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Reduction of Lipid Accumulation in HepG2 Cells by Luteolin is associated with Activation of AMPK and Mitigation of Oxidative Stress

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The present study was carried out to investigate the lipid-lowering effect of luteolin by using a cell model of steatosis induced by palmitate. Incubation of HepG2 cells with palmitate markedly increased lipid accumulation (Oil Red O staining), the genes involved in lipogenesis, including fatty acid synthase (FAS) and its upstream regulator sterol regulatory element binding protein 1c (SREBP-1c), and reactive oxygen species (ROS) production. Luteolin enhanced the phosphorylation of AMP-activated protein kinase α (AMPKα) and its primary downstream targeting enzyme, acetyl-CoA carboxylase (ACC), up-regulated gene expression of carnitine palmitoyl transferase 1 (CPT-1), which is the rate-limiting enzyme in mitochondrial fatty acid β-oxidation, and down-regulated SREBP-1c and FAS mRNA levels in the absence and presence of palmitate. In addition, luteolin significantly decreased ROS production and ameliorated lipid accumulation in HepG2 cells caused by palmitate. Furthermore, intracellular triglyceride (TG) measurement indicated that the luteolin-mediated reduction of enhanced TG caused by palmitate was blocked by pretreatment with the AMPK inhibitor, compound C. The results suggested that the lipid-lowering effect of luteolin might be partially mediated by the up-regulation of CPT-1 and down-regulation of SREBP-1c and FAS gene expression, possibly by activation of the AMPK signaling pathway, and partially might be through its antioxidative actions.

Keywords: luteolin; AMPK; SREBP-1c; FAS; CPT-1; reactive oxygen species.

INTRODUCTION

Obesity and its associated conditions, such as type 2 diabetes, coronary heart disease, as well as nonalcoholic fatty liver disease (NAFLD), are currently a worldwide health problem (Dunn and Schwimmer, 2008). There is accumulating evidence that central obesity is associated with an impaired free fatty acid (FFA) metabolism (Boden, 2008). An excessive release of FFA from adipocytes in visceral fat has been suggested to contribute to the development of NAFLD, which is emerging as an obesity-related disorder in obese patients (Gentile and Pagliassotti, 2008).

A large body of evidence indicates that AMP-activated protein kinase (AMPK) is involved in regulating hepatic lipogenesis and may be a therapeutic target for treating fatty liver disease (Schimmack *et al*., 2006). The AMPK complex is an evolutionally conserved serine/threonine heterotrimer kinase complex consisting of α-, β- and γ-subunits and acts as a 'master switch' for lipid metabolism (Hardie, 2007). Activation of hepatic AMPK (through phosphorylation of its α-subunit on Thr172) switches off fatty acid synthesis acutely via increased phosphorylation and inactivation of acetyl-CoA carboxylase (ACC), and also switches off fatty acid synthesis chronically, by decreased transcriptional activation of sterol regulatory element binding protein 1c (SREBP-1c) (Hardie, 2007). SREBP-1c is the most important transcription factor regulating genes involved in *de novo* lipogenesis, including fatty acid synthase (FAS), in the liver (Ahmed and Byrne, 2007). In addition, inactivation of ACC reduces the synthesis of malonyl-CoA, which in turn de-represses carnitine palmitoyl transferase 1 (CPT-1) and activates fatty acid oxidation (McGarry and Brown, 1997).

Studies in cell lines and animal models have shown that dietary flavonoids, including polyphenolic compounds, potentially have protective roles against obesity and fatty liver disorders through the activation of the AMPK signaling pathway (Baur *et al*., 2006; Guo *et al*., 2009; Lin *et al*., 2007; Zang *et al*., 2006). Luteolin (3′,4′,5,7-tetrahydroxyflavone) is one of the most common flavonoids present in edible plants and in plants used in medicinal herbs, and has been found to have antitumorigenic, antioxidant and antiinflammatory properties (Lopez-Lazaro, 2009). Experimental data also indicate that luteolin reduces cholesterol levels *in vitro* (Andrikopoulos *et al*., 2002; Gebhardt, 2002) and that some plants with this flavonoid have antiobesity effects by reducing plasma triacylglycerol levels in animal models (Han *et al*., 2003) and may prevent diabetes by reducing glucose levels (Cunha *et al*., 2008; Andrade-Cetto and Wiedenfeld, 2001). However, the cellular mechanism of the lipid-lowering effect of luteolin is unknown.

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Herein the hypolipidaemic effects of luteolin were investigated by examining the effect of luteolin on reduction in fat deposition in HepG2 cells treated with palmitate. The results demonstrated that luteolin enhances the phosphorylation of AMPK, decreases gene expression of SREBP-1c and FAS and increases CPT-1 gene in the absence and presence of palmitate, leading to a decrease in the intracellular lipid levels in HepG2 cells. In addition, luteolin also ameliorates palmitate-induced oxidative stress.

MATERIALS AND METHODS

Materials. Luteolin (HLPC content 98%) was purchased from Shanghai Winherb Medical S & T Development Co. Ltd (Shanghai, China). Anti-phospho-AMPKα (Thr 172) and AMPKα antibodies, anti-phospho-ACC (Ser 79) and β-actin antibody were purchased from Cell Signaling Technology (MA, UAS). Anti-mouse and anti-rabbit antibodies conjugated to horseradish peroxidase were obtained from Kirkegaard and Perry Laboratories (Gaithersburg, MD). Minimum essential medium (MEM), fetal bovine serum, penicillin–streptomycin solution and sodium pyruvate were purchased from Invitrogen. AICA-Riboside (AICAR), compound C and protease inhibitor cocktail set I were purchased from Calbiochem. Palmitate and *N*-acetyl-L-cysteine (NAC) were purchased from Sigma. All other reagents were of analytical grade.

Cell culture and treatment. Human hepatoma HepG2 cells obtained from the American Type Culture Collection (ATCC) were cultured in MEM medium containing 10% (vol/vol) fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin and 1 mM sodium pyruvate at 37 °C in a humidified atmosphere containing 5% CO_2. The cells were grown to 70% confluence and incubated in serum-free medium overnight before treatments. The cells were exposed to various concentrations (10–20 μM) of luteolin or to vehicle (DMSO) for the indicated times. When the cells were grown in media supplemented with fatty acid (FA), sodium palmitate was dissolved in pre-heated 0.1 N NaOH and diluted in MEN containing 1.76% (w/v) bovine serum albumin (BSA), to give a final palmitate concentration of 0.4 mM. All cells were cultured in a 5% CO_2 atmosphere at 37 °C.

Cell viability assay. Cell viability was assessed with the MTT assay performed according to the manufacturer's suggestions (Sigma). HepG2 cells were grown in 96-well plates at a density of 1×10^4 cells per well with 100 μL culture medium. After the cells were attached, the medium was refreshed with different concentrations of luteolin (from 10 to 100 μM) or 0.4 mM sodium palmitate. For the control group, the same concentration of vehicle was added to the medium. After being cultured for 24 h, the cells were incubated with MTT for another 4 h at 37°C. Subsequently, the medium was removed and DMSO was added to each well. The absorbance of the samples was measured at 570 nm using a FlexStation 3 microplate reader (Molecular Devices). All experiments were performed independently in triplicate.

Western blot analysis. After treatment, HepG2 cells were harvested in a lysis buffer (20 mM Tri, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 μg/mL leupeptin, 1 mM PMSF). Samples were sonicated three times for 5 s with 15 s breaks between cycles, and centrifuged at $16000 \times \mathbf{g}$ for 60 min at 4°C. The protein concentrations of the supernatants were determined with a protein assay kit (Bio-Rad). Equal amounts of total cellular proteins were resolved by 10% SDS-PAGE transferred onto polyvinylidene difluoride membranes (Amersham Biosciences) and then probed with primary antibody followed by secondary antibody conjugated with horseradish peroxidase. The immunocomplexes were visualized with enhanced chemiluminescence kits (Amersham Biosciences).

Reverse transcription-PCR analysis. Total RNA was isolated from the liver tissue of each rat using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). For each sample, 1 μg of total RNA was subjected to RT-PCR amplification using the Access RT-PCR System (Promega, Madison, USA) according to the manufacturer's directions. The following primers were used, with the size of the amplification in bracket: SREBP-1c, forward-cagctcatcaacaaccaagac and reverse-ttgtggc tcctgtgtctgtc (667bp); FAS, forward-tgcaactgtgcgtt agccacc and reverse-tgtttcaggggagaagagacc (721bp); β-actin, forward-atgccatcctgcgtctggacctggc and reverse-agcatttgcggtgcacgatggaggg (606bp). The number of amplification cycles was determined according to a kinetic profile. β-actin was used as an internal control. PCR products were subjected to electrophoresis on 1.5% agarose gel containing ethidium bromide. All PCR reactions were normalized against β-actin expression.

Determination of intracellular lipid content. The total intracellular lipid content was evaluated by Oil Red O staining (Ramirez-Zacarias *et al*., 1992). Briefly, the cells were fixed in 4% paraformaldehyde in PBS for 30 min, stained with Oil Red O for 1 h at room temperature, and then rinsed with water. The cell images were captured under a microscope. For quantitative analysis of cellular lipids, 1 mL isopropanol was added to every hole of the stained culture plate. The extracted dye was removed immediately by gentle pipetting and its absorbance was monitored by a spectrophotometer at 510 nm. Intracellular triglyceride (TG) content was determined in cell lysates by an enzymatic colorimetric method using a commercially available kit (Shanghai Mind Bioengineering Co, China) and normalized by protein content as described (Guo *et al*., 2009). Data are represented as percentages of control cells.

Measurement of reactive oxygen species (ROS). Cellular ROS levels were measured using a cell-permeable probe 2′,7′-dichiorofluorescin diacetate (DCFDA) (Sigma). The cells were loaded with 10 μM DCFDA in PBS for 30 min. After washing the cells with PBS twice, fluorescence was measured by a FlexStation 3 microplate reader (Molecular Devices) at excitation of 485 nm and emission of 525 nm. All the readings were normalized to protein levels (mg/mL) by the Bradford assay.

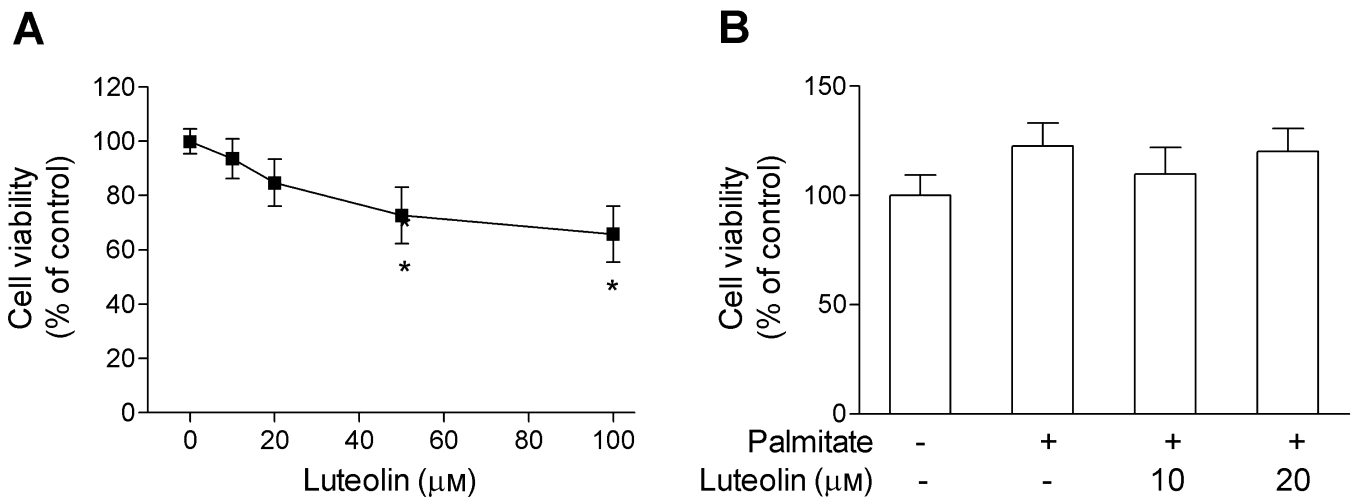

Figure 1. Dose dependent reduction effects of luteolin on cell survival. HepG2 cells were exposed to various concentrations of luteolin in the absence (A) or presence (B) of palmitate (0.4 mM). For the control group, the cells were treated with the respective vehicle (see "MATERIALS AND METHODS"). After being incubated for 24 h, the cell viability was determined by MTT tests. The data represent mean ± SE of three independent experiments with triplicate wells. * $p < 0.05$ vs control.

Statistical analysis. All data are presented as mean ± SEM. Student's test (unpaired) was used to determine the statistical significance ($p < 0.05$) of obtained data.

RESULTS

Luteolin inhibited cellular lipid accumulation induced by palmitate

First of all, the effect of luteolin on cell viability was determined by MTT. In HepG2 cells, luteolin at 50 and 100 μM significantly decreased the cell viability (Fig. 1A). Then doses (10 and 20 μM) were selected for further study. To investigate the ability of luteolin to prevent the lipid accumulation, the HepG2 cells were incubated in medium containing palmitate to induce FA overloading conditions. Cultured HepG2 cells were exposed to 0.4 mM palmitate for 24 h and the total lipid levels were detected by Oil Red O staining. HepG2 cells exposed to the FA mixtures developed a clear increase of lipid accumulation in the cytosol compared with the BSA control (Fig. 2A and B), indicating that the cell model of steatosis was successfully induced by palmitate. Co-treatment of HepG2 cells with palmitate (0.4 mM) and luteolin (20 μM) significantly prevented cellular lipid accumulation (Fig. 2A and B). These results were further confirmed by quantification of the intracellular TG content (Fig. 6). Palmitate (0.4 mM) alone or combined with luteolin (10 and 20 μM) did not show cytotoxic effects (Fig. 1B).

Luteolin increased phosphorylation of AMPKα in HepG2 cells

Having observed that luteolin had an effect on hepatocyte lipid levels, the possible mechanisms responsible for this effect were assessed. AMPK is a metabolic master switch that responds to changes in cellular energy status (Hardie, 2007) and has been suggested to play a crucial role in regulating fat metabolism in the liver. Therefore, the effect of luteolin on AMPK activation was examined in HepG2 cells. As the activity of AMPK correlates tightly with phosphorylation at Thr-172 (pAMPKα), the activation of AMPK was assessed by determining phosphorylation of AMPKα and its primary downstream targeting enzyme, ACC, using immunoblots with specific phospho-Thr-172 and phospho-Ser-79 antibodies. As shown in Fig. 3A and B, luteolin (10 and 20 μM) significantly increased the levels of AMPKα phosphorylation at 1 h, which continued to increase at 24 h after treatment with luteolin. AICAR, an AMP analogue and known AMPK activator, was used as a positive control. No change in the expression of endogenous AMPKα protein was noted by immunoblotting with AMPKα antibody (Fig. 3A). Furthermore, increased levels of AMPKα phosphorylation stimulated by luteolin were accompanied by enhanced phosphorylation of ACC, indicating that luteolin activated AMPK leading to inhibition of ACC (Fig. 3A and B). In addition, the phosphorylation of AMPKα and ACC was not affected by palmitate (0.4 mM), but was markedly up-regulated by luteolin at 1 h in FA-overloaded HepG2 cells (Fig. 3C, D, and Fig. 5B).

Luteolin down-regulated SREBP-1c and FAS and up-regulated CPT-1 mRNA expression in HepG2 cells

SREBP-1c is a critical transcriptional factor known to regulate the expression of lipogenic enzymes in the hepatic lipogenic pathway (Shimano *et al.*, 1999) and has been reported to play an important role in the pathogenesis of NAFLD (Ahmed and Byrne, 2007). Our previous studies showed that high-fat-diet-induced hepatic steatosis and obesity down-regulated the phosphorylation of AMPK in liver, leading to up-regulation of hepatic SREBP-1c and FAS mRNA (Guo *et al.*, 2009). The effects of luteolin on gene expression of

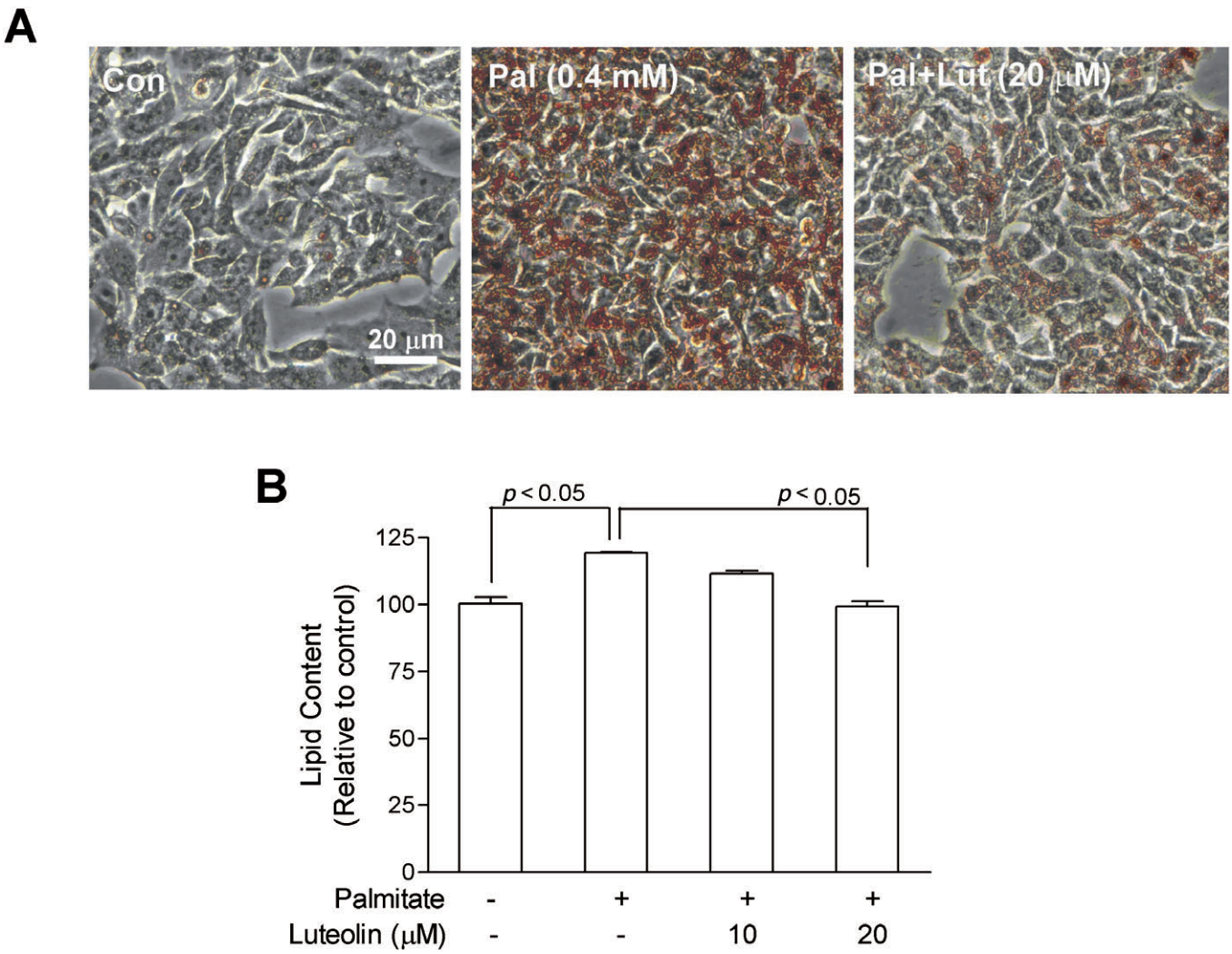

Figure 2. Luteolin decreases intracellular lipid content in HepG2 cells. The cells were treated with palmitate (0.4 mM) in the absence or presence of luteolin (10 and 20 μM) for 24 h. The control cells were treated with 1.76% bovine serum albumin for 24 h. (A) Images of cells were captured by microscope at 400× original magnification showing fat accumulation in cells stained by Oil Red O; (B) Quantitative analysis of lipid deposition in cells by Oil Red O staining. The data represent mean ± SE of three independent experiments. Con, control; Pal, palmitate; Lut, luteolin. This figure is available in colour online at wileyonlinelibrary.com/journal/ptr

SREBP-1c and its target gene, FAS, in HepG2 cells were examined by PCR analysis. HepG2 cells exposed to palmitate (0.4 mM) for 24 h dramatically enhanced the gene expression of SREBP-1c and FAS, which were restored by luteolin treatment at 6 h (Fig. 4C and D). In addition, in the absence of palmitate, SREBP-1c and FAS mRNA were also down-regulated after exposure to luteolin (20 μM) for 24 h in HepG2 cells (Fig. 4A and B).

Activation of AMPK is associated with an increase in fatty acid oxidation. Mitochondria are the main sites for fatty acid β-oxidation, with CPT-1 as the rate-limiting enzyme. AMPK causes phosphorylation and inhibition of ACC, which reduces the production of malonyl-CoA, an allosteric inhibitor of CPT-1 (Park *et al.*, 2002). Thus AMPK activation appears to be important in regulating CPT-1 activity. As expected, associated with an increase in the phosphorylation of AMPKα and ACC, the mRNA level of CPT-1 was enhanced by luteolin at 1 and 24 h in HepG2 cells (Fig. 4A and B). Surprisingly, CPT-1 mRNA tended to be elevated by 0.4 mM palmitate at 24 h, however, luteolin still induced the promotion of CPT-1 mRNA expression in the presence of palmitate (Fig. 4C and D).

Luteolin inhibited palmitate-induced ROS generation

Palmitate is known as a lipototoxic fatty acid increasing oxidative stress and ROS production that induced insulin resistance in hepatocytes (Nakamura *et al.*, 2009). Indeed, ROS levels are increased in clinical conditions associated with insulin resistance, such as obesity and type 2 diabetes (Furukawa *et al.*, 2004; Keaney, Jr. *et al.*, 2003). Increased cellular ROS levels are known to stimulate SREBP-1c activity (Sekiya *et al.*, 2008). The antioxidant properties of luteolin are widely acknowledged (Lopez-Lazaro, 2009). Thus, the effect of luteolin on palmitate-induced ROS production was investigated by using DCFDA to detect cellular ROS levels. As illustrated in Fig. 5A, incubating HepG2 cells with palmitate (0.4 mM) did not induce an increase in the cellular ROS level at 8 h or 16 h, but a significant increase was observed at 24 h, which was in

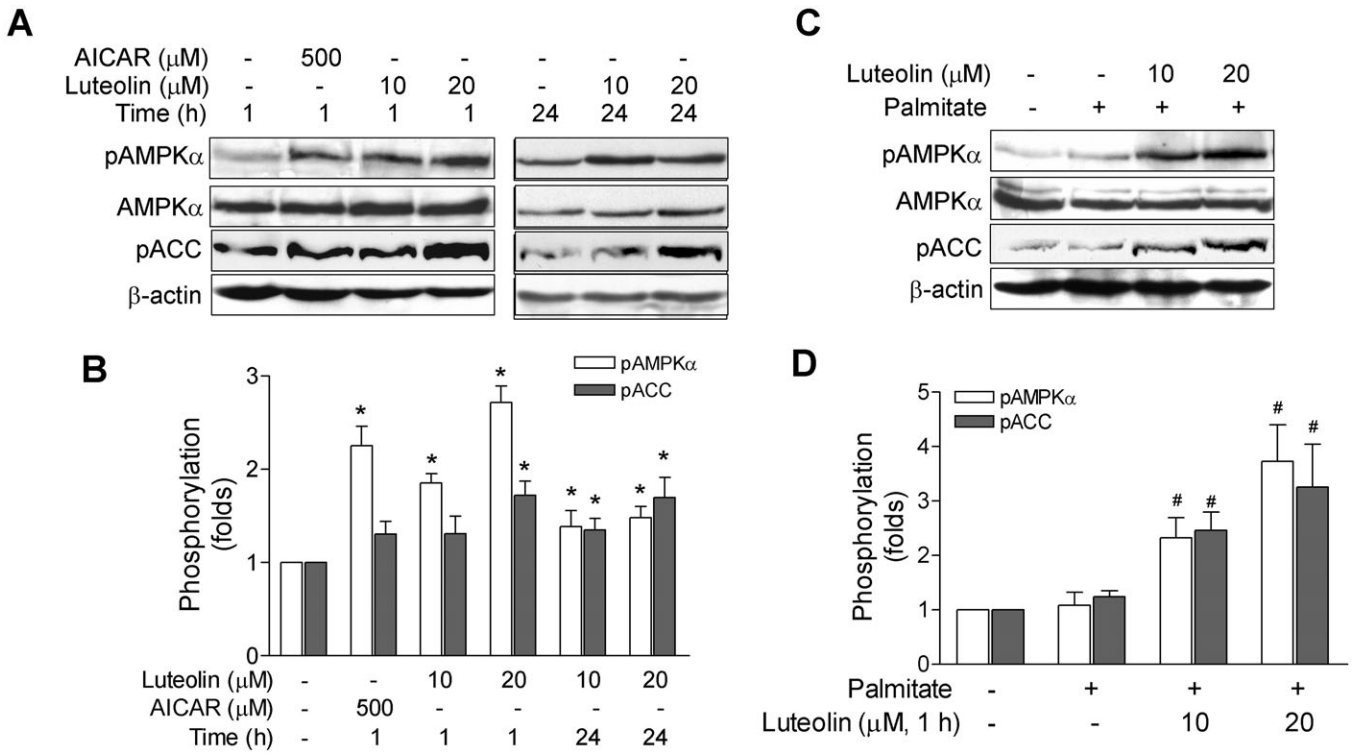

Figure 3. Luteolin stimulates the phosphorylation of AMPKα and ACC in HepG2 cells. Cells were incubated with 10 and 20 μM luteolin for the indicated times in the absence (A and B) or presence (C and D) of palmitate (0.4 mM, 24 h). (A) and (C) Representative immunoblots for phosphorylation of AMPKα and ACC, total AMPKα and β-actin in HepG2 cells. AICAR was used as a positive control. (B) and (D) Densitometric analysis of AMPKα and ACC phosphorylation levels. Data were expressed as mean ± SE relative to the basal phosphorylation level from at least four independent experiments. * $p < 0.05$ vs respective control; # $p < 0.05$ vs respective untreated palmitate control.

parallel with enhanced SREBP-1c and FAS gene expression (Fig. 4C and D). Treatment with luteolin (10 and 20 μM) attenuated the ROS generation in the palmitate-stimulated HepG2 cells in a time- and dose-dependent manner (Fig. 5A).

It has been shown previously that oxidative stress associated with hypertension inhibits the LKB1/AMPK signaling axis (Dolinsky *et al*., 2009). However, in the present studies, palmitate (0.4 mM) caused oxidative stress by increasing cellular ROS and enhanced the phosphorylation of AMPKα slightly (Fig 5B). The antioxidant reagent NAC at 20 mM significantly suppressed palmitate-induced ROS production (Fig. 5A), but unaffected the phosphorylation of AMPKα (Fig. 5B), which is similar to a previous report (Lin *et al*., 2007). In contrast, in parallel with decreased ROS levels, luteolin markedly increased the phosphorylation of AMPKα in palmitate-stimulated HepG2 cells (Fig. 3C and D, Fig. 5B). Thus, luteolin did not appear to activate AMPK in palmitate-induced cellular steatosis via the ROS pathway.

AMPK inhibition retarded luteolin-induced reduction in lipid accumulation

The functional relationship between AMPK activation and lipid content was further examined after inhibition of AMPK activity by a selective AMPK inhibitor compound C, which has been used widely to evaluate the roles that AMPK plays in various cellular processes (Hwang *et al*., 2007; Zhou *et al*., 2001). As shown in Fig. 6, HepG2 cells pre-treated with compound C (40 μM) for 30 min significantly impeded the luteolin-induced prevention of increased intracellular TG caused by palmitate. Interestingly, in the presence of NAC TG accumulation following palmitate treatment in HepG2 cells was also attenuated, which was not retarded by compound C (Fig. 6).

DISCUSSION

Flavonoids are ubiquitous dietary components and have been suggested to be consistently associated with a reduced risk of developing chronic diseases, including diabetes mellitus, cardiovascular disease, carcinogenesis as well as NAFLD and other diseases (Eyre *et al*., 2004; Stickel and Schuppan, 2007). As a member of the flavonoid family, luteolin has been shown in a number of previous studies to reduce cholesterol levels *in vitro* (Andrikopoulos *et al*., 2002; Gebhardt, 2002), and, as a component in extracts of *Salix matsudanda* leaves, have antiobesity effects by reducing plasma triacylglycerol levels in animal models (Han *et al*., 2003). Several flavonoids have been reported to reduce hyperlipidaemia and hyperglycaemia, and their effects on lipogenesis have been suggested to be mediated via activation of AMPK (Baur *et al*., 2006; Lin *et al*., 2007; Zang *et al*., 2006). Recently, it was also reported that baicalin, a flavonoid, protected against the development of hepatic steatosis and obesity in rats induced by a long-term high-fat diet and its protective effect is associated

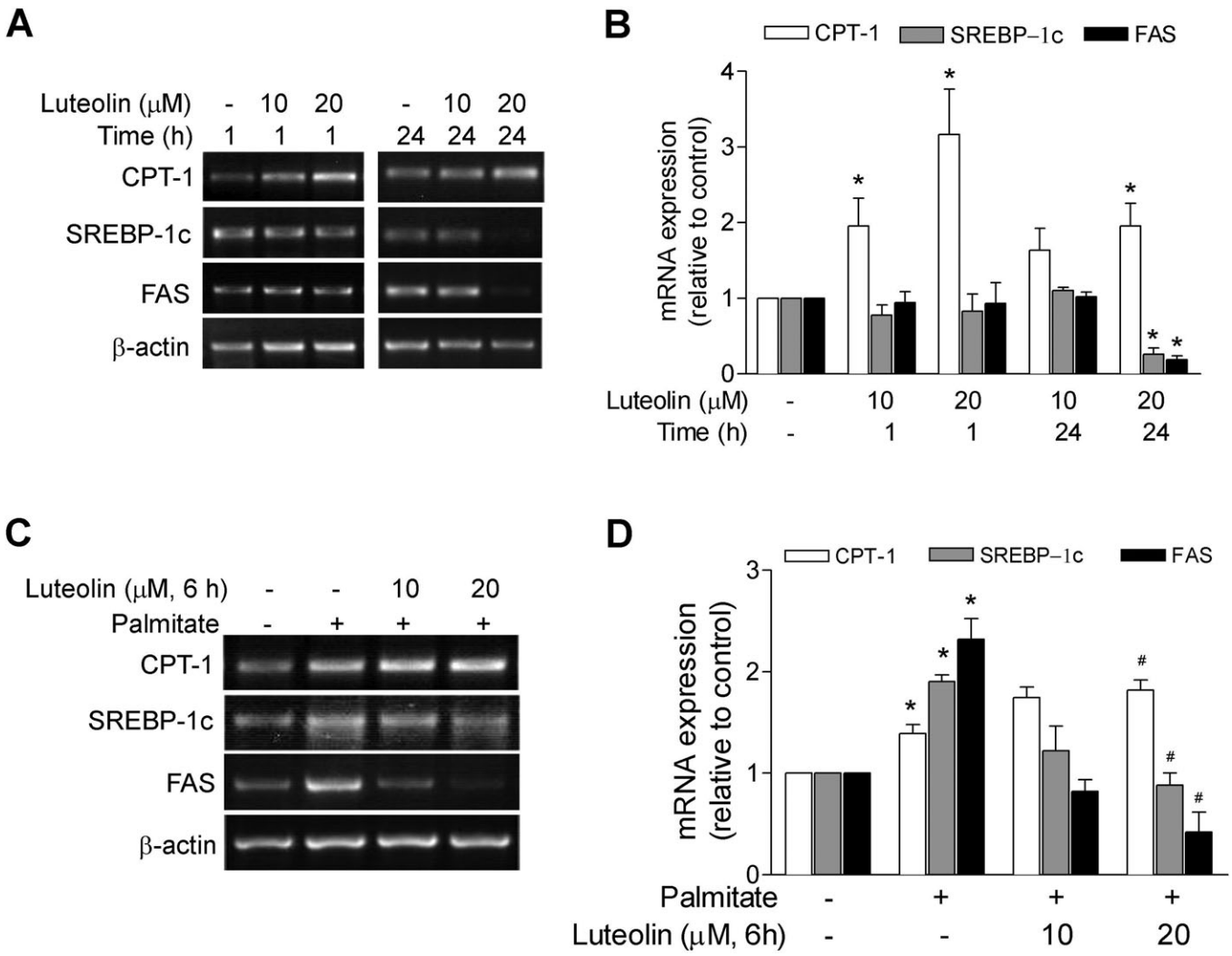

Figure 4. Luteolin up-regulates mRNA expression of CPT-1 and down-regulates the mRNA expression of SREBP-1c and FAS in HepG2 cells. The cells were incubated with 10 and 20 μM luteolin for the indicated times in the absence (A and B) or presence (C and D) of palmitate (0.4 mM, 24 h). The expressions of CPT-1, SREBP-1c and FAS mRNA in cells were assayed by reverse transcription PCR. All PCR reactions were normalized for β-actin expression. Data were expressed as mean ± SE relative to the corresponding basal mRNA level from at least three independent experiments. * $p < 0.05$ vs respective control; # $p < 0.05$ vs respective untreated palmitate control.

mainly with a significant enhancement of hepatic AMPK activation and suppression of hepatic SREPB-1c and FAS gene expression (Guo *et al.*, 2009). The structure of luteolin, 3′,4′,5,7-tetrahydroxyflavone, is similar to baicalin (5,6,7-trihydroxyflavone). It will be very interesting to study whether the lipid-lowering effect of luteolin is mediated by regulating AMPK activation.

The present study investigated the ability of luteolin to prevent fat deposition by using a cell model of steatosis induced by palmitate. The results showed that 0.4 mM palmitate treatment resulted in a remarkable fat accumulation in HepG2 cells demonstrated by Oil Red O staining (Fig. 2) as well as by cellular TG measurement (Fig. 6). Luteolin (20 μM) significantly reduced the amount of lipid almost to basal level (Fig. 2) without producing a cytotoxic effect (Fig. 1). In addition, palmitate markedly increased the genes involved in lipogenesis, including FAS and its upstream regulator SREBP-1c (Fig. 4C and D), suggesting that palmitate incorporation into TG in HepG2 cells appeared to be the SREBP-1c pathway. Luteolin enhanced the phosphorylation of AMPK and ACC (Fig. 3), up-regulated CPT-1 gene expression and down-regulated the gene expression of SREBP-1c and FAS in HepG2 cells in the absence (Fig. 4A and B) or presence of palmitate (Fig. 4C and D). Furthermore, inhibition of AMPK activation by compound C, a selective AMPK inhibitor, retarded luteolin-induced reduction in lipid accumulation caused by palmitate (Fig. 6). The results suggested that the lipid-lowering effect of luteolin is, at least in part, mediated by the up-regulation of CPT-1 and down-regulation of SREBP-1c and FAS gene expressions, possibly via activation of the AMPK signaling pathway.

SREBP-1c is the most important transcription factor regulating *de novo* lipogenesis in the liver and is responsible primarily for the regulation of genes involved in fatty acid biosynthesis including FAS (Shimano *et al.*, 1999). SREBP-1c plays a considerable role in the pathogenesis of NAFLD (Ahmed and Byrne, 2007). Increased SREBP-1c levels have been found in patients with histologically diagnosed NAFLD (Kohjima *et al.*, 2008), and in the fatty livers of obese (ob/ob) mice (Ahmed and Byrne, 2007) and obese rats induced by fat-diet feeding (Madsen *et al.*, 2003). It is known that SREBP-1c is negatively regulated by AMPK (Zhou

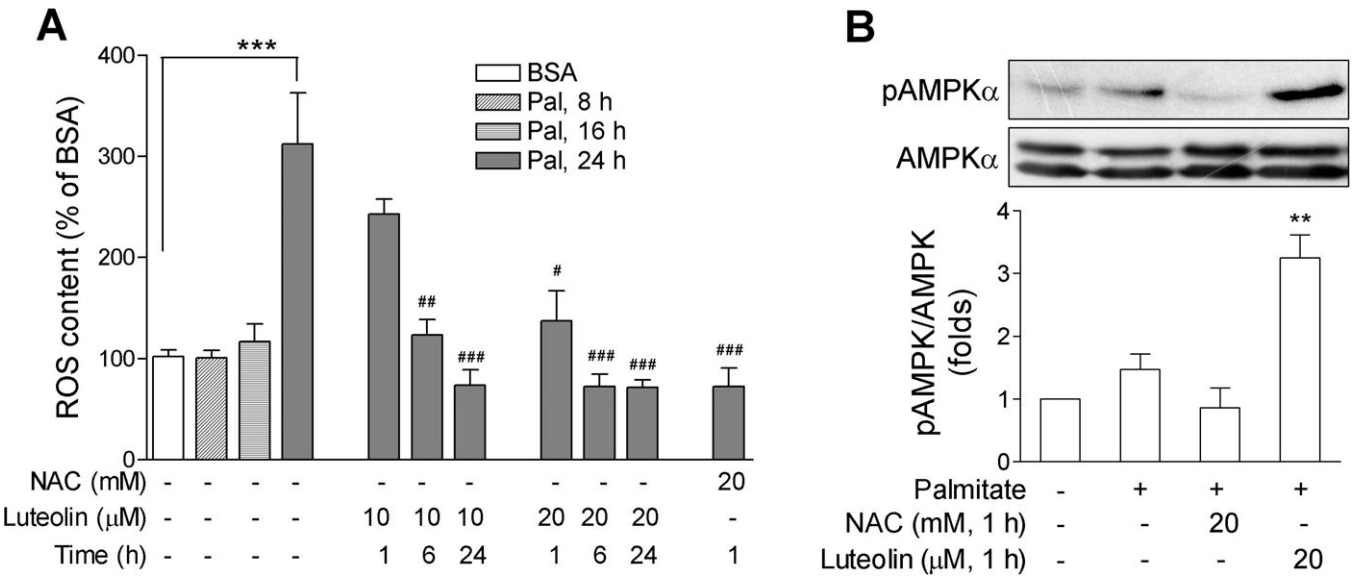

Figure 5. Effects of luteolin and *N*-acetyl-L-cysteine (NAC) on palmitate (Pal)-induced ROS production and AMPKα phosphorylation. HepG2 cells were incubated with 0.4 mM Pal for 8 or 16 h in the absence, or for 24 h in the presence of luteolin (10 and 20 μM) or NAC (20 mM) for the indicated times. Control cells were incubated with 1.76% bovine serum albumin (BSA) for 8, 16 or 24 h, respectively. (A) Intracellular ROS production quantified using the fluorescent probe DCFDA. (B) Western blot analysis for the phosphorylation of AMPKα and total AMPKα. Note the different concentration units between NAC and luteolin. Data were expressed as mean ± SE relative to BSA control from at least four independent experiments. ** $p < 0.01$ and *** $p < 0.001$ vs BSA control; # $p < 0.05$ and ### $p < 0.001$ vs untreated Pal (24 h) control.

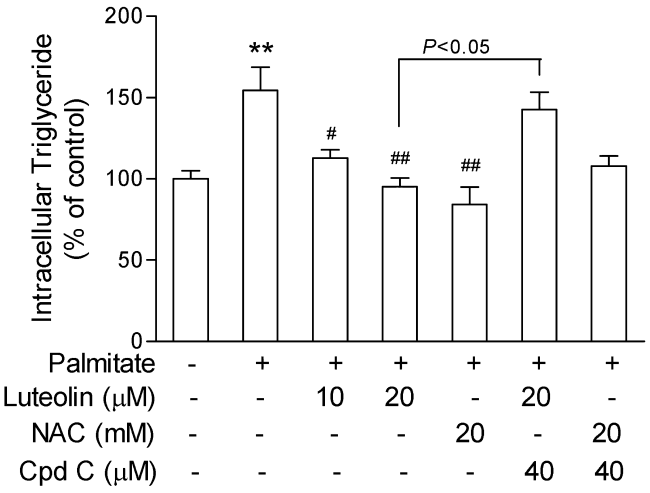

Figure 6. Treatment with AMPK inhibitor compound C blunts luteolin-induced reduction in intracellular triglyceride accumulation in HepG2 cells triggered by palmitate. The cells were treated with luteolin (10 and 20 μM) or *N*-acetyl-L-cysteine (NAC, 20 mM) in the presence of 0.4 mM palmitate for 24 h without or with pre-incubated with 40 μM compound C (Cpd C) for 30 min. The control cells were incubated with 1.76% bovine serum albumin (BSA) for 24 h. The histogram represents the mean of the percentage of the vehicle control ± SE from at least three independent experiments. ** $p < 0.01$ vs BSA control; # $p < 0.05$ and ## $p < 0.01$ vs untreated palmitate control.

et al., 2001). AMPK is a key enzyme related to energy adjustment in the cells. Two major classes of insulin-sensitizing drug, metformin and the thiazolidinediones, activate AMPK in hepatocytes and in turn reduce hepatic SREBP-1c levels and lower lipid accumulation in the livers of insulin-resistant rats (Fryer *et al*., 2002; Ye *et al*., 2004; Zhou *et al*., 2001). In the present study, it was found that 0.4 mM palmitate significantly increased SREBP-1c and FAS expression in parallel with AMPK phosphorylation was unchanged, indicating that the negative feedback regulation of SREBP-1c via AMPK failed in FA-overloading conditions. Luteolin at 20 μM markedly enhanced AMPK phosphorylation and decreased SREBP-1c and FAS expression in HepG2 cells. Furthermore, under FA-overloading conditions, luteolin was still able to activate AMPK and suppressed the increased gene expression of SREBP-1c and FAS caused by palmitate, implying that activation of AMPK stimulated by luteolin may lead to down-regulation of SREBP-1c and FAS expression, which may contribute to luteolin-mediated reduction of lipid accumulation in FA-overloaded HepG2 cells. Previous studies have also shown that luteolin is able to inhibit lipogenesis in prostate and breast cancer cells probably via blockage of the enzymatic activity of FAS (Brusselmans *et al*., 2005).

Palmitate known as a lipototoxic fatty acid might increase oxidative stress and ROS production and induce insulin resistance in hepatocytes (Nakamura *et al*., 2009). Indeed, ROS levels are increased in clinical conditions associated with insulin resistance, such as obesity and type 2 diabetes (Furukawa *et al*., 2004; Keaney, Jr. *et al*., 2003). Increased cellular ROS levels are known to stimulate SREBP-1c activity (Sekiya *et al*., 2008). The results also showed that treatment of HepG2 cells with 0.4 mM palmitate for 24 h induced cellular oxidative stress and ROS generation (Fig. 5A), which was in parallel with increased SREBP-1c and FAS mRNA expression and was associated with intracellular lipid accumulation. These results suggest that saturated FA, palmitate, can cause ROS production and oxidative stress, which might also contribute to the stimulation of SREBP-1c activity and lead to cellular steatosis. Luteolin exhibits obvious antioxidative effect and has been shown to be able to scavenge ROS (Harris *et al*., 2006). Consistently, it was found that suppression of SREBP-1c and FAS gene expression by luteolin is in parallel with the inhibition of ROS production in

palmitate-stimulated HepG2 cells (Fig. 5A). The results indicate that in addition to the activation of AMPK by luteolin, a decrease in the ROS production may also contribute to a reduction of lipid accumulation in HepG2 cells induced by palmitate.

CPT-1, regulating acyl-CoA inflow and β-oxidation in the mitochondrial outer membrane, is a rate-limiting step for fatty acid oxidation (McGarry and Brown, 1997). AMPK causes phosphorylation and inhibition of ACC, which reduces the production of malonyl-CoA, an allosteric inhibitor of CPT-1 (McGarry and Brown, 1997). Thus AMPK activation appears to be important in regulating CPT-1 activity. The results showed that, concomitant with enhancement of AMPK activity by promoting AMPK phosphorylation, and inhibition of ACC activation by increasing ACC phosphorylation, luteolin up-regulated CPT-1 gene expression in HepG2 cells, suggesting that luteolin may also reduce lipid levels via promotion of hepatic fatty acid oxidation. Moreover, AMPK inhibitor compound C significantly impeded luteolin-induced prevention of increased intracellular TG accumulation caused by palmitate, implying an important role of AMPK activation in the lipid-lowering effect of luteolin. The details of the basis for luteolin-mediated activation of AMPK remain unknown.

In summary, it was demonstrated that luteolin decreases the gene expression of SREBP-1c and FAS, and enhances CPT-1 expression in HepG2 cells. The results suggest that the activation of the AMPK signaling pathway may play a critical role in the suppression effect of luteolin on SREBP-1c and FAS as well as in the promotion effect of luteolin on CPT-1. The results also indicate that the lipid-lowering effect of luteolin might be due partly to its strong antioxidant actions. These findings may provide molecular evidence for the use of luteolin as a therapy for the management of NAFLD.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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