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Perilla Leaf Extract Inhibits 3T3-L1 Preadipocytes Differentiation

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Abstract Effects of perilla leaf extracts (PLE) on adipocytes differentiation of 3T3-L1 cells were examined. Ethanol extract of PLE treatment significantly decreased lipid accumulation, a marker of adipogenesis, in a dose-dependent manner. Moreover, gene expression levels of peroxisome proliferator-activated receptor γ (PPARγ), the key adipogenic transcription factor, were markedly decreased by PLE. PLE also suppressed adipocyte fatty acid binding protein (aP2) and glycerol-3-phosphate dehydrogenase (GPDH), which are adipogenic marker proteins. These results suggest that PLE treatment suppressed differentiation of 3T3-L1 adipocytes, in part by down-regulating expression of adipogenic transcription factor and other specific target genes.

Keywords: perilla leaf, 3T3-L1 cell, peroxisome proliferator-activated receptor γ (PPARγ), adipocyte fatty acid binding protein (aP2), glycerol-3-phosphate dehydrogenase (GPDH)

Introduction

Adipocytes play a central role in maintaining lipid homeostasis and energy balance by storing triglyceride (TG) or releasing free fatty acids in response to changes in energy demands (1,2). The preadipocytes differentiate into mature adipocytes and increasing fat mass. Therefore, adipocyte differentiation and the extent of subsequent fat accumulation are closely related to the occurrence and advancement of various diseases such as obesity and coronary artery disease (3,4).

Peroxisome proliferator-activated receptors (PPARs) are one class of transcription factors that belongs to the nuclear receptor super family (5). They have a pivotal role in the regulation of intermediary metabolism, both in liver and adipose tissue, where they control the expression of several genes associated with lipid and glucose homeostasis (6,7).

PPARγ is expressed primarily in adipose tissue and promotes fat storage by increasing adipocyte differentiation and transcription of a number of lipogenic proteins (8,9). During the adipocyte differentiation process, PPARγ and C/EBPa in the sequential expression of genes involved in creating and maintaining the adipocyte phenotype, including the genes for adipocyte fatty acid binding protein (aP2), glucose transporter 4 (GLUT4), lipoprotein lipase, and leptin (10). The cytosolic enzyme glycerol-3-phosphate dehydrogenase (GPDH) plays an important role in the conversion of glycerol into TG.

Perilla [*Perilla frutescens* (L.) Britt. Var. *japonica*] is an annual herbaceous plant native to Asia, and its leaves are often used in Asian gourmet food. Perilla leaves, usually accompanied with seafood, are believed to prevent food poisoning (11). Recently, considerable attention has been given to the anti-allergic, anti-inflammatory, and anti-tumor promoting substances (12-14) in perilla leaf and seeds. These researches showed that glycoprotein isolated from perilla extract (11) and rosmarinic acid, one of phenolic constituents in perilla leaves extract (15), show anti-allergic activity *in vivo* and *in vitro*. Furthermore, rosmarinic acid of perilla leaves extract has an antidepressant property in animal models of depression (16). However, the anti-obesity effect of perilla leaf has not been reported. Therefore, the present study was performed to examine the effects of perilla leaf extract (PLE) on adipocyte differentiation in 3T3-L1 cells.

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Materials and Methods

Chemicals Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and antibiotics were obtained from Life Technologies Inc. (Grand Island, NY, USA). Insulin, dexamethasone, 3-isobutyl-1-methylxanthine (IBMX), Oil Red O, and TG assay kit were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of perilla leaf extract Perilla leaf, purchased from Karak market (Seoul, Korea), were dried, powdered and extracted with 70% ethanol (yield: 12.3% of dry wt.). The extract was filtered and freeze-dried at −40°C. The perilla leaf extract (PLE) dissolved in ethanol.

Cell viability assay 3-(4,5-Methylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to determine the number of viable cells in culture. 3T3-L1 preadipocyte cells were obtained from American Type Culture Collection (Rockville, MD, USA) and maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C in 5% CO_2. 3T3-L1 preadipocytes were seeded in 96-well plates at a density of 2,500 cells/mL/well and PLE (1, 10, 25, 50, 100, 200, and 500 μg/mL) was added at 24 hr after cell seeding, and further cultured for another 24 hr. MTT (5.0 mg/mL) solution was added to each well and incubated for 4 hr at

37°C. Formazan formed by viable cells was dissolved with dimethyl sulfoxide (DMSO) and absorbance at 570 nm was determined by microplate reader (Bio-Tek, Winooski, VT, USA).

Adipocyte differentiation of 3T3-L1 preadipocyte cells 3T3-L1 preadipocyte cells maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C in 5% CO_2. After 2 days of post-confluent, cells were exposed to differentiation medium consisting of DMEM supplemented with 10% FBS, 5 μg/mL insulin, 1 μM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthin including theophylline 1 mM, PLE 25, 50, and 100 μg/mL, respectively for 2 days. Cells were then incubated for additional 3 days in DMEM containing 10% FBS and 5 μg/mL insulin including theophylline 1 mM, PLE 25, 50, and 100 μg/mL, respectively.

Oil Red O staining Oil Red O staining was used to monitor lipid accumulation in differentiated adipocytes. Monolayer cells were washed with phosphate buffered saline (PBS), fixed with 10% isopropanol in PBS (pH 7.4), and stained with 0.5% Oil Red O-isopropanol for 1 hr and excess of stain was washed by 70% ethanol and water. Stained oil droplets was dissolved with isopropanol and quantified by spectrophotometrical analysis at 510 nm. Results were represented as a relative percentage of fully differentiated cells.

Triacylglyceride assay Intracellular triacylglyceride (TG) content was determined using a colorimetric assay. Differentiated 3T3-L1 cells were washed with PBS, scraped on ice in 100 μL of saline solution [2 M NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA), 50 mM sodium phosphate, pH 7.4], sonicated to homogenize the cell suspension, and assayed for TG (GPO-Trinder; Sigma-Aldrich).

Reverse transcription-polymerase chain reaction (RT-PCR) analysis Total RNA was isolated from cells with Trizol reagent (Sigma- Aldrich) according to the manufacturer's instruction. The reverse transcription (RT) was performed at 42°C for 1 hr, and heated at 95°C for 5 min to inactivate the avian myeloblastosis virus reverse transcriptase (AMV-RT) enzyme. The following primers were used: peroxisome proliferator-activated receptor γ (PPARγ), forward (5'-AAAGACCCAGCTCTACAACA-3') and reverse (5'-TCGTAGATGACAAATGGTGA-3'); adipocyte fatty acid binding protein (aP2), forward (5'-CCTCGAAGGTTTAC AAAATGTGTGA-3') and reverse (5'-AAACTCTTGTGG AAGTCACGCCTTT-3'); glycerol-3-phosphate dehydrogenase (GPDH), forward (5'-GAACTAAGGAGCAGCTCAAAG GTTC-3') and reverse (5'-CAGTTGACTGACTGAGCAA ACATAG-3'). β-Actin (forward: 5'-AGGTATCCTGACCC TGAAGTACCCC-3'; reverse: 5'-GTTGCCAATAGTGAT GACCTGGCCG-3') was used as an internal standard.

The PPARγ amplification was performed by denaturing at 94°C for 30 sec, annealing at 59°C for 30 sec, and extension at 72°C for 30 sec for 30 cycles and finally 72°C for 5 min. The aP2 amplification was performed by denaturing at 94°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec for 30 cycles and finally 72°C for 5 min. The GPDH amplification was performed by denaturing at 94°C for 30 sec, annealing at 57°C for 30 sec, and extension at 72°C for 30 sec for 30 cycles and finally 72°C for 5 min. The β-actin amplification was performed by denaturing at 94°C for 30 sec, annealing at 63°C for 30 sec, and extension at 72°C for 30 sec for 25 cycles and finally 72°C for 5 min. The PCR products were loaded onto 1.5% agarose gel stained with ethidium bromide. The relative level of RT-PCR reaction products was measured with Forg gel image analysis system (spot densitometry program, Core Bio, Korea) under UV light.

Statistical analysis Results were presented as mean± standard error mean (SEM), and the data were analyzed by analysis of variance (ANOVA) followed by Turkey HSD's post-hoc test.

Results and Discussion

Cytotoxicities of various PLE concentrations To exclude the possible cytotoxic effect of PLE, cell viability was measured at 3T3-L1 preadipocytes. PLE were not cytotoxic at doses of 1-200 μg/mL. But, PLE exerted cellular toxicity at higher concentration (500 μg/mL) exhibiting 39.0±3.6% cell viability (Fig. 1). These results show that PLE can be used safe. A variety of naturally occurring leaf extracts have been found to have beneficial effects on health, and these compounds have drawn attention because of their relative safeness and accumulated evidence of physiological properties (15-17).

Inhibitory effect on PLE in 3T3-L1 adipocytes differentiation As shown in Fig. 2 and 3, dose-dependent decrease in 3T3-L1 preadipocytes differentiation was observed. Addition of PLE (25, 50, and 100 μg/mL) decreased intracellular TG content, visualized by Oil Red O staining and quantified as TG assay, in 3T3-L1 adipocytes. Theophyline (1 mM) was used as a positive control. The prevalence of obesity and obesity-related disorders has led to major research interests in the influence of adipose mass (18). The 3T3-L1 cell line is widely used as a model of adipocytes differentiation and adipose biology. Wang and Jones (19) indicated that the decreased adipocytic lipogenesis is one of the mechanisms of proposed anti-obesity. In the present study, we first investigated the potential inhibitory effects of perilla leaf extract on adipocytes differentiation as assessed by lipid

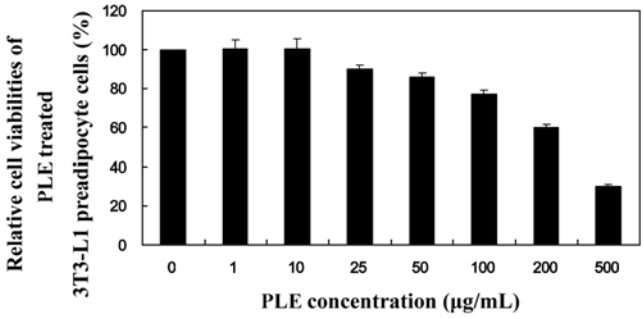

Fig. 1. Relative cell viabilities of perilla leaf extract (PLE) on 3T3-L1 preadipocyte cells.

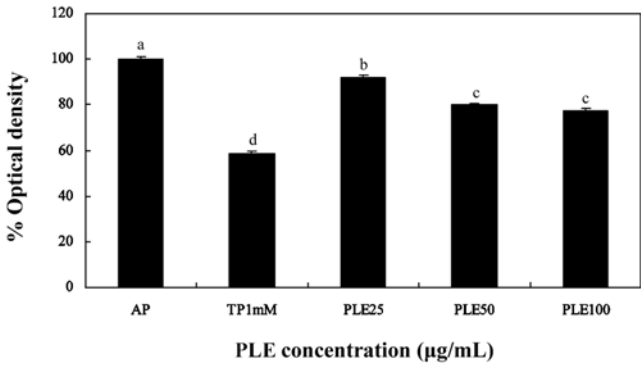

Fig. 2. Effect of perilla leaf extract (PLE) on 3T3-L1 adipocyte differentiation. AP, adipocyte; TP 1 mM, adipocyte treated with 1 mM theophyline; PLE 25, 50, and 100 were adipocyte treated with 25, 50, and 100 μg/mL PLE, respectively. The data are 3 independent experiments. Different letters denote significant differences within each group ($p<0.05$).

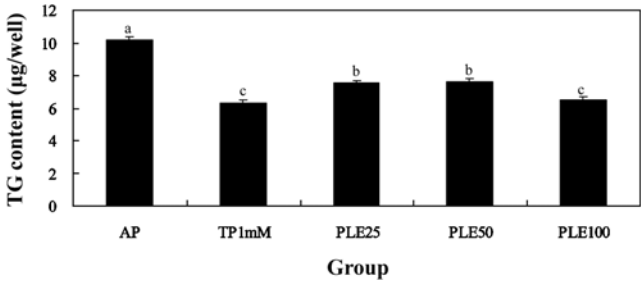

Fig. 3. Effect of perilla leaf extract (PLE) on triglyceride content in 3T3-L1 adipocytes. AP, adipocyte; TP 1 mM, adipocyte treated with 1 mM theophyline; PLE 25, 50, and 100 were adipocyte treated with 25, 50, and 100 μg/mL PLE, respectively. The data are 3 independent experiments. Different letters denote significant differences within each group ($p<0.05$).

accumulation. As shown in Fig. 2 and 3, dose-dependent decrease in 3T3-L1 preadipocytes differentiation was observed. Addition of 25, 50, and 100 μg/mL PLE decreased intracellular triglyceride content visualized by Oil Red O staining and quantified as TG content in 3T3-L1 adipocytes. These results suggest that PLE can be used for obesity prevention, and concentrations of 100 μg/mL might be appropriate for the treatment of 3T3-L1 adipocyte cells.

Expression of PPARγ, aP2, and GPDH In 3T3-L1 cells treated with PLE (25, 50, and 100 μg/mL), decreased differentiation was accompanied by decreased mRNA expression for PPARγ, aP2, and GPDH in a dose-dependent manner (Fig. 3). The effects of PLE (100 μg/mL) on these gene expression was more potent than theophyline which is widely used as an anti-obesity drug. Theophylline is a member of the methylxanthine group of chemicals and a nonselective inhibitor of phosphodiesterase, which implies that the cAMP cascade remains fully active. Arner *et al.* (20) reported that effect of theophylline in lean subjects on adipose tissue increased lipolysis rate. The mRNA expressions of PPARγ, aP2, and GPDH were significantly decreased by 56.7, 48.5, and 65.9%,

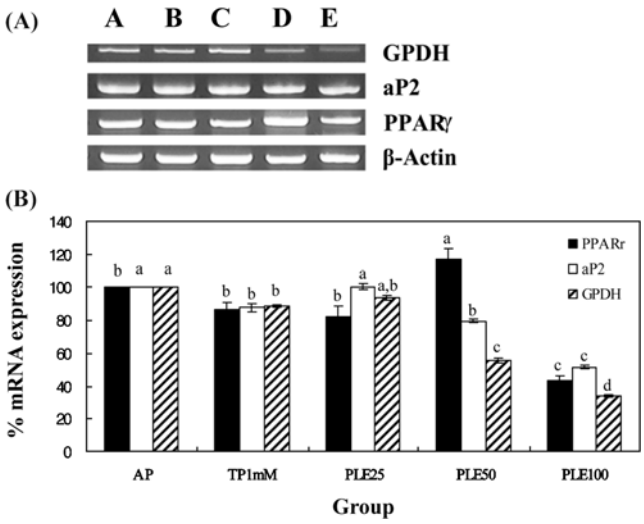

Fig. 4. Effects of perilla leaf extract (PLE) on the expression of PPARγ, aP2, and GPDH mRNA in 3T3-L1 adipocytes. A, RT-PCR analysis; B, % expression levels relative to adipocytes group. AP, adipocyte; TP 1 mM, adipocyte treated with 1 mM theophyline; PLE 25, 50, and 100 were adipocyte treated with 25, 50, and 100 μg/mL PLE, respectively. The data are 3 independent experiments. Different letters denote significant differences within each group ($p<0.05$).

respectively, following 100 μg/mL of PLE exposure when compared to the adipocytes group without PLE treatment. To elucidate the molecular mechanism underlying PLE-induced inhibition of adipgenesis, we further examined the expression of adipogenic transcription factors and adipocyte characteristic proteins using RT-PCR method. The adipocytes differentiation process is regulated by transcriptional activators such as PPARγ. The promoters of adipogenic genes such as aP2 and leptin are regulated by PPARγ transcription factors (10). The aP2 gene is central to the pathway that links obesity to insulin resistance, possibly by linking fatty acid metabolism and expression of tumor necrosis factor α (TNF-α) (21). Ueda *et al.* (22) suggested that perilla leaf extract suppressed the TNF-α production *in vitro* system. The cytosolic GPDH appears to have an important role in the TG synthesis pathway, at the point where it branches from the glycolytic pathway (23).

In conclusion, the inhibitory effects of PLE on adipocyte differentiation, as indicated by a decrease in lipid accumulation, appears to be mediated through the down-regulated expression of adipogenic transcription factor, PPARγ, and adipogenic specific genes, aP2 and GPDH. These results indicate that perilla leaf may play a role in the control of adipogenesis and they might have further implication in *in vivo* antiobesity effects. To our knowledge, this is the first report showing an inhibitory effect of perilla leaf on adipocytes differentiation.

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