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Lotus Leaf Ethanol Extract and Nuciferine Suppress Adipocyte Differentiation by Regulating Akt-mTORC1 Signaling in 3T3-L1 Cells

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Abstract Lotus leaf has been reported to exert anti-inflammatory, hypolipidemic, and hepatoprotective effects. However, the effect of lotus leaf on adipocyte differentiation and its action mechanism have not been clarified. In this study, 3T3-L1 preadipocytes were incubated with or without lotus leaf ethanol extract (EEN) for 8 days. Microscopic inspection and Oil Red O staining indicated that EEN treatment significantly reduced adipogenesis in 3T3-L1 cells. EEN also downregulated the protein levels of adipogenic transcription factors including sterol regulatory element binding protein 1 (SREBP1), peroxisome proliferator-activated receptor-gamma (PPARγ), and CCAAT/enhancer binding protein α (C/EBPα), and target genes such as adipocyte binding protein 2 (aP2) and fatty acid synthase (FAS) in a dose-dependent manner. In order to understand whether nuciferine, the primary active component of EEN contributed to the anti-adipogenic activity of EEN, we examined the effect of nuciferine on adipogenesis related gene expression. Nuciferine significantly reduced expression of adipogenic transcription factors and target genes. Notably, nuciferine downregulated the phosphorylation of Akt, mammalian target of rapamycin complex 1 (mTORC1), S6K, and 4EBP1. These results suggest that lotus leaf ethanol extract exerts anti-adipogenic activity, and could be partially mediated through the regulation of the Akt-mTORC1 signaling pathway by nuciferine.

Keywords: *lotus leaf, nuciferine, adipogenesis, Akt-mTORC1 signaling, 3T3-L1 cell*

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1. Introduction

Obesity is the leading public health issue worldwide. According to the World Health Organization, the prevalence of obesity has almost tripled over the past 30 years and is now assumed more than 650 million people in the world [1]. It is characterized by a significant increase in adiposity that depends on the expansion of pre-existing adipocytes and/or generation of new adipocytes (adipogenesis) [2,3]. Additionally, obesity leads to enhanced comorbid metabolic and chronic diseases including type-2 diabetes mellitus, heart diseases, and cancer [4].

Several transcription factors regulate a process of adipogenic differentiation and promote mature adipocyte formation [5]. The sterol regulatory element binding protein 1 (SREBP1) [6], peroxisome proliferator-activated receptor-gamma (PPARγ) [7], and CCAAT/enhancer binding protein α (C/EBPα) are the key regulators of adipogenic differentiation that modulate the genes expression related to lipid and cholesterol homeostasis. It has been suggested that this phenomenon is related to the impairment of signaling molecules, such as Akt or protein kinase B (PKB)-mammalian target of rapamycin (mTOR). Akt-mediated phosphorylation of tuberous sclerosis protein 2 (TSC2) inhibits its ability to act as a GTPase activating protein for Ras homolog enriched in brain (Rheb) within cells, allowing Rheb-GTP to accumulate and activate mTOR complex 1 (mTORC1) [8,9]. The activation of mTORC1 promotes adipogenesis, which result in lipid storage [10,11].

To date, there are several reports studying the efficacy of dietary plants on obesity prevention because they are largely free from side effects [12,13,14]. Sacred lotus is an ornamental plant that is also a dietary staple in eastern Asia. Lotus leaves have long been used to extend shelf life and enhance taste of meat in China. Several active constituents of lotus responsible for its medicinal properties have been isolated from the leaf, flower, rhizome, and seed. Extracts from different parts have shown antioxidant (rhizome [15], stamens [16,17], seed [18,19]), anticancer (seed [20,21]), anti-inflammatory (rhizomes [22]), hepatoprotective (flower [23]), and

hypoglycemic (rhizomes [24,25]) properties. Several studies have demonstrated that lotus leaf has a beneficial effect on metabolic syndromes including obesity, hyperlipidemia, and diabetes [26,27,28,29]. Nuciferine (C19H21NO2), an aromatic ether-containing alkaloid and major active aporphine has been identified as a major compound responsible for the activity of lotus leaf extracts. [30,31]. The main pharmacological effects of nuciferine include hyperlipidemia amelioration, stimulation of insulin secretion and vasodilation, hypotension induction, and anti-hepatic steatosis activity [32,33,34,35]. However, the effect of lotus leaf on adipocyte differentiation and its action mechanism remain unclear. Therefore, the present study demonstrated the effect of lotus leaf ethanol extract (EEN) on adipogenic differentiation, and to clarify the possible mechanism by investigating adipogenesis specific transcription factors and target genes. We also examined the effect of nuciferine on adipogenesis related gene expression.

2. Materials and Methods

2.1. Chemicals

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Calbiochem (San Diego, California, USA). Isobutyl-3-methylxanthine (IBMX), dexamethasone, insulin, and Oil Red O were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Hyclone (Logan, UT, USA). Bovine calf serum and penicillin/streptomycin/glutamine (PSG) antibiotics were obtained from Gibco BRL-Life Technologies (Burlington, ON, Canada). Antibodies against SREBP1, PPARγ, C/EBPα, phospho-Akt, Akt, phospho-mTOR, mTOR, phospho- p70 ribosomal protein S6 kinase (S6K), S6K, phospho-eukaryotic initiation factor 4E binding protein 1 (4E-BP1), 4E-BP1, and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and two antibodies against fatty acid-binding protein 4 or adipocyte binding protein 2 (aP2) and fatty acid synthase (FAS) were purchased from Cell Signaling Technology (Danvers, MA, USA). Nuciferine was purchased from TRC (Toronto Research Chemicals Inc., Toronto, ON, Canada).

2.2. Sample Preparation and HPLC Conditions

Dried lotus leaves were collected from Haenam, Jeonnam, South Korea. Dried lotus leaves (1 kg) were extracted in 70% ethanol (10 L) at 80°C for 3 h. Following extraction, the sample was filtered through filter paper (Whatman Grade No. 2, New Jersey, USA), concentrated under a vacuum at 37°C, then freeze-dried. The dried extract (173.3 g) was stored at -20°C.

HPLC was conducted on an Ultimate 3000 HPLC system (Thermo Dionex, CA, USA) and data was collected using Chromeleon version 6.8 software. Samples were separated on an INNO C-18 column (4.6 × 250 mm, 5 μm, Youngjin Biochrom, Korea). The mobile phase consisted of buffer A (0.3% trifluoroacetic acid in distilled water) and buffer B (acetonitrile). The linear gradient profile consisted of 20% B from 0 to 1 min, 90% B from 1 to 20 min, 90% B from 20 to 25 min, 20% B from 25 to 26 min, then 20% B in 26 to 30 min with a 1 ml/min flow rate and 10 μl injection volume. Absorbance was measured at 270 nm using a Diode Array Detector scanning from 190 to 400 nm. Peaks were identified by standard retention time comparison.

2.3. Cell Culture and Differentiation

The 3T3-L1 mouse fibroblast cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in DMEM containing 10% calf serum (CS), 1% PSG at 37°C under a 5% CO_2 atmosphere. At day 2 after the cells had reached confluence, cell differentiation was performed with a differentiation medium (DMEM with 10% FBS and 1% PSG antibiotics containing 0.5 mM IBMX, 1 μM dexamethasone, and 1 μg/ml insulin (MDI)) for 2 days. The cells were cultured for another 2 days in DMEM with 10% FBS and 1% PSG antibiotics containing 1 μg/ml insulin. Thereafter, the cells were maintained in postdifferentiation medium (DMEM with 10% FBS and 1% PSG antibiotics containing 1 μg/ml insulin), and the medium was replaced every 2 days. To test the effect of EEN and nuciferine on preadipocyte differentiation, the cells were treated with differentiation medium in the presence of various concentrations of EEN and nuciferine. On day 8, when differentiation was completed, the cells were harvested.

2.4. MTT Assay

3T3-L1 cells were allowed to adhere and spread for 24 h in a 96-well plate. 3T3-L1 preadipocytes were incubated with various concentrations of EEN and nuciferine for 24h at 37°C. Twenty microliters of MTT solution were added into each well and incubated for 4 h. Then, the MTT solution was removed and replaced with 200 μl of dimethyl sulfoxide (DMSO) until the crystals had dissolved. Cell viability was determined by measuring absorbance at 570 nm using a 96-well plate reader.

2.5. Oil Red O Staining and Cell Quantification

3T3-L1 preadipocytes were maintained in adipocyte induction media and exposed to 50 or 100 μg/ml EEN and 25 or 50 μM nuciferine for 8 days. Then, the differentiated 3T3-L1 adipocytes were washed twice with phosphate-buffered saline (PBS). Cells were fixed with 10% formalin for 1 h, dried, and stained with Oil Red O (0.2% Oil Red O in 60% isopropanol) for 10 min. The cells were then washed with water and dried. The lipid content of the stained cells was visualized using an Olympus IX71 microscope. The stained oil droplets were dissolved in DMSO and quantified by spectrophotometric analysis at 500 nm.

2.6. Western Blot Analysis

The harvested cells were sonicated for 5 second at 40W. Cell lysates were centrifuged at 13,000 xg at 4°C for 10

min. Protein determination by bicinchoninic acid (BCA) method was performed with a Thermo Scientific Pierce BCA Protein Assay kit (Rockford, IL, USA) using bovine serum albumin as the standard. Total protein (20 μg per lane) was analyzed by SDS-polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membrane was incubated with a blocking buffer of tris-buffered saline containing 5% skim milk and 0.1% Tween 20 (Amresco Inc., Solon, OH, USA) for 1 h at room temperature. After overnight incubation in primary antibodies at 4°C, membranes were washed three times in TBS buffer containing 0.1% Tween 20 and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Immunodetection was performed using electrochemiluminescence detection reagent (Amersham Biosciences, Uppsala, Sweden). All figures that show quantitative analysis results (Image J, National Institutes of Health) include data from at least three independent experiments.

2.7. Statistical Analysis

Data are expressed as the mean ± SD. Statistical analyses were performed with GraphPad Prism, version 7.04 software (San Diego, CA, USA). One-way analysis of variance was used to compare more than two groups, followed by the Bonferroni post-hoc test to detect differences between groups ($p < 0.05$).

3. Results

3.1. EEN Nuciferine Content

HPLC method was used to identify nuciferine, the major component of EEN, by a retention time of 15.85 min (data not shown). The total amount of nuciferine in EEN was 1.87 mg/g EEN.

3.2. EEN Inhibits 3T3-L1 Preadipocyte Differentiation

Cell viability and cytotoxicity were investigated to examine toxic effects of EEN on 3T3-L1 preadipocytes. We observed a 90% or higher cell viability at 400 μg/ml EEN (Figure 1A). Investigation into whether EEN inhibited adipocyte differentiation showed that EEN treated cells dose-dependently reduced lipid droplets, in contrast to DMSO treated-cells (MDI) (Figure 1B). Lipid content was also significantly reduced by 29% and 42% on Oil Red O stained sections treated with 50 and 100 μg/ml EEN, respectively (Figure 1C). This result suggests that EEN inhibits adipogenic differentiation in 3T3-L1 cells.

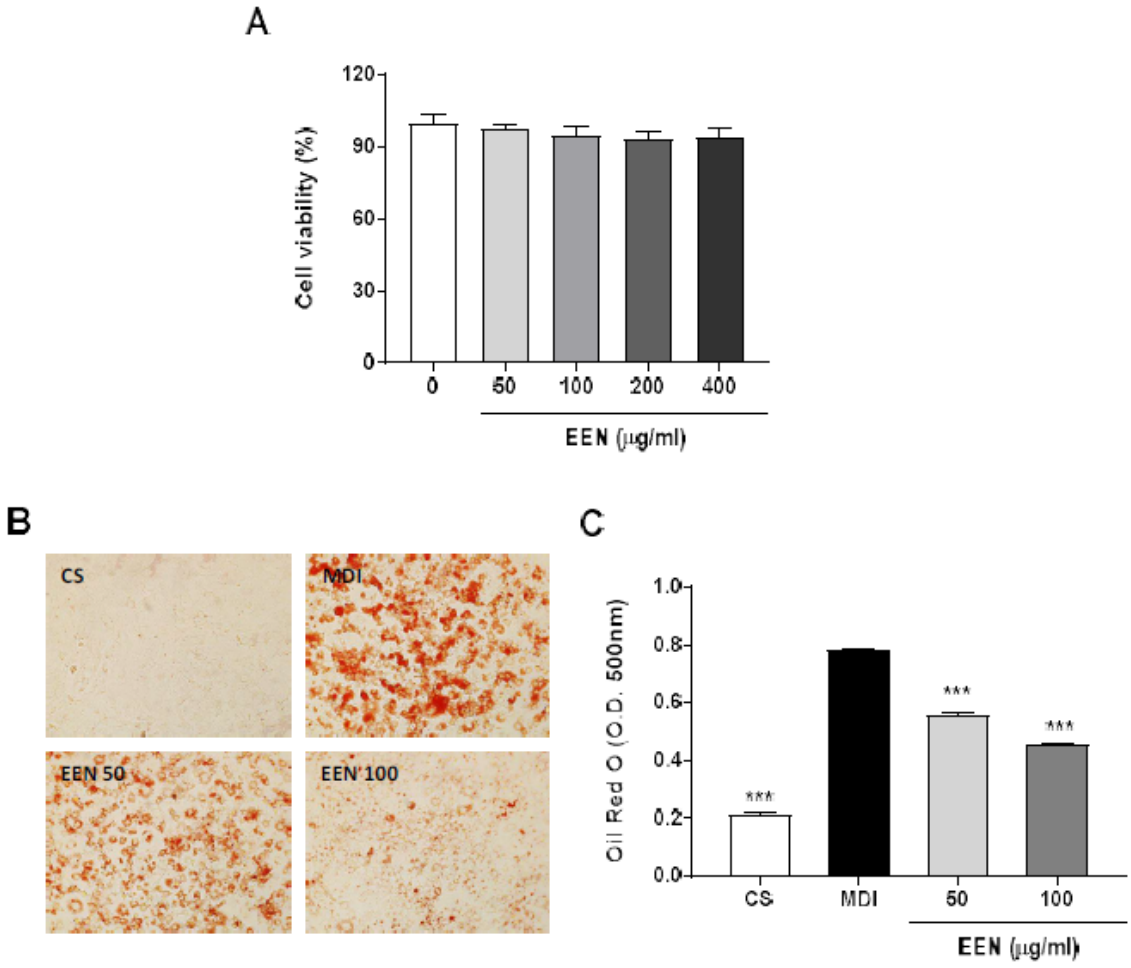

Figure 1. Effect of EEN on 3T3-L1 preadipocyte differentiation

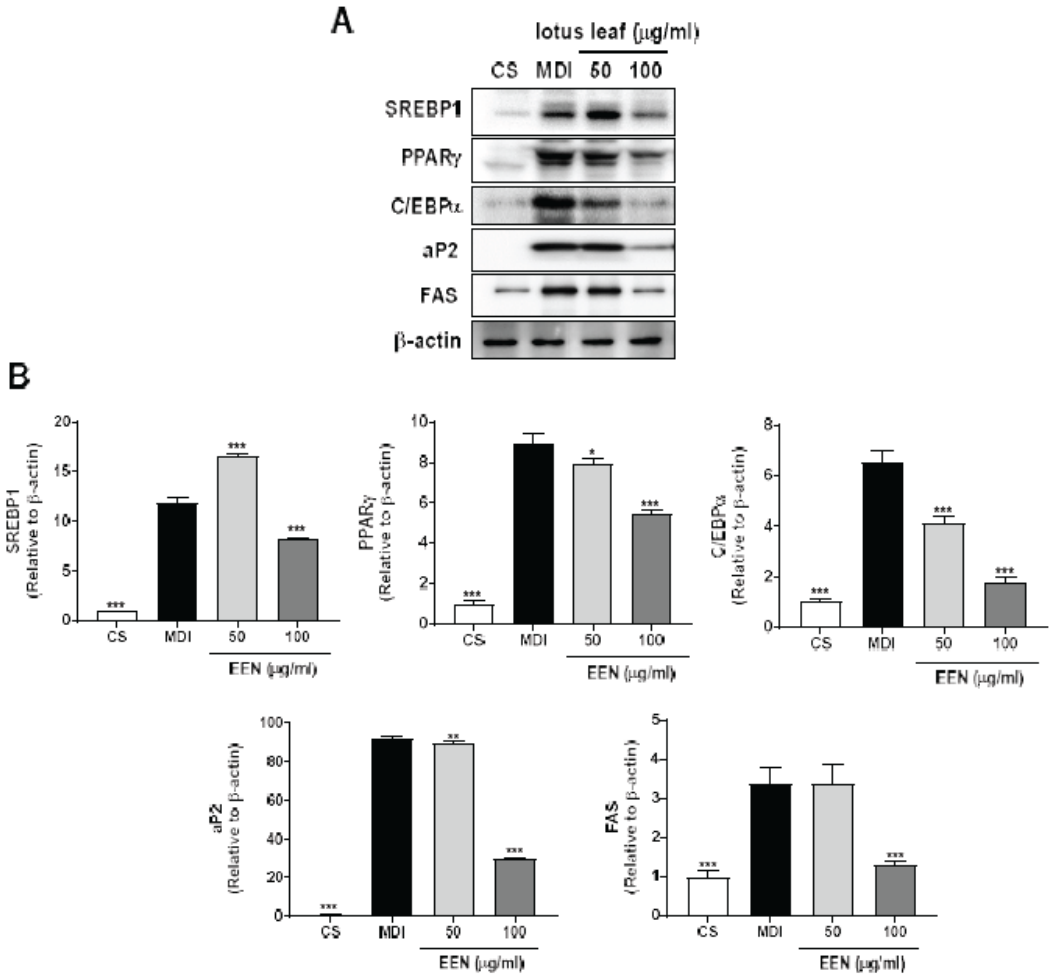

Figure 2. Effect of EEN on MDI-induced adipogenic transcription factors and target gene expression in 3T3-L1 adipocytes

3.3. EEN Inhibits Adipogenesis Related Factors in 3T3-L1 Cells

Western blot results showed that SREBP1, PPARγ, C/EBPα, aP2, and FAS proteins were highly upregulated in MDI-induced adipocyte differentiation. However, 50 and 100 μg/ml EEN treatment inhibited adipogenic transcription factors and target genes in a dose-dependent manner (Figure 2A and B). These results indicate that EEN attenuates lipid accumulation in 3T3-L1 cells through the regulation of adipogenic transcription factors and target genes.

3.4. Nuciferine Suppresses 3T3-L1 Adipogenic Differentiation

MTT assay showed that no significant cytotoxicity was detected in 3T3-L1 preadipocytes after 24 h incubation with 50 μM of nuciferine (Figure 3A). Furthermore, nuciferine treatment strongly suppressed 3T3-L1 preadipocyte differentiation induced by MDI (Figure 3B and C). Nuciferine treatment dose-dependently reduced the number of lipid droplets (Figure 3B), and the lipid content in 3T3-L1 cells was significantly reduced by 42% and 73% on Oil Red O stained sections treated with 25 and 50 uM nuciferine, respectively (Figure 3C). Therefore, nuciferine significantly inhibited 3T3-L1 adipogenic differentiation.

3.5. Nuciferine Attenuates Adipogenesis Related Factors in 3T3-L1 Cells

Next, the protein expressions of adipogenic transcription factors including SREBP1, PPARγ and C/EBPα, and their target genes FAS and aP2 were measured. The MDI-induced increase of SREBP1, PPARγ, and C/EBPα were markedly attenuated by nuciferine treatment. Furthermore, expression of key adipogenic enzymes FAS and aP2 was also reduced by nuciferine treatment during adipogenesis (Figure 4A and B). These results indicate that nuciferine blocks lipid accumulation in 3T3-L1 cells by regulating adipogenic genes.

3.6. Nuciferine Suppresses Adipocyte Differentiation through Regulating Akt-mTORC1 Pathway

To investigate the mechanism underlying nuciferine induced suppression of adipogenic differentiation, the effect of nuciferine on Akt-mTORC1 pathway was tested. MDI treatment increased phospho-Akt, and it was recovered by nuciferine in a dose-dependent manner (Figure 5). Based on the nuciferine-induced decrease in Akt phosphorylation, we hypothesized that the mTORC1 signaling could be inhibited in 3T3-L1 cells by nuciferine. mTORC1 phosphorylation was decreased by nuciferine treatment (Figure 5). Nuciferine also

inhibited S6K and 4EBP1 phosphorylation (Figure 5). These results indicate that nuciferine suppresses adipogenic differentiation in 3T3-L1 cells via attenuating Akt-mTORC1.

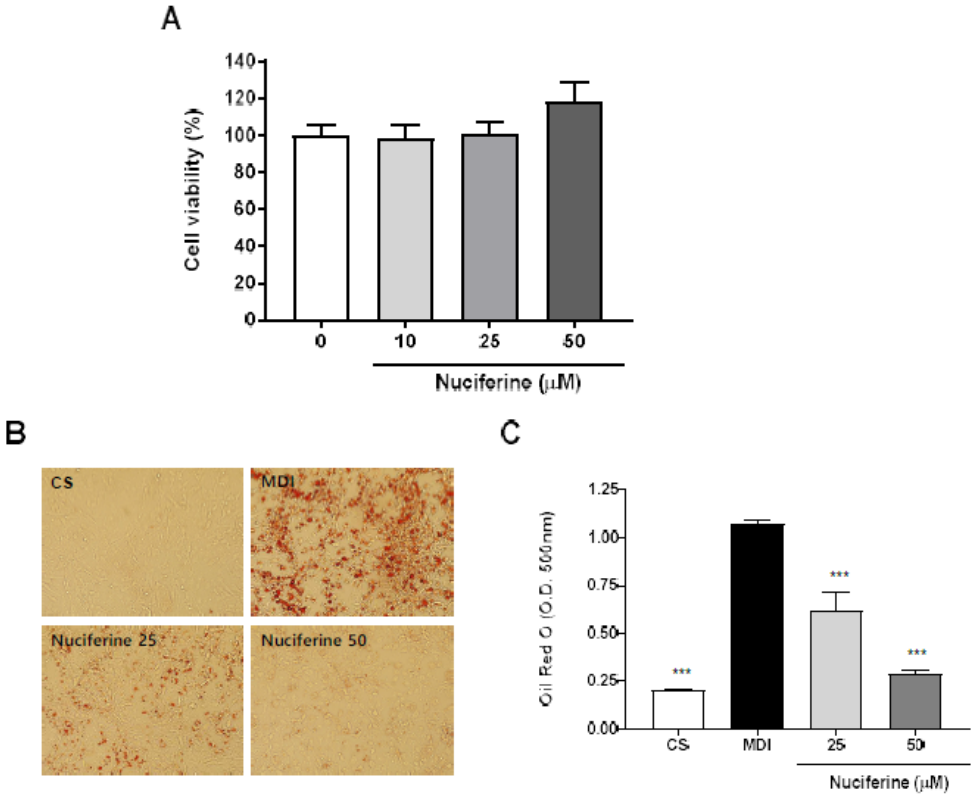

Figure 3. Effect of nuciferine on 3T3-L1 preadipocyte differentiation

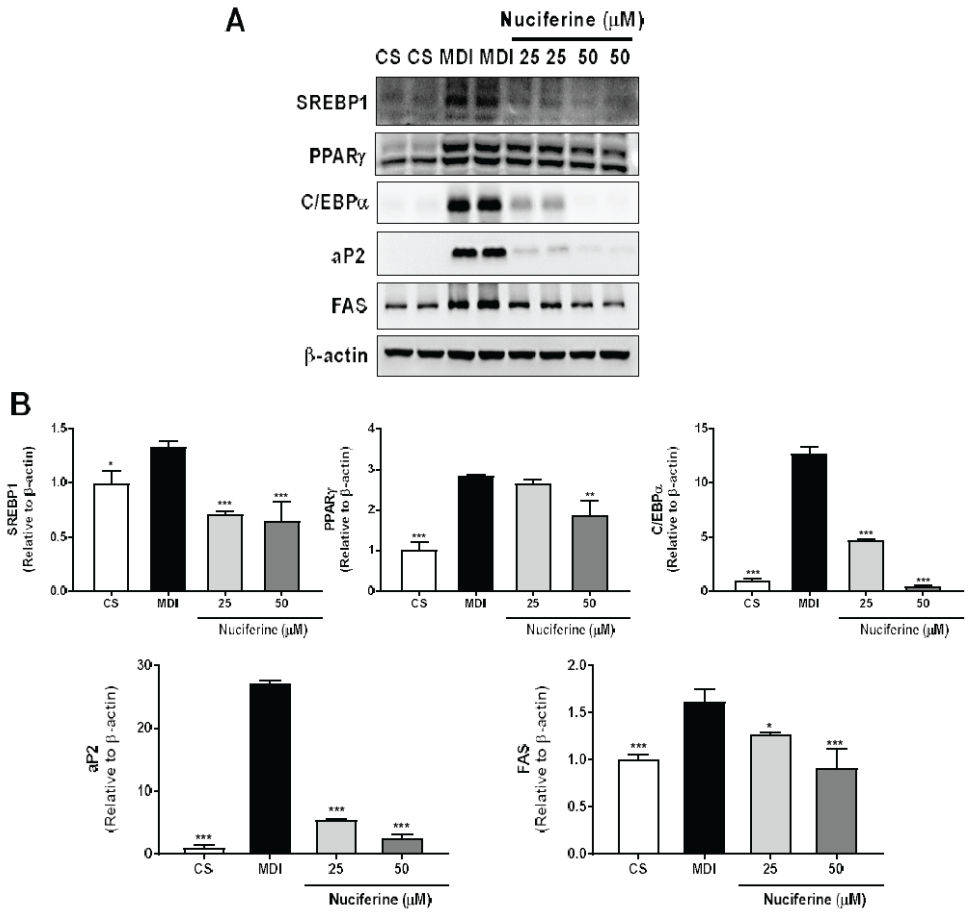

Figure 4. Effect of nuciferine on MDI-induced adipogenic protein expression in 3T3-L1 adipocytes

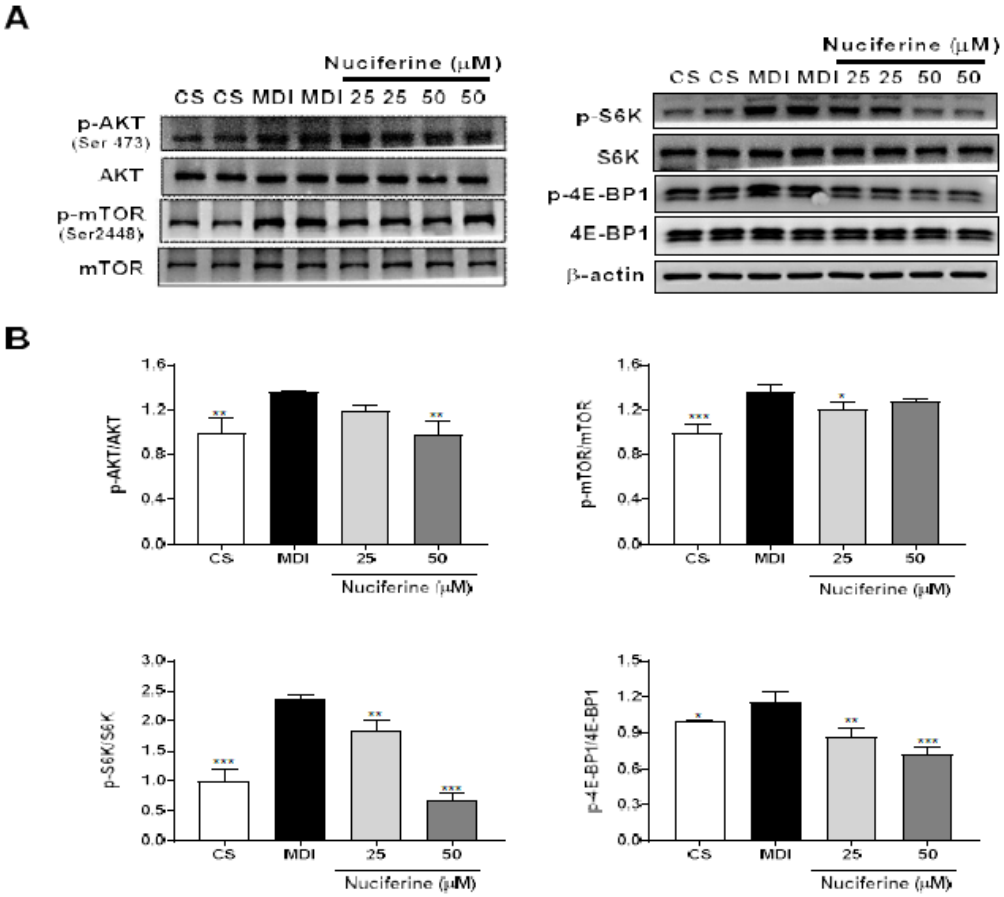

Figure 5. Effect of nuciferine on Akt-mTOR signaling in 3T3-L1 adipocytes

4. Discussion

Previous reports have demonstrated that plant extracts and some phytochemicals from plants can attenuate adipogenesis [36], and lotus leaf extract has been reported to attenuate digestive enzymes, plasma lipid levels, and body weight in mice [26]. Our results showed that EEN suppressed the 3T3-L1 adipocyte differentiation, and that its active component, nuciferine, also inhibited this process by regulating adipogenesis related gene expression.

Adipogenesis is a process by which undifferentiated preadipocytes are converted to mature adipocytes [36,37]. Constitutive Akt activation or insulin treatment promotes nuclear accumulation of SREBP1 and lipid synthesis related-genes expressions [38,39]. C/EBPβ and C/EBPδ trigger C/EBPα, which subsequently induces PPARγ expression [40]. The activation of PPARγ promotes the expression of several genes regulating fatty acid synthesis, esterification, and storage in adipocytes [41]. Our results revealed that the protein levels of SREBP1, PPARγ, and C/ EBPα were downregulated by EEN and nuciferine (Figure 2 and Figure 4). These results indicated that EEN and nuciferine suppressed the differentiation of 3T3-L1 cells by downregulating adipocyte-specific transcription factors and target genes.

SREBP1, PPARγ, and C/EBPα synergistically activate the expression of downstream aP2 and FAS, adipocyte specific genes [5,6]. aP2 is a terminal differentiation marker expressed in adipocytes, and promotes the cellular uptake of long-chain fatty acids in a pathway related to obesity [42]. FAS facilitates the synthesis of long-chain fatty acids in the cytosol and then cytoplasmic storage of massive amounts of triglycerides [43]. In the present study, EEN and nuciferine decreased aP2 and FAS protein levels. Microscopic observation and Oil Red O staining also indicated that EEN and nuciferine treatment markedly decreased lipid accumulation. Therefore, reduced lipid content and reduction of adipogenic protein expression were an obvious consequence of the anti-adipogenic effect of EEN and nuciferine in fully differentiated adipocytes.

mTORC1 phosphorylates the translational regulators S6K and 4E-BP1 to coordinately upregulate protein biosynthesis [44]. The present study showed that nuciferine treatment inhibited mTORC1, and downstream targets S6K and 4E-BP1 in adipocytes (Figure 5). These two substrates are critical factor to promotes cell proliferation [44,45]. Adipogenic differentiation requires increased protein biosynthesis to enhance cell growth and mitotic clonal expansion production. Therefore, the ability of nuciferine to attenuate mTORC1 phosphorylation would negatively affect PPARγ and C/EBPα expressions.

Akt is an important regulator of cell proliferation and metabolism. Adipose tissue was decreased in Akt 1/2 deficient mice [46], and Akt 2 knockout decrease lipid accumulation in obese mice model [47]. Furthermore, mTORC1 is a critical regulator in the progression of obesity identified in the model of high-fat diet supplementation [48]. Our study showed that nuciferine regulated Akt-mTORC1 pathway (Figure 5). It is noticeable that inhibition of Akt-mTORC1 by nuciferine suppresses adipogenic differentiation, and that nuciferine may be responsible for anti-adipogenic activity of EEN.

5. Conclusions

In conclusion, the present study shows that lotus leaf ethanol extract and its active component, nuciferine, suppress 3T3-L1 adipogenic differentiation. Nuciferine may contribute to the anti-adipogenic activity of lotus leaf by regulating adipogenesis related gene expression including Akt-mTORC1 signaling as summarized in Figure 6. From these findings, we propose that lotus leaf might be helpful to prevent obesity. To confirm this, follow-up studies are needed.

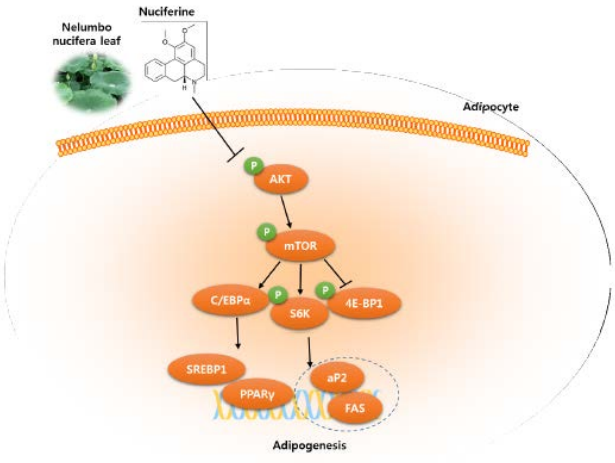

Figure 6. Proposed mechanism for anti-adipogenic effect of lotus leaf and nuciferine in adipocyte.

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Statement of Competing Interests

The authors declare no conflict of interest.

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