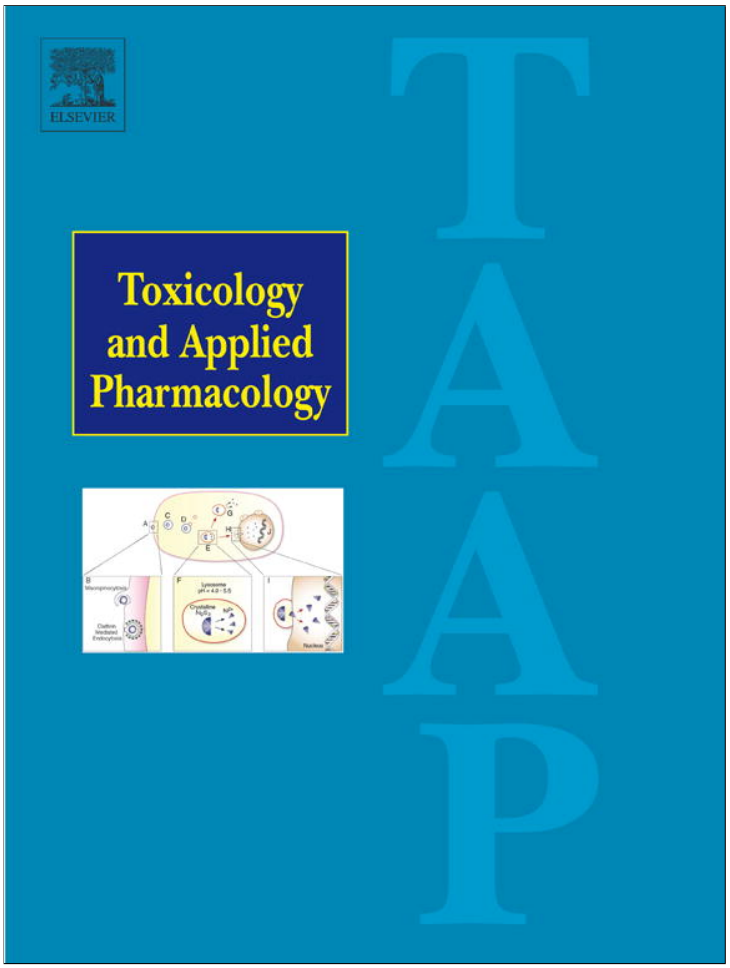

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Saponins, especially platycodin D, from *Platycodon grandiflorum* modulate hepatic lipogenesis in high-fat diet-fed rats and high glucose-exposed HepG2 cells

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ABSTRACT

AMP-activated protein kinase (AMPK) plays a central role in controlling hepatic lipid metabolism through modulating the downstream acetyl CoA carboxylase (ACC) and sterol regulatory element-binding protein-1c (SREBP-1c) pathway. Saponins, particularly platycodin D, from the roots of *Platycodon grandiflorum* (Changkil saponins, CKS) have a variety of pharmacological properties, including antioxidant and hepatoprotective properties. The aim of this study was to investigate the effects of CKS on hepatic lipogenesis and on the expression of genes involved in lipogenesis, and the mechanisms involved. CKS attenuated fat accumulation and the induction of the lipogenic genes encoding SREBP-1c and fatty acid synthase in the livers of HFD-fed rats and in steatotic HepG2 cells. Blood biochemical analyses and histopathological examinations showed that CKS prevented liver injury. CKS and platycodin D each increased the phosphorylation of AMPK and acetyl-CoA carboxylase in HFD-fed rats and HepG2 cells. The use of specific inhibitors showed that platycodin D activated AMPK via SIRT1/CaMKKβ in HepG2 cells. This study demonstrates that CKS or platycodin D alone can regulate hepatic lipogenesis via an AMPK-dependent signalling pathway.

Introduction

Maintaining energy balance depends on the efficiency of tightly regulated mechanisms of energy intake and expenditure. Excessive fat accumulation in the body causes obesity and results in an increased risk for many serious diseases, including diabetes, cardiovascular diseases, nonalcoholic fatty liver disease (NAFLD), hypertension, and hyperlipidaemia, as well as other health problems (Kopelman, 2000). To control body weight, many different approaches besides diet therapy and exercise have been suggested. They include drugs for weight loss or loss of appetite, and food supplements. Lipid-lowering drugs have been used to inhibit hepatic steatosis; however, these drugs can cause serious side effects such as vomiting, headache, stomach ache, and heart attack (Ioannides-Demos et al., 2006). Some medicinal herb extracts with less significant side effects have been reported to be useful for the control of fat liver, hyperlipidaemia, and blood glucose concentrations (Vermaak et al., 2011). Recently, various saponin-containing natural products have emerged as potential lead compounds, drug alternatives, and/or nutritional supplements (Osbourn et al., 2011; Sparg et al., 2004).

Adenosine monophosphate (AMP)-activated protein kinase (AMPK) has been identified as a major regulator of glucose metabolism, including glucose transport, lipogenesis, gluconeogenesis, and lipolysis (Hardie, 2004). AMPK is a serine-threonine kinase that is activated following a rise in the intracellular AMP/ATP ratio. Numerous studies have characterised the effect of AMPK activation on liver metabolism (Winder and Thomson, 2007). Classical targets for the system include acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), and 3-hydroxy-3-methylglutaryl-CoA reductase, which catalyse the key regulatory steps in fatty acid and sterol syntheses. AMPK activation leads to concomitant inhibition of fatty acid synthesis and activation of fatty acid oxidation (Viollet et al., 2007). Thus, it has emerged as a therapeutic target for metabolic disorders (Zhang et al., 2009).

Sterol regulatory element-binding proteins (SREBPs) are transcription factors that regulate the expression of lipogenic enzymes such as ACC, FAS, and 3-hydroxy-3-methylglutaryl-CoA reductase (Goldstein et al., 2002). Studies have reported that increased SREBP expression was strongly associated with fatty liver in two mouse models of diabetes mellitus (Horton et al., 2002; Yahagi et al., 2002). Recently, it was reported that AMPK inactivates SREBP-1 and inhibits hepatic steatosis

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in high-fat diet-induced animal models (Kim et al., 2010). AMPK and SREBPs are particularly promising therapeutic targets to prevent fatty liver disease. Furthermore, SREBP-1c expression is transcriptionally regulated by the nuclear receptors, liver X receptor (LXR)-α and -β, a family of transcription factors involved in the control of glucose, lipid, and cholesterol metabolism (Calkin and Tontonoz, 2012). A previous study showed that D-glucose and D-glucose-6-phosphate are direct agonists of both LXRα and LXRβ (Mitro et al., 2007).

Platycodi radix, the root of *Platycodon grandiflorum* (*P. grandiflorum*) A. DC. (Campanulaceae family), has been used as a food and traditional oriental medicine to treat chronic adult diseases (e.g., bronchitis, asthma, pulmonary tuberculosis, hyperlipidaemia, and hypercholesterolemia) and inflammatory diseases (Kim et al., 1995; Takagi and Lee, 1972). Recently, saponins from the root of *P. grandiflorum* (Changkil saponins, CKS) showed novel pharmacological potential as a treatment for metabolic diseases such as hyperlipidaemia and diabetes (Han et al., 2000; Kim et al., 1995; Noh et al., 2010). In our earlier study, CKS inhibited ethanol-induced liver injury through AMPK activation (Khanal et al., 2009). A previous study reported that CKS contains triterpenoid saponins such as deapio-platycoside E, platycoside E, deapio-platycodin D3, platycodin D3, polygalacin D2, platyconic acid A, platycodin D2, platycodin D, and 2′-*O*-acetylpolygalacin D2 (Noh et al., 2010). Platycodin D, a naturally occurring saponin in the roots of *P. grandiflorum*, has various pharmacological activities, including anti-inflammatory, anti-hyperlipidemic, and anti-obesity effects (Ahn et al., 2005; Lee et al., 2010; Zhao et al., 2006). Furthermore, Ahn et al. reported that *P. grandiflorum* saponins inhibited iNOS and cyclooxygenase 2 (COX-2) by suppressing NF-κB and that among the triterpenoid saponins from *P. grandiflorum*, platycodin D had the most potent biological effect (Ahn et al., 2005). CKS and platycodin D have been shown to be effective against obesity and hyperlipidaemia. However, insufficient information is available regarding the CKS-induced mechanism related to AMPK signalling and the regulation of lipid disorders.

It is well known that high-fat diets (HFDs) are responsible for the high global prevalence of obesity or NAFLD (Feldeisen and Tucker, 2007). Rodents fed a lard-based HFD showed visceral adiposity, hyperglycemia, dyslipidaemia, hyperinsulinemia, and hepatic steatosis, which are distinctly linked with human obesity and NAFLD (Hariri and Thibault, 2010). The present study investigated whether CKS inhibits HFD-induced hepatic steatosis and its mechanistic basis. Our findings demonstrate that CKS and platycodin D, which was isolated from CKS, have the ability to prevent SREBP-1c-dependent lipogenesis in the liver via AMPK activation, thereby reversing hepatic steatosis. Moreover, treatment of hepatocytes with CKS or platycodin D activated AMPK via calcium/calmodulin-dependent protein kinase kinase β (CaMKKβ) and silent information regulator T1 (SIRT1). The beneficial effects of CKS and platycodin D suggest their potential for therapeutic application in lipogenesis and other metabolic dysfunctions.

Materials and methods

Materials. Compound C, Nile red, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), nicotinamide, and STO-609 were purchased from Sigma Chemical Co. (St. Louis, MO). A lactate dehydrogenase (LDH) release detection kit and a BCA protein assay kit were obtained from Roche Applied Science (Indianapolis, IN) and Bio-Rad Laboratories, Inc. (Hercules, CA), respectively. An enhanced chemiluminescence (ECL) system and polyvinylidene difluoride (PVDF) membrane were supplied by Amersham Pharmacia Biotech (Uppsala, Sweden). The plasmid pCMV-β-gal was purchased from Clontech (Palo Alto, CA).

Lipofectamine™ 2000 and a SYBR® Safe DNA Gel Stain kit were from Invitrogen Co. (Carlsbad, CA). Oligonucleotide PCR primers were custom-synthesised by Bioneer Co. (South Korea). Primary antibodies against p-AMPKα (Thr172), p-ACC (Ser79), p-CaMKIIα (Thr286), AMPKα, ACC, FAS, SREBP-1c, SIRT1, and β-actin as well as horseradish-peroxidase (HRP)-linked anti-rabbit and anti-mouse IgG were obtained from Cell Signaling Technologies (Beverly, MA) and Santa Cruz Biotechnology (Santa Cruz, CA). Platycodin D was kindly provided by Dr. Hyun-Sun Lee (KRIBB, Daejeon, Korea). All other chemicals and reagents were of analytical grade.

Preparation of CKS and platycodin D. CK, the aqueous extract from 20-year-old Platycodi Radix root, was supplied by Jangsaeng Doraji Co., Ltd. (Jinju, South Korea) and prepared as described previously (Lee and Jeong, 2002). The composition of CK has been reported (Kim et al., 2010). CK was subjected to column chromatography over Amberlite XAD-2, Diaion MCl Gel HP20, or Kogel BG4600. After removing the saccharides and amino acids with water, the column was eluted with methanol to obtain CKS, which is the saponin fraction of CK, as described previously (Noh et al., 2010). Platycodin D was purified by high-speed countercurrent chromatography as previously described, and its purity was determined to be 96.3% by HPLC coupled with an evaporative light scattering system (Noh et al., 2010). Purified CKS and platycodin D were stored at −20 °C until use and were dissolved in distilled water immediately prior to administration.

Animals and diets. Four-week-old male Sprague–Dawley (SD) rats were obtained from Samtako (Osan, Korea). A control diet (normal diet, ND) and a HFD (60% kcal fat; D12492) were obtained from Research Diets, Inc. (New Brunswick, NJ). All animals were housed under controlled temperature (22 °C ± 2 °C) and humidity (50% ± 5%) with a 12-h light:12-h dark cycle and had free access to food and water. The SD rats were divided into two groups and fed the ND ($n = 5$) or the HFD ($n = 20$). After 4 weeks of feeding to induce obesity, the 20 animals in the HFD group were divided into four experimental groups of five rats each: group 1 received the HFD and groups 2, 3, and 4 received the HFD plus CKS at 0.5, 1, or 2 mg/kg/day, respectively. CKS was dissolved in saline and administered at the prescribed dose (approximately 200 μl per rat) by oral gavage daily for 4 weeks. Rats that did not receive CKS (the ND and HFD groups) received an equal volume of saline. During the experiment, the body weights were recorded. At the end of the experimental period, all animals were anesthetised with ether, and blood was collected by cardiac puncture. The livers were excised, weighed, and examined histopathologically. The animal protocols and experiments were performed according to the rules and regulations of the animal ethics committee of Chungnam National University.

Cell culture and measurement of cell cytotoxicity. HepG2 cells were maintained in DMEM containing normal glucose (5.5 mM D-glucose) and supplemented with 10% foetal bovine serum (FBS), 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine (Invitrogen), at 37 °C in a humidified atmosphere of 5% CO_2 in air. HepG2 cells were cultured in complete medium containing 10% FBS until they reached 70% confluence and were used for assays after overnight serum depletion. For treatments, CKS or platycodin D was dissolved in DMSO and added to the medium. The final concentration of DMSO did not exceed 0.1%, which did not affect cell cytotoxicity or AMPKα phosphorylation. A cell model of high-glucose-induced accumulation of intracellular lipids was created by exposing HepG2 cells to a high concentration of glucose (30 mM) for 24 h, as described previously (Zang et al., 2004). Briefly, HepG2 cells were cultured in serum-free DMEM overnight and then incubated in DMEM containing either normal (5.5 mM) or high (30 mM) D-glucose. Cell cytotoxicity was examined by MTT reduction assay and LDH release, using an assay kit according to the manufacturer's instructions.

Determination of cholesterol and triglyceride levels. Intracellular triglyceride and total cholesterol contents were measured in HepG2 cell lysates as previously described (Zang et al., 2004) and are expressed as micrograms of lipid per milligramme of cellular protein. Hepatic cholesterol and triglyceride concentrations were analysed using an

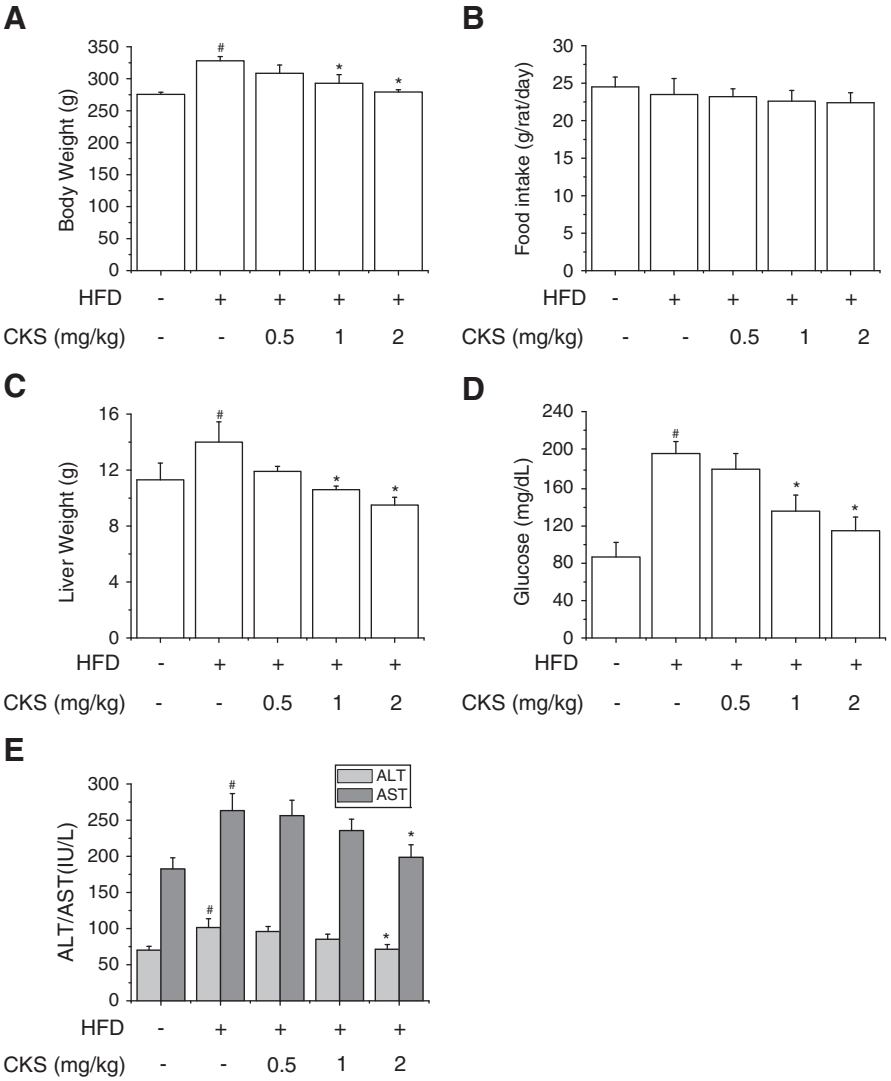

Fig. 1. CKS attenuates HFD-induced weight gain and serum glucose levels. Male SD rats were fed a normal diet (ND) or a high-fat diet (HFD) for 8 weeks. During the final 4 weeks of feeding, CKS (0.5, 1, or 2 mg/kg/day) was intragastrically (i.g.) administered to rats seven times per week. (A and C) The effects of CKS on body and liver weights in HFD-fed rats. (B) The effect of CKS on food intake by HFD-fed rats. (D) Serum glucose levels in obese rats. (E) The effects of CKS on HFD-induced hepatotoxicity. Hepatotoxicity was determined by quantifying the serum activity of ALT and AST. Results are expressed as means ± SD ($n = 5$). $^{\#}p<0.05$ vs. control group; $^{*}p<0.05$ vs. HFD-fed group.

enzymatic kit (Asan Pharm Co., Seoul, Korea). Liver tissue (100 mg) was homogenised in PBS (1 ml), and the protein concentration was determined. Homogenates were extracted with 5 ml of chloroform: methanol (2:1, v/v) by vigorously vortex-mixing the sample and allowing the two phases to separate, followed by centrifugation at 2000 rpm for 10 min at 4 °C. An aliquot of the organic phase was evaporated to dryness under nitrogen gas. Hepatic total cholesterol and triglyceride concentrations were determined and normalised to the protein concentration. Concentrations are expressed as milligrammes of lipid per gramme of tissue protein (Lee and Jeong, 2002).

Nile red staining assay. Platycodin D was added to cells in fresh DMEM with 0.1% dimethyl sulfoxide (DMSO; final concentration). After 24-h exposure, cells were washed with Hank's buffered salt solution (HBSS), and background fluorescence was detected with a spectrofluorometer (Varioskan; Thermo Electron Co.) by measuring the emission at 535 nm after excitation at 580 nm. Nile red was then freshly diluted from a 1 mM stock solution in DMSO with 1% Pluronic F127 (Sigma) to 1 mM in HBSS, and 100 ml was added to each culture well. After a 1-h incubation at room temperature in the dark, Nile red was removed, and cells were washed once with HBSS. After further incubation for 1 h in HBSS at room temperature in the dark, fluorescence was again determined, as above, and the background fluorescence was subtracted to determine the fluorescence due to bound Nile red.

Transient transfection and reporter gene assays. Luciferase reporter constructs containing the wild-type SREBP-1c promoter (pSREBP (−1516/+40)-luciferase) were gifts from Dr. Cagen (University of Tennessee, TN). Cells were co-transfected with 0.5 μg of a plasmid containing the wild-type *SREBP-1* promoter linked to the firefly luciferase reporter gene and 25 ng of the control plasmid phRL-TK, containing the *Renilla* luciferase reporter gene, using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA, USA). Relative luciferase activity was calculated by normalising *SREBP-1* promoter-driven firefly luciferase activity to *Renilla* luciferase activity.

Western blotting. Treated cells were collected, washed with PBS and lysed on ice for 30 min in 100 μl of lysis buffer (120 mM NaCl, 40 mM Tris, pH 8, 0.1% NP40). Lysates were clarified by centrifugation at 13,000 ×*g* for 15 min. The supernatants were collected, and the protein concentration was determined using a BCA protein assay. Lysates samples containing 80 μg of protein were subject to 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE),

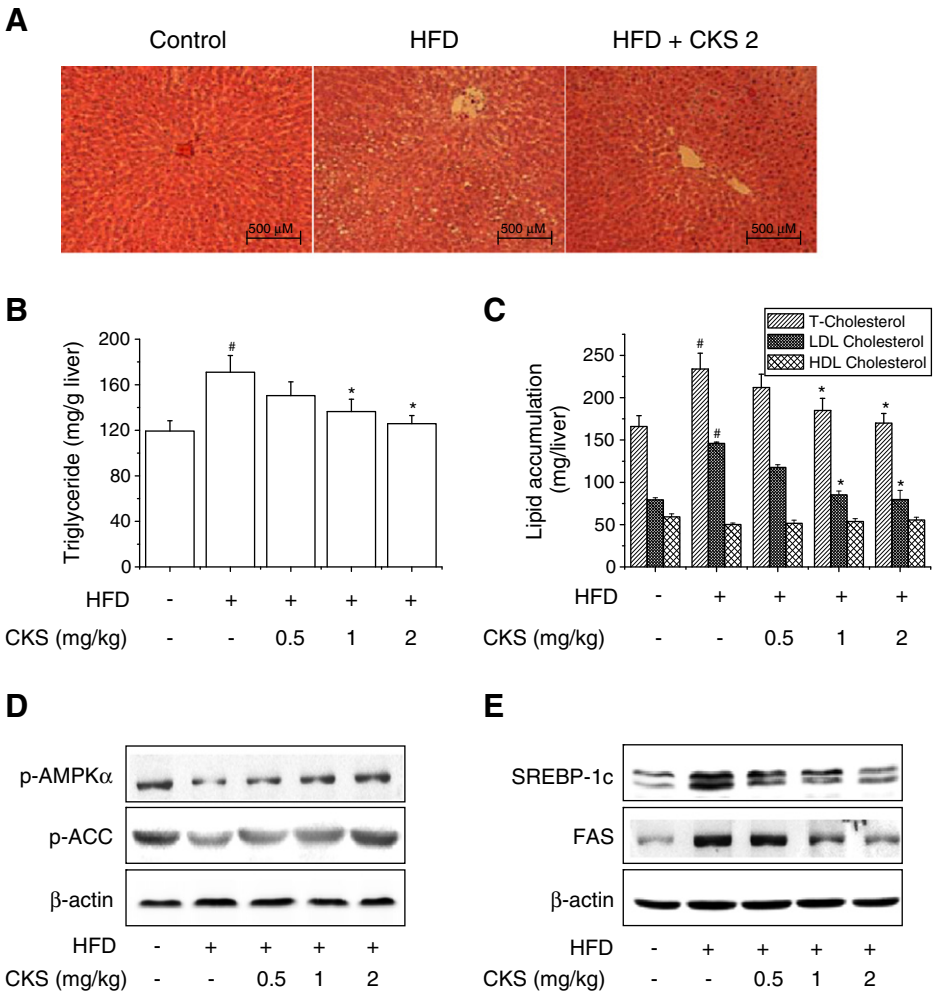

Fig. 2. CKS prevents high-fat diet-induced liver steatosis in rats. (A) The effect of CKS on fatty droplet accumulation in the livers of ND- and HFD-fed rats. Photomicrographs of liver tissue sections stained with hematoxylin and eosin are shown at a magnification of 100×. (B and C) Anti-steatotic effect of CKS. The extent of hepatic triglyceride, total cholesterol, LDL cholesterol, and HDL cholesterol accumulation was assessed in HFD-fed rats treated with vehicle or CKS. Results are expressed as means ± SD ($n = 5$). $^{\#}p<0.05$ vs. control group; $^{*}p<0.05$ vs. HFD-treated group. (D) The effect of CKS on AMPKα and ACC phosphorylation in rats with HFD-induced liver steatosis. The Western blots shown are representative of three independent experiments. (E) Western blot analysis of SREBP-1c and its target gene FAS in the liver. The Western blots shown are representative of three independent experiments.

electrotransferred to a PVDF membrane, and immunoblotted with rabbit polyclonal antibodies specific for phospho-AMPKα, phospho-ACC, p-CaMKIIα, AMPKα, ACC, FAS, or SREBP-1c, and a mouse monoclonal antibody against β-actin. The membranes were then probed with HRP-conjugated secondary antibodies, and immunoreactive protein bands were detected by enhanced chemiluminescence.

Quantitative real-time RT-PCR (qRT-PCR). PCR product formation was monitored continuously during amplification using Sequence Detection System software version 1.7 (Applied Biosystems, Foster City, CA, USA). The accumulated products detected directly by monitoring the signal from the reporter dye, SYBR® Green. The mRNA expression levels of *FAS* and *SREBP-1c* were compared between treated and control cells at each time point using the comparative cycle threshold (Ct) method. The following primers (5′→3′) were used: SREBP-1c forward, GCC ATG GAT TGC ACT TT; SREBP-1c reverse, CAA GAG AGG AGC TCA ATG; FAS forward, GAA ACT GCA GGA GCT GTC; FAS reverse, CAC GGA GTT GAG GCG CAT; β-actin forward, TGG CAC CCA GCA CAA TGA A; and β-actin reverse, CTA AGT CAT AGT CCG CCT AGA AGC A. The quantity of each transcript was calculated as described in the instrument manual and normalised to the mRNA expression of the housekeeping gene β-actin.

NAD^+/NADH ratio determination. Cells were treated with different concentrations of platycodin D for 30 min and lysed with NAD^+/NADH extraction buffer by freezing and thawing twice. Samples were vortex-mixed and then centrifuged for 5 min. Supernatants were transferred to new tubes. Nicotinamide nucleotides were assayed using a NAD^+/NADH Quantification Kit (BioVision, Mountain View, CA).

Statistical analysis. Results are presented as means ± standard deviation (SD). Statistical analyses were performed using SPSS 9.0 (SPSS Inc., Chicago, IL). Significance was assessed by analysis of various followed by Duncan's test. Significance levels were considered as $p<0.01$ and $p<0.05$.

Results

CKS ameliorated HFD-induced lipid accumulation

This study was carried out to investigate effects of CKS on fatty liver inhibition in HFD-fed rats. The rats were treated with CKS by oral gavage for 4 weeks, and then several parameters were assessed. After 56 days on a HFD, the mean body weight and liver weight in the HFD group were higher than the corresponding values in the normal

diet group, indicating that the HFD induced obesity. CKS administration (2 mg/kg/day) significantly decreased mean body weight and liver weight in the HFD + CKS group relative to the non-CKS-treated HFD group (Figs. 1A and C). The average food intake did not significantly differ among all the groups during final 4 weeks (Fig. 1B). The serum glucose concentration was significantly suppressed in the CKS group compared with the HFD group (Fig. 1D). These results suggest that CKS reduces HFD-induced body weight gain by a mechanism not attributable to a change in food intake. CKS administration also significantly reduced the levels of ALT and AST, which are markers of cell damage, in HFD-fed rats. Serum ALT and AST levels in the HFD rats that received CKS at 2 mg/kg/day were significantly lower than the levels in the HFD rats that did not receive CKS (Fig. 1E).

The gross appearance and hematoxylin and eosin staining of the liver revealed fatty infiltration in the HFD control group compared with rats fed the normal diet. However, fatty infiltration was not observed in the livers of CKS-treated HFD rats (Fig. 2A). As fat accumulation in the liver is the main histological feature of fatty liver diseases such obesity and NAFLD, hepatic lipid accumulation was measured in the rats. The levels of hepatic triglycerides and total cholesterol were increased in the livers of rats fed the HFD (Figs. 2B and C). The levels of hepatic triglycerides, total cholesterol, and low-density lipoprotein (LDL) cholesterol were significantly decreased by treatment with CKS at a daily dose of 1 or 2 mg/kg (Figs. 2B and C). The high-density lipoprotein (HDL) cholesterol level did not differ between the HFD group and the CKS-treated HFD groups (Fig. 2C).

To determine the molecular mechanism of the hypolipidemic effects of CKS, the expression of proteins involved in lipid metabolism were analysed on Western blots. CKS dose-dependently increased the phosphorylation of AMPK and ACC in liver tissue compared with phosphorylation levels in the HFD control group (Fig. 2D). CKS also

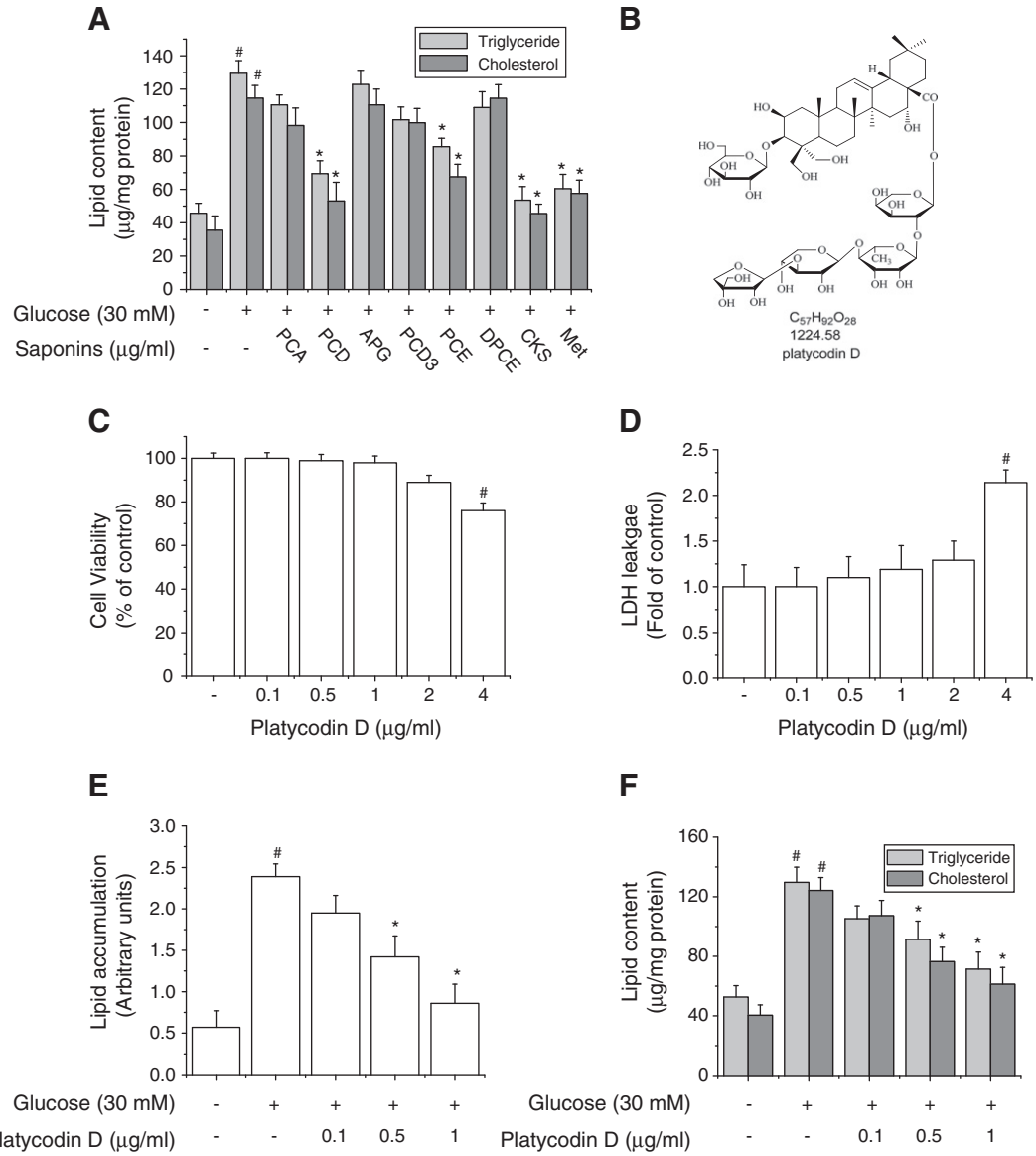

Fig. 3. Platycodin D inhibits high-glucose-induced lipid accumulation in HepG2 cells. (A) Intracellular triacylglycerol and cholesterol levels in cells treated with triterpenoid saponins (1 μg/ml), CKS (1 μg/ml), or metformin (1 mM) for 24 h. Metformin was used as a positive control for lipid-lowering effect. All data are expressed as means ± SD of three independent experiments. $^{\#}p<0.01$ vs. control cells; $^{*}p<0.01$ vs. high-glucose-treated cells. (B) The chemical structure of platycodin D. (C and D) The effect of platycodin D on cell cytotoxicity. HepG2 cells were treated with 0.1–1 μg/ml platycodin D for 24 h, and cytotoxicity was determined by MTT and LDH release assays. All data are expressed as means ± SD of three independent experiments. (E) Intracellular lipids were stained with Nile red and visualized by fluorescence. (F) Intracellular triglyceride and total cholesterol contents were measured. All data are expressed as means ± SD of three independent experiments. $^{\#}p<0.01$ vs. control cells; $^{*}p<0.01$ vs. high-glucose-treated cells.

decreased the levels of FAS and mature SREBP-1c in the liver (Fig. 2E). These results suggest that CKS may inhibit HFD-induced hepatic lipogenesis.

Platycodin D reduces high-glucose-induced lipid accumulation in HepG2 cells

This study was carried out to investigate the hypolipidemic effects of CKS in vitro. First, the cytotoxic effects of the CKS triterpenoid saponins such as platyconic acid (PCA), platycodin D (PCD), 2′-*O*-acetylpolygalacin D2 (APG), platycodin D3 (PCD3), platycoside E (PCE), deapio platycoside E (DPCE) were determined in HepG2 cells. HepG2 cells were treated with 0–4 μg/ml triterpenoid saponins for 24 h, and cytotoxicity was determined by MTT and LDH release assays. Treatment with each triterpenoid saponin at concentrations of 0–4 μg/ml for 24 h showed that saponin concentrations <2 μg/ml were not cytotoxic as determined by the MTT and LDH assays (data not shown). Thus, triterpenoid saponins at concentrations ranging from 0.1 to 1 μg/ml were used in this subsequent experiment. Next, the lipid-lowering effects of the CKS terpenoid saponins were examined in HepG2 cells in the absence and presence of high glucose (30 mM) for 24 h. Among the terpenoid saponins tested, platycodin D and platycoside E significantly decreased triglyceride and total cholesterol accumulations in HepG2 cells in the presence of high glucose (Fig. 3A). Previous studies have shown that platycodin D is the most potent CKS triterpenoid saponin with regard to its effect on peroxyl radical-scavenging activity and its pharmacological effects (Ryu et al., 2011). Intracellular levels of triacylglycerol and cholesterol in HepG2 cells exposed to platycodin D (0, 0.1, 0.5, and 1 μg/ml) for 24 h were also measured. Platycodin D dose-dependently reduced intracellular lipid accumulation (Fig. 3E) without causing cytotoxicity (Figs. 3C and D). As shown in Fig. 3F, platycodin D significantly decreased triglyceride and total cholesterol accumulations in a dose-dependent manner.

Platycodin D reduces high-glucose-induced SREBP-1c and FAS expression in HepG2 cells

To explore the mechanism(s) underlying the lipid-lowering action of platycodin D in HepG2 cells, the expression of SREBP-1c and FAS, important regulators of hepatic lipogenesis, was measured by Western blotting and qRT-PCR. *SREBP-1* promoter activity was also assessed. Both the protein expression (Fig. 4A) and the mRNA expression (Fig. 4B) of FAS and SREBP-1c were reduced in platycodin D-treated HepG2 cells. *SREBP-1*-luciferase gene activity was enhanced by exposure of the cells to a high glucose concentration, and this response was prevented by platycodin D treatment (Fig. 4C). These results indicate that platycodin D treatment decreased the levels of the lipogenic proteins SREBP-1c and FAS in HepG2 cells, suggesting that reduced intracellular lipid accumulation is the mechanism of the lipid-lowering effect of platycodin D in HepG2 cells.

Platycodin D reduces lipid accumulation and SREBP-1c and FAS expression caused by high glucose in an AMPK-dependent manner

To explore platycodin D-induced AMPK activation under high-glucose conditions, HepG2 cells grown in serum-free medium with a normal glucose concentration were pretreated with platycodin D (0.1–1 μg/ml, 1 h) and then exposed to high glucose for an additional

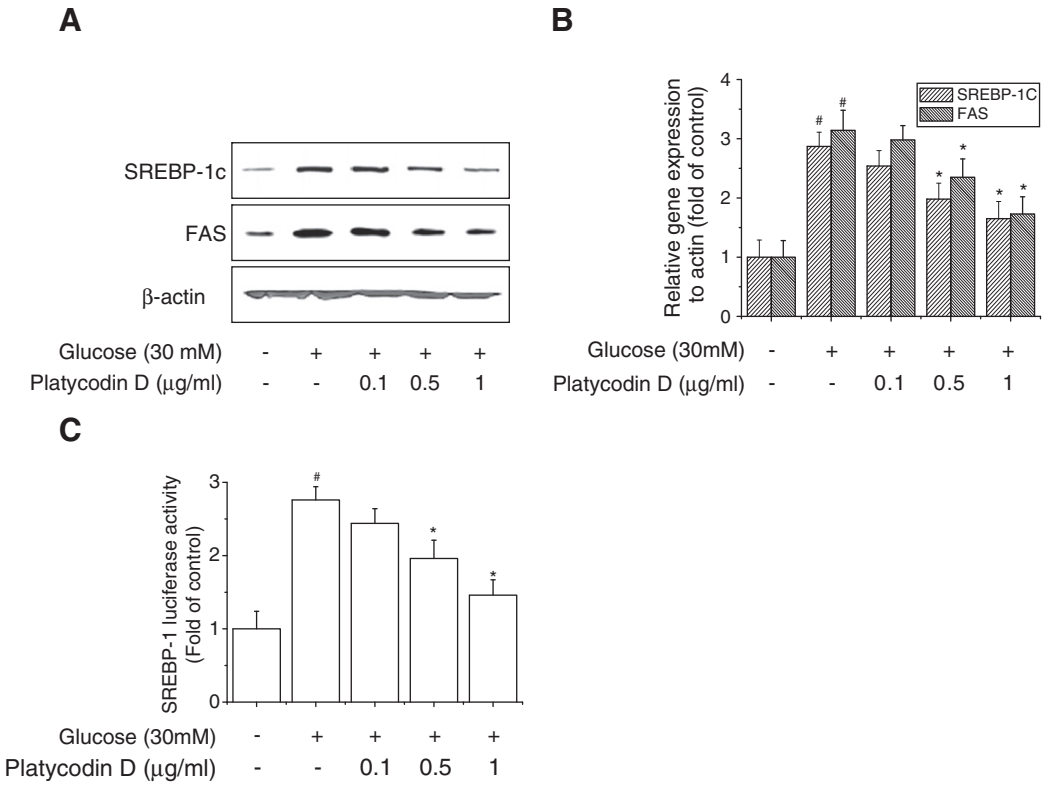

Fig. 4. Platycodin D inhibits high-glucose-induced SREBP-1c and FAS expression. (A–C) HepG2 cells were grown in serum-free medium overnight and incubated in normal or high glucose concentrations in the absence or presence of platycodin D (0.1–1 μg/ml) for an additional 24 h. (A) SREBP-1c and FAS protein levels in cell lysates were measured by Western blotting. (B) Total RNA was extracted and the expression of FAS and SREBP-1c mRNA was measured by qRT-PCR. All data are expressed as means ± SD of three independent experiments. $^{\#}p<0.01$ vs. control cells; $^{*}p<0.01$ vs. high-glucose-treated cells. (C) The effect of platycodin D on *SREBP-1* promoter activity. The cells were transfected with a pSREBP-1c-luciferase construct (500 ng/well) and then treated with 30 mM glucose and/or platycodin D (0.1–1 μg/ml). All data are expressed as means ± SD of three independent experiments. $^{\#}p<0.01$ vs. control cells; $^{*}p<0.01$ vs. high-glucose-treated cells.

1 h. As shown in Fig. 5A, the phosphorylation of AMPKα and ACC was significantly attenuated in cells grown in high glucose (30 mM), and platycodin D dose-dependently restored the phosphorylation of AMPKα and ACC. To confirm the role of AMPK in the regulation of metabolic gene expression, cells were pretreated with compound C (a specific AMPK inhibitor; 1 μM) for 1 h before treatment with 1 μg/ml platycodin D for an additional 1 or 24 h under high-glucose conditions. Compound C completely abolished platycodin D-mediated activation of AMPKα and ACC phosphorylation (Fig. 5B). Compound C pretreatment also attenuated the inhibitory effects of platycodin D on lipid synthesis and SREBP-1c and FAS expression (Figs. 5C and D). Moreover, platycodin D significantly decreased lipid accumulation in cells exposed to high glucose; compound C abolished this inhibitory effect of platycodin D (Fig. 5E). These results suggest that platycodin D inhibits SREBP-1c activation and FAS expression by activating AMPKα in HepG2 cells.

CaMKKβ-dependent AMPK activation by platycodin D

AMPK is also activated by CaMKKβ in response to a rise in the intracellular calcium ion concentration, without detectable changes in the AMP/ATP ratio (Hurley et al., 2005). To evaluate the upstream kinase in platycodin D-induced AMPK activation, the phosphorylation of CaMKIIα were measured by immunobloting in HepG2 cells under normal glucose conditions. Treatment with platycodin D strongly induced the phosphorylation of CaMKIIα in a concentration-dependent manner (Fig. 6A). To confirm the role of AMPK in the regulation of metabolic gene expression, cells were pretreated with STO-609 (a CaMKKβ inhibitor; 1 μM) for 1 h before treatment with 1 μg/ml platycodin D for an additional 24 h under high-glucose conditions. Treatment with 1 μM STO-609 abolished the platycodin D-induced suppression of SREBP-1c and FAS expression (Fig. 6B), the platycodin D-induced suppression of SREBP-1c

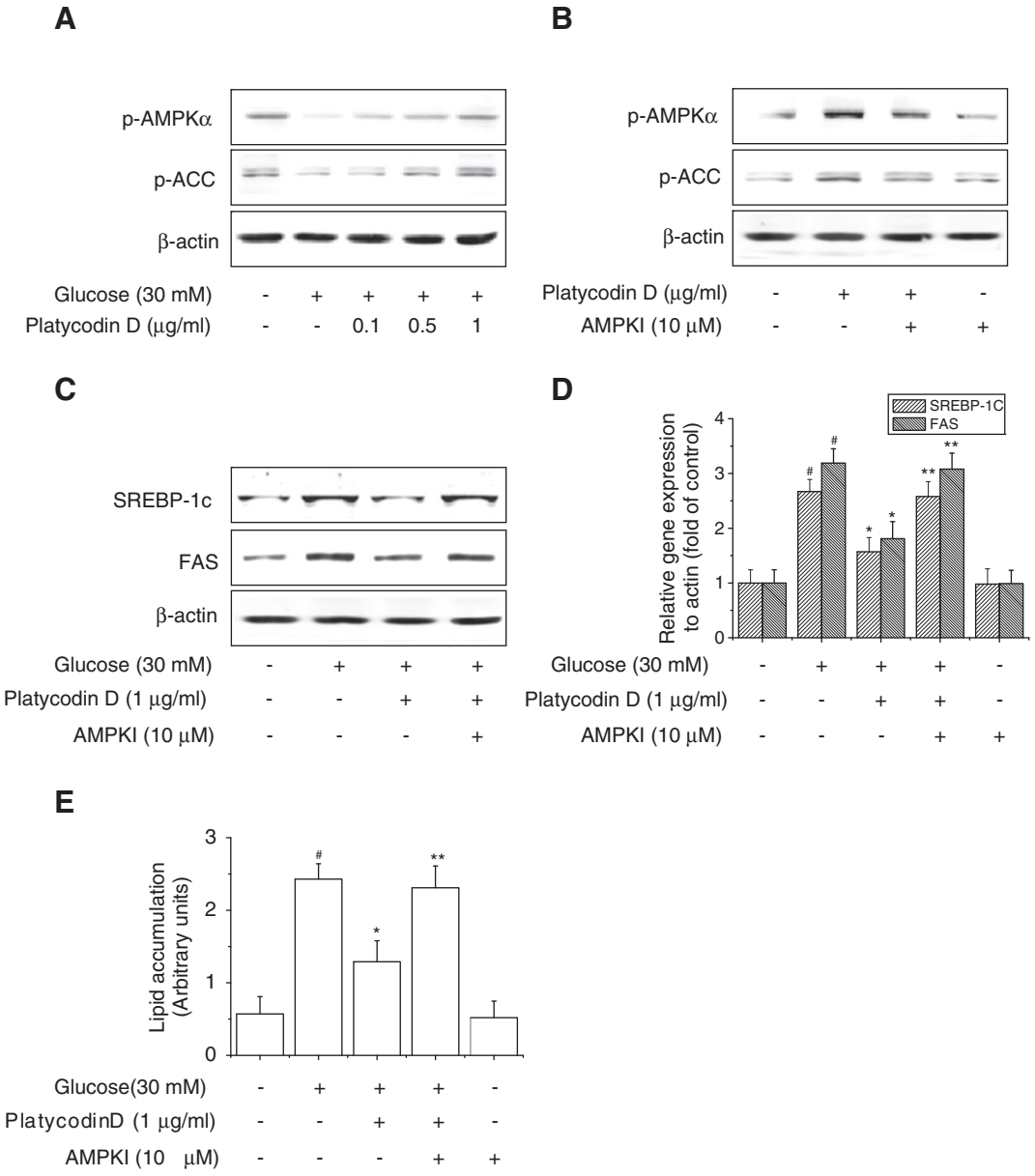

Fig. 5. Platycodin D inhibits high-glucose-induced lipid accumulation and increased SREBP-1c and FAS protein expression in an AMPK-dependent manner. (A) The effect of platycodin D on AMPKα and ACC phosphorylation. (A) Western blot analysis with anti-p-AMPKα (Thr172), anti-p-ACC (Ser79), and anti-β-actin antibodies. β-actin was the protein loading control. (B–E) HepG2 cells were treated with 1 μM compound C, an AMPK inhibitor, for 1 h and then incubated with or without platycodin D (1 μg/ml) for an additional 1 h (B) or 24 h (C–E). (B and C) Western blot analysis of p-AMPKα (Thr172), p-ACC (Ser79), SREBP-1c, and FAS. β-actin was the protein loading control. (D) Total RNA was extracted, and the expression of FAS and SREBP-1c mRNA was measured by qRT-PCR. (E) Lipid-bound Nile red fluorescence was measured using a microplate fluorescence reader. All data are expressed as means ± SD of three independent experiments. $^{\#}p<0.01$ vs. control cells; $^{*}p<0.01$ vs. high-glucose-treated cells; $^{**}p<0.01$ vs. cells treated with high glucose plus platycodin D.

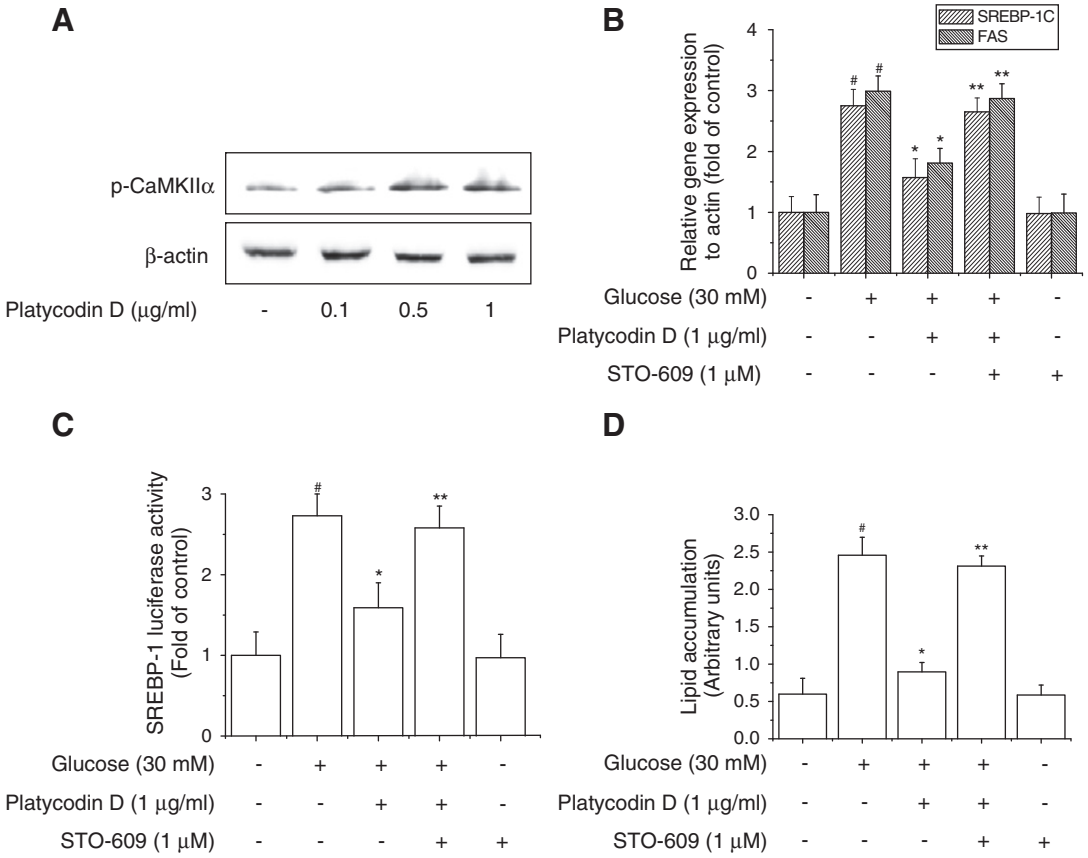

Fig. 6. Platycodin D-induced activation of AMPK is mediated by CaMKKβ. (A) The effects of platycodin D on the phosphorylation of CaMKIIα in HepG2 cells. The cells were incubated in the absence or presence of platycodin D (0.1–1 μg/ml) for 30 min. Whole-cell lysates were extracted and analysed by Western blotting with anti-p-CaMKIIα and anti-β-actin antibodies. β-actin was the protein loading control. (B–D) HepG2 cells were treated with the CaMKKβ inhibitor STO-609 (1 μM) for 1 h and then incubated with or without platycodin D (1 μg/ml) for an additional 24 h. (B) Total RNA was extracted, and the expression of FAS and SREBP-1c mRNA was measured by qRT-PCR. All data are expressed as means ± SD of three independent experiments. (C) *SREBP-1* luciferase activity was measured. (D) Lipid-bound Nile red fluorescence was measured using a microplate fluorescence reader. All data are expressed as means ± SD of three independent experiments. $^{\#}p<0.01$ vs. control cells; $^{*}p<0.01$ vs. high-glucose-treated cells; $^{**}p<0.01$ vs. cells treated with high glucose plus platycodin D.

promoter activity (Fig. 6C), and the platycodin D-induced decrease in lipid accumulation (Fig. 6D). These findings suggest that CaMKKβ may be required for AMPK-dependent SREBP-1c and FAS expression and lipid accumulation.

Pharmacological inhibition of SIRT1 attenuates platycodin D-induced AMPK activation

To investigate the role of another upstream kinase in platycodin D-induced AMPK activation, the effect of platycodin D on SIRT1 activity were also measured in HepG2 cells. Recent studies have suggested that SIRT1 is closely associated with lipid metabolism (Kume et al., 2010; Lomb et al., 2009). Resveratrol, a well-known antioxidant, has been shown to stimulate SIRT1 activity (Howitz et al., 2003). Platycodin D is a naturally occurring antioxidant with potent reactive oxygen species-scavenging activity (Han et al., 2000). Given that SIRT1 is a NAD^+-dependent enzyme and acts as a metabolic sensor of NAD^+, the NAD^+/NADH ratio were measured in HepG2 cells. Treatment of HepG2 cells with platycodin D for 30 min significantly increased the NAD^+/NADH ratio (Fig. 7A). Moreover, platycodin D-induced AMPKα and ACC phosphorylation was abolished by treatment with nicotinamide (5 mM), a SIRT1 inhibitor (Fig. 7B), as were the platycodin D-induced decreases in SREBP-1c expression (Fig. 7C) and lipid accumulation (Fig. 7D). These results indicate that SIRT1 may be required for AMPK-dependent SREBP-1c and FAS expression and hepatic lipogenesis.

Discussion

The lipid lowering effects of many natural compounds involve diverse mechanisms, including the regulation of appetite, food intake, lipogenesis, fatty acid oxidation, and adipogenesis (Rayalam et al., 2008). The root of *P. grandiflorum*, which is used as a food and a traditional medicine in Korea, has a wide range of beneficial health effects, including anti-inflammatory actions and the prevention of hyperlipidaemia and diabetes. Platycodin D, a potent and abundant saponin found in the root of *P. grandiflorum*, has various pharmacological activities such as anti-inflammatory, anti-tumour, anti-hyperlipidaemia, and anti-obesity effects (Ahn et al., 2005; Lee et al., 2010; Zhao et al., 2006). In addition, Lee et al. reported that platycodin D inhibits adipogenesis in 3 T3-L1 adipocytes through activation of the WNT/β-catenin pathway (Lee et al., 2010, 2011; Zhao et al., 2006). However, no lipogenic effect has yet been reported for platycodin D. The primary goal of this study was to investigate the effects of CKS, and specifically platycodin D, on hepatic lipogenesis through AMPK signalling and on the expression of genes involved in lipogenesis, and the mechanisms involved. In our animal model, body weight gain, adipose tissue weight, and serum triglycerides significantly lowered by CKS administration, which did not alter food intake.

In general, the development of fatty liver is strongly associated with obesity and NAFLD (James and Day, 1999; Kopelman, 2000). HFD feeding induced the accumulation of numerous fatty droplets, which is typical of fatty liver. CKS administration reduced the accumulation of lipid droplets and the signs of liver pathology, and protected

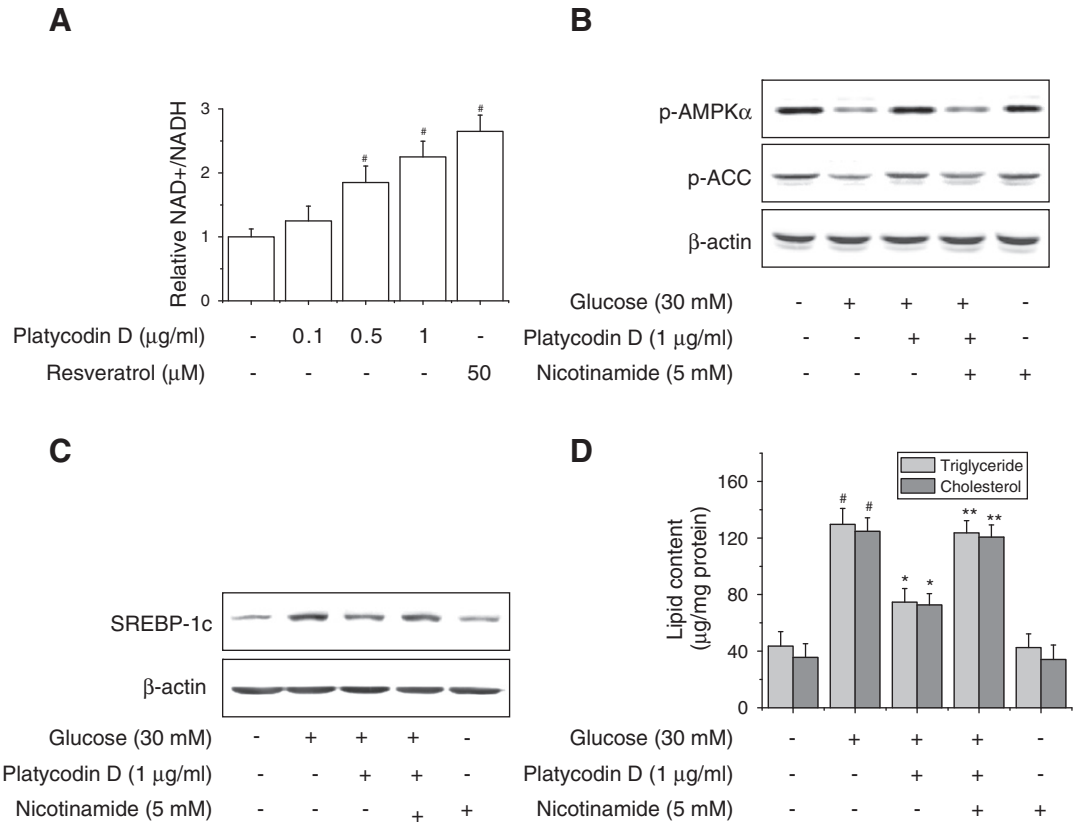

Fig. 7. SIRT1-dependent activation of AMPK by platycodin D. (A) The effect of platycodin D on the cellular NAD^+/NADH ratio. The concentrations of NAD^+ and NADH were measured in cells for 30 min. All data are expressed as means ± SD of independent experiments. $^{\#}p<0.01$ vs. control cells. (B–D) The cells were treated with 5 mM nicotinamide for 30 min and then incubated with 1 μg/ml platycodin D for 1 h (B) or 24 h (C and D). (B and C) Western blot performed with anti-p-AMPKα (Thr172), anti-p-ACC (Ser79), anti-SREBP-1c, anti-FAS, and anti-β-actin antibodies. (D) Intracellular triglyceride and total cholesterol levels were measured using spectrophotometric assays. All data are expressed as means ± SD of three independent experiments. $^{\#}p<0.01$ vs. control cells; $^{*}p<0.01$ vs. high-glucose-treated cells; $^{**}p<0.01$ vs. cells treated with high glucose plus platycodin D.

against an increase in liver tissue weight. It also significantly reduced the serum levels of ALT and AST, which are biochemical markers of liver function. These results suggest that CKS protects against the development of HFD-induced fatty liver, although the underlying molecular mechanisms are unknown.

AMPK activation is associated with metabolic organs, including the liver, skeletal muscle, and pancreas, as well as adipose tissue (Gruzman et al., 2009). In the present study, HFD feeding significantly depressed the activation of AMPK and ACC in SD rat liver tissue, and phosphorylated AMPK and ACC levels returned to normal after the administration of CKS for 28 days, suggesting that CKS has a beneficial role in activating the AMPK signalling pathway.

SREBP transcription factors regulate the expression of lipogenic enzymes, including ACC, FAS, and HMGA (Goldstein et al., 2002; Horton et al., 2002). AMPK activation reduces lipogenesis by decreasing SREBP-1c activation, thereby downregulating ACC and FAS expression (Kim et al., 2010). CKS reduced the expression of SREBP-1c and FAS in HFD-fed rats. These results strongly suggest that CKS exerts beneficial effects in rats with HFD-induced lipid accumulation by decreasing de novo lipogenesis in liver tissue via activation of the AMPK signalling pathway.

As potent scavengers of reactive oxygen species, triterpenoid saponins isolated from *P. grandiflorum* can protect against various types of chemical-induced hepatotoxicity (Khanal et al., 2009; Lee et al., 2008). Among these terpenoid saponins, platycodin D is the most abundant and has the highest radical scavenging activity and pharmacological efficacy (Ahn et al., 2005). Consistent with our CKS data, platycodin D significantly increased the phosphorylation of AMPK and ACC in HepG2 cells. It also decreased SREBP-1c and FAS expression and significantly decreased high-glucose-induced lipid accumulation and intracellular triacylglycerol and cholesterol levels in HepG2 cells. These results indicate that similar to CKS, platycodin D inhibits *de novo* lipogenesis in liver tissue.

In mammals, CaMKKβ has been identified as an upstream kinase that phosphorylates Thr172 of AMPK to activate it (Hurley et al., 2005). Emerging evidence suggests that SIRT1 cooperates with AMPKα to regulate energy metabolism (Hou et al., 2008). SIRT1, a NAD^+-dependent deacetylase, is a principal modulator of pathways downstream of calorie restriction and has beneficial effects on glucose homeostasis and insulin sensitivity (Rutanen et al., 2010). Furthermore, SIRT1 exerts effects similar to those of AMPK on cellular energy metabolism and mitochondrial biogenesis (Philp et al., 2011; Schug and Li, 2011). In the present study, platycodin D activated AMPK through CaMKβKβ, implying that CaMKKβ acts upstream of AMPK activation induced by platycodin D. Our results showing that platycodin D increased the NAD^+/NADH ratio and that SIRT1 inhibitor treatment inhibited platycodin D-induced AMPK activation support the concept that SIRT1 is necessary for the AMPK-dependent inactivation of SREBP-1c by platycodin D.

In conclusion, CKS administration in rats with HFD-induced lipid accumulation reduced body weight gain, liver weight, and fatty droplet accumulation in the liver. Additionally, CKS increased AMPK and ACC phosphorylation in liver tissues. In HepG2 cells, CKS and its major component, platycodin D, activated the SIRT1 and CaMKKβ/AMPK signalling pathways and inhibited the expression of SREBP-1c. Taken together, our

findings indicate that CKS and platycodin D may inhibit HFD- and/or high-glucose-induced lipid accumulation by inhibiting lipogenesis in liver tissue and HepG2 cells.

Conflict of interest

The authors have declared no conflicts of interest.

Acknowledgments

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