



Original Article

Icaritin Attenuates Lipid Accumulation by Increasing Energy Expenditure and Autophagy Regulated by Phosphorylating AMPK

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Abstract

Background and Aims: Lipid accumulation is the major characteristic of non-alcoholic fatty liver disease, the prevalence of which continues to rise. We aimed to investigate the effects and mechanisms of icaritin on lipid accumulation. **Methods:** Cells were treated with icaritin at 0.7, 2.2, 6.7, or 20 μM for 24 h. The effects on lipid accumulation in L02 and Huh-7 cells were detected by Bodipy and oil red O staining, respectively. Mitochondria biogenesis of L02 cells was detected by MitoTracker Orange staining. Glucose uptake and adenosine triphosphate content of 3T3-L1 adipocytes and C2C12 myotubes were detected. The expression levels of proteins in the adenosine 5'-monophosphate-activated protein kinase (AMPK) signaling pathway, biomarkers of autophagy, and mitochondria biogenesis were measured by western blotting. LC3 puncta were detected by immunofluorescence. **Results:** Icaritin significantly attenuated lipid accumulation in L02 and Huh-7 cells and boosted the mitochondria biogenesis of L02 cells. Icaritin enhanced glu-

cose uptake, decreased adenosine triphosphate content, and activated the AMPK signaling pathway in 3T3-L1 adipocytes and C2C12 myotubes. Icaritin boosted autophagy and also enhanced the initiation of autophagic flux in 3T3-L1 preadipocytes and C2C12 myoblasts. However, icaritin decreased autophagy and promoted mitochondria biogenesis in 3T3-L1 adipocytes and C2C12 myotubes. **Conclusions:** Icaritin attenuates lipid accumulation by increasing energy expenditure and regulating autophagy by activating the AMPK pathway.

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Introduction

The global prevalence of non-alcoholic fatty liver disease (NAFLD) is estimated to be about 24% to 25% of the population, which has steadily rose over recent decades.¹ NAFLD represents a range of diseases, from simple hepatic steatosis to steatohepatitis that eventually leads to cirrhosis and hepatocellular carcinoma.² Lipid accumulation in the liver is the major characteristic of NAFLD. Lipid accumulation in skeletal muscles is associated with the development of insulin resistance, which is an early marker and significantly contributes to NAFLD.³ Adipose acts as a fuel reservoir, which controls the mobilization of lipids.⁴ As such, we employed hepatic cells (L02 and Huh-7 cells), C2C12 myoblasts, C2C12 myotubes, 3T3-L1 preadipocytes, and 3T3-L1 adipocytes in this study.

No effective medical interventions can completely reverse NAFLD, other than lifestyle and dietary changes, to date. Although some drugs show benefits, such as vitamin E and pioglitazone, no drugs have been approved by the US Food and Drug Administration.⁵ NAFLD results from the

Keywords: Icaritin; Lipid accumulation; NAFLD; AMPK; Autophagy.

Abbreviations: ACC, acetyl-CoA carboxylase; AMPK, adenosine 5'-monophosphate-activated protein kinase; ATCC, American type culture collection; ATP, adenosine triphosphate; CaMKKβ, Calcium/calmodulin-dependent protein kinase β; CQ, chloroquine; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LKB1, liver kinase B1; NAFLD, non-alcoholic fatty liver disease; NBSCS, newborn calf serum; NRF1, nuclear respiratory factor 1; p-ACC, phospho-ACC; p-AMPK, phospho-AMPK; PBS, phosphate buffer saline; PFA, paraformaldehyde; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; p-LKB1, phospho-LKB1; PVDF, polyvinylidene fluoride; RT, room temperature; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SIRT1, silent mating type information regulation 2 homolog 1; TFAM, transcription factor A; 2-NBDG, 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose.

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chronic energy imbalance. Increasing energy expenditure is an effective strategy to attenuate lipid accumulation and combat NAFLD. There is an urgent need to develop candidate agents to combat NAFLD through enhancing energy expenditure and attenuating lipid accumulation.

Icaritin is a naturally bioactive flavonoid of the traditional Chinese herbal medicine *Herba Epimedii*. Flavonoids of *Herba Epimedii* have been widely used for their metabolic regulation, anti-oxidation, and hepatoprotective effects.⁶ Icaritin is also the metabolite of the major flavonoid of *Herba Epimedii*, icariin (Fig. 1A).⁶ Icariin's lipid-lowering effects have attracted widespread attention recently.⁷ Icaritin inhibited intravascular thrombosis and extravascular lipids deposition.⁸ However, icaritin's effects on lipid accumulation have not been explored. In this study, we investigated the effects and mechanisms of icaritin on lipid accumulation.

Methods

Cell culture and treatments

Human hepatic L02 cells were obtained from the American Type Culture Collection (commonly known as the ATCC). L02 cells were cultured in RPMI-1640 medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) (Biological Industries, Cromwell, CT, USA) and 1% penicillin-streptomycin (HyClone). Huh-7 cells were a kind gift from Prof. Lang Bai of West China Hospital, Sichuan University. Huh-7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (HyClone) supplemented with 10% FBS and 1% penicillin-streptomycin.

3T3-L1 preadipocytes were obtained from the ATCC, cultured in DMEM supplemented with 10% newborn calf serum (referred to herein as "NBCS") (Biological Industries) and 1% penicillin-streptomycin. When the cells reached 50% confluence, they were induced to differentiate into adipocytes by changing the medium to a differentiation medium which was composed of DMEM supplemented with 10% NBCS, 0.5 mM 3-isobutyl-1-methylxanthine, 1 μ M dexamethasone, and 10 μ g/mL insulin, for 48 h. Then, the cells were cultured with DMEM supplemented with 10% FBS and 1% penicillin-streptomycin for another 48 h. After being induced for 8 to 10 days, more than 90% of the 3T3-L1 preadipocytes differentiated into adipocytes, which showed features of insulin resistance and a fat cellular phenotype filled with lipid droplets.

C2C12 myoblasts were cultured in the DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. When the cells reached 80% confluence, they were induced to differentiate into myotubes by changing the medium to a differentiation medium which was composed of DMEM containing 2% horse serum (HyClone). C2C12 myoblasts were cultured in the differentiation medium for 4 to 6 days, with medium change every 48 h, until they differentiated into insulin-resistant mature myotubes. Numerous mature myotubes formed and aligned regularly as observed by optical microscope (Zeiss, Oberkochen, Germany). All cells were cultured at 37 °C in an atmosphere of 5% CO₂.

Icaritin (3,7-dihydroxy-8-prenyl-4'-methoxychrysin, PubChem CID: 5318980, CAS: 118525-40-9) with purity above 99% was commercially obtained from Shanghai Yuanye Biotechnology (Cat# B21277; Shanghai, China).

Determination of lipid accumulation

Huh-7 and L02 cells were seeded in 24-well plates and treated with sodium oleate at 100 μ M for 24 h when 40–

50% confluence was reached. Then, the cells were treated with icaritin for 24 h. For Bodipy staining, L02 cells were stained with 4 μ M Bodipy staining solution (Thermo Fisher Scientific, Waltham, MA, USA) in dark at 37 °C for 15 m after treatments.⁹ Then, the cells were washed twice with phosphate buffer saline (PBS) and fixed with 4% paraformaldehyde (PFA) (Sangon, Shanghai, China) for 20 m. Finally, the nucleus was stained with Hoechst 33258 (Sigma, St. Louis, MO, USA) for 3 m. For oil red O staining, L02 and Huh-7 cells were washed with PBS and fixed with 4% PFA for 20 m at room temperature (RT). Then, the 4% PFA was discarded and the cells were washed with PBS. Following, 60% oil red O solution was added for staining for 45 m at RT. The cells were then washed with 60% isopropanol in PBS, and the nucleus was stained with hematoxylin for 5 m. Finally, the cells were washed with PBS and double-distilled water, and sealed with glycerin. Lipid droplets in the cells were observed and photographed by an optical microscope (Zeiss).

Determination of mitochondria biogenesis

L02 cells, C2C12 myotubes, and 3T3-L1 adipocytes were seeded on coverslips in 24-well plates for 24 h. Then, the cells were incubated with icaritin at 0.7, 2.2, 6.7, or 20 μ M for 24 h. The number of mitochondria in the cells was evaluated by MitoTracker Orange staining.¹⁰ The cells were first stained with a staining solution containing MitoTracker probe (Thermo Fisher Scientific) for 30 m. Then, the cells were fixed with 4% PFA for 20 m and permeabilized with 0.5% Triton X-100 (BBI, Shanghai, China) for 15 m. The nucleus was stained with Hoechst 33258 for 3 m. Finally, cells were sealed with an anti-fluorescence quenching agent and stored at –20 °C. The biogenesis and morphology of mitochondria were observed and photographed by confocal microscopy (Zeiss).

Glucose uptake analysis

Both C2C12 myotubes and 3T3-L1 adipocytes were seeded on coverslips in 24-well plates. After reaching 80% confluence, the cells were treated with icaritin (2.2, 6.7, or 20 μ M), metformin (2.5 mM), or phloretin (100 μ M) for 4 h. Phloretin, a glucose uptake inhibitor, was employed as the negative control and metformin as the positive control. The 2-NBDG glucose uptake assay kit (BioVision, San Francisco, CA, USA) was employed to measure the amount of glucose uptake.¹¹ The cells were incubated with the glucose uptake mix for 30 m after washing. Then, the cells were washed, fixed, and observed. Amounts of 2-NBDG taken-up were determined by fluorescence microscopy (Olympus, Tokyo, Japan).

Adenosine triphosphate (ATP) content analysis

C2C12 myotubes and 3T3-L1 adipocytes were treated with icaritin at 0.7, 2.2, 6.7, or 20 μ M, or metformin at 2.5 mM, for 24 h, and harvested with trypsin. Total ATP content was measured using an ATP colorimetric/fluorometric assay kit (BioVision).¹² A 100 μ L aliquot of ATP assay buffer was added. Interfering proteins were removed by perchloric acid. Then, samples were centrifuged at 13,000 rpm for 15 m at 4 °C. A 50 μ L aliquot of supernatant was added to each well of the 96-well plate and mixed with 50 μ L reaction mix in dark for 30 m. The absorbance was measured at 570 nm using a microplate reader (Thermo Fisher Scientific).

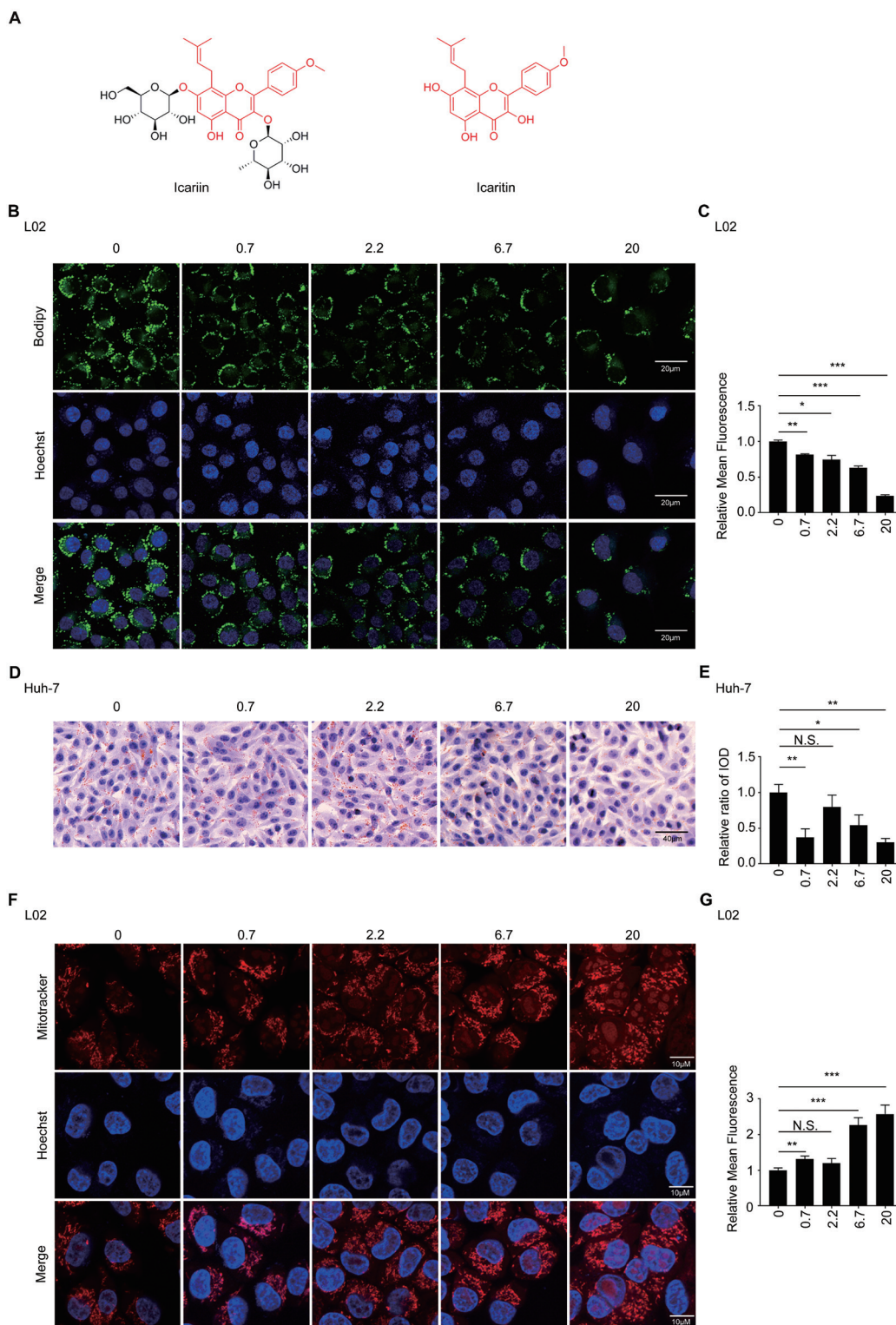


Fig. 1. Icaritin attenuated lipid accumulation in sodium oleate-induced L02 and Huh-7 cells, and enhanced the mitochondria biogenesis of L02 cells. (A) The chemical structure of icaritin and icaritin. (B, C) L02 cells were induced by sodium oleate at 100 μ M for 24 h to establish a NAFLD cell model. Then, L02 cells were treated with icaritin at 0.7, 2.2, 6.7, or 20 μ M for 24 h. Lipid accumulation in L02 cells was attenuated by icaritin, as visualized by Bodipy staining and observed by a confocal microscope. (D, E) Huh-7 cells were induced by sodium oleate at 100 μ M for 24 h to establish a NAFLD cell model. Then, Huh-7 cells were treated with icaritin at 0.7, 2.2, 6.7, or 20 μ M for 24 h. Lipid accumulation in Huh-7 cells was attenuated by icaritin, as visualized by oil red O staining. (F, G) L02 cells were induced by sodium oleate at 100 μ M for 24 h and treated with icaritin for 24 h. The mass of mitochondria in L02 cells was enhanced by icaritin, as detected by MitoTracker Orange staining (red). Abbreviation: NAFLD, non-alcoholic fatty liver disease.

Western blot analysis

C2C12 myoblasts, C2C12 myotubes, 3T3-L1 preadipocytes, and 3T3-L1 adipocytes were incubated with icaritin at 0.7, 2.2, 6.7, or 20 μM , or metformin at 2.5 mM, for 24 h. Cells were harvested by trypsin and lysed by 1 \times sodium dodecyl sulfate-polyacrylamide gel electrophoresis (commonly known as SDS-PAGE) loading buffer. Then, samples were boiled at 98 $^{\circ}\text{C}$ for 10 m. Proteins were separated by SDS-PAGE and transferred to a polyvinylidene fluoride (commonly known as PVDF) membrane. Non-specific binding was blocked with 5% skim milk (BBI) for 1 h. Then, the PVDF membranes were incubated with primary antibodies, including CaMKK β (D262840; Sango), LKB1 (D163053; Sango), p-LKB1 (D151527; Sango), AMPK (2532s; Cell Signaling Technology, Danvers, MA, USA), p-AMPK (2535; Cell Signaling Technology), ACC (3662; Cell Signaling Technology), p-ACC (11818; Cell Signaling Technology), p-62 (PM045; MBL, Tokyo, Japan), LC3 (M186-3; MBL), SIRT1 (sc-135791; Santa Cruz Biotechnology, Dallas, TX, USA), PGC-1 α (66369-1; Proteintech, Rosemont, IL, USA), NRF1 (66832-1; Proteintech), TFAM (22586-1; Proteintech), and GAPDH (sc-32233; Santa Cruz Biotechnology), for 2 h at RT. Then, the PVDF membranes were incubated with a secondary antibody to rabbit or mouse IgG conjugated to horseradish peroxidase at RT for 1 h after washing. Blots were developed using enhanced chemiluminescence detection reagents (Mei5bio, Beijing, China) and images were taken by chemiluminescence imager (Clinx, Shanghai, China).

Immunofluorescence analysis

C2C12 myotubes, C2C12 myoblasts, 3T3-L1 preadipocytes, and 3T3-L1 adipocytes were seeded on round coverslips in 24-well plates for 24 h. Then, the cells were treated with icaritin (6.7 μM) alone, or in combination with chloroquine (CQ) at 10 μM for 24 h. After, the cells were fixed with 4% PFA for 20 m, permeabilized with 0.5% Triton X-100 for 15 m, and blocked with 4% bovine serum albumin (BioFroxx, Einhausen, Germany) for 1 h. Then, the cells were incubated with anti-LC3 antibody (MBL) at RT for 2 h, and with the secondary antibody conjugated to fluorescein isothiocyanate in dark at RT for 1 h. Finally, nuclei were stained with Hoechst 33258 for 3 m after washing. The intensity of fluorescence was observed and pictured by confocal microscopy (Zeiss).

Statistical analysis

All experiments were performed in triplicate. Results were expressed as the mean \pm standard deviation. All data were analyzed with *t*-test using GraphPad Prism 7.0 (GraphPad Software Inc., La Jolla, CA, USA). A *p*-value of <0.05 was defined as statistically significant.

Results

Icaritin attenuated lipid accumulation in L02 and Huh-7 cells, and enhanced the mass of mitochondria of L02 cells

L02 and Huh-7 cells were treated with sodium oleate at 100 μM for 24 h to establish the NAFLD cell model. Icaritin attenuated lipid accumulation in L02 cells at 0.7, 2.2, 6.7, and 20 μM , as detected by Bodipy staining (Fig. 1B, C). As

shown in Fig. 1 D and E, icaritin significantly attenuated lipid accumulation in Huh-7 cells, as detected by oil red O staining. Besides, the mass and fluorescence intensity of mitochondria in L02 cells was enhanced by icaritin (Fig. 1F, G).

Icaritin enhanced the glucose uptake, decreased the ATP content, and activated the LKB1/AMPK/ACC pathway in 3T3-L1 adipocytes and C2C12 myotubes

Icaritin significantly enhanced glucose uptake of both 3T3-L1 adipocytes (Fig. 2A, B) and C2C12 myotubes (Fig. 2C, D) in a dose-dependent manner, compared with the untreated cells. Meanwhile, icaritin decreased the ATP content in 3T3-L1 adipocytes (Fig. 2E) and C2C12 myotubes (Fig. 2F). Icaritin significantly enhanced the expression of CaMKK β , p-LKB1, p-AMPK, and p-ACC in both 3T3-L1 adipocytes (Fig. 3A–F) and C2C12 myotubes (Fig. 3G–L). The changes of p-LKB1 and p-AMPK in 3T3-L1 adipocytes occurred in a dose-dependent manner (Fig. 3C, E). As for C2C12 myotubes, the changes of expression of CaMKK β , p-LKB1, and p-ACC also occurred in a dose-dependent manner (Fig. 3G–L). Interestingly, the constitutive expression of AMPK was decreased in both 3T3-L1 adipocytes and C2C12 myotubes (Fig. 3D, J).

Icaritin enhanced autophagy and promoted the initiation of autophagy in autophagic flux in both 3T3-L1 preadipocytes and C2C12 myoblasts

Icaritin enhanced the number of endogenous LC3 puncta in both 3T3-L1 preadipocytes (Fig. 4A, B) and C2C12 myoblasts (Fig. 4C, D) at 0.7, 2.2, 6.7, and 20 μM , as detected by immunofluorescence. For 3T3-L1 preadipocytes, the enhanced trend of LC3 puncta slightly decreased at 20 μM , while it was still higher than that observed in the untreated cells. Meanwhile, the expression of p62 was decreased by icaritin in both 3T3-L1 preadipocytes (Fig. 4E, F) and C2C12 myoblasts (Fig. 4H, I), compared to the untreated cells. The ratio of LC3II/LC3I in both cells was increased by icaritin (Fig. 4E, G, H, J). To investigate the role of icaritin on autophagic flux, we employed CQ. As shown in Fig. 5A and B, the combination of CQ and icaritin further increased LC3 puncta than icaritin alone (Fig. 5A, B, F, G). Consistent with the above observations, the ratio of LC3-II/I in 3T3-L1 preadipocytes (Fig. 5C, E) and C2C12 myoblasts (Fig. 5H, J) was also increased by icaritin alone or in combination with CQ. Meanwhile, the expression of p62 was also increased by icaritin in combination with CQ (Fig. 5D, I).

Icaritin inhibited autophagy and enhanced the mitochondrial biogenesis in 3T3-L1 adipocytes and C2C12 myotubes

Given that icaritin induced autophagy in undifferentiated 3T3-L1 preadipocytes and C2C12 myoblasts, we wondered whether icaritin was able to work uniformly in insulin-resistant cells, 3T3-L1 adipocytes, and C2C12 myotubes. The western blotting analysis showed that the expression of p62 was increased by icaritin in both 3T3-L1 adipocytes (Fig. 6A, B) and C2C12 myotubes (Fig. 6D, E). Accordingly, the ratio of LC3-II/LC3-I in 3T3-L1 adipocytes and C2C12 myotubes was decreased by icaritin to some extent (Fig. 6A, C, D, F). Icaritin significantly boosted the mass and density of mitochondria of 3T3-L1 adipocytes (Fig. 7A, B) and C2C12 myotubes (Fig. 7H, I). The expression of several proteins (SIRT1, PGC-1 α , NRF1, and TFAM) which play

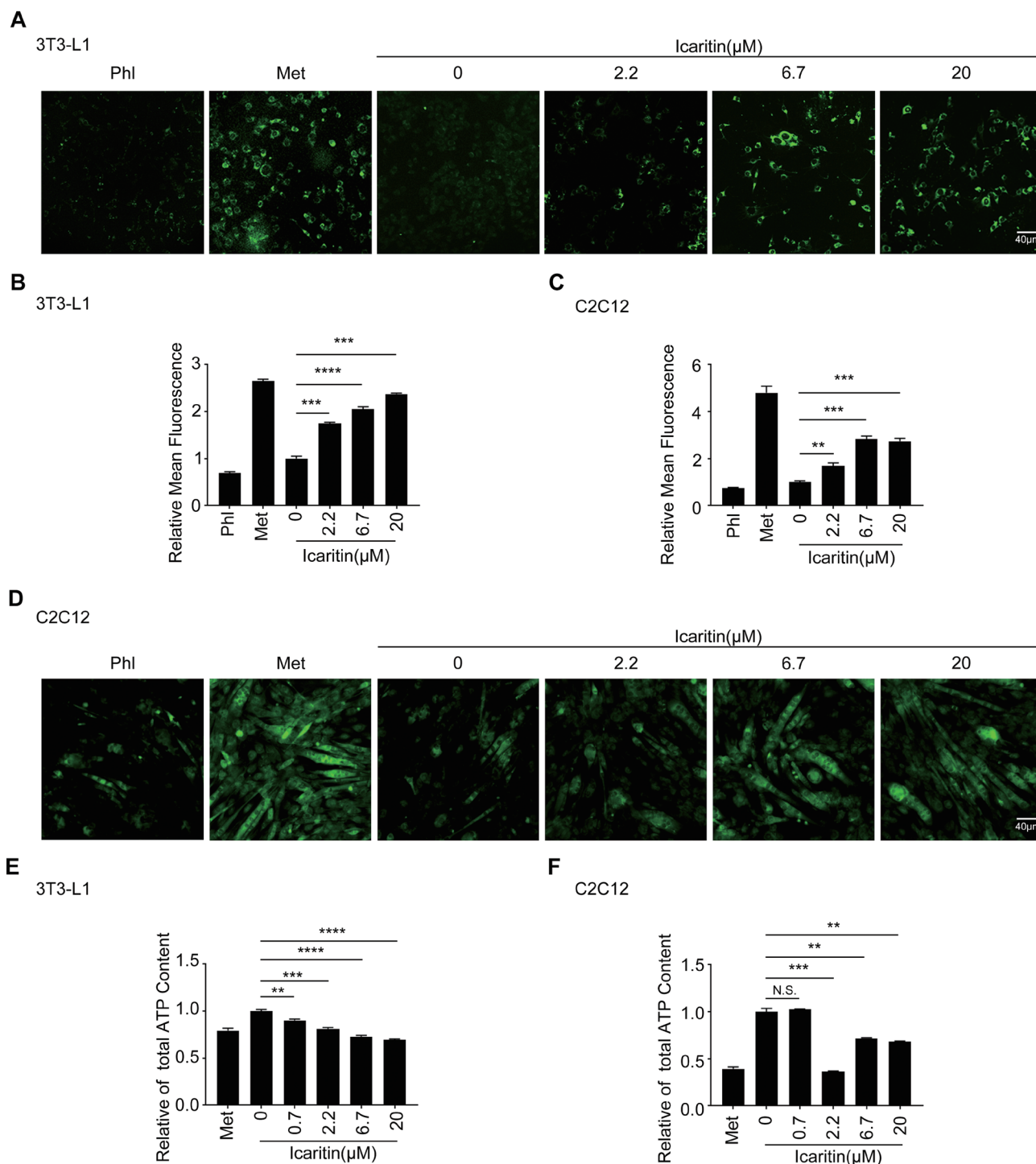


Fig. 2. Icaritin increased the glucose uptake and decreased the ATP content in 3T3-L1 adipocytes and C2C12 myotubes. (A, B) 3T3-L1 adipocytes were treated with icaritin (0, 2.2, 6.7, or 20 μM), metformin (2.5 mM), or phloretin (100 μM) for 4 h. Glucose uptake in 3T3-L1 adipocytes was dose-dependently increased by icaritin, as observed by fluorescence microscopy after being stained with 2-NBDG. (C, D) C2C12 myotubes were treated as in (A). Icaritin increased the glucose uptake of C2C12 myotubes. (E, F) 3T3-L1 adipocytes and C2C12 myotubes were treated with icaritin (0, 2.2, 6.7, or 20 μM), or metformin (2.5 mM) for 24 h. The ATP content was decreased by icaritin, as determined using an ATP calorimetric assay kit. All values are presented as mean±standard deviation (n=3). **p*<0.05; ***p*<0.01; ****p*<0.001 vs. untreated cells. Abbreviations: ATP, adenosine triphosphate; Met, metformin; Phl, phloretin; 2-NBDG, 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose.

prominent roles in mitochondria biogenesis, were all significantly increased by icaritin in both cells (Fig. 7C–G, J–N).

However, icaritin significantly enhanced the expression of TFAM in 3T3-L1 adipocytes only at 6.7 μM (Fig. 7C, G).

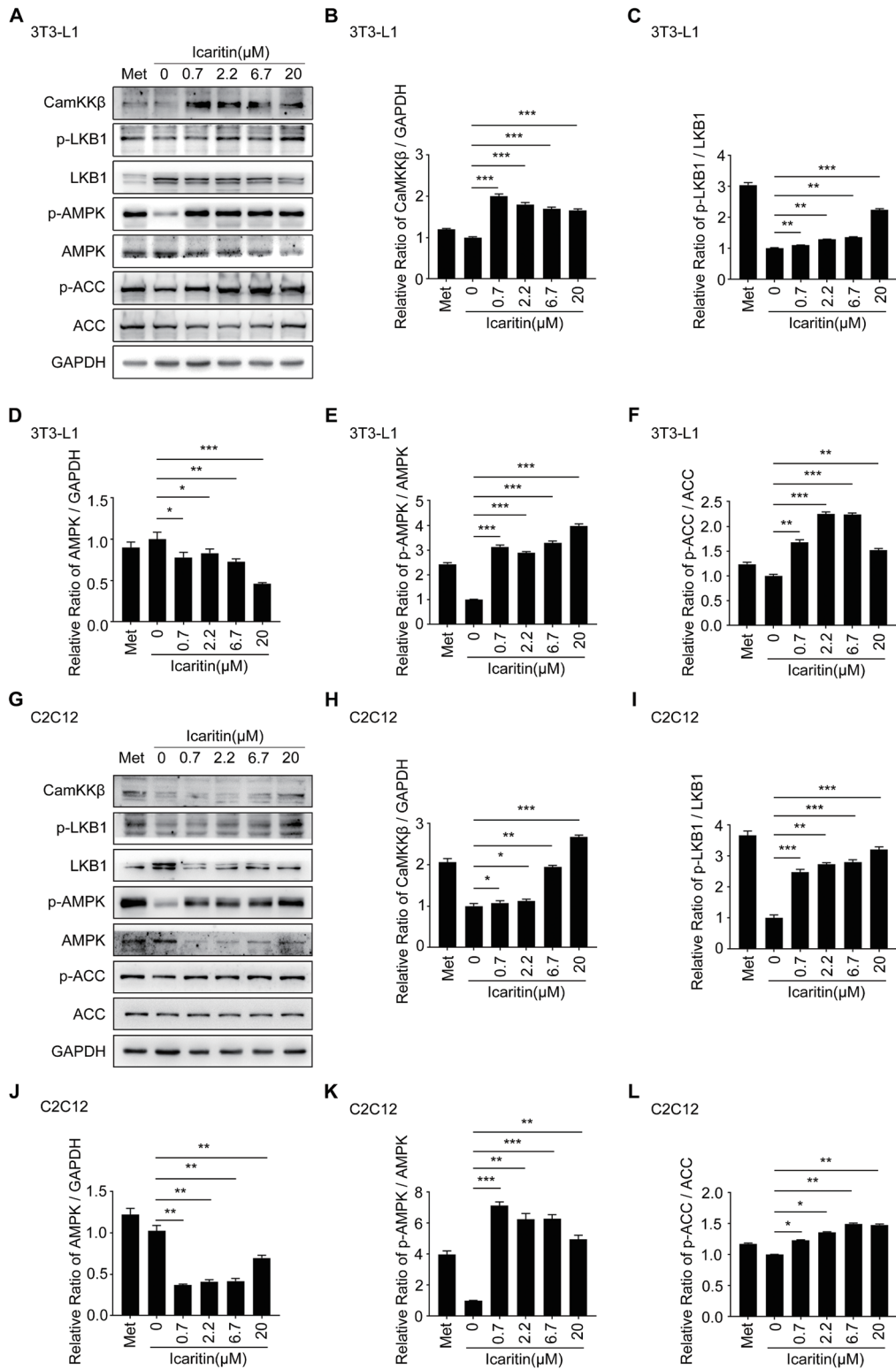


Fig. 3. Icaritin activated the AMPK pathway in 3T3-L1 adipocytes and C2C12 myotubes. 3T3-L1 adipocytes and C2C12 myotubes were treated with icaritin (0, 0.7, 2.2, 6.7, or 20 μM), or metformin (2.5 mM) for 24 h. (A–F) Icaritin increased the expression of CaMKKβ, the ratio of p-LKB1/LKB1, p-AMPK/AMPK, and p-ACC/ACC in 3T3-L1 adipocytes, as detected by western blotting. (G–L) Icaritin increased the expression of CaMKKβ, the ratio of p-LKB1/LKB1, p-AMPK/AMPK, and p-ACC/ACC in C2C12 myotubes, as detected by western blotting. Values are presented as mean±standard deviation (*n*=3). **p*<0.05; ***p*<0.01; ****p*<0.001 vs. untreated cells. Abbreviations: ACC, Acetyl-CoA carboxylase; AMPK, adenosine 5′-monophosphate-activated protein kinase; CaMKKβ, Calcium/calmodulin-dependent protein kinase kinase β; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LKB1, liver kinase B1; p-ACC, phospho-ACC; p-AMPK, phospho-AMPK; p-LKB1, phosphor-LKB1.

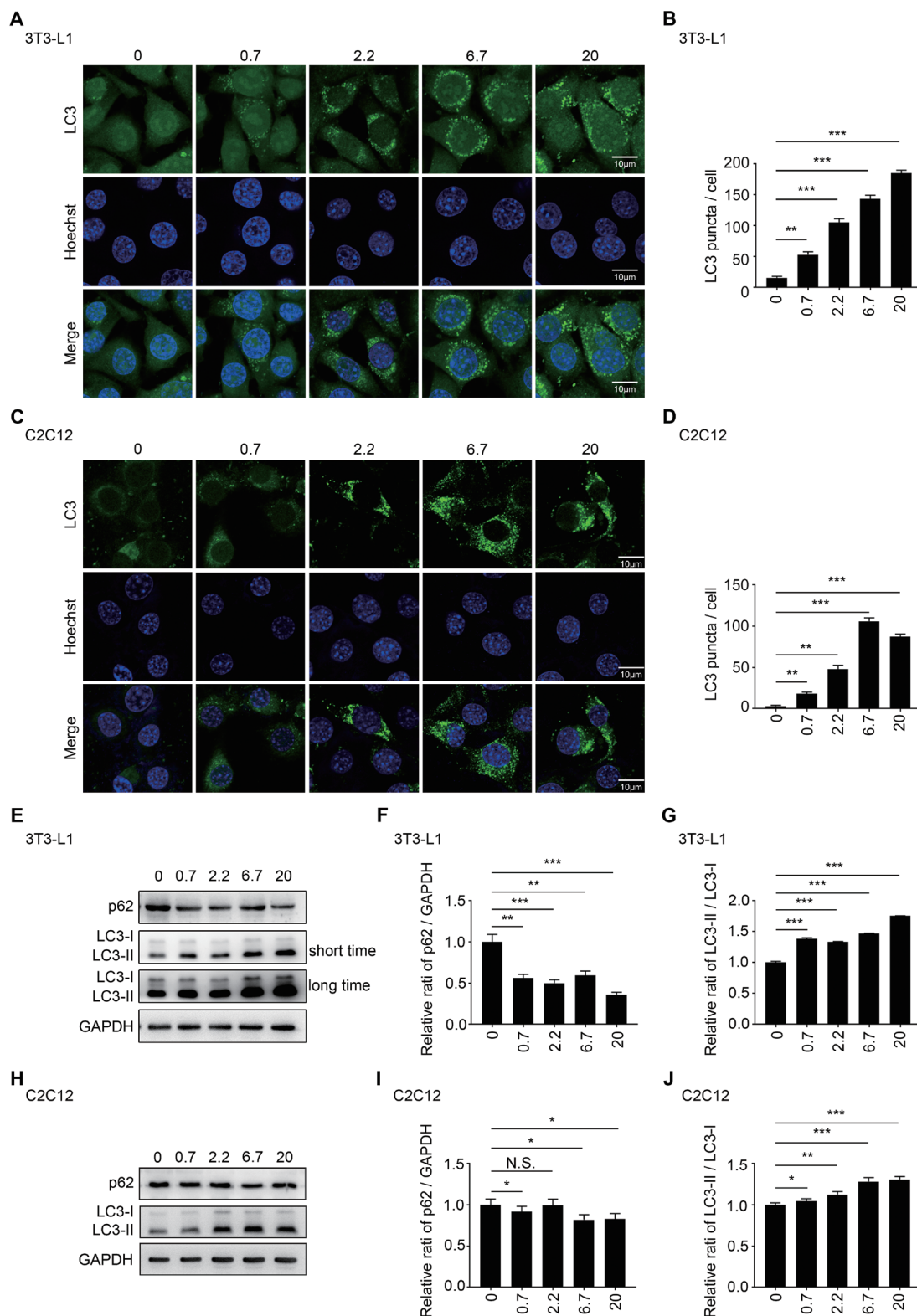


Fig. 4. Icaritin promoted autophagy in 3T3-L1 preadipocytes and C2C12 myoblasts. 3T3-L1 preadipocytes and C2C12 myoblasts were treated with icaritin (0, 0.7, 2.2, 6.7, or 20 μM) for 24 h. (A–D) 3T3-L1 preadipocytes and C2C12 myoblasts were incubated with an anti-LC3 antibody and the secondary antibody conjugated to fluorescein isothiocyanate. Icaritin increased endogenous LC3 puncta in 3T3-L1 preadipocytes and C2C12 myoblasts, as observed by confocal microscopy. (E–J) Icaritin decreased the expression of p62 and increased LC3II/LC3I in 3T3-L1 preadipocytes and C2C12 myoblasts, as detected by western blotting. All values were presented as mean±standard deviation ($n=3$). * $p<0.05$; ** $p<0.01$; *** $p<0.001$ vs. untreated cells. Abbreviation: GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

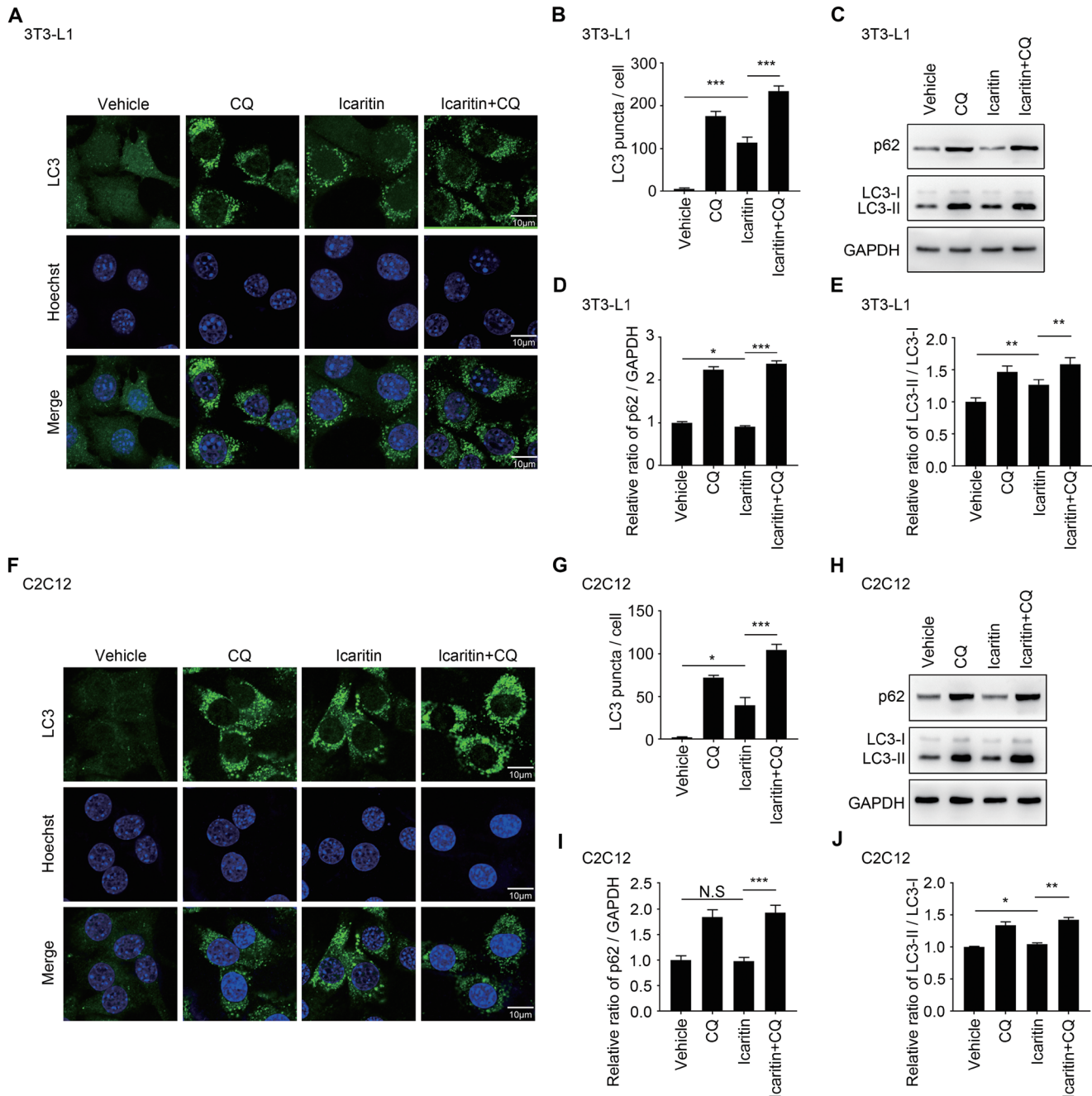


Fig. 5. Icaritin promoted initiation of autophagy in autophagic flux of 3T3-L1 preadipocytes and C2C12 myoblasts. 3T3-L1 preadipocytes and C2C12 myoblasts were treated with icaritin (6.7 μ M) alone or in combination with CQ (10 μ M) for 24 h. (A, B) 3T3-L1 preadipocytes were incubated with an anti-LC3 antibody and the secondary antibody conjugated to FITC. Icaritin increased the endogenous LC3 puncta in 3T3-L1 preadipocytes, which was further increased by the combination of CQ, as observed by confocal microscopy. (C-E) Icaritin increased the expression of p62 and enhanced LC3II/LC3I of 3T3-L1 preadipocytes, which was further increased by the combination of CQ, as detected by western blotting. (F, G) C2C12 myoblasts were treated as in (A). Icaritin increased the endogenous LC3 puncta in C2C12 myoblasts, which was further increased by the combination of CQ. (H-J) Icaritin increased the expression of p62 and enhanced LC3II/LC3I of C2C12 myoblasts, which was further increased by the combination of CQ, as detected by western blotting. Values are presented as mean \pm standard deviation ($n=3$). * $p<0.05$; ** $p<0.01$; *** $p<0.001$ vs. vehicle or as indicated. Abbreviations: CQ, chloroquine; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Discussion

Accumulating evidence has demonstrated the anti-cancer effects of icaritin. However, there have been no reports on its roles in NAFLD, so far. Enhancing energy expenditure

and inhibiting lipid accumulation is an effective strategy to combat NAFLD. Our results showed that icaritin significantly attenuated sodium oleate-induced lipid accumulation in L02 and Huh-7 cells. Icaritin increased the number and fluorescence intensity of mitochondria in L02 cells. Accordingly, icaritin decreased the ATP content of 3T3-L1 adipo-

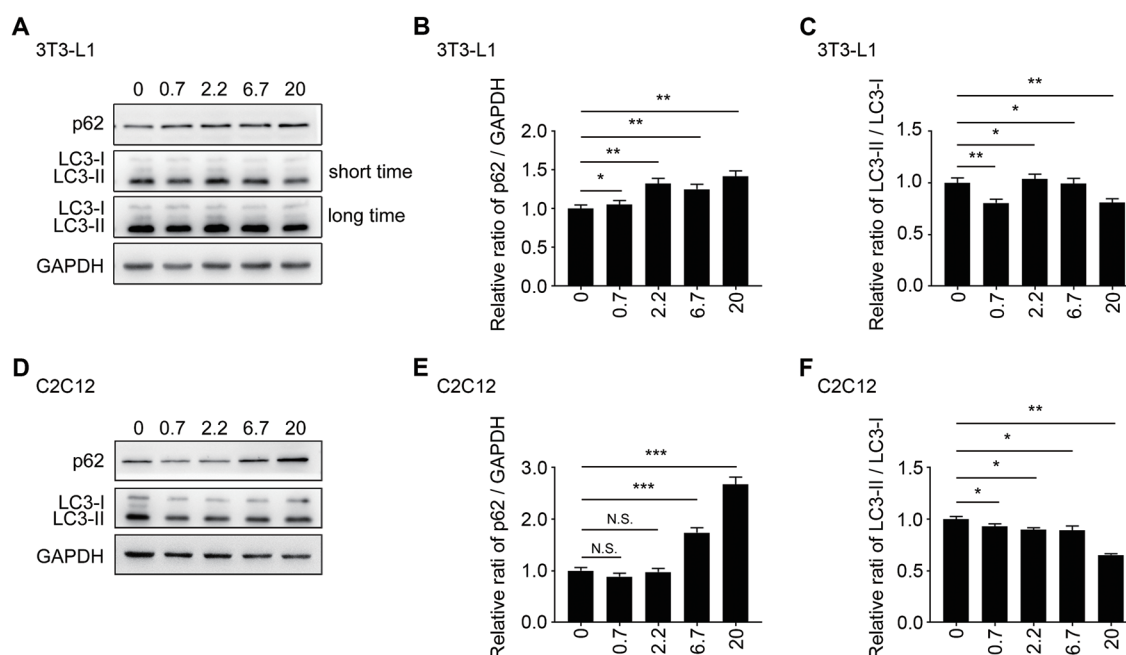


Fig. 6. Icaritin inhibited autophagy in 3T3-L1 adipocytes and C2C12 myotubes. 3T3-L1 adipocytes and C2C12 myotubes were treated with icaritin (0, 0.7, 2.2, 6.7, or 20 μ M) for 24 h. (A–C) Icaritin increased the expression of p62 and decreased LC3II/LC3I to some extent in 3T3-L1 adipocytes, as detected by western blotting. (D–F) Icaritin increased the expression of p62 and decreased LC3II/LC3I to some extent in C2C12 myotubes, as detected by western blotting. Values are presented as mean \pm standard deviation ($n=3$). * $p<0.05$; ** $p<0.01$; *** $p<0.001$ vs. untreated cells. Abbreviation: GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

cytes and C2C12 myotubes. Our results demonstrated that icaritin does attenuate lipid accumulation through increasing energy expenditure, making it a promising candidate for NAFLD.

As a well-recognized energy sensor, AMPK is in the central position of energy status in the eukaryotic cytoplasm, which activates multiple signaling pathways to regulate mitochondrial function.¹³ It is worth noting that enhancing AMPK activity has been considered a feasible strategy for increasing energy expenditure and improving NAFLD.¹⁴ Our study demonstrated that icaritin activates the LKB1/AMPK/ACC pathway. Interestingly, it also decreased the constitutive expression of AMPK α . Decreased expression of AMPK α may be compensation or adaptation to the changed energy metabolism due to changed cellular status.¹⁵ Chronic energy imbalance is the common ground for many metabolic diseases, such as obesity, type 2 diabetes mellitus, and NAFLD. Since icaritin can increase energy expenditure by activating AMPK, we hypothesized that it may be a promising candidate for these metabolic diseases, including NAFLD.

Autophagy acts as a dynamic recycling system to generate new components and energy for cell repair and energy homeostasis.¹⁶ Autophagy stimulates cholesterol efflux, which in turn inhibits lipid accumulation.¹⁷ Our results showed that icaritin regulates autophagy according to the cellular status. Icaritin increased autophagy in 3T3-L1 preadipocytes and C2C12 myoblasts. Similar to our results, icaritin induced autophagy in human GBM cell line U87 cells at 10 and 20 μ M.¹⁸ The process between fusing with the lysosome and then degrading is termed as “the initiation of autophagy”.¹⁹ CQ has been widely used to inhibit the last stage of autophagy, functioning through blocking lysosomal degradation.¹⁹ Our results showed that the LC3 puncta were significantly increased in both 3T3-L1 preadipocytes and C2C12 myoblasts when treated with icaritin and CQ. Our results, thus, indicate that icaritin promotes the initiation of autophagy. However, activating autophagy also poses some risks since excess autophagy

induces cell death.²⁰ Icaritin rescued 3T3-L1 adipocytes and C2C12 myotubes out of this danger, which reduced the autophagy to a normal level. Fig. 8 summarizes our findings.

Our study has a couple of limitations. The effects of icaritin on lipid accumulation were explored by *in vitro* experiments, which should be further confirmed in animal models. Besides energy expenditure, whether or not icaritin decreases energy intake should also be explored in animal models. Dysregulated autophagy leads to both impaired mitochondrial biogenesis and mitochondrial dysfunction. The effects of icaritin on mitochondrial dysfunction should be measured in further studies.

In conclusion, our study demonstrated that icaritin attenuates lipid accumulation by enhancing energy expenditure and regulating autophagy. These effects were induced by activating the LKB1/AMPK/ACC pathway.

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Conflict of interest

The authors declare that they have no conflicts of interest

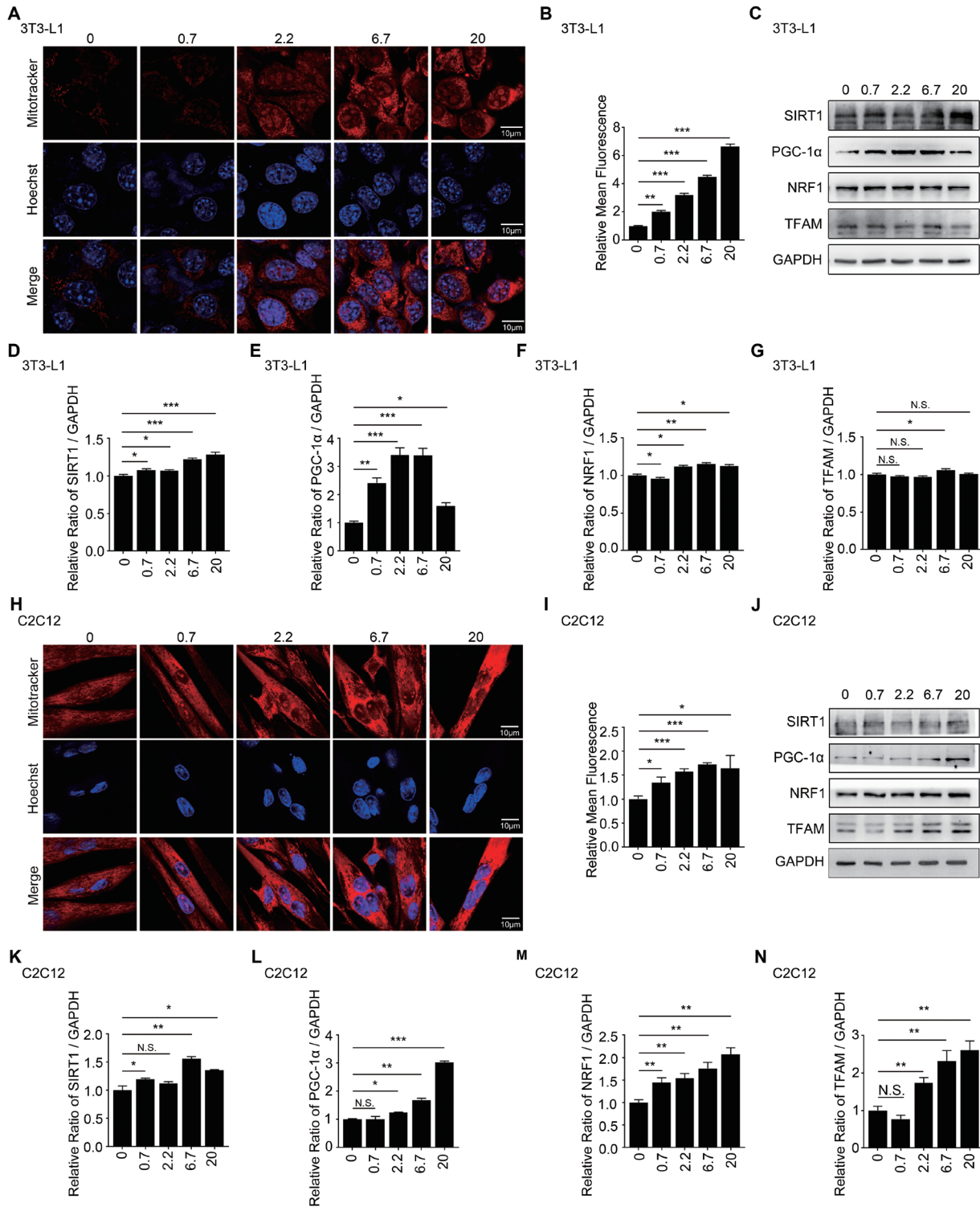


Fig. 7. Icaritin promoted mitochondria biogenesis of 3T3-L1 adipocytes and C2C12 myotubes. 3T3-L1 adipocytes and C2C12 myotubes were treated with icaritin (0, 0.7, 2.2, 6.7, or 20 μM) for 24 h. (A, B) Icaritin enhanced the mitochondria biogenesis of 3T3-L1 adipocytes, as detected by MitoTracker Orange staining (red). (C-G) Expression of SIRT1, PGC-1α, NRF1, and TFAM was increased by icaritin in 3T3-L1 adipocytes, as detected by western blotting. Icaritin enhanced the ratio of SIRT1/GAPDH, PGC-1α/GAPDH, NRF1/GAPDH, and TFAM/GAPDH in 3T3-L1 adipocytes. (H, I) Icaritin enhanced mitochondria biogenesis of C2C12 myotubes, as detected by MitoTracker Orange staining (red). (J-N) Icaritin enhanced the ratio of SIRT1/GAPDH, PGC-1α/GAPDH, NRF1/GAPDH, and TFAM/GAPDH in C2C12 myotubes. Values are presented as mean±standard deviation (n=3). *p<0.05; **p<0.01; ***p<0.001 vs. untreated cells. Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NRF1, nuclear respiratory factor 1; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; SIRT1, silent mating type information regulation 2 homolog 1; TFAM, transcription factor A.

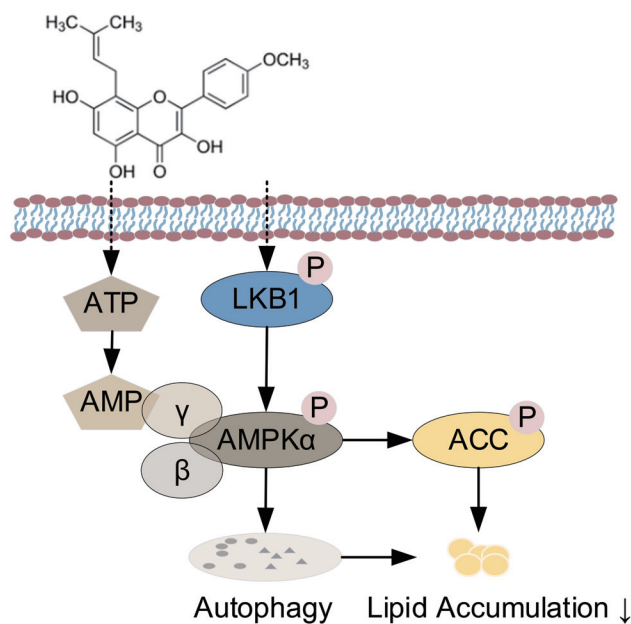


Fig. 8. Proposed mechanisms of icaritin on lipid accumulation. Icaritin attenuates lipid accumulation by enhancing energy expenditure and regulating autophagy. These effects were induced by activating the LKB1/AMPK/ACC pathway. Abbreviations: ACC, Acetyl-CoA carboxylase; AMPK, adenosine 5'-monophosphate-activated protein kinase; LKB1, liver kinase B1.

related to this publication.

Author contributions

Optimized and performed the experiments (YW, YY, FL), drafted the manuscript (YW) performed the immunofluorescence experiment (JZ, YHW, MXX), helped with part of the experimentation (YLW, RXL, YTS) optimized the experiments (SL), designed the study and edited the manuscript (YYZ, XDS). All authors analyzed the data and contributed to the study.

Data sharing statement

All data are available upon request.

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