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Anti-adipogenic effect of mulberry leaf ethanol extract in 3T3-L1 adipocytes

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BACKGROUND/OBJECTIVES: Adipogenesis is part of the cell differentiation process in which undifferentiated fibroblasts (pre-adipocytes) become mature adipocytes with the accumulation of lipid droplets and subsequent cell morphological changes. Several transcription factors and food components have been suggested to be involved in adipogenesis. The aim of this study was to determine whether mulberry leaf ethanol extract (MLEE) affects adipogenesis in 3T3-L1 adipocytes.
MATERIALS/METHODS: The 3T3-L1 adipocytes were treated with different doses of MLEE for 8 days starting 2 days post-confluence. Cell viability, fat accumulation, and adipogenesis-related factors including CCAAT-enhancer-binding protein alpha (C/EBPα), peroxisome proliferator-activated receptor gamma (PPARγ), PPARγ coactivator 1 alpha (PGC-1α), fatty acid synthase (FAS), and adiponectin were analyzed.
RESULTS: Results showed that MLEE treatments at 10, 25, 50, and 100 μg/ml had no effect on cell morphology and viability. Without evident toxicity, all MLEE treated cells had lower fat accumulation compared with control as shown by lower absorbances of Oil Red O stain. MLEE at 50 and 100 μg/ml significantly reduced protein levels of PPARγ, PGC-1α, FAS, and adiponectin in differentiated adipocytes. Furthermore, protein level of C/EBPα was significantly decreased by the treatment of 100 μg/ml MLEE.
CONCLUSION: These results demonstrate that MLEE treatment has an anti-adipogenic effect in differentiated adipocytes without toxicity, suggesting its potential as an anti-obesity therapeutic.

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INTRODUCTION

Obesity is a global epidemic caused by abnormal energy metabolism and is associated with increased metabolic diseases worldwide. Obesity represents an imbalance between fat synthesis and fat degradation. Differentiation of undifferentiated fibroblasts (pre-adipocytes) to mature adipocytes, which is termed adipogenesis, is a central area of obesity research [1]. The main characteristics of cellular adipogenesis are continuous fat mobilization and subsequent cell morphological changes in size and shape. During adipogenesis, fat droplets accumulate in adipocytes and cells become more insulin-responsive [2]. Also, gene expressions of fat-related factors are changed; for example, expressions of adiponectin and fatty acid synthase (FAS) increase as adipogenesis progresses [3,4]. Adipogenesis is tightly regulated by the network of transcription factors and other effector proteins [2]. CCAAT-enhancer-binding proteins (C/EBPs), peroxisome proliferator-activated receptor gamma (PPARγ), and PPARγ coactivator 1 alpha (PGC-1α) stimulate adipogenesis [2,5,6]. However, Wnt and beta-catenin impair adipocyte differentiation by inhibiting adipocyte development from mesenchymal precursors and by repressing C/EBPβ and PPARγ [7,8,9]. The balance between adipogenic factors and anti-adipogenic factors decides the fate of adipocyte development and the extent of adiposity.

Mulberry has been used as a part of traditional oriental medicine. It contains phenolic compounds including 1-deoxynojirimycin, rutin, quercetin, isoquercitrin, and resveratrol [10,11]. Raw material and extracts of mulberry leaves and fruits are commonly consumed in the diet as a type of herbal tea and dietary supplement. Water and ethanol extracts of mulberry leaves and fruits exert anti-oxidative and anti-diabetic effects in cell culture and animal models [12-15]. Moreover, vascular protective effects of mulberry water extract were observed in rats fed an atherogenic diet, as shown as reduced levels of blood pressure and acetylcholine-induced relaxation of aortic rings [16]. These protective effects were accompanied by improvements in plasma lipid profiles and cell adhesion molecules expression associated with vascular dysfunction in the aorta [16].

Previous reports demonstrated the anti-obesity effects of

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mulberry in rodents [11,17,18]. In diet-induced obese mice, ethanol extract of mulberry leaves inhibited weight gain acting as a melanin-concentrating hormone-1 antagonist [17]. And combined treatment of mulberry leaf and fruit ethanol extract decreased body weight gain and obesity-related inflammation [11]. Mulberry water extract treatment for 12 weeks decreased weight gain and adiposity, and serum and liver lipids; especially, hepatic fat was reduced with the alterations of lipogenesis- and lipolysis-related gene expression [18].

Compared to the anti-obesity effects of mulberry, evidence regarding mulberry and adipogenesis is limited. We hypothesized that mulberry leaf ethanol extract (MLEE) alters adipogenesis and related markers in 3T3-L1 adipocytes. To test the hypothesis, 3T3-L1 adipocytes were treated with different doses of MLEE for 8 days starting at 2 days after post-confluence, and cell viability, fat accumulation, and markers of adipogenesis were analyzed.

MATERIALS AND METHODS

Plant extraction

Mulberry leaves were collected from Yang Pyeong Agricultural Development & Technology Center (Yang Pyeong-gun, South Korea). The dried leaves (1.0 kg) were extracted with 70% ethanol. The mixture was filtered, evaporated in rotary evaporator and lyophilized. Using this procedure, the yield was 20% of the starting dry weight of mulberry leaves. The obtained ethanol extract of mulberry leaves was kept at -20°C until used.

Cell culture and differentiation

The 3T3-L1 fibroblasts (American Type Culture Collection, Manassas, VA, USA) were cultured to confluence in Dulbecco's modified Eagle medium (DMEM; GIBCO, New York, NY, USA) supplemented with 10% (v/v) bovine calf serum (BCS; GIBCO) and 1% penicillin-streptomycin (GIBCO) in a CO_2 incubator at 37°C. On day 2 post-confluence (designated as day 0), cells were induced to differentiate with DMEM containing 10% fetal bovine serum (FBS; GIBCO), 5 μg/ml insulin (Sigma, St. Louis, MO, USA), 1 μmol/L dexamethasone (Sigma), and 0.05 mmol/L 3-isobutyl-1-methylxanthine (IBMX; Sigma). After 2 days, the medium was replaced with DMEM supplemented with 10% FBS and 5 μg/ml insulin. The cells were subsequently re-fed every 48 h with DMEM containing 10% FBS. To examine the anti-adipogenic effect of mulberry leaf, MLEE was added to medium at different concentrations (10, 25, 50, and 100 μg/ml) during medium changes. Cells without the MLEE treatment were considered as a control.

MTT assay

The 3T3-L1 fibroblasts were seeded at a density of 1×10^4 cells/well in 96-well plates. The cells were treated with different concentrations of dimethyl sulfoxide (DMSO) or MLEE for 48 h. After completion of the treatment, the cells were incubated with 0.5 mg/ml MTT (3-4,5-dimethylthiazol-2-yl-2, 3-diphenyl tetrazolium bromide) for 4 h at 37°C. The supernatants were carefully aspirated and 100 μL of DMSO was added to dissolve the formazan crystal product. Absorbances were measured at 560 nm using a microplate reader.

Oil Red O staining

Adipocyte cell monolayers were gently rinsed twice with phosphate-buffered saline (PBS), fixed in a with 4% paraformaldehyde-PBS solution for 1 h at room temperature, stained with the 0.5% Oil Red O-isopropyl alcohol for 1 h, and then washed with distilled water. The cells were checked by a bright-field optical microscope (HS-100, OPTICAL, China). The cells were eluted with isopropyl alcohol and absorbances were measured at 520 nm with a microplate reader.

Preparation of nuclear fractions

Treated 3T3-L1 adipocytes were homogenized with buffer A [10 mM HEPES-KOH (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), and 0.2 mM phenylmethanesulfonyl fluoride (PMSF)] and centrifuged. Pellets were resuspended in buffer C [20 mM HEPES-KOH (pH 7.9), 25% glycerol, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT, and 0.2 mM PMSF] followed by incubation on ice for 20 min. After vortex mixing, the resulting suspension was centrifuged, and the supernatant was stored at -80°C after determination of protein concentrations.

Western blot analysis

Cells were harvested by scraping in 120 μL lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and protease inhibitor cocktail]. They were then incubated on ice for 20 min and centrifuged at 13,000 rpm for 15 min. The supernatant was then transferred to a fresh tube. Protein concentrations were determined using a NanoPhotometer (Implen, Germany). Equal concentrations of protein (30 μg per lane) were loaded in the wells of 6-12% polyacrylamide gels. After the electrophoretic run, proteins on gels were transferred to a polyvinylidene difluoride membrane (Millipore, Marlborough, MA, USA) and incubated in 5% non-fat milk at room temperature. The membrane was incubated with polyclonal antibody against FAS (Abcam, Cambridge, UK, 1:500), adiponectin (Cell Signaling Technology, Danvers, MA, USA, 1:1000), PGC-1α (Santa Cruz Biotechnology, CA, USA, 1:2000), PPARγ (Abcam, 1:500), C/EBPα (Cell Signaling Technology, 1:1000), and β-actin (Santa Cruz Biotechnology, 1:200) overnight at 4°C. The blots were incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) for 1 h. The blots were developed by enhanced chemiluminescence (Santa Cruz Biotechnology). The chemiluminescence signal was recorded and quantified with the Syngene G box (Syngene, Cambridge, UK).

Statistical analyses

Results are expressed as mean ± SD. Statistical significance was determined by one-way analysis of variance followed by a Duncan's test for multiple comparisons using SPSS 20. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of MLEE on cell viability

On day 2 of post-confluence, 3T3-L1 cells were induced to differentiate and treated with various concentrations of MLEE