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### Cover Page Footnote

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# Apigenin ameliorates hepatic lipid accumulation by activating the autophagy-mitochondria pathway

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## Abstract

Apigenin, a flavonoid isolated from plants, provides protection against non-alcoholic fatty liver disease. However, the mechanism by which apigenin decreases lipid accumulation in the liver is unclear. In this study, we investigated the molecular mechanism underlying the beneficial effect of apigenin on the hepatic deregulation of lipid metabolism. Oleic acid (OA)-induced lipid accumulation in human hepatoma cells (Huh7 cells) was used as an *in vitro* model. Western blot analysis was used for evaluating protein expression. Oil red O staining, Nile red staining, and conventional assay kits were used to assess the level of lipids. Immunocytochemistry was performed to observe mitochondrial morphology. Seahorse XF analyzer was used to measure mitochondrial bioenergetics. Treatment with OA induced lipid accumulation in Huh7 cells, which was attenuated by apigenin. Mechanistically, treatment with apigenin increased the expression of autophagy-related proteins including Beclin1, autophagy related gene 5 (ATG5), ATG7, and LC3II, and the formation of autophagolysosomes, leading to an increase in intracellular levels of fatty acids. Inhibition of autophagy by bafilomycin A1 or chloroquine abolished the protection of apigenin in OA-induced lipid accumulation. Apigenin up-regulated the protein expression related to the  $\beta$ -oxidation pathway including acyl-CoA synthetase long chain family member 1, carnitine palmitoyltransferase 1 $\alpha$ , acyl-CoA oxidase 1, peroxisome proliferator activated receptor (PPAR)  $\alpha$ , and PPAR $\gamma$  coactivator 1- $\alpha$ . Moreover, apigenin increased the mitochondrial network structure and mitochondrial function by increasing the protein expression related to the process of mitochondria fusion and mitochondrial function. Collectively, our findings suggest that apigenin ameliorates hepatic lipid accumulation by activating the autophagy-mitochondrial pathway.

**Keywords:** Apigenin, Autophagy, Hepatic lipid metabolism, Mitochondria

## 1. Introduction

Apigenin, a flavonoid component, is a natural constituent of many vegetables and fruits, especially parsley and celery [1,2]. During the past decade, considerable efforts have been made towards unveiling the potential of apigenin in treating many human diseases, particularly cancers, inflammatory diseases, and metabolic

diseases [3-5]. For instance, apigenin induces cell death in human papillary thyroid carcinoma cells [6]. Apigenin exerts anti-inflammatory effects in acute pancreatitis by inhibiting the expression of tumor necrosis factor- $\alpha$  [7]. In addition, apigenin has the atheroprotective effect of increasing the cholesterol efflux in apoE-deficient mice [8]. Moreover, apigenin prevents high-fat diet (HFD)-induced hyperlipidemia and non-alcoholic fatty

*Abbreviations:* apoE, apolipoprotein E; ATG, autophagy related protein; ATP, adenosine triphosphate; ACSL1, long-chain fatty acid coenzyme A ligase 1; ACOX1, acyl-coenzyme A oxidase 1; BafA1, bafilomycin A1; CPT1 $\alpha$ , carnitine palmitoyltransferase 1; CQ, chloroquine; Drp1, dynamin-related protein 1; LC3, microtubule-associated protein 1A/1B-light chain 3; MTCO1, cytochrome c oxidase subunit 1; Mff, mitochondrial fission factor; Mfn2, mitofusin 2; MMP, mitochondrial membrane potential; NAFLD, non-alcoholic fatty liver disease; OA, oleic acid; OPA1, mitochondrial dynamin like GTPase; PPAR $\alpha$ , peroxisome proliferator-activated receptor  $\alpha$ ; PGC-1 $\alpha$ , peroxisome proliferator-activated receptor  $\gamma$  coactivator 1- $\alpha$ ; ROS, reactive oxygen species; SQSTM1, sequestosome 1.

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liver disease (NAFLD) in mice [9]. Although the protective effects of apigenin on metabolic diseases have been studied extensively *in vitro* and *in vivo* [10], there is little information about the molecular mechanism underlying the protective effect of apigenin on the deregulation of lipid metabolism.

Autophagy is a conserved self-eating process that is important for the intracellular homeostasis of lipid metabolism upon stress conditions [11]. Autophagy is known to elicit the hydrolysis of triglycerides to fatty acids and attenuate the progression of NAFLD [12]. When the excess triglycerides are accumulated in the cells, the process of autophagy is initiated and triglycerides are encapsulated, and subsequently fused with the lysosome [13,14]. Lipids are broken down into fatty acids within the lysosome and then transported to mitochondria for  $\beta$ -oxidation and to produce adenosine triphosphate (ATP) [15]. However, whether apigenin induces autophagy and regulates the turnover of triglycerides to fatty acids is largely unknown. Nevertheless, the molecular mechanism behind the catabolic metabolism of lipid droplets to fatty acids contributing to apigenin-mediated protection in the lipid homeostasis of hepatocytes requires further investigation.

Mitochondria are the energy factories that provide the cell with energy by the biochemical processes of cellular respiration [16]. Disruption of this highly controlled process results in the deficiency in bioenergetics [17]. In response to different stimuli and metabolic demands of the cells, the number, shape, and structure of mitochondria are dynamically regulated by the balance of fusion and fission [18]. Indeed, growing evidence suggests that mitochondrial dysfunction is associated with metabolic diseases such as NAFLD [19,20]. The excess accumulation of lipids in hepatocytes creates the environmental cues and enhances the inputs of the electron transport chain, which in turn causes inefficient electron transport and results in the production of mitochondrial reactive oxygen species (ROS) [21,22]. Such metabolic insult-induced mitochondrial ROS disturbs the mitochondrial dynamics by deregulating the balance of fusion and fission, leading to the impairment of mitochondrial bioenergetics [23]. However, less is known about the effect of apigenin on the excess lipid-induced production of mitochondrial ROS and bioenergetics.

Given the beneficial effect of apigenin on lipid metabolism, we aimed to investigate the effect of apigenin as well as its molecular mechanism in oleic acid (OA)-induced lipid accumulation in hepatocytes.

We first investigated the effect of apigenin on OA-induced lipid accumulation and then examined whether apigenin affected the autophagy and the morphology and function of mitochondria in hepatocytes. Our findings suggest that treatment with apigenin induces autophagy, facilitates mitochondrial function, and results in an increase in the turnover of lipid droplets, ultimately leading to the amelioration of OA-induced lipid accumulation in the hepatocytes.

## 2. Materials and methods

### 2.1. Reagents

Apigenin, OA, acridine orange, bafilomycin A1 (BafA1), chloroquine (CQ), and oligomycin were from Cayman Chemical (Ann Arbor, MI, USA). Rabbit antibodies for Beclin1 (BECN1, sc-11427), long-chain fatty acid coenzyme A ligase 1 (ACSL1, sc-98925), and peroxisome proliferator-activated receptor  $\alpha$  (PPAR $\alpha$ , sc-9000) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit antibodies for microtubule-associated protein 1A/1B-light chain 3 (LC3, #4108), sequestosome 1 (SQSTM1, #5114), mitofusin 2 (Mfn2, #9482), and dynamin-related protein 1 (Drp1, #8570) were from Cell Signaling Technology (Beverly, MA, USA). Rotenone and rabbit antibodies for autophagy related 7 (ATG7, ab133528), acyl-coenzyme A oxidase 1 (ACOX1, ab59964), and peroxisome proliferator-activated receptor  $\gamma$  coactivator 1- $\alpha$  coactivator-1 $\alpha$  (PGC-1 $\alpha$ , ab54481) and mouse antibodies for carnitine palmitoyltransferase 1 $\alpha$  (CPT1 $\alpha$ , ab128568) and cytochrome c oxidase subunit 1 (MTCO1, ab14705) were from Abcam (Cambridge, MA, UK). Rabbit antibody for autophagy related 5 (ATG5, nb110-53818) was from Novus Biologicals (Littleton, CO, USA). Rabbit antibody for mitochondrial fission factor (Mff, 17090-1-AP) was from Proteintech (Rosemont, IL, USA). Mouse antibody for mitochondrial dynamin like GTPase (OPA1, 612606) was from BD Bioscience (San Jose, CA, USA). Nile red and mouse antibody for  $\alpha$ -tubulin were from Sigma-Aldrich (St Louis, MO, USA). The colorimetric/fluorometric kit for free fatty acids was obtained from BioVision (Milpitas, CA, USA). JC-10 was from AAT Bioquest (Sunnyvale, CA, USA). MitoSOX Red and MitoTracker Green were all obtained from Invitrogen (Eugene, OR, USA).

### 2.2. Cell culture

Human hepatoma cells Huh7, Huh7.5 (stable LC3-GFP-expressing), HepG2 and murine hepatocyte

cell line AML12 were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and penicillin (100 U/mL)/streptomycin (100 µg/mL) in a humidified incubator at 37 °C, 95% air, and 5% CO<sub>2</sub>.

### 2.3. Lipid staining

At the end of experiments, the confluence of Huh7 cells was ~95%. Intracellular lipid accumulation was assessed by oil red O and Nile red staining. Cells were fixed with 4% paraformaldehyde and then stained with 0.5% oil red O or Nile red (1 µg/mL) for 15 min at room temperature. Hematoxylin was used as a counterstain for oil red O staining. The fluorescence of Nile red was determined by fluorescence fluorometry (Molecular Devices, Sunnyvale, CA, USA) using 540-nm excitation and 590-nm emission wavelengths. The fluorescence was normalized to the total protein concentration. The fold of vehicle was defined as level of fluorescence intensity relative to the vehicle group set as 1. Photomicrographs were digitally captured under a Leica DMIRB Microscope with LAS V4.12 software (Wetzlar, Germany).

### 2.4. Western blot analysis

Aliquots of cell lysates were separated on SDS-PAGE and then transblotted onto the PVDF membrane (Millipore, Bedford, MA). After being blocked with 5% skim milk, the blotting membrane was then incubated with the primary antibodies, followed by the corresponding horseradish peroxidase-conjugated secondary antibodies. Bands were visualized by using an enzyme-linked chemiluminescence detection kit (PerkinElmer, Waltham, MA, USA), and the band density was measured using the TotalLab 1D (Newcastle Upon Tyne, UK).

### 2.5. Visualization of acidic vesicular organelles

Intracellular acidic vacuoles including lysosomes, endosomes, and autophagosomes were assessed by acridine orange staining. Living cells were stained by acridine orange (2.7 µM) in culture medium for 15 min at 37 °C. The fluorescence of acridine orange staining was determined by fluorometry (Molecular Devices) using the excitation/emission spectra of 502/525 nm, 460/650 nm, and 475/590 nm. Photomicrographs were digitally captured under a Leica DMIRB Microscope.

### 2.6. The measurement of cellular free fatty acids

The lipids in the cells were extracted by use of n-hexane/isopropanol (3:2, v/v) for 1 h on a shaker. After drying, the extracted lipids were dissolved by using the commercial solutions, and the levels of intracellular free fatty acids were assessed by using fluorescence assay kits (BioVision, Milpitas, CA, USA). The fluorescence intensity of cell lysates was analyzed at 530 nm excitation and 590 nm emission wavelengths by SpectraMax i3x fluorometry (San Jose, CA, USA).

### 2.7. Immunocytochemistry

Cells were seeded into 4-well plates, incubated with the test reagents, and then fixed with 4% paraformaldehyde for 30 min. After being permeabilized by 70% alcohol for 2 h at 4 °C, cells were blocked in 2% BSA for 30 min at 37 °C. Cells were incubated with primary antibody for 2 h at room temperature and then with the corresponding secondary antibody for overnight at 4 °C. Photomicrographs were digitally captured under a Leica DMIRB Microscope.

### 2.8. Mitochondrion-selective probes

The detection of mitochondria was performed using MitoTracker reagent. Cells were incubated with medium containing MitoTracker reagent (250 nM) at 37 °C for 30 min. Photomicrographs were digitally captured under a Leica DMIRB Microscope.

### 2.9. Mitochondrial superoxide indicator

The production of mitochondria superoxide was measured by MitoSOX reagent. Cells were incubated with medium containing MitoSOX reagent (5 µM) at 37 °C for 10 min. The fluorescence of MitoSOX was determined by fluorometry (Molecular Devices) using the excitation/emission spectra of 510/580 nm. Photomicrographs were digitally captured under a Leica DMIRB Microscope.

### 2.10. Mitochondria membrane potential

The membrane potential of mitochondria was assessed by mitochondria-specific probe JC-10. Cells were incubated with medium containing JC-10 (3 µM) at 37 °C for 30 min and then stimulated

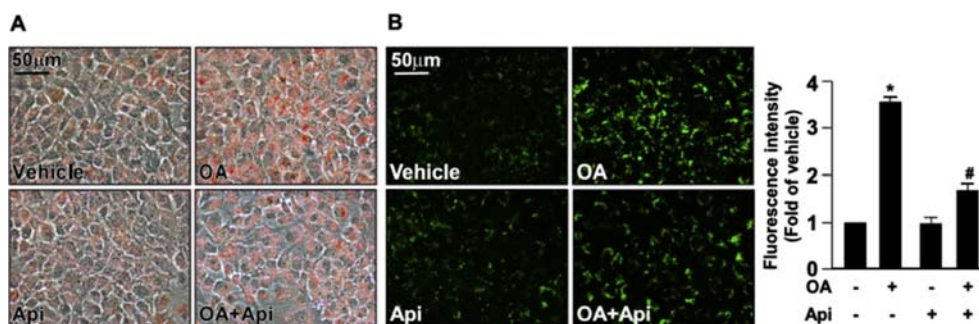


Fig. 1. Apigenin prevents oleic acid (OA)-induced lipid accumulation in Huh7 cells. Huh7 cells were pretreated with OA (125  $\mu\text{g}/\text{mL}$ ) for 24 h and then with or without apigenin (20  $\mu\text{M}$ ) for an additional 18 h. (A) Representative images of oil red O staining of Huh7 cells. (B) Representative images and the quantitative intensity of Nile red staining. Data are the mean  $\pm$  SEM from 5 independent experiments. \* $P < 0.05$  vs. the vehicle-treated group; # $P < 0.05$  vs. the OA-treated group.

with test reagents for 10 min. The fluorescence of JC-10 was determined by fluorometry (Molecular Devices) using the excitation/emission spectra of 490/525 nm and 540/590 nm. Photomicrographs were digitally captured under a Leica DMIRB Microscope.

### 2.11. Mitochondrial bioenergetics

The measurement of mitochondrial energy pathways was conducted according to the manufacturer's protocol by using the Seahorse XFe24 instrument and analyzed by software version Wave 2.2 (Seahorse Bioscience, Billerica, MA, USA).

### 2.12. Statistical analysis

The results are presented as the mean  $\pm$  SEM from 5 independent experiments and the sample size used for each statistical analysis is 5. For comparisons of data of the two groups, the Mann-Whitney U test was used. For comparisons of data of more than two groups, one-way analysis of variance (ANOVA) followed by the Dunnett's multiple comparisons or Mann-Whitney U test was performed. SPSS software v8.0 (SPSS Inc., Chicago, IL) was used for all statistical analyses. Differences were considered statistically significant at  $P < 0.05$ .

## 3. Results

### 3.1. Apigenin prevents OA-induced lipid accumulation in Huh7 cells

Previous studies indicate that excessive lipids accumulate in the hepatocytes during the development of NAFLD [12,19]. To clarify the potential effect of apigenin in regulating the lipid metabolism in hepatocytes, we used OA-induced intracellular

lipid accumulation in Huh7 cells as our *in vitro* model. We first studied the cytotoxic effect of apigenin on the cell viability. The exposure of Huh7 cells to various concentrations (0, 2.5, 5, 10, 20, 40, 80, and 160  $\mu\text{M}$ ) of apigenin for 18 h and the results of the MTT assay showed that apigenin had no cytotoxic effect on cell viability at the concentrations ranging from 2.5 to 20  $\mu\text{M}$ . However, apigenin induced cell death at concentrations starting from 40  $\mu\text{M}$  (Supplementary Fig. S1A). Thus, 20  $\mu\text{M}$  apigenin was chosen as the standard exposure for subsequent studies. Oil red O and Nile red staining showed that the intracellular levels of lipids in Huh7 cells were increased in response to OA challenge (Fig. 1A and B). Importantly, the OA-induced increase in intracellular lipid accumulation was abolished by the treatment with apigenin (Fig. 1A and B). In addition, such an inhibitory effect on lipid accumulation was observed in HepG2 and AML12 cells (Supplementary Fig. S2 and S3). These findings suggest that apigenin has a protective effect on lipid metabolism of hepatocytes.

### 3.2. The activation of autophagy flux is required for the apigenin-conferred protection in OA-induced deregulation of lipid metabolism

Autophagy is known to play a crucial role in the degradation of intracellular lipid droplets [11,12]. We then examined the effect of apigenin on autophagy and found that exposure of OA-treated Huh7 cells to apigenin induced an increase in the expression of autophagy pathway-related proteins including Beclin 1, ATG5, ATG7, LC3-I, LC-3-II, and SQSTM1 in a concentration-dependent manner (Fig. 2A–F). Additionally, treatment with apigenin time-dependently induced the formation of autophagosomes as evidenced by the appearance of

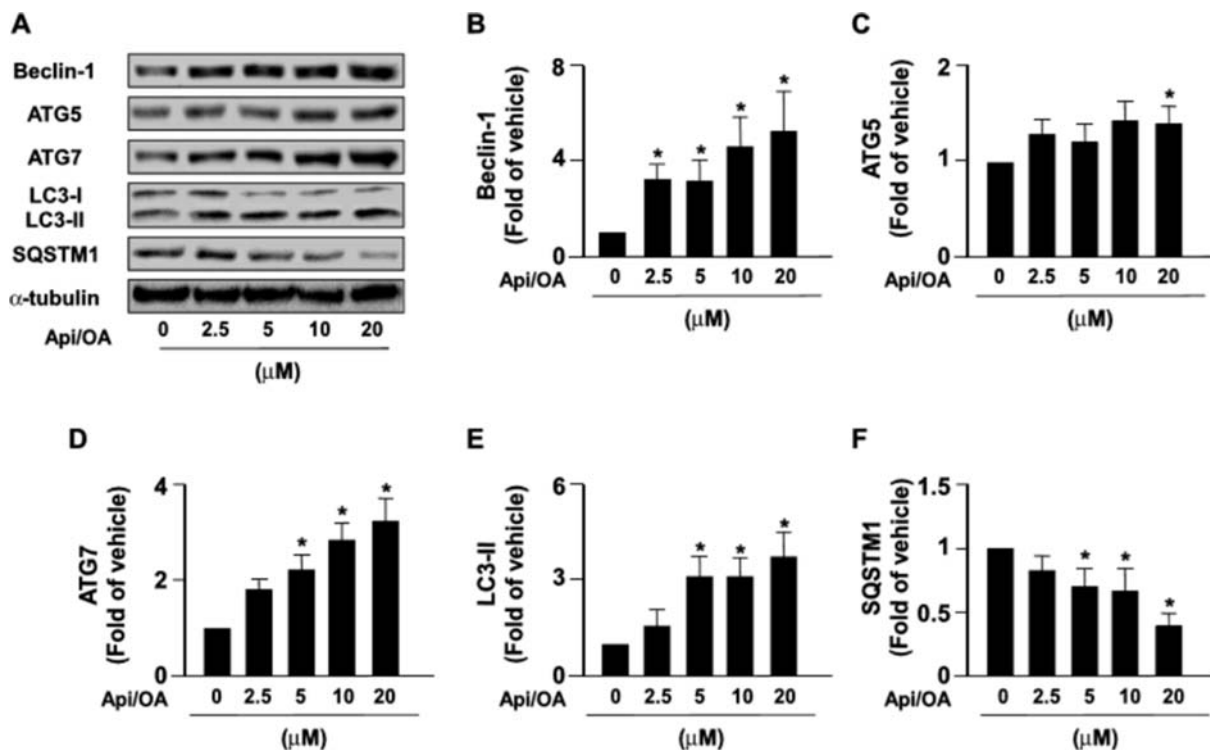


Fig. 2. Apigenin induces autophagy in Huh7 cells. (A–F) Huh7 cells were pretreated with OA (125 μg/mL) for 24 h and then apigenin (20 μM) for the indicated concentrations. Western blot analysis of protein levels of Beclin1, ATG5, ATG7, LC3, SQSTM1, and  $\alpha$ -tubulin. Data are the mean  $\pm$  SEM from 5 independent experiments. \* $P < 0.05$  vs. the 0 μM group.

GFP-LC3 puncta as early as 6 h after treatment (Fig. 3A). Moreover, the results of acridine orange staining showed that apigenin time-dependently increased the autolysosome lysosomal activity as evidence by the increased intracellular levels of acidic vacuoles within 6–9 h after treatment (Fig. 3B). Finally, the intracellular levels of free fatty acids were increased after apigenin treatment (Fig. 3C). We next assessed whether the activation of autophagy was involved in the beneficial effect of apigenin on OA-induced lipid accumulation in hepatocytes. Pretreatment with BafA1 or CQ, two autophagy inhibitors for blocking the formation of autolysosome, abolished that protective effect of apigenin on the OA-induced lipid accumulation (Fig. 4A–D). Collectively, these findings suggest the activation of the autophagy pathway is essential for the beneficial effect of apigenin on the degradation of lipid droplets induced by OA in hepatocytes.

### 3.3. Apigenin activates the $\beta$ -oxidation of free fatty acids in Huh7 cells

The activity of  $\beta$ -oxidation of free fatty acids in mitochondria plays a central role for maintaining the homeostasis of energy metabolism [16,19]. Inhibition of fatty acid oxidation damages energy

metabolism and accelerates the development of NAFLD [16,19]. We next investigated the effect of apigenin on the  $\beta$ -oxidation pathway; we found that apigenin concentration-dependently increased the expression of  $\beta$ -oxidation pathway-related proteins including ACSL1, CTP1 $\alpha$ , ACOX1, PPAR $\alpha$ , and PGC1 $\alpha$  in OA-treated Huh7 cells (Fig. 5A–F). These findings suggest that apigenin may promote the activity of  $\beta$ -oxidation of free fatty acids to reduce the intracellular levels of lipids.

### 3.4. Apigenin rescues the OA-induced mitochondrial dysfunctions in Huh7 cells

We next investigated whether the increased activity of  $\beta$ -oxidation by apigenin was attributed to the altered mitochondrial dynamics. Our data revealed that apigenin prevented the OA-induced increase in the number of mitochondria as evidenced by the expression of MTCO1, a key enzyme in the mitochondrial electron transport chain (Fig. 6A and B). In addition, apigenin up-regulated the expression of mitochondrial fusion-related proteins, such as OPA1 and Mfn2; however, it had no effect on the expression of fission-related proteins, including Mff and Drp1 (Fig. 6C–G). Moreover, the results of fluorescent microscopy showed that OA

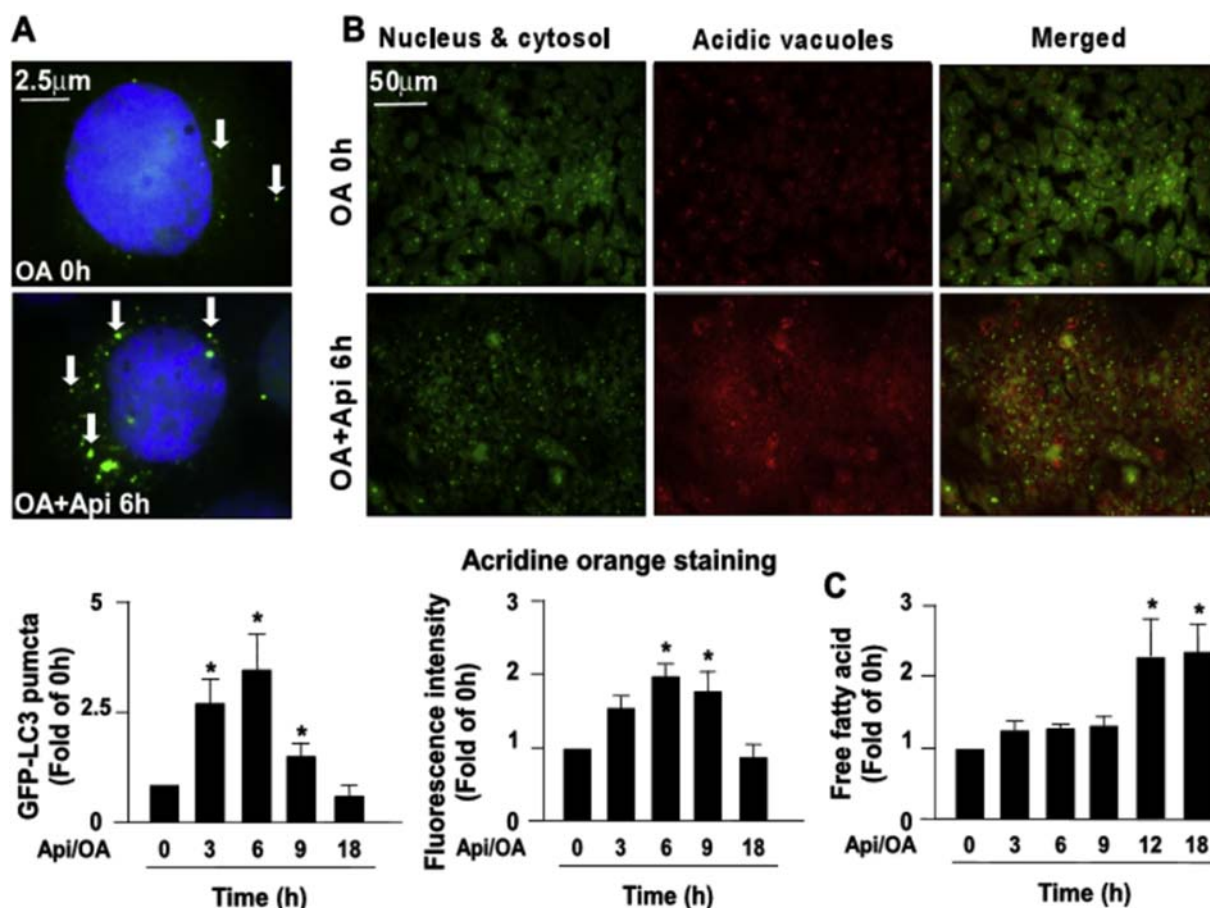


Fig. 3. Apigenin induces the formation of GFP-LC3 puncta and activates lysosome-dependent autophagy pathway. Huh7.5 cells were pre-treated with OA (125  $\mu\text{g}/\text{mL}$ ) for 24 h and then apigenin (20  $\mu\text{M}$ ) for the indicated times. (A) Representative images and the quantitative result of GFP-LC3 puncta. (B) Representative images, acidic vacuoles (red fluorescence), the nucleus and cytoplasm (green fluorescence), and the quantitative intensity of acridine orange staining of Huh7 cells. (C) The intracellular levels of free fatty acids in Huh7 cells with apigenin (20  $\mu\text{M}$ ) for indicated times. Data are the mean  $\pm$  SEM from 5 independent experiments. \* $P < 0.05$  vs. the 0 h group.

inhibited the mitochondrial fusion as evidenced by an increase in fragmented round forms of mitochondria; in contrast, apigenin promoted mitochondrial fusion as evidenced by an increase in elongated tubular forms of mitochondria and prevented the OA-induced inhibition of mitochondrial fusion (Fig. 6H).

Increased levels of mitochondrial ROS deregulate the mitochondrial dynamics resulting in the impairment of mitochondrial membrane potential (MMP) and mitochondrial function [23]. We then explored the effect of apigenin on the production of mitochondrial ROS elicited by OA. Our results revealed that treatment with apigenin abolished the OA-induced increase in the production of mitochondrial ROS (Fig. 7) and prevented the OA-induced damage in MMP (Fig. 8). Furthermore, the results of Seahorse XFe24 analysis demonstrated that treatment with apigenin prevented the detrimental effect of OA on mitochondrial function

including cellular basal respiration, ATP production, maximal respiration, and proton leak, all of which were abolished by treatment with BafA1 or CQ in hepatocytes (Fig. 9A–E). To ensure the involvement of mitochondrial function in the apigenin-mediated protection in lipid metabolism, Huh7 cells were pretreated with oligomycin or rotenone, the specific inhibitor for the ATPase and mitochondrial complex I, respectively, and the results showed that inhibition of mitochondrial function diminished the apigenin-conferred protection on OA-induced lipid accumulation in Huh7 cells (Fig. 10A–D). Collectively, these results indicate that apigenin has a protective effect against OA-induced mitochondrial dysfunction.

#### 4. Discussion

Apigenin is known to have protective effects against metabolic diseases [5,24]; however, the



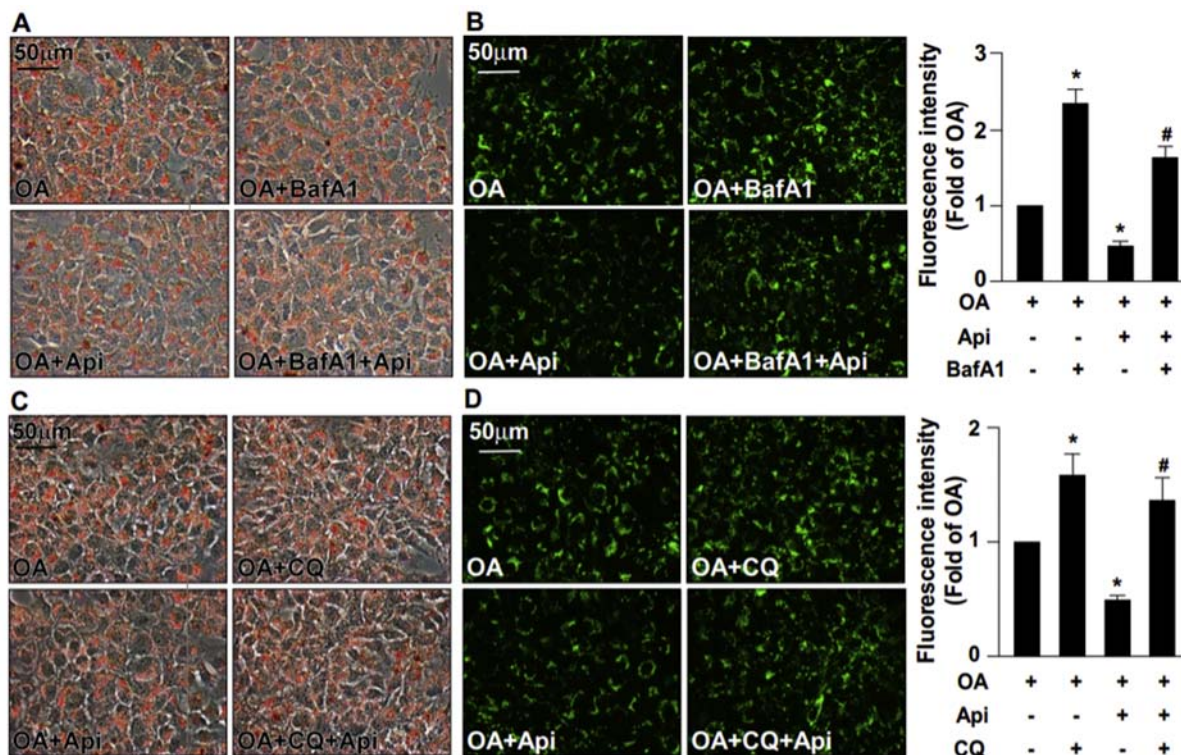


Fig. 4. Inhibition of autophagy abolishes the apigenin-mediated protection from OA-induced lipid accumulation. Huh7 cells were pretreated with OA (125  $\mu\text{g}/\text{mL}$ ) for 24 h and then with or without autophagy inhibitors (bafilomycin A1, BafA1, 100 nM, or chloroquine, CQ, 40  $\mu\text{M}$ ) for 2 h, followed by apigenin (20  $\mu\text{M}$ ) for an additional 18 h. (A and C) Representative image of oil red O staining. (B and D) The representative images and the quantitative intensity of Nile red staining. Data are the mean  $\pm$  SEM from 5 independent experiments. \* $P < 0.05$  vs. the OA-treated group; # $P < 0.05$  vs. the OA + apigenin-treated group.

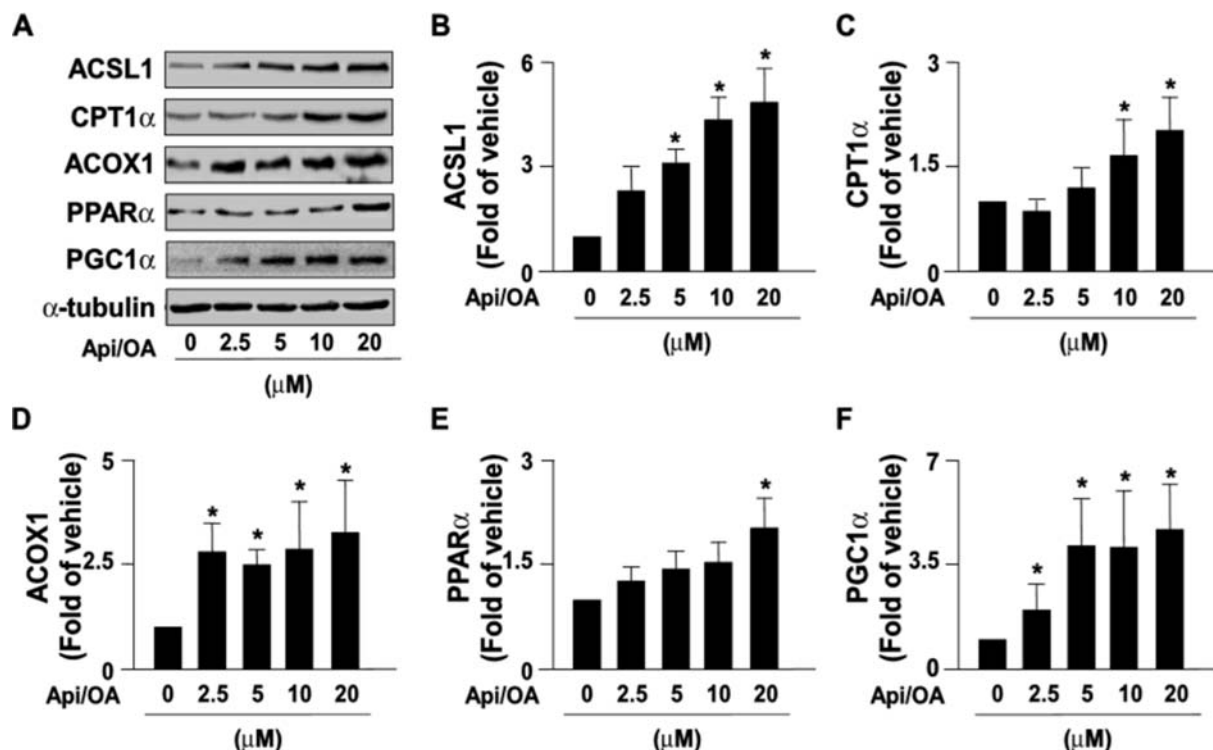


Fig. 5. Apigenin activates the  $\beta$ -oxidation pathway. (A-F) Huh7 cells were pretreated with OA (125  $\mu\text{g}/\text{mL}$ ) for 24 h and then apigenin (20  $\mu\text{M}$ ) for the indicated concentrations. Western blot analysis of protein levels of ACSL1, CPT1 $\alpha$ , ACOX1, PPAR $\alpha$ , PGC1 $\alpha$ , and  $\alpha$ -tubulin. Data are the mean  $\pm$  SEM from 5 independent experiments. \* $P < 0.05$  vs. the 0  $\mu\text{M}$  group.

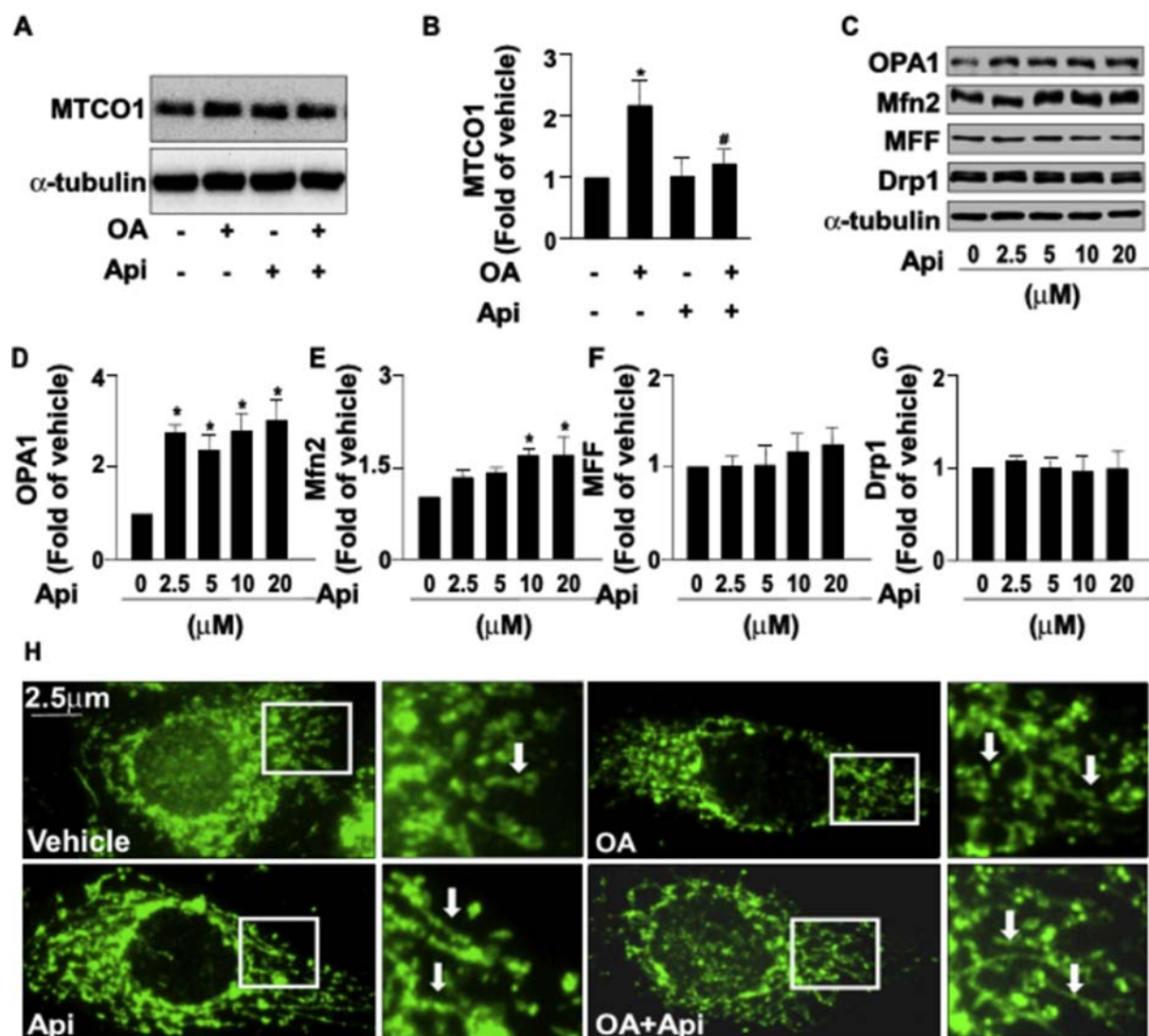


Fig. 6. Effect of apigenin on the mitochondrial biogenesis and dynamics in hepatocytes. (A and B) Huh7 cells were treated with OA (125 μg/mL) for 24 h in the absence or presence of apigenin (20 μM). Western blot analysis of protein levels of MTCO1 and α-tubulin. (C–G) Huh7 cells were treated with apigenin (20 μM) for the indicated concentrations. Western blot analysis of protein levels of OPA1, Mfn2, Mff, Drp1, and α-tubulin. (H) Representative images of mitochondrial morphologies as indicated by arrows. Data are the mean ± SEM from 5 independent experiments. \*P < 0.05 vs. the vehicle and 0 μM group.

molecular mechanism underlying the protective effect of apigenin on the deregulation of lipid metabolism has not yet been fully investigated. In this study, we used OA-induced lipid accumulation as our *in vitro* model and characterized a potential mechanism for apigenin in decreasing the lipid accumulation in hepatocytes. In Huh7 cells, exposure to excess OA induced the deregulation of lipid metabolism, which was attenuated by apigenin. Mechanistically, apigenin elicited the autophagy flux and increased the β-oxidation of fatty acids and mitochondrial bioenergetics and ultimately decreased the lipid accumulation. Additionally, apigenin promoted mitochondrial function by reducing the production of

mitochondrial ROS and regulating its biogenesis, dynamics, and membrane potential. Ultimately, apigenin accelerated the conversion the lipids into energy to maintain the metabolic balance of hepatocytes (Fig. 11). Our findings regarding the benefits of apigenin in activating the autophagy-mitochondria pathway were in agreement with the previous reports that increasing the function of autophagy and mitochondria has therapeutic value in treating lipid metabolism disorders and related metabolic diseases [25,26]. Based on these observations in this study, we provided new insights into the protective effect and the underlying molecular mechanisms of apigenin in hepatic lipid metabolism.

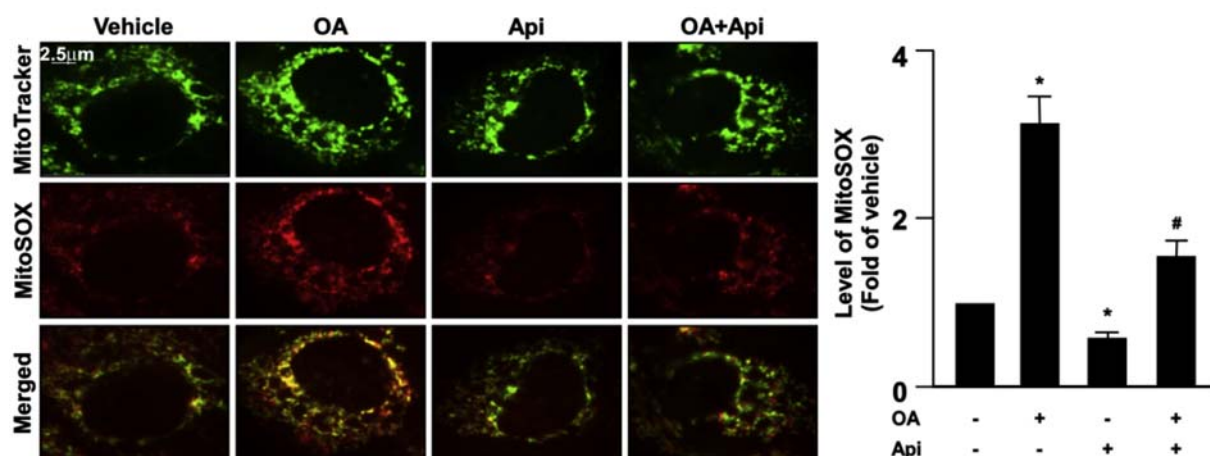


Fig. 7. Apigenin prevents OA-induced mitochondrial superoxide production in hepatocytes. Huh7 cells were pretreated with OA (125  $\mu\text{g}/\text{mL}$ ) for 24 h and then apigenin (20  $\mu\text{M}$ ) for 18 h. The MitoTracker and MitoSOX assay kits were used to measurement of the mitochondrial superoxide production. Data are the mean  $\pm$  SEM from 5 independent experiments. \* $P < 0.05$  vs. the vehicle-treated group; # $P < 0.05$  vs. the OA-treated group.

Interestingly, Chen et al. reported that apigenin elicits the activation of autophagy and induces programmed cell death, leading to the inhibition of tumor growth [27], which is in line with our finding that autophagy plays a crucial role in the beneficial effect of apigenin on lipid metabolism. However, to our knowledge, the detailed molecular mechanism behind the protective effect of apigenin on autophagy flux is not fully understood. Importantly, AMP-activated protein kinase (AMPK) is known to be a key player in regulating autophagy and lipid metabolism [28,29]; AMPK positively regulates the initiation of autophagy and promotes the clearance of intracellular lipid droplets [30]. Activation of AMPK signaling by pharmacological activators increases autophagy and leads to the decrease in the lipid accumulation in the liver [31]. Moreover, Lu

et al. suggested that apigenin activates the AMPK signaling pathway and improves the deregulation of lipid metabolism, leading to the alleviation of lipid accumulation in HepG2 cells [32]. In contrast, the mammalian target of rapamycin (mTOR), a well-known inhibitor for autophagy, plays a central role in the deregulation of lipid metabolism and development of NAFLD [33]. Zhou and Ye reported that the inhibition of the mTOR pathway by rapamycin increases autophagy and attenuates hepatic steatosis in rats with type 2 diabetes [34]. Recently, Farah et al. reported that pharmacological induction of autophagy by rapamycin improves glucose-6-phosphatase deficiency-induced hepatic lipid metabolism in mice [35]. Nevertheless, whether the AMPK pathway or the mTOR signaling involved in the beneficial effect of apigenin on lipid metabolism

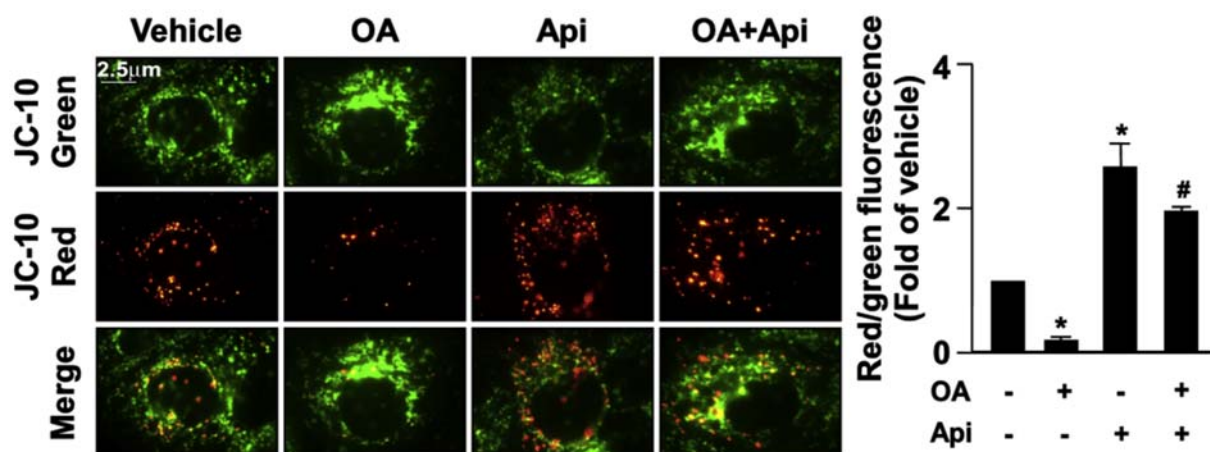


Fig. 8. Apigenin prevents the OA-induced damage on mitochondrial membrane potential in hepatocytes. Huh7 cells were pretreated with OA (125  $\mu\text{g}/\text{mL}$ ) for 24 h and then apigenin (20  $\mu\text{M}$ ) for 18 h. The JC-10 assay was used to detect the mitochondrial membrane potential. Data are the mean  $\pm$  SEM from 5 independent experiments. \* $P < 0.05$  vs. the vehicle-treated group; # $P < 0.05$  vs. the OA-treated group.

in hepatocytes is unclear. To this end, further investigations for clarifying the molecular mechanism underlying the protective effect of apigenin on lipid metabolism are warranted.

Maintenance of mitochondrial function is a key event in regulating the normal function of hepatic lipid metabolism [36]. It is known that dynamic regulation in mitochondrial biogenesis, morphological changes, ROS production, and membrane potential are important for maintaining mitochondrial hemostasis [37–40]. It is well-established that mitochondrial dysfunction is highly associated with metabolic diseases, including NAFLD, hyperlipidemia, and atherosclerosis [20,41]. Disturbance of mitochondrial function induces the deregulation of lipid metabolism and accelerates the progression of metabolic diseases [19,41]. Rescuing the mitochondrial dysfunction by pharmacological reagents or supplementation with nutrients in foods alleviates the progression of metabolic diseases [42,43], suggesting that improving the mitochondrial dysfunction has therapeutic value in treating metabolic

diseases. Interestingly, Choi et al. demonstrated that apigenin ameliorates obesity-induced skeletal muscle atrophy by attenuating mitochondrial dysfunction *in vivo* and *in vitro* [44]. The notion was further supported by our findings that apigenin decreases the excessive fatty acid-induced lipid accumulation in hepatocytes by increasing the mitochondrial biogenesis and fusion, decreasing mitochondrial ROS production, and correcting the deregulation of membrane potential of mitochondria. Intriguingly, we found that OA also increased mitochondrial biogenesis in our *in vitro* model, which is in line with the results of Rehman et al., who found that OA at a low dose could elicit the biogenesis of mitochondria against the diabetic insults in endothelial cells [45]. Therefore, we thought that increased mitochondrial biogenesis by OA in this study was a compensatory protective response for the deregulation of lipid metabolism in hepatocytes. In addition to the beneficial effect on mitochondrial functions, our data demonstrated that apigenin increased the expression of proteins

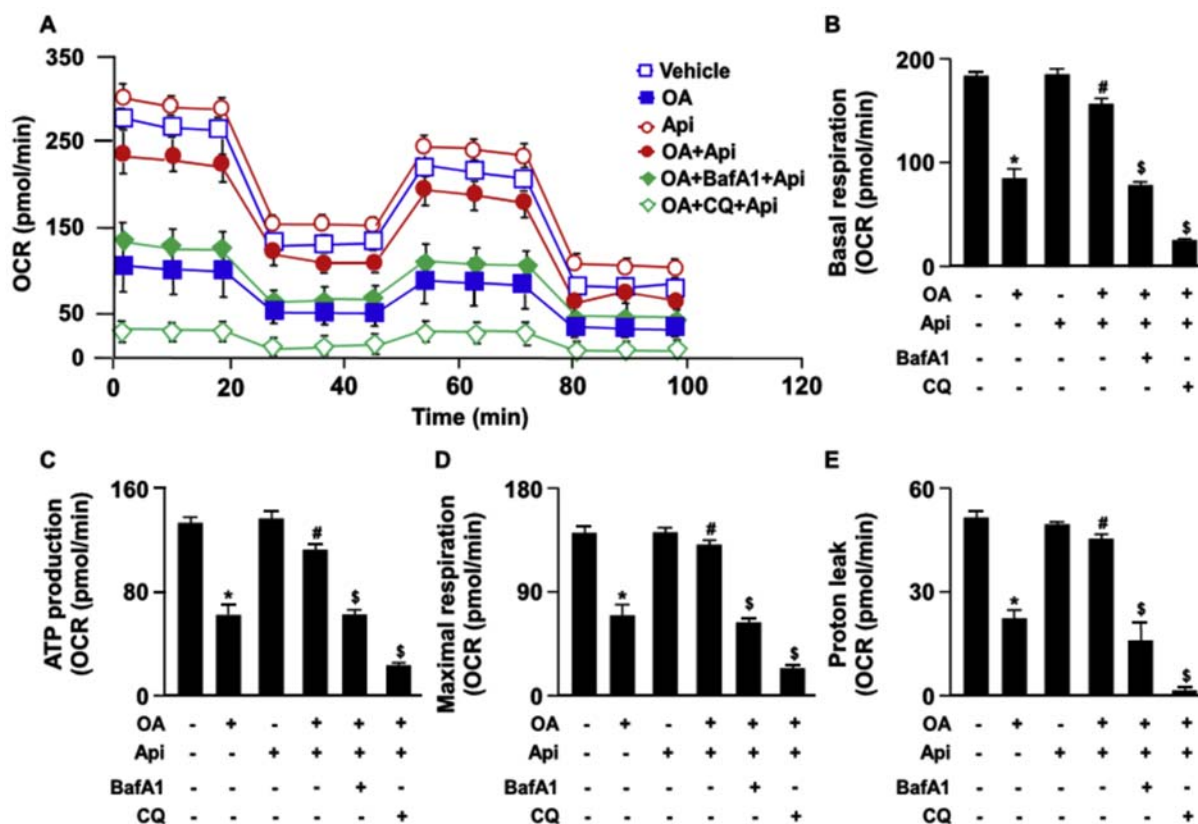


Fig. 9. Apigenin rescues the OA-induced mitochondrial dysfunction in hepatocytes. Huh7 cells were pretreated with OA (125 µg/mL) for 24 h and then with or without autophagy inhibitors (bafilomycin A1, BafA1, 100 nM, or chloroquine, CQ, 40 µM) for 2 h, followed by apigenin (20 µM) for an additional 18 h. (A–E) The Seahorse XFe24 instrument was used for the measurement of mitochondrial energy pathways including the cellular basal respiration, ATP production, maximal respiration, and proton leak. Data are the mean ± SEM from 5 independent experiments. \*P < 0.05 vs. the vehicle-treated group; #P < 0.05 vs. the OA-treated group; \$P < 0.05 vs. the OA + apigenin-treated group.

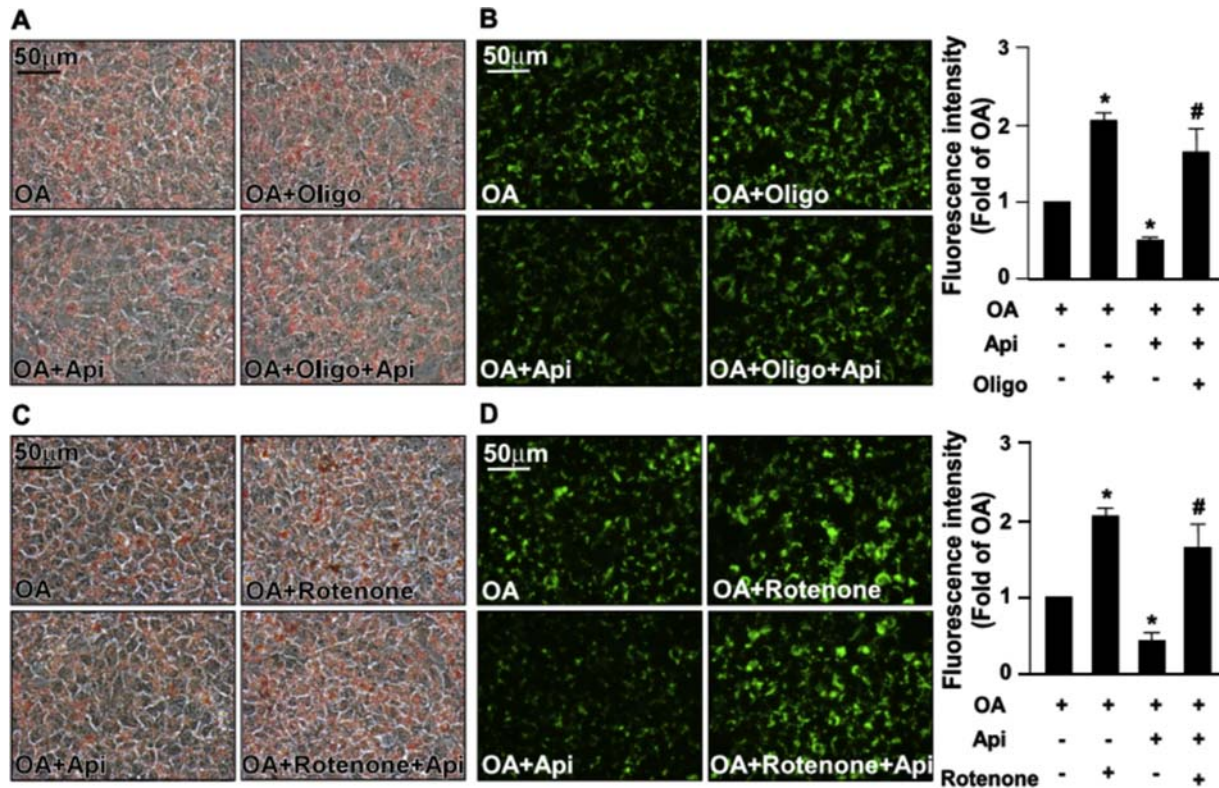


Fig. 10. Inhibition of mitochondrial function diminishes the apigenin-conferred protection from OA-induced lipid accumulation. Huh7 cells were pretreated with OA (125  $\mu\text{g}/\text{mL}$ ) for 24 h, autophagy inhibitor with or without mitochondrial inhibitors (oligomycin, Oligo, 0.5  $\mu\text{M}$ , or Rotenone, 0.5  $\mu\text{M}$ ) for 2 h, and then with or without apigenin (20  $\mu\text{M}$ ) for an additional 18 h. (A and C) Representative images of oil red O staining of Huh7 cells. (B and D) Representative images of the intracellular lipid accumulation and the quantitative results of Nile red staining. Data are the mean  $\pm$  SEM from five independent experiments. \* $P < 0.05$  vs. the OA-treated group; # $P < 0.05$  vs. the OA + apigenin-treated group.

related to the  $\beta$ -oxidation of free fatty acids, which is in agreement with the previous findings that supplementation with flavonoids promoted free fatty acid oxidation and protection from metabolic diseases [46]. Collectively, these observations suggest that apigenin prevents excess fatty acid-induced mitochondrial dysfunction and promotes the  $\beta$ -oxidation of free fatty acids, and these two events may work in concert to decrease the lipid accumulation in hepatocytes.

ROS is an important secondary messenger for regulating cellular physiological functions and the development of metabolic diseases [47]. In the initial stage of metabolic diseases, ROS-mediated oxidative stress disturbs intracellular signaling cascades, deregulates physiological functions, and leads to the progression of metabolic disease [48]. Mitochondrial ROS is mainly generated by the electron transport chain located on the inner mitochondrial membrane during the process of oxidative phosphorylation and is involved in the regulation of lipid metabolism, inflammatory response, and apoptosis/autophagy pathways [49,50]. Increasing lines of evidence

suggest that targeting the ROS pathway with antioxidants has been considered as an effective therapeutic strategy for preventing oxidative stress-mediated metabolic events in clinical trials and animal studies [51]. Recently, Feng et al. reported that apigenin ameliorates HFD-induced NAFLD by modulating the oxidative stress in the liver [10]. Moreover, Zhong et al. demonstrated that apigenin attenuates apoptosis by decreasing ROS-mediated mitochondrial dysfunction [52]. This notion is further supported by our findings that treatment with apigenin abrogated the OA-induced lipid accumulation by decreasing the production of mitochondrial ROS in Huh7 cells. Conceivably, these findings suggest that the antioxidative property of apigenin may have therapeutic value in the treatment of metabolic diseases. Nonetheless, determining whether apigenin prevents the ROS-mediated detrimental effects on metabolic diseases and its underlying molecular mechanism requires further investigations and clinical trials.

Our study contains several limitations because we did not use animal models to mimic the

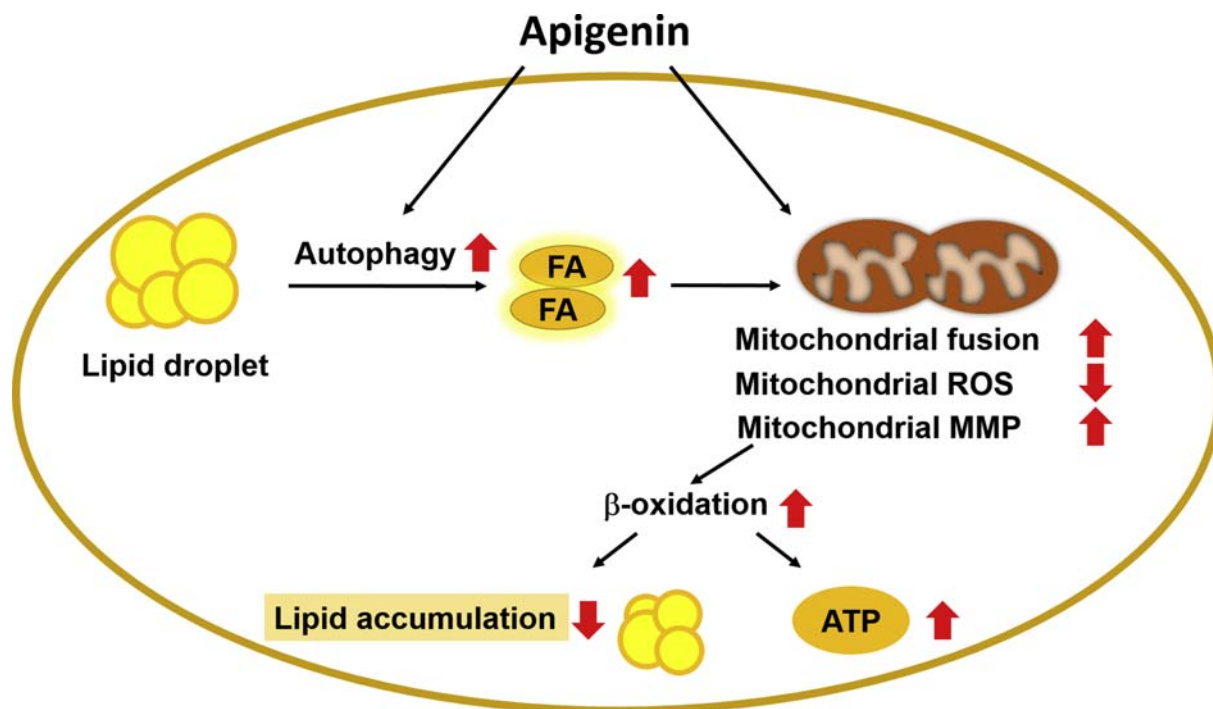


Fig. 11. Schematic illustration of the proposed molecular mechanisms by which apigenin protects the hepatocytes from the excess fatty acid-induced lipid accumulation. As shown, treatment with apigenin ameliorates OA-induced deregulation of lipid metabolism by activating the autophagy flux and rescuing mitochondrial dysfunction, leading to an increase in  $\beta$ -oxidation of fatty acids and ultimately decreasing the lipid accumulation in hepatocytes.

pathogenesis of metabolic diseases and to delineate the molecular mechanism underlying the apigenin-conferred protection from lipid disorders. Using the obesity or NAFLD mouse models with genetic approaches will be helpful to clarify the effects and molecular mechanisms of apigenin on lipid disorders under pathological conditions. Moreover, we do not have clinical data to support our observations from *in vitro* studies. To this end, further *in vivo* studies or clinical trials describing the implications of apigenin in the deregulation of hepatic lipid metabolism and related metabolic diseases are warranted.

In conclusion, this study demonstrates the unique mechanisms of apigenin on hepatic lipid metabolism, which involves activation of autophagy flux, reduction of mitochondrial ROS production, rectification of mitochondrial function, and promotion of free fatty acid  $\beta$ -oxidation, and ultimately leads to alleviation of lipid accumulation in hepatocytes. Here, we provide new insight into the beneficial effects of apigenin on the deregulation of hepatic lipid metabolism, which broadens the biomedical implications of apigenin in the prevention or treatment of metabolic diseases.

### Conflicts of interest

The authors declare no conflict of interest.

### Author contributions

Man-Chen Hsu, Bei-Chia Guo, Chia-Hui Chen, and Po-An Hu performed the experiments and analyzed the data; TS Lee designed the experiments and wrote the paper.

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## Appendix A. Supplementary material

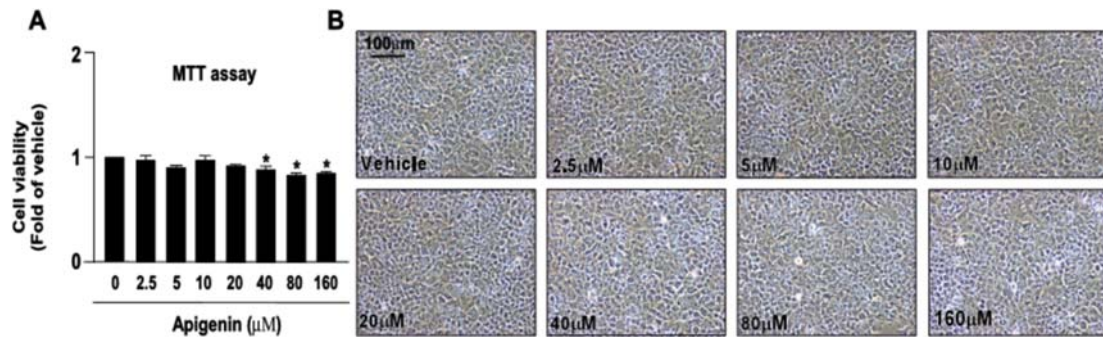


Fig. S1. Effect of increasing dose of apigenin on Huh7 cells. Huh7 cells were incubated with various concentrations (0, 2.5, 5, 10, 20, 40, 80 and 160  $\mu\text{M}$ ) of apigenin for 18 h. (A) Cell viability was determined by MTT assay. (B) Bright field images of cells treated with various dose of apigenin to observe the morphologic changes in Huh7 cells. Data are the mean  $\pm$  SEM from 5 independent experiments. \* $P < 0.05$  vs. the vehicle-treated group.

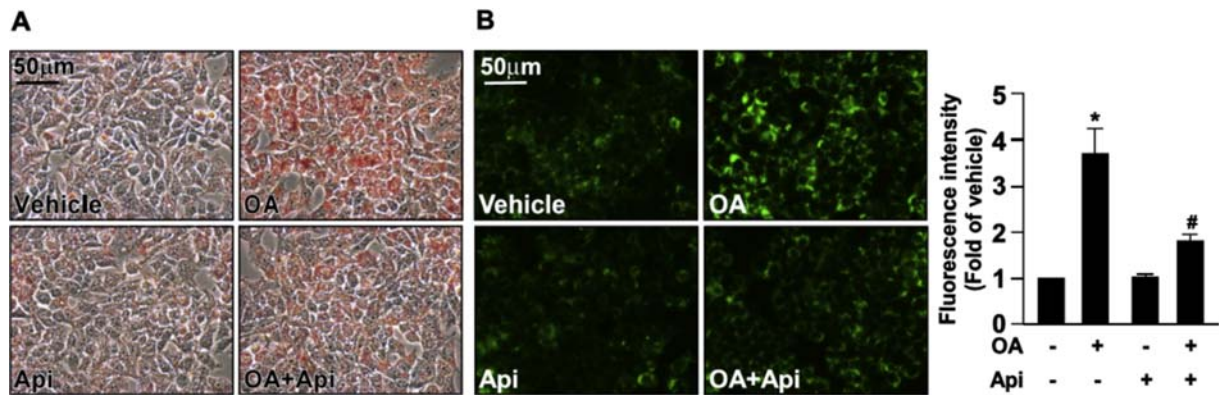


Fig. S2. Apigenin prevents oleic acid (OA)-induced lipid accumulation in HepG2 cells. HepG2 cells were pretreated with OA (125  $\mu\text{g}/\text{mL}$ ) for 24 h and then with or without of apigenin (20  $\mu\text{M}$ ) for additional 18 h. (A) Representative images of oil red O staining of HepG2 cells. (B) Representative images and the quantitative intensity of Nile red staining. Data are the mean  $\pm$  SEM from 5 independent experiments. \* $P < 0.05$  vs. the vehicle-treated group; # $P < 0.05$  vs. the OA-treated group.

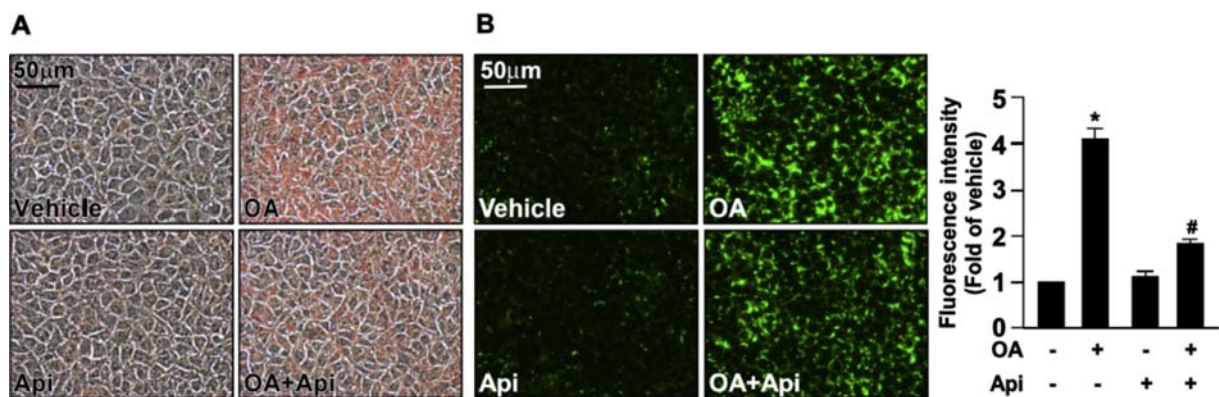


Fig. S3. Apigenin prevents oleic acid (OA)-induced lipid accumulation in AML 12 cells. AML 12 cells were pretreated with OA (125  $\mu\text{g}/\text{mL}$ ) for 24 h and then with or without of apigenin (20  $\mu\text{M}$ ) for additional 18 h. (A) Representative images of oil red O staining of AML 12 cells. (B) Representative images and the quantitative intensity of Nile red staining. Data are the mean  $\pm$  SEM from 5 independent experiments. \* $P < 0.05$  vs. the vehicle-treated group; # $P < 0.05$  vs. the OA-treated group.

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