Ferulic Acid Alleviates Lipotoxicity-induced Hepatocellular Death Through SIRT1 Activating-regulated Autophagy Pathway, and Independently of AMPK and Akt in AML-12 Hepatocytes

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Research

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Abstract

Background: Lipotoxicity-induced cell death plays a detrimental role in the pathogenesis of metabolic diseases. Ferulic acid, widespread in vegetative food, is conferred as a radical scavenger with multiple bioactivities. However, the benefits of Ferulic acid against hepatic lipotoxicity are largely unclear. Here, we investigated the protective role of ferulic acid on palmitate-induced lipotoxicity and clarify its potential mechanisms in AML-12 hepatocytes.

Methods: AML-12 mouse hepatocyte was employed and exposed to palmitate to mimic lipotoxicity. Different doses (25, 50, and 100 μM) of ferulic acid were added 2 h before palmitate induction. Cell viability was detected by the measurements of lactate dehydrogenase release, nuclear staining, and the expression of cleaved-caspase3. Intracellular reactive oxygen species and mitochondrial membrane potential were analyzed by fluorescent probe. The potential mechanisms were explored by molecular biological methods, including Western-blot and quantitative real-time PCR, and further verified by siRNA interference.

Results: Our data showed that ferulic acid significantly reversed palmitate-induced cell death, rescued mitochondrial membrane potential, reduced reactive oxidative species accumulation, and improved inflammatory factors activation, including IL-6 and IL-1beta. Ferulic acid significantly stimulated autophagy in hepatocytes, while, autophagy suppression blocked ferulic acid-protected lipotoxicity. Ferulic acid-activated autophagy, which was triggered by Sirt1 upregulation, was mechanistically involved in its anti-lipotoxicity benefits. Sirt1 silencing blocked ferulic acid-induced profitable alterations.

Conclusions: We demonstrated that ferulic acid was a protective phytochemical against hepatic lipotoxicity in plant-based food, through Sirt1/autophagy pathway. Increasing ferulic acid-enriched food intake is a potential strategy to prevent and/or improve metabolic diseases with lipotoxicity as a typical pathological feature.

1. Introduction

Metabolic diseases, including non-alcoholic fatty liver disease (NAFLD), diabetes mellitus, and obesity, are worldwide epidemic and commonly featured with hyperlipidemia as a hallmark. Under normal physiological condition, adipose tissues have a high ability to store excessive fat, and maintain lipolysis homeostasis; however, in the pathological state of insulin resistance, enhanced lipolysis of adipose tissue leads to elevated free fatty acids (FFAs) heterotopic deposition in non-adipose tissues, such as liver, skeletal muscle, and pancreas, resulting in cell dysfunction and even cell death, which is called lipotoxicity or lipoapoptosis [1]. As the central organ of metabolism, hepatic cell death induced by lipotoxicity accelerates the occurrence and development of metabolic diseases. We previously reported that alleviating hepatic lipotoxicity effectively improved high-fat diet-induced NAFLD in mice [2]. FFAs are chemically divided into saturated fatty acids (SFAs) and unsaturated fatty acids (USFAs). Among which, lipotoxicity-caused cell death is commonly induced by SFAs via stimulating oxidative stress and

endoplasmic reticulum stress [3, 4], whereas USFAs tend to improve SFA-induced lipotoxicity [5]. Palmitate acid (PA, C16:0) is the most abundant natural SFA in food and human body and is widely used as lipotoxicity inducer for scientific investigations [6, 7].

Autophagy is a highly conserved physiological process by which intracellular components can be degraded and removed for quality and nutritional purposes. People with lipids metabolic disorder of liver are usually suffered from impaired autophagy, such as NAFLD patients [2]. Recent studies including ours have confirmed that activating autophagy is an effective way to improve lipotoxicity-induced hepatocellular injury in both cultured cells and animal models [2, 8]. Although the penetrating mechanisms behind autophagy-regulated lipotoxicity are not fully illustrated, autophagy activation helped to eliminate damaged organelles, such as mitochondria and endoplasmic reticulum, which were termed as mitophagy and reticulophagy, respectively, was involved in its lipotoxicity protective role via improving oxidative stress and endoplasmic reticulum stress [9, 10]. Additionally, the activation of autophagy degraded intracellular accumulation of triglycerides, which was termed as lipophagy [11], and in turn alleviating obesity-associated metabolic disorder in liver [12].

Several mechanisms have been identified implicating in the regulation of autophagy. Among which, adenosine monophosphate-activated protein kinase (AMPK) activation acts as a positive regulator of autophagy via inhibiting mammalian target of rapamycin (mTOR) in the state of intracellular energy deficiency [13]. Sirtuin 1 (SIRT1), a homologue of mammalian silencing information regulator 2, is a NAD^+ dependent deacetylase that regulates protein activity via modifying the acetylation of molecules, transcriptional factors and enzymes. It is known that SIRT1 participated in the regulation of autophagy via affecting autophagy-related genes 3 (Atg3), Atg7 and microtubule-associated protein 1 (MAP1) light chain 3 (LC3) deacetylation. LC3, an initiator of autophagosomes, can be deacetylated by SIRT1 in nucleus, by which LC3 will be selectively activated and allowed to engage in autophagy [14, 15]. We previously reported that SIRT1 activation protected lipotoxicity-induced hepatic cell death via stimulating autophagy [16]. Akt (known as protein kinase B, PKB), a key regulator for cellular survival, has also been confirmed participating in the regulation of autophagy and lipotoxicity-induced hepatic cell death [17, 18].

There are no safe and effective clinical drugs to treat metabolic diseases so far. Accumulating evidence supports that patients with abnormal liver metabolism enhanced their physical health indicators by adjusting dietary structure, like increasing the intakes of fruits, vegetables, and whole grains [19]. Ferulic acid (FA)„ chemically known as 4-hydroxy-3-methoxy cinnamic acid, is mainly cross-linked with cytoderm polysaccharides and lignin to form part of cytoderm in plants. FA is commonly spread in the seeds of whole grains (bran, rice, wheat, etc.), vegetables (tomato, celery, spinach, etc.), and fruits (pineapple, grape, blackberry, etc.), with the highest content in whole grains (up to 1000 mg/kg in rye) [20–22]. An accurate nutrition survey about FA intake is lacking. Consumption of food source FA can be estimated in daily intake of 150–200 mg [23]. Several biological functions of FA have been reported, such as anti-diabetes, anti-oxidation, anti-inflammation, anti-cancer, and lowering blood lipid. FA is more easily absorbed by the body than other phenolic acids and stays in the blood for longer [24]. FA acts as antioxidant due to its scavenging on radicals, instead of the formation of phenoxyl radicals, as well as

inhibition of reactive oxygen species (ROS) generation through donation of a hydrogen atom. Previous studies showed that FA improved thioacetamide- and CCl4-induced hepatic fibrosis [25, 26] and diosbulbin B-, cadmium-, and streptozotocin-induced liver damage [27–29]. FA supplementation significantly improved hepatic lipids metabolic disorder and decreased liver injury in high-fat diet-induced obese mice [30, 31]. However, limited study has been conducted to analyze the effect of FA on lipotoxicity-induced hepatic cell death, and the mechanisms are largely unclear.

The present study was designed to emphasize the influence of FA on lipotoxicity-induced hepatocytes impairments, including apoptosis, mitochondrial function, oxidative stress, and its potential molecular mechanisms. PA was chosen to establish hepatic lipotoxicity model *in vitro*. We observed that FA incubation markedly ameliorated PA-induced apoptosis, LDH leakage, mitochondrial membrane potential (MMP) reduction, ROS generation, and inflammatory activation. FA triggered SIRT1 upregulation, which in turn activated autophagy, was mechanistically involved in its beneficial role against lipotoxicity. Hence, our study reveals that FA is a potential effective phytochemical compound resistant to hepatic lipotoxicity.

2. Material And Methods

2.1 Chemicals

All of chemicals, including PA, bovine serum albumin (BSA), dimethylsulfoxide (DMSO), and choloroquine (CQ) were purchased from Sigma-Aldrich (St. Louis, MO). FA was provided by Chengdu Herbpurify Co., Ltd (Sichuan, China). PA-BSA conjugates were prepared as described previously [16]. All experiments contained a control group/vehicle, which was exposed to a same amount of solvent (e.g. BSA, DMSO).

2.2 Cell Culture

AML-12, a non-transformed mouse hepatocyte cell line, was obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in Dulbecco's Modified Eagle Medium/Ham's Nutrient Mixture F-12, 1:1 (DMEM/F-12, Hyclone, Logan, Utah) including 10% (v/v) fetal bovine serum, 10 μg/mL insulin, 5.5 μg/mL transferrin, 5 ng/mL sodium selenite, 40 ng/mL dexamethasone, 100 U/mL penicillin, 100 μg/mL streptomycin at 37°C in a humidified O_2/CO_2 (19:1) atmosphere.

2.3 Cell death assays

Cells were seeded into plate and allowed to grow to approximate 80% confluency, and then were incubated with indicated treatments. Cell viability was detected by MTT test, lactate dehydrogenase (LDH) release, and Hoechst staining. For MTT assay, MTT (Solarbio, Beijing, China) was added to each well to final 5 mg/mL and maintained at room temperature for 4 hours. Then DMSO was added and incubated on a plate shaker for 10 min. The absorption was detected at 470 nm using microplate reader (Dynatech, El Paso, Texas, MR-4100). For LDH assay, the medium was collected for LDH analyzing using LDH kit (Pierce, Rockford, lL) according to the manufacturer's instructions. The absorption values were

measured at 340 nm using microplate reader (Dynatech, El Paso, Texas, MR-4100). Cell viability was also assessed by Hoechst 33342 (Sigma-Aldrich, St. Louis, MO) staining. The plate was washed twice with chilled phosphate buffered saline (PBS) after staining for 30 min. The nuclear morphological changes were examined by fluorescence microscope (Nikon, Tokyo, Japan, TE2000-U).

2.4 ROS detection

ROS was determined by 2,7-dichlorodi-hydrofluorescein diacetate (DCFH-DA, Sigma, St. Louis, MO) probe. DCFH-DA (10 μM as final concentration) was added to each well and stained at room temperature for 30 min. Chilled PBS were used to wash cells for three times. The fluorescence intensity was measured with inverted fluorescent microscope (Nikon, Tokyo, Japan, TE2000-U). Image J 1.51 software was used to quantify the mean fluorescence intensity (MFI) from five random fields.

2.5 MMP assay

MMP was assessed via fluorescent dye Rhodamine 123 (Rh123, Solarbio, Beijing, China) staining. Cells were stained with Rh123 (100 μg/mL as final concentration) for 45 min at room temperature. Then the cells were washed by chilled PBS to remove excess dye. The fluorescence intensity was measured with inverted fluorescent microscope (Nikon, Tokyo, Japan, TE2000-U). Image J 1.51 software was used to analyze MFI from five random fields.

2.6 RNA interference

Small interfering RNA (siRNA) for mouse SIRT1 was purchased from GenePharma Co., Ltd (Shanghai, China). SiRNA-Mate (GenePharma, Shanghai, China) was utilized to deliver siRNA to the targeted cells according to the manufacturer's protocol. Scrambled siRNA (GenePharma, Shanghai, China) was applied in negative control group. Silencing efficiency was verified by quantitative real-time PCR and western-blot analysis.

2.7 RNA extraction and quantitative real-time PCR

Intracellular total RNA was harvested by Trizol (Invitrogen, Carlsbad, CA). qRT-PCR was performed with StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA). The data were analyzed by $2^{-(\triangle\triangle Ct)}$ method. *Rn18s* was used as house-keeping gene for calibration. Primers' sequences were listed in Table 1.

2.8 Western-blot analysis

Western-blot was performed as previously described [16]. The primary antibodies were used as followed: anti-cleaved-caspase3, anti-Bcl2, anti-Bax, anti-phosphorylated-AMPK, anti-AMPK, anti- phosphorylated-Akt, anti-Akt, anti-LC3B, anti-p62, anti-Beclin1, and anti-SIRT1 were from Cell Signaling Technology Inc (Danvers, MA); anti-β-tubulin1 was from Boster Biological Technology (Wuhan, China). The secondary antibodies were provided by Boster Biological Technology (Wuhan, China).

2.9 Analysis of autophagic flux

The autophagic flux was measured as previously described [32]. In brief, chloroquine (CQ) was added prior to the FA treatment to inhibit lysosome acidification. The autophagic flux was determined by detecting GFP-LC3 puncta with laser scanning confocal microscope (Zeiss, Jena, German, LSM880) as well as LC3-II expression by western blot. For GFP-LC3 fluorescence detection, cells were transiently infected with recombinant GFP-LC3 lentivirus (GeneChem, Shanghai, China).

2.10 Statistical analysis

All experimental data were from at least three independent experiments and shown as mean ± SD. Statistical significance was compared by Student's t-test or one-way ANOVA followed by Tukey's multiple comparisons test. All tests were performed with SPSS 19.0. A value of $p < 0.05$ was considered significant.

3. Results

3.1 FA protects against hepatocyte cell death induced by PA.

The cytotoxicity of FA was initially determined on AML12 hepatocytes. There was no significant cytotoxicity observed at dose up to 800 μM (Fig. 1A). Combined with previous studies [8, 33], an incremental dose of FA (0, 25, 50, and 100 μM) were selected to evaluate the protective effect of FA on palmitate-induced lipotoxicity. The result indicated that FA significantly reversed PA-induced hepatocyte cell death in a dose-dependent manner (Fig. 1B). FA exposure also prevented PA-induced caspase-3 cleavage, a typical indicator of apoptosis, with an optimal dose at 100 μM (Fig. 1C). In addition, PA-induced DNA condensation and nuclei fragmentation were also reversed by FA pretreatment (Fig. 1D). To test the generality of our finding, we conducted the similar experiments in human hepatocytes cell line (HepG2). Our results showed that FA incubation markedly reversed PA-induced cell death in HepG2 cells (Fig. S1). All these results suggested that FA exhibited a strong protective role against hepatic lipotoxicity-induced by PA.

3.2 FA improves lipotoxicity-induced mitochondrial dysfunction in hepatocytes.

In view of the strong antioxidant effect of FA, we subsequently tested protective ability of FA on mitochondrial, the main source of intracellular ROS. Our results showed that PA exposure significantly potentiated intracellular ROS levels, while FA pretreatment effectively reversed PA-triggered excessive ROS generation (Fig. 2A). Meanwhile, FA pretreatment also dramatically recovered MMP suppressed by PA (Fig. 2B). Moreover, the alteration of apoptosis-related mitochondrial proteins, including Bcl-2 and Bax, was significantly reversed by FA pretreatment (Fig. 2C).

3.3 Autophagy activation contributes to FA-inhibited lipotoxicity.

We previously reported that activation of autophagy protects hepatocytes from PA-induced lipotoxicity [8]. In this study, we found that FA exposure significantly stimulated hepatic autophagy in AML12 cells, evidenced by the observation of increased Beclin1 and LC3 II conversion, the well-established markers of autophagy induction, whereas reduced p62, a protein specifically degraded by autophagy, in a dose-dependent and time-course manner (Fig. 3A & B). Moreover, FA incubation markedly stimulated autophagic flux, which was confirmed by increased LC3-II conversion and LC-3 puncta formation in the presence of chloroquine (Fig. 3C & D). Importantly, FA-protected lipotoxicity was robustly blocked by autophagy inhibition (Fig. 3E-G), which indicating autophagy activation participated in the beneficial role of FA against lipotoxicity.

3.4 SIRT1 upregulation participates in FA-induced autophagy.

Subsequently, we analyzed upstream regulators by which FA-stimulated autophagy. Our data showed that FA treatment did not increased expressions of phosphorylated-AMPK and -Akt (Fig. 4A). However, SIRT1 was upregulated by FA intervention in a dose-dependent and time-course manner (Fig. 4A & B). PA blunted SIRT1 protein abundance was obviously reversed by FA incubation (Fig. 4C). Importantly, FA treatment failed to stimulated autophagy in SIRT1-silenced hepatocytes (Fig. 4D-F), indicating that SIRT1 was participated in FA-stimulated autophagy. We also observed that more LC3 II was detected in SIRT1-knockingdown cells (Fig. 4F), which probably due to the fact that SIRT1 deficiency led to the impediment of autophagic flux and in hence blocked LC3 II degradation. To further certified that, we tested the expression of p62, which could be specially degraded by autophagy. In support of our hypothesis, p62 was obviously accumulated in Sirt1-knockingdown cells (Fig. S2). This phenomenon was in agree with a previous study [14].

3.5 Sirt1-regulated autophagy is mechanistically involved in FA-alleviated lipotoxicity.

We then analyzed the participation of Sirt1-mediated autophagy in FA-protected lipotoxicity in AML12 hepatocytes. The results showed that genetically knocking-down Sirt1 significantly blocked FA-protected lipotixicity-induced by PA treatment, evidenced by detections of LDH release, cleaved-caspase3 expression, and nuclear staining (Fig. 5A-C).

3.6 FA improves PA-induced proinflammatory cytokines activation in hepatocytes.

The anti-inflammatory role of FA was investigated in this study. Our results showed that PA exposure significantly transcriptionally stimulated pro-inflammatory factors, including IL-1beta and IL-6, while FA pretreatment markedly reversed PA-induced inflammatory reactions (Fig. 6A & B).

4. Discussion

In this study, we identified that FA, a safe phenolic acid, exerted strong anti-apoptosis role in PA-induced hepatic lipotoxicity. FA intervention significantly alleviated lipotoxicity-caused mitochondrial dysfunction

and inflammation in AML-12 hepatocytes. Our data suggested that Sirt1-mediated autophagy signaling pathway contributed to the beneficial roles of FA mentioned above.

Lipotoxicity plays an essential pathological role in the development of several metabolic diseases [34]. In liver, excessive FFAs may initiate hepatocyte injury, inflammation, and even apoptosis, which in turn leads to liver dysfunction and promotes the occurrence of various metabolic diseases [35, 36]. It is commonly recognized that the major detrimental role of lipotoxicity was not sourced from neutral triglyceride deposition, but originated from excessive free SFAs, among which, PA was the most selected lipotoxicity-inducer [5, 7, 37]. Accumulated evidence has proved that strategy on alleviating hepatic lipotoxicity effectively improved metabolic diseases, such as NAFLD [38]. To date, no safe and efficient clinical drug targeting on lipotixicity prevention have been officially approved. Plenty of epidemiological studies have reported that improving dietary habits, especially increasing the intake of plant foods, such as whole-grain food, was benefit for the prevention of hepatic metabolic disorder in NAFLD patients [39]. Therefore, phytochemicals extracted from plant foods or medical herbs provide a feasible alternative for the treatment of lipotixicity-related metabolic diseases. FA, which is widely existing in whole grains with a dose of 1000 mg/kg in rye [22], has recently been reported to improve high-fat diet-induced hepatic metabolic disorder in experimental animal [30, 31, 40]. Several studies have demonstrated that FA was a strong scavenger of excessive ROS, which induction was mechanistically involved in lipotoxicity-induced apoptosis [41, 42]. However, limited study has conducted to investigate the protective role of FA on lipotoxicity-induced cell death in hepatocytes. In this study, we reported for the first time that FA intervention significantly alleviated PA-induced apoptosis in hepatocytes, which was confirmed by the detections of LDH release, caspase-3 cleavage, and nuclear morphology.

We subsequently analyzed the potential mechanisms behind the protective effects of FA. Autophagy is a conservative and complex quality control pathway that plays a crucial part in eliminating damaged proteins and organelles [43]. Upon autophagy induction, LC3 (microprotein 1 light chain 3), a homologue of Atg8 in mammalian cells, controls the formation of autophagosomes and lysosome, as well as the degradation of substrates. LC3-I is regulated with phosphatidylethanolamine at the glycine residue, and becomes LC3-II, which is bound to both the outer and the inner membrane of the autophagosome [44]. Beclin1 (also termed as BECN1), a homologue of yeast Atg6, not only participates in the formation of autophagosomes, but also regulates autophagy activity [45]. SQSTM1 (sequestrome1, also known as p62) is a selective autophagy receptor, which transports ubiquitinated targets to autophagosomes [46]. It has been well-considered that autophagic flux was damaged in the liver of metabolic diseases, such as NAFLD [47–49]. The activation of autophagy could improve hepatic metabolic disorders by removing excessive lipids droplets from hepatocytes, and alleviate liver injury [50]. We previous reported that autophagy activation protected hepatocyte from SFAs-induced hepatic apoptosis [8]. Therefore, we hypothesized that autophagy activating contributed to the beneficial role of FA. In support of our vision, FA incubation markedly stimulated autophagy in hepatocytes, based on the observations of increased autophagic flux, along with enhanced Beclin1 expression and LC3-II conversion, and reduced p62 expression. In line with our observations, FA has also been reported to stimulate autophagy in other types of cells, including renal cells, brain microvascular endothelial cells, and myocardial cells [51–53].

Importantly, the inhibition of autophagy significantly blocked the protective role of FA against PA-induced apoptosis, which indicating autophagy induction was mechanistically involved in FA-alleviated lipotoxicity in hepatocytes.

Lipotoxicity-induced mitochondrial dysfunction, excessive ROS production, and even programmed apoptosis played a critical role in the pathological process in hepatic metabolic diseases [4]. Selective degradation of damaged mitochondrial by autophagy was also termed as mitophagy, which helped to maintain the integrity of the cell function. Recent evidence has provided that the induction of mitophagy prevented high-fat diet-induced liver injury [54, 55]. Besides, PA-decreased mitophagy, leading to mitochondrial dysfunction as characterized by extensive mito-ROS production and loss of MMP [56]. Data from our study clearly revealed that FA treatment significantly abrogated PA-induced mitochondrial dysfunction, by the facts of improved MMP and intracellular ROS level. However, more direct evidence on mitophagy of FA-treated hepatocytes was still needed in the future studies.

Several mechanisms have been identified in the regulation of autophagy. Among which, Sirt1, an NAD^+-dependent deacetylase, regulated autophagy initiation via mediating LC3 deacetylation [57]. Sirt1 activation exerted positive effect in the regulation of liver lipids metabolism, oxidation and inflammation [58], whereas, Sirt1 depletion accelerated hepatic injury in the pathogenesis of NAFLD [58, 59]. We recently reported the protective effect of Sirt1 induction on PA-induced hepatocellular death [16]. These evidences promoted us to hypothesize that Sirt1-regulated autophagy contributed to FA-protected lipotoxicity. This notion was indeed supported by the following evidences. Firstly, FA treatment obviously stimulated Sirt1 expression in a dose-dependent and time-course manner. Such performance was also observed in FA-treated skeletal muscle cells, bone, and testis [60–62]. Secondly, genetically knocking-down Sirt1 robustly abolished FA-stimulated autophagy induction. Last but importantly, FA-protected lipotoxicity was strongly blocked in Sirt1-silenced hepatocytes. Additionally, AMPK, a central sensor of intracellular energy, is a key regulator of autophagy via inhibiting the downstream targets mTOR complex 1, which is a negative regulator of autophagy. Several studies have reported the reciprocal regulatory relationship between Sirt1 and AMPK [63]. The activation of AMPK can significantly eliminate lipotoxicity-induced hepatocyte death [64]. FA was showed to active AMPK in skeletal muscle cells and cardiac myocytes [60, 65]. We therefore analyzed the involvement of phosphorylated-AMPK in FA-treated hepatocytes. Beyond our expectation, FA incubation did not activate AMPK phosphorylation in AML12 hepatocytes, which excluded the participation of AMPK in FA-protected lipotoxicity. Phosphatidylinositol 3-kinase (PI3K)/Akt pathway, which activation promoted cell survival under lipotoxicity, was mechanistically involved in autophagy induction [17]. The reports on the regulation of FA on PI3K/Akt were inconsistent. It seemed like that PI3K/Akt was inhibited by FA in tumor cells [66] but stimulated in detrimental stimuli-induced cellular dysfunction [67]. In this study, we observed that Akt phosphorylation was not significantly regulated by FA treatment in AML12 hepatocytes, implying that PI3K/Akt pathway was not participated in FA-induced autophagy and further beneficial roles against lipotoxicity.

Pro-inflammatory factors-triggered cytotoxicity played a detrimental role in the development of hepatic metabolic disorders. Accumulated studies including ours have indicated that PA exposure

transcriptionally stimulated expressions of pro-inflammatory factors, including IL-1beta and IL-6 [68–70]. In the present study, we observed that FA intervention markedly reversed PA-caused activation of pro-inflammatory factors in hepatocytes, which was in line with the facts that FA-inhibited pro-inflammatory reactions in other types of cells [71, 72]. Therefore, we presumed that FA-blocked inflammation might be mechanistically participated in its lipotoxicity-alleviative role. In view of the crosstalk between oxidative stress and inflammation, we are still not sure whether FA inhibited lipotoxicity by directly acting on the inflammatory signaling pathway or by improving oxidative stress, which needs further investigations.

5. Conclusions

In summary, our study reported that via activating Sirt1/autophagy pathway, FA exposure in hepatocytes protects lipotoxicity-induced apoptosis. Our findings provided a new mechanism that may help to understand the biological values of FA in hepatic metabolic diseases. We highlighted the potential value of FA as a dietary supplement in preventing and/or treating liver diseases with lipotoxicity as a typical pathological feature.

Abbreviations Used

FA, ferulic acid;

NAFLD, non-alcoholic fatty liver disease;

FFAs, free fatty acids;

SFAs, saturated fatty acids;

USFAs, unsaturated fatty acids;

PA, palmitate acid;

AMPK, adenosine monophosphate-activated protein kinase;

mTOR, mammalian target of rapamycin;

SIRT1, Sirtuin 1;

Atg, autophagy-related genes;

LC3, microtubule-associated protein 1 light chain 3;

PKB, protein kinase B;

SQSTM1 (also known as p62), sequestrome1;

MMP, mitochondrial membrane potential;

ROS, reactive oxygen species;

CQ, choloroquine;

LDH, lactate dehydrogenase;

DCFH-DA, 2,7-dichlorodi-hydrofluorescein diacetate;

Rh123, Rhodamine 123.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

The authors declare no conflict of interest.

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Authors' contributions

XD, SL and HC designed of the work; TX made contributions to the acquisition and analysis; QS drafted the manuscript; LZ, HN and QQ made contributions to the interpretation of data; QH and HP revised the manuscript. All authors read and approved the final manuscript.

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Table

Table 1
List of primers.

Gene	Forward primer (5’-3’)	Reverse primer (5’-3’)
SIRT1	GGAGCAGATTAGTAAGCGGCTTG	GTTACTGCCACAGGAACTAGAGG
IL-6	CCGGAGAGGAGACTTCACAGC	AGAATTGCCATTGCACAAC
IL-1beta	TCCAGGATGAGGACATGAGCAC	GAACGTCACACACCAGCAGGTTA
Rn18s	GAATGGGGTTCAACGGGTTAA	GGTCTGTGATGCCCTTAGA

Figures

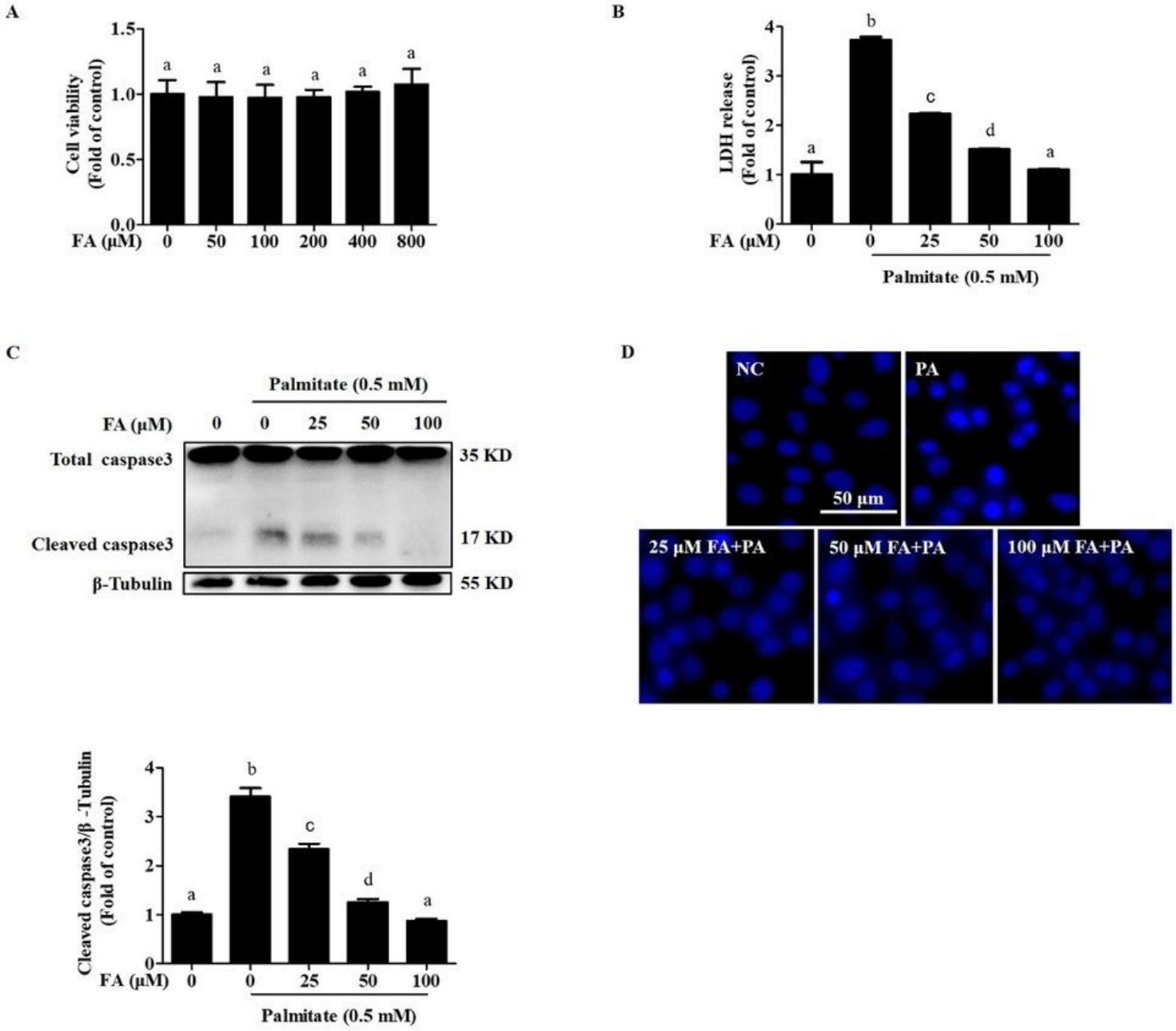

Figure 1

Ferulic acid protects hepatocytes against palmitate-induced cell death. (A) AML-12 cells were treated with 0, 50, 100, 200, 400, and 800 μM ferulic acid (FA) at 80% confluence for 24 h. Cell viability was analyzed by MTT assay. (B) AML-12 cells were incubated with 0.5 mM palmitic acid (PA) for 12 h at 80% confluence, with 0, 25, 50, or 100 μM FA pretreatment for 2 h before PA exposure. LDH release was measured. (C) Total cellular lysates were detected by immunoblotting assay for cleaved-caspase3. (D) Nuclear morphology was detected by Hoechst staining. All values are denoted as means ± SD from three or more independent batches of cells. Bars with different superscripts are significantly different at p 0.05.

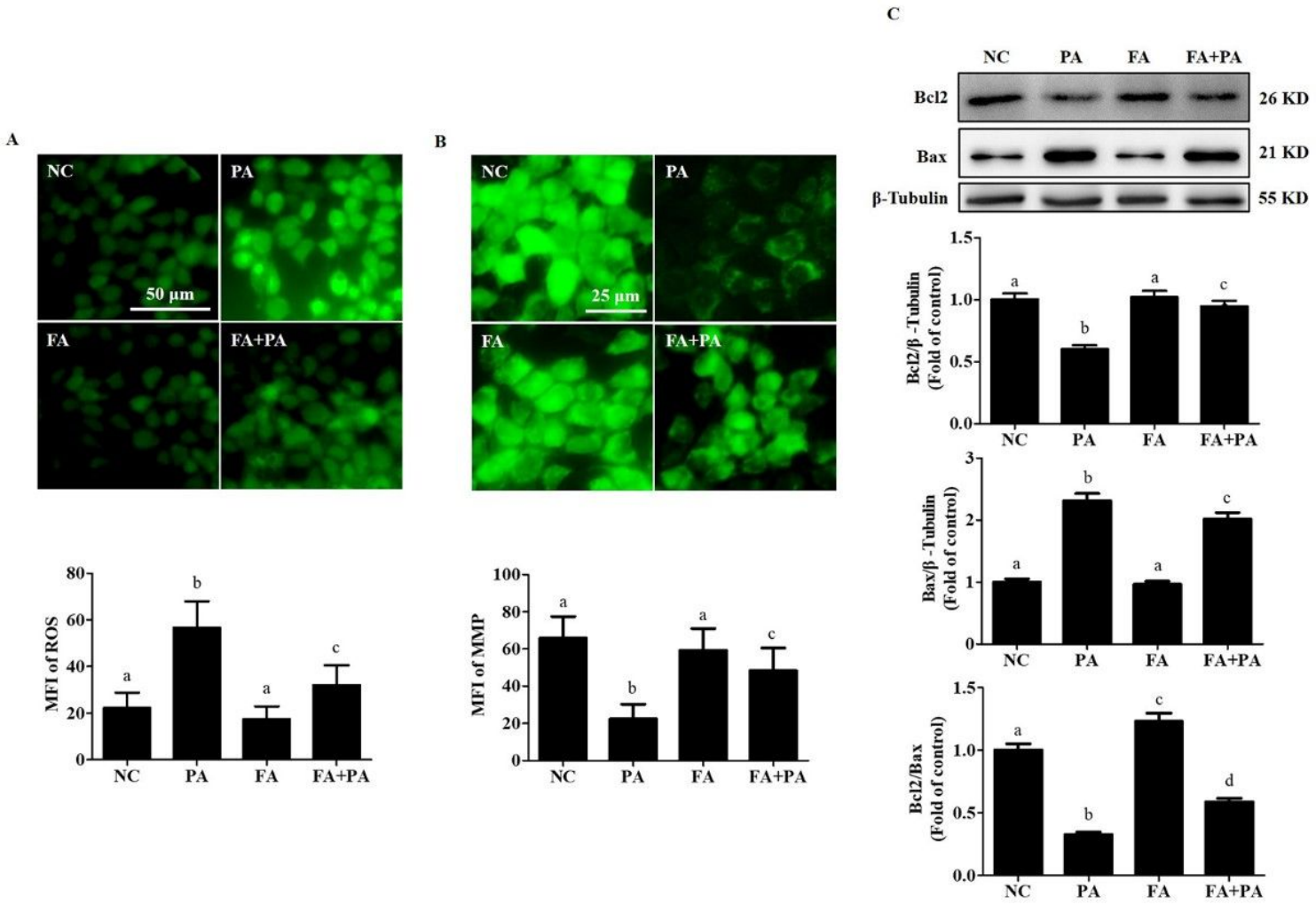

Figure 2

Ferulic acid improves lipotoxicity-induced mitochondrial dysfunction in hepatocytes. AML-12 cells were treated with 0.5 mM palmitic acid (PA) with or without 100 μM ferulic acid (FA) pretreatment for 2 h. (A) Intracellular reactive oxygen species (ROS) was analyzed by DCFH-DA staining. (B) Mitochondrial membrane potential (MMP) was detected by Rh123 staining. (C) Bcl-2 and Bax were detected by immunoblotting. All values are denoted as means ± SD from three or more independent batches of cells. Bars with different superscripts are significantly different at p 0.05.

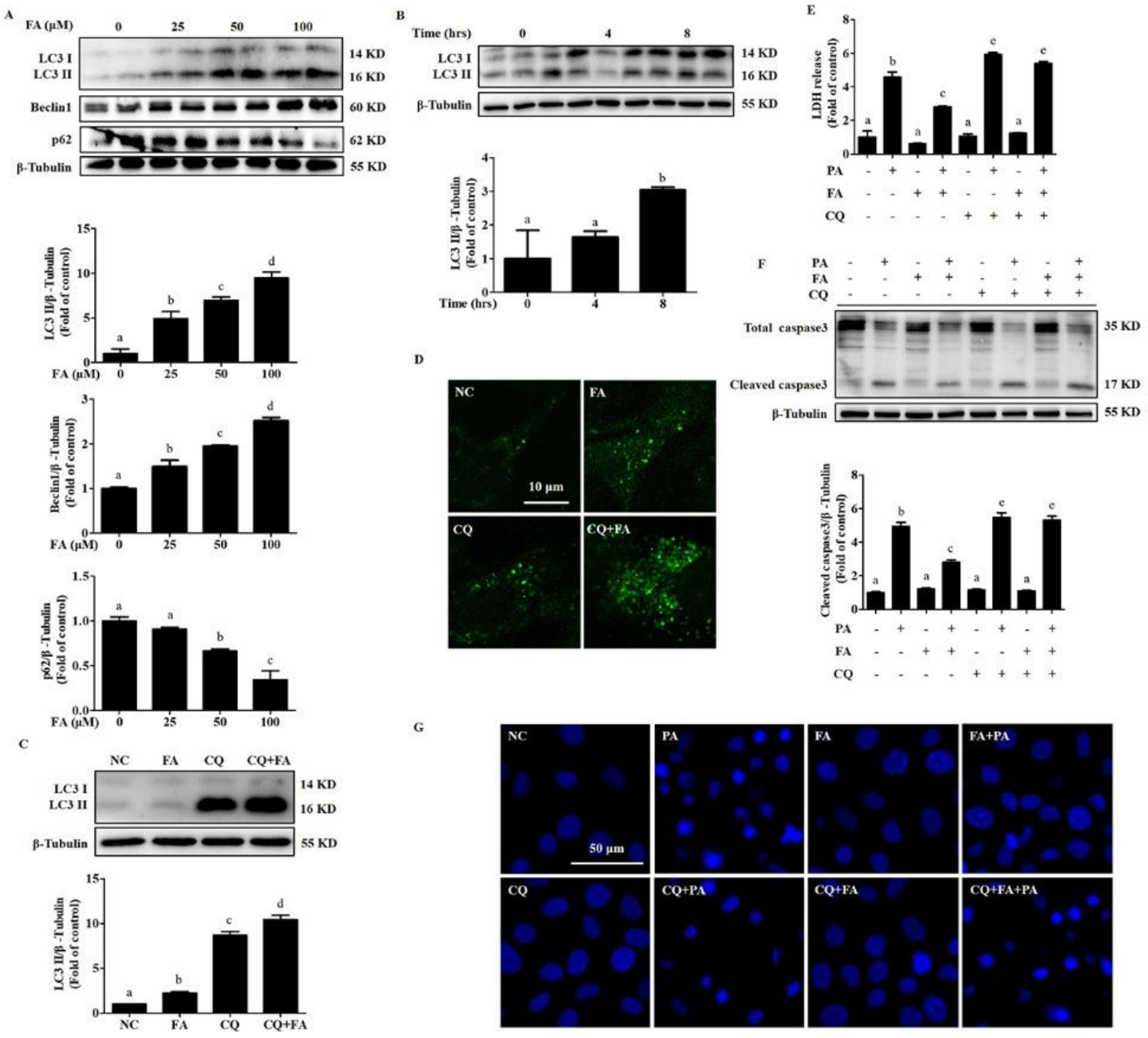

Figure 3

Autophagy activation contributes to ferulic acid-induced lipotoxicity. (A) AML-12 cells were treated with 0, 25, 50, or 100 μM ferulic acid (FA) for 12 h. LC3, Beclin1, and p62 were detected by immunoblotting. (B) AML-12 cells were treated with 100 μM FA for 0, 4, or 8 h. LC3 were detected by immunoblotting. (C) AML-12 cells were pretreated with chloroquine (CQ, lysosomal acidification blocker, 20 μM) for 1 h with or without 100 μM FA exposure for 12 h. LC3 were detected by immunoblotting. (D) AML-12 cells were transfected with mRFP-GFP-LC3 plasmid before chemical exposure. LC3 puncta was detected by confocal microscopy after the indicated treatments. (E) Cells were pretreated with CQ for 1 h before FA exposure. 100 μM FA was added 2 h before palmitic acid (PA) exposure. LDH release was measured. (F) Caspase3 cleavage was detected. (G) Nuclear morphology was detected by Hoechst staining. All values

are denoted as means ± SD from three or more independent batches of cells. Bars with different superscripts are significantly different at p ⍰ 0.05.

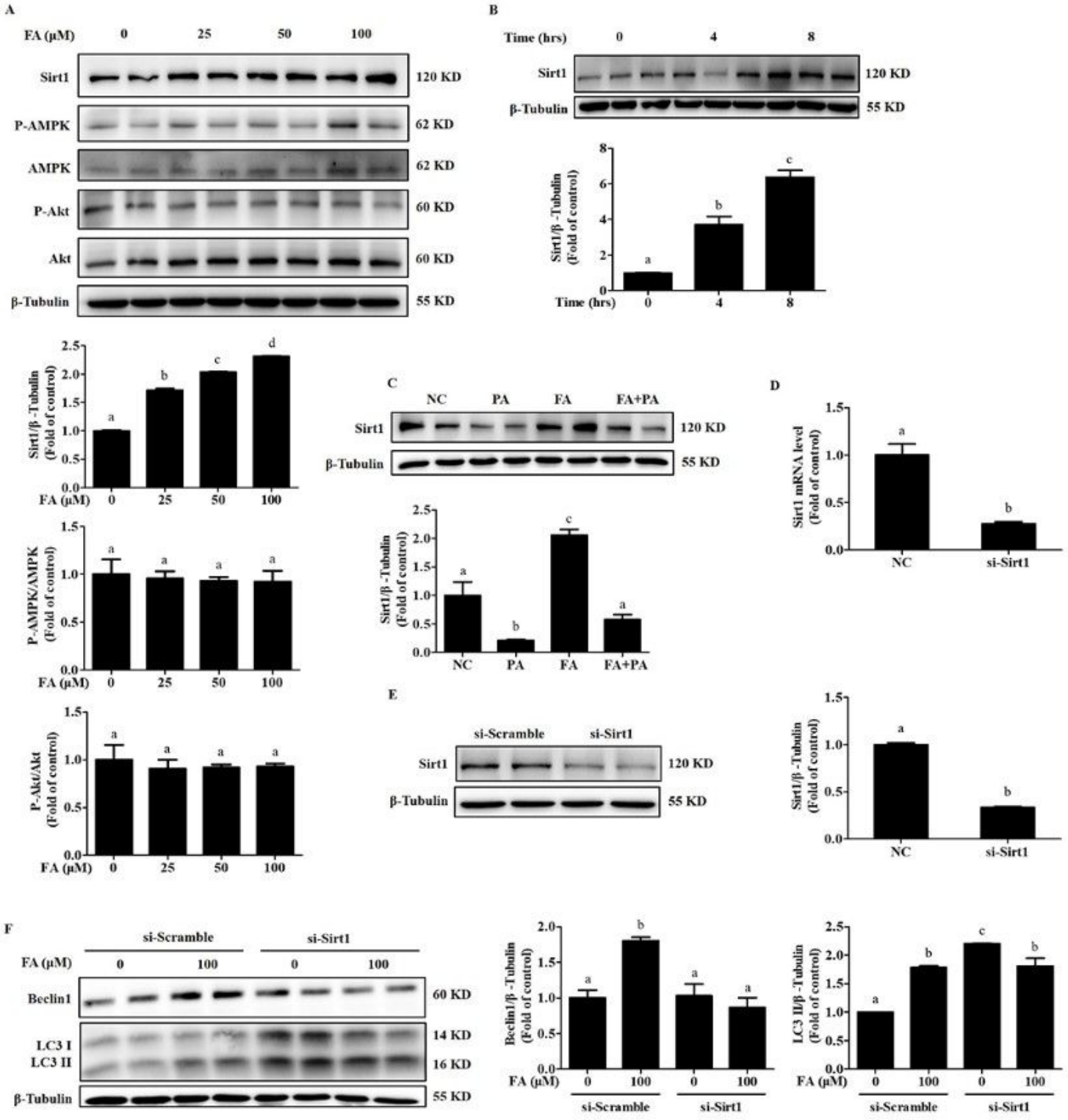

Figure 4

SIRT1 upregulation participated in ferulic acid-induced autophagy. (A) AML-12 cells were treated with 0, 25, 50, and 100 μM ferulic acid (FA) for 12 h. Total cell lysate were detected by immunoblotting assay for SIRT1, phosphorylated-AMPK, and phosphorylated-Akt. (B) Cells were treated with 100 μM FA for 0, 4, and

8 h. SIRT1 protein abundance was detected. (C) 100 μM FA was added 2 h before 0.5 mM palmitic acid (PA) exposure. After 12 h incubation, SIRT1 was detected by immunoblotting. (D, E & F) AML-12 cells were transfected with si-SIRT1 or scramble siRNA. Silencing efficiency was verified by SIRT1 mRNA and protein expression. Autophagy related proteins, including Beclin1 and LC3, were detected by immunoblotting. All values are denoted as means ± SD from three or more independent batches of cells. Bars with different superscripts are significantly different at p < 0.05.

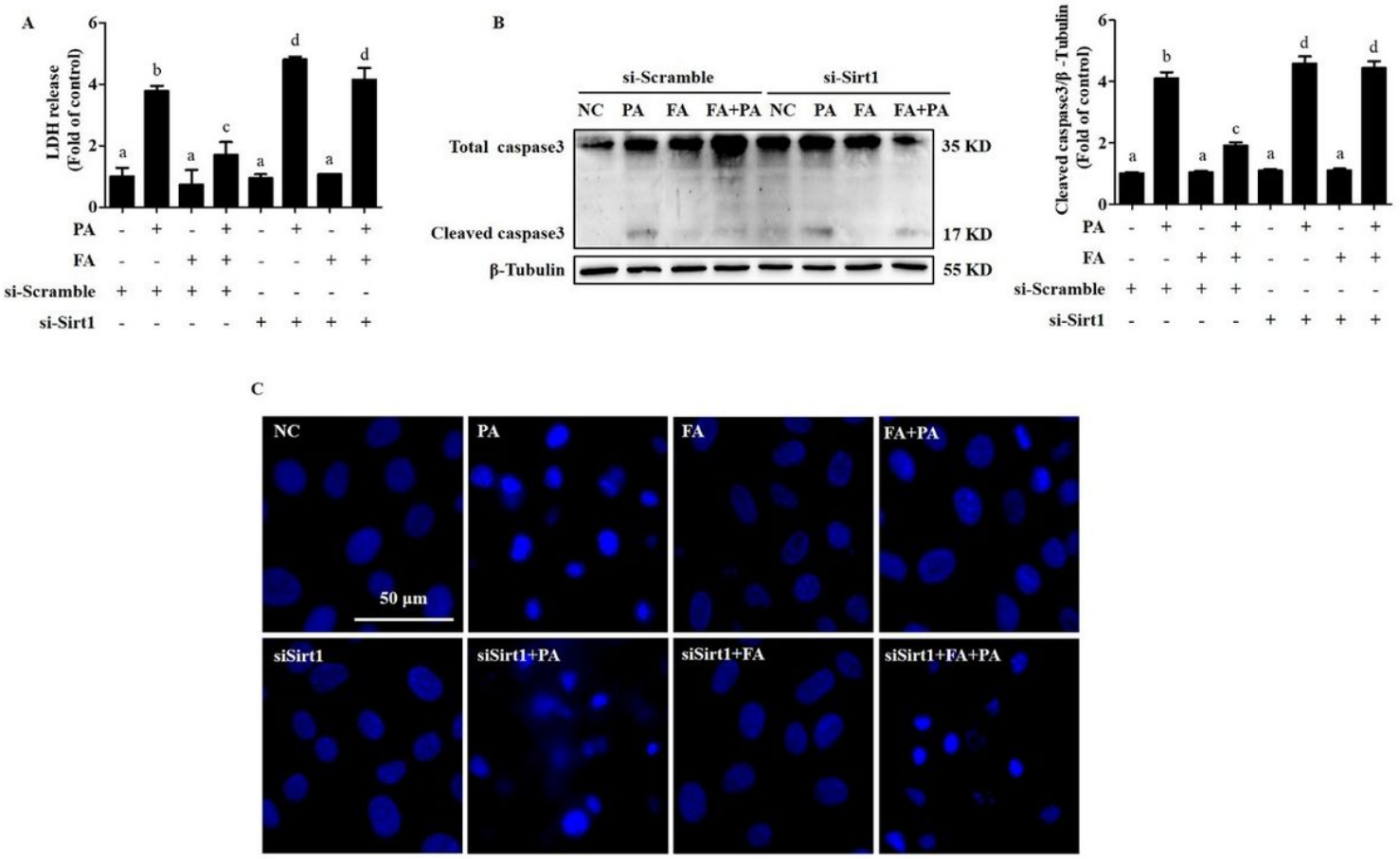

Figure 5

SIRT1-regulated autophagy is mechanistically involved in ferulic acid-alleviated lipotoxicity. AML-12 cells were transfected with si-SIRT1 or scramble siRNA before ferulic acid (FA) exposure. 100 μM FA was added 2 h before 0.5 mM palmitic acid (PA) exposure (12 h). (A & B) LDH release and caspase3 cleavage were detected. (C) Nuclear morphology was observed by Hoechst staining. All values are denoted as means ± SD from three or more independent batches of cells. Bars with different superscripts are significantly different at p < 0.05.

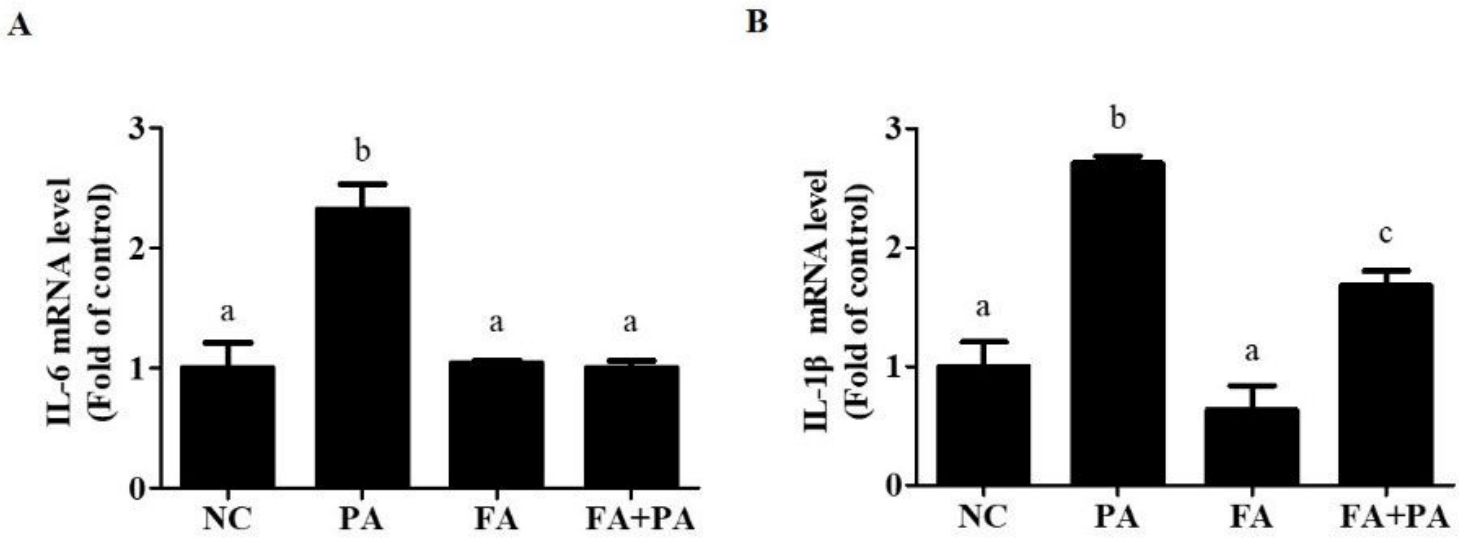

Figure 6

Ferulic acid improves PA-induced proinflammatory cytokines activation in hepatocytes. AML-12 cells were pretreated with or without ferulic acid (FA, 100 μM) for 2 h before 12 h palmitic acid (PA, 0.5 mM) exposure. Gene expressions of proinflammatory factors were all analyzed by quantitative real-time PCR. (A) IL-6 mRNA. (B) IL-1β mRNA. All values are denoted as means ± SD from three or more independent batches of cells. Bars with different superscripts are significantly different at p 0.05.

Supplementary Files

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