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Inhibitory Effect of Ethyl Acetate Extract of White Peach Pericarp on Adipogenesis of 3T3-L1 Preadipocyte Cells

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Abstract In order to determine whether peach contains compounds to regulate adipocyte differentiation, extracts of flesh/pericarp of yellow/white peach were prepared in water, ethyl acetate (EtOAc), or *n*-butanol solvent and determined for effects on adipocyte differentiation in C3H10T1/2 or 3T3-L1 cells. Interestingly, none of peach extracts has statistically significant stimulatory effect on the adipocyte differentiation in C3H10T1/2. Furthermore, the presence of EtOAc extract of white peach pericarp (WPP) was found to inhibit lipid accumulation in 3T3-L1 cells both by microscopic examination of Oil Red O-stained lipid droplets and by spectrophotometric quantification of extracted stain, indicating a significant inhibitory effect on adipocyte differentiation. The inhibition of lipid accumulation was accompanied by a significant decrease in the expression levels of adipocyte molecular markers-peroxisome proliferator-activated receptor γ, CAAT enhancer binding protein α, and fatty acid-binding protein. Thus, this study determined that WPP EtOAc extract contains the inhibitory compound(s) on adipogenesis.

Keywords: *Prunus persica*, peach, obesity, C3H10T1/2, 3T3-L1

Introduction

Adipocytes are the major cellular component in fat tissue and excessive growth, differentiation, and hypertrophy of adipocytes are the fundamental processes affecting obesity. Maturation of adipocytes can occur among cells from a pre-existing pool of adipocyte progenitor cells (preadipocytes) that are present irrespective of age (1). Obesity poses a serious health risk, contributing to the increased prevalence of a host of other diseases including hypercholesterolemia, type-2 diabetes, hyperlipidemia, and hypertension (2). Many drugs were originally derived from herbs and other natural resources, and in many regions of the world, natural medicine has been used for treating diseases and disorders for thousands of years. For example, Ginseng has a long history of medicinal usage traditionally in the oriental region as a general tonic to promote health. There are extensive reports that ginseng has many pharmacological effects on immune, cardiovascular, central nervous systems, and endocrine (3,4). Recently, the extract of *Panax ginseng* berry and ginsenoside Re were also reported to have anti-diabetic effect in an *ob/ob* mouse model (5). *Prunus persica* L. Batsch (Rosaceae, peach) has been used as an ingredient in a variety of Chinese medicine preparations to treat women's diseases in Japan, China, Korea, and other Asian countries (6-11). For example, peach seed-water extracts have been used for the treatment of degenerative disorders such as hypermenorrhea, dysmenorrheal, leiomyoma, and infertility (12-15).

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Two types of faithful *in vitro* model systems have been extensively used over the past 20 years to study adipocyte differentiation. In general, much higher percentage (60-70%) of 3T3-L1 cells was differentiated to adipocytes, following 8 days under the standard adipogenic induction protocol, than the percentage (5-10%) of C3H10T1/2 cells that was seen to differentiate under the same conditions. Therefore, in this study, C3H10T1/2 cells were used as a model system to inspect stimulatory effects, while 3T3-L1 cells were utilized to examine inhibitory effects on adipocyte differentiation. To determine whether peaches contain fraction that can regulate adipocyte differentiation, the effect of various peach extracts on adipocyte differentiation in C3H10T1/2 and 3T3-L1 cells was investigated.

Materials and Methods

Preparation of *P. persica* extracts White and yellow peaches (*Prunus persica* L. Batsch) were purchased at the Kyeonggi Dong-Boo Fruit Agricultural Cooperative (Icheon, Korea). The seeds of the washed peaches were removed. The collected flesh (100 g) or pericarp (100 g) of white or yellow peaches was extracted 3 times in 5 volumes (w/v) of 80% ethanol for 48 hr at room temperature. The extracts were filtered, and concentrated in a rotary vacuum evaporator. The concentrated extracts was then freeze-dried to give solvent-free extracts of white flesh (30.4 g), white pericarp (12.7 g), yellow flesh (27.3 g), and yellow pericarp (9.2 g). The dried extracts of the flesh and pericarp were fractioned with water, ethyl acetate (EtOAc), and *n*-butanol (BuOH). The dried extracts and fractions were stored at −20°C until needed.

Cell culture and adipocyte differentiation 3T3-L1 and

C3H10T1/2 cells (American Type Culture Collection, Manassas, VA, USA) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were differentiated according to a well-established standard adipocyte differentiation protocol (16,17). Briefly, adipogenic differentiation of 2-day post-confluence cells (referred to as day 0) were stimulated to differentiate by addition of a standard adipogenic induction media including 5 μg/mL insulin, 1 μM dexamethasone, and 0.5 mM methylisobutylxanthine (IBMX) in DMEM supplemented with 10% FBS, and then cultured for 2 days. Next, the induction medium was replaced and cultured for 2 days with DMEM supplemented with 10% FBS and 5 μg/mL insulin. Cells were re-fed with DMEM supplemented with 10% FBS for 2 days. During adipocyte differentiation, 3T3-L1 and C3H10T1/2 cells were treated with 100 μg/mL of the indicated extract from day 0 to day 8. For a positive control, 1 μM rosiglitazone (Rosi) was added to the standard adipogenic induction media. All assays were performed in triplicate and at least 2 separate assays were performed.

Oil Red O staining To determine the degree of differentiation and lipid accumulation, accumulation of cytoplasmic triglycerides in cells was detected by staining with Oil Red O. C3H10T1/2 and 3T3-L1 cells were fixed with 10% formalin for 60 min at 4°C and stained with Oil Red O (Sigma-Aldrich, St. Louis, MO, USA). The stained cells were photographed using an Olympus CKX41 inverted microscope (Osaka, Japan) system at 100× magnification. To measure the amount of the stained Oil Red O, the stain was extracted from the cells with isopropanol and the absorbance was determined spectrophotometrically using a MRX II microplate reader (Dynatech Labs., Chantilly, VA, USA) at 570 nm.

Reverse transcription-polymerase chain reaction (RT-PCR) 3T3-L1 cells were cultured in 60 mm dishes and treated with or without EtOAc extract of white peach pericarp. Total RNA was isolated with Trizol reagent (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's instructions. Two μg of total RNA was converted to cDNA using an RT premix kit (Bioneer Corp., Seoul, Korea). The resulting cDNA population was amplified using a PCR premix kit (Intron Biotech., Seongnam, Korea) to assay the levels of adipocyte molecular markers -peroxisome proliferator-activated receptor γ (PPARγ), CAAT enhancer binding protein α (C/EBPα), and fatty acid-binding protein (aP2), which were compared with concurrently measured β-actin expression levels. The PCR primers used are listed in Table 1.

Table 1. PCR primer sequences

Gene[1)]	Primer sequence
C/EBPα	5' GGGTGAGTTCATGGAGAATGG 3'
	5' CAGTTTGGCAAGAATCAGAGCA 3'
PPARγ	5' AGGCCGAGAAGGAGAAGCTGTTG 3'
	5' TGGCCACCTCTTTGCTCTGCTC 3'
aP2	5' TCTCACCTGGAAGACAGCTCCTCCTCG 3'
	5' TTCCATCCAGGCCTCTTCCTTTGGCTC 3'
β-Actin	5' GGACTCCTATGGTGGGTGACGAGG 3'
	5' GGGAGAGCATAGCCCTCGTAGAT 3'

[1)]C/EBPα, CAAT enhancer binding protein α; PPARγ, peroxisome proliferator-activated receptor γ; aP2, fatty acid-binding protein.

Statistical analysis SPSS 12.0 statistical package program (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. The unpaired *t*-test was performed to assess the statistical difference between groups. A p value of <0.05 was considered to indicate a statistically significant difference.

Results and Discussion

Effects of white or yellow peach extracts on adipocyte differentiation in C3H10T1/2 cells The C3H10T1/2 cell line is one of multipotent stem cell lines that have not undergone commitment to the adipocyte lineage and the most commonly used pluripotent mesenchymal cell line (18,19), which was established in 1973 from 14- to 17-day-old C3H mouse embryos. Treatment of 10T1/2 cells with a demethylating agent generates cells exhibiting morphological and biochemical features of muscle, adipose, bone, or cartilage cells, demonstrating the multipotent character of these cells (20). To investigate the stimulatory effect of various extracts from white or yellow peach pericarp (WPP, YPP) or flesh (WPF, YPF) on adipocyte differentiation, C3H10T1/2 cells were differentiated into adipocytes in standard induction media including 100 μg/mL of the indicated extract (WPP, YPP, WPF, or YPF) in water, EtOAc, or BuOH (Fig. 1). After 8 days, cells were stained with Oil Red O and adipocyte differentiation was assessed by microscopic examination of Oil Red O-stained lipid droplets and by spectrophotometric quantification of extracted stain. Less than 10% of the control cells showed lipid droplets via Oil Red O staining, resulting in 0.14 of an optical density for the extracted Oil Red O. For the positive control, Rosi, a ligand for PPARγ, was administered and over 70% of cells were observed as differentiated, resulting in 0.25 of an optical density. All extracts, especially the WPP BuOH extract, demonstrated a very slight increase (if any) in the presence of lipid droplets and extracted stain optical density values when compared to control cells (Fig. 1A and 1B). However, this increase was not statistically significant in any case (Fig. 1B). Therefore, the peach extracts do not show a statistical significant stimulatory effect on the adipogenesis in C3H10T1/2 cells.

Effects of white or yellow peach extracts on adipocyte differentiation in 3T3-L1 cells The preadipocyte cell lines that have already been committed to the adipocyte lineage and can be induced to terminally differentiate into adipocytes, but not into other cell types. The 3T3-L1 preadipocyte cell line is the most widely characterized and is also of murine origin (21-25). To investigate the inhibitory effect of the extracts (WPP, YPP, WPF, or YPF) in water, EtOAc, or BuOH on adipocyte differentiation, 3T3-L1 cells were differentiated into adipocytes for 8 days in the standard induction media including 100 μg/mL of the indicated extract (Fig. 2). The presence of the EtOAc extract of WPP was found to significantly inhibit lipid accumulation in 3T3-L1 cells, both by microscopic

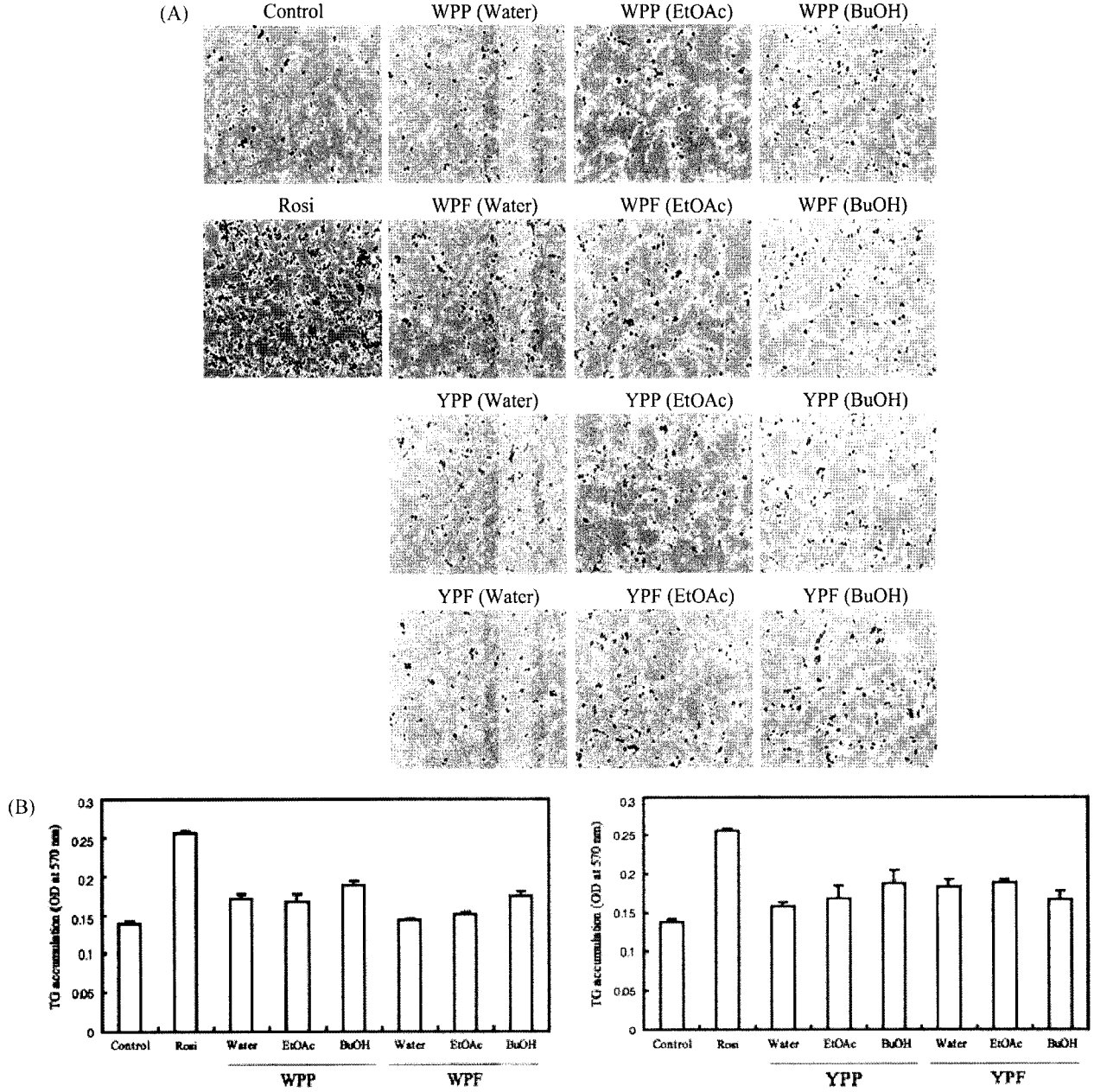

Fig. 1. Effects of white or yellow peach extracts on adipocyte differentiation in C3H10T1/2 cells. (A) C3H10T1/2 cells were induced to differentiate for 8 days in the standard adipogenic induction media containing 100 μg/mL of the indicated extract from white or yellow peach pericarp (WPP, YPP) or flesh (WPF, YPF) in water, EtOAc, or BuOH. The stained cells with Oil Red O were photographed at 100× magnification. (B) The Oil Red O stain was extracted with isopropanol. The absorbance was determined spectrophotometrically at 570 nm to measure triglyceride (TG) accumulation. The data is presented as the mean±SE for the 3 cultures.

examination of Oil Red O stained lipid droplets (Fig. 2A) and by spectrophotometric quantification of the extracted stain (Fig. 2B), indicating inhibition of adipocyte differentiation. In addition, the EtOAc extract of WPP decreased the expression levels of adipocyte molecular markers-PPARγ, C/EBPα, and aP2, confirming the inhibition of adipocyte differentiation (Fig. 2C). Of note, the EtOAc extract of YPP didn't demonstrate any inhibitory effect on adipocyte differentiation which is contrast to the inhibitory effect observed in WPP EtOAc extract treatment group. Taken together, these data suggest that the EtOAc extract of WPP has inhibitory compound(s) that affect adipocyte differentiation in 3T3-L1 cells.

Based on the current understanding of adipocyte development, which is largely derived from studies of the aforementioned cell culture models of adipogenesis, the increase in adipocyte number contributing to adipose tissue mass during obesity is initiated by the commitment of pluripotent stem cells to the adipocyte lineage, followed by the induction of these preadipocytes to undergo mitotic clonal expansion and differentiation into mature adipocytes (26-29). It is worth noting that the EtOAc extract of WPP didn't show any inhibitory effect on adipocyte differentiation in C3H10T1/2 pluripotent mesenchymal cells (though this is not an optimal system to examine inhibitory effects), but did demonstrate an inhibitory effect in 3T3-L1 preadipocytes.

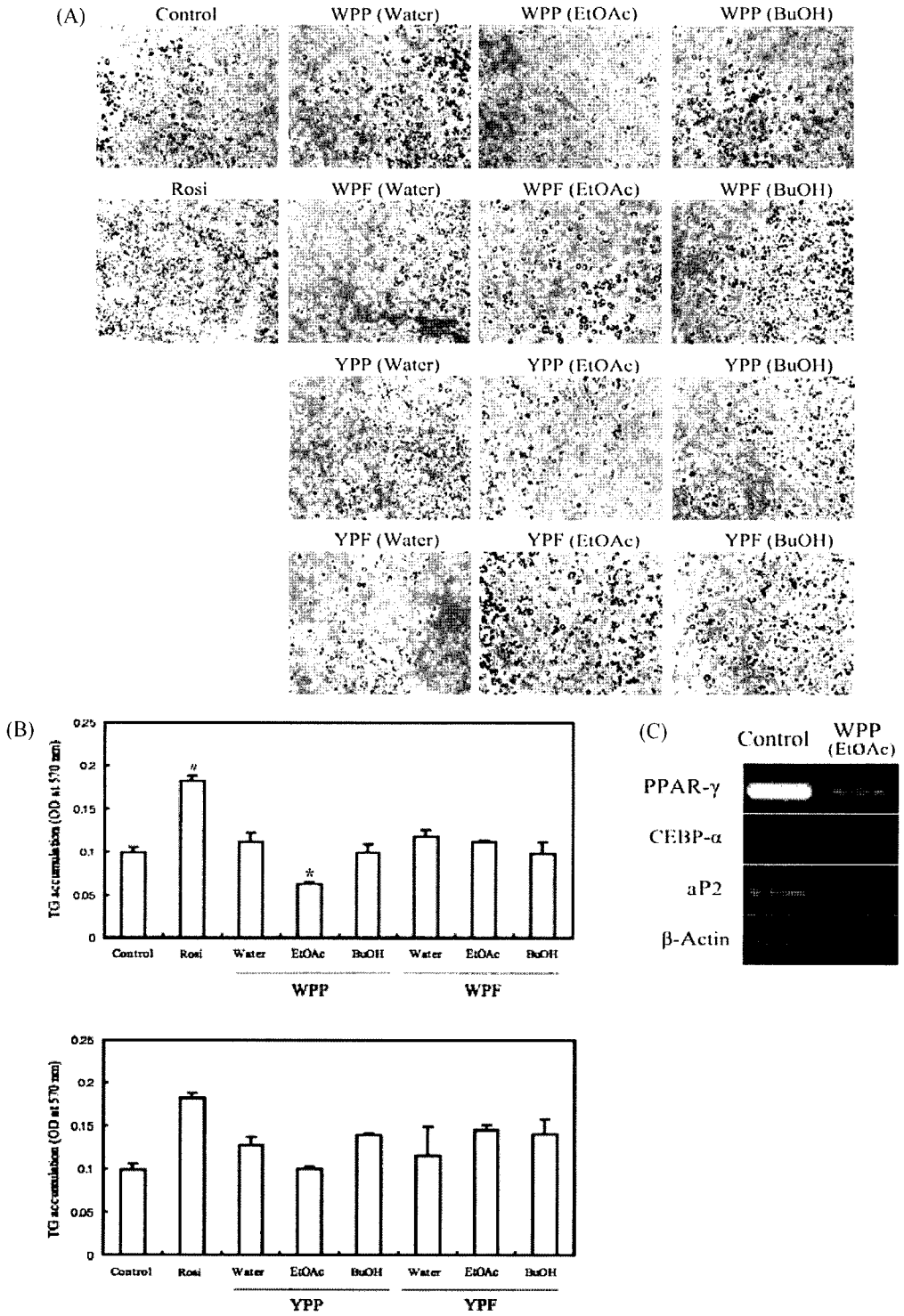

Fig. 2. Effects of white or yellow peach extracts on adipocyte differentiation in 3T3-L1 cells. (A) 3T3-L1 cells were induced to differentiate for 8 days in the standard adipogenic induction media containing 100 μg/mL of the indicated extract (WPP, YPP, WPF, or YPF) in water, EtOAc, or BuOH. Adipogenesis of 3T3-L1 cells was visualized by Oil Red O staining and was photographed at 100× magnification. (B) The absorbance was determined spectrophotometrically at 570 nm to measure triglyceride (TG) accumulation. The data is presented as the mean±SE for the 3 cultures. The symbols (*, #) indicate significant differences at $p<0.001$, when compared the control value. (C) mRNA expression of adipocyte marker genes (PPARγ, C/EBPα, and aP2) at day 8 was detected by RT-PCR using the gene-specific primers listed in Table 1.

Thus, it seems that EtOAc extract of WPP conferred its inhibitory effect at the mature stage of adipocyte differentiation, but not at the earlier commitment stage. In addition, none of the extracts (WPP, YPP, WPF, or YPF) in water, EtOAc, or BuOH induce adipocyte differentiation. Thus, peaches, especially white peaches, could be recommended for a restricted diet. This study motivates the further investigation to identify the compound(s) in the EtOAc extract of WPP that demonstrates this regulatory effect on adipogenesis.

Acknowledgments

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