tukey's test of one-way ANOVA. The different letters indicate significant differences among the groups. * $p < 0.05$ and ** $p < 0.001$ between the UA group and the HFD-fed control group.

results demonstrated that UA ameliorates the plasma glucose concentrations by improving glucose tolerance and insulin sensitivity in HFD-fed mice.

3.4 UA activates PPAR α and the expression of its target genes and proteins in mouse liver

PPAR α is a key transcriptional regulator in hepatic lipid and glucose metabolism, and our previous study suggested that UA is a PPAR α activator because it reduces the intracellular lipid concentrations in hepatocytes [10]. To investigate the modes of action of UA on lipid and glucose metabolism, the expression levels of PPAR α and its responsive genes were assessed in mouse livers. Both mRNA and protein expression levels of PPAR α were induced significantly in the livers

of mice administered UA compared with those found in the HFD-fed mice (Figs. 4A and B). The mRNA and protein expression levels of PPAR α target genes were assessed. FATP4, which regulates fatty acid uptake, were upregulated significantly in the UA200 livers, and the expressions of ACS1, CPT1, and ACOX1, which mediate fatty acid β -oxidation, were upregulated significantly in these mice compared with HFD-fed mice (Figs. 4A and B). However, the gene expression levels of SREBP1c, as well as those of its responsive genes FAS and SCD1, which regulate lipogenesis in the liver, were downregulated in the liver following UA administration (Fig. 4A). These results suggest that the hypolipidemic effects of UA are achieved primarily by the activation of hepatic PPAR α and its responsive genes associated with both fatty acid uptake and β -oxidation. Downregulation of hepatic lipogenic genes may contribute to the reduction of lipid accumulation as well.

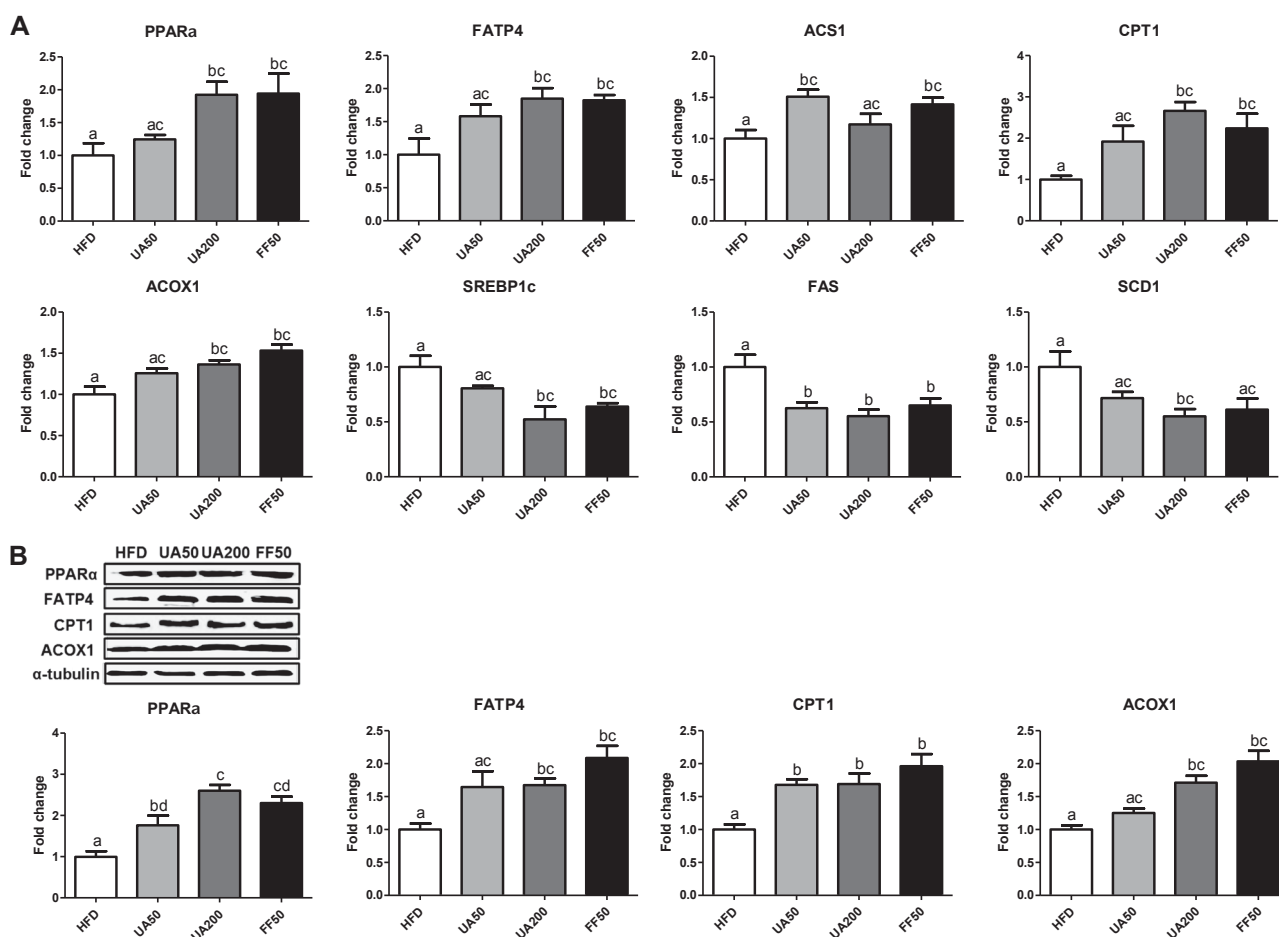


Figure 4. UA induces PPAR α gene and protein expression and regulates its responsive genes associated with hepatic lipid metabolism. (A) The mRNA expression levels of PPAR α and its responsive genes involved in fatty acid uptake and β -oxidation were upregulated, whereas the PPAR α -responsive genes associated with lipogenesis were downregulated. (B) Protein expression levels of PPAR α and its responsive genes involved in fatty acid uptake and β -oxidation were upregulated. PPAR α , peroxisome proliferator-activated receptor alpha; FATP4, fatty acid transport protein 4; ACS1, Acetyl-CoA synthetase 1; CPT1, carnitine palmitoyltransferase 1; ACOX1, peroxisomal acyl-coenzyme A oxidase 1; SREBP1c, sterol regulatory element binding protein 1c; FAS, fatty acid synthase; SCD1, stearoyl-CoA desaturase 1. The data are presented as the means \pm SEM. The group ($n = 5-6$) comparisons were performed with a Tukey's test of one-way ANOVA. The different letters indicate significant differences among the groups.

3.5 UA induces the hepatic autophagy pathway

Hepatic autophagy is tightly regulated by multiple mechanisms. It is known that activation of hepatic autophagy plays major roles in both lipid and glucose metabolisms, thus, we investigated whether the hepatic autophagy pathway is activated in the livers of UA-administered mice. LC3-II protein is a critical marker of autophagosome formation. In the tandem mRFP-GFP-LC3 vector-transfected HEK293 cells, the autophagosomes are shown in yellow puncta while autolysosomes are observed as red puncta [32]. UA administration induced the formation of autophagosomes (yellow puncta) and autolysosomes (red puncta) in a dose-dependent manner (Fig. 5A). The protein expression of LC3-II, a key protein in the formation of autophagy, was increased significantly in the livers of the UA-treated groups compared with those

of the controls (Fig. 5B). In addition, major proteins in the autophagy pathway, beclin1, were commonly induced in the liver following UA administration (Fig. 5B). These results suggest that UA induces the hepatic autophagy pathway and this could contribute to hypolipidemic and hypoglycemic effects of UA in HFD-fed mice.

4 Discussion

UA is a natural pentacycliterpenoid and a major bioactive compound in several medicinal herbs, including *Cornus officinalis*. It has been reported the UA has diverse biological activities, including the regulation of lipid and glucose metabolism. Previously, we reported that UA is a PPAR α activator activating fatty acid β -oxidation in cultured

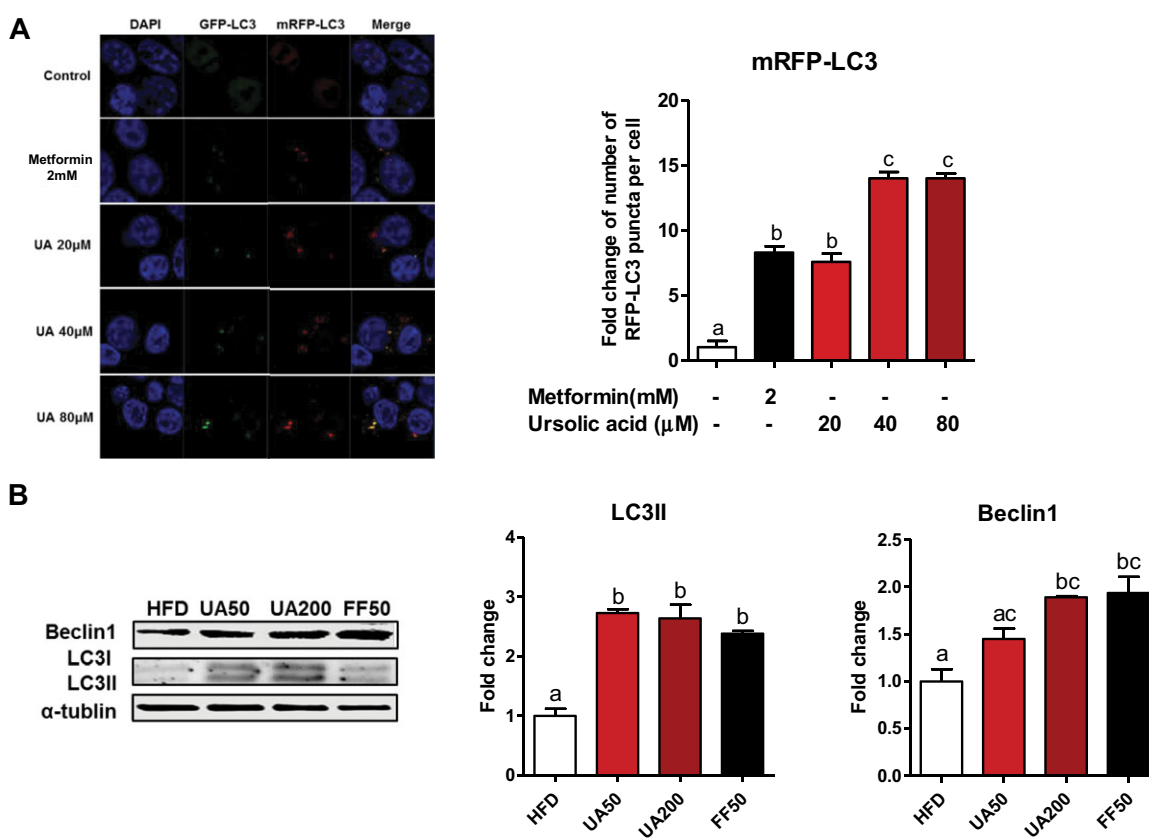


Figure 5. UA activates the hepatic autophagy pathway. (A) Formation of autophagosomes (yellow puncta) and autolysosomes (red puncta) in HEK293 cells transfected with the tandem mRFP-GFP-LC3 expression vector, and quantification of autolysosomes. (B) Protein expression levels of LC3-II and beclin1 in the liver. The expression level of LC3II in HEK293 cells transfected with the fluorescent mRFP-GFP-LC3 plasmid and treated with serial concentrations of UA and in the UA-treated livers of HFD-fed mice were investigated. LC3, microtubule-associated protein 1A/1B-light chain 3. Data are presented as the means \pm SEM. Group ($n = 4$) comparisons were performed with a Tukey's test of one-way ANOVA. The different letters indicate significant differences among the groups.

hepatocytes [10]. To investigate the metabolic effects of UA in vivo, we investigated the biological mechanisms of UA in the liver associated with PPAR α activation. The oral administration of UA for 8 wk significantly reduced the plasma and hepatic triglyceride concentrations, increased HDL-cholesterol, and reduced the liver weight. A reduction of lipid accumulation was found in adipose tissue, thus, the adipose size and weight were significantly reduced in UA-fed mice compared with the HFD controls. These suggest that hypolipidemic activities of UA are primarily mediated by the activation of PPAR α and the regulation of its responsive genes associated with fatty acid and lipoprotein metabolism (Fig. 6). The results of this study are in line with previous data suggesting hepatic lipid reduction by UA [9] and provide additional data for the elucidation of the mechanism underlying the liver protection induced by UA [7,8].

In this study, UA was found to regulate two key adipokines in metabolism: adiponectin and leptin. The plasma concentrations of adipokines are tightly associated with both lipid and glucose metabolisms, and leptin regulates energy intake

and expenditure [33], thus, the concentration of circulating leptin is proportional to the total amount of white adipose tissue in the body. The reduced plasma leptin concentration obtained after UA administration in this study confirmed a reduction in the adipose tissue mass. Adiponectin mediates several metabolic processes, such as fatty acid oxidation and glucose regulation [34], thus, increased plasma concentrations increase lipid catabolism and glucose metabolism, demonstrating that UA administration resulted in hypoglycemic and hypolipidemic activities, at least in part, via the regulation of these adipokines.

In HFD-fed mice, UA showed the potential for anti-obesity effects by reducing lipid accumulation in adipose tissue, which is in line with a previous report [6]. One potential mechanism underlying this finding is that UA is a phosphodiesterase inhibitor that enhances lipolysis in adipocytes [5]. The results showed that UA administration significantly reduced the fat/muscle weight ratio compared with those of HFD-fed mice, which is in accordance with a previous research study that showed that UA increases skeletal muscle and brown fat mass, thus increasing energy expenditure

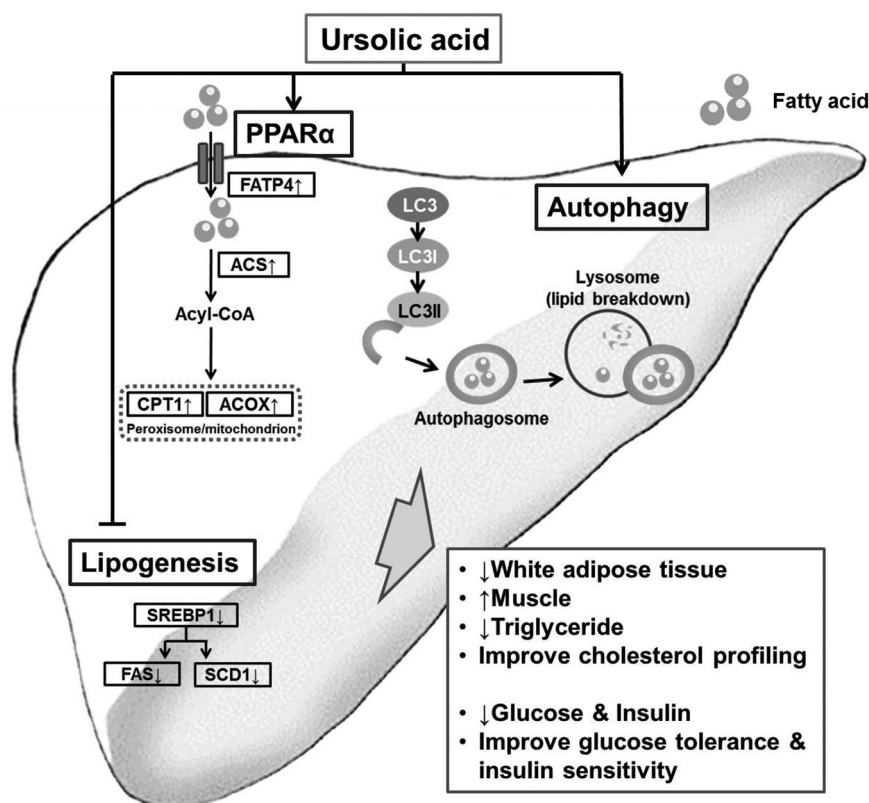


Figure 6. UA regulates lipid and glucose metabolism primarily via activation of PPAR α and hepatic autophagy. UA activates PPAR α , thus upregulating the expression of FATP4 to increase hepatic fatty acid uptake and inducing ACS1, CPT1, and ACOX1 to improve hepatic β -oxidation. UA represses SREBP1c and its responsive genes, FAS and SCD1, thus reducing hepatic lipogenesis as well. Hepatic autophagy is induced by UA administration, as increasing the expression of the autophagic proteins LC3II and beclin1 in vitro and in vivo. Therefore, UA improves lipid profiling, the muscle and adipose tissue ratio, glucose tolerance, and insulin sensitivity primarily via the activation of PPAR α and hepatic autophagy. PPAR α , peroxisome proliferator-activated receptor alpha; FATP4, fatty acid transport protein 4; ACS1, Acetyl-CoA synthetase; CPT1, carnitine palmitoyltransferase 1; ACOX1, peroxisomal acyl-coenzyme A oxidase; SREBP1c, sterol regulatory element binding protein 1c; FAS, fatty acid synthase; SCD1, stearoyl-CoA desaturase 1; LC3, microtubule-associated protein 1A/1B-light chain 3.

to reduce obesity, improve glucose tolerance, and decrease hepatic steatosis in a diet-induced obese mouse model [35]. The investigation of the mechanism of UA should be a topic for future research.

We previously reported that UA induced transactivation of PPAR α , but did not directly bind with ligand-binding domain of PPAR α protein [10]. These results suggested that UA activated PPAR α via indirect mechanisms potentially by activating endogenous ligand synthesis or increasing availability of endogenous ligand. In line with previous results, this study showed that the expression of PPAR α and its responsive genes in lipid metabolism was significantly regulated in the liver following UA administration (Fig. 6). PPAR α mediates the transcription of genes involved in fatty acid uptake and the oxidation of fatty acids in hepatic lipid metabolism. Fatty acids are transported into cells by the activation of FATP4 [14] and esterified to fatty acyl-CoAs via the activation of ACS1 in a PPAR α -dependent manner in rodent livers [36]. Then, the fatty acyl-CoAs transfer to the peroxisomal/mitochondrial for β -oxidation catalyzed by CPT1 and ACOX1, which are directly activated by PPAR α in the liver [37]. The regulation of these genes by UA activated hepatic fatty acid uptake and β -oxidation thus reduced plasma and hepatic triglyceride concentrations in HFD fed mice. The results of the animal experiments performed in this study demonstrate that UA administration downregulated the gene expression levels of SREBP1, FAS, and SCD1, thus reducing hepatic fatty acid synthesis

to decrease the triglyceride concentrations in the circulatory system and lipid accumulation in the liver.

Autophagy plays a critical role in hepatic lipid accumulation. Autophagy is suggested to degrade lipid droplets in hepatocytes by a specific autophagy-related process, namely “lipo(macro)autophagy” or lipophagy, digesting lipid droplets as the mitochondria and peroxisome turn over by autophagy, which occurs in a regulated fashion under physiological (increased exposure of fatty acids) and pathophysiological (overloaded high-fat) conditions [24]. Atg15 (Cvt17 in yeast) is predicted to deliver vacuolar hydrolases from the cytoplasm to the vacuole-targeting pathway as a putative lipase, thereby hydrolyzing neutral lipids, such as triglycerides, according to the autophagic sequence [38]. As a result, the UA-induced reduction of the plasma triglyceride concentrations and hepatic lipid accumulation may act via the induction of the autophagy pathway as indicated by the in vitro and in vivo results.

Autophagy is also associated with carbohydrate homeostasis. For example, a deficiency in autophagy (with decreased beclin1 expression) revealed defective glucose metabolism in skeletal muscle [28], which indicates that autophagy mediates glucose metabolism via increased skeletal muscle glucose uptake/utilization efficiency to increase insulin sensitivity [39]. It has been identified that UA significantly reduces the plasma glucose levels and improves glucose tolerance and insulin sensitivity via preserving pancreatic β -cells in high-fat-fed diabetic mice [2]. Our results showed that UA

administration improves glucose tolerance and insulin sensitivity and that this could be achieved, at least in part, via the activation of autophagy. Autophagy is regulated by multiple mechanisms. For example, it has been shown that activation of Akt by phosphorylation suppresses autophagy activity, thus blocking mammalian target of rapamycin (mTOR) pathway, which is reported to suppress activation of autophagy pathway [40, 41]. In addition, it is well known that AMP-activated protein kinase activation suppressed mTOR thus activating autophagy pathway. It was reported that UA could activate AMP-activated protein kinase [42]. Alternatively, activation of PGC1 α , a PPAR α target gene, is known to induce autophagy in several tissues. Therefore, we suggest that UA may activate hepatic autophagy by AMP-activated protein kinase- or PGC1 α -dependent manners. The exact mechanism behind this should be investigated in the future.

In this research, UA improved triglyceride and glucose metabolism to a lesser degree of the effects of FF. Although there are minor differences between the effects of UA and FF, it is not surprising because the metabolic activity and side effects are vary even among different fibrates drugs. In this study, the somewhat notable differences between UA and FF effects were found in gene and protein expressions of autophagy markers. It is possible that these are due to the potency of PPAR α activation and the detailed mechanisms for these differences between UA and FF on autophagy regulation is currently unknown and should be examined in the future.

In conclusion, this study suggests that the hypolipidemic and hypoglycemic activities of UA are primarily mediated through PPAR α activation and induction of hepatic autophagy. The regulation of PPAR α -responsive genes and the activation of the autophagic pathway are a suggested mechanism of UA on lipid and glucose metabolism. The induction of plasma adiponectin may also contribute to the improved metabolic parameters. In addition, this also demonstrated that UA accumulation reduces lipid accumulation in adipose tissue, suggesting potential anti-obesogenic activities. Collectively, the appropriate intake of UA in food or medicinal plants or in an isolated form may exert hypotriglyceridemic and hypoglycemic effects, which indicates that UA exhibits potential for the prevention and treatment of hyperlipidemia and glucose tolerance simultaneously.

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The authors have declared no conflict of interest.

5 References

- [1] Somova, L. O., Nadar, A., Rammanan, P., Shode, F. O., Cardiovascular, antihyperlipidemic and antioxidant effects of oleanolic and ursolic acids in experimental hypertension. *Phytomedicine* 2003, 10, 115–121.
- [2] Jang, S. M., Yee, S. T., Choi, J., Choi, M. S. et al., Ursolic acid enhances the cellular immune system and pancreatic beta-cell function in streptozotocin-induced diabetic mice fed a high-fat diet. *Int. Immunopharmacol.* 2009, 9, 113–119.
- [3] Liu, J., Pharmacology of oleanolic acid and ursolic acid. *J. Ethnopharmacol.* 1995, 49, 57–68.
- [4] Azevedo, M. F., Camsari, C., Sa, C. M., Lima, C. F. et al., Ursolic acid and luteolin-7-glucoside improve lipid profiles and increase liver glycogen content through glycogen synthase kinase-3. *Phytother. Res.* 2010, 24(Suppl 2), S220–S224.
- [5] Kim, J., Jang, D. S., Kim, H., Kim, J. S., Anti-lipase and lipolytic activities of ursolic acid isolated from the roots of *Actinidia arguta*. *Arch. Pharmacol. Res.* 2009, 32, 983–987.
- [6] Rao, V. S., de Melo, C. L., Queiroz, M. G., Lemos, T. L. et al., Ursolic acid, a pentacyclitriterpene from *Sambucus australis*, prevents abdominal adiposity in mice fed a high-fat diet. *J. Med. Food* 2011, 14, 1375–1382.
- [7] Ma, X. H., Zhao, Y. C., Yin, L., Xu, R. L. et al., Studies on the preventive and therapeutic effects of ursolic acid (UA) on acute hepatic injury in rats. *Yao xue xue bao* 1986, 21, 332–335.
- [8] Shukla, B., Visen, P. K. S., Patnaik, G. K., Tripathi, S. C. et al., Hepatoprotective activity in the rat of ursolic acid isolated from eucalyptus hybrid. *Phytother. Res.* 1992, 6, 74–79.
- [9] Jayaprakasam, B., Olson, L. K., Schutzki, R. E., Tai, M. H. et al., Amelioration of obesity and glucose intolerance in high-fat-fed C57BL/6 mice by anthocyanins and ursolic acid in Cornelian cherry (*Cornus mas*). *J. Agric. Food Chem.* 2006, 54, 243–248.
- [10] Jia, Y., Bhuiyan, M. J. H., Jun, H. J., Lee, J. H. et al., Ursolic acid is a PPAR-alpha agonist that regulates hepatic lipid metabolism. *Bioorg. Med. Chem. Lett.* 2011, 21, 5876–5880.
- [11] Desvergne, B., Wahli, W., Peroxisome proliferator-activated receptors: nuclear control of metabolism. *Endocr. Rev.* 1999, 20, 649–688.
- [12] Aoyama, T., Peters, J. M., Iritani, N., Nakajima, T. et al., Altered constitutive expression of fatty acid-metabolizing enzymes in mice lacking the peroxisome proliferator-activated receptor alpha (PPARalpha). *J. Biol. Chem.* 1998, 273, 5678–5684.
- [13] Reddy, J. K., Nonalcoholic steatosis and steatohepatitis - III. Peroxisomal beta-oxidation, PPAR alpha, and steatohepatitis. *Am. J. Physiol-Gastr. L.* 2001, 281, G1333-G1339.
- [14] Motojima, K., Passilly, P., Peters, J. M., Gonzalez, F. J. et al., Expression of putative fatty acid transporter genes are regulated by peroxisome proliferator-activated receptor alpha and gamma activators in a tissue- and inducer-specific manner. *J. Biol. Chem.* 1998, 273, 16710–16714.
- [15] Harano, Y., Yasui, K., Toyama, T., Nakajima, T. et al., Fenofibrate, a peroxisome proliferator-activated receptor alpha agonist, reduces hepatic steatosis and lipid peroxidation in fatty liver Shionogi mice with hereditary fatty liver. *Liver Int.* 2006, 26, 613–620.
- [16] Nakajima, T., Tanaka, N., Kanbe, H., Hara, A. et al., Bezafibrate at clinically relevant doses decreases serum/liver

- triglycerides via down-regulation of sterol regulatory element-binding protein-1c in mice: a novel peroxisome proliferator-activated receptor alpha-independent mechanism. *Mol. Pharmacol.* 2009, *75*, 782–792.
- [17] Leone, T. C., Weinheimer, C. J., Kelly, D. P., A critical role for the peroxisome proliferator-activated receptor alpha (PPARalpha) in the cellular fasting response: the PPARalpha-null mouse as a model of fatty acid oxidation disorders. *Proc. Natl. Acad. Sci. USA* 1999, *96*, 7473–7478.
- [18] Kersten, S., Seydoux, J., Peters, J. M., Gonzalez, F. J. et al., Peroxisome proliferator-activated receptor alpha mediates the adaptive response to fasting. *J. Clin. Invest.* 1999, *103*, 1489–1498.
- [19] Li, A. C., Palinski, W., Peroxisome proliferator-activated receptors: how their effects on macrophages can lead to the development of a new drug therapy against atherosclerosis. *Annu. Rev. Pharmacol. Toxicol.* 2006, *46*, 1–39.
- [20] Choi, A. M., Ryter, S. W., Levine, B., Autophagy in human health and disease. *N. Engl. J. Med.* 2013, *368*, 1845–1846.
- [21] Wang, K., Klionsky, D. J., Mitochondria removal by autophagy. *Autophagy* 2011, *7*, 297–300.
- [22] Marino, G., Lopez-Otin, C., Autophagy: molecular mechanisms, physiological functions and relevance in human pathology. *Cell. Mol. Life Sci.* 2004, *61*, 1439–1454.
- [23] Mizushima, N., Autophagy: process and function. *Genes Dev.* 2007, *21*, 2861–2873.
- [24] Singh, R., Kaushik, S., Wang, Y., Xiang, Y. et al., Autophagy regulates lipid metabolism. *Nature* 2009, *458*, 1131–1135.
- [25] Lamark, T., Johansen, T., Aggrephagy: selective disposal of protein aggregates by macroautophagy. *Int. J. Cell Biol.* 2012, *2012*, 736905.
- [26] Yang, L., Li, P., Fu, S. N., Calay, E. S. et al., Defective hepatic autophagy in obesity promotes ER stress and causes insulin resistance. *Cell Metab.* 2010, *11*, 467–478.
- [27] Ebato, C., Uchida, T., Arakawa, M., Komatsu, M. et al., Autophagy is important in islet homeostasis and compensatory increase of beta cell mass in response to high-fat diet. *Cell Metab.* 2008, *8*, 325–332.
- [28] He, C. C., Bassik, M. C., Moresi, V., Sun, K. et al., Exercise-induced BCL2-regulated autophagy is required for muscle glucose homeostasis. *Nature* 2012, *481*, 511–515.
- [29] Chetiveaux, M., Nazih, H., Ferchaud-Roucher, V., Lambert, G. et al., The differential apoA-I enrichment of pre beta(1) and alpha HDL is detectable by gel filtration separation. *J. Lipid Res.* 2002, *43*, 1986–1993.
- [30] Jia, Y. Y., Kim, J. Y., Jun, H. J., Kim, S. J. et al., Cyanidin is an agonistic ligand for peroxisome proliferator-activated receptor-alpha reducing hepatic lipid. *Bba-Mol. Cell Biol. L.* 2013, *1831*, 698–708.
- [31] Hoang, M. H., Houng, S. J., Jun, H. J., Lee, J. H. et al., Barley intake induces bile acid excretion by reduced expression of intestinal ASBT and NPC1L1 in C57BL/6J mice. *J. Agric. Food Chem.* 2011, *59*, 6798–6805.
- [32] Kimura, S., Noda, T., Yoshimori, T., Dissection of the autophagosome maturation process by a novel reporter protein, tandem fluorescent-tagged LC3. *Autophagy* 2007, *3*, 452–460.
- [33] Brennan, A. M., Mantzoros, C. S., Drug Insight: the role of leptin in human physiology and pathophysiology—emerging clinical applications. *Nat. Clin. Pract. Endocrinol. Metab.* 2006, *2*, 318–327.
- [34] Diez, J. J., Iglesias, P., The role of the novel adipocyte-derived hormone adiponectin in human disease. *Eur. J. Endocrinol.* 2003, *148*, 293–300.
- [35] Kunkel, S. D., Elmore, C. J., Bongers, K. S., Ebert, S. M. et al., Ursolic acid increases skeletal muscle and brown fat and decreases diet-induced obesity, glucose intolerance and fatty liver disease. *PloS One* 2012, *7*, e39332.
- [36] Martin, G., Poirier, H., Hennuyer, N., Crombie, D. et al., Induction of the fatty acid transport protein 1 and acyl-CoA synthase genes by dimer-selective retinoids suggests that the peroxisome proliferator-activated receptor-retinoid X receptor heterodimer is their molecular target. *J. Biol. Chem.* 2000, *275*, 12612–12618.
- [37] Fan, C. Y., Pan, J., Usuda, N., Yeldandi, A. V. et al., Steatohepatitis, spontaneous peroxisome proliferation and liver tumors in mice lacking peroxisomal fatty acyl-CoA oxidase. Implications for peroxisome proliferator-activated receptor alpha natural ligand metabolism. *J. Biol. Chem.* 1998, *273*, 15639–15645.
- [38] Teter, S. A., Eggerton, K. P., Scott, S. V., Kim, J. et al., Degradation of lipid vesicles in the yeast vacuole requires function of Cvt17, a putative lipase. *J. Biol. Chem.* 2001, *276*, 2083–2087.
- [39] Ryder, J. W., Chibalin, A. V., Zierath, J. R., Intracellular mechanisms underlying increases in glucose uptake in response to insulin or exercise in skeletal muscle. *Acta Physiol. Scand.* 2001, *171*, 249–257.
- [40] Seldin, M. M., Lei, X., Tan, S. Y., Stanson, K. P. et al., Skeletal muscle-derived myonectin activates the mammalian target of rapamycin (mTOR) pathway to suppress autophagy in liver. *J. Biol. Chem.* 2013, *288*, 36073–36082.
- [41] Sharp, Z. D., Bartke, A., Evidence for down-regulation of phosphoinositide 3-kinase/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR)-dependent translation regulatory signaling pathways in Ames dwarf mice. *J. Gerontol. A Biol. Sci. Med. Sci.* 2005, *60*, 293–300.
- [42] Yang, Y., Zhao, Z., Liu, Y., Kang, X. et al., Ursolic acid via LKB1-AMPK signaling suppresses oxidative stress and improves liver functions in mice. *J. Gastroenterol. Hepatol.* 2014, doi: 10.1111/jgh.12723