



Special Issue

Aster yomena (Kitam.) Honda Inhibits Adipocyte Differentiation in 3T3-L1 Cells

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SUMMARY

Background: Obesity has become a global health problem and causes serious complications. In this study, we investigated the effect of ethyl acetate fraction from *Aster yomena* (Kitam.) Honda (EFAY) on obesity in 3T3-L1 cells.

Methods: To examine the effect of EFAY on adipocyte differentiation, we conducted oil red O staining assay in mature adipocytes. 3T3-L1 preadipocytes were differentiated with EFAY (25, 50, and 100 µg/mL) for 4 days. On day 9 of differentiation, the number of lipid droplets was measured by oil red O. The underlying mechanisms of the action of EFAY against differentiation in 3T3-L1 cells were investigated using Western blot analysis.

Results: In oil red O staining assay, differentiated adipocytes generated massive lipid droplets compared with preadipocytes. However, treatment with EFAY significantly reduced the generation of lipid droplets compared to EFAY non-treated adipocytes. Western blot analysis revealed that EFAY down-regulated adipogenesis-related protein expressions, such as peroxisome proliferator-activated receptor γ , CCAAT/enhancer-binding protein (C/EBP) α , and C/EBP β . Furthermore, EFAY down-regulated levels of lipogenesis-related protein expressions such as fatty acid synthase and acetyl-CoA carboxylase. On the contrary, EFAY promoted lipolysis by activating phospho-hormone-sensitive lipase and adipose triglyceride lipase. Phospho-AMP-activated protein kinase (p-AMPK) and phospho-acetyl-CoA carboxylase were up-regulated by treatment of 3T3-L1 cells with EFAY, subsequently up-regulating GLUT4 levels, indicating that EFAY could regulate glucose uptake as well as lipid metabolism.

Conclusions: EFAY reduced adipocyte differentiation by suppressing adipogenesis and lipogenesis, and provoking lipolysis and AMPK pathway. These results imply that EFAY could be employed as a natural agent to ameliorate obesity.

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1. Introduction

The prevalence of obesity has risen steadily, and now obesity has become a major health problem worldwide. According to the World Health Organization, about 13% of adults (more than 650 million) were obese in 2016, and the percentage has nearly tripled since 1975.¹ Obesity is a state of excessive adipose accumulation resulting from sustained positive energy balance, which arises due to an imbalance between energy intake and expenditure.^{2,3}

Obesity is characterized by increased adipose tissue mass; henceforth adipocytes are widely used to study obesity at the cellular level.⁴ In particular, to investigate mechanisms related to adipogenesis under *in vitro* model, 3T3-L1 cell lines are widely employed.⁵ The differentiation process of 3T3-L1 cells is controlled by various transcription factors. During the early phase of adipocyte differentiation, CCAAT/enhancer-binding protein (C/EBP) family mediates the expression of

peroxisome proliferator-activated receptor γ (PPAR γ) and C/EBP α , which can induce adipocyte differentiation.⁶ The target genes for adipogenesis are associated with lipogenesis and glucose uptake. In the terminal phase of differentiation, *de novo* lipogenesis is markedly increased in adipocytes.⁶ The lipogenic enzymes such as fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC) are activated with an increase in the levels of glucose and insulin.⁷ AMP-activated protein kinase (AMPK) pathway was implicated in the regulation of fatty acid metabolism as well as glucose transport in 3T3-L1 cells.⁷ The activated AMPK could inhibit phosphorylation of ACC, causing a reduction in lipogenesis and regulation of phosphorylation of hormone-sensitive lipase (HSL), which modulates lipolysis.⁸ HSL and adipose triglyceride lipase (ATGL) are known as participants in lipolysis and catabolism of stored TGs for mobilization of fatty acid.⁹

There exist numerous medications for obesity management, but they are associated with serious adverse effects.¹⁰ Apparently, recent focus has been on the development of non-toxic and natural agents for the development of anti-obesity medications. *Aster* species including *Aster yomena* (Kitam.) Honda (AY) were used as traditional medicines for antibacterial and insecticidal medicines.¹¹ According to previous studies, AY exerted several biological activities

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which are beneficial for metabolism in human.^{12,13} AY demonstrated the anti-inflammatory effect in lipopolysaccharide-stimulated RAW 264.7 by suppressing Toll-like receptor-mediated nuclear factor κ B signaling pathway.¹⁴ Furthermore, it inhibited the biomarkers of asthma by suppressing Th2 responses in a murine asthma model, which was induced by ovalbumin.¹⁵ It was reported that ethyl acetate fraction from AY (EFAY) exerted high antioxidant activity compared to other extract and fractions due to the presence of high phenolic contents.¹⁶ Several evidences demonstrated the preventive effects of phenolic compounds against obesity,¹⁷ therefore EFAY could effectively inhibit obesity. However, the protective effect of EFAY against adipocyte differentiation and obesity has not been studied yet. In this study, we evaluated the anti-obesity effect of an active fraction from AY in 3T3-L1 cells and elucidated the regulatory mechanisms of EFAY on adipocyte differentiation.

2. Materials and methods

2.1. Reagents and materials

3T3-L1 cells were purchased from the Korean Cell Line Bank (Seoul, Korea). Dulbecco's modified eagle's medium (DMEM), penicillin-streptomycin solution, bovine calf serum (BCS), fetal bovine serum (FBS), and trypsin-EDTA solution were manufactured by Welgene Inc. (Daegu, Korea). 3-Isobutyl-1-methylxanthine (IBMX), dexamethasone, and insulin were purchased from Sigma Chemical Co. (St Louis, MO, USA). Radioimmunoprecipitation assay (RIPA) buffer was obtained from Elpis Biotech. (Daejeon, Korea). Polyvinylidene fluoride (PVDF) membrane was purchased from Millipore Co. (Billerica, USA). Antibodies were purchased from Cell Signaling Tech. (Beverly, USA).

2.2. Preparation of sample

AY was obtained from Gurye-gun (Jeollanam-do, Korea). The extraction was executed with methanol (MeOH) in a reflux cooling system at 65–75 °C using the leaf part of AY (1,645 g). Consequently, 393.9 g of AY MeOH extract was obtained. This extract was partitioned in order from non-polar solvent to polar solvent using *n*-hexane, dichloromethane, ethyl acetate (EtOAc), and *n*-butanol. Finally, 4.2 g of EFAY was obtained.

2.3. Cell culture

3T3-L1 cells were maintained in DMEM supplemented with 10% BCS and 100 units/mL penicillin-streptomycin. In order to induce differentiation into adipocytes, after 2 days from the confluent state in each well, cells were fed with DMEM supplemented with 10% FBS and MDI (0.5 mM IBMX, 1 μ M dexamethasone, and 10 μ g/mL insulin). After 2 days, cells were fed with DMEM containing 10% FBS and 10 μ g/mL insulin for further 2 days. Subsequently, the media was replaced with DMEM containing 10% FBS every other day.

2.4. Oil red O staining assay

Oil red O staining assay was carried out to assess the amount of lipid accumulation in 3T3-L1 cells. The 3T3-L1 cells were grown in 6-well plate at a density of 1×10^5 cells/mL. EFAY (25, 50, and 100 μ g/mL) was added to media for 4 days when differentiation was induced. On day 9 of differentiation, the cells that had been washed with phosphate buffered saline (PBS, pH 7.4) were fixed for 1 h with 10% formaldehyde at room temperature. Typically, 0.35% oil red O solution (diluted with water at 3:2 ratio) was added for 30 min to

stain the lipid droplets. The cells were washed with PBS to remove extra oil red O. The photographs were taken using an $\times 100$ immersion lens on optical microscopy.

2.5. Western blot analysis

Cells were collected and subsequently extracted with RIPA buffer containing protease inhibitor cocktail. The extracted proteins were equally quantified and separated by 8–10% sodium dodecyl sulfate-polyacrylamide gels electrophoresis. The separated proteins were transferred to PVDF membranes. The membranes were incubated with 5% skim milk solution with tween 20 for 50 min and then incubated with primary antibodies overnight at 4 °C as follows: [β -actin (1:1000, Cell Signaling Tech.); PPAR γ (1:500, Cell Signaling Tech.); C/EBP α (1:500, Cell Signaling Tech.); C/EBP β (1:500, Cell Signaling Tech.); FAS (1:500, Cell Signaling Tech.); ACC (1:500, Cell Signaling Tech.); p-HSL (1:500, Cell Signaling Tech.); ATGL (1:500, Cell Signaling Tech.); p-AMPK (1:500, Cell Signaling Tech.); AMPK (1:500, Cell Signaling Tech.); p-ACC (1:500, Cell Signaling Tech.); glucose transporter 4 (GLUT4, 1:500, Cell Signaling Tech.)]. After incubation with appropriate HRP-conjugated secondary antibodies for 1 h, immunocomplexes were visualized with Davinci-chemiluminescent imaging system (CoreBio, Seoul, Korea).

2.6. Statistical analysis

All data are expressed as mean \pm standard deviation (SD) and statistical comparisons for determining significance were conducted using one-way ANOVA and Duncan's post-hoc-tests. $p < 0.05$ was considered a statistically significant difference.

3. Results

3.1. Effect of EFAY on the differentiation of 3T3-L1 cells

Lipid accumulation is considered as a marker of adipocyte differentiation in 3T3-L1 cells. The content of accumulated lipid was measured using oil red O staining assay. Considerable generation of lipid droplets was observed in the differentiated adipocytes compared to preadipocytes (Figure 1). Treatment of the cells with EFAY during differentiation, especially at a concentration of 100 μ g/mL, significantly reduced the production of lipid droplets compared to non-treated controls, showing 65.47% and 49.45% production at the concentrations of 50 and 100 μ g/mL, respectively.

3.2. Effects of EFAY on adipogenesis-related protein expressions in 3T3-L1 cells

To investigate the effect of EFAY on adipocyte differentiation in 3T3-L1 cells, Western blot analysis was performed. The mature 3T3-L1 adipocytes elevated the expressions of PPAR γ , C/EBP α , and C/EBP β compared to 3T3-L1 preadipocytes (Figure 2). After treatment with EFAY, however, protein expression of PPAR γ and C/EBP β was significantly reduced compared to adipocytes. In particular, EFAY effectively inhibited C/EBP α expression in 3T3-L1 adipocytes. These results suggested that EFAY could inhibit adipogenesis during differentiation in 3T3-L1 adipocytes by modulating PPAR γ , C/EBP α , and C/EBP β protein expressions.

3.3. Effects of EFAY on lipogenesis-related protein expressions in 3T3-L1 cells

The effect of EFAY on lipogenesis in 3T3-L1 cells was observed.

The protein expression of FAS and ACC was significantly increased in 3T3-L1 adipocyte compared to preadipocytes, but it was decreased

by treatment with EFAY (Figure 3). EFAY effectively diminished the ACC expression. These results proposed that lipogenesis pathway was discouraged by EFAY via reductions in the expressions of FAS and ACC.

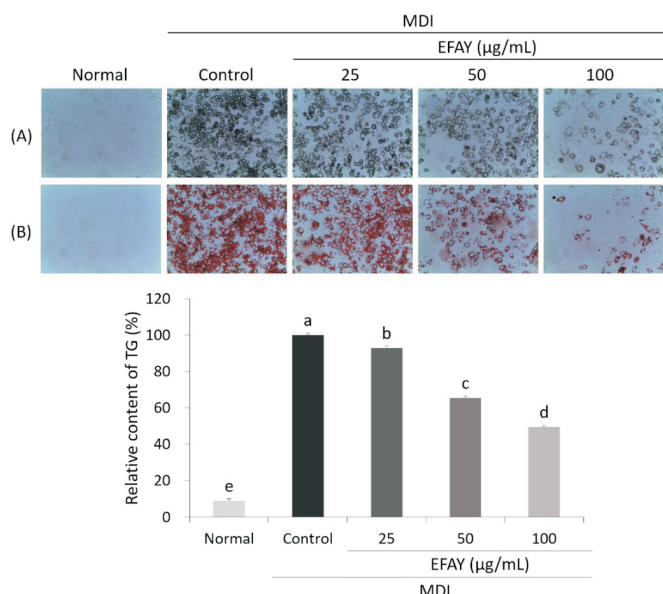


Figure 1. Effect of EtOAc fraction from *Aster yomena* (Kitam.) Honda on the differentiation of 3T3-L1 cells. (A); Light microscopy (magnification, $\times 100$) (B); Stained with oil red O, light microscopy (magnification, $\times 100$). Values are mean \pm SD. ^{a-e} Means with the different letters are significantly different ($p < 0.05$) by Duncan's multiple range test.

3.4. Effects of EFAY on lipolysis-related protein expressions in 3T3-L1 cells

We examined whether EFAY inhibits lipolysis in 3T3-L1 adipocytes. Treatment with EFAY resulted in an obvious increase in p-HSL and ATGL protein expressions, compared to non-treated 3T3-L1 adipocytes (Figure 4). These results indicated that EFAY promoted lipolysis in the adipocytes by stimulating production of p-HSL and ATGL.

3.5. Effects of EFAY on AMPK pathway-related protein expressions in 3T3-L1 cells

The effect of EFAY on AMPK pathway in 3T3-L1 adipocytes was observed. Treatment with EFAY promoted phosphorylation of AMPK and ACC compared to MDI-treated control group, indicating that EFAY treatment activated AMPK pathway (Figure 5). EFAY also increased protein expression of GLUT4, which is one of the downstream targets of AMPK pathway. These results showed that EFAY enhanced AMPK pathway by phosphorylating AMPK and ACC. Furthermore, EFAY increased GLUT4 protein expression.

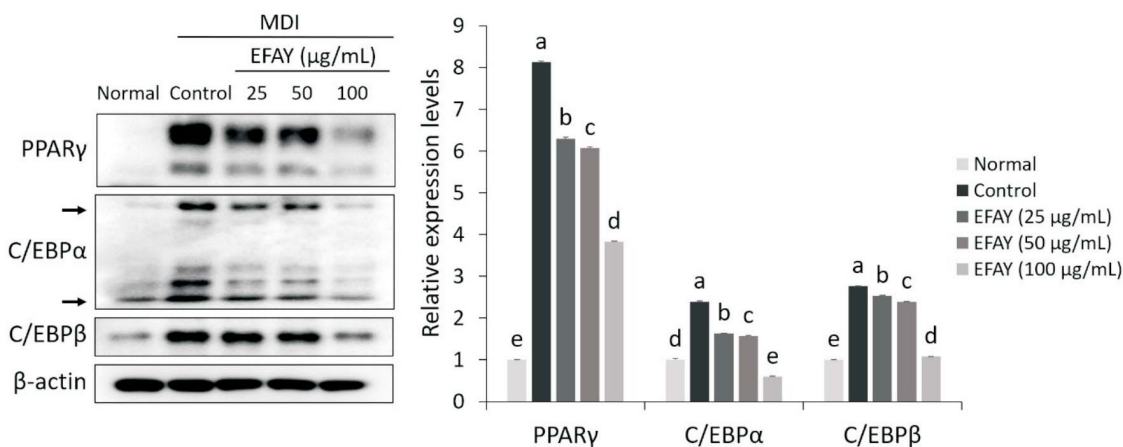


Figure 2. Effects of EtOAc fraction from *Aster yomena* (Kitam.) Honda on adipogenesis-related protein expressions in 3T3-L1 cells. Values are mean \pm SD. ^{a-e} Means with the different letters are significantly different ($p < 0.05$) by Duncan's multiple range test.

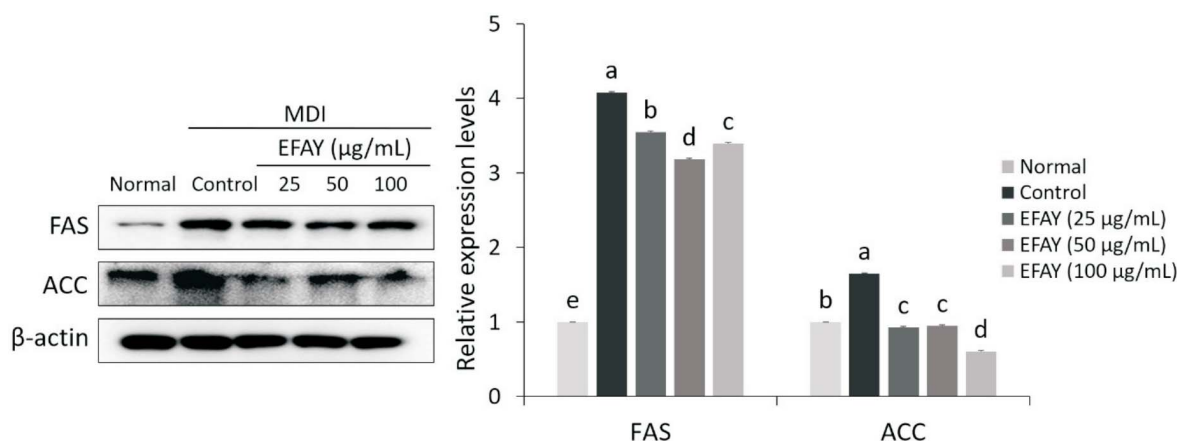


Figure 3. Effects of *Aster yomena* (Kitam.) Honda on lipogenesis-related protein expressions in 3T3-L1 cells. Values are mean \pm SD. ^{a-e} Means with the different letters are significantly different ($p < 0.05$) by Duncan's multiple range test.

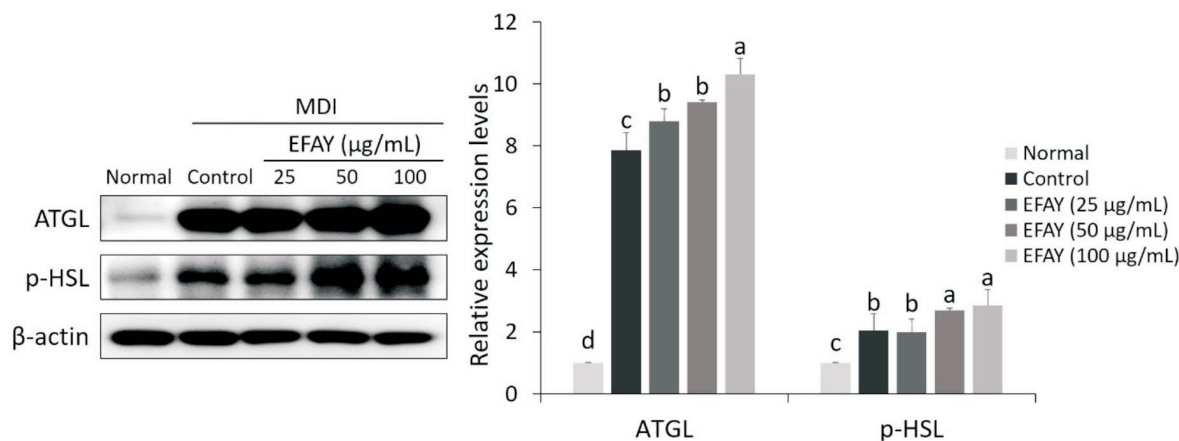


Figure 4. Effects of *Aster yomena* (Kitam.) Honda on lipolysis-related protein expressions in 3T3-L1 cells. Values are mean \pm SD. ^{a-d} Means with the different letters are significantly different ($p < 0.05$) by Duncan's multiple range test.

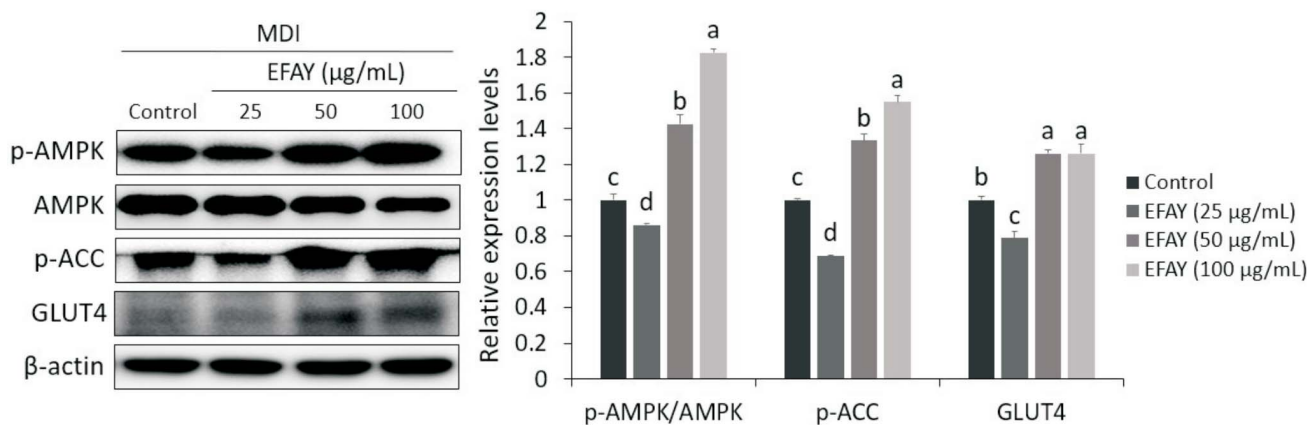


Figure 5. Effects of *Aster yomena* (Kitam.) Honda on glucose uptake-related protein expressions in 3T3-L1 cells. Values are mean \pm SD. ^{a-d} Means with the different letters are significantly different ($p < 0.05$) by Duncan's multiple range test.

4. Discussion

Obesity and its complications possess a major threat to our health. As adipose tissue grows, adipocytes that are components of adipose tissue are gradually activated and release more adipocytokines.¹⁸ It is known that differentiation from preadipocytes to adipocytes and subsequent lipid accumulation in adipocytes are closely related to obesity.^{19,20} Our previous study revealed that EFAY showed greatest pharmacological activities among other extracts and fractions.²¹ Therefore, in the present work, we evaluated the anti-obesity activity of EFAY on adipocyte differentiation in 3T3-L1 cells.

Lipid droplets are accumulated in the cytoplasm when 3T3-L1 preadipocytes differentiate into adipocytes. Staining of accumulated lipid with oil red O, which is a dye for specifically staining triglycerides, is considered as a useful method to quantitate the extent of differentiation.²² Our results showed that treatment of preadipocytes with MDI induced generation of lipid droplets, indicating successive differentiation of preadipocytes into adipocytes. However, treatment with EFAY significantly reduced lipid accumulation in 3T3-L1 adipocytes in a concentration-dependent manner, suggesting that EFAY inhibited adipocyte differentiation. Previous studies reported that esculetin and caffeic acid present in EFAY reduced lipid content in confluent 3T3-L1 preadipocytes during the lipid filling stage.^{23,24} Apparently, our results suggest that EFAY and its active compounds inhibit adipocyte differentiation in 3T3-L1 cells.

It is well-known that the exposure of post-confluent 3T3-L1

preadipocytes to MDI promotes adipogenesis.²⁵ Adipogenesis is the development of mature adipocytes from preadipocytes and a complex process accompanied by many signaling pathways and transcription factors.²⁶ C/EBP β is the first transcription factor to get activated during adipogenesis which is responded to dexamethasone in the early stage of differentiation.²⁷ The C/EBP β mediates the activation of PPAR γ and C/EBP α which are the key transcription factors of adipogenesis by inducing transcription of many adipocyte genes alone and in combination.²⁷ In the present study, treatment of post-confluent 3T3-L1 preadipocytes with MDI promoted PPAR γ , C/EBP α , and C/EBP β protein expressions, and EFAY significantly down-regulated the expressions of the specific genes in 3T3-L1 adipocytes. Previous researches demonstrated that the down-regulation of PPAR γ , C/EBP α , and C/EBP β during differentiation plays a key role in inhibiting differentiation of 3T3-L1 cells, which has been proven by several studies on the use of dietary agents.^{26,28,29} Our results propose that EFAY exerts an inhibitory effect on adipocyte differentiation in 3T3-L1 cells by regulation of adipogenesis-related protein expression.

It is known that transcription factors including PPAR γ and C/EBP α regulate the synthesis of fatty acid during adipocyte differentiation.³⁰ FAS and ACC are the key enzymes in *de novo* lipogenesis, a metabolic pathway for the synthesis of fatty acids.³¹ FAS synthesizes fatty acids by adding malonyl-CoA units to acetyl-CoA, and ACC carboxylates acetyl-CoA to produce malonyl-CoA.³² Our data showed upregulation of protein expressions of FAS and ACC, resulting in lipogenesis in mature adipocytes compared with preadipocytes. On

the contrary, EFAY significantly lowered FAS and ACC expressions in adipocytes. These results suggest that EFAY suppresses lipogenesis by down-regulating FAS and ACC derived from the inhibited expression of adipogenic transcription factors during adipocyte differentiation.

In the lipolysis process, triglyceride (TG) that are stored in cellular lipid droplets are degraded and produce fatty acids.³³ HSL and ATGL sequentially catabolize TG in adipose tissue. HSL hydrolyze TG to diacylglycerol (DG) and subsequently, ATGL converts DG to monoacylglycerol.³⁴ The present study confirmed that lipolysis-related protein expressions including p-HSL and ATGL were up-regulated in the presence of EFAY during differentiation into 3T3-L1 adipocytes. These results indicate that EFAY activated lipolysis in 3T3-L1 adipocytes by enhancing protein expressions of p-HSL and ATGL.

AMPK, a sensor of energy status in cells gets activated when energy status is lowered.³⁵ A drop in cellular ATP/AMP ratio triggers activation of AMPK which participates in various metabolic reactions by phosphorylation.³⁶ ACC is a downstream target of AMPK, as well as lipogenesis-related protein. However, the lipogenic ability of ACC is inactivated by phosphorylation in adipocytes.³⁷ Our data indicate that EFAY promotes activation of AMPK followed by phosphorylation of ACC, which attributes to inhibition of lipogenesis. Furthermore, it was observed that the level of GLUT4, a glucose transporter in adipocytes, was up-regulated by EFAY. Previous studies demonstrated that AMPK cascades could promote glucose uptake while inhibiting adipogenesis- and lipolysis-related protein expressions and this was distinguished from insulin activity in 3T3-L1 cells.^{38,39} Activated AMPK accelerates GLUT4 translocation to membrane resulting in stimulation of glucose uptake.⁴⁰ The present results demonstrated that EFAY stimulated AMPK pathway in 3T3-L1 cells. Furthermore, EFAY could promote glucose uptake by enhancing GLUT4 protein expression via the AMPK pathway, suggesting that EFAY could ameliorate complications of obesity such as type 2 diabetes.

In summary, we examined the potential of EFAY in exerting regulatory effects on obesity in 3T3-L1 cells. EFAY reduced lipid accumulation during differentiation of preadipocytes to adipocytes, suppressed the adipogenesis-related protein expressions of PPAR γ , C/EBP α , and C/EBP β , and inhibited the expressions of FAS and ACC which caused lipogenesis. Furthermore, EFAY promoted lipolysis that was mediated by p-HSL and ATGL, and AMPK pathway including p-AMPK and p-ACC, and activated GLUT4 that was downstream of AMPK pathway. These results suggest that AY could play beneficial roles in exerting a protective effect on obesity, therefore we propose AY as a potential natural anti-obesity agent.

Disclosure of conflicts of interest

None of the authors have any financial or other potential conflict of interest for this study.

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