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Basic nutritional investigation

Partially purified *Peucedanum japonicum* Thunb extracts exert anti-obesity effects in vitro

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ABSTRACT

Objective: Crude ethanolic extract of *Peucedanum japonicum* Thunb (PJT) has been identified as an antiobesity and antidiabetic candidate. The aim of this study was to assess the mechanisms related to the antiobesity activity of partially purified PJT ethanol extract in vitro.
Methods: PJT ethanol extract (EE) was partitioned into hexane phase (HP) and residual water phase. The effect of PJT extracts on triacylglycerol (TG) content, lipid metabolism-related gene expression, and energy expenditure was assessed in vitro in 3T3-L1, HepG2, and C2C12 cells. Furthermore, the active components in PJT extracts were partially profiled by high-performance liquid chromatography (HPLC) analysis.
Results: The HP significantly down-regulated lipogenic gene expressions in hepatocytes, inhibited TG accumulation, and decreased the size of 3T3-L1 adipocytes. Moreover, the inhibition of lipid accumulation was at maximum during the adipocyte maturation stage. Furthermore, we found an increase in fat hydrolysis by HP in 3T3-L1. In C2C12 myotubes, the HP tended to enhance energy expenditure. HPLC analysis demonstrated that hydrophobic compounds available in the HP were responsible for antiobesity, which corresponded to the latter peaks on the HPLC chromatogram of EE.
Conclusion: Our study demonstrated that the HP plays a crucial role in regulating lipid metabolism-related gene expressions and energy expenditure in vitro. The results thus provide insight on the activity of HP involved in suppressing obesity and its chemical entities.

Introduction

Obesity has become one of the most critical medical issues worldwide due to its close relationship with type 2 diabetes, fatty liver disease, and other critical metabolic disorders [1,2]. There are several pharmacologic substances available globally as antiobesity drugs, yet they often have hazardous side effects [3,4]. Natural products, however, have been used for treating obesity in many Asian countries for centuries.

Peucedanum japonicum Thunb (PJT), belonging to the family Apiaceae (Umbelliferae), originates from southern Japan, China, and Taiwan. PJT leaves have been used traditionally to treat cold and cough on Okinawa Island, Japan. Previous studies have reported on the antiobesity properties of PJT powder [5]. In this context, studies [6,7] further demonstrated antiobesity activity of PJT with the lipase inhibition, and thus ethanol extract of PJT (EE) being characterized as antidiabetic activity [8]. However, it remains unexplored whether the phytochemicals in PJT itself alter the lipid metabolism to attenuate adiposity and body weight gain of high-fat diet-fed mice.

We have unequivocally confirmed the up-regulation of peroxisome proliferator activated receptor gamma (*PPARγ*) gene expression [6,7], indicating an elevation in adipocyte

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differentiation and energy metabolism [9]. Furthermore, PJT down-regulated a key lipogenic activator, sterol regulatory element-binding protein-1 (*SREBP1 c*) and adipocyte size marker gene, paternally expressed gene 1/mesoderm-specific transcript (*PEG1/MEST*) in adipose tissue in vivo. Moreover, the adipose tissue weight was significantly reduced by PJT [6,7]. Additionally, PJT diet suppressed lipogenic indicators such as fatty acid synthase (*FAS*) [10] and *SREBP1 c* [11], in the liver [8]. Genes such as carnitine palmitoytransferase 1 alpha (*CPT1* α) and uncoupling protein 3 (*UCP3*), which are respectively crucial for fatty acid β-oxidation [12] and energy metabolism [13], showed increased expression levels in the muscle tissue [7]. Collectively, these results imply that the antiobesity activity of PJT is due to additional mechanisms related to the modulation of lipid metabolism apart from the inhibition of lipid absorption. On the other hand, a persisting limitation is that PJT crude extracts restrict in-depth investigations on the mechanisms related to antiobesity.

Therefore, in the present study, we investigated the inhibitory effects of partially purified EE on obesity in vitro. To the best of our knowledge, this is the first report of a detailed in vitro study on lipid metabolism in adipocytes, hepatocytes, and myotubes cultured with PJT extracts. We also examined the target stage of adipocyte differentiation for the maximum inhibition of lipid accumulation and related gene modulation pattern in adipogenesis. Furthermore, PJT extracts were analyzed by high-performance liquid chromatography (HPLC) to partially identify the active compounds in the extract.

Materials and methods

Preparation of PJT extracts

The origin of PJT used in this study, ground, and extracted was as described elsewhere [8]. The yield of EE was ~11% of the starting material. EE was further suspended in water and partitioned into *n*-hexane (4 × 1: 1 v/v). The approximate yields of the hexane phase (HP) and the residual water phase (WP) were 3.3% and 7.0%, respectively. All extracts were stored at −80°C for further use.

Cell culture

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Heat-inactivated horse serum (HS) and bovine calf serum (BCS) were purchased from Life Technologies (Grand Island, NY, USA). All cultured cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). Cell lines excluding 3T3-L1, were maintained in DMEM supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% FBS in an atmosphere of 5% CO_2 at 37°C.

3T3-L1 preadipocytes were grown in 10% BCS and avoided complete confluence before initiating differentiation. For adipogenesis, the confluent fibroblasts were maintained for another 2 d (defined as day 0). Differentiation was induced with standard differentiation inducers as mentioned previously [14] for 48 h (from day 0–2). On day 2, the culture medium was changed to the maintenance medium containing DMEM, 10% FBS, 10 μg/mL insulin, 25 or 50 μg/mL EE, 6 or 11 μg/mL HP, or 19 or 37 μg/mL WP from day 2 to 6. The control group was maintained in the basic maintenance medium without PJT treatments. The non-differentiated (ND) cells were maintained in DMEM supplemented with 10% FBS from day 0 to 6 with no differentiation induction. To study the acting stage of HP in the adipocyte development, cell cultures were supplemented with HP (50 μg/mL) at different time intervals.

HepG2 cells were incubated in complete medium to 70% confluence, and maintained in serum-free DMEM overnight, as described elsewhere [15]. HepG2 cells were then cultured with or without insulin (1 μM) for 12 h and subsequently treated with or without insulin and/or EE (50 μg/mL), HP (11 μg/mL), or WP (37 μg/mL) for another 12 h.

For C2C12 cells, DMEM supplemented with 2% HS was used to induce differentiation, as previously described [16]. For the measurement of glucose consumption, the culture medium was replaced by DMEM supplemented with 0.25% (w/v) bovine serum albumin (BSA). Then, cells were treated with PJT extracts for 24 h. Insulin (0.1 μmol/L) was used as the positive control. The glucose concentration in the medium was determined as mentioned elsewhere [17].

Cell viability was determined by using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) solution as previously described [18].

Triacylglycerol assay

At the end of the treatment period, 3T3-L1 and HepG2 cells were washed with phosphate buffer solution (PBS; Wako Pure Chemical Industries Ltd.) and harvested with the use of accutase (Innovative Cell Technologies, Inc., San Diego, CA, USA). Total lipids were extracted according to the Bligh and Dyer method [19], and triacylglycerol (TG) content was quantified using a commercial enzymatic kit (Wako Pure Chemical Industries Ltd.) according to the manufacturer's instructions. The content of cellular protein was determined using the Quant-iT protein assay kit (Life Technologies).

Quantitative real-time reverse transcriptase-polymerase chain reaction

First-strand cDNA was synthesized with 2 μg of total RNA as a template using high-capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, USA). The quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) was performed on the Step One Plus™ Real-Time PCR System (Applied Biosystems) with the following parameters: One cycle of 95°C for 20 sec, 40 cycles of 95°C for 3 sec, and 60°C for 30 sec. A melting curve analysis was performed starting at 95°C for 15 sec, 60°C for 60 sec and increasing by 0.3°C every 15 sec to determine primer specificity (specific primers are listed in supplementary material: Tables 1 and 2). The mRNA levels of all genes were normalized using actin, beta, cytoplasmic (*ACTB*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and cyclophilin a (*CYPa*) as the internal controls for 3T3-L1, HepG2, and C2C12, respectively.

HPLC analyses for PJT extracts

EE, HP, and WP were dissolved in 50% methanol, applied to an octa decyl silyl (ODS) column (ODS-HG-5, 150 × 4.6 mm I.D., Nomura Chemical, Seto, Japan) and analyzed using the Shimadzu HPLC 10 A system (Shimadzu, Kyoto, Japan). The mobile phases were 0.2% trifluoroacetic acid (TFA) in 10% methanol as the starting eluent and 0.2% TFA in 70% methanol as the limiting eluent. Samples were analyzed using the gradient program of the limiting eluent as follows: 0 to 5 min, 50% to 100%, and 5 to 40 min, 100% with a flow rate of 0.5 mL/min at a wavelength of 323 nm.

Statistical analyses

All results were expressed as the mean ± SEM. A normality test was performed by the use of Shapiro-Wilk test to confirm the Gaussian distribution of data. The statistical significance of the difference between the means of control and treatment groups was determined by Dunnett's test. The significance of the difference between the means of two groups was determined by Student's *t* test. Differences were considered significant at $P < 0.05$.

Results

Effect of PJT extracts on 3T3-L1 adipocytes

Our preliminary results indicated that EE inhibited lipid accumulation in adipocytes in a dose-dependent manner (data not shown). A dose-dependent inhibition in the lipid accumulation was observed in EE and HP compared with that of the control (Fig. 1A). When the HP concentration was doubled, lipid accumulation was further reduced by 37.6% ($P < 0.05$). Under the conditions of our study, the treatments produced no detectable cell toxicity for all cell types.

In concordance with our most recent in vivo results [8], EE-treated cells showed a 2.4-fold increase in *PPARγ* expression. In HP treatment, *PPARγ* showed a 2.6-fold increase (Fig. 1B). Fatty acid transport gene, fatty acid-binding protein 4 (*FABP4*) [20], showed a 3.4-fold increase by EE and HP, and a twofold increase by WP treatment. Lipoprotein lipase (*LPL*) showed 2.7-fold increase due to the EE and HP treatments. CCAAT/enhancer binding protein alpha (*C/EBPα*) expression was up-regulated significantly by EE, and uncoupling protein 2 (*UCP2*), involved