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The activation of PPAR-α and Wnt/β-catenin by *Paeonia lactiflora* root supercritical carbon dioxide extract

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Abstract : The root of *Paeonia lactiflora* has been used in Chinese medicine. We conducted to check the comparative qualities of ethanol solvent extraction (PLE) and supercritical carbon dioxide extraction (PLS) of *P. lactiflora* root. PLE had higher antioxidant and polyphenol contents than PLS. But, PLS were significantly increased peroxisome proliferator-activated receptor (PPAR)-α. In addition, PLS inhibited the adipocyte differentiation of 3T3-L1 cells. When treated with the extract at a concentration of 100 μg/mL, the Wnt/β-catenin pathway reporter luciferase activity of HEK 293-TOP cells increased approximately by 3-folds compared to that of the untreated control group. These results indicate that *P. lactiflora* supercritical carbon dioxide extract may serve as a cosmeceutical for improving skin barrier function and the treatment of obesity.

Keywords : Paeonia lactiflora, Supercritical carbon dioxide extraction, Peroxisome proliferators activated receptors, Wnt/β-catenin, Adipocyte differentiation

1. Introduction

Medicinal plants are used in traditional medicine and synthesise hundreds of biological compounds for functions including defence against insects, fungi, diseases, and herbivorous mammals. Despite profound therapeutic benefits of many biological compounds, most medicinal plants have been studied mostly for the development of drugs and there are only a few reports on cosmeceutical agents. Additionally, more effective and less toxic medicinal compounds are still required. Ethanol, water and supercritical carbon dioxide are generally considered as safe solvents. Especially, supercritical carbon dioxide (CO_2) extraction method is an eco-friendly and has been successfully used to isolate the essential oil from natural products[1].

PPAR-α promotes the synthesis of cholesterol and ceramides in keratinocytes during epidermal differentiation[2]. In terms of keratinocyte differentiation and epidermal permeability barrier, PPAR-α agonists have been known extensively, and it has been studied that treatment with PPAR ligands promotes differentiation of the murine epidermis[3]. Therefore, a search for new agonists is necessity to determine whether activators of PPAR-α can alter the rate of keratinocyte differentiation. Wnt/β-catenin

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signaling pathway plays a role in adipose cell communication and the inhibition of adipogenesis[4]. The understanding of molecular and cellular mechanisms regulating adipogenesis is essential for designing rational therapies for treatment of obesity[5].

Paeonia lactiflora is a species of herbaceous perennial flowering plant in the family Paeoniaceae, native to central and eastern Asia. Also, the root of its plant has been used in Chinese medicine[6]. Several studies have reported that the root of *P. lactiflora* shows positive biological effects, such as neuroprotective activity[7], anti-inflammation effects[6], and skin-depigmentation[8]. However, the effects of its root extract as a skin therapeutic agent for skin barrier function and as a nutraceutical product for obesity treatment have not been studied. In this study, we examined PPAR-α activity and adipogenesis activity with *P. lactiflora* root extract.

2. Experimental

2.1. Sample preparation

The *P. lactiflora* root was purchased from the Jeonnam herbal farming cooperative (Hwasun-gun, Jeollanam-do, Korea). For supercritical co-solvent modified carbon dioxide extraction (PLS), the system and required components were acquired at 250 bar 50℃ 150 min at a flow rate of ethyl alcohol 3 mL/min for 90min from Nano bio research center (Janseong-gun, Jeollanam-do, Korea). The extraction was performed by a method previously described[9]. In case of PLE, *P. lactiflora* root was soaked in 99.9% ethyl alcohol for 7 days and then was dried by speed vacuum at 50℃.

2.2. Cell culture and materials

3T3-L1 preadipocytes, which were obtained from Yonsei University, were cultured by a method previously described[10]. Human normal keratinocyte and HEK 293-TOP cells were purchased from American Type Culture Collection (ATCC, USA) and cultured by a method previously described[11].

2.3. Cell viability

Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Human normal keratinocytes were treated with different concentrations of *P. lactiflora* root (PL) for 48 h at 37° C, followed by the addition of 50 μL of 2 mg/mL MTT (Sigma-Aldrich, St. Louis, MO, USA) solution to respective wells, and then incubation for 3 h at 37° C. The procedure was conducted by the method previously described[12].

2.4. DNA constructs, transient transfection, and PPRE transactivation assay

PPAR-α transcription activity was performed using the PPAR response element (PPRE) transactivation method[13] with slight modifications. PPAR-α transcription activity was performed using the PPRE transactivation method (Kim *et al.*, 2006) with slight modifications. A commercial PPAR-α expression vector was purchased from Promega (Mannheim, Germany) and transformed into *Escherichia coli* competent cells. PPAR-α DNA constructs were extracted using a DNA preparation kit (Qiagen, Hilden, Germany). The reporter construct (SA Bioscience, Hilden, Germany) and PPAR-α expression vector were co-transfected into CV-1 cells by using Lipofectamine (Invitrogen, Carlsbad, CA, USA). Transactivation assays were performed using the Dual-Luciferase Reporter Assay System (Promega, Mannheim, Germany), and normalized luciferase activity was determined.

2.5. Luciferase assay

HEK 293-TOP cells (3×10^4) were seeded into 96-well plates and incubated in a medium with 10% FBS for one day. The procedure was carried out as previously

described[11]. HEK 293-TOP cells (3 x 10^4) were seeded into 96-well plates and incubated in medium with 10% FBS for one day. Total cell lysates were extracted with 25 μl 1x reporter lysis buffer (Promega, Madison, WI) per each well and luciferase activities were measured by adding 25 μl luciferin (USB, Cleveland, OH) per each well using a Microplate Luminometer (BMG Labtech, Offenburg, Germany).

2.6. Adipocyte differentiation and Oil Red O staining

The 3T3-L1 preadipocytes were cultured in Dulbecco's modified Eagle's medium (DMEM) with high glucose, 110 mg/L pyruvate supplemented with heat inactivated 10% (v/v) calf serum (Gibco, CA, USA), 100 μg/mL penicillin, 100 μg/mL streptomycin in a CO_2 incubator at 37 ° C. To induce adipocyte differentiation, 3T3-L1 cells were cultured with DMEM plus 10% (v/v) heat inactivated fetal bovine serum (FBS) (Gibco) containing 520 μM isobutylmethylxanthine, 1 μM dexamethasone and 167 nM insulin. After 2 days, the media was then changed, and 167 ❍ M of insulin. On day 4, medium was replaced with DMEM containing 10% FBS, and changed with fresh identical medium every 2 days. To measure the anti-differentiation effects of each drug, 3T3-L1 preadipocytes were induced to differentiate in the presence of different concentrations of LiCl, plant extracts. Adipocyte cell layers were washed with PBS, fixed with 4% paraformaldehyde in PBS, stained with the Oil Red O dye solution for 1 hour, and then washed with distilled water. Cells were checked by bright-field optical microscope (Nikon TE-200U, Tokyo, Japan).[10]

2.7. Antioxidant assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was performed to determine the antioxidant capacity of LC. The SC_{50} (concentration required to obtain a 50% antioxidant effect), a commonly used parameter to indicate the antioxidant capacity, was also measured[14].

2.8. Total polyphenol content assay

Total phenolic content and other oxidation substrates were examined using the Folin Ciocalteau (FC) reagent. We used a previously described method[15]. The FC assay relies on the transfer of electrons in alkaline medium from phenolic compounds to phosphomolybdic/phosphotungstic acid complexes, which are determined at 765 nm. Antioxidant reducing capacity is expressed as gallic acid equivalents (GAE).

2.9. Statistical analysis

All data are presented as the mean ± standard error of the mean (S.E.M). Statistical analyses were conducted with GraphPad Prism 5.0 (GraphPad Software, Inc. San Diego, CA, USA). Comparisons between multiple groups were performed using the one-way analysis of variance with Bonferroni post-hoc test. P-value of less than 0.05 was considered significant.

3. Results and Discussion

3.1. Effect of PL on cell viability

We examined the effect of PL on cell viability. After 24 h treatment of Human normal keratinocytes with PL (10, 50, and 100 μg/mL), cell viability was assessed by the MTT assay. Cell viability was not affected by 100 μg/mL of PLE (Figure 1). In case of PLS, there was no cell toxicity up to 10 μg/mL.

3.2. Antioxidant effect and total polyphenol content of PL

The radical scavenging activities of PL increased in a concentration-dependent manner. The radical scavenging activity SC_{50} of PLE was 141.7 μg/ml and that of PLS was 265.2 μg/ml, therefore PLE has better

antioxidant activity. The polyphenol content of PLE was 25.27 GAE mg/g and that of PLS was 20.02 GAE mg/g. So, This seems to be due to the higher polyphenol content of PLE (Fig. 2A, B).

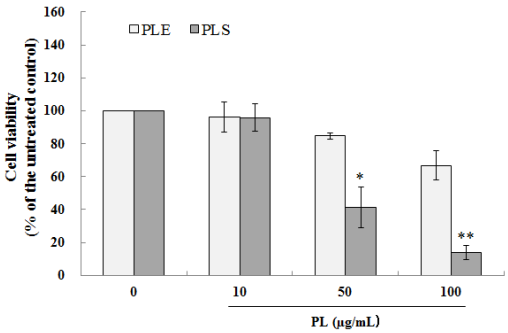

Fig. 1. Effect of PL on cell viability. Ethanol solvent extraction and supercritical carbon dioxide extraction from *P. lactiflora root* indicate PLE and PLS respectively. No treatment was used as a negative control. Values are presented as means ± standard error of the mean (SEM).*p 〈 0.05, **p 〈 0.01 compared to the untreated control group.

3.3. Transactivation of PPRE

As shown in Fig. 3, the concentration of PL largely influenced PPAR-α ligand binding activity in the treated group, with 50 μg/mL of PLS showing an increased binding compared to that in the untreated control group. In addition, the PLE-treated groups showed low levels of transactivation activity compared to the WY14643-treated groups. These results show that PLS has the potential as a novel PPAR-a agonist. Therefore, PLS may serve as potential compounds for improving skin barrier function.

3.4. Effect of PLS on the adipocyte differentiation and Wnt/β -catenin activity

The reporter activity was increased approximately by 3-folds with the treatment of PLS at concentrations of 100 μg/mL, compared to that of the non-treated control group (Fig. 4). Additionally, PLS inhibited adipocyte differentiation of 3T3-L1 cells in a concentration dependent manner (Fig. 5). These results indicate that PLS induces differentiation of 3T3-L1 cells, showing the role of the Wnt/β-catenin activator in the adipocyte differentiation. Wnt/β-catenin

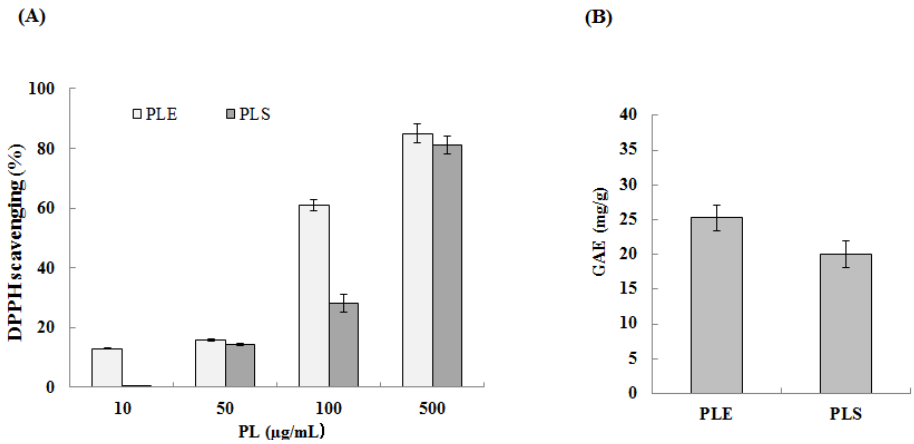

Fig. 2. (A) Antioxidant effect on PL. PL indicate *P. lactiflora* root extract. (B) Total polyphenol content of PLE and PLS GAE indicate gallic acid equivalent in mg/g. Values are presented as means ± standard error of the mean (SEM).

signaling has been known to modulate additional developmental processes of adipocytes and to play a role in the suppression of differentiation of pre-adipocyte cells of 3T3-L1 cells[16]. At present, there are some reports about the interaction between PPAR-α and Wnt/β-catenin signaling. PPAR-α activation, with its agonist clofibrate, inhibits Wnt/β-catenin signaling in renal fibrosis and pancreatic cancer cells[17]. Also, Wnt/β-catenin activation promotes PPAR-α expression in Alzheimer disease[18]. Totally, our study indicate that PLS promotes PPAR-α and inhibits adipocyte differentiation in relation to the Wnt/β-catenin signaling pathway. Here, we report that PLS can be used in the development of cosmeceutical products for skin barrier dysfunction such as atopic dermatitis (AD) and obesity. Therefore, natural substances derived from plants influencing adipogenesis are receiving attention for the treatment of obesity.

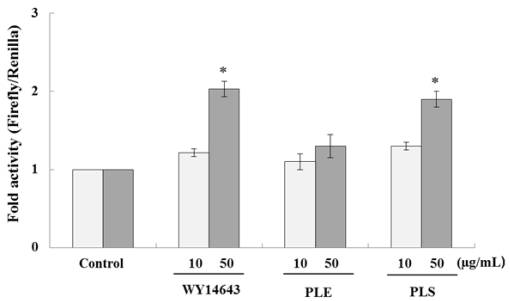

Fig. 3. Transactivation of peroxisome proliferator-ctivated receptor (PPAR)-esponsive element (PPRE) by different concentrations of PLE and PLS. HEK293 cells were transfected with PPRE luciferase construct. WY14643 was used as a positive control and no treatment was used as a negative control. Values are presented as means ± standard error of the mean (SEM). *p < 0.01 compared to the untreated control group.

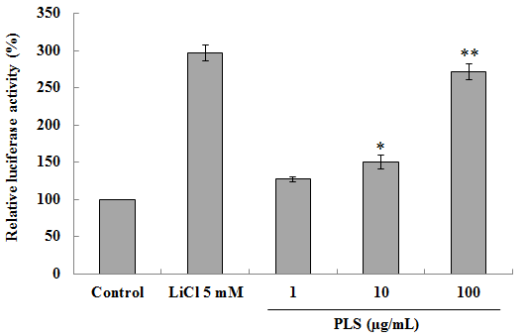

Fig. 4. Effect of PLS on Luciferase reporter activity. HEK293 cells containing pTOPFlash reporter gene in its chromosome were cultured and treated with different LC at 1, 10 or 100 μg/mL concentration, and cellular extract was prepared after 24 h as described in the Materials and methods. PLS indicate supercritical carbon dioxide extraction from *P. lactiflora* root. *p < 0.05, **p < 0.01 compared to the untreated control group.

4. Conclusion

This study shows that supercritical carbon dioxide extraction (PLS) of *Paeonia lactiflora* root can increase peroxisome proliferator-activated receptor (PPAR)-α and inhibite the adipocyte differentiation of 3T3-L1 cells. When treated with the extract at a concentration of 100 μg/mL, the Wnt/β-catenin pathway reporter luciferase activity of HEK 293-TOP cells was increased approximately by 3-folds compared to that of the untreated control group. This study suggests that PLS have potential as cosmeceutical agents for improving skin barrier functions for atopic dermatitis and the treatment of obesity.

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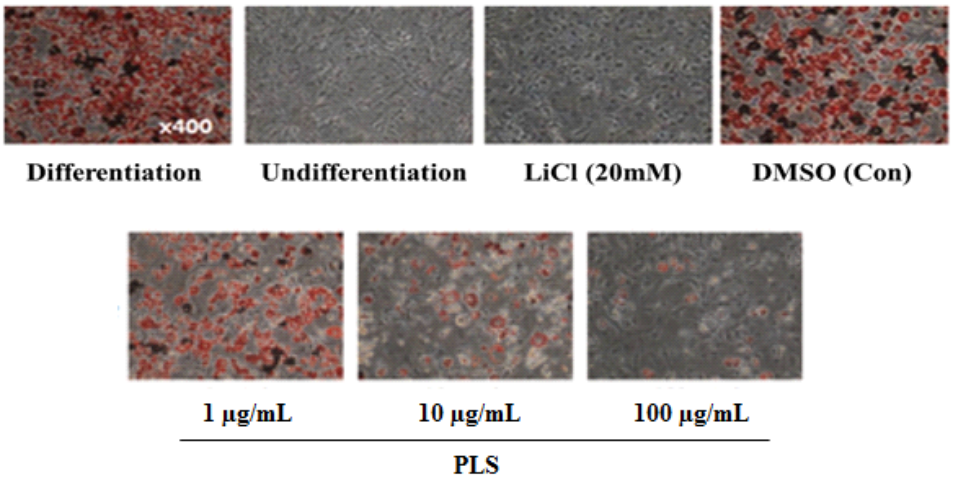

Fig. 5. Effect of PLS on the adipocyte differentiation of 3T3-L1 preadipocyte cells. 3T3-L1 cells were induced to differentiation for 7 days with or without 20 mM LiCl. Intracellular lipids of 3T2-L1 cells were visualized by Oil Red O staining as described in the Materials and methods. Original magnification: x400. PLS indicate supercritical carbon dioxide extraction from *P. lactiflora* root.

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