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

Tiantian Xu
Zhejiang Chinese Medical University

Qing Song
Zhejiang Chinese Medical University

Li Zhou
Zhejiang Chinese Medical University

Hua Ning
Zhejiang Chinese Medical University

Qianyu Qian
Zhejiang Chinese Medical University

Hui Chai
Zhejiang Chinese Medical University

Qiang Han
Zhejiang Chinese Medical University

Hongzhi Pan
Shanghai University of Medicine and Health Sciences

Xiaobing Dou
Zhejiang Chinese Medical University

Songtao Li (lisontao@vip.126.com)
Zhejiang Chinese Medical University

Research

Keywords: Ferulic acid, Sirt1, autophagy, lipotoxicity, metabolic diseases

DOI: https://doi.org/10.21203/rs.3.rs-97741/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
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 phenoxy 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 chloroquine (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 O₂/CO₂ (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, IL) 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^(-ΔΔCt) 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 lipotoxicity-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 lipotoxicity 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 lipotoxicity-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 deacylation [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-induced autophagy and further benecial roles against 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), sequestome1;
MMP, mitochondrial membrane potential;
ROS, reactive oxygen species;
CQ, chloroquine;
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.

Funding
This work was supported by the Natural Science Foundation of China [grant numbers 81973041, 81773981]; Zhejiang Natural Science Foundation for Distinguished Young Scholars [grant number LR20H260001]; and Research Fund of Zhejiang Chinese Medical University [grant number 771200F027].

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.

Acknowledgements
Not applicable.

References
1. van Herpen NA, Schrauwen-Hinderling VB. Lipid accumulation in non-adipose tissue and lipotoxicity. Physiol Behav. 2008; 94:231-241.

2. Li S, Dou X, Ning H, Song Q, Wei W, Zhang X, Shen C, Li J, Sun C, Song Z. Sirtuin 3 acts as a negative regulator of autophagy dictating hepatocyte susceptibility to lipotoxicity. Hepatology. 2017; 66:936-952.

3. Han J, Kaufman RJ. The role of ER stress in lipid metabolism and lipotoxicity. J Lipid Res. 2016; 57:1329-1338.

4. Hauck AK, Bernlohr DA. Oxidative stress and lipotoxicity. J Lipid Res 2016, 57:1976-1986.

5. Alkhouri N, Dixon LJ, Feldstein AE. Lipotoxicity in nonalcoholic fatty liver disease: not all lipids are created equal. Expert Rev Gastroenterol Hepatol. 2009; 3:445-451.

6. de Almeida IT, Cortez-Pinto H, Fidalgo G, Rodrigues D, Camilo ME. Plasma total and free fatty acids composition in human non-alcoholic steatohepatitis. Clin Nutr. 2002; 21:219-223.

7. Ogawa Y, Imajo K, Honda Y, Kessoku T, Tomeno W, Kato S, Fujita K, Yoneda M, Saito S, Saigusa Y, et al. Palmitate-induced lipotoxicity is crucial for the pathogenesis of nonalcoholic fatty liver disease in cooperation with gut-derived endotoxin. Sci Rep. 2018; 8:11365.

8. Li S, Li J, Shen C, Zhang X, Sun S, Cho M, Sun C, Song Z. tert-Butylhydroquinone (tBHQ) protects hepatocytes against lipotoxicity via inducing autophagy independently of Nrf2 activation. Biochim Biophys Acta. 2014; 1841:22-33.

9. Zhang NP, Liu XJ, Xie L, Shen XZ, Wu J. Impaired mitophagy triggers NLRP3 inflammasome activation during the progression from nonalcoholic fatty liver to nonalcoholic steatohepatitis. Lab Invest. 2019; 99:749-763.

10. Pang L, Liu K, Liu D, Lv F, Zang Y, Xie F, Yin J, Shi Y, Wang Y, Chen D. Differential effects of reticulophagy and mitophagy on nonalcoholic fatty liver disease. Cell Death Dis. 2018; 9:90.

11. Singh R, Kaushik S, Wang Y, Xiang Y, Novak I, Komatsu M, Tanaka K, Cuervo AM, Czaja MJ. Autophagy regulates lipid metabolism. Nature. 2009; 458:1131-1135.

12. Yang L, Li P, Fu S, Calay ES, Hotamisligil GS. Defective hepatic autophagy in obesity promotes ER stress and causes insulin resistance. Cell Metab. 2010; 11:467-478.

13. Kim J, Kundu M, Viollet B, Guan KL. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. Nat Cell Biol. 2011; 13:132-141.

14. Ou X, Lee MR, Huang X, Messina-Graham S, Broxmeyer HE. SIRT1 positively regulates autophagy and mitochondria function in embryonic stem cells under oxidative stress. Stem Cells. 2014; 32:1183-1194.

15. Morselli E, Marino G, Bennetzen MV, Eisenberg T, Megalou E, Schroeder S, Cabrera S, Benit P, Rustin P, Criollo A, et al. Spermidine and resveratrol induce autophagy by distinct pathways converging on the acetylproteome. J Cell Biol. 2011; 192:615-629.

16. Shen C, Dou X, Ma Y, Ma W, Li S, Song Z. Nicotinamide protects hepatocytes against palmitate-induced lipotoxicity via SIRT1-dependent autophagy induction. Nutr Res. 2017; 40:40-47.
17. Ning H, Sun Z, Liu Y, Liu L, Hao L, Ye Y, Feng R, Li J, Li Y, Chu X, et al. Insulin Protects Hepatic Lipotoxicity by Regulating ER Stress through the PI3K/Akt/p53 Involved Pathway Independently of Autophagy Inhibition. Nutrients. 2016; 8:227.

18. Wu YT, Tan HL, Huang Q, Ong CN, Shen HM. Activation of the PI3K-Akt-mTOR signaling pathway promotes necrotic cell death via suppression of autophagy. Autophagy. 2009; 5:824-834.

19. Riazi K, Raman M, Taylor L, Swain MG, Shaheen AA. Dietary Patterns and Components in Nonalcoholic Fatty Liver Disease (NAFLD): What Key Messages Can Health Care Providers Offer? Nutrients. 2019; 11.

20. Zdunska K, Dana A, Kolodziejczak A, Rotsztejn H. Antioxidant Properties of Ferulic Acid and Its Possible Application. Skin Pharmacol Physiol. 2018; 31:332-336.

21. Kumar N, Pruthi V. Potential applications of ferulic acid from natural sources. Biotechnol Rep (Amst). 2014; 4:86-93.

22. Rybka K, Sitarski J, Raczenska-Bojanowska K. Ferulic acid in rye and wheat grain and grain dietary fiber. Cereal Chemistry. 1993; 70:55-55.

23. Zhao Z, Moghadasian MH. Chemistry, natural sources, dietary intake and pharmacokinetic properties of ferulic acid: A review. Food Chem. 2008; 109:691-702.

24. Tee-ngam P, Nunant N, Rattanarat P, Siangproh W, Chailapakul O. Simple and rapid determination of ferulic acid levels in food and cosmetic samples using paper-based platforms. Sensors (Basel). 2013; 13:13039-13053.

25. Hussein RM, Anwar MM, Farghaly HS, Kandeil MA. Gallic acid and ferulic acid protect the liver from thioacetamide-induced fibrosis in rats via differential expression of miR-21, miR-30 and miR-200 and impact on TGF-beta1/Smad3 signaling. Chem Biol Interact. 2020; 324:109098.

26. Mu M, Zuo S, Wu RM, Deng KS, Lu S, Zhu JJ, Zou GL, Yang J, Cheng ML, Zhao XK. Ferulic acid attenuates liver fibrosis and hepatic stellate cell activation via inhibition of TGF-beta/Smad signaling pathway. Drug Des Devel Ther. 2018; 12:4107-4115.

27. Niu C, Sheng Y, Zhu E, Ji L, Wang Z. Ferulic acid prevents liver injury induced by Diosbulbin B and its mechanism. Biosci Trends. 2016; 10:386-391.

28. Sanjeev S, Bidanchi RM, Murthy MK, Gurusubramanian G, Roy VK. Influence of ferulic acid consumption in ameliorating the cadmium-induced liver and renal oxidative damage in rats. Environ Sci Pollut Res Int. 2019; 26:20631-20653.

29. Thyagaraju BM. Ferulic acid supplements abrogate oxidative impairments in liver and testis in the streptozotocin-diabetic rat. Zoolog Sci. 2008; 25:854-860.

30. Naowaboot J, Piyabhan P, Munkong N, Parklak W, Pannangpetch P. Ferulic acid improves lipid and glucose homeostasis in high-fat diet-induced obese mice. Clin Exp Pharmacol Physiol. 2016; 43:242-250.

31. Ma Y, Chen K, Lv L, Wu S, Guo Z. Ferulic acid ameliorates nonalcoholic fatty liver disease and modulates the gut microbiota composition in high-fat diet fed ApoE(-/-) mice. Biomed Pharmacother. 2019; 113:108753.
32. Mizushima N, Yoshimori T, Levine B. Methods in mammalian autophagy research. Cell. 2010; 140:313-326.
33. Doss HM, Samarpita S, Ganesan R, Rasool M. Ferulic acid, a dietary polyphenol suppresses osteoclast differentiation and bone erosion via the inhibition of RANKL dependent NF-kappaB signalling pathway. Life Sci. 2018; 207:284-295.
34. Ertunc ME, Hotamisligil GS. Lipid signaling and lipotoxicity in metaflammation: indications for metabolic disease pathogenesis and treatment. J Lipid Res. 2016; 57:2099-2114.
35. Paschos P, Paletas K. Non alcoholic fatty liver disease and metabolic syndrome. Hippokratia. 2009; 13:9-19.
36. Liu J, Han L, Zhu L, Yu Y. Free fatty acids, not triglycerides, are associated with non-alcoholic liver injury progression in high fat diet induced obese rats. Lipids Health Dis. 2016; 15:27.
37. Listenberger LL, Han X, Lewis SE, Cases S, Farese RV, Jr, Ory DS, Schaffer JE. Triglyceride accumulation protects against fatty acid-induced lipotoxicity. Proc Natl Acad Sci USA. 2003; 100:3077-3082.
38. Trauner M, Arrese M, Wagner M. Fatty liver and lipotoxicity. Biochim Biophys Acta. 2010; 1801:299-310.
39. Ross AB, Godin JP, Minehira K, Kirwan JP. Increasing whole grain intake as part of prevention and treatment of nonalcoholic Fatty liver disease. Int J Endocrinol. 2013; 2013:585876.
40. de Melo TS, Lima PR, Carvalho KM, Fontenele TM, Solon FR, Tome AR, de Lemos TL, da Cruz Fonseca SG, Santos FA, Rao VS, de Queiroz MG. Ferulic acid lowers body weight and visceral fat accumulation via modulation of enzymatic, hormonal and inflammatory changes in a mouse model of high-fat diet-induced obesity. Braz J Med Biol Res. 2017; 50:e5630.
41. Picone P, Nuzzo D, Di Carlo M. Ferulic acid: a natural antioxidant against oxidative stress induced by oligomeric A-beta on sea urchin embryo. Biol Bull. 2013; 224:18-28.
42. Kelainy EG, Ibrahim Laila IM, Ibrahim SR. The effect of ferulic acid against lead-induced oxidative stress and DNA damage in kidney and testes of rats. Environ Sci Pollut Res Int. 2019; 26:31675-31684.
43. Glick D, Barth S, Macleod KF. Autophagy: cellular and molecular mechanisms. J Pathol. 2010; 221:3-12.
44. Gimenez-Xavier P, Francisco R, Platini F, Perez R, Ambrosio S. LC3-I conversion to LC3-II does not necessarily result in complete autophagy. Int J Mol Med. 2008; 22:781-785.
45. Kang R, Zeh HJ, Lotze MT, Tang D. The Beclin 1 network regulates autophagy and apoptosis. Cell Death Differ. 2011; 18:571-580.
46. Shaid S, Brandts CH, Serve H, Dikic I. Ubiquitination and selective autophagy. Cell Death Differ. 2013; 20:21-30.
47. Madrigal-Matute J, Cuervo AM. Regulation of Liver Metabolism by Autophagy. Gastroenterology. 2016; 150:328-339.
48. Gonzalez-Rodriguez A, Mayoral R, Agra N, Valdecantos MP, Pardo V, Miquilena-Colina ME, Vargas-Castrillon J, Lo Iacono O, Corazzari M, Fimia GM, et al. Impaired autophagic flux is associated with increased endoplasmic reticulum stress during the development of NAFLD. Cell Death Dis. 2014; 5:e1179.

49. Yamada E, Singh R. Mapping autophagy on to your metabolic radar. Diabetes. 2012; 61:272-280.

50. Martinez-Lopez N, Singh R. Autophagy and Lipid Droplets in the Liver. Annu Rev Nutr. 2015; 35:215-237.

51. Chowdhury S, Ghosh S, Das AK, Sil PC. Ferulic Acid Protects Hyperglycemia-Induced Kidney Damage by Regulating Oxidative Insult, Inflammation and Autophagy. Front Pharmacol. 2019; 10:27.

52. Chen JL, Duan WJ, Luo S, Li S, Ma XH, Hou BN, Cheng SY, Fang SH, Wang Q, Huang SQ, Chen YB. Ferulic acid attenuates brain microvascular endothelial cells damage caused by oxygen-glucose deprivation via punctate-mitochondria-dependent mitophagy. Brain Res. 2017; 1666:17-26.

53. Luo C, Zhang Y, Guo H, Han X, Ren J, Liu J. Ferulic Acid Attenuates Hypoxia/Reoxygenation Injury by Suppressing Mitophagy Through the PINK1/Parkin Signaling Pathway in H9c2 Cells. Front Pharmacol. 2020; 11:103.

54. Liu P, Lin H, Xu Y, Zhou F, Wang J, Liu J, Zhu X, Guo X, Tang Y, Yao P. Frataxin-Mediated PINK1-Parkin-Dependent Mitophagy in Hepatic Steatosis: The Protective Effects of Quercetin. Mol Nutr Food Res. 2018; 62:e1800164.

55. Ma X, McKeen T, Zhang J, Ding WX. Role and Mechanisms of Mitophagy in Liver Diseases. Cells. 2020; 9.

56. Yang S, Xia C, Li S, Du L, Zhang L, Zhou R. Defective mitophagy driven by dysregulation of rheb and KIF5B contributes to mitochondrial reactive oxygen species (ROS)-induced nod-like receptor 3 (NLRP3) dependent proinflammatory response and aggravates lipotoxicity. Redox Biol. 2014; 3:63-71.

57. Huang R, Xu Y, Wan W, Shou X, Qian J, You Z, Liu B, Chang C, Zhou T, Lippincott-Schwartz J, Liu W. Deacetylation of nuclear LC3 drives autophagy initiation under starvation. Mol Cell. 2015; 57:456-466.

58. Ding RB, Bao J, Deng CX. Emerging roles of SIRT1 in fatty liver diseases. Int J Biol Sci. 2017; 13:852-867.

59. Colak Y, Ozturk O, Senators E, Tuncer I, Yorulmaz E, Adali G, Doganay L, Enc FY. SIRT1 as a potential therapeutic target for treatment of nonalcoholic fatty liver disease. Med Sci Monit. 2011; 17:HY5-9.

60. Chen X, Guo Y, Jia G, Zhao H, Liu G, Huang Z. Ferulic acid regulates muscle fiber type formation through the Sirt1/AMPK signaling pathway. Food Funct. 2019; 10:259-265.

61. Hou T, Zhang L, Yang X. Ferulic acid, a natural polyphenol, protects against osteoporosis by activating SIRT1 and NF-kappaB in neonatal rats with glucocorticoid-induced osteoporosis. Biomed Pharmacother. 2019; 120:109205.

62. El-Mesallamy HO, Gawish RA, Sallam AM, Fahmy HA, Nada AS. Ferulic acid protects against radiation-induced testicular damage in male rats: impact on SIRT1 and PARP1. Environ Sci Pollut
63. Ruderman NB, Xu XJ, Nelson L, Cacicedo JM, Saha AK, Lan F, Ido Y. AMPK and SIRT1: a long-standing partnership? Am J Physiol Endocrinol Metab. 2010; 298:E751-760.

64. Kim DS, Jeong SK, Kim HR, Chae SW, Chae HJ. Metformin regulates palmitate-induced apoptosis and ER stress response in HepG2 liver cells. Immunopharmacol Immunotoxicol. 2010; 32:251-257.

65. Panneerselvam L, Raghunath A, Ravi MS, Vetivel A, Subramaniam V, Sundarraj K, Perumal E. Ferulic acid attenuates arsenic-induced cardiotoxicity in rats. Biotechnol Appl Biochem. 2020; 67:186-195.

66. Wang T, Gong X, Jiang R, Li H, Du W, Kuang G. Ferulic acid inhibits proliferation and promotes apoptosis via blockage of PI3K/Akt pathway in osteosarcoma cell. Am J Transl Res. 2016; 8:968-980.

67. He S, Guo Y, Zhao J, Xu X, Song J, Wang N, Liu Q. Ferulic acid protects against heat stress-induced intestinal epithelial barrier dysfunction in IEC-6 cells via the PI3K/Akt-mediated Nrf2/HO-1 signaling pathway. Int J Hyperthermia. 2019; 35:112-121.

68. Haversen L, Danielsson KN, Fogelstrand L, Wiklund O. Induction of proinflammatory cytokines by long-chain saturated fatty acids in human macrophages. Atherosclerosis. 2009; 202:382-393.

69. Bunn RC, Cockrell GE, Ou Y, Thrailkill KM, Lumpkin CK, Jr., Fowlkes JL. Palmitate and insulin synergistically induce IL-6 expression in human monocytes. Cardiovasc Diabetol. 2010; 9:73.

70. Staiger H, Staiger K, Stefan N, Wahl HG, Machicao F, Kellerer M, Haring HU. Palmitate-induced interleukin-6 expression in human coronary artery endothelial cells. Diabetes. 2004; 53:3209-3216.

71. Liu YM, Shen JD, Xu LP, Li HB, Li YC, Yi LT. Ferulic acid inhibits neuro-inflammation in mice exposed to chronic unpredictable mild stress. Int Immunopharmacol. 2017; 45:128-134.

72. Zheng X, Cheng Y, Chen Y, Yue Y, Li Y, Xia S, Deng H, Zhang J, Cao Y. Ferulic Acid Improves Depressive-Like Behavior in Prenatally-Stressed Offspring Rats via Anti-Inflammatory Activity and HPA Axis. Int J Mol Sci. 2019; 20.

### Table

| Gene | Forward primer (5’-3’) | Reverse primer (5’-3’) |
|------|------------------------|------------------------|
| SIRT1 | GGAGCAGATTAGTAAGCGGCTTG | GTTACTGCCACAGGAACAGAGG |
| IL-6  | CCGGAGGAGGAGGACTTCACAGC | AGAATTGCCATTGCACAAC  |
| IL-1beta | TCCAGGATGAGGACATGACGCAC | GAACGTCAACACACCGAGGTAA |
| Rn18s | GAATGGGGGTCAACCGGTTAA | GGTCTGTGATGCCCCCTAGA |