

Platycodin D Inhibits Adipogenesis of 3T3-L1 Cells by Modulating Kruppel-like Factor 2 and Peroxisome Proliferator-Activated Receptor γ

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In this study, platycodin D was found to inhibit intracellular triglyceride accumulation in 3T3-L1 cells with an IC₅₀ of 7.1 μ M. The expression levels of genes involved in lipid metabolism such as fatty-acid-binding protein 4 and lipoprotein lipase were significantly downregulated following treatment with platycodin D. Treatment with platycodin D also resulted in a reduction of Peroxisome proliferator-activated receptor(PPAR) γ expression and its binding to target DNA sequence. Among the various upstream regulators of PPAR γ , the expression of Kruppel-like factor(KLF)2, an anti-adipogenic factor, was significantly upregulated following platycodin D treatment. When the upregulation of KLF2 was inhibited by KLF2 siRNA, the expression and binding of PPAR γ to its target sequence were significantly recovered under these conditions. The results of this study suggested that anti-adipogenic effect of platycodin D involves the upregulation of KLF2 and subsequent down-regulation of PPAR γ . Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: obesity; adipogenesis; platycodin D; PPAR γ ; KLF2; 3T3-L1.

INTRODUCTION

Platycodin saponins are bioactive components of *Platycodi Radix*, and have anti-inflammatory, anti-allergy, antitumor, apoptosis-inducing, and immune-stimulating activities (Choi *et al.*, 2001; Kim *et al.*, 2001; Ahn *et al.*, 2005). Recently, it was reported that platycodin saponins are beneficial in the treatment of metabolic disorders including obesity and hyperlipidemia (Han *et al.*, 2002). It has also been reported that platycodin saponins increase fecal triglyceride excretion and reduce hepatic and serum triglyceride levels in Sprague-Dawley rats fed with a high-fat diet (Zhao *et al.*, 2005). Platycodin D, one of the major platycodin saponins, was reported to inhibit pancreatic lipase activity in a competitive manner with a K_i of 180 μ M (Zhao and Kim, 2004). It was also reported that platycodin D demonstrates a lowering effect of triglyceride as well as a reduction in the body-weight gain in a dose-dependent manner after 8 week's administration in ICR mice (Zhao *et al.*, 2006). To date, however, the mechanism for the anti-obesity activity of platycodin saponins or platycodin D has not been fully elucidated at the cellular and molecular level.

Adipogenesis is a differentiation process by which undifferentiated preadipocytes are converted to fully

differentiated adipocytes, i.e., fat cells. Adipogenesis is known to be closely related to the etiologies of obesity and obesity-related metabolic disorders (Spiegelman *et al.*, 1993). A mouse preadipocyte cell line 3T3-L1, originally derived from a mouse embryo, can be differentiated into adipocytes, and has been widely used as an *in vitro* model for studying obesity (Green and Meuth, 1974; Harmon and Harp, 2001; Madsen *et al.*, 2005; Rahman *et al.*, 2008). During adipogenesis, 3T3-L1 cells change from fibroblast-like preadipocytes to spherical adipocytes and accumulate large fat droplets containing triglycerides (Jessen and Stevens, 2002). Decades of studies have helped identify the major genes involved in the formation of fat cells. During adipogenesis, Peroxisome proliferator-activated receptor(PPAR) γ and CAAT/enhancer binding protein(C/EBP) α activate the expression of lipid metabolizing enzymes such as fatty-acid-binding protein (FABP) 4, lipoprotein lipase (LPL), fatty acid synthase (FAS) and others (Rosen and MacDougald, 2006). In this study, the ability of platycodin D to inhibit the accumulation of intracellular fat was documented, and its mechanism of action was characterized by analyzing the expression levels of genes known to be involved in adipogenesis.

MATERIALS AND METHODS

Chemicals and reagents. Platycodin D was purified by high-speed countercurrent chromatography as previously described. Its purity was determined to be 96.3% by high performance liquid chromatography (HPLC) coupled with evaporative light scattering system (Ha and Kim, 2009). It was stored in -20°C and was dis-

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solved in distilled water just before the treatment. Cell culture reagents were obtained from Life Technologies Inc. (Gibco, Grand Island, NY, USA). Anti-C/EBP α , anti-C/EBP β , anti-C/EBP δ , and anti-SREBP1C antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-PPAR γ , anti-FABP4, anti- β -Actin antibodies, and anti-rabbit secondary antibody were purchased from Cell Signaling (Beverly, MA, USA). KLF2 siRNA and control siRNA were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The lipofectamine RNAiMAX transfection reagent was purchased from Invitrogen (Carlsbad, CA, USA). All other chemicals were purchased from Sigma (St Louis, MO, USA).

Cell culture. 3T3-L1 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Two days after reaching confluence (day 0), 3T3-L1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 1 μ g/mL insulin, 0.25 μ M dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine and 10% fetal bovine serum (differentiation-induction medium) for 2 days. Next, cells were maintained in DMEM containing 1 μ g/mL insulin and 10% fetal bovine serum (differentiation maintenance medium). The differentiation maintenance medium was changed every 2 days until the cells were harvested. To test the effects of platycodin D on adipogenesis, it was added to the differentiation induction medium and the differentiation maintenance medium until the cells were harvested. Differentiated cells in each well of 6-well plates were harvested in 500 μ L of phosphate-buffered saline, frozen, and sonicated. The triglyceride contents of the cell lysates were measured using the TG-S reaction kit (Asan Pharm. Co., Seoul, Korea) according to the manufacturer's instructions. Cytotoxicity was measured using the 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT) method as described (Freshney, 1994).

Real-time reverse transcription polymerase chain reaction. Total RNA was extracted using the RNeasy kit (Qiagen, Hilden, Germany). The PCR primer and probe sequences for C/EBP α and C/EBP β were kindly provided by Gustafson and Smith (2006), and the assay-on-demand gene expression products (Applied Biosystems, Inc., Foster City, CA, USA) were used for other genes: FABP4, Mm00445880_m1; LPL, Mm00434764_m1; PPAR γ , Mm00440945_m1; C/EBP δ , Mm00786711_s; SREBP1C, Mm00550338_m1; KLF5, Mm00456521_m1; KLF2, Mm01244979_g1. The level of 18S rRNA was used as an endogenous control as described (Gustafson and Smith, 2006). Briefly, for each sample, the levels of each mRNA were normalized to the abundance of 18S rRNA, and ratios of normalized mRNA amounts of each samples to that of the preadipocyte (day 0) were determined by using the comparative *Ct* method (Livak and Schmittgen, 2001).

PPAR γ DNA binding activity assay. 3T3-L1 cells were scraped in phosphate buffered saline (PBS) and centrifuged for 10 min at 2,000 \times g. The pelleted cells were dissolved in buffer A containing 25 mM Tris-HCl (pH 7.5), 50 mM KCl, 2 mM MgCl₂, 1 mM EDTA, and 5 mM dithiothreitol, and homogenized in a tight homogenizer. The nuclei were pelleted by centrifugation at

3,300 \times g for 10 min and washed twice with buffer A. High salt extraction of nuclear proteins was performed by incubating the nuclei with buffer B containing 25 mM Tris-HCl (pH 7.5), 420 mM NaCl, 1.5 mM MgCl₂, 1 mM dithiothreitol, 0.5 mM EDTA, and 25% sucrose, for 30 min on ice. After a 30-min centrifugation at 20,000 \times g, the supernatants were used as nuclear extracts. The ability of PPAR γ in the nuclear extracts to bind to its target DNA sequence, i.e., peroxisome proliferators response element (PPRE:5'-AACTAGGTCAAAGGTCA-3') was measured using the TransAM PPAR γ ELISA kit (ActiveMotif, Carlsbad, CA, USA).

SiRNA transfection experiment. Two days after reaching confluence, 3T3-L1 cells were cultured in serum-free medium for 1 h and transfected with 60 nM of KLF2 siRNA or 60 nM of control siRNA using the Lipofectamine RNAiMAX transfection reagent. Six hours later, the transfected cells were differentiated according to the differentiation protocol. After 4 days, total RNA and nuclear extracts were prepared for real-Time RT-PCR experiments and the PPAR γ DNA binding activity assay, respectively.

Statistical analysis. These data were expressed as means \pm SE from at least three independent experiments. Statistical significances for comparisons between the platycodin D-treated samples and corresponding untreated samples were determined using the unpaired *t*-test. All analyses were performed using SPSS ver. 14 (SPSS Inc., Chicago, IL, USA).

RESULTS

Platycodin D inhibits the accumulation of triglycerides and the expression of genes involved in lipid metabolism

The triglyceride content of cells increased roughly 14-fold during 7 days of adipogenesis, and the addition of 10 μ M of platycodin D into the medium almost completely blocked triglyceride accumulation (Fig. 1A). The inhibitory effect of platycodin D on triglyceride accumulation occurred in a dose-dependent manner and the half inhibitory concentration (IC₅₀) was determined to be 7.1 μ M (Fig. 1B). To test the cytotoxic effects, MTT assays were conducted after the treatment of various concentrations of platycodin D for 7 days during the adipogenesis of 3T3-L1 cells. The results of the MTT assay showed that platycodin D did not decrease the cell viability indicating that its ability to inhibit triglyceride accumulation was not a result of cytotoxicity (data not shown). FABP4, which binds fatty acids with high affinity and transports them to various compartments in the cell, is a key mediator of fatty acid metabolism in adipose tissues. FABP4 is highly expressed during adipogenesis and comprises up to 6% of total cytosolic proteins in mature fat cells (Spiegelman and Green, 1980). Expression of the FABP4 mRNA was upregulated more than 1600-fold during adipogenesis; however, when cells were treated with 10 μ M platycodin D, the level of FABP4 protein was significantly reduced (Fig. 1C). Lipoprotein lipase

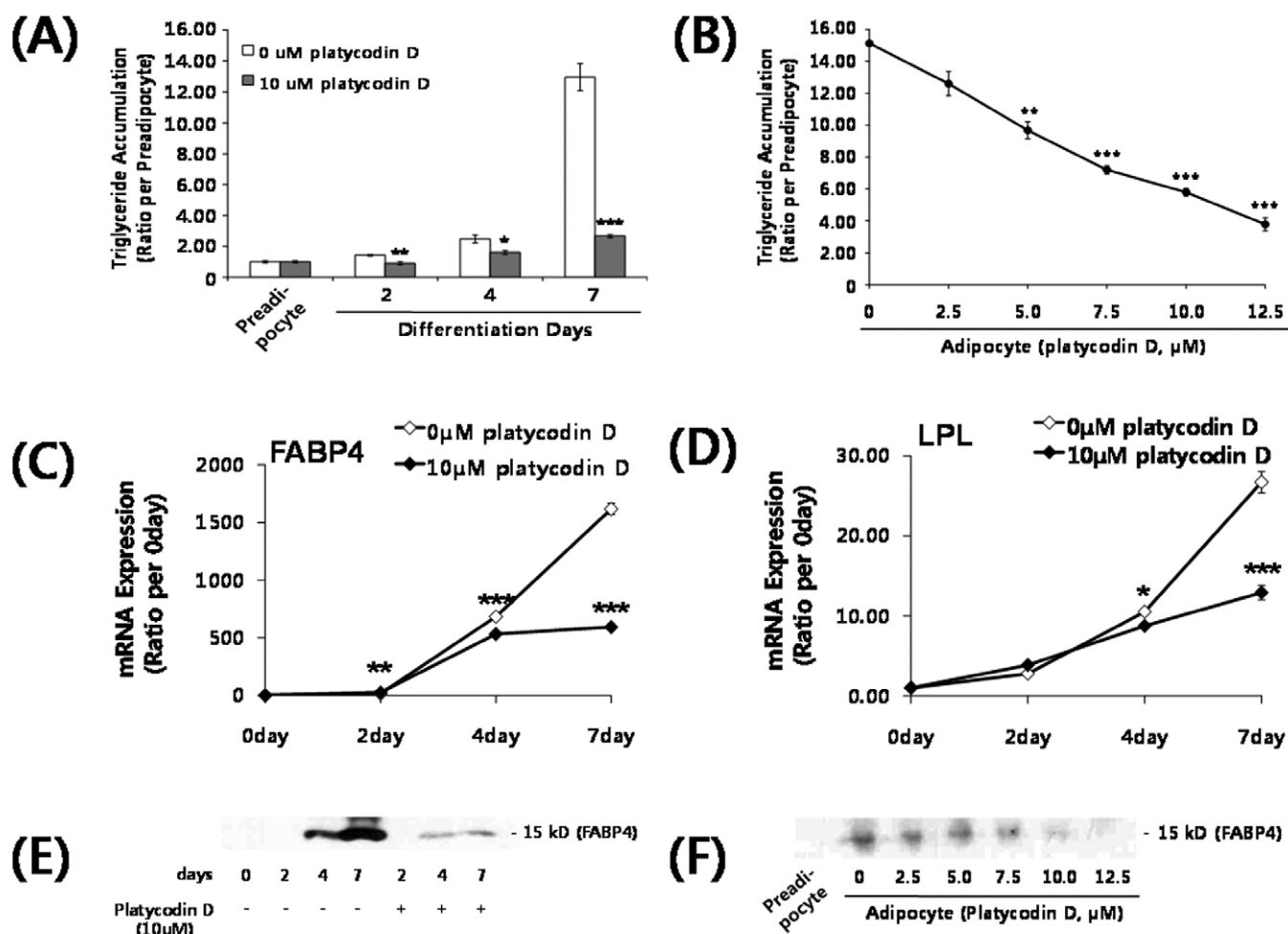


Figure 1. The accumulation of intracellular triglycerides and the expression of genes involved in lipid metabolism are inhibited in 3T3-L1 cells following treatment with platycodin D. (A) The triglyceride content of 3T3-L1 cells differentiated for 2, 4 or 7 days (in the presence or absence of 10 μM platycodin D) are presented. (B) The inhibition of triglyceride accumulation following platycodin D treatment, which was measured at day 7, occurs in a dose-dependent manner. (C) FABP4 mRNA expression levels in differentiated 3T3-L1 cells in the presence or absence of platycodin D. (D) LPL mRNA expression levels in differentiated 3T3-L1 cells in the presence or absence of platycodin D. (E) FABP4 protein expression in differentiated 3T3-L1 cells in the presence or absence of platycodin D. (F) Platycodin D alters the expression of the FABP4 protein in a dose-dependent manner. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with untreated adipocyte.

(LPL) hydrolyses triglycerides circulating in lipoprotein particles, allowing for the uptake of fatty acids into fat cells (Fielding and Frayn, 1998). Expression of LPL increased more than 25-fold during adipogenesis, but was significantly inhibited following platycodin D treatment (Fig. 1D). The effect of platycodin D treatment on FABP4 protein levels mirrored its effect on the mRNA levels (Figs 1E and 1F).

Platycodin D downregulates PPAR γ and C/EBP α , the major transcription factors of adipogenesis

PPAR γ is a master regulator of adipogenesis and is both necessary and sufficient for adipogenesis (Rosen *et al.*, 2000). The expression of PPAR γ alone is sufficient to induce adipogenesis in fibroblasts (Tontonoz *et al.*, 1994). In this study, we found that the binding of PPAR γ to its target DNA sequence, PPRE (5'-AACTAGGTCAAAGGTCA-3'), increased more than 20-fold during adipogenesis (Fig. 2A). Platycodin D treatment significantly reduced the amounts of PPAR γ bound to PPRE. Reduced binding of PPAR γ to PPRE

can be explained by a reduction of its mRNA and protein levels as shown in Figs 2B and 2C. The inhibitory effect of platycodin D on PPAR γ expression was dose-dependent (Fig. 2D). It is reported that functional PPRES exist in the promoter regions of genes involved in lipid metabolism, including, among others, FABP4 (Schachtrup *et al.*, 2004), LPL (Schoonjans *et al.*, 1995), and acyl-CoA synthetase (Schoonjans *et al.*, 1995). The platycodin D-induced reduction of FABP4 and LPL mRNA levels, as shown in Figs 1C and 1D, can be explained by a reduction of PPAR γ binding to PPRE within the promoter regions of these genes. Expression of C/EBP α mRNA was also upregulated more than 20-fold during adipogenesis, however, its expression was significantly inhibited following treatment with 10 μM platycodin D (Fig. 2E). C/EBP α protein levels showed a similar expression pattern to the levels of its mRNA (Fig. 2F), and the inhibitory effect of platycodin D on C/EBP α expression was dose-dependent (Fig. 2G). The platycodin D-induced reduction of C/EBP α levels can also be explained, at least in part, by a decrease in PPAR γ levels following platycodin D treatment, because PPAR γ induces the expression of C/

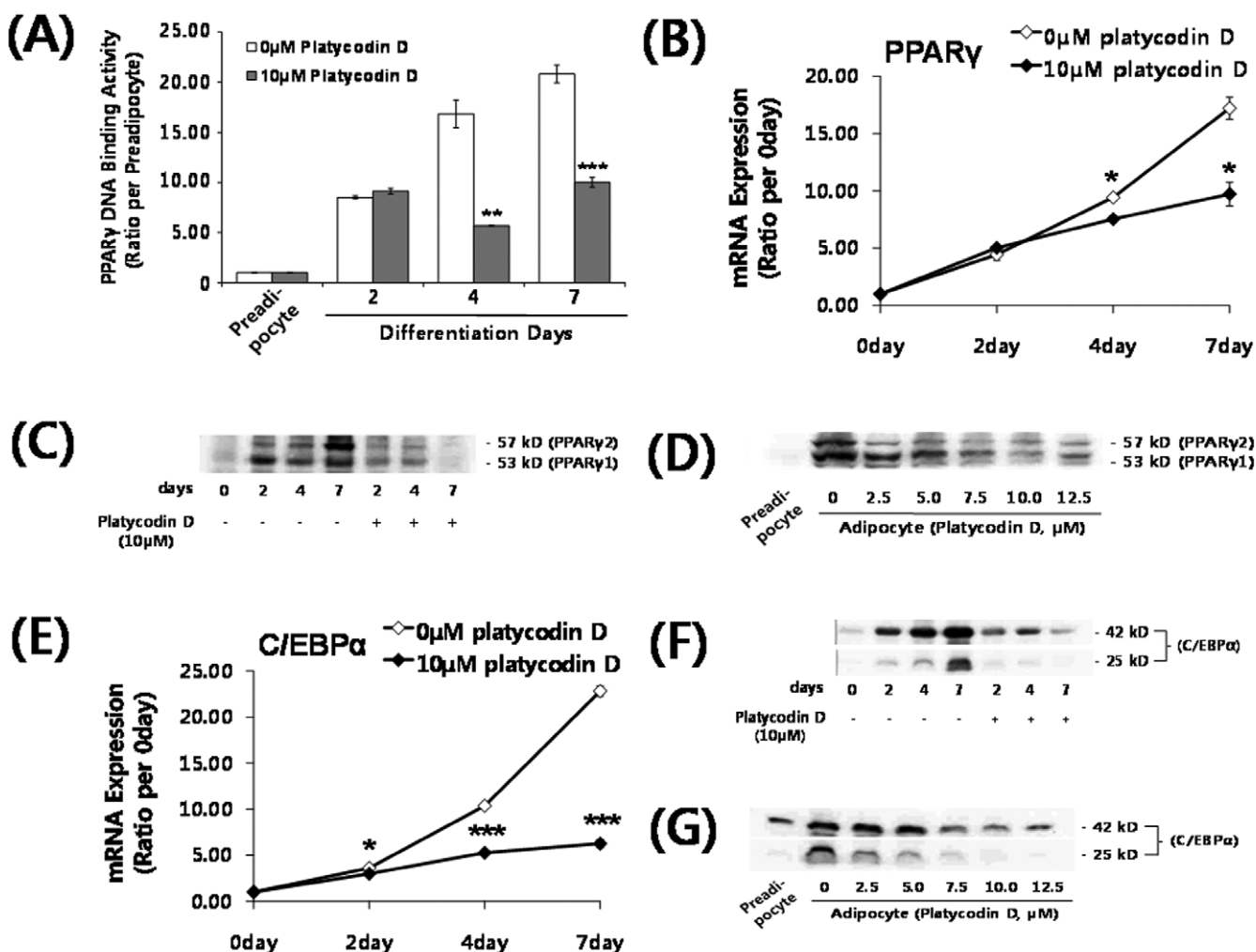


Figure 2. Effects of platycodin D treatment on PPAR γ and C/EBP α , the major transcription factors of adipogenesis. (A) Binding of PPAR γ to the PPRE sequence. Nuclear extracts were prepared during adipogenesis and the ability of their protein contents to bind to the PPRE sequence was determined. These data were normalized to the value of preadipocytes. (B) Effects of platycodin D on the abundance of PPAR γ mRNA. Real-time PCR experiments were conducted using primers which amplify 3' part of PPAR γ mRNA contained in both PPAR γ 1 and PPAR γ 2. (C) Effects of platycodin D on the abundance of PPAR γ protein. (D) Platycodin D treatment effects PPAR γ protein expression in a dose-dependent manner. (E) Effects of platycodin D on the abundance of C/EBP α mRNA. (G) Effects of platycodin D on the abundance of C/EBP α protein. (H) Platycodin D effects C/EBP α protein expression in a dose-dependent manner. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with untreated adipocytes.

EBP α by binding to its promoter region (Rosen *et al.*, 2002). PPAR γ and C/EBP α are two major transcription factors of adipogenesis and their expressions are mutually reinforced by forming a positive feedback loop between themselves. Among them, however, PPAR γ has a more crucial role than C/EBP α . PPAR γ can promote adipogenesis in the absence of C/EBP α , but C/EBP α has no ability to promote adipogenesis in the absence of PPAR γ (Rosen *et al.*, 2000).

Platycodin D has no effect on upstream regulators such as C/EBP β , C/EBP δ , or SREBP1C

C/EBP β , C/EBP δ , and Sterol regulatory element-binding protein (SREBP)1C are well-known upstream regulators of PPAR γ in the adipogenesis pathway (Rosen *et al.*, 2000). To identify upstream regulators involved in platycodin D-induced downregulation of PPAR γ , the expression levels of C/EBP β , C/EBP δ , and SREBP1C were characterized during the adipogenesis of 3T3-L1 cells in the presence and absence of platycodin D. The

transcription factors C/EBP β and C/EBP δ are expressed in the early phase of adipogenesis, and their major function is to induce the expression of PPAR γ (Wu *et al.*, 1996). Interestingly, the mRNA and protein levels of C/EBP β , C/EBP δ were unchanged following treatment with platycodin D (Figs 3A–3D). SREBP1C is a basic helix-loop-helix transcription factor that binds to an E-box motif located in the promoter of its target gene. The PPAR γ gene has three promoters, γ 1, γ 2 and γ 3; SREBP1C was shown to activate PPAR γ expression by binding to E-box motifs in the γ 1 and γ 3 promoters (Fajas *et al.*, 1999). The results indicate that the mRNA and protein levels of SREBP1C changed only slightly following treatment with platycodin D (Figs 3E and 3F).

Platycodin D upregulates KLF2, an anti-adipogenic factor, and leads to a reduction in PPAR γ expression and the binding of PPAR γ to its target sequence

Kruppel-like factor (KLF) family members are zinc-finger type transcription factors that regulate adipogen-

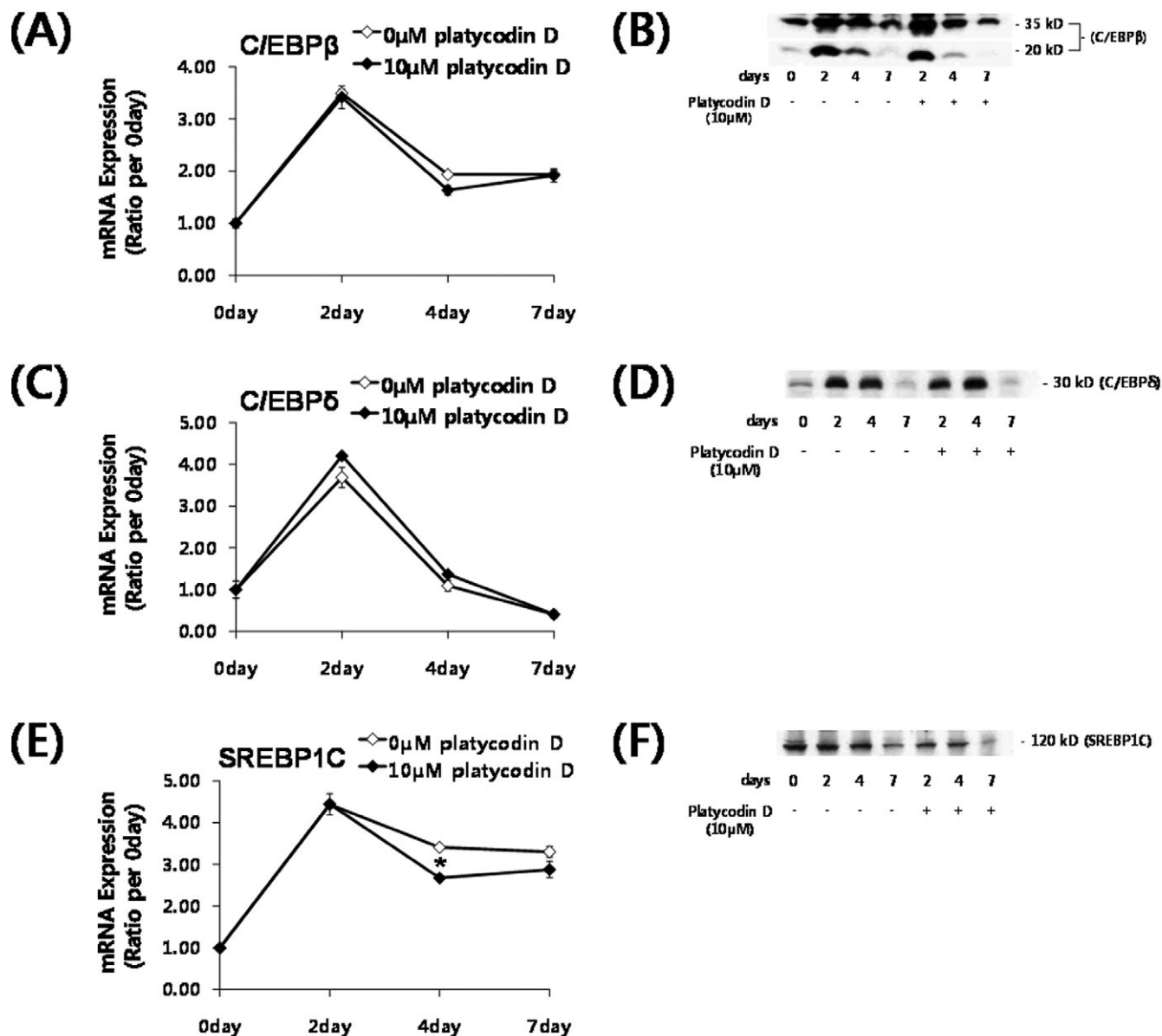


Figure 3. Effects of platycodin D on various upstream regulators of the adipogenesis pathway. The abundance of C/EBPβ, C/EBPδ, and SREBP1C mRNA (A,C, and E) and protein (B,D, and F) were measured by real time PCR and western blotting, respectively. **p* < 0.05 compared with untreated adipocytes.

esis and it was reported that multiple KLFs regulate PPARγ expression by binding to its promoter region during adipocyte differentiation (Rosen and MacDougald, 2006). KLF5 is a pro-adipogenic factor induced during early adipogenesis and is reported to bind to and activate the γ2 promoter of the PPARγ gene (Oishi *et al.*, 2005). KLF2, on the other hand, is an anti-adipogenic factor which binds to and represses the PPARγ promoter (Banerjee *et al.*, 2003). The effects of platycodin D on these two KLFs were different; the expression of the KLF5 mRNA was not significantly altered (Fig. 4A), but that of KLF2 was increased about 8 fold following platycodin D treatment (Fig. 4B). These results suggest that platycodin D differentially regulates KLF family members. To prove a causal relationship between the upregulation of KLF2 and downregulation of PPARγ, siRNA transfection experiments were conducted using control siRNA and KLF2 siRNA during adipogenesis in the presence or absence

of platycodin D. Compared with control siRNA-treated cells, KLF2 siRNA-treated cells showed significantly decreased KLF2 mRNA expression levels (Supplementary data 1). When the upregulation of KLF2 was inhibited by treatment with KLF2 siRNA, the mRNA expression of PPARγ, which was downregulated by treatment with platycodin D, was significantly recovered (Fig. 4C). The binding of PPARγ to its target DNA sequence showed a similar pattern to its mRNA levels (Fig. 4D). It is easy to speculate that the downregulation of genes involved in lipid metabolism shown in Fig. 1 results from an upregulation of the anti-adipogenic KLF2 gene following platycodin D treatment since their expression requires the binding of PPARγ to their promoter regions. In this study, platycodin D was shown to upregulate the expression of KLF2 resulting in a reduced binding of PPARγ to its target DNA sequence, which causes downregulations of lipid metabolizing enzymes and reduced intracellular triglyceride accumulation.

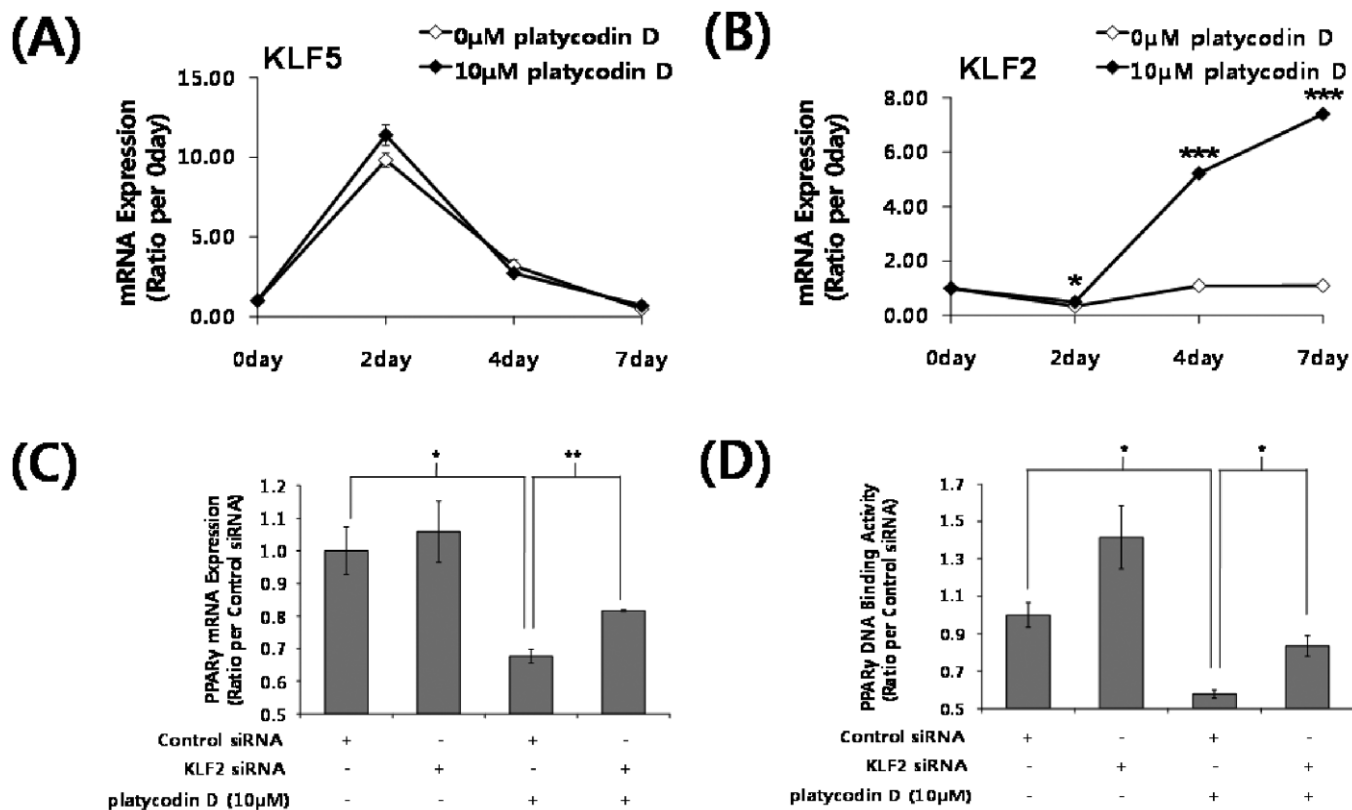


Figure 4. KLFs are differentially regulated during platycodin D-mediated inhibition of adipogenesis. (A) Effects of platycodin D on the expression of KLF5. (B) Effects of platycodin D on the expression of KLF2. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with untreated adipocytes. (C) Effects of KLF2 siRNA on the expression of PPAR γ mRNA in the presence or absence of platycodin D. (D) Effects of KLF2 siRNA on the binding of PPAR γ to its target sequence in the presence or absence of platycodin D. * $p < 0.05$, ** $p < 0.01$.

DISCUSSION

The anti-obesity effects of many natural compounds are mediated through the regulation of fat cells. For instance, epigallocatechin gallate (EGCG), genistein, esculetin, DHA, berberine, resveratrol, guggulsterone, conjugated linoleic acid (CLA), capsaicin, baicalein, and procyanidins were reported to inhibit adipogenesis (Rayalam *et al.*, 2008). Studies suggest that these compounds inhibit adipogenesis in a variety of ways regulating the upstream portion of the adipogenesis pathway. Genistein, EGCG, and capsaicin were shown to inhibit adipogenesis by activating AMP-activated protein kinase (Hwang *et al.*, 2005). Polyunsaturated fatty acids were shown to suppress adipogenesis by downregulation of sterol regulatory element-binding protein (Worgall *et al.*, 1998). Resveratrol was reported to increase the expression of sirtuin 1, a gene which represses the expression of PPAR γ (Bai *et al.*, 2008). Baicalein increases the expression of cyclooxygenase-2, a gene which is normally downregulated during adipogenesis (Cha *et al.*, 2006). In this study, platycodin D was shown to upregulate the expression of KLF2, an event which leads to the subsequent downregulation of the expression and binding of PPAR γ to its target DNA sequence. Ultimately, these events lead to the inhibition of adipogenesis.

KLFs are transcription factors implicated in many biological processes, including proliferation, apoptosis, differentiation, and development. The characteristic feature of this family is the presence of three zinc fingers that bind to CACCC elements and GC-rich regions of

DNA, to mediate activation and/or repression of transcription (Pearson *et al.*, 2008). Each zinc finger has 21 to 23 amino acid residues and chelates a single zinc ion coordinated by two cysteine and two histidine residues. Until now, 17 members of the KLF family were found in mammalian species (Suske *et al.*, 2005). Some members of the KLF family were reported to be involved in adipogenesis (Pearson *et al.*, 2008). KLF5 is a pro-adipogenic transcription factor which binds to and activates the $\gamma 2$ promoter of the PPAR γ gene (Oishi *et al.*, 2005). On the contrary, KLF2 is an anti-adipogenic transcription factor that binds to and represses the PPAR γ promoter (Banerjee *et al.*, 2003). The physiological roles of KLFs during adipogenesis were well reported, but, until now, few reports were found about the modulation of KLFs by pharmaceutically active compounds. In this study, it was shown that KLF2 can be effectively upregulated by treatment with platycodin D, a small compound with well-known anti-obesity effects. This study characterizes the molecular mechanism of action of platycodin D, a compound which effectively inhibits triglyceride accumulation in adipocytes. These findings also suggest that KLF2 may be a useful target for pharmaceutically active compounds in the treatment of obesity and related diseases.

Acknowledgements

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