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Roots Extract of *Adenophora triphylla* var. *japonica* Inhibits Adipogenesis in 3T3-L1 Cells through the Downregulation of IRS1

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The purpose of this study was to investigate the action mechanism of the roots of *Adenophora triphylla* var. *japonica* extract (ATE) in 3T3-L1 adipocytes. Cell toxicity test by MTT assay and lipid accumulation was performed to evaluate the inhibitory effect on the differentiation of adipocyte from preadipocytes induced by MDI differentiation medium, while adipogenesis related proteins expression level were evaluated by western blotting. As a result, ATE inhibited MDI-induced adipocyte differentiation in 3T3-L1 cells dose-dependently without cytotoxicity. Our results showed that ATE inhibited the phosphorylation of IRS1, thereby decreasing the expression of PI3K110α and reducing the phosphorylation of AKT and mTOR, resulting in attenuated protein expression of C/EBPα, PPARγ, ap2 and FAS in 3T3-L1 cells. These results suggest anti-adipogenic functions for ATE, and identified IRS1 as a novel target for ATE in adipogenesis.

keywords : Roots of *Adenophora triphylla* var. *japonica* extract, 3T3-L1 Adipocyte, Adipogenesis, Anti-obesity

Introduction

Obesity is considered a complex and multifactorial chronic disease. It is related to increased risks of various diseases, such as cancer, respiratory complications, osteoarthritis, type 2 diabetes, hyperlipidemia, hypertension, arteriosclerosis, fatty liver, and cardiovascular diseases.[1,2] For this reason, obesity has become a serious worldwide problem in human health[3]. Most studies have demonstrated the main cause of obesity in the lipid storage of adipocytes to be adipogenesis, and the 3T3-L1 cell line is primarily used to study the molecular mechanisms of adipogenesis.[4]

Adipogenesis involves the differentiation of preadipocytes into adipocytes, and the development of mature adipocytes. The mechanism of adipogenesis is regulated by several transcription factors.[5] Insulin binding of insulin receptor phosphorylates its substrates, such as insulin receptor substrates (IRS), and then activates phosphatidylinositol 3-kinase (PI3K), and induces the activation of downstream signaling molecules like AKT.[6,7] In addition, mammalian target of rapamycin (mTOR) signaling pathway, which is regulated by the upstream AKT pathway, has been demonstrated to be associated with adipocyte differentiation.[8-11] Adipocyte differentiation is closely linked to the mammalian target of rapamycin (mTOR) pathway. It has been previously demonstrated that treatment with rapamycin during 3T3-L1 adipocyte differentiation resulted in the reduction of protein levels of PPARγ and C/EBPα, thereby indicating that mTOR inactivation prevents adipocyte differentiation.[12]

Adenophora triphylla (A. triphylla) var. *japonica* (Korean name: Jan-dae, Japanese name: lady bell, English name: Three-leaf ladybell) belongs to the *Adenophora* species (Campanulaceae), which has been used as an oriental medicinal plant in Korea, China, and Japan for anti-inflammatory, anti-tussive, and hepatoprotective effects.[13]

Previous studies found that *Adenophora triphylla* var. *japonica* extract (ATE) inhibited lipid accumulation, and affected the expression level of adipogenesis-related proteins, such as PPARγ, adipocyte fatty acid-binding protein 2 (ap2), and fatty acid synthase (FAS) in 3T3-L1 adipocytes. Moreover, they explained that additional studies of the upstream mechanism of adipogenesis were needed.[14] Thus, the present study investigates the possible upstream mechanism of adipogenesis, such as IRS/PI3K/AKT and mTOR signaling in 3T3-L1 cells.

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# Both authors contributed equally to this work

Materials and Methods

1. Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM), Bovine Calf Serum (BCS), and penicillin-streptomycin were purchased from Gibco BRL (Grand Island, NY, USA). Fetal Bovine Serum (FBS) was purchased from ATLAS Biologicals (Fort Collins, CO). The 3-isobutylmethylxanthine, insulin, and dexamethasone were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Oil-red O (ORO) solution was purchased from Sigma-Aldrich (MO, USA). Isopropanol was obtained from Daejung Chemical (Seoul, Korea). Primary antibodies against β-actin, CCAAT/enhancer-binding protein (C/EBP) α, Peroxisome proliferator-activated receptor (PPAR) γ, Fatty acid synthase (FAS), Mammalian target of rapamycin (mTOR), p-mTOR, AKT, p-AKT, Insulin receptor substrates 1 (IRS1), p-IRS1 and Phosphoinositide 3-kinase (PI3K) 110α subunit were purchased from Cell Signaling Technology (Danvers, MA, USA). Adipocyte fatty acid-binding protein 2 (ap2) was purchased from Invitrogen (Carlsbad, CA, USA). Horseradish peroxidase (HRP)-linked anti-rabbit IgG and HRP-linked anti-mouse IgG were purchased from GenDEPOT (Barker, TX, USA).

2. 3T3-L1 mouse preadipocytes culture and differentiation

The 3T3-L1 mouse preadipocytes were purchased from the American Type Culture Collection (Rockville, MD, USA), and maintained in DMEM containing 10% BCS with 1% penicillin-streptomycin at 37 ℃ in an incubator containing a humidified atmosphere with 5% CO_2. To differentiate 3T3-L1 preadipocytes into mature adipocytes (defined as Day 0), fully confluent 3T3-L1 preadipocytes were treated with differentiation medium containing DMEM, 10% fetal bovine serum, 0.5 mM 3-isobutylmethylxanthine, 5 μg/mL insulin, and 1 μM dexamethasone (MDI differentiation medium). After two days incubation, the cell medium was replaced by DMEM supplemented with 10% FBS and 5 μg/mL of insulin (Day 2). After another two days, the medium was changed with DMEM containing 10% FBS (Day 4). These cells were completely differentiated into mature adipocytes on Day 6. To determine the effect of ATE on adipogenesis, differentiated preadipocytes were treated with presence or absence of ATE for 4 d. Roots of *Adenophora triphylla* var. *japonica* extract was prepared by same method as previously reported[14]. Dried *A. triphylla* var. *japonica* was extracted with 70% aqueous ethyl alcohol for 24 hours at 70 ℃ and was then filtered. The filtered extract was concentrated with a vacuum evaporation and dried.

3. Oil-red O staining

After differentiation (Day 8), 3T3-L1 cells were washed with phosphate-buffered saline (PBS), fixed with 10% (v/v) formalin solution (Sigma-Aldrich, MO, USA) for 1 h, and dried. Fresh Oil-red O working solutions were prepared by mixing stock solution with distilled water (6:4), followed by incubation for 1 h at room temperature (RT), and further filtration. Cells were stained with Oil-red O working solution for 4 h, washed with distilled water, and photographed with ECLIPSE Ts2 microscope (Nikon Corporation, Tokyo, Japan). Stained lipid droplets were extracted with isopropanol, and the absorbance was measured at 520 nm by microplate reader (Tecan, Mannedorf, Switzerland).

4. Cell cytotoxicity activity

Cell viability was determined by MTT assay. The cells were treated with various concentrations of ATE (0, 100, 300, and 500 μg/mL) for 24 h. MTT solution (5 mg/mL) was added to each of the wells, and the cells were incubated for 3 h at 37 ℃. The supernatants were removed, and DMSO was added to dissolve the formazan crystals. Absorbance at 570 nm was measured by microplate plate reader (Tecan, Mannedorf, Switzerland).

5. Protein extraction and Western blot analysis

3T3-L1 cells were lysed in CelLytic buffer (Sigma-Aldrich, MO, USA). The lysates were centrifuged at 12,000 rpm for 15 min at 4 ℃, and the protein concentrations were measured by Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). The proteins were separated by 10% SDS-PAGE, and transferred to Immobilon-P membrane (Millipore, Bedford, Mass). The membranes were initially blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) for 1 h, and then incubated with specific primary antibodies against β-actin, C/EBPα, PPARγ, ap2, FAS, mTOR, p-mTOR, AKT, p-AKT, IRS1, p-IRS1 and PI3K110α for overnight at 4 ℃. The membranes were then incubated in the corresponding horseradish peroxidase-conjugated anti-rabbit, anti-mouse immunoglobulin G (GenDEPOT, Barker, TX, USA) for 1 h at 23 ℃. Detection was performed with ECL solution (GenDEPOT, Barker, TX, USA), and the intensity of bands was detected by LuminoGraph (Atto, Tokyo, Japan).

6. Statistical analysis

All data were presented as mean ± standard deviation. The values were compared using Student's *t*-test. One-way

analysis of variance (ANOVA) was conducted using the software Origin 7 (Microcal Software, USA). Values of $P < 0.05$ were considered to indicate statistical significance.

Results

1. Effects of ATE on cell viability in 3T3-L1 preadipocytes

The potential effects of ATE on the 3T3-Ll preadipocytes and cell viability were evaluated by MTT assay. ATE had no cytotoxic activity at various concentrations (0, 100, 300, and 500 μg/mL) (Fig. 1A).

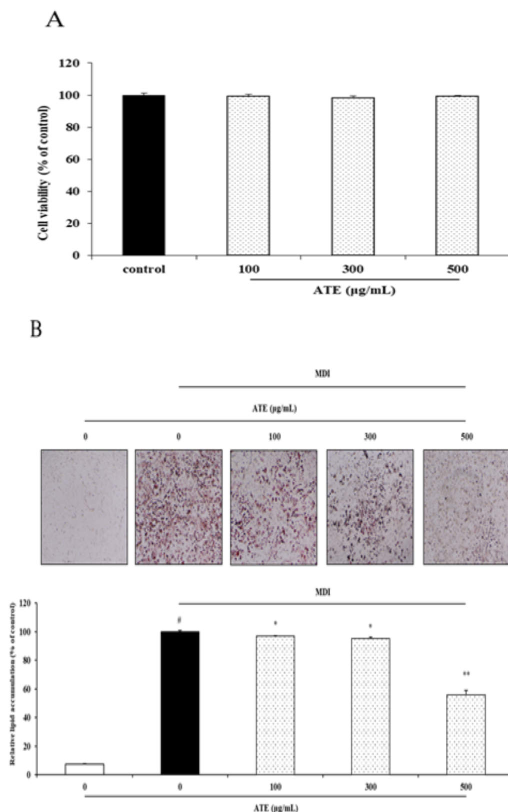


Fig. 1. Effects of ATE on cell viability and adipocyte differentiation in 3T3-L1 preadipocytes. (A) 3T3-L1 cells were treated with various concentrations of ATE (0, 100, 300, and 500 μg/mL) for 24 h. The cell viability was evaluated by MTT assay, as described in the Materials and methods. (B) Lipid droplets were photographed after Oil-Red O staining, and lipid content was then quantified by measuring absorbance. Each percentage value in treated cells was calculated with respect to control cells. #$P < 0.05$, compared with the vehicle control. *$P < 0.05$ and **$P < 0.01$, when compared to only MDI differentiation medium treated-control group.

2. ATE inhibits adipocyte differentiation in 3T3-L1 preadipocytes

Previous study[14] has found that ATE treatment inhibited lipid accumulation, and affected the expression levels of adipogenesis-related proteins in 3T3-L1 adipocyte. That study also explained that further studies were needed on the expression levels of other genes related to adipogenesis. Therefore, to investigate the effects of ATE on adipogenic differentiation, 3T3-L1 preadipocytes were treated with various concentrations of ATE (0, 100, 300, and 500 μg/mL). Adipogenesis was tested with Oil-red O staining of lipid droplets and contents. The results show that ATE decreased the lipid accumulation in 3T3-L1 adipocytes without cell cytotoxicity (Fig. 1B). A reduction was observed in the lipid accumulation in the percentage of 3.15-44.05%.

3. Effects of ATE on the expression of adipogenesis related proteins

Expression levels of adipogenic key transcriptional factors, such as C/EBPα and PPARγ, and other adipocyte-specific gene, such as ap2 and FAS, were also examined by western blotting. The results of western blotting analysis showed that C/EBPα, PPARγ, ap2 and FAS protein levels in MDI-induced 3T3-L1 adipocytes were significantly increased over the control, whereas ATE inhibited the expression levels of C/EBPα, PPARγ, ap2 and FAS by 89.94%, 85.78%, 69.10%, and 75.43% respectively (Fig. 2).

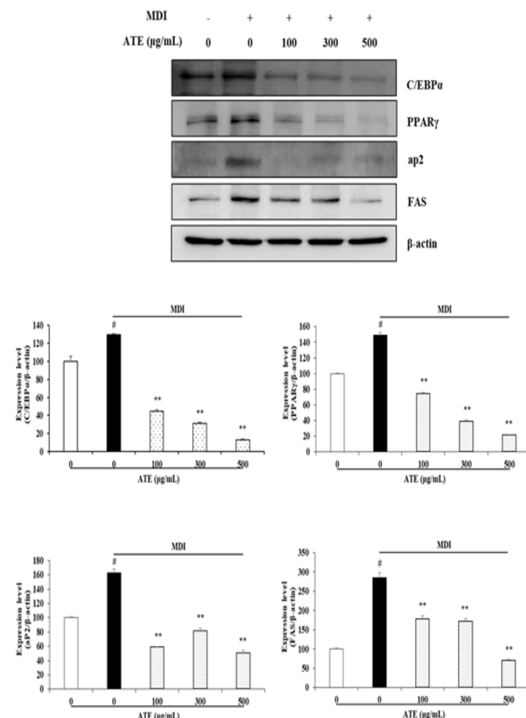
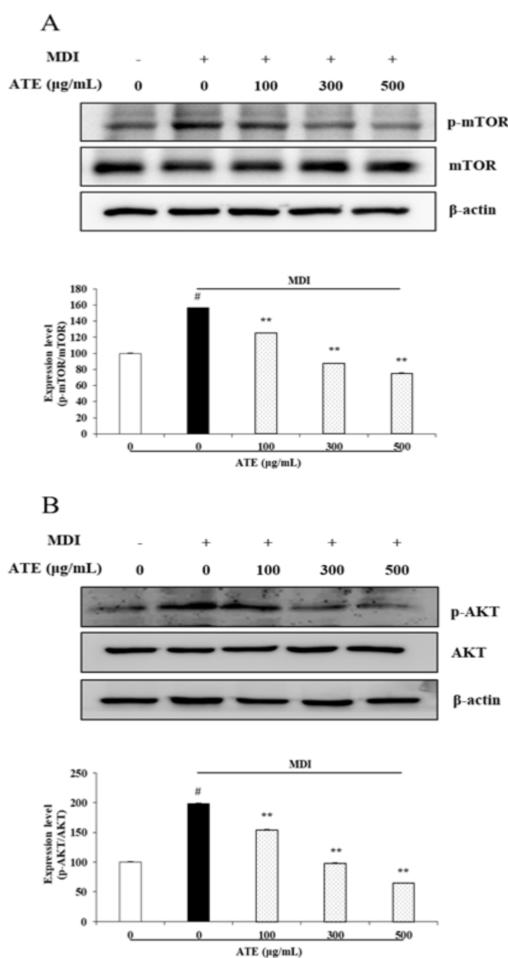


Fig. 2. Effects of ATE on the expression of adipogenesis related proteins. Western blot analysis was applied to investigate the expression of adipogenesis related proteins. Proteins were extracted at day 8 from the cells treated with or without ATE during differentiation. Adipogenesis related protein expression levels of C/EBPα, PPARγ, ap2 and FAS. Western blot bands were analyzed using Image J software. β-actin was used as a loading control. #$P < 0.05$, compared with the vehicle control. *$P < 0.05$ and **$P < 0.01$, when compared to only MDI differentiation medium treated-control group.

4. Effects of ATE on the IRS/PI3K/AKT and mTOR signaling pathway.

To further investigate the mechanism of ATE in adipogenesis, we examined the expression of mTOR signaling pathway proteins, which have important roles in adipogenesis. mTOR is one of the upstream factors of C/EBPα and PPARγ, which stimulates adipogenesis. Fig. 3A shows that ATE downregulates the phosphorylation of mTOR with an inhibition percentage of 52.09%, without affecting total mTOR expression. To further investigate the upstream mechanism of ATE in adipogenesis, we confirmed the IRS/PI3K/AKT signaling pathway proteins, which constitute the upper mechanism of mTOR signaling. Proteins expression levels were determined via western blot analysis. The stimulation with MDI caused the phosphorylation of IRS1, induction of PI3K110α, and phosphorylation of AKT. However, the phosphorylation of AKT, (Fig. 3B), IRS1, and the protein expression levels of PI3K110α (Fig. 3C) were significantly decreased in ATE treated cells by 67.24%, 63.31%, and 71.66%, respectively. Whereas, ATE did not show any effect on the expression of total IRS1 and AKT.



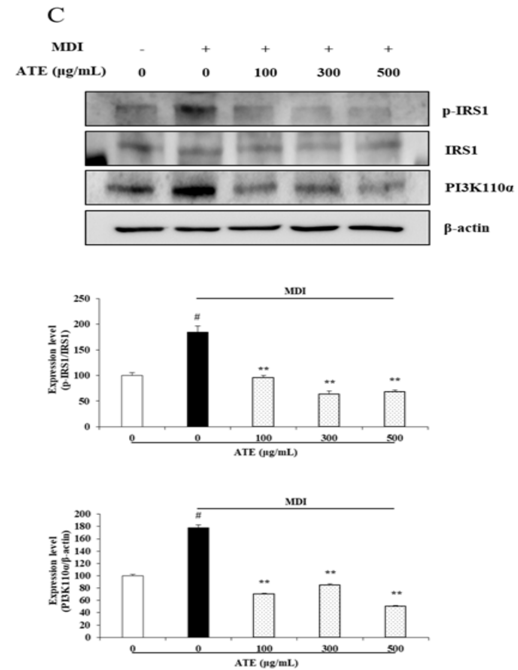


Fig. 3. Effects of ATE on the IRS/PI3K/AKT and mTOR signaling pathway. Western blot analysis was applied to investigate the expression of IRS/PI3K/AKT and mTOR signaling factors. (A) The protein expression levels of mTOR and phosphorylation of mTOR. (B) The protein expression levels of AKT and phosphorylation of AKT. (C) The protein expression levels of AKT signaling upstream factors, such as IRS1, p-IRS1, and PI3K110 α. Western blot bands were analyzed using Image J software. β-actin was used as a loading control. #P < 0.05, compared with the vehicle control. *P < 0.05 and **P < 0.01, when compared to only MDI differentiation medium treated-control group.

Discussion

Recent studies have shown that extract from the roots of *Adenophora triphylla* has anti-oxidant, anti-bacterial, and anti-cancer effects.[15-18] In particular, other studies have shown *Adenophora triphylla* var. *japonica* extract has an anti-obesity effect.[19]

In the previous study, ATE treatment inhibited lipid accumulation, and decreased the expression levels of PPAR γ, ap2, and FAS in 3T3-L1 adipocyte. That study also explained that additional studies of the upstream mechanism of adipogenesis were needed.[14] Therefore, to investigate the upstream mechanism of adipogenesis, we examined the possible upstream mechanism of adipogenesis, such as IRS/PI3K/AKT and mTOR signaling in 3T3-L1 cells.

In the present studies, we evaluated the effect of ATE on the accumulation of lipid in 3T3-L1 cells. This was examined by the 3T3-L1 adipocytes differentiation system. To differentiate 3T3-L1 preadipocytes into mature adipocytes, fully confluent 3T3-L1 preadipocytes were treated with MDI differentiation medium containing various concentration of ATE (0, 100, 300, and 500 μg/mL). The results show that

ATE also decreased the lipid accumulation in 3T3-L1 adipocytes without cell cytotoxicity (Fig. 1A and 1B).

Expression levels of adipogenic key transcriptional factors, such as C/EBPα and PPARγ, were confirmed by western blotting. ap2, a downstream target of PPARγ which is an adipocyte-specific gene, and involved in maintaining adipocyte phonotype,[20] was also examined using western blotting. As well as, FAS downregulation may significantly help reduce weight and treat obesity.[21] The present study confirmed the same results as previous studies[14] that PPARγ, ap2 and FAS proteins expression were inhibited in ATE-treated 3T3-L1 cells (Fig. 2). Additionally, our results showed that C/EBPα expression was reduced in ATE-treated 3T3-L1 cells (Fig. 2). Differentiation of preadipocytes is controlled by a complex network of transcription factors, and the center of this network is nuclear receptor PPARγ and members of the C/EBP family.[22] Mammalian target of rapamycin (mTOR) signaling pathway, which is regulated by the upstream AKT pathway, has been demonstrated to be associated with adipocyte differentiation.[8-11] Recent studies have revealed that the mTOR signaling pathway plays a critical role in the regulation of adipose tissue function, including adipogenesis, lipid metabolism, thermogenesis, and adipokine synthesis/secretion. mTOR has been reported to be involved in adipogenesis, and regulates the expression of PPARγ.[23] Therefore in this study, we further evaluated mTOR, an upstream mechanism of C/EBPα and PPARγ. ATE attenuated the phosphorylation of mTOR, whereas ATE did not show any effect on the expression of total mTOR (Fig. 3A). These results revealed that p-mTOR inhibited by ATE downregulates the expression of C/EBPα, PPARγ, ap2 and FAS expression.

In adipogenesis, insulin signaling pathway plays an important role. AKT are downstream kinases of the insulin signaling pathway that are critically involved in adipocyte differentiation. Previous studies have demonstrated that AKT can induce mTOR signaling through phosphorylation.[24,25] We assessed the phosphorylation of AKT, the upper mechanism of mTOR. Our results show that the phosphorylation of AKT was significantly decreased in ATE-treated cells (Fig. 3B).

When insulin receptor (IR) is activated by insulin, it phosphorylates IRS1, and then PI3K is activated by complexing with the IRS1.[26,27] Destruction of IRS1 in mice delays body mass gain and decreases insulin stimulation glucose uptake, but does not incur diabetes, due to increased insulin secretion to compensate for mild resistance to insulin.[28-30] For this reason, inhibition of IRS1 is an effective anti-obesity mechanism without causing diabetes. Following our results, the stimulation with MDI caused the phosphorylation of IRS1 and induction of PI3K110α in 3T3-L1 cells. However, the phosphorylation of IRS1 and the protein expression levels of PI3K110α were significantly decreased in ATE-treated cells, whereas ATE did not show any effect on the expression of total IRS1 (Fig. 3C).

In conclusion, we found that ATE inhibited adipogenesis in 3T3-L1 cells by reducing the phosphorylation of IRS1, thereby decreasing the expression of PI3K110α and inhibiting the phosphorylation of AKT and mTOR, resulting in repressed adipogenesis related proteins expression of C/EBPα, PPARγ, ap2 and FAS.

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