Compound K, Intestinal Metabolite of Ginsenoside, Attenuates Hepatic Lipid Accumulation via AMPK Activation in Human Hepatoma Cells

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Compound K (CK) is a major intestinal metabolite of ginsenosides derived from ginseng radix. Although antidiabetic and antihyperlipidemic activities of CK have been investigated in recent years, action mechanism of CK remains poorly understood. Therefore, we examined whether CK affects the lipid metabolism in insulin-esistant HepG2 human hepatoma cells. In this study, a significant increase in AMP-activated protein kinase (AMPK) was observed when the cells were treated with CK. Activation of AMPK was also demonstrated by measuring the phosphorylation of acetyl-CoA carboxylase (ACC), a substrate of AMPK. CK attenuated gene expression of sterol regulatory element-binding protein 1c (SREBP1c) in time- and dose-dependent manners. Genes for fatty acid synthase (FAS) and stearoyl-CoA desaturase 1 (SCD1), well-known target molecules of SREBP1c, were also suppressed. In contrast, gene expressions of peroxisome proliferator-activated receptor α (PPAR-α) and CD36 were increased. These effects were reversed by treatment of compound C, an AMPK inhibitor. However, there were no differences in gene expressions of SREBP2, hydroxymethyl glutaryl CoA reductase (HMGR), and low-density-lipoprotein receptor (LDLR). Taken together, AMPK mediates CK induced suppression and activation of SREBP1c and PPAR-α, respectively, and these effects seem to be one of antidiabetic and/or antihyperlipidemic mechanisms of CK in insulin-resistant HepG2 human hepatoma cells.

KEYWORDS: Compound K; diabetes; hyperlipidemia; AMPK; ACC; SREBP1c; PPAR-α; HepG2 hepatoma cells

INTRODUCTION

Hepatic metabolism plays a key role in the regulation of whole-body energy status because the liver is the major site for storage and release of glucose and lipid. Therefore, accumulations of lipid within liver have been proposed to cause obesity, insulin resistance, and type 2 diabetes and are subject to nutritional influences (*1*).

Recent data indicate that AMP-activated protein kinase (AMPK) has been implicated as a key regulator of glucose metabolism including glucose transport, gluconeogenesis, and lipolysis (*2*). AMPK is serine−threonine kinase that is activated following a rise in the intracellular AMP:ATP ratio. Numerous studies have characterized the effect of AMPK activation on liver metabolism (*3*). Two of the classical targets for the system are acetyl-CoA carboxylase (ACC) and 3-hydrox-3-methylglu-

taryl-CoA reductase (HMGR), catalyzing the key regulatory steps in fatty acid and sterol synthesis, respectively. Therefore, the overall effects of AMPK activation in the liver would be decreases in fatty acid, triglyceride, and sterol synthesis and increases in fatty acid oxidation and ketogenesis (*4, 5*).

Compound K (CK) is the main metabolite of protopanaxadiol type ginseng saponins in the intestine after oral administration and also is the major form of protopanaxadiol saponins absorbed to the body (**Figure 1**). Recently, CK has received increasing attention because various pharmacological actions including
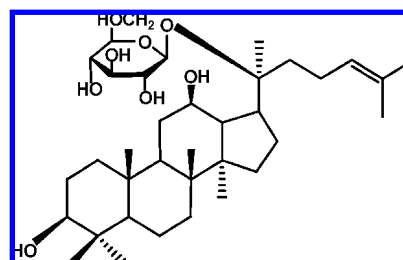

Figure 1. Chemical structure of compound K.

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anticancer (*6, 7*), anti-inflammation, (*8*) and antidiabetes (*9, 10*) were shown to be mediated by this compound. However, there is none demonstrating the activity of this compound against diabetes and hyperlipidemia through the AMPK pathway. In this study, we examined whether CK activates AMPK and affects lipid metabolism in insulin-resistant human hepatoma HepG2 cells cultured in high glucose media.

MATERIALS AND METHODS

Chemicals. CK was obtained from the Central Research Center, IL HWA Pharmaceutical Co. (Guri, Korea), and dissolved in 0.1% DMSO. Antibodies against phospho-LKB1, AMPK, phospho-AMPK, ACC, and phospho-ACC were from Cell Signaling Technology (Beverly, MA), and antiactin was from Santa Cruz Biotechnology (Santa Cruz, CA). Reverse transcriptase, Taq polymerase were supplied by Promega (Mannheim, Germany), and compound C (an AMPK inhibitor) was from Calbiochem (Darmstadt, Germany). Protein extraction kit, EASY-BLUE total RNA extraction kit and ECL-reagent kit were from Intron Biotechnology Inc. (Beverly, MA). SYBR Premix Ex Taq was from Takara Bio Inc. (Dalian, China). Other reagents and chemicals were of analytical grade.

Cell Culture and Preparation of Whole Cell Lysates. Human hepatoma HepG2 cell line was purchased from Korean Cell Line Bank (Seoul, Korea). HepG2 cell were cultured in DMEM (GibcoBL, Grand Island, NY) containing 10% fetal bovine serum, 100 unit/mL penicillin, and 100 μg/mL streptomycin in an atmosphere of 95% air and 5% CO_2 at 37 °C. For preparation of whole cell lysates to detect phosphoproteins, cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed in protein extraction kit. Insoluble protein was removed by centrifugation at 15000 rpm for 20 min. Protein concentrations in cell lysates were measured using a Bio-Rad protein assay kit (Hercules, CA).

Cytotoxicity Test. For MTS assay, HepG2 cells were cultured in 96-well culture plate and treated with indicated concentrations of CK for 24 h. The cytotoxicity of CK was determined by CellTiter 96 AQ$_{ueous}$ One solution Cell Proliferation Assay kit (Promega, Madison, WI).

Western Blot. Equal amounts of protein (50 μg/lane) were resolved by 8% SDS-polyacrylamide gel electrophoreses (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Millipore, MA). The membrane was further incubated with respective specific antibodies such as pLKB1 (1:2000), p-AMPK (1:1000), AMPK (1:1000), pACC (1:2000), ACC (1:2000), and actin (1:5000). The membrane was continuously incubated with appropriate secondary antibodies coupled to horseradish peroxidase and developed in the ECL Western detection reagents. The immunoreactive bands were visualized by an enhanced chemiluminescence and then were quantified by a densitometric analysis.

RNA Extraction, RT-PCR and Real-Time PCR. Total RNA was isolated using an EASY-BLUE total RNA extraction kit according to the manufacturer's instruction. First-strand cDNA synthesis was performed as described previously (*11*) using 5 μg of RNA, oligo (15)-dT primers, and reverse transcriptase in total volume of 50 μL. PCR reactions were performed in a total volume of 20 μL comprising 2 μL of cDNA product, 0.2 mM of each dNTP, 20 pmol of each primer, and 0.8 unit of Taq polymerase. Oligonucletide primer sequences used in PCR amplication are as follow; GAPDH forward, 5′-TCCACCAC-CCTGTTGCTGTA-3′ and reverse, 5′-ACCACAGTCCATGCCAT-CAC-3′ SREBP1c forward, 5′-GTGGCGGCTGCATTGAGAGT-GAAG-3 and reverse, 5′-AGGTACCCGAGGGCATCCGAGAAT-3′; SREBP2 forward, 5′-CGCCACCTGCCCCTCTCCTTCC-3′ and reverse, 5′-TGCCCTGCCACCTATCCTCTCACG-3″; FAS forward, 5′-CAAGAACTGCACGGAGGTGT-3′ and reverse, 5′-AGCTGCCA-GAGTCGGAGAAC-3′; SCD1 forward, 5′-TTGCCAGCTCTAGCCTTTAAATTC-3′ and reverse, 5′-TCCTGGTAGCATTATTCAGTAGTT-3′; CD36 forward, 5′-GGGCTATAGGGGATCCATTTTTG-3′ and reverse 5′-CCTTTCAGATTAACGTCGGATTC-3′; PPAR-α forward, 5′-TC-CGACTCCGTCTTCTTGAT-3′ and reverse, 5′-GCCTAAGGAAAC-CGTTCTGTG-3′; HMGR forward, 5′-TACCATGTCAGGGGTTACGTC-3′ and reverse, 5′-CAAGCCTAGAGACATAATCATC-3′; LDLR forward, 5′-CCCCGCAGATCAAACCCCCACC-3′ and reverse, 5′-

AGACCCCCAGGCAAAGGAGACGA-3′. PCR was performed at 95 °C for 30 s, followed by 50 °C (FAS, PPAR-α, HMGR), 52 °C (GAPDH), 56 °C (SCD1, CD36, LDLR, SREBP2), or 64 °C (SREBP1c) for 30 s, and 72 °C for 1 min. The last cycle was followed by a final extension step at 72 °C for 10 min. The RT-PCR products were electophoresed in 0.8% agarose gels under 100 V and were stained with 0.5 μg/mL ethidium bromide. Scanning densitometry was performed with i-MAX gel image analysis system (Core-Bio, Seoul, Korea). SREBP1c, SCD1, FAS, CD36, and PPAR-α mRNA levels were measured by LightCyclerReal-Time PCR System (Roche Applied Science, Indianapolis, IN) using SYBR Premix Ex Taq reagent and normalized to the endogenous GAPDH. Data were analyzed according to the manufacturer's instructions.

Statistical Analysis. All data were expressed as a mean ± standard error (SE) and differences between groups were analyzed using Student's *t* test. Mean values were considered significantly different when $p < 0.05$.

RESULTS

CK Stimulates AMPK Phosphorylation. To examine cellular toxicity of CK, various concentrations of CK (5−40 μM) were treated on HepG2 cell for 24 h. CK did not show any cellular toxicity up to 40 μM concentrations (data not shown). Thus we employed less than 40 μM of CK in following experiments. To investigate whether phosphorylation of AMPK was induced by CK, HepG2 cells were treated with 20 μM of CK for up to 24 h (**Figure 2A**). After each time, the cells were harvested and the total cell lysates were extracted. The phosphorylation state of the α subunit of AMPK (AMPKα) was noted by immunoblotting phospho-Thr-172 AMPK antibody. Compared to the basal level (0 time), CK markedly stimulated the phosphorylation of AMPK in a time-dependent manner (**Figure 2A**). No change in the expression of endogenous AMPK α was noted by immunoblotting AMPK α antibody. LKB1, a well-known upstream AMPK kinase in cultured liver, was also phosphorylated at Thr189 in a time-dependent manner. Being a primary target of AMPK, we also examined whether ACC is phosphorylated by CK treatment. As shown in **Figure 2A**, ACC was significantly phosphorylated at Ser79 in a time-dependent manner, parallel with AMPK phosphorylation. Next, HepG2 cells were exposed to the indicated concentrations of CK for 24 h, and **Figure 2B** showed that AMPK and ACC were both phosphorylated by CK in dose dependent manners. In addition, to confirm whether CK activates AMPK, we attempted to inhibit the phosphorylations of AMPK and ACC by a pharmacological approach. Pretreatment of HepG2 cells with compound C, an AMPK inhibitor, significantly attenuated phosphorylations of AMPK and ACC in dose-dependent fashions (**Figure 2C,D**). This result suggests that AMPK activation is required for the phosphorylation of ACC.

Effects of CK on Lipogenic and Lipolytic Gene Expressions. It has been reported that lipid accumulation is regulated via AMPK signal pathway (*12*). Therefore, we investigated the effects of CK on the expression of genes associated with lipid metabolism. As shown in parts **A** and **B** of **Figure 3**, CK attenuated gene expression of SREBP1c, central to the intracellular surveillance of lipid catabolism and de novo biogenesis, in time- and dose-dependent manners. Genes for SCD1 and FAS, well-known target molecules of SREBP1c, were also suppressed in time- and dose-dependent manners. Similarly, gene expressions of PPAR-α and CD36, responsible for inducing lipid uptake and catabolism, were increased in time- and dose-dependent manners (**Figure 3C,D**). Because of favorable effects on lipogenic and lipolytic gene expressions, CK is expected to reduce triglyceride accumulation in the liver. However, there were no differences in gene expressions of
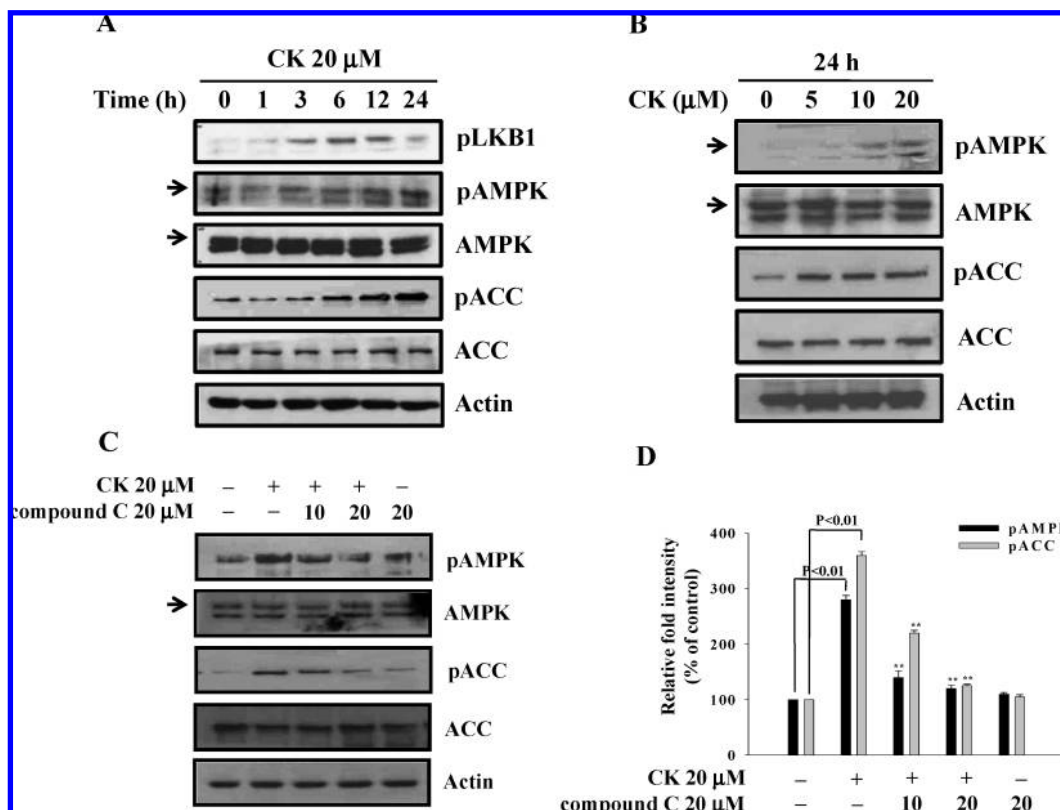
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Figure 2. Effects of CK on AMPK and ACC phosphorylations in HepG2 cells. HepG2 cells were treated with 20 μM of CK for the indicated times (**A**) and were treated with different concentrations (5−20 μM) of CK for 24 h (**B**). Phosphorylations of AMPK and ACC were also examined in the presence of compound C, an AMPK inhibitor (**C,D**). HepG2 cell were pretreated for 2 h with compound C, and then treated with CK for 24 h. Whole cell lysate (50 μg) was subjected to SDS-PAGE. Western blotting using antibodies against phospho-LKB (pLKB1), phospho-AMPK (pAMPK), AMPK, phospho-ACC (pACC), ACC, and actin was carried out as described in Materials and Methods. Actin was used as an internal control to evaluate relative expression of protein. The data are represented as the mean ± SE ($n = 3$).

SREBP2, HMGR, and LDLR when compared to basal level (**Figure 3E**,**F**). These data suggest that CK attenuates triglyceride storage through regulation of expression of genes involved in lipogenesis and lipolysis but no effect on cholesterol synthesis and cellular uptake into liver cells.

Compound C Reversed CK Induced Suppression of Lipid Accumulation. Next, we determined whether CK induced AMPK activation is necessary for the CK induced-reduction of lipid accumulation using the compound C. As shown in parts **A** and **C** of **Figure 4**, CK induced decreases in SREBP1c, SCD1, and FAS gene expressions were abrogated by treatment of compound C. In contrast, CK induced increases in PPAR-α and CD36 gene expressions were significantly suppressed by treatment of compound C (**Figure 4B**,**D**). These results strongly suggest that CK regulates lipogenic and lipolytic gene expressions via AMPK signal pathway.

DISCUSSION

The exact mechanisms that link obesity, impaired glucose metabolism, hepatic lipid accumulation, and insulin resistance are unknown, but fatty liver is considered to occur commonly in type 2 diabetes, with estimates of prevalence ranging from 21 to 78% (*13*). Recent work has shown that insulin resistance in type 2 diabetes is strongly associated with accumulation of excess triglyceride in liver (*14*). In addition, Kim et al. evidently reported that accumulation of intracellular fatty acid metabolites such as long-chain fatty acyl-CoAs reduces the activity of insulin-induced phosphatidyl inositol-3-kinase (PI-3-K)—a key

step in the insulin signaling cascade and may thus interfere with insulin signaling (*15*).

CK is an intestinal metabolite of panaxadiol ginsenosides. Although *Panax ginseng* is known to have antidiabetic and antihyperlipidemic activities, the active ingredient is not yet fully identified. Recently, we reported that compound K significantly decreased the fasting blood glucose levels in C57BL/KsJ db/db mice through enhancing insulin secretion and improving insulin resistance (*10*). In the meantime, nobody attempts to explore whether CK stimulates AMPK phosphorylation in HepG2 cells. AMPK plays a key role in regulating carbohydrate and fat metabolism, serving as a metabolic master switch in response to alterations in cellular energy charge. On the basis of this, AMPK cascades have emerged as novel targets for the treatment of obesity and fatty liver. AMPK phosphorylates multiple targets in the liver in order to acutely switch on alternative catabolic pathways and switch off anabolic pathways. ACC and HMGR were the first enzymes shown to be downstream targets for AMPK (*16*). ACC is an important rate-controlling enzyme for the synthesis of malonyl-CoA, which is both a critical precursor in the biosynthesis of fatty acids and a potent inhibitor of mitochondrial fatty acid oxidation. Inhibition of ACC by AMPK through phosphorylation leads to a fall in malonyl-CoA content and a subsequent decrease in fatty acid synthesis and increase in mitochondrial fatty acid oxidation via the allosteric regulation of carnitine palmitoyltransferase-1 (CPT-1), which catalyzes the entry of long-chain fatty acyl-CoA into mitochondria.
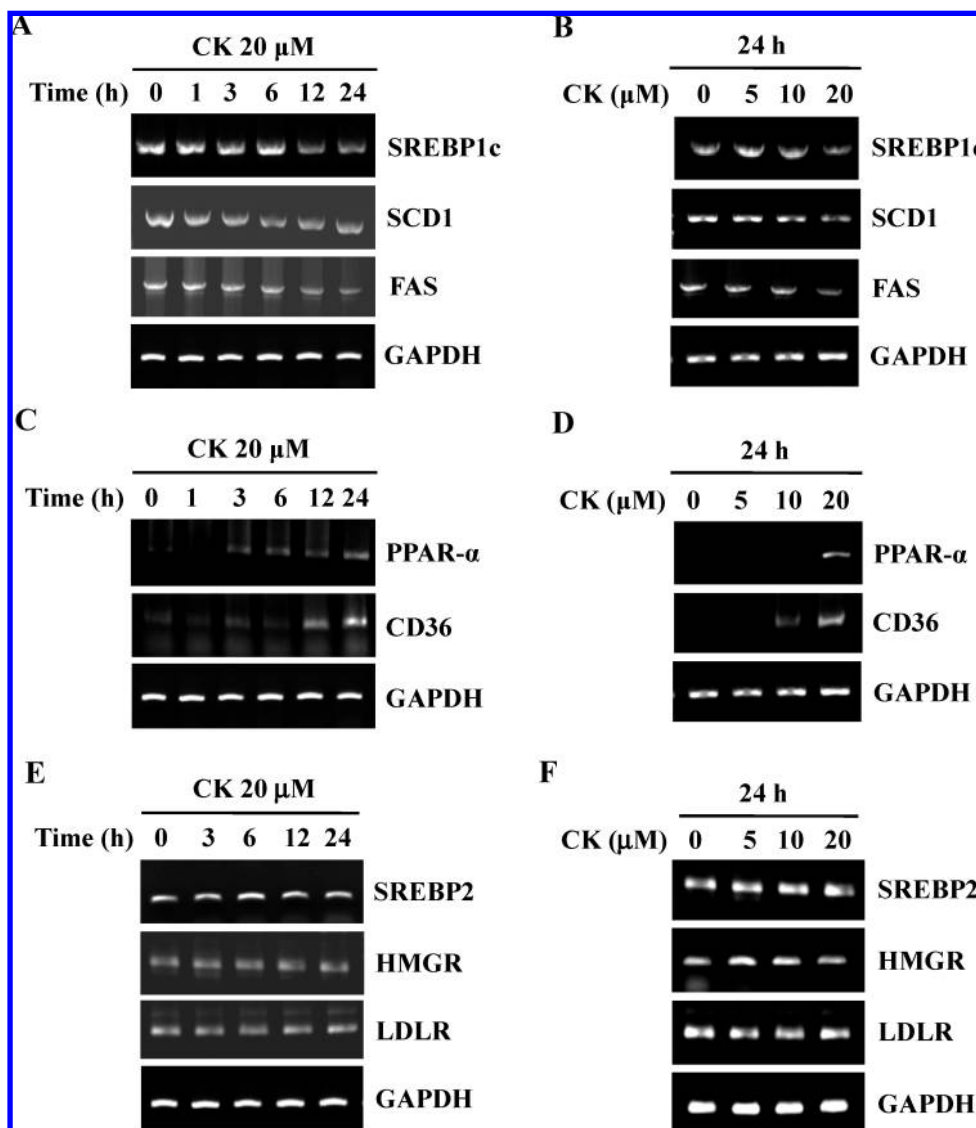
Figure 3. Effects of CK on lipogenic (**A**,**B**) and lipolytic (**C**,**D**) gene expressions in HepG2 cells. HepG2 cells were treated with 20 μM of CK for indicated times or treated with different concentrations of CK for 24 h. Effects of CK on gene expressions responsible for cholesterol synthesis and cellular uptake were also evaluated under the same experimental conditions (**E**,**F**). Total RNA was subjected to RT-PCR as described in Materials and Methods.

As shown in **Figure 2**, CK stimulated AMPK and ACC phosphorylations in time- and dose-dependent fashions. In addition, these phosphorylations were significantly attenuated in the presence of compound C, an AMPK inhibitor (**Figure 2C**). Thus, the activation of AMPK by CK inhibits ACC, decreases malonyl-CoA levels, and leads to stimulation of fatty acid oxidation. Recently, LKB1 was demonstrated to phosphorylate and activate the catalytic α subunit of AMPK at its T-loop residue Thr 172 in a cell-free system (*17*). The major AMPK kinase activity in the liver seems to correspond to LKB1 because it has been shown that deletion of LKB1 in the liver results in a proportional decrease of AMPK phosphorylation, rendering AMPK insensitive to stimuli which normally activate it (*18*). Therefore, we also tested whether CK phosphorylates LKB1 in HepG2 cells. As shown **Figure 2A**, CK phosphorylated LKB1 in a time-dependent manner, and phosphorylations of LKB1, AMPK, and ACC occurred sequentially.

Although the action of AMPK in systemic energy balance is achieved by rapid and direct phosphorylation of metabolic enzymes, long-term effects have also been clearly demonstrated on gene expression. It is now clearly established that

AMPK plays an important role in the repression of glycolytic and lipogenic gene expression in the liver. The intracellular and membrane levels of fatty acids and cholesterol are under constant surveillance, coordinated with de novo lipid biosynthesis controlled by ER-bound SREBPs (*19*). The SREBP families of basic-helix—loop—helix—leucine zipper (bHLH-LZ) transcription factors consists of SREBP-1a, SREBP-1c, and SREBP-2 protein and differ in their tissue-specific expression, their target-gene selectivity, and the relative potencies of their transactivation domains (*20*). SREBP1a and SREBP2 are responsive to change in sterol balance, whereas SREBP1c is primarily responsive to changes in carbohydrate intake and insulin. Therefore, gene expression of SREBP, a transcriptional factor for triglyceride and cholesterol biosynthesis, was evaluated in HepG2 cells. Gene expressions of SREBP1c and its target proteins, such as SCD1 and FAS, were decreased, but gene expressions of PPAR-α and CD36, a transcriptional regulator for lipid uptake and catabolism in the liver, were markedly enhanced in time- and dose-dependent manners (**Figure 3A,D**). These effects suggest that CK is expected to inhibit triglyceride deposition. However, there were no differences in gene expression of SREBP2,
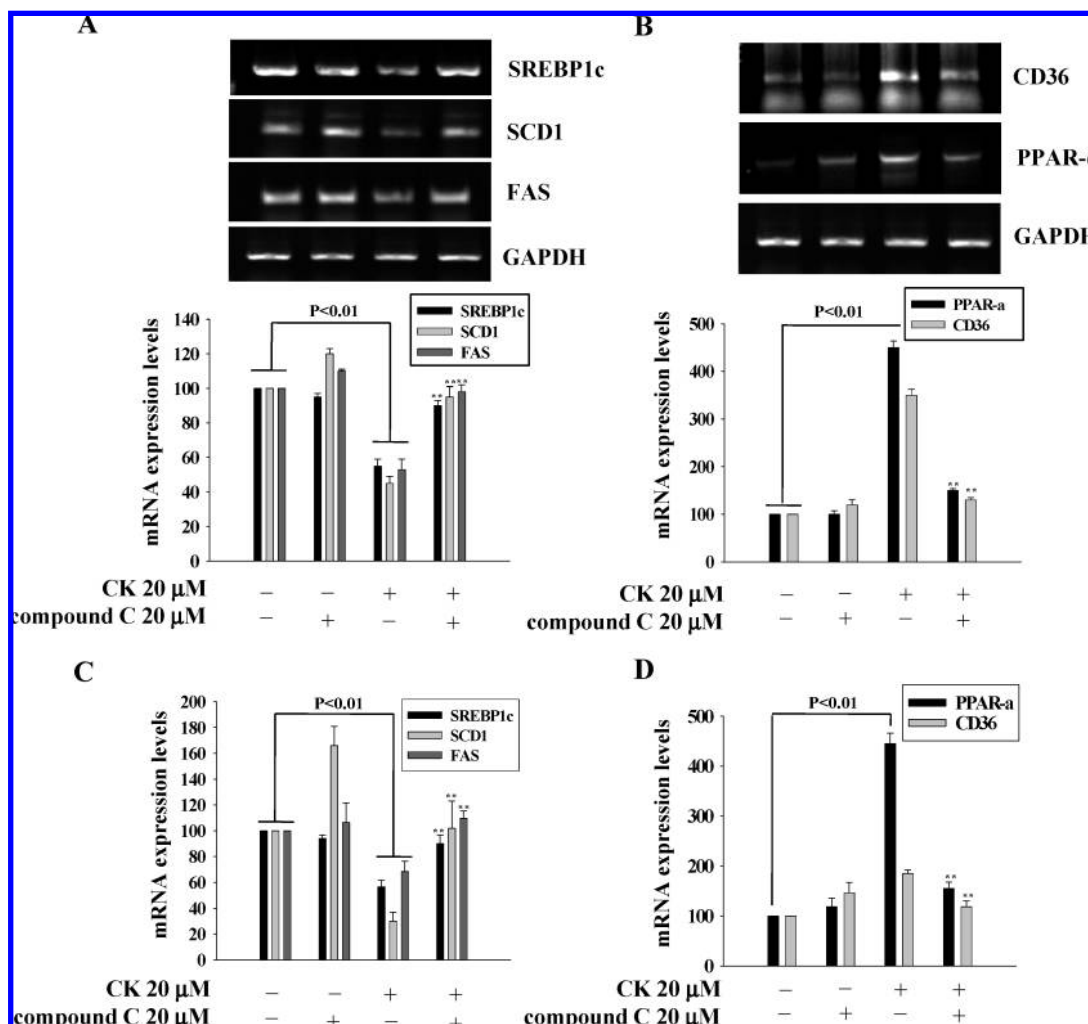
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Figure 4. Effects of compound C on lipogenic (**A**,**C**) and lipolytic (**B**,**D**) gene expressions in HepG2 cells. HepG2 cells were pretreated for 2 h with compound C, and then treated with CK for 24 h. Total RNA was subjected to RT-PCR as described in Materials and Methods (**A**,**B**). Transcripts of lipogenic (**C**) and lipolytic genes (**D**) were quantified by the real-time PCR and normalized to GAPDH. The data are represented as the mean $\pm$ SE (n = 3). ** $p < 0.01$ vs cells treated with CK.

HMGR, and LDLR, proteins responsible for cholesterol synthesis and cellular uptake (**Figure 3E**,**F**). In addition, CK induced decrease in SREBP1c gene expression and increases in PPAR-α and CD36 gene expressions were reversed by treatment of compound C in Real-Time PCR experiments (**Figure 4C**,**D**). These results suggest that CK regulates lipogenic and lipolytic gene expressions via AMPK cascade.

In conclusion, CK plays a significant role in reducing HepG2 cellular triglyceride accumulation by stimulating AMPK phosphorylation, and LKB1/AMPK/ACC signaling module may be involved in this process. Therefore, CK could represent a promising agent to reduce fatty liver or reverse hepatic disorders linked to type 2 diabetes as a monotheraphy or in combination with other existing drugs.

ABBREVIATIONS USED

CK, compound K; AMPK, AMP-activated protein kinase; ACC, acetyl-CoA carboxylase; TG, triacylglycerol; SREBP1c, sterol regulatory element-binding protein 1c; SREBP2, sterol regulatory element-binding protein 2; FAS, fatty acid synthase; SCD, stearoyl-CoA desaturase; PPAR-α, peroxisome proliferator-activated receptor α; HMGR, hydroxymethyl glutaryl CoA reductase; LDLR, low-density-lipoprotein receptor.

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