Adipogenesis Inhibitory Effects of *Limonium tetragonum* in Mouse Bone Marrow Stromal D1 Cells

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Abstract Unbalanced differentiation of bone marrow cells into adipocytes rather than osteoblasts causes severe bone-related disorders, such as osteoporosis. Natural products that can act against unbalanced differentiation are targeted for development of functional dietary supplements. *Limonium tetragonum*, a halophyte known for anti-oxidant constituents, was studied for beneficial effects in prevention of adipogenesis of bone marrow stromal D1 cells *in vitro*. Solvent-partitioned extract fractions of *L. tetragonum* prevented differentiating D1 cells from accumulating lipids. Expression of the key adipogenic markers PPAR γ , SREBP1c, and C/EBP α were inhibited at mRNA and protein levels. Suppressed protein levels of the phosphorylated MAPK-related factors ERK, JNK, and p-38 indicated inhibition of PPAR γ pathway-linked adipogenesis in D1 cells. The *n*-BuOH fraction was the most effective fraction for bioactive substances. *L. tetragonum* inhibited adipogenesis in D1 bone marrow stromal cells due to the presence of bioactive flavonoids.

Keywords: adipogenesis, bone marrow, D1 cell, *Limonium tetragonum*, PPAR γ

Introduction

Bone related issues are a major cause of a low quality of life, morbidity, and mortality (1). Osteoporosis, which is associated with an imbalance in bone mass, is one of the most common bone related disorders. Patients aged above 50 years suffering from bone fractures are commonly diagnosed with osteoporosis (2,3). Current strategies for treatment of osteoporosis include balancing differentiation of stromal cells in bone marrow while preventing possible causes of bone volume loss, such as elevated bone marrow adipogenesis and obesity, to name a few. Prevention of differentiation of bone marrow stromal cells into adipocytes has been reported to be beneficial for relief of deterioration in the bone mass balance (4). Differentiation of cells into either osteocytes or adipocytes is mainly regulated by PPAR γ pathways. Studies have demonstrated that, depending on the ligand of the PPAR γ outcome, cell differentiation into both osteocytes and adipocytes can elevate and suppress differentiation (5). In addition, PPAR γ pathways are notable targets for treatment of obesity-related complications, which also include bone mass loss due to involvement of PPAR-related signaling in energy metabolism (6). Obesity-related complications can be suppressed by early control of body weight gain due to an elevated adipogenesis level. Apart from

commercial drugs, natural products and functional foods are of much interest due to remedial properties and natural origins.

In this context, halophytes have been of much interest due to known abilities to withstand harsh environments via production of different metabolites. Some halophyte species already have been reported to exert health beneficial effects (7-9). *Limonium tetragonum* is a common species of salt-tolerant halophytes that on salt marshes and muddy seashores throughout the western coastal area of South Korea. However, no reports of potential health beneficial effects and bioactive constituents of *L. tetragonum* are known, except for an anti-oxidant activity, which is common for most halophytes (10). Considering the potential of halophytes as part of a prominent research trend for development of novel substances from natural plants for nutraceutical purposes, *L. tetragonum* was examined in this study for beneficial effects against obesity *in vitro* using a mouse bone marrow stromal cell model.

Materials and Methods

Plant materials Specimens of *L. tetragonum* were collected by hand from the seashore of Yulchon-myeon, Yeosu, Jeonnam, Korea

in May of 2015 and dried outside under shade for 3 days prior to grinding with an electric food grinder (FM-808; Hanil Electric Co., Ltd., Seoul, Korea). Dried and grinded *L. tetragonum* samples were subjected to extraction twice first using 3 L of CH_2Cl_2 , then using 3 L of MeOH. Obtained crude extracts were dried *in vacuo* to retrieve a residue of 24.41 g, which was later solvent-partitioned between 3 L of CH_2Cl_2 and 3 L of H_2O . The CH_2Cl_2 layer was further separated using *n*-Hexane and 85% aqueous MeOH fractionation. The H_2O layer was further fractionated between *n*-BuOH and H_2O . Subsequently, the 4 solvent-partitioned fractions of *n*-Hexane (1.70 g), 85% aqueous MeOH (0.34 g), *n*-BuOH (3.08 g), and H_2O (18.92 g) were collected. Solvent-partitioned samples of these fractions were dissolved in 10% dimethyl sulfoxide (DMSO) prior to use in subsequent assays. Isolation of bioactive substances was carried out following a previous method (11).

Cell viability Evaluation of fraction cytotoxicity towards D1 cells was performed using a formazan-based cell viability assay (10). D1 cells were grown in 96-well plates until an approximate density of 5×10^3 cells per well was reached. Subsequent to confluence, samples of the crude extract of *L. tetragonum* and solvent-partitioned fractions were introduced into culture media at concentrations of 10, 50, and 100 $\mu\text{g}/\text{mL}$ and incubation was continued for another 48 h. Cells were then supplied with 100 μL of 1 mg/mL MTT reagent prior to another 4 h of incubation. Lastly, dissolution of retained formazan salt was achieved using addition of 100 μL of DMSO. Cell viability was confirmed and measured based on absorbance values at 540 nm using a microplate reader (Genios; Tecan Austria GmbH, Grodig, Austria).

Cell culture and adipocyte differentiation Mouse D1 bone marrow stromal cells were seeded in 6-well plates to attain a cell density of 2×10^5 cells/well and supplied with Dulbecco's Modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) until confluence in a CO_2 (5%) incubator at 37°C . The first day after cells reached confluence was designated as day 0 and adipogenic differentiation of cells was induced using introduction of a differentiation-mix consisting of 0.5 mM 3-isobutyl-1-methylxanthine (I5879; Sigma-Aldrich, St. Louis, MO, USA), 0.25 μM dexamethasone (D4902; Sigma-Aldrich), and 5 $\mu\text{g}/\text{mL}$ insulin (I6634; Sigma-Aldrich) in DMEM containing 10% FBS. Subsequent to differentiation-mix introduction on day 2, the cell culture medium was removed and replaced with DMEM containing 10% FBS and 5 $\mu\text{g}/\text{mL}$ insulin. This medium was renewed every 2 days until day 6 when the medium was replaced with DMEM containing 10% FBS until day 8. *L. tetragonum* solvent-partitioned fractions of crude extracts were supplied with every medium change from day 0 to day 8.

Oil Red O staining Fully differentiated D1 cells were fixed on 6-well plates for 1 h of incubation on a counter at room temperature ($20\text{--}22^\circ\text{C}$) in 10% formaldehyde after the cell culture medium was

removed and cells were washed gently with PBS. Staining of lipid droplets was carried out using a paper-filtered (Whatman #1; GE Healthcare, Little Chalfont, UK) 0.5% Oil Red O solution of 60% isopropanol and 40% water (w/v). After 1 h of incubation on a counter at room temperature ($20\text{--}22^\circ\text{C}$) for staining, the staining solution was removed from wells, followed by adding and removing of 1 mL PBS (70013032; Life Technologies, Carlsbad, CA, USA) to wells and air-drying on a counter at room temperature ($20\text{--}22^\circ\text{C}$). Stained lipid droplet images of fully differentiated D1 adipocytes were captured using an Eclipse TS100 inverted microscope (Nikon Instruments, Tokyo, Japan). For calculation of lipid accumulation, dye absorbed by lipid droplets was eluted using isopropanol. Quantification of eluted dye was performed based on optical absorbance values at 500 nm using a microplate reader.

RNA extraction and reverse transcription (RT) PCR analysis Total RNA was isolated from mature D1 adipocytes both treated and not treated with solvent-partitioned fractions using Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA). For synthesis of cDNA, 2 μg of RNA was added to RNase-free water and oligo (dT) (Bio-Rad Laboratories, Hercules, CA, USA), denatured at 70°C for 5 min, then cooled immediately in an ice bath to room temperature ($20\text{--}22^\circ\text{C}$). RNA was reverse transcribed using a master mix (Bio-Rad Laboratories) containing 1x RT buffer, 1 mM dNTPs, 500 ng of oligo (dT), 140 U of M-MLV reverse transcriptase, and 40 U of RNase inhibitor at 42°C for 60 min and at 72°C for 5 min using an automatic thermos-cycler (T100; Bio-Rad Laboratories). Target cDNA was amplified using sense and antisense primers of forward 5'-TTTT CAAGGGTGCCAGTTTC-3' and reverse 5'-AATCCTTGCCCTCTGAGAT-3' for PPAR γ ; forward 5'-TGTTGGCATCTGCTATCTG-3' and reverse 5'-AGGGAAAGCTTTGGGGTCTA-3' for SREBP1c; forward 5'-TTACAAC AGGCCAGGTTTCC-3' and reverse 5'-GGCTGGCGACATACAGTACA-3' for C/EBP; and forward 5'-GCCACCCAGAAGACTGTGGAT-3' and

Fig. 1. Cytotoxic effects of an *L. tetragonum* extract and solvent-partitioned fractions in D1 cells. Values are reported as a mean $\pm$ SD of 3 separate experiments.

reverse 5'-TGGTCCAGGGTTTCTTACTCC-3' for β -actin. Amplification cycles were carried out at 95°C for 45 sec, 60°C for 1 min, and 72°C for 45 sec. After 30 cycles, PCR products were separated using electrophoresis (Mini-Sub; Bio-Rad Laboratories) on 1.5% agarose gel for 30 min at 100 V. Gels were then stained with 1 mg/mL ethidium bromide and visualized using UV light with a Davinch-Chemi CAS-400SM Imager (Davinch-K, Seoul, Korea) and AlphaEase® gel image

analysis software (Alpha Innotech, San Leandro, CA, USA). RT-PCR gel images were quantified with quantification process of AlphaEase gel image analysis software (Alpha Innotech) and normalized to β -actin levels by dividing the obtained values to that of β -actin band quantifications of each well.

Western blot analysis Immunoblotting was carried out following

Fig. 2. Effect of *L. tetragonum* solvent-partitioned fractions on lipid accumulation in D1 cells undergoing adipogenesis depicted using (A) Oil red O staining and (B) eluted dye quantification. Values represent a mean $\pm$ SD of 3 separate experiments.

Fig. 3. mRNA expression levels of key adipogenic factors in differentiated mature D1 adipocytes following treatment with *L. tetragonum* solvent-partitioned fractions [(A) H₂O, (B) *n*-BuOH, (C) 85% aq. MeOH, and (D) *n*-Hexane] assayed using RT-PCR. Values represent a mean $\pm$ SD of 3 separate experiments with significance compared with a control. ^{a-e}Means with the different letters are significantly different ($p < 0.05$) by Duncan's multiple range test.

standard procedures (10). Lysates were prepared from differentiated D1 cells using RIPA lysis buffer (Sigma-Aldrich) at 4°C for 30 min. Obtained lysates were then subjected to 12% sodium dodecyl sulfate (SDS) gel electrophoresis (Mini-PROTEAN; Bio-Rad Laboratories) for separation, followed by transfer of separated proteins to polyvinylidene fluoride membranes (GE Healthcare). Blocking of membranes was achieved by incubation of membranes in a 5% skim milk (70166; Sigma-Aldrich) with an orbital shaker (SH30; FINEPCR, Gunpo, Korea) prior to hybridization through incubation on an orbital shaker with primary antibodies (1:1,000 diluted with distilled water). Proteins were detected after incubation on an orbital shaker with horseradish-peroxidase-conjugated secondary antibody at room temperature using a commercial electrochemiluminescence (ECL) assay kit (RPN2232; GE Healthcare) following the kit manual. Immunoreactive protein bands were observed using a Davinch-Chemi CAS-400SM Imager.

Statistical analysis Graphs in all figures were plotted with mean $\pm$ standard deviation (SD) values based on at least 3 separate experiments. Statistical differences between individual groups were analyzed using the analysis of variance (ANOVA) method of Statistical Analysis System software, SAS v 9.1 (SAS Institute, Cary, NC, USA) coupled with Duncan's multiple range test. Statistical significance was defined at $p < 0.05$.

Results and Discussion

In order to provide insights anti-adipogenic effects of *L. tetragonum* extract fractions, D1 mouse bone marrow stromal cells were induced to undergo adipogenesis in the presence and absence of *L. tetragonum* extracts.

Fig. 4. Protein levels of key adipogenic factors in differentiated D1 mature adipocytes following treatment with *L. tetragonum* solvent-partitioned fractions [(A) H₂O, (B) *n*-BuOH, (C) 85% aq. MeOH, and (D) *n*-Hexane] assayed based on Western blotting. Values represent a mean $\pm$ SD of 3 separate experiments with significance compared with a control. ^{a-e}Means with the different letters are significantly different ($p < 0.05$) by Duncan's multiple range test.

Cytotoxicity Extract fraction toxicity was evaluated based on cell viability assays (10). No fractions were toxic to D1 cells at concentrations of 10, 50, and 100 μ g/mL (Fig. 1). D1 stromal cells accumulated large amounts of lipids via adipogenesis, which was dose-dependently hindered by *L. tetragonum* fractions (Fig. 2A). Lipid accumulation levels in D1 cells (Fig. 2B) were affected in order of strength by the MeOH, *n*-Hexane, H₂O, and *n*-BuOH fractions based on efficiency for reduction of lipid accumulation.

Effect of *L. tetragonum* extracts on the PPAR γ pathway For evaluation of a possible mechanism behind the anti-adipogenic effect of *L. tetragonum* fractions, mRNA expression and protein levels of crucial adipogenesis (PPAR γ , C/EBP α , and SREBP1c) and transcription factors (p38, p-ERK, and p-JNK) were observed following treatments with different fractions. Previous studies have linked obesity with complications throughout the body, including bone-related diseases. Adipogenic induction of bone marrow stromal

cell causes obesity-related bone diseases as adipose tissue-dependent factors induce PPAR γ related adipogenesis stimulation in stromal cells (12). Expression of mRNA for all adipogenesis-related factors was decreased in a dose-dependent manner with treatment (Fig. 3). All fractions caused a decrease in mRNA expression levels. However, the *n*-BuOH (Fig. 3B) and *n*-Hexane (Fig. 3D) fractions were more active than other fractions (Fig. 3A and 3C). For confirmation of PPAR γ pathway interaction, protein levels of PPAR γ , C/EBP α , and SREBP1c were assessed based on immunoblotting (Fig. 4). Protein levels of all factors were decreased dose-dependently with the *n*-BuOH (Fig. 4B) fraction suppressing protein translation more than other fractions, followed by the MeOH (Fig. 4C), H₂O (Fig. 4A), and *n*-Hexane (Fig. 4D) fractions.

Effect of *L. tetragonum* extracts on the MAPK pathway In order to understand intervention in the function of the PPAR γ pathway, levels of the MAPK-related proteins p38, p-ERK, and p-JNK were evaluated.

Fig. 5. Levels of key MAPK pathway proteins in differentiated D1 mature adipocytes following treatment with *L. tetragonum* solvent-partitioned fractions assayed based on Western blotting. [(A) H₂O, (B) *n*-BuOH, (C) 85% aq. MeOH, and (D) *n*-Hexane].

The ERK and JNK pathways are also activated during adipogenesis via early PPAR γ signaling in a MAPK-independent manner, including elevated levels of phosphorylated ERK, JNK, and p38 proteins (13). D1 cells were affected in a manner that hindered the PPAR γ pathway via MAPKs, preventing phosphorylation of ERK, JNK, and p38, resulting in interruption of the PPAR γ transcription factor cascade. In a dose-dependent manner, *L. tetragonum* fractions lowered phosphorylated ERK, JNK, and p38 levels, indicating inhibition of transcription factor relocation into the nucleus, which lead to PPAR γ -stimulated adipogenesis (Fig. 5A-5D).

Bone marrow and pre-adipocyte cell adipogenesis share the same adipogenic pathways although stimuli and mature adipocytes can exhibit differences (14). *L. tetragonum* fractions prevented bone marrow stromal cells from undergoing adipogenic differentiation. Insight was, thus, obtained into development of natural products against obesity and obesity-related complications, especially for osteoporosis, considering the role of bone marrow cell adipogenesis in hindering development of an excess amount of adipose tissue. Adipose tissue also secretes adipokines, which are known to exert harmful effects on the body and to stimulate body weight gain.

Isolation of bioactive compounds from *L. tetragonum* fractions The *n*-BuOH fraction of *L. tetragonum* was significantly ($p < 0.05$) more effective for prevention of adipogenesis in stromal cells, including lipid accumulation and adipogenesis pathway interaction, than other fractions. The *n*-BuOH fraction exerted the strongest inhibitory effect

against lipid accumulation, mRNA synthesis, and protein expression. Hence, for elucidation of *L. tetragonum* constituents that exerted anti-adipogenic effects, the *n*-BuOH fraction was subjected to an isolation process reported in an earlier study (11) that produced 4 known compounds. Following isolation, the chemical compounds myricetin 3-galactoside, quercetin 3-*O*-beta-galactopyranoside, quercetin 3-*O*-beta-glucopyranoside, and myricetin 3-*O*-alpha-rhamnopyranside were obtained (Fig. 6). These compounds and derivatives from *Limonium densiflorum*, *Inula helenium*, and *Sedum caespitosum* exhibited anti-oxidant, anti-tumor, anti-viral, and anti-inflammation activities (15-19). *L. tetragonum* contained these substances in amounts large enough for efficient inhibition of PPAR γ transcription factor level adipogenesis.

Prevention of adipose tissue formation is a promising avenue for control of early stage obesity. In this context, *L. tetragonum* is a potential source for active metabolites that can be developed into efficient anti-adipogenic substances. *L. tetragonum* is a valuable new source of anti-adipogenic agents with different chemical structures and can be an important part of functional natural product development for use against obesity onset via prevention of adipose tissue formation.

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Fig. 6. Compounds isolated from the active *n*-BuOH fraction of *L. tetragonum*.

Disclosure The authors declare no conflict of interest.

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