Original Article

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Ethanol extracts of *Aster yomena* (Kitam.) Honda inhibit adipogenesis through the activation of the AMPK signaling pathway in 3T3-L1 preadipocytes

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Summary **The leaves of *Aster yomena* (Kitam.) Honda have long been used as a traditional herb for treating disorders including coughs, asthma, and insect bites. According to recent studies, *A. yomena* leaf extracts have several pharmacological properties, including anti-inflammatory, antioxidant, and anti-asthmatic activities. However, little information is available regarding their anti-obesity effect. In this study, we investigated the inhibitory effect of the ethanol extracts of *A. yomena* leaves (EEAY) on adipocyte differentiation and adipogenesis using 3T3-L1 preadipocytes. When 3T3-L1 preadipocytes were treated with various concentrations of EEAY (ranging from non-toxic), the number of lipid droplets, lipid content, and triglyceride production, the typical characteristics of adipocytes, were suppressed in a concentration-dependent manner. During this process, EEAY significantly reduced the expression of adipogenic transcription factors, including peroxisome proliferator-activated receptor-γ, CCAAT/enhancer-binding protein α and β, and sterol regulatory element-binding protein-1c. In addition, EEAY was also found to potently inhibit the expression of adipocyte-specific genes, including adipocyte fatty acid-binding protein and leptin. In particular, EEAY treatment effectively enhanced the activation of the AMP-activated protein kinase (AMPK) signaling pathway; however, the co-treatment with compound C, an inhibitor of AMPK, significantly restored the EEAY-induced inhibition of pro-adipogenic transcription factors and adipocyte-specific genes. These results indicate that EEAY may exert an anti-obesity effect by controlling the AMPK signaling pathway, suggesting that the leaf extract of *A. yomena* may be a potential anti-obesity agent.**

Keywords: *Aster yomena* (Kitam.), 3T3-L1 preadipocytes, adipocyte differentiation, adipogenesis, AMPK

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1. Introduction

Obesity is a metabolic disorder caused by an imbalance in the energy intake and expenditure. It is a major risk factor for several chronic diseases, including hypertension, atherosclerosis, and type 2 diabetes

(*1,2*). Obesity is characterized by an overgrowth of the adipocytes, indicated by an increase in the number of adipocytes, resulting from their division and differentiation. Adipocytes also play a crucial role in storing excess energy in the form of triglycerides (TGs) and releasing energy in the form of glycerol and fatty acids (*3,4*). In particular, pre-adipocytes differentiate into mature adipocytes owing to high caloric intake and various stimulatory factors such as insulin and glucocorticoids (*5,6*). The differentiation of pre-adipocytes into mature adipocytes is accompanied by the sequential expression and activation of adipogenic transcription factors, including peroxisome proliferator-activated receptor-γ (PPARγ), CCAAT/enhancer-binding proteins (C/EBPs), and sterol regulatory element-binding protein-1c (SREBP-1c) in a coordinated manner (*6,7*). In the final stages of the differentiation, adipocytes secrete adipose tissue-specific products, including adipocyte-specific fatty acid binding protein (aP2) and leptin (*8-11*). After differentiation, they regulate the lipid metabolism by synthesizing lipolytic enzymes such as fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC), and hormone-sensitive lipase (*12,13*). Therefore, the inhibition of the differentiation of preadipocytes into adipocytes would reduce the mass of adipose tissue and reduce the incidence of obesity. Further, the identification of a factor that inhibits differentiation would be useful for treating obesity and related metabolic diseases.

Furthermore, the AMP-activated protein kinase (AMPK), a serine/threonine kinase, is a key sensory protein that controls energy metabolism (*14,15*). Once AMPK is activated *via* phosphorylation by the upstream AMPK kinases, it functions as a cellular energy sensor through the regulation of fatty acid and glucose homeostasis (*16,17*). The activated AMPK increases glucose transport and fatty acid oxidation; it also inhibits the energy-consuming processes such as lipogenesis, protein synthesis, and gluconeogenesis (*18,19*). AMPK also induces inactivation by phosphorylating ACC, the key enzyme for fatty acid oxidation and biosynthesis (*13,20*). Further, when AMKP is activated, it may protect preadipocyte differentiation with the inhibition of the transcription factors such as PPARγ and C/EBPs. In particular, low levels of AMPK activation are associated with the onset of obesity and diabetes (*21,22*); therefore, AMPK has been identified as a potential target for treating obesity and the related metabolic disorders (*14,22,23*).

Plant-based materials have been used since a long time as traditional medicines in many cultures and are well-known sources for developing anti-obesity compounds. In this study, *Aster yomena* (Kitam.) Honda was selected from among traditional Korean medicine resources for the development of a new anti-obesity active substance. *A. yomena* is a perennial herb widely distributed throughout Asia that has been used in traditional medicine to treat several diseases such as cough, asthma, and insect bites (*24*). Several previous studies have shown that the leaf extracts or compounds of *A. yomena* have many pharmacological activities, including anti-microbial (*25,26*), antioxidant (*27-29*) and anti-coagulant (*30*) actions. Kim *et al*. (*31*) showed that the phenolic compounds extracted from the leaves of this plant significantly inhibited the production of interleukin-6 by stimulating tumor necrosis factor-α in the osteoblasts, indicating an anti-inflammatory activity. We recently reported that the ethanol extract of *A. yomena* leaves (EEAY) inhibited lipopolysaccharide-induced inflammatory responses in the macrophage model owing to the deactivation of the toll like receptor-mediated nuclear factor-κB signaling pathway (*32*). However, to our knowledge, the efficacy of EEAY as anti-obesity agents and the underlying molecular mechanisms are still unclear. Therefore, we designed this study to evaluate the anti-obesity effect of EEAY using 3T3-L1 preadipocytes and attempted to study the mechanism associated with the inhibition of the adipogenesis of 3T3-L1 cells.

2. Materials and Methods

2.1. *Cell culture*

The murine 3T3-L1 preadipocytes were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM, Welgene, Daegu, Republic of Korea) containing 4.5 g/L D-glucose (Welgene) supplemented with 10% bovine calf serum (BCS, Welgene) and 1% penicillin/streptomycin (Welgene) at 37°C in a humidified atmosphere comprising 5% CO_2 and 95% air.

2.2. *Preparation and treatment of EEAY*

The dried leaves of *A. yomena* used in this study were provided by the Gurye Wild Flower Institute (Gurye, Republic of Korea). The EEAY were prepared as per the method described by Kang *et al*. (*32*). The prepared EEAY were dissolved in dimethylsulfoxide (DMSO; Sigma-Aldrich Chemical Co. St. Louis, MO, USA) to achieve a final concentration of 200 mg/mL (extract stock solution).

2.3. *Differentiation of 3T3-L1 preadipocytes*

To differentiate the 3T3-L1 preadipocytes into adipocytes, 3T3-L1 preadipocytes were grown in culture plates for 2 d to full confluency. Thereafter, the cells were cultured in a differentiation medium (MDI) containing 5 μg/mL insulin, 1 mM dexamethasone, and 0.5 mM isobutylmethylxanthine (Sigma-Aldrich Chemical Co.). After 2 d, the medium was changed to DMEM, containing 10% BCS and 5 μg/mL insulin for 2 d and subsequently cultured in a normal medium for

4 d. Various concentrations of EEAY were added along with the MDI, and 3T3-L1 preadipocytes that induced 8-d differentiation were used for various experimental analyses.

2.4. *Assessment of cell viability*

To evaluate the cytotoxic ability of EEAY, 3T3-L1 preadipocytes were seeded in 6-well plates at a density of 1×10^4 cells per well. After 24 h, the cells were treated with different concentrations of EEAY for 72 h. Thereafter, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich Chemical Co.) was added to each well at a concentration of 0.5 mg/mL, followed by incubation at 37°C in a dark environment for 3 h. The MTT solution was removed, and DMSO (200 µL) was added to dissolve the formazan complex. The viable cells were detected by reading the absorbance of formazan at 540 nm using an enzyme-linked immunosorbent assay (ELISA) microplate reader (Dynatech Laboratories, Chantilly, VA, USA). The optical density of the formazan formed in the control (untreated) cells was used to represent 100% viability.

2.5. *Oil red O staining*

The cells were washed gently with ice-cold phosphate-buffered saline (PBS) twice and fixed with 3.7% formalin (Sigma-Aldrich Chemical Co.) at room temperature for 1 h. Thereafter, the cells were stained with Oil Red O solution (0.5% in propylene glycol, Sigma-Aldrich Chemical Co.) for 20 min and rinsed with 70% ethanol and PBS. The stained lipid droplets in the cells were then observed under a microscope (X100, Olympus, Tokyo, Japan).

2.6. *Measurement of the intracellular TG content*

The cellular TG contents were measured using a commercial TG assay kit (Sigma-Aldrich Chemical Co.) according to the manufacturer's instructions. Briefly, the harvested adipocytes were washed twice with ice-cold PBS and lysed in RIPA lysis buffer. Thereafter, the cells were centrifuged at 13,000 g for 20 min at 4°C, and the supernatants were used to measure the intracellular TG content at 550 nm using a microplate reader.

2.7. *Protein isolation and Western blot analysis*

As described in a previous study (*33*), the cells were collected and lysed with a cell lysis buffer. Then, their protein concentrations were determined by using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). For Western blotting, equal amounts of protein samples were electrophoretically transferred onto polyvinylidene difluoride membranes (Schleicher & Schuell, Keene, NH, USA) following electrophoretic separation on sodium-dodecyl sulfate (SDS) gel. Subsequently, the membranes were blocked with 5% non-fat dry milk/Tris-buffered saline containing 0.1% Triton X-100 (TBST, Sigma-Aldrich Chemical Co.) for 1 h and probed overnight with specific primary antibodies at 4°C. After washing the primary antibodies with TBST, the membranes were incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (Amersham Biosciences, Westborough, MA, USA) for 2 h at room temperature. The protein bands were detected using an enhanced chemiluminescence (ECL) kit (Amersham Biosciences) as per the manufacturer's instructions.

2.8. *Statistical analyses*

All numerical data are expressed using mean ± standard deviation (SD) values. The significance of the differences between the mean values was analyzed using the Student's *t*-test. All statistical analyses were conducted using the Statistical Package for the Social Sciences version 17.0 software (SPSS Inc., Chicago, IL, USA). Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. *Effect of EEAY on 3T3-L1 preadipocyte viability*

3T3-L1 preadipocytes were exposed to various concentrations of EEAY to measure its effect on cell viability using an MTT assay. Our data indicate that EEAY had no cytotoxic effect on the 3T3-L1 cells at concentrations ≤ 500 µg/mL (Figure 1), and the cells did not show morphological changes (data not shown). Therefore, the maximum concentration of EEAY was set at 200 µg/mL for future research.

3.2. *EEAY inhibits the differentiation of 3T3-L1 preadipocytes*

To measure the effects of EEAY on adipogenesis, 3T3-

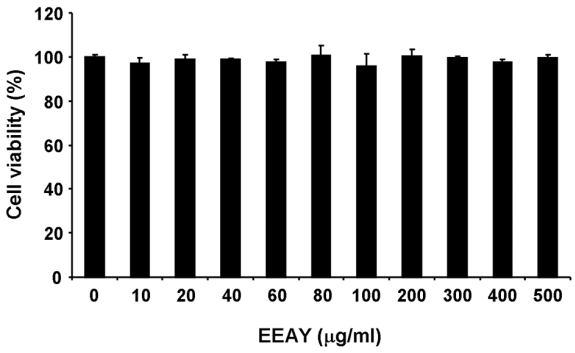

Figure 1. Effects of EEAY on the proliferation of 3T3-L1 mouse preadipocytes. Cells were treated with the indicated concentrations of EEAY for 72 h. Cell viability was determined using an MTT assay. Data of the three independent experiments are expressed as mean ± SD values.

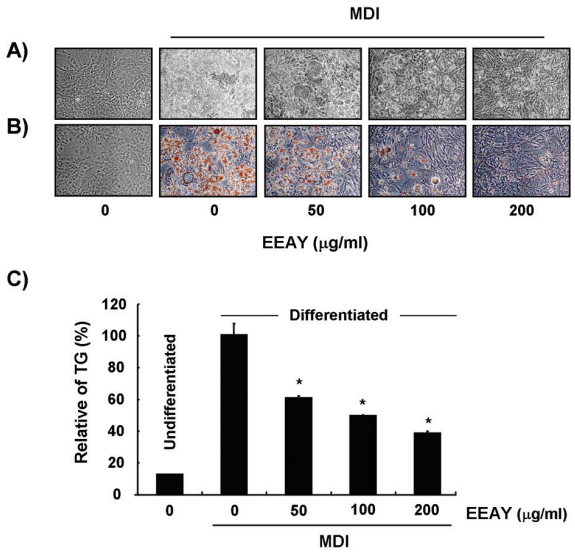

Figure 2. Effects of EEAY on intracellular lipid accumulation and TG content in the differentiated 3T3-L1 cells. The 3T3-L1 preadipocytes were treated with various concentrations of EEAY during differentiation. (A and B) On Day 8, the cells were directly observed via light microscopy **(A)** or the cells were fixed and stained with Oil Red O to visualize lipid droplets **(B)** (Magnification, ×200). **(C)** The TG content was measured to quantify the intracellular lipid content at 500 nm wavelength using the ELISA reader. All values of the three independent experiments are presented as means ± SD values (*$p < 0.05$ *vs*. the differentiated cells without treatment).

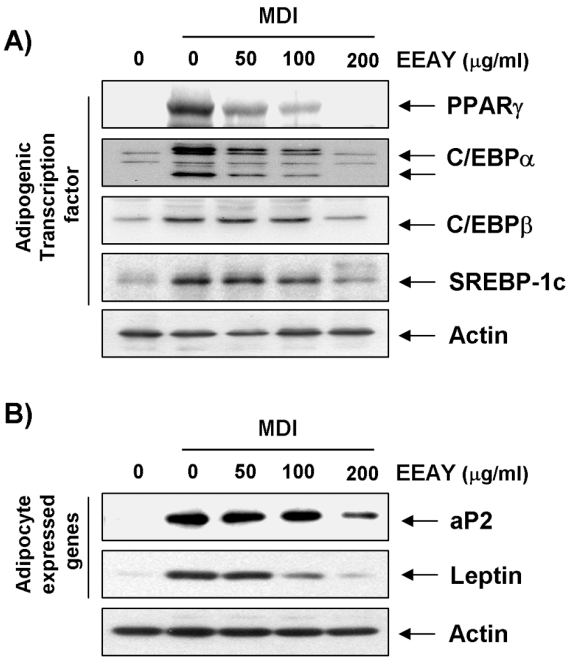

Figure 3. Effects of EEAY on the levels of adipogenic transcription factors (A) and adipocyte expressed genes (B) expression in the differentiated 3T3-L1 cells. At day 0, confluent 3T3-L1 preadipocytes were treated with various concentrations of EEAY in the absence or presence of an MDI differentiation medium for 48 h. On day 8, completely differentiated cells were lysed to extract the total protein. The cellular proteins were separated electrophoretically using SDS-polyacrylamide gels and transferred onto membranes. The membranes were probed with the indicated antibodies. The proteins were visualized using an ECL detection system. Actin was used as an internal control.

L1 preadipocytes were differentiated in the presence or absence of EEAY. As shown in Figure 2A and B, EEAY reduced lipid accumulation in 3T3-L1 adipocytes, as evidenced by the decrease in the cell size and number of lipid droplets in the mature adipocytes in a concentration-dependent manner. Further, it is noteworthy that there was about 60% lipid reduction after treatment with 200 μg/mL EEAY (Figure 2C). These data suggest that EEAY could exert an anti-adipogenic effect through inhibition or delay in the differentiation and adipogenesis of the 3T3-L1 preadipocytes.

3.3. *EEAY attenuates the expression of adipogenic transcription factors*

The differentiation of preadipocytes into adipocytes is coordinated by a complex process that involves the sequential activation of multiple adipogenic transcription factors, including PPARγ, C/EBPα/β, and SREBP-1c (*6,7*). To investigate the role of EEAY in reducing fat accumulation during the adipogenesis of 3T3-L1 preadipocytes, we examined the effects of EEAY on the expression of these adipogenic transcription factors. The immunoblotting results indicated that the increased expression of the transcription factors was largely inhibited following EEAY treatment in a concentration-dependent manner (Figure 3A). These results suggest that the inhibition of lipid accumulation by EEAY is associated with the suppression of the expression of adipogenic transcription factors.

3.4. *EEAY suppress the expression of adipocyte-related genes*

We further examined the influence of EEAY on the expression of adipocyte-related markers such as aP2 and leptin (*9,11*). As shown in Figure 3B, the increased expression of aP2 and leptin protein in differentiated 3T3-L1 cells decreased in a concentration-dependent manner compared to that in the control group after EEAY administration. These results support the hypothesis that the anti-adipogenic effect of EEAY is associated with the downregulation of the expression of adipocyte-related markers involved in lipid metabolism.

3.5. *EEAY induces the phosphorylation of AMPK and ACC*

AMPK plays a central role in regulating the cellular metabolism and energy balance in the adipose tissue. The activation of AMKP inhibits preadipocyte differentiation and adipogenesis through increased phosphorylation of ACC, a substrate of AMPK (*13,17*). To investigate whether AMPK is activated by EEAY during 3T3-L1 differentiation, we analyzed the levels of phosphorylated AMPK and ACC and found that EEAY remarkably enhanced the phosphorylation of AMPK and ACC

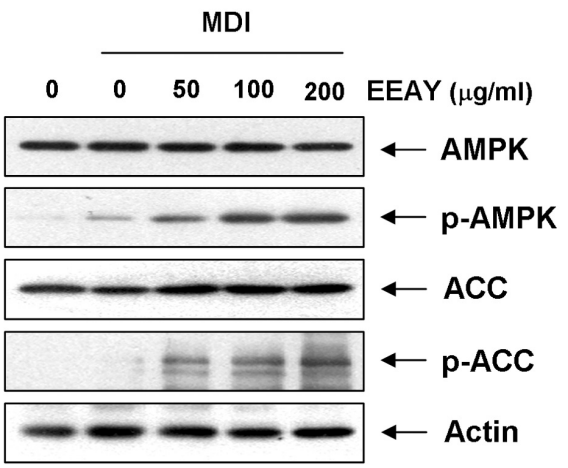

Figure 4. Effects of EEAY on the phosphorylation of AMPK and ACC in the differentiated 3T3-L1 cells. The proteins were isolated cells grown under the same conditions (as shown in Figure 3), and the total protein levels of AMPK and ACC, and their phosphorylated states (p-AMPK and p-ACC, respectively) were determined using the indicated antibodies and an ECL detection system. Actin was used as an internal control.

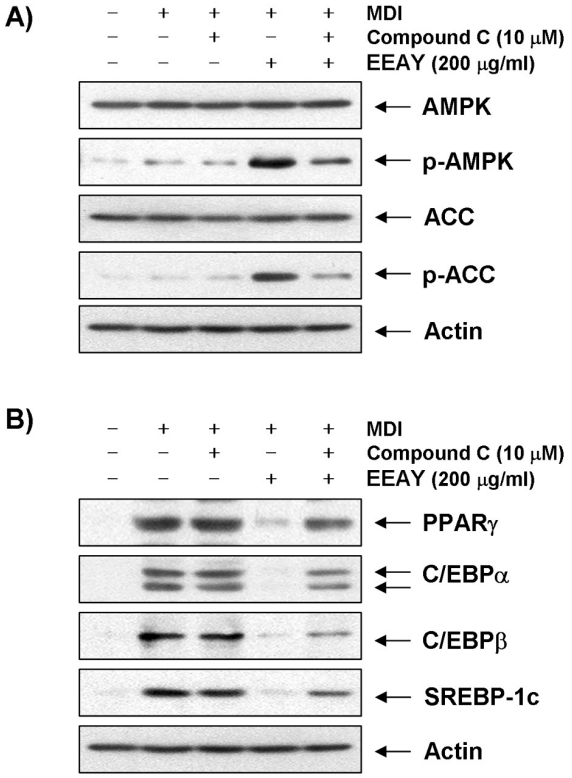

Figure 5. Effects of AMPK inhibitor on the EEAY-induced reduction in the expression of adipogenic transcription factors in the differentiated 3T3-L1 cells. Cells were pre-treated with compound C, an AMPK inhibitor, for 1 h, and then treated with 200 mg/mL EEAY. On day 8, completely differentiated cells were lysed, and the cellular proteins were separated electrophoretically using SDS-polyacrylamide gels and transferred onto membranes. The membranes were probed with the indicated antibodies. Proteins were observed using an ECL detection system. Actin was used as an internal control.

without altering the expression of their total protein expression, indicating the activation of the AMPK pathway. (Figure 4). However, when compound C, an AMPK specific inhibitor, was pretreated in the 3T3-L1 cells, the phosphorylation of both, AMPK and ACC induced by EEAY was significantly reduced (Figure 5A).

3.6. *Activation of the AMPK signaling pathway is involved in the inhibitory effects of EEAY on 3T3-L1 adipogenesis*

We aimed to analyze the relationship between the activation of the AMPK signaling pathway and EEAY-mediated inhibition of adipogenesis. Therefore, we evaluated the EEAY-induced reduction in the effect of compound C on the expression of adipogenic transcription factors such as PPARγ, C/EBPα, C/EBPβ, and SREBP-1c. As shown in Figure 5B, the expression of these transcription factors that was repressed by EEAY was increased again in the presence of compound C. These results suggest that the EEAY-induced inhibition of adipogenesis in the 3T3-L1 cells is achieved by the activation of at least the AMPK signaling pathway.

4. Discussion

In this study, we investigated the anti-adipogenesis effect of EEAY using 3T3-L1 preadipocytes. According to the results of this study, the lipid accumulation and TG content in the cytoplasm of differentiated 3T3-L1 cells was actively increased and was significantly inhibited by EEAY treatment in a concentration-dependent manner. Adipocyte differentiation is accompanied by lipid and TG accumulation; therefore, the results indicate that EEAY treatment significantly suppressed the differentiation of 3T3-L1 preadipocytes.

The differentiation of preadipocytes into adipocytes is regulated by a complicated process that is sequentially co-ordinated by various transcription factors. Among them, C/EBPβ is induced in the early stages of differentiation and is known to induce the expression of PPARγ and C/EBPα, major regulators of adipogenesis and lipogenesis in the late stage differentiation (*6,7*). In addition, SREBP-1c is involved in lipid metabolism and is a further regulator of fatty acid synthesis enzymes. This transcription factor also enhances the differentiation of preadipocytes and the expression of down-stream genes associated with fatty acid metabolism (*34,35*). In addition to the activation of adipogenic transcription factors, transactivating adipocyte-specific genes such as aP2 and leptin are also critically important for the differentiation of preadipocytes into mature adipocytes (*8,10*). Therefore, the inhibition of adipogenic transcription and adipocyte-specific factors would decrease the adipocyte differentiation associated biosynthesis of fatty acids and TGs. In order to investigate the role of EEAY in regulating the expression level of the adipogenic transcription factors during adipogenesis of 3T3-

L1 cells, we compared their expression levels in the presence and absence of EEAY. As shown in Figure 3A, our immunoblotting data indicated that EEAY treatment of 3T3-L1 adipocytes significantly diminished the expression of the four transcription factors that were examined during adipocyte differentiation. These results indicated that EEAY blocked adipocyte differentiation by suppressing the expression of transcriptional factors involved in the different stages of differentiation. In addition, EEAY also reduced the expression of adipocyte markers aP2 and leptin on the 3T3-L1 cells. aP2 is a carrier protein that can trigger the accumulation of lipid droplets in the cytoplasm of differentiating adipocytes (*36,37*), and leptin upregulates the adipocyte genes involved in lipid oxidation, enhancing lipid accumulation in the adipocytes (*38,39*); therefore, our results also suggest that EEAY strongly suppress the de novo synthesis of TGs and differentiation of adipocytes.

Accumulating evidence suggests that the AMPK signaling pathway is a target for the energy balance and metabolic disorders involved in the maintenance of lipid and cholesterol homeostasis. During preadipocyte differentiation and adipogenesis, AMPK is inactivated by lower phosphorylation levels (*17,19*). In addition, AMPK activation by phosphorylation can reduce the degree of obesity by inhibiting adipocyte differentiation by changing the expression and activity of the enzymes and proteins involved in lipid metabolism (*16,19*). Furthermore, ACC, a downstream substrate of AMPK, is a rate-limiting enzyme that limits the critical rates in fatty acid synthesis and oxidation, reducing the fatty acid and lipid synthesis to inhibit the onset and progression of obesity (*13,18*). Therefore, AMPK signaling has gained the attention of researchers as a molecular target for fighting obesity. To determine whether AMPK activation is involved in EEAY-mediated attenuation of adipocyte differentiation and adipogenesis, we examined whether EEAY can activate AMPK. According to our results, EEAY markedly elevates the phosphorylation level of AMPK in a dose-dependent manner. Moreover, ACC phosphorylation was also increased, indicating that the AMPK signaling pathway was activated following EEAY administration. To investigate whether EEAY directly weakens adipocyte differentiation and adipogenesis through AMPK activation, the effect of compound C, an AMPK inhibitor, on the reduced adipogenic transcription factors in cultured 3T3-L1 cells in the presence of EEAY was evaluated. As expected, the elevated phosphorylation of ACC and AMPK was inhibited simultaneously by treatment with compound C (Figure 5A). In addition, the EEAY-induced reduction in the expression of transcription factors such as PPARγ, C/EBP members, and SREBP-1c was reversed by the suppression of AMPK activity by compound C (Figure 5B). The results collectively indicate that EEAY stimulated AMPK activity, suppressing the adipocyte differentiation regulators and consequently their target lipogenic enzymes and proteins, ultimately resulting in reduced lipid accumulation.

In summary, our results indicate that EEAY reduced adipocyte differentiation and adipogenesis in the 3T3-L1 cells by suppressing the adipogenic transcriptional factors and their downstream target genes without cytotoxicity. Further, EEAY also increased the phosphorylation of AMPK and ACC; however, the artificial blockage of AMPK activity suppressed the inhibitory effects of EEAY on the expressions of adipogenic transcriptional factors, demonstrating that EEAY has significant anti-adipogenic effect that function *via* the AMPK pathway. Thus, the activation of the AMPK pathway by EEAY may be a potential strategy for preventing obesity.

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