Food Sci. Biotechnol. 26(1): 263-269 (2017)
DOI 10.1007/s10068-017-0035-0

Food Science
and
Biotechnology

Inhibitory effect of esculetin on free-fatty-acid-induced lipid accumulation in human HepG2 cells through activation of AMP-activated protein kinase

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Received September 30, 2016
Revised December 8, 2016
Accepted December 16, 2016
Published online February 28, 2017

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pISSN 1226-7708
eISSN 2092-6456

Abstract This study aimed to determine the lipid-lowering effect of esculetin (6,7-dihydroxycoumarin), a coumarin derivative, using a cell model of steatosis induced by a mixture of free fatty acids (FFAs). Esculetin dose-dependently inhibited intracellular lipid accumulation by down-regulating the protein expression of lipogenic genes such as sterol regulatory element-binding protein-1c (SREBP1c) and fatty acid synthase (FAS) in FFAs-induced HepG2 cells. Moreover, esculetin significantly elevated the activation of the adenosine monophosphate-activated protein kinase (AMPK) signaling pathways in HepG2 hepatocytes. The anti-lipogenic effects of esculetin mediated by AMPK activation were abolished when FFAs-induced HepG2 cells were treated with a specific inhibitor of AMPK, i.e., compound C. These results suggest that esculetin attenuates hepatic lipid accumulation by inhibiting lipogenesis through the modulation of AMPK signaling pathway on FFAs-induced steatosis in HepG2 cells and may be used for the prevention of nonalcoholic fatty liver disease (NAFLD).

Keywords: esculetin, lipogenesis, AMPK, NAFLD, HepG2 cells

Introduction

Nonalcoholic fatty liver disease (NAFLD), one of the main causes of chronic liver disease, has emerged as a major public health concern (1). NAFLD is characterized by triglyceride (TG) accumulation in hepatocytes in the absence of significant ethanol consumption, which can lead to more severe diseases such as nonalcoholic steatohepatitis (NASH), hepatitis, and liver cirrhosis (2). Impaired free fatty acid (FFA) metabolism can lead to excessive hepatic lipid accumulation correlating with disease severity (3). Numerous studies have suggested that FFA influx and *de novo* lipogenesis are the major pathogenic processes in the development of nonalcoholic fatty liver (4). Therefore, the prevention of elevated circulating FFA levels and *de novo* lipogenesis in the liver may be therapeutic strategies against multiple stages of NAFLD.

Several studies indicate that activation of adenosine monophosphate-activated protein kinase (AMPK) plays an important role in hepatic lipid metabolism for the treatment of NAFLD (5,6). AMPK, a serine/threonine heterotrimer kinase complex comprising α-, β-, and γ-subunits, acts as an intracellular sensor for energy homeostasis (7). AMPK activation leads to inhibition of lipogenesis and stimulation of fatty-acid oxidation through acetyl-CoA carboxylase (ACC) phosphorylation in liver (8). Moreover, it has been reported that hepatic AMPK activation suppresses the expression of sterol regulatory element-binding protein-1 (SREBP1) in diet-induced insulin-resistance mice (9). SREBP1, a major transcription factor for lipogenesis in hepatocytes, modulates the genes involved in fatty acid and TG synthesis, e.g., fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC) (10,11). Thus, AMPK cascades may be therapeutic targets in the treatment of fatty liver.

Recent studies in food science have focused on dietary therapy that can be helpful in the prevention and treatment of NAFLD for health improvements. Esculetin (6,7-dihydroxycoumarin) is a natural coumarin compound isolated from various vegetables and fruits such as *Ceratostigma willmottianum*, *Cichorium intybus L.*, *Artemisia capillaris*, and *Citrus limonia* (12-14). Previous studies have demonstrated that esculetin exerts several pharmacological actions such as anti-inflammatory, anti-oxidant, anti-cancer, and anti-adipogenic effects (15-17). In addition, the major coumarins in dietary chamomile (*Matricaria chamomilla* L.) tea are herniarin,

umbelliferone, and esculetin, which comprise approximately 0.1% of total polyphenolic compounds. Among these coumarins, esculetin contributes to the hyperglycemic effect (18). However, the effect of esculetin on lipid metabolism in HepG2 hepatocytes has not been investigated. This study was conducted to demonstrate the beneficial effect of esculetin, especially intracellular lipid accumulation and lipogenesis through AMPK cascade on FFA-induced steatosis in human hepatocytes, in the prevention of NAFLD.

Materials and Methods

Chemicals Esculetin (6,7-dihydroxycoumarin) (purity 98%), quercetin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), sodium oleate, Oil Red O, dimethyl sulfoxide (DMSO), sodium palmitate, and fatty-acid-free bovine serum albumin (BSA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), phosphate buffered saline (PBS), trypsin-ethylenediaminetetraacetic acid (EDTA), fetal bovine serum (FBS), and penicillin–streptomycin antibiotic solution were obtained from Gibco BRL (Gaithersburg, MD, USA). The lactate dehydrogenase (LDH) activity assay kit was purchased from Roche Applied Science (Indianapolis, IN, USA). Antibodies against β-actin, SREBP1c, and FAS were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). AMPKα, phospho-AMPKα (T172), ACC, and phospho-ACC antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Peroxidase-conjugated secondary antibodies and SuperSignal® West Pico Chemiluminescent Substrate were purchased from Thermo Scientific (Tewksbury, MA, USA). All other chemicals were of the highest purity grade commercially available.

Cell culture and FFAs treatment Human hepatoma HepG2 cells (American Type Culture Collection, Rockville, MD, USA) were cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 unit/mL penicillin, and 50 μg/mL streptomycin. The HepG2 cells were cultured at 37°C with 5% CO_2. To induce cellular fat-overloading, the HepG2 cells were maintained at 75% confluence with serum-free DMEM overnight and then treated with different concentrations of esculetin (25, 50, and 100 μM) or quercetin (50 μM) as positive control (19) in DMEM containing an FFA mixture (oleate and palmitate at a ratio of 2:1) for 24 h. FFAs were mixed with 10% fatty-acid-free BSA, as described previously (3).

Cell viability and cytotoxicity Cell viability and cytotoxicity were determined using the MTT assay (20) and LDH cytotoxicity detection kit, respectively. The HepG2 cells were cultured in an serum-free medium overnight to serum depletion and were then exposed to different concentrations of esculetin (25, 50, and 100 μM) in an serum-free medium containing an FFA mixture for 24 h in 96-well plates. To measure cell viability of esculetin on the cells, MTT reagent was added to each well for 4 h. The culture medium was removed; subsequently, intracellular formazan products were dissolved in DMSO, and absorbance was measured at 550 nm using a spectrophotometer (BioTek, Inc., Winooski, VT, USA). The cytotoxicity of esculetin was estimated using the LDH cytotoxicity detection kit as per the manufacturer's instructions. LDH leakage was expressed as a percentage of total activity [(activity in the medium)/(activity in the medium+activity of the cells)]×100.

Oil Red O staining The HepG2 cells were exposed to 500 μM of FFAs with indicated concentrations of esculetin or quercetin for 24 h. To stain lipid droplets in the HepG2 cells, the cells were washed twice with cold PBS and fixed with 10% formaldehyde for 30 min. The fixed cells were washed thrice with water and then stained with 0.5% Oil red O in a 60% isopropanol working solution for 20 min at room temperature. Cell images were obtained using an Olympus CKX41 microscope (Tokyo, Japan) in conjunction with a digital camera at 100× magnification.

Intracellular TG level HepG2 cell lysates were extracted using 5 mL of chloroform and methanol (2:1, v/v). The mixture was vortexed vigorously and then centrifuged at 240x*g* for 10 min at 4°C to separate it into two phases. The organic phase was evaporated under nitrogen gas until dry. The pellet was dissolved in PBS containing 1% Triton X-100, and protein concentration was measured using a Take3™ Multi-Volume Plate (BioTek, Inc.). The intracellular TG concentration was measured using an enzymatic reagent kit (Asan Pharm. Co., Seoul, Korea) according to the manufacturer's protocol.

Western blot analysis Cell lysates were prepared on ice using a Pro-Prep™ sample buffer (iNtRON Biotechnology, Seongnam, Korea). Protein concentration was quantified using Take3™ Multi-Volume Plate in an Epoch plate reader. Equal amounts of proteins were separated on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels and then transferred onto nitrocellulose membranes (GE Healthcare, Buckinghamshire, UK). The membranes were blocked in Tris-buffered saline/Tween 20 (TBS-T) containing 5% skim milk for 1 h and incubated with primary antibodies against SREBP1c, FAS, phospho-AMPKα (Thr172), AMPKα, phospho-ACC (Ser79), ACC, and β-actin for 3 h. After washing with the TBS-T buffer, the membrane was incubated using a horseradish-conjugated anti-goat, anti-mouse, and anti-rabbit immunoglobulin G (IgG) secondary antibody. Specific bands were visualized on an X-ray film activated by chemiluminescence using ECL™ detection reagents (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical analysis The results are reported as means±standard error. Statistical comparisons are determined using one-way ANOVA followed by Duncan's multiple comparison test using SAS version 8.1 (SAS Institute, Cary, NC, USA). A p-value of <0.05 was considered as significant.

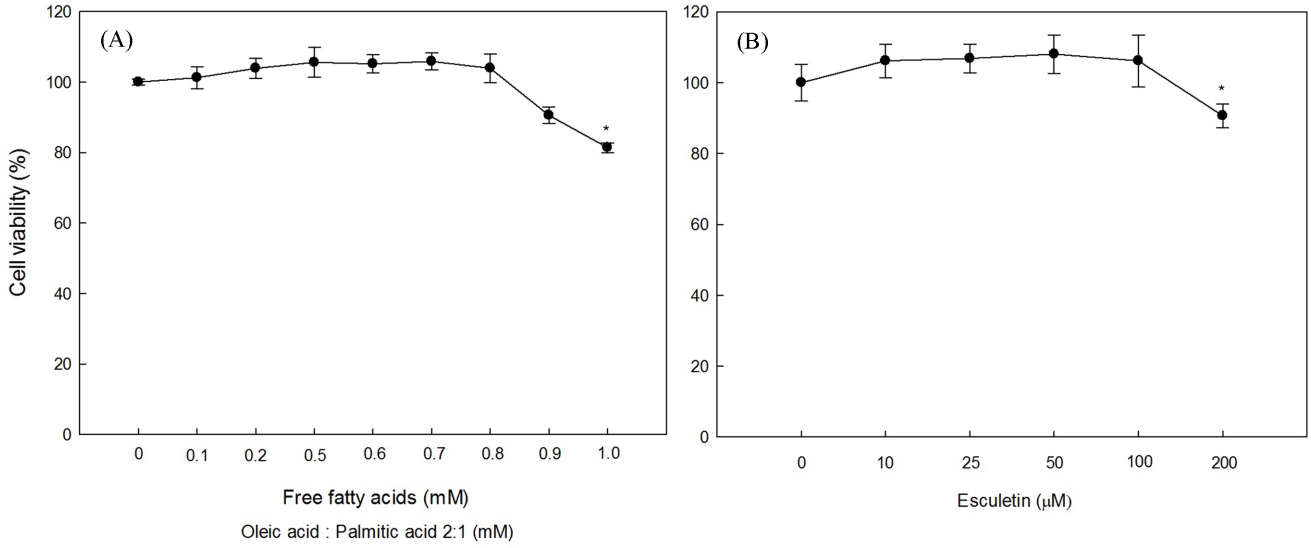

Fig. 1. Effects of FFAs and esculetin on HepG2 cell growth. The cells were treated with the indicated concentrations of FFA (A) and esculetin (B) for 24 h, and cell viability was measured using the MTT assay. Values are expressed as the mean±SE (*n*=3). **p*<0.05 in comparison to the control.

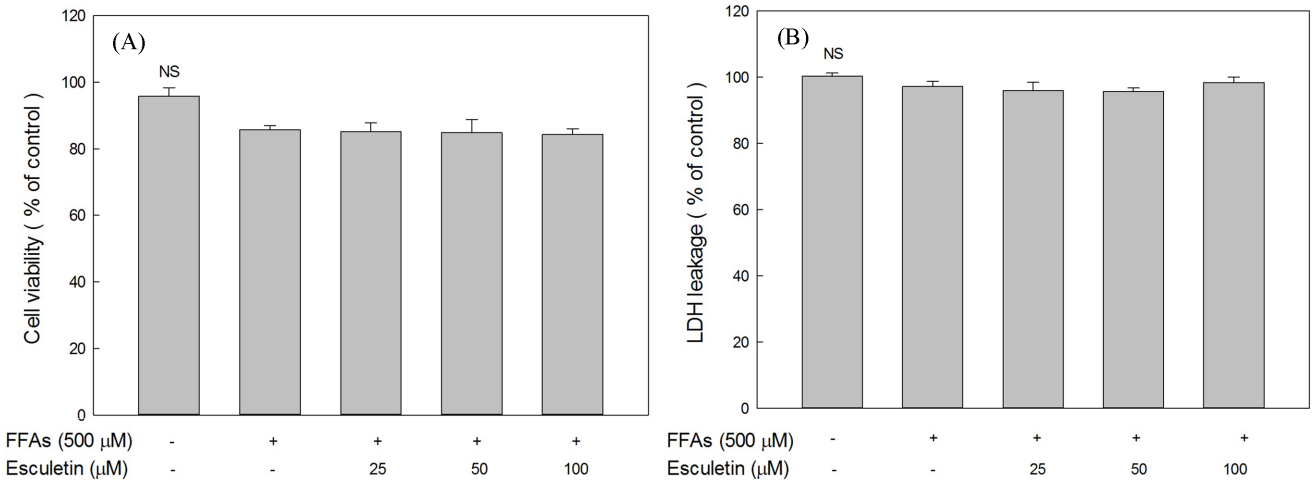

Fig. 2. Effect of esculetin on the cell viability and cytotoxicity of FFA-stimulated HepG2 cells. The cells were treated with the indicated concentrations of esculetin in the absence or presence of FFAs for 24 h. Cell viability (A) and cytotoxicity (B) were determined by the MTT and LDH assays, respectively. Each bar represents the mean±SE (*n*=3). NS= no significant difference between control and all treatments (*p*<0.05).

Results and Discussion

Effects of FFAs and esculetin on HepG2 cell growth NAFLD appears linked with liver manifestation of the metabolic syndrome. Although the pathogenesis of NAFLD has not been completely described, lipid accumulation from increased FFA influx to the liver is the main pathological characteristic of a human liver with NAFLD (21). Plasma-high FFA levels may exacerbate hepatic fat accumulation by disrupting lipid metabolism in NAFLD patients. Nearly 60–80% and 25% of liver TG are derived from circulating FFAs and *de novo* lipogenesis, respectively (22). Therefore, agents with the ability to decrease high lipid levels and *de novo* lipogenesis may be a potential therapeutic choice for NAFLD. Generally, high-fat diet-fed animals have been used to study NAFLD, but this *in vivo* model is difficult to prepare and reproducibility of the experimental results is often problematic (23). To explore the alternatives, *in vitro* models of fatty liver disease were established using HepG2 cells treated with FFAs; these models can reliably reproduce the key features of hepatic steatosis in humans (4,24). Palmitate and oleate are common dietary long-chain FFAs and the most abundant FFAs in liver in both normal subjects and NAFLD patients (25). Thus, HepG2 cells incubated with a mixture of oleate/palmitate (2:1 ratio) has been commonly utilized to develop *in vitro* cellular mechanisms involved in FFA-mediated hepatic steatosis (23,26). The present study focuses on the beneficial effect of esculetin, a widely available plant-derived coumarin, on hepatic steatosis and the underlying mechanisms *in vitro*.

In this study, the effects of FFAs and esculetin on cell viability were measured via an MTT assay to determine the optimal concentration

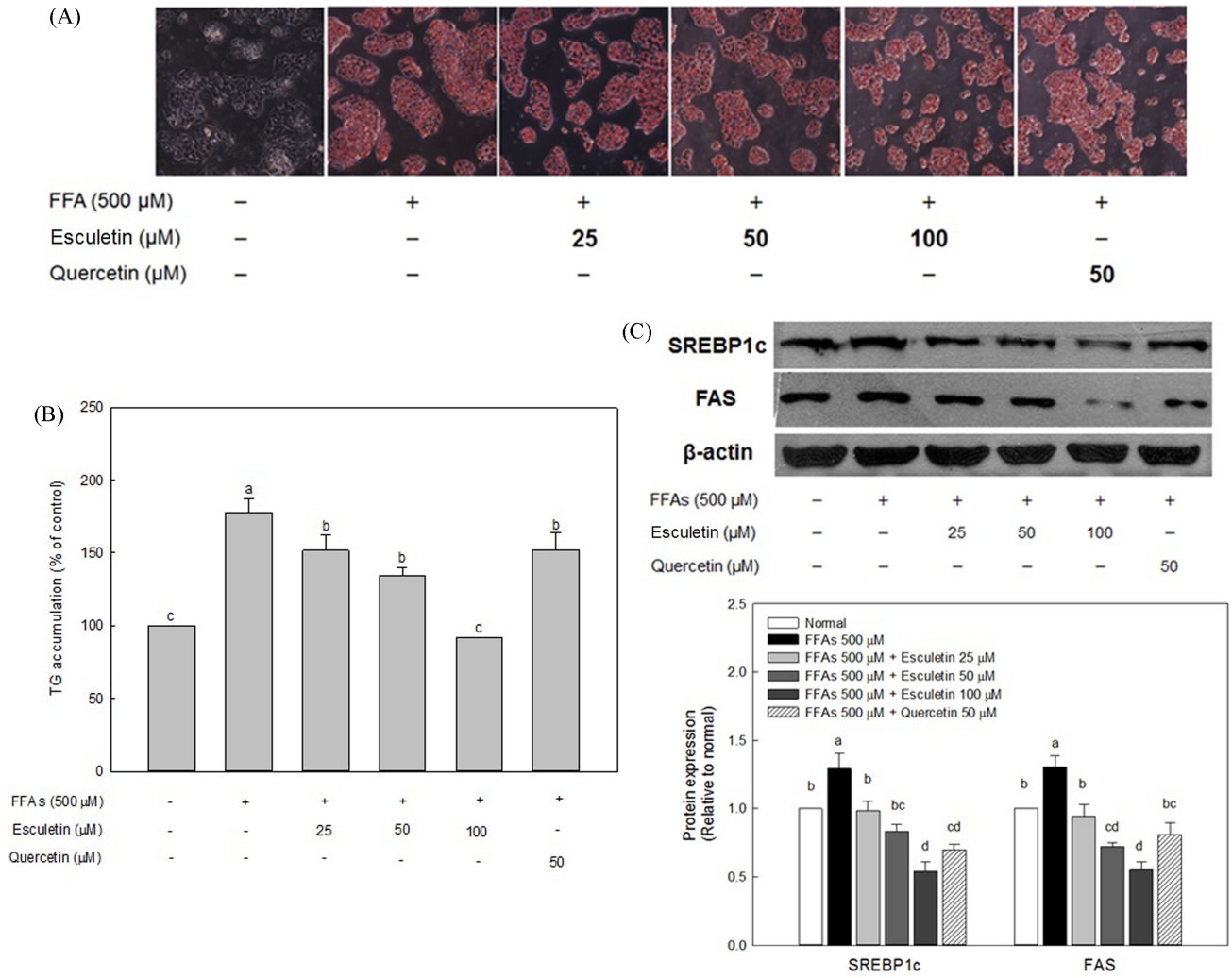

Fig. 3. Effects of esculetin on the intracellular lipid accumulation and lipogenic-related protein expression in FFA-stimulated HepG2 cells. The cells were treated with the indicated concentrations of esculetin and quercetin in the absence or presence of FFAs for 24 h. The cells were fixed with formalin, and intracellular lipids were stained with Oil red O solution (A). Quantification of intracellular TGs was performed using the enzymatic colorimetric method (B). The protein expression levels of SREBP1c and FAS were analyzed via western blotting (C). β-Actin was used as a loading control, and blots are representative of at least three independent experiments. Each bar represents the mean±SE (n=3). Different letters indicate a significant difference at the p<0.05 level.

of FFAs and esculetin. Cell viabilities were not affected by up to 0.8 mM of FFAs and 100 μM of esculetin, respectively (Fig. 1A and 1B). Therefore, 500 μM of FFAs and 10–50 μM of esculetin were employed. The cell viability and cytotoxicity of esculetin in the presence of 500 μM FFAs for 24 h were measured using the MTT and LDH assays, respectively. Co-treatment with FFAs and esculetin did not influence the cell viability and cytotoxicity of HepG2 cells at the indicated concentrations (Fig. 2A and 2B).

Effects of esculetin on intracellular lipid accumulation in FFA-exposed HepG2 cells Cellular lipid accumulation was assessed using Oil Red O staining and an enzymatic kit to investigate the effect of esculetin on FFA-induced cellular steatosis in the HepG2 cells. The intracellular lipid droplets in FFA-treated cells became larger with a deeper red color; however, these droplets were reduced by esculetin or quercetin treatment (Fig. 3A). The intracellular TG content in HepG2 cells was significantly increased by the 500-μM FFA treatment to the extent of 77% compared with that in FFA untreated HepG2 cells (Fig. 3B), whereas the intracellular TG content was significantly decreased by esculetin or quercetin treatment when compared with that in the FFA-treated group. Using this hepatic steatosis model, esculetin treatment dose-dependently attenuated lipid droplets and hepatocellular TG levels in FFA-stimulated HepG2 cells without inducing cell cytotoxicity.

Effects of esculetin on lipogenesis in FFA-exposed HepG2 cells It is well-known that hepatic *de novo* lipogenesis is mediated by activating the expression of the major genes of fatty-acid synthesis (ACC, FAS, and others) under the transcriptional regulation of SREBP1c (27). In this study, the protein expression levels of SREBP1c and FAS, the key

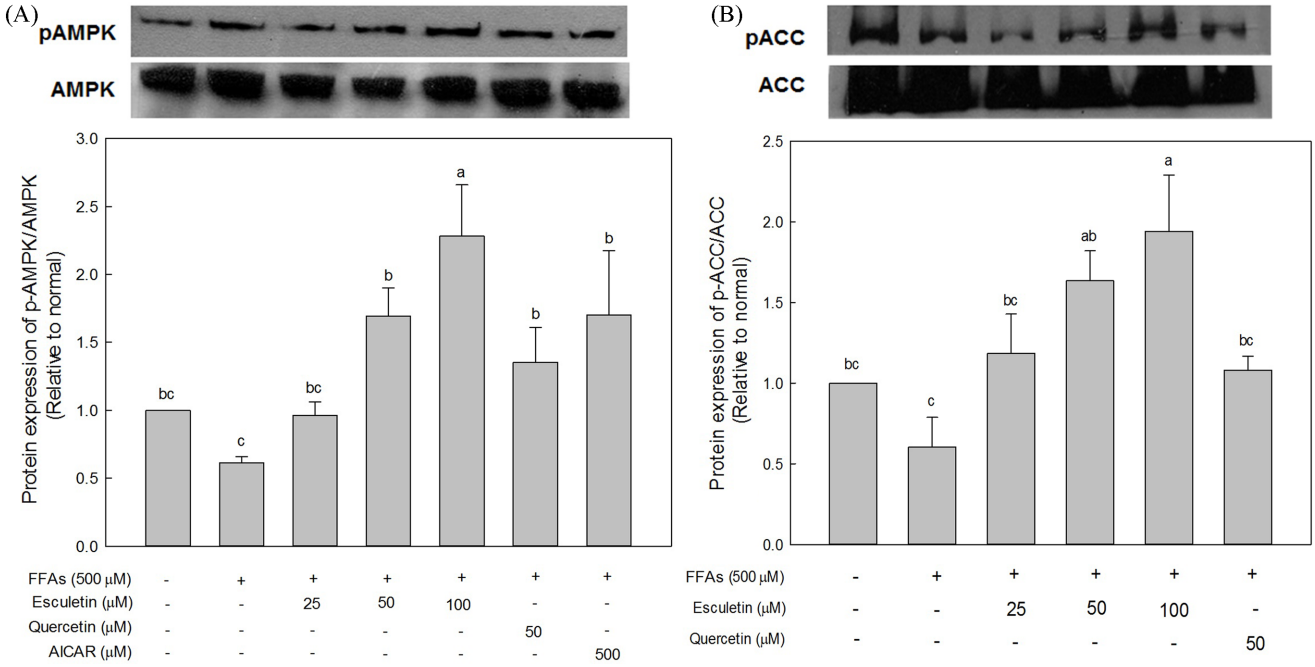

Fig. 4. Effects of esculetin on the AMPK pathway in FFA-stimulated HepG2 cells. The cells were treated with the indicated concentrations of esculetin, quercetin, and AICAR in the absence or presence of FFAs for 24 h. The protein expression levels of AMPK (A) and ACC (B) phosphorylation were analyzed via western blotting. AICAR was used as a positive control. AMPK and ACC were used as loading controls, and blots are representative of at least three independent experiments. Each bar represents the mean±SE (n=3). Different letters indicate a significant difference at the p<0.05 level.

regulators of hepatic lipogenesis, were examined via the western blot analysis to explore the underlying mechanism of esculetin-mediated lipid-lowering effects in FFA-stimulated HepG2 cells. Esculetin dose-dependently declined FFA-induced increases of the SREBP1c and FAS protein expressions in FFA-stimulated HepG2 cells (Fig. 3C). The protein expressions of these lipogenic genes were also significantly down-regulated by quercetin treatment. These results indicate that esculetin ameliorates cellular-lipid formation through inhibition of *de novo* lipogenesis in hepatocytes.

Effects of esculetin on AMPK and ACC phosphorylation AMPK activation plays an important role in cellular-energy homeostasis and is regarded as a potential therapeutic target for the prevention and treatment of NAFLD (28). The inactivation of ACC through AMPK phosphorylation reduces malonyl-CoA, which is not only a potent inhibitor in mitochondrial fatty-acid oxidation but also a critical precursor in FAS (29). In addition, a recent study demonstrated that AMPK represses the target gene expression of SREBP1c through direct suppression of the SREBP1c cleavage and nuclear translocation in hepatocytes exposed to high fat/high sucrose; this leads to inhibited lipogenesis and hepatic lipid content (9). Therefore, AMPK plays a crucial role in the regulation of hepatic lipid metabolism.

When the HepG2 cells were incubated with esculetin (25, 50, and 100 μM) in the presence of FFAs for 24 h, esculetin treatment notably increased the phosphorylation of AMPKα (Thr172) and its downstream kinase, i.e., ACC (Ser79) in a concentration-dependent manner (Fig. 4). As expected, quercetin and AICAR (5-aminoimidazole-4-carboxamide-1-β--ribofuranoside), a well-characterized AMPK activator, significantly evoked the phosphorylation of AMPKα in FFA-stimulated HepG2 cells. These data suggest the contribution of AMPK to the effects of esculetin on decreased fat accumulation in hepatocytes. To confirm whether esculetin-mediated lipid reduction is mediated by AMPK signaling, the HepG2 cells were pre-incubated for 1 h with a selective AMPK inhibitor, compound C, prior to esculetin treatment. Compound C declined the esculetin-stimulated phosphorylation levels of AMPKα and ACC (Fig. 5A). The protein-expression levels of SREBP1c and FAS were significantly decreased by esculetin treatment; however, these effects were blunted in the presence of compound C (Fig. 5B). Moreover, the suppressive effect of esculetin on the intracellular TG content was reversed nearly to the level of FFA-treated control cells by treatment with compound C (Fig. 6).

Many phytochemicals such as *S*-Allyl cycteine, monascin, and ankaflavin can inhibit hepatic steatosis by activating AMPK and can therefore inhibit SREBP1c (30,31). These findings are in agreement with those of the present study, indicating that esculetin dramatically phosphorylated AMPK and its direct substrate ACC in FFA-stimulated HepG2 cells. Moreover, the inhibitory effects of esculetin on lipid accumulation and lipogenic protein expression were rescued by compound C pretreatment. Thus, the AMPK signaling pathway plays a crucial role in mediating the suppressive effect of esculetin on FFA-induced *de novo* lipogenesis in HepG2 hepatocytes.

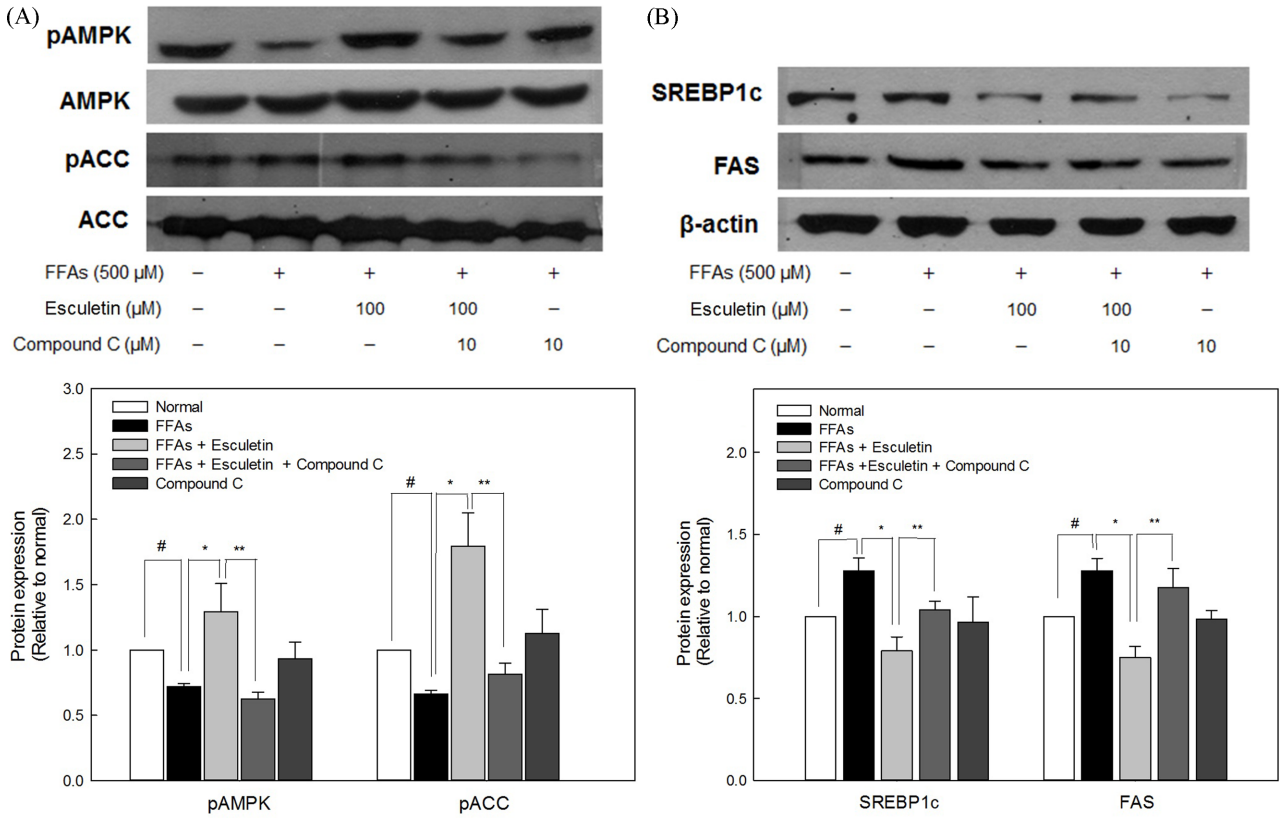

Fig. 5. The anti-lipogenic effects of esculetin on AMPK activation in FFA-stimulated HepG2 cells. The cells were treated with esculetin and compound C (a specific AMPK inhibitor) in the absence or presence of FFAs for 24 h. The protein-expression levels of AMPK and ACC phosphorylation (A) and lipogenic-related genes (B) were analyzed via western blotting. AMPK and ACC were used as loading controls for pAMPK and pACC β-actin was used as a loading control for SREBP1c and FAS. Blots are representative of at least three independent experiments. Each bar represents the mean±SE (n=3). $^{\#}p$<0.05, vs control cells; *p<0.05, vs cells treated with FFAs; **p<0.05, vs cells treated with FFAs and esculetin.

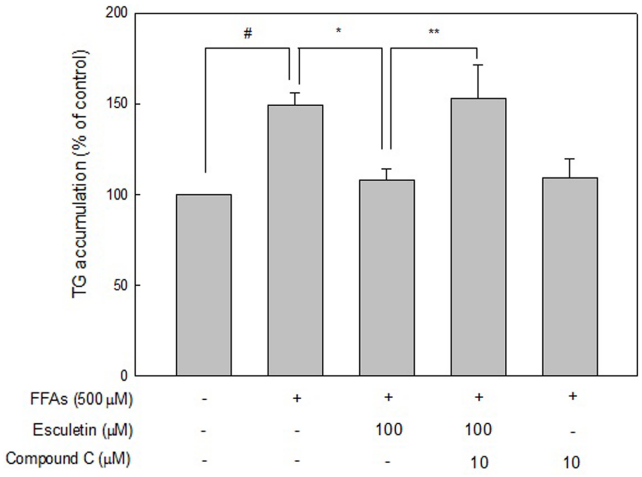

Fig. 6. Lipid-lowering effects of esculetin on AMPK activation in FFA-stimulated HepG2 cells. The cells were treated with esculetin and compound C (a specific AMPK inhibitor) in the absence or presence of FFAs for 24 h. Quantification of intracellular TGs was measured using the enzymatic colorimetric method. Each bar represents the mean±SE (n=3). $^{\#}p$<0.05, vs control cells; *p<0.05, vs cells treated with FFAs; **p<0.05, vs cells treated with FFAs and esculetin.

In conclusion, esculetin plays an important role in preventing hepatic lipid accumulation by modulating *de novo* hepatic lipogenesis on FFA-induced cellular fatty liver in HepG2 cells. The anti-lipogenic properties of esculetin could be mediated by the AMPK signaling pathway. Although further studies are required to confirm these results, this study establishes that esculetin may be used a therapeutic agent to prevent and manage NAFLD.

Acknowledgment This study was supported by the Rural Development Administration (Project number PJ0108382016).

Disclosure The authors declare no conflict of interest.

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