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Cydonia oblonga Miller fruit extract exerts an anti-obesity effect in 3T3-L1 adipocytes by activating the AMPK signaling pathway

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ABSTRACT

BACKGROUND/OBJECTIVES: The fruit of Cydonia oblonga Miller (COM) is used traditionally in Mediterranean region medicine to prevent or treat obesity, but its mechanism of action is still unclear. Beyond a demonstrated anti-obesity effect, the fruit was tested for the mechanism of adipogenesis in 3T3-L1 preadipocytes.

MATERIALS/METHODS: 3T3-L1 preadipocytes were cultured for 8 days with COM fruit extract (COME) at different concentrations (0–600 μg/mL) with adipocyte differentiation medium. The cell viability was measured using an MTT assay; triglyceride (TG) was stained with Oil Red O. The expression levels of the adipogenesis-related genes and protein expression were analyzed by reverse transcription polymerase chain reaction and Western blotting, respectively.

RESULTS: COME inhibited intracellular TG accumulation during adipogenesis. A COME treatment in 3T3-L1 cells induced upregulation of the adenosine monophosphate-activated protein kinase (AMPK)α phosphorylation and downregulation of the adipogenic transcription factors, such as sterol regulatory element-binding protein 1c, peroxisome proliferator-activated receptor γ, and CCAAT/enhancer binding protein α. The COME treatment reduced the mRNA expression of fatty acyl synthetase, adenosine triphosphate-citrate lyase, adipocyte protein 2, and lipoprotein lipase. It increased the mRNA expression of hormone-sensitive lipase and carnitine palmitoyltransferase I in 3T3-L1 cells.

CONCLUSIONS: COME inhibits adipogenesis via the AMPK signaling pathways. COME may be used to prevent and treat obesity.

Keywords: 3T3-L1 cells; AMP-activated protein kinase; adipogenesis; lipogenesis; lipolysis

INTRODUCTION

Obesity is a serious global health problem associated with increased morbidity and mortality from many major chronic diseases, including cardiovascular diseases, metabolic syndrome, type 2 diabetes, some types of cancers, and neurodegenerations [1,2]. Therefore, the prevention and treatment of obesity are important for improving health by managing chronic diseases.

Obesity occurs when adipocytes increase excessively in number (hyperplasia) and size (hypertrophy), characterized by fat accumulation in the adipose tissue. Fat accumulation in adipose tissue is controlled by the dynamic processes of adipogenesis (differentiation of preadipocytes into mature adipocytes), lipogenesis, and lipolysis, which involve many transcription factors, enzymes, and proteins [3,4]. Therefore, several mechanisms, including inhibition of adipogenesis and lipogenesis and stimulation of lipolysis, have been considered effective targets for treating obesity [3,5].

Drugs used to treat obesity typically work by inhibiting appetite (e.g., phentermine) or by inhibiting fat absorption (e.g., Orlistat) [6]. Although these drugs are highly effective, they cause unwanted and adverse side effects and weight regain when the medication is stopped [7,8]. Therefore, there is a need for effective and safe anti-obesity agents to treat obesity. Recently, food- and plant-derived bioactive ingredients, such as hydroxycitric acid [9,10] and catechin [11,12], have been explored and developed as effective and safe anti-obesity agents.

Cydonia oblonga Miller (COM) is a plant of the Rosaceae family, Cydonia genus, known by various names, including quince, aiva, bier, and marmelo [13,14]. The fruit of COM has mainly been consumed in the Western world as jams, jellies, and marmalade. In addition, the fruit of COM has been used mainly as a traditional medicine for diabetes, hypertension, cardiovascular diseases, respiratory disorders, ulcers, and hemolysis [15].

The fruit of COM contains numerous polyphenolic compounds, including chlorogenic acid, cryptochlorogenic acid, neochlorogenic acid, isochlorogenic acid, quercetin 3-rutinoside, quercetin 3-galactoside, quercetin 3-glucoside, kaempferol 3-glucoside, kaempferol 3-glycoside, and kaempferol 3-rutinoside. These compounds have been associated with various beneficial physiological activities, making the fruit of COM unique for medicinal purposes [16,17]. Several *in vitro* and *in vivo* studies have reported that different COM fruit extracts exhibit various beneficial physiological effects, such as antioxidant [18,19], anti-inflammatory [18], anti-cancer [19,20], anti-diabetic [21], anti-hypertensive [22], and hypolipidemic effects [23].

Lee *et al.* [24] reported that the COM fruit extract (COME) had an anti-obesity effect on obese C57BL/6 mice induced by a high-fat diet. On the other hand, studies on the anti-obesity effect of COME and its mechanism are lacking. Therefore, this study examined the effects of COME on adipogenesis, lipogenesis, and lipolysis and their mechanisms of action *in vitro* using 3T3-L1 adipocytes to explore the anti-obesity effects and the underlying mechanisms of COME.

MATERIALS AND METHODS

Chemicals and materials

The chemicals and materials used in this study were acquired from the following suppliers listed: Dulbecco's Modified Eagle's Medium (DMEM) and various additional cell culture reagents from Welgene (Daegu, Korea); bovine calf serum (BCS) and fetal bovine serum from Thermo Fisher Scientific Inc. (Waltham, MA, USA); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 3-isobutyl-1-methyxanthine (IBMX), dexamethasone, insulin, and Oil red O from Sigma-Aldrich Co. (St. Louis, MO, USA); primary antibodies against adenosine monophosphate (AMP)-activated protein kinase (AMPK)α, phospho-AMPK, acetyl coenzyme A carboxylase (ACC), phospho-ACC, and β-actin from Cell Signaling Technology

(Beverly, MA, USA); Luminata™ Forte Western HRP substrate from Millipore (Billerica, MA, USA); Trizol from Invitrogen Life Technologies (Carlsbad, CA, USA); HyperScript™ RT master mix kit from GeneAll Biotechnology (Seoul, Korea); QuantiNova SYBR Green PCR kit from Qiagen (Valencia, CA, USA); triglyceride (TG) assay kit and free glycerol assay kit from Biomax (Seoul, Korea).

Preparation of COME

COME were prepared by BnG Inc. (Chuncheon, Korea) according to the method described previously [24]. Briefly, the COM fruits from which the juice was removed by compression were extracted twice with 30% ethanol. The extracts were filtered, concentrated using a rotary vacuum evaporator, and powdered using a spray dryer. The powder obtained was used as COME.

Cell culture and adipocyte differentiation induction

Murine 3T3-L1 preadipocytes were obtained from the Korea Cell Line Bank (Seoul, Korea). 3T3-L1 preadipocytes were expanded in DMEM supplemented with 10% BCS, 100 U/mL penicillin, and 100 µg/mL streptomycin in a humidified atmosphere containing 5% CO_2 and 95% air at 37°C. Two days after confluence (denoted as day 0), 3T3-L1 were exposed to adipocyte differentiation medium (ADM)-I (DMEM containi ng 10% FBS, 0.5 mM IBMX, 1 µM dexamethasone, and 5 µg/mL insulin) for 2 days to induce adipocyte differentiation. On day 2, the medium was changed to ADM-II (DMEM containing 10% FBS and 5 µg/mL insulin). On day 4, the medium was changed to DMEM containing 10% FBS, and the cells were cultured for the next 4 to 8 days for full differentiation. The cells were incubated in ADM in the absence or presence of various concentrations of COME to examine the effect of COME on adipocyte differentiation, lipogenesis, and lipolysis.

Cell viability assay

3T3-L1 cells were seeded on 24-well plates at a density of 3×10^4 cells/well. After 24 h-incubation, the cells were treated with different concentrations of COME and incubated for 72 h. The cell viability was measured using a MTT assay, as described previously [25].

Oil red O staining

The 3T3-L1 cells were induced to differentiate and treated with different concentrations of COME for 8 days as stated above. The fully differentiated 3T3-L1 cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 1 h, washed with PBS, and stained with an Oil-red O solution for 90 min. The stained cells were visualized using a microscope (AxioImager, Carl Zeiss, Jena, Germany). The stained oil droplets were dissolved in isopropanol, and the absorbance was measured at 490 nm.

Measurement of cellular TG contents

After differentiation and treatment with COME for 8 days, the total lipids in the cells were isolated, according to the conventional extraction procedure, with a slight modification [26]. The resulting lipid pellet was reconstituted in 20 µL chloroform, and the TG content of the lipid pellet was measured using a TG assay kit (Biomax) according to the manufacturer's protocol.

Measurement of glycerol contents in conditioned medium

The 3T3-L1 cells were induced to differentiate and treated with different concentrations of COME, as stated above. After 8 days of treatment, cell culture media conditioned for 24 h was collected. The free glycerol content in the 24-h conditioned medium was estimated using a free glycerol assay kit (Biomax) according to the manufacturer's protocol.

Real-time reverse transcription-polymerase chain reaction (RT-PCR)

After 8 days of differentiation and treatment with COME, the total RNA in the cells was extracted, reverse transcribed, and real-time RT-PCR was conducted using a QuantiNova SYBR Green PCR kit (Qiagen) and a Rotor-gene 3000 PCR (Corbett Research, Mortlake, Australia), as reported elsewhere [27]. **Table 1** lists the sequences of the PCR primers utilized in this study. The results were analyzed using the Rotor-Gene 6000 Series System Software program, version 6 (Corbett Research), and the relative expression of the target genes was calculated relative to that of glyceraldehyde-3-phosphate dehydrogenase.

Western blot analysis

After differentiation and treatment with COME for 8 days, the cells were lysed and Western blot analyses were performed as reported elsewhere [28]. The immunoreactive bands were visualized using Luminata™ Forte Western HRP Substrate (Millipore). The images were acquired, and the intensity of each protein band was quantified using the ImageQuant™ LAS 500 imaging system (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The protein expression levels were calculated relative to those of β-actin.

Statistical analysis

The collected data were analyzed using Statistical Analysis System for Windows version 9.4 (SAS Institute, Cary, NC, USA). All data are presented as the mean ± SEM. A student's *t*-test was used to analyze the differences between the undifferentiated and differentiated groups. An analysis of variance followed by a Duncan's multiple comparison test was performed to assess the differences between the treatment groups and the differentiated groups. The $P < 0.05$ was considered statistically significant.

RESULTS

Cytotoxicity of COME on 3T3-L1 preadipocyte

The effects of COME on cell viability were determined before examining its influence on adipocyte differentiation. The effects of COME on the cell viability of 3T3-L1 preadipocytes were determined using an MTT assay with 100, 200, 300, 400, 600, and 800 µg/mL of COME. There was no significant difference in cell viability up to 600 µg/mL, but it decreased significantly at 800 µg/mL (**Fig. 1**). Therefore, COME was used at concentrations of 0–600 µg/mL in subsequent experiments to exclude the possibility that the COME treatment was cytotoxic to 3T3-L1 cells.

Table 1. Primer sequences used in this study

Target gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
ACL	TGGATGCCACAGCTGACTAC	GGTTCAGCAAGGTCAGCTTC
aP2	GGATTTGGTCACCATCCGGT	TTCACCTTCCTGTCGTCTGC
C/EBP-α	TGGACAAGAACAGCAACGAGTAC	GCAGTTGCCCATGGCCTTGAC
CPT1	CCTGGAAGAAACGCCTGATT	CAGGGTTTGGCGAAAGAAGA
FAS	AGGGGTCGACCTGGTCCTCA	GCCATGCCCAGAGGGTGGTT
HSL	CCGTTCCTGCAGACTCTCTC	CCACGCAACTCTGGGTCTAT
LPL	CCAATGGAGGCACTTTCCA	CACGTCTCCGAGTCCTCTCT
PPAR-γ	CAAAACACCAGTGTGAATTA	ACCATGGTAATTTCTTGTGA
SREBP-1c	CACTTCTGGAGACATCGCAAAC	ATGGTAGACAACAGCCGCATC
GAPDH	TGGGTGTGAACCATGAGAAG	GCTAAGCAGTTGGTGGTGC

ACL, adenosine triphosphate-citrate lyase; aP2, adipocyte protein 2; C/EBP-α, CCAAT/enhancer binding protein α; CPT1, carnitine palmitoyltransferase 1; FAS, fatty acid synthetase; HSL, hormone-sensitive lipase; LPL, lipoprotein lipase; PPAR-γ, peroxisome proliferator-activated receptor γ; SREBP-1c, sterol regulatory element-binding protein 1c; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
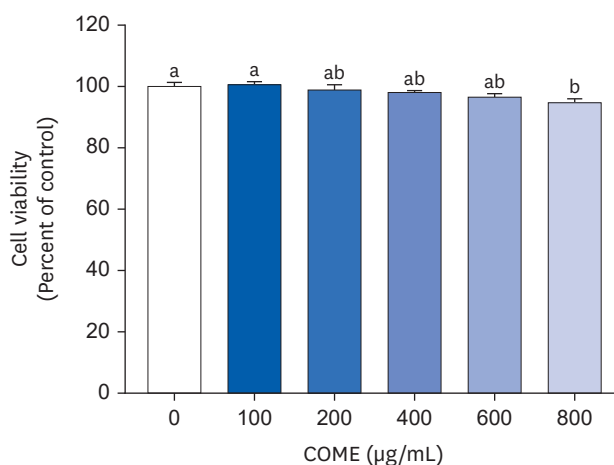
Fig. 1. Effects of COME on the cell viability in 3T3-L1 preadipocyte. 3T3-L1 cells were plated at 3×10^4 cells/well and incubated for 24 h. After 24 h incubation, the cells were incubated for 72 h in a medium containing COME concentrations ranging from 0 to 800 µg/mL. The cell viability was measured using a MTT assay. The values are expressed as the mean ± SEM. Means with different letters are significantly different ($P < 0.05$). COME, *Cydonia oblonga* Miller fruit extract.

## COME inhibits the differentiation of 3T3-L1 preadipocytes into adipocytes

The effects of COME on adipocyte differentiation and adipogenesis were examined by culturing post-confluent 3T3-L1 cells with ADM and different concentrations of COME. The accumulation of lipid droplets in differentiated 3T3-L1 cells was visualized and quantified by Oil Red O staining. After 8 days of culture, it was confirmed that lipid droplets accumulated in 3T3-L1 cells (**Fig. 2A**). On the other hand, lipid accumulation was reduced significantly by the COME treatment in a concentration-dependent manner by 7.6%, 10.3%, and 14.9% at 200, 400, and 600 µg/mL, respectively (**Fig. 2B**). These results show that COME inhibits adipocyte differentiation and adipogenesis in 3T3-L1 preadipocytes.

COME inhibits lipogenesis and promotes lipolysis in 3T3-L1 adipocytes

The effects of COME on lipogenesis were examined by differentiating the differentiated 3T3-L1 preadipocytes into adipocytes and determining the intracellular TG contents after treatment with 200, 400, and 600 µg/mL of COME. The intracellular TG contents of 3T3-L1 cells differentiated into adipocytes were increased remarkably compared to the non-ADM-treated (undifferentiated) cells, which was decreased significantly by the COME treatment. On the other hand, there was no significant difference according to the COME concentration (**Fig. 2C**). In addition, the free glycerol content in the 24-h conditioned medium was measured to determine if COME promotes lipolysis. The free glycerol content in the 24-h conditioned medium significantly increased with adipocyte differentiation and increased in a dose-dependent manner with the COME treatment (**Fig. 2D**). These results suggest that COME inhibits lipogenesis and promotes lipolysis in 3T3-L1 adipocytes.

COME regulates the expression of adipogenic transcription factors and their target genes

3T3-L1 cells were cultured by exposure to different concentrations of COME for 8 days and measured by real-time RT-PCR to determine if COME affects the mRNA expression of adipogenic transcription factors and their target genes. The mRNA expression of sterol regulatory element-binding protein 1c (SREBP-1c), peroxisome proliferator-activated receptor γ (PPAR-γ), and CCAAT/enhancer binding protein α (C/EBP-α) were markedly higher

(A)

|				
ADM	−	+	+	+	+
COME (μg/mL)	−	−	200	400	600

(B)

(C)

(D)
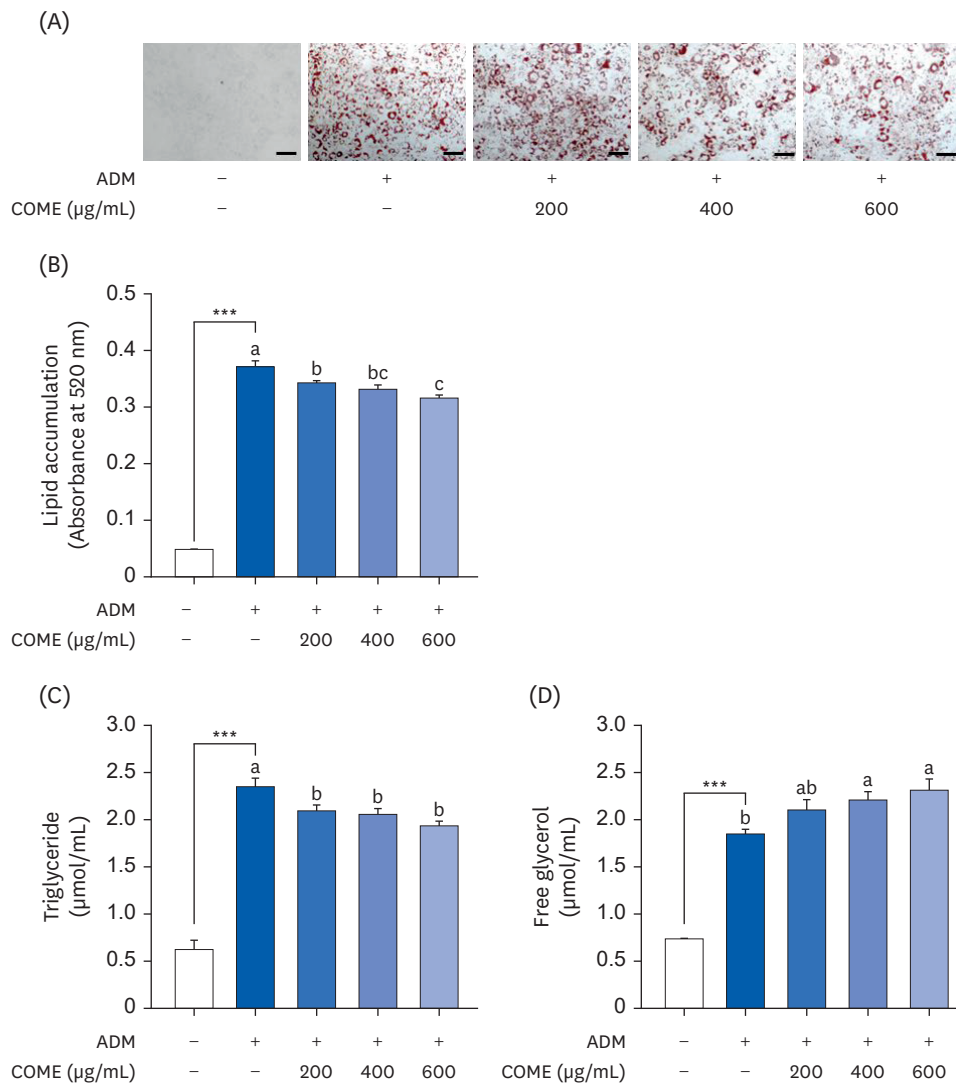
Fig. 2. Effects of COME on lipid accumulation, cellular TG content, and glycerol contents in the 3T3-L1 cells. 3T3-L1 preadipocytes were incubated in an ADM in the absence or presence of various COME concentrations for 8 days. (A) Differentiated 3T3-L1 cells were stained with Oil-red O solution and observed by optical microscopy. Scale bar, 50 μm. (B) Accumulated lipid droplets in stained 3T3-L1 adipocytes with Oil red O solution were quantified. (C) 3T3-L1 cells were differentiated and treated with COME for 8 days. The intracellular TG accumulation in differentiated 3T3-L1 adipocytes was measured. (D) Cells were differentiated and treated with COME for 8 days. The 24-h conditioned media were collected, and the free glycerol contents in the 24-h conditioned media were measured. The values are expressed as mean ± SEM. The means with different letters are significantly different ($P < 0.05$).
ADM, adipocyte differentiation medium; COME, *Cydonia oblonga* Miller fruit extract.
[***]$P < 0.001$ significantly different from that of non-ADM-treated (undifferentiated) group.

in the ADM-treated (differentiated) cells than the non-ADM-treated (undifferentiated) cells, which was decreased significantly by the COME treatment. At 600 μg/mL, COME decreased the mRNA expressions of SREBP-1c, PPAR-γ, and C/EBP-α by 37.7%, 47.1%, and 33.5%, respectively, compared to the non-COME-treated control group (**Fig. 3**). The mRNA expression of the adipogenesis and lipogenesis-related genes regulated by these transcription factors was next examined. The mRNA expression of adenosine triphosphate (ATP)-citrate lyase (ACL), fatty acid synthetase (FAS), adipocyte protein 2 (aP2), and lipoprotein lipase (LPL) were significantly higher in the ADM-treated (differentiated) cells compared to the non-ADM-treated (undifferentiated) cells, as shown in **Fig. 4**. The increased levels of ACL, FAS, aP2, and LPL mRNA expression were decreased significantly by the COME treatment. The ADM treatment did not affect the mRNA expression of hormone-sensitive lipase
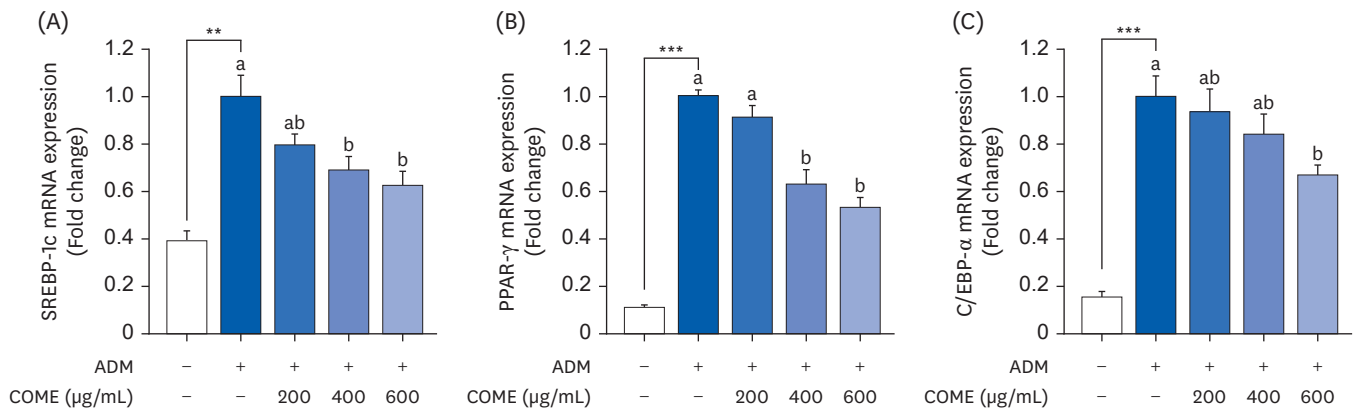
Fig. 3. Effects of COME on the expression of adipogenic transcription factors in 3T3-L1 cells. 3T3-L1 preadipocytes were incubated in an ADM in the absence or presence of various concentrations of COME for 8 days. The total RNA in 3T3-L1 cells was isolated. Real-time RT-PCR of (A) SREBP-1c, (B) PPARγ, (C) C/EBP-α was conducted. The values are expressed as the mean ± SEM. Means with different letters are significantly different ($P < 0.05$).

COME, *Cydonia oblonga* Miller fruit extract; SREBP-1c, sterol regulatory element-binding protein 1c; PPAR-γ, peroxisome proliferator-activated receptor γ; C/EBP-α, CCAAT/enhancer binding protein α; ADM, adipocyte differentiation medium.

[**]$P < 0.01$, [***]$P < 0.001$ significantly different from that of non-ADM-treated (undifferentiated) group.
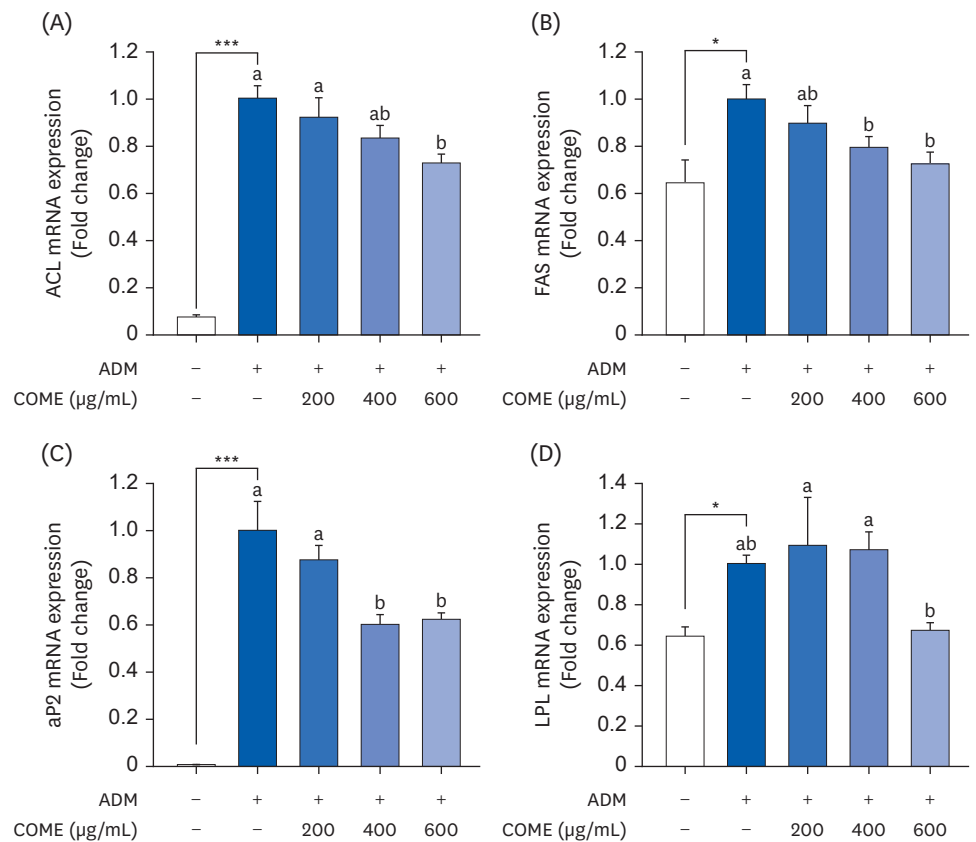
Fig. 4. Effects of COME on the expression of adipogenesis and lipogenesis-related genes in 3T3-L1 cells. Eight days after the induction of differentiation and treatment with COME, the total RNA in 3T3-L1 cells was isolated. Real-time reverse transcription polymerase chain reaction of (A) ACL, (B) FAS, (C) aP2, and (D) LPL was performed. The values are expressed as mean ± SEM. Means with different letters are significantly different ($P < 0.05$).

COME, *Cydonia oblonga* Miller fruit extract; ACL, ATP-citrate lyase; FAS, fatty acid synthetase; aP2, adipocyte protein 2; LPL, lipoprotein lipase; ADM, adipocyte differentiation medium.

[*]$P < 0.05$, [***]$P < 0.001$ significantly different from that of non-ADM-treated (undifferentiated) group.

(HSL), which regulates lipolysis. On the other hand, the mRNA expression of HSL was up-regulated by the COME treatment (**Fig. 5A**). In the ADM-treated (differentiated) cells, the mRNA expression of carnitine palmitoyltransferase 1 (CPT1), which is involved in fatty acid oxidation, was considerably decreased, compared to the non-ADM-treated (undifferentiated) cells. The decreased CPT1 mRNA expression was elevated significantly by the COME treatment (**Fig. 5B**). These results show that COME effectively inhibits adipogenesis and lipogenesis by down-regulating the expression of adipogenic transcription factors, followed by regulating the expression of their target genes.

COME induces the activation of AMPK

3T3-L1 cells were cultured with ADM and various concentrations of COME, and AMPK activation was assessed by Western blot analysis to determine if the AMPK pathway mediates the inhibitory effect of COME on adipogenic differentiation. AMPK protein expression was not altered by the COME treatment. On the other hand, phospho-AMPK expression was increased significantly by the COME treatment. The phospho-AMPK/AMPK ratio increased in a dose-dependent manner up to a COME concentration of 400 μg/mL, and there was no significant difference between COME at 400 μg/mL and 600 μg/mL (**Fig. 6A and B**). ACC, downstream of an AMPK signaling pathway, tended to increase its phosphorylation upon the COME treatment. Unlike other proteins, ACC is inactivated upon phosphorylation. The phospho-ACC/ACC ratio increased in a dose-dependent manner from COME at 200–600 μg/mL (**Fig. 6C and D**). These results suggest that the AMPK pathway mediates the anti-angiogenic and anti-lipogenic effects of COME.

DISCUSSION

This study confirmed the anti-obesity effects of COME in 3T3-L1 preadipocytes. The anti-obesity effect of COME was previously reported in a study of high-fat diet-induced obese C57BL/6 mice [24]. Adipogenesis is the differentiation of fibroblasts from preadipocytes into adipocytes, which is a multi-step process. Depending on the stage of adipogenesis,
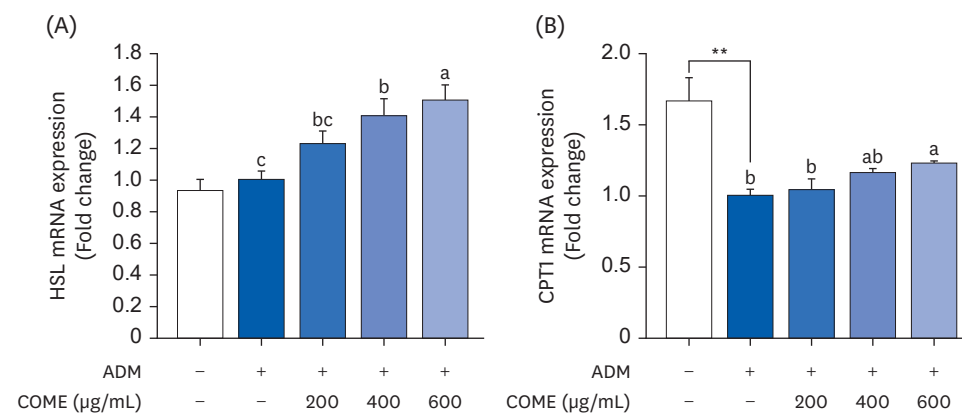
Fig. 5. Effects of COME on the expression of lipolysis-related genes in 3T3-L1 cells. Eight days after the induction of differentiation and treatment with COME, the total RNA in 3T3-L1 cells was isolated. Real-time reverse transcription polymerase chain reaction of (A) HSL and (B) CPT1 was performed. The values are expressed as the mean ± SEM. Means with different letters are significantly different ($P < 0.05$).
HSL, hormone-sensitive lipase; CPT1, carnitine palmitoyltransferase 1; ADM, adipocyte differentiation medium; COME, *Cydonia oblonga* Miller fruit extract.
**$P < 0.01$ significantly different from that of the non-ADM-treated (undifferentiated) group.

(A)

(B)

(C)

(D)
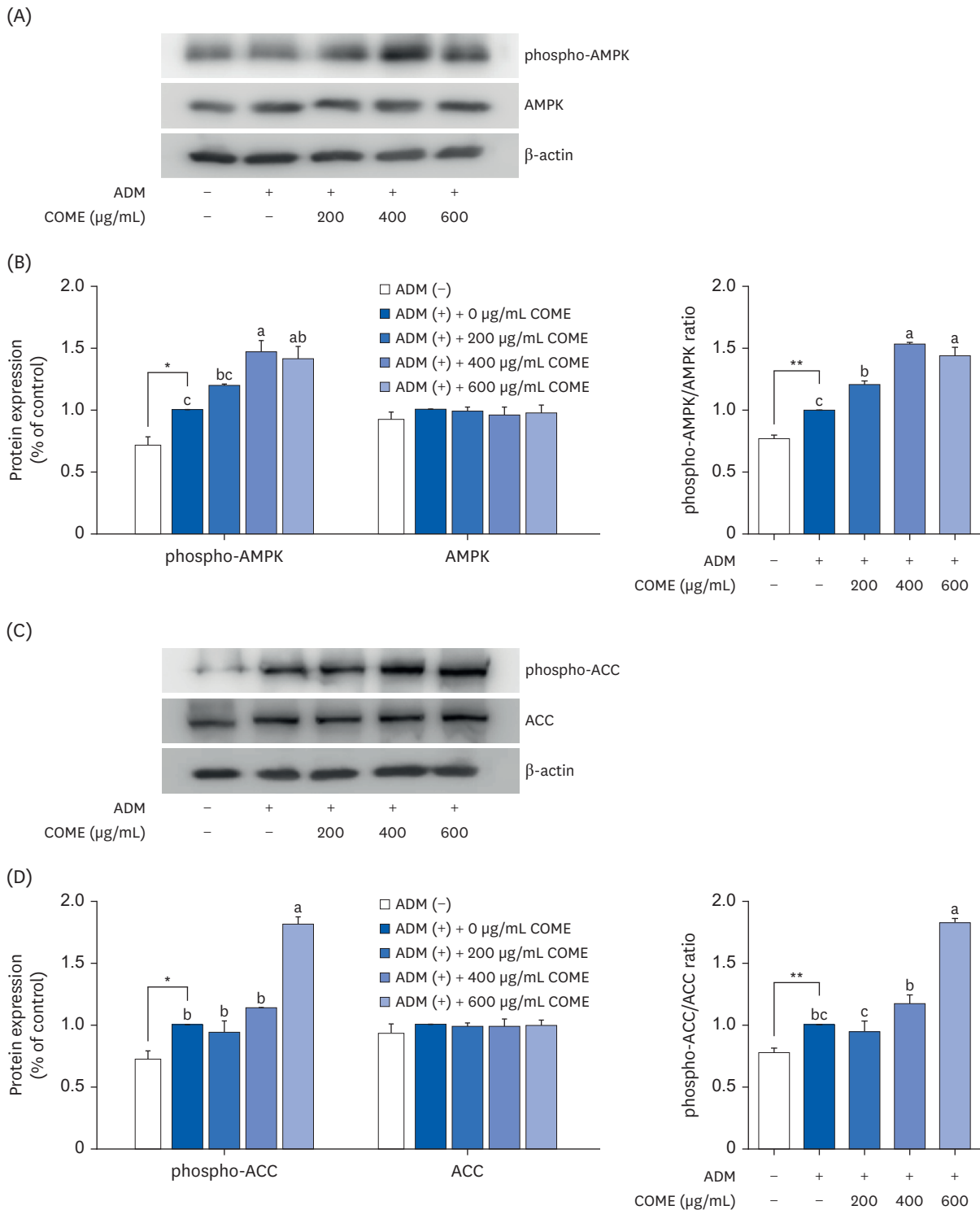
Fig. 6. Effects of COME on the expression of AMPK and ACC in 3T3-L1 cells. 3T3-L1 preadipocytes were incubated in an ADM in the absence or presence of various concentrations of COME for 8 days. The total lysates were prepared and analyzed by Western blotting using the indicated antibodies. (A, C) Images of Western blots representative of 3 independent experiments are shown. (B, D) Quantitative analysis of Western blot results. The protein expression levels were normalized to β-actin and are expressed relative to those of the non-ADM-treated (undifferentiated) group. Means with different letters are significantly different ($P < 0.05$). ADM, adipocyte differentiation medium; COME, *Cydonia oblonga* Miller fruit extract; AMPK, adenosine monophosphate-activated protein kinase; ACC, acetyl coenzyme A carboxylase.
[*]$P < 0.05$, [**]$P < 0.01$ significantly different from that of non-ADM-treated (undifferentiated) cells.

the expression patterns of transcripts and proteins involved in adipogenesis are regulated. Preadipocyte differentiation in an *in vivo* system is very complex, and the results can vary depending on several other factors. Therefore, a study of the molecular mechanism of adipogenesis in an *in vitro* system is needed [29]. This study is significant because it showed the anti-obesity effect of COME not only in previous *in vivo* studies and *in vitro* studies. In addition, this study identified the molecular biological mechanism of the anti-obesity effect of COME.

Intracellular fat accumulation was inhibited when 3T3-L1 preadipocytes were cultured with COME (**Fig. 2**). Other studies in which 3T3-L1 cells were treated with various polyphenolic compounds consistently reported a reduction in cell number and size and an inhibition of the rate of differentiation of preadipocytes into adipocytes [30,31]. In a previous study, COME had a particularly high chlorogenic acid content among the polyphenolic compounds, suggesting that this was the main component exhibiting anti-obesity effects [24].

Altering the level of adipocyte cell proliferation is a major area of research in adipogenesis. Adipogenesis occurs sequentially through a complex series of processes, such as cell cycle arrest, clonal expansion, and differentiation. Several transcription factors, PPAR-γ, C/EBP-α, SREBP-1c, and their target genes, are involved in this process of adipogenesis [30,32]. This process is initiated by SREBP family transcription factors [32]. The SREBP family directly regulates genes involved in TG and cholesterol synthesis [33]. C/EBPβ and C/EBPᵈ are expressed during early differentiation and prevent normal adipose tissue growth. The next important transcription factors are C/EBP-α and PPAR-γ. When PPAR-γ transcription is activated, it directly affects other genes involved in adipogenesis [34]. In the present study, COME reduced the mRNA expression of PPAR-γ, C/EBP-α, and SREBP-1c significantly in 3T3-L1 cells (**Fig. 3**). These transcription factors regulate the genes involved in adipogenesis and lipogenesis in adipose tissue. In this study, the mRNA expression of ACL, FAS, aP2, and LPL, which are genes related to adipogenesis and lipogenesis, was also reduced significantly by the COME treatment (**Fig. 4**). FAS is a downstream target gene of SREBP-1c. FAS plays a catalytic role in synthesizing long-chain fatty acids from acetyl CoA and malonyl CoA. The gene plays a vital role in the long-term regulation of lipogenesis [35]. FAS is a primary enzyme that regulates fatty acid synthesis and adipocyte differentiation by controlling the cellular fatty acid metabolism. Therefore, the reduction of FAS mRNA expression by the COME treatment is an essential indication of the anti-adipogenic activity of COME. ACL is an enzyme that converts citrate and CoA to acetyl-CoA and oxaloacetate. Acetyl-CoA is a precursor for fat and cholesterol synthesis [36]. aP2 is a type of fatty acid-binding protein and an early biomarker of several metabolic syndromes, including obesity [37]. A decrease in aP2 mRNA is a target for treating metabolic syndrome [37]. LPL is an enzyme that catalyzes the breakdown of TGs in lipoproteins. Depending on the energy state, the products broken down by LPL can be stored in adipose tissue or used as fuel in muscle. When adipose LPL is increased, the free fatty acid produced by lipoprotein hydrolysis stimulates the PPAR transcription factor, leading to fat synthesis. Therefore, the downregulation of the LPL gene means the inhibition of lipogenesis [29]. In this study, the mRNA expression of CPT1, which is involved in fatty acid oxidation, and HSL, which is involved in lipolysis, was increased by the COME treatment (**Fig. 5**). These results show that COME inhibits the early and late stages of the adipogenic process and promotes lipolysis.

This study examined whether the anti-obesity effect of COME treatment was related to AMPK activation. AMPK maintains the cellular energy balance and TG and cholesterol

homeostasis. When the AMP/ATP and adenosine diphosphate/ATP ratios increase, the body activates AMPKα, preventing TG accumulation in the liver and inactivating gluconeogenic enzymes. Furthermore, in the muscle, satiety is caused by leptin released from fat cells, which promotes fatty acid oxidation [38]. AMPK inhibits ACC, activates malonyl-CoA decarboxylase, and promotes the lipid metabolism. Cytoplasmic malonyl-CoA is a major substrate for fatty acid synthesis regulated by 2 enzymes: malonyl-CoA decarboxylase and ACC. Obesity induces a chronic inflammatory state in the body, leading to leptin resistance. Such leptin resistance inhibits the AMPK pathway, disrupting systemic metabolic homeostasis and inducing obesity and inflammation. In addition, AMPK dysfunction leads to insulin resistance and promotes obesity-related diseases [39,40]. Therefore, activating the AMPK pathway is an important target for treating or preventing obesity [41]. In this study, the COME treatment increased the phosphorylation of AMPK and ACC in 3T3-L1 cells (**Fig. 6**). Hence, COME activates AMPK and inactivates ACC. Activated AMPK can reduce the expression of genes related to adipogenesis and lipogenesis [42]. In other words, it inhibits the synthesis of fats - TGs, fatty acids, and cholesterol, increasing fatty acid uptake and β-oxidation. In the present study, the COME treatment reduced the mRNA expression of transcriptional genes, such as PPAR-γ, C/EBP-α, SREBP-1c, and adipogenesis and lipogenesis-related genes (ACL, FAS, aP2, and LPL). The expression levels of genes related to lipolysis (HSL and CPT1) were reduced by the COME treatment. These results show that COME suppresses the expression and activity of adipogenic transcription factors and their related genes by promoting the activation of AMPK and inhibiting the function of ACC, thereby inhibiting preadipocyte differentiation and lipid accumulation.

In conclusion, COME had anti-obesity effects, such as a significant decrease in cell differentiation and fat accumulation in 3T3-L1 adipocytes. This effect of COME was attributed to the increase in AMPK phosphorylation by the COME treatment, which reduces the expression of the transcriptional genes PPAR-γ, C/EBP-α, SREBP-1c, and their target genes. This study is significant because it elucidates the mechanism through an *in vitro* study of the anti-obesity effect of COME in a previous *in vivo* study. These results suggest that COME may be an anti-obesity agent, even though further research is needed before it can be used as a functional food ingredient with anti-obesity effects in humans.

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