Zanthoxylum schinifolium leaf ethanol extract inhibits adipocyte differentiation through inactivation of the extracellular signal regulated kinase and phosphoinositide 3-kinase/Ark signaling pathways in 3T3-L1 pre-adipocytes

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Abstract. Zanthoxylum schinifolium is widely used as a food flavoring in east Asia. Although this plant has also been used in traditional oriental medicine for the treatment of the common cold, toothache, stomach ache, diarrhea and jaundice, its anti-obesity activity remains to be elucidated. The present study investigated the effects of ethanol extract from the leaves of Z. schinifolium (EEZS) on adipocyte differentiation, and its underlying mechanism, in 3T3-L1 pre-adipocytes. The results demonstrated that EEZS effectively suppressed intracellular lipid accumulation at non-toxic concentrations, and was associated with the downregulation of several adipocyte-specific transcription factors, including peroxisome proliferation-activity receptor γ (PPARγ), CCAAT/enhancer binding protein (C/EBP)α and C/EBPβ, in a concentration-dependent manner. Furthermore, it was observed that EEZS markedly inactivated the extracellular signal-regulated protein kinase (ERK) and phosphatidylinositide 3-kinase (PI3K)/Ark pathways, which act upstream of PPARγ and C/EBPs in adipogenesis. These results suggested that EEZS inhibited lipid accumulation by downregulating the major transcription factors involved in the pathway of adipogenesis, including PPARγ, C/EBPα and C/EBPβ, via regulation of the ERK and PI3K/Ark signaling pathways in 3T3-L1 adipocyte differentiation. This indicated the potential use of EEZS as an anti-obesity agent.

Introduction

Obesity is one of the most prevalent physiological disorders associated with a variety of conditions, including hypertension (1), dyslipidemia (2), atherosclerosis (3), type II diabetes (4), non-alcoholic fatty liver disease (5), periodontal disease (6), asthma (7), cardiovascular disease (8) and certain types of cancer (9,10). Additionally, the number of obese individuals is continuing to increase and is becoming a serious health problem worldwide (11,12). Therefore, there is a continuing requirement for novel and safe substances to overcome this problem, with a view to combating obesity-associated health problems (13).

Adipogenesis is regulated by a number of transcription factors, including the CCAAT/enhancer binding proteins (C/EBPs) and peroxisome proliferator-activated receptor γ (PPARγ) (14,15). C/EBPβ and C/EBPδ rapidly induce the expression levels of PPARγ and C/EBPα. The PPARγ and C/EBPα proteins activate the expression of a number of genes involved in adipocyte differentiation, including genes responsible for lipid accumulation and insulin sensitivity (16). In addition, the activation of the extracellular signal regulated kinase (ERK) and phosphatidylinositide 3-kinase (PI3K)/Ark pathways is necessary for adipogenesis (16,17). ERKs regulate cell proliferation and are required for initiating the differentiation process in pre-adipocytes. The phosphorylation of ERK is increased during the early stages of adipocyte differentiation in embryonic stem cells. Several previous studies have reported that inhibition of the ERK pathway in the early stages of differentiation inhibits adipogenesis, and there is increasing evidence demonstrating the importance of the ERK pathway in adipogenesis (18,19).
The involvement of Akt in adipocyte differentiation is better understood, and inhibition of the PI3K/Akt pathway has been demonstrated to reduce adipogenesis (20,21).

Zanthoxylum schinifolium, which belongs to the Rutaceae family of plants, is an aromatic plant widely used as a pungent condiment and seasoning in Korea and other countries in east Asia (22). Z. schinifolium has also been used in traditional Chinese medicine for the treatment of several symptoms, including the common cold, diarrhea, toothache and jaundice. Additionally, various pharmacological activities, including the inhibition of lipid peroxidation (23), and anti-oxidant (24), anti-platelet aggregation (25), anti-inflammatory (26) and antitumor (23,27,28) effects, have been reported by this plant. However, to the best of our knowledge, the potential anti-obesity activity of Z. schinifolium remains to be elucidated. Therefore, the aim of the present study was to determine the inhibitory ability of ethanol extract from the leaves of Z. schinifolium (EEZS) on adipocyte differentiation, using the 3T3-L1 murine pre-adipocyte model, determined by measuring the levels of lipid accumulation and the expression levels of adipocyte marker genes. The effects EEZS on the ERK and PI3K/Akt pathways were also examined to investigate the possible underlying molecular mechanisms.

Materials and methods

Preparation of EEZS. Z. schinifolium was obtained from Dongeui Oriental Hospital, Dongeui University College of Korean Medicine (Busan, Republic of Korea). Dried leaves from Z. schinifolium (40 g) were cut into small pieces, ground into a fine powder and then soaked with 500 ml 100% ethanol (Sigma-Aldrich, St. Louis, MO, USA) for 2 days. The extracted liquid was filtered through Whatman No. 3 filter paper (Sigma-Aldrich) twice to remove any insoluble materials and was then concentrated using a rotary evaporator (EYELA N-1000; Rikakikai Co., Ltd., Tokyo, Japan). The extracts were redissolved in dimethylsulfoxide (DMSO; Sigma-Aldrich) to a final concentration of 200 mg/ml (extract stock solution) and were subsequently diluted with Dulbecco’s modified Eagle’s medium (DMEM; WelGENE) and 1% penicillin-streptomycin (Sigma-Aldrich), to the desired concentration prior to use.

Cell culture and induction of adipocyte differentiation. The 3T3-L1 cell line was obtained from American Type Culture Collection (Manassas, VA, USA). The cells were cultured in DMEM, supplemented with 10% fetal bovine serum (FBS; WelGENE) and 1% penicillin-streptomycin (Sigma-Aldrich) at 37°C in a humidified atmosphere of 5% CO₂. To initiate differentiation of the 3T3-L1 pre-adipocytes into adipocytes, the cells were stimulated with differentiation inducers, including 0.5 mM isobutylmethylxanthine (Sigma-Aldrich), 10 µg/ml insulin (MDI; Sigma-Aldrich) and 1 µM dexamethasone, which were added to the DMEM, containing 10% FBS, for 48 h. Following differentiation, the adipocytes were incubated with post-differentiation medium, which consisted of DMEM, containing 10% FBS and 10 µg/ml insulin. The medium was replaced every other day for up to 8 days (29).

Cell viability. To assess the cell viability in the 3T3-L2 adipocytes, the cells were plated into 24-well plates (1x10⁵ cells/ml) and incubated for 24 h at 37°C until confluent. The cells were subsequently treated with the indicated concentrations (100-600 µg/ml) of EEZS for 72 h at 37°C. The control cells were supplemented with complete medium, containing 0.5% DMSO, as a vehicle control. Subsequent to incubation for 72 h at 37°C, the medium was removed and the cells were incubated with 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) solution for 3 h at room temperature. The supernatant was subsequently discarded and the formazan blue, which had formed in the cells, was then dissolved in 100% DMSO. The DMSO concentration did not exceed 0.05%. The optical density was measured at 540 nm using a microplate reader (Dynatech Laboratories, Chantilly, VA, USA) and the effects of EEZS on the inhibition of cell growth were assessed as the percentage of cell viability, in which the vehicle-treated cells were considered to be 100% viable.

Oil Red-O staining. The intercellular lipid accumulation within adipocytes was assessed using Oil Red-O solution. Following the induction of adipocyte differentiation, the cells were washed twice with phosphate-buffered saline (PBS; WelGENE) and fixed at room temperature with 10% formalin (Junsei Chemical Co., Ltd., Tokyo, Japan) for 1 h. The cells were dried and stained with Oil Red-O (0.5% Oil Red-O in 100% aqueous 2-isopropanol; Sigma-Aldrich) for 20 min at room temperature. Following staining of the lipid droplets, the Oil Red-O staining solution was removed, the plates were rinsed twice with PBS and 60% isopropanol and were then dried. Images of the stained oil droplets in the 3T3-L1 cells were captured under light microscopy. To quantify the intracellular lipids, the stained lipid droplets were dissolved in isopropanol, containing 4% Nonidet P-40 (NP-40; Sigma-Aldrich). The extracted dye was transferred into a 96-well plate, and the absorbance was measured using a microplate reader (MR5000; Dynatech Laboratories, Chantilly, VA, USA) at 500 nm. The difference in absorbance between the samples with and without dye solution was calculated (30).

Protein extraction and western blot analysis. The cells were harvested and washed once with ice-cold PBS, and were gently lysed for 20 min in ice-cold lysis buffer containing 40 mM Tris (pH 8.0), 120 mM NaCl, 0.5% NP-40, 0.1 mM sodium orthovanadate, 2 µg/ml leupeptin and 100 µg/ml phenylmethylsulfonyl fluoride (all Sigma-Aldrich). The supernatants were collected and the protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Equal quantities of the protein extracts were denatured by boiling at 95°C for 5 min in sample buffer (Bio-Rad Laboratories, Inc.), containing 0.5 M Tris-HCl (pH 6.8), 4% sodium dodecyl sulphate (SDS), 20% glycerol, 0.1% bromophenol blue and 10% β-mercaptoethanol, at a ratio of 1:1. The samples were either stored at -80°C or were used immediately for immunoblotting. Aliquots, containing 30 µg total protein, were separated on 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Amersham Life Sciences, Arlington Heights, IL, USA). The membranes were subsequently blocked with 5% non-fat milk and incubated overnight at 4°C with primary antibodies, probed with enzyme-linked
secondary antibodies at room temperature for a further 1 h and then detected using an enhanced chemiluminescence detection system (Amersham Life Sciences), according to the manufacturer's instructions. The primary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and Cell Signaling Technology, Inc. (Danvers, MA, USA) (Table I). The horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (IgG, sc-2004; 1:1,000), anti-mouse IgG (sc-2005; 1:1,500) and anti-goat IgG (sc-2350; 1:1,500) were purchased from Santa Cruz Biotechnology, Inc. β-actin was used as an internal control.

Statistical analysis. The data are expressed as the mean ± standard deviation. Comparisons between the groups were made using analysis of variance and the significance between differences were analyzed using Duncan's multiple range test. Statistical analyses were performed using SPSS version 18.0 (SPSS Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistical significant difference.

Results

Cytotoxic effects of EEZS on 3T3-L1 cells. To determine the cytotoxicity of EEZS, the 3T3-L1 cells were treated with various concentrations of EEZS and the cell viability was assessed using an MTT assay. The data demonstrated that EEZS exhibited no cytotoxic effects at concentrations ≤200 µg/ml (Fig. 1). Therefore, concentrations of 50, 100, 150 and 200 µg/ml were selected for use in the subsequent experiments.

EEZS inhibits adipogenesis in 3T3-L1 cells. As increased lipid accumulation during the differentiation of pre-adipocytes into adipocytes is a typical phenomenon, which occurs in 3T3-L1 cells and is used as a marker of differentiation, the 3T3-L1 pre-adipocytes were treated with various concentrations of EEZS in the presence of MDI or with MDI alone for 8 days. Lipid accumulation was subsequently measured in the cells using Oil Red-O staining. As shown in Fig. 2, oil droplets were not visible in the undifferentiated 3T3-L1 cells, however, several lipid droplets were visible in the fully differentiated cells treated with MDI. Microscopic observations of the Oil Red-O staining revealed a reduction in the number of lipid droplets as the concentrations of EEZS increased. The inhibitory effects of EEZS on the triglyceride contents of the differentiated adipocytes were measured, which revealed that the triglyceride contents of the adipocytes increased markedly during 8 days incubation with MDI (Fig. 3). However, treatment with EEZS decreased the triglyceride levels significantly, in a concentration-dependent manner. These results demonstrated that EEZS inhibited adipocyte differentiation in the 3T3-L1 cells at non-cytotoxic concentrations.

Table I. List of primary antibodies.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Catalog no.</th>
<th>Species of origin and supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>C/EBPα</td>
<td>1:1,000</td>
<td>2295s</td>
<td>Rabbit polyclonal; Cell Signaling Technology, Inc.</td>
</tr>
<tr>
<td>C/EBPβ</td>
<td>1:1,000</td>
<td>3087s</td>
<td>Rabbit polyclonal; Cell Signaling Technology, Inc.</td>
</tr>
<tr>
<td>PPARγ</td>
<td>1:1,000</td>
<td>2430s</td>
<td>Rabbit polyclonal; Cell Signaling Technology, Inc.</td>
</tr>
<tr>
<td>ERK</td>
<td>1:1,000</td>
<td>sc-154</td>
<td>Rabbit polyclonal; Santa Cruz Biotechnology, Inc.</td>
</tr>
<tr>
<td>p-ERK</td>
<td>1:500</td>
<td>9106s</td>
<td>Mouse monoclonal; Cell Signaling Technology, Inc.</td>
</tr>
<tr>
<td>PI3K</td>
<td>1:1,000</td>
<td>sc-7176</td>
<td>Rabbit polyclonal; Santa Cruz Biotechnology, Inc.</td>
</tr>
<tr>
<td>p-PI3K</td>
<td>1:500</td>
<td>sc-293115</td>
<td>Rabbit polyclonal; Santa Cruz Biotechnology, Inc.</td>
</tr>
<tr>
<td>Akt</td>
<td>1:500</td>
<td>sc-8312</td>
<td>Rabbit polyclonal; Santa Cruz Biotechnology, Inc.</td>
</tr>
<tr>
<td>p-Akt</td>
<td>1:500</td>
<td>sc-101629</td>
<td>Rabbit polyclonal; Santa Cruz Biotechnology, Inc.</td>
</tr>
<tr>
<td>β-actin</td>
<td>1:1,000</td>
<td>sc-1616</td>
<td>Goat polyclonal; Santa Cruz Biotechnology, Inc.</td>
</tr>
</tbody>
</table>

CEBP, CCAAT/enhancer binding protein; PPARγ, peroxisome peroxisome proliferator-activated receptor γ; ERK, extracellular signal-regulated protein kinase; p, phosphorylated; PI3K, phosphatidylinositide 3-kinase. Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; Cell Signaling Technology, Inc., Danvers, MA, USA.
EEZS inhibits the expression of adipogenic transcription factors during adipocyte differentiation in 3T3-L1 cells. Adipogenesis is accompanied by the increased expression of adipogenic transcription factors and adipocyte-specific genes. To investigate the anti-adipogenic mechanism underlying EEZS, the fully differentiated adipocytes were treated with different concentrations of EEZS, and the protein expression levels of PPARγ, C/EBPα and C/EBPβ were determined by western blot analysis. As expected, the levels of these three proteins were significantly upregulated during the process of differentiation (Fig. 4). However, treatment with EEZS suppressed the expression levels of PPARγ, C/EBPα and C/EBPβ compared with the fully differentiated control adipocytes, and this occurred in a concentration-dependent manner. This result suggested that EEZS inhibited adipogenesis by reducing the expression of C/EBPβ, which lead to a downregulation in the expression levels of C/EBPα and PPARγ.

Anti-adipogenic effects of EEZS are associated with inactivation of the ERK and PI3K/Akt pathways in 3T3-L1 cells. The control of adipogenesis requires two well-established signaling mechanisms, the ERK and PI3K/Akt pathways, which are important upstream of adipocyte differentiation (17). The 3T3-L1 cells were pretreated with either ERK- or PI3K-specific inhibitors for 1 h prior to incubation with MDI. The cells were lysed and the cellular proteins were separated on sodium dodecyl sulphate-polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were probed with the indicated antibodies, and the proteins were visualized using an enhanced chemiluminescence detection system. β-actin was used as an internal control. EEZS, ethanol extract of leaves from Zanthoxylum schinifolium; MDI, insulin; PPAR, peroxisome proliferator-activated protein; C/EBP, CAAT/enhancer binding protein.
Figure 5. Effects of EEZS on the phosphorylation of ERK during the early stages of 3T3-L1 cell differentiation. (A) 3T3-L1 cells were either treated with MDI in the presence or absence of 200 µg/ml EEZS or were (B) pre-treated with 10 µM PD98059 for 1 h prior to incubation with MDI in the presence or absence of 200 µg/ml EEZS. The cells were harvested at the indicated time-points and equal quantities of cell lysate were resolved on sodium dodecyl sulphate-polyacrylamide gels, transferred onto nitrocellulose membranes and probed with anti-ERK and anti-p-ERK antibodies. The proteins were visualized using an enhanced chemiluminescence detection system. β-actin was used as an internal control. EEZS, ethanol extract of leaves from Zanthoxylum schinifolium; MDI, insulin; ERK, extracellular signal regulated kinase; p-, phosphorylated.

Figure 6. Effects of EEZS on the phosphorylation of PI3K and AKT during the early stages of 3T3-L1 cell differentiation. The 3T3-L1 cells were either treated with MDI in the presence or absence of (A) 200 µg/ml EEZS or were (B) pre-treated with 10 µM LY294002 for 1 h prior to incubation with MDI in the presence or absence of 200 µg/ml EEZS. The cells were harvested at the indicated time-points and equal quantities of the cell lysate were resolved on sodium dodecyl sulphate-polyacrylamide gels, transferred onto nitrocellulose membranes and were probed with the anti-PI3K, anti-p-PI3K, anti-Akt and anti-p-Akt antibodies. The proteins were visualized using an enhanced chemiluminescence detection system. β-actin was used as an internal control. EEZS, ethanol extract of leaves from Zanthoxylum schinifolium; MDI, insulin; p-, phosphorylated; PI3K, phosphoinositide 3-kinase.
However, treatment with EEZS effectively suppressed the MDI-induced phosphorylation of ERK. The specific ERK inhibitor, PD98059, induced significant inhibition of the phosphorylation of ERK and used as a positive control for treatment with EEZS (Fig. 5B). As shown in Fig. 6, treatment with DMI rapidly induced the phosphorylation of PI3K and Akt, and this continued for 6 h subsequent to treatment. However, the levels of phosphorylated-PI3K and Akt were markedly attenuated following treatment with EEZS, similar to the results, which were obtained using LY294002, a PI3K-specific inhibitor. These results suggested that the inhibition of adipocyte differentiation by EEZS was associated with inactivation of the ERK and PI3K/Akt signaling pathways.

Discussion

Increased consumption of high calorie foods containing sugars and fats, and lack of physical activity lead to obesity, which is a major risk factor for serious chronic diseases, including diabetes, cardiovascular disease and hypertension (1-11). Adipocyte differentiation is an adaptive response to excess energy intake, inducing obesity and metabolic diseases (17,31). Accordingly, adipocytes are a therapeutic target in treating obesity and investigations are being performed to prevent obesity through regulating adipogenesis. During adipogenesis, undifferentiated fibroblast-like pre-adipocytes become spherical fat cells and accumulate as lipid droplets (32,33). The present study, as part of an ongoing investigation to identify anti-obesity agents from traditional medicine sources, investigated whether EEZS possessed anti-obesity activity using a 3T3-L1 pre-adipocyte model.

To investigate suppressive effects of EEZS on adipocyte differentiation, the effects of EEZS on the accumulation of intracellular lipids were determined using Oil Red-O staining and triglyceride content analysis in 3T3-L1 pre-adipocytes. The data demonstrated that EEZS significantly inhibited adipocyte differentiation and lipid accumulation in a concentration-dependent manner (Figs. 2 and 3). The concentrations of EEZS used to inhibit adipocyte differentiation exhibited no affect on cell viability, as assessed using an MTT assay (Fig. 1), indicating that the promising anti-obesity potential of EEZS in 3T3-L1 cells was not simply due to a cytotoxic effect.

Adipocyte differentiation is highly regulated by several transcription factors. The PPAR and C/EBP families are important in adipocyte differentiation. More specifically, the adipocyte marker transcription factors, PPARγ and C/EBPα, have been reported to be important in differentiation and lipid storage, and in the coordinated expression of genes involved in creating or maintaining the phenotype of adipocytes (14,15). Additionally, C/EBPβ, which is considered to mediate the expression levels of PPARγ and C/EBPα during adipogenesis, is the first transcription factor induced following exposure of pre-adipocytes to differentiation medium and, therefore, may be involved in directing the differentiation process (32,33). The present study revealed that EEZS significantly down-regulated the protein expression levels of PPARγ, C/EBPα and C/EBPβ, induced by differentiation medium, in the 3T3-L1 cells (Fig. 4). Therefore, EEZS appeared to inhibit adipogenesis, which may be attributable to its ability to downregulate the expression levels of adipocyte marker proteins.

It has also been reported that activation of the ERK and PI3K/Akt pathways is necessary for adipogenesis (18,19). Activation of these pathways during adipogenesis promotes differentiation by activating factors that regulate the expression levels of PPARγ and C/EBPs. Several previous studies have demonstrated that the activation of ERK and Akt induces differentiation by activating factors, which regulate the expression levels PPARγ and C/EBPs during the early-stages of adipogenesis (14,15). In addition, there is increasing evidence that the inhibition of the ERK and Akt pathways in adipocyte differentiation inhibits adipogenesis (16,34,35). Therefore, to further assess the effect of EEZS on the upstream signaling pathways of PPARγ and C/EBPs, the present study investigated the effects of EEZS on the expression levels of phosphorylated-ERK and Akt. As the phosphorylation of Akt is regulated by PI3K, as an upstream kinase of Akt (36,37), the effects of EEZS on the levels of PI3K were also determined. The data demonstrated that the phosphorylation of ERK, PI3K and Akt were significantly activated during the early stages of adipogenesis, and the activation continued for 3 or 6 h following the induction of adipocyte differentiation by MDI (Figs. 5 and 6). However, EEZS significantly inhibited the phosphorylation of ERK, in a time-dependent manner. The protein expression levels of total PI3K and Akt, as with ERK, remained unchanged throughout the experiment. EEZS also attenuated the protein expression levels of phosphorylated-PI3K and Akt (Fig. 6). Although the precise molecular signaling mechanism underlying EEZS remains to be elucidated, these results suggested that EEZS inhibited the activation of the ERK and PI3K/Akt pathways at an early stage and inhibited the expression of adipogenic transcription factors by modulating the ERK- and PI3K/Akt-mediated signaling pathways during adipocyte differentiation.

In conclusion, the present study demonstrated that EEZS suppressed adipogenesis in the 3T3-L1 cells by downregulating the expression levels of PPARγ, C/EBPα and C/EBPβ, through inactivation of the ERK and PI3K/Akt signaling pathways in the early stages of adipogenesis. These findings suggested the possible use of EEZS as a therapeutic substance or as a lead in the development of therapeutic substances for the prevention and management of obesity.

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References