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Anti-obesity effects of glabridin-rich supercritical carbon dioxide extract of licorice in high-fat-fed obese mice

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ABSTRACT

Licorice (*Glycyrrhiza glabra* Linne) is a well-known medicinal plant and glabridin is an isoflavan isolated from licorice. In this study, we investigated the anti-obesity effect of glabridin and glabridin-rich supercritical fluid extract of licorice (LSC). Glabridin effectively inhibited adipogenesis in 3T3-L1 cells. Moreover, LSC showed inhibitory effect on adipogenesis in a dose-dependent manner. The inhibitory effect of LSC resulted from inhibiting the induction of the transcriptional factors CCAAT enhancer binding protein alpha and peroxisome proliferator-activated receptor gamma. Then we fed mice with high-fat diet containing none, 0.1% and 0.25% LSC for 8 weeks to explore the anti-obesity effect of LSC *in vivo*. LSC significantly reduced weight gain by high-fat diet in a dose-dependent manner. The reductions of the hypertrophy of white adipose tissue and of fat cell size were also observed. In the liver, LSC supplementation effectively inhibited high-fat diet-induced hepatic steatosis through downregulation of gluconeogenesis related phosphoenolpyruvate carboxykinase and glucose 6-phosphatase and upregulation of the β-oxidation related carnitine palmitoyltransferase 1. Taken together, our results suggest that glabridin and glabridin-rich licorice extract would be effective anti-obesity agents.

1. Introduction

Obesity is closely associated with life-style-related diseases, such as hyperlipidemia, hypertension, arteriosclerosis, type 2 diabetes mellitus, and cancer (Wu et al., 2010). It is a widely accepted fact that obesity results from the disequilibrium between energy intake and expenditure and is highly likely to be the cause of chronic diseases such as heart disease, type 2 diabetes, high blood pressure, stroke, and some forms of cancer (Larsson et al., 1981). The prevalence of obesity is rapidly rising. Excess energy is conserved in the form of fat in adipose tissue, leading to obesity and obesity-associated fatty liver disease (Evans et al., 2004). As a result, there is increased urgency to develop strategies that will be effective for both the prevention and treatment of obesity (Park et al., 2009).

In addition to diet therapy and exercise, many different approaches to treat and control obesity have been suggested, such as drugs for weight loss, appetite suppressants, and food supplements (Park et al., 2007). However, some of the treatments have been reported to have serious side effects, such as vomiting, headaches, stomach pain, and heart attacks (Ho et al., 2006). Some recent studies have focused on the search for functional food ingredients or herbal extracts that can suppress weight gain and body fat accumulation induced by a high-fat diet with less significant side effects (Wu et al., 2010).

Licorice, the root of *Glycyrrhiza glabra* Linne, has long been used worldwide as an herbal medicine and natural sweetener. There are several species of licorice, including *Glycyrrhiza uralensis* Fischer, *G. glabra* Linne, and *G. inflate* Batalin, each of which contains species-specific flavonoids (Shibata, 2000). A large number of components have been isolated from licorice, including triterpenes, saponins, flavonoids, isoflavonoids, and chalcones. Anti-ulcer, anti-inflammatory, anti-diuretic, anti-epileptic, anti-viral, anti-allergic, and anti-oxidant pharmacological activities have been attributed to the major licorice compound, glycyrrhizic acid (Visavadiya et al.,

Abbreviations: LSC, glabridin-rich supercritical fluid extract of licorice; LHW, hot water extract of licorice; LET, 70% ethanol extract of licorice; ISL, isoliquritigenin; GA, glycyrrhizic acid; GB, glabridin; AP2, adipocyte protein 2; PEPCK, phosphoenolpyruvate carboxykinase; G6Pase, glucose-6-phosphatase; CPT-1α, carnitine palmitoyltransferase 1; C/EBPα, CCAAT enhancer binding protein alpha; PPARγ, peroxisome proliferator-activated receptor gamma; SREBP-1c, sterol regulatory element-binding protein-1c; FAS, fatty acid synthase.

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2009). Glycyrrhizic acid was also reported to improve dyslipidemia and insulin resistance (Eu et al., 2010). Licorice flavonoid oil (Kamisoyama et al., 2008) and several licorice chalcones (Birari et al., 2011) have been studied to determine the source of anti-obesity properties. However, the anti-obesity effect of glabridin, one of the well-defined flavonoids of licorice, has not been reported yet.

Supercritical CO_2 extraction techniques are considered non-toxic, free of residues, moderate, and comparatively environmentally harmless among liquid–solid extraction operations. This is a potential replacement for the use of many undesirable organic solvents that present a threat to the environment, health, and safety in the work place (Klejdus et al., 2005). Furthermore, the supercritical CO_2 extraction method has been proposed for the preparation of antioxidant-fortified extracts from certain herbs (Marongiu et al., 2004) and it may serve as a very promising process in the food industry.

In this study, we first compared the anti-adipogenic activity of the major flavonoid ingredients occurring in *G. glabra* Linne and found that glabridin markedly inhibited adipogenesis in 3T3-L1 cells. HPLC analysis showed that supercritical CO_2 extraction is the appropriate method to prepare glabridin-rich extract from licorice. Moreover, we applied the glabridin-rich supercritical extract of licorice (LSC) to high-fat diet-induced obese C57BL/6J mice and investigated the anti-obesity effect of LSC *in vivo*.

2. Methods and materials

2.1. Preparation of the plant extract

Dried Ukraine licorice (*G. glabra* Linne) was supplied by Sam Do P&F (Seoul, Korea) and identified by Professor Y.M. Park, Department of Life Science, Cheongju University. Voucher specimens (KFRI-GG04005) were preserved in Korea Food Research Institute. Dried licorice was cut into pieces and used for the experiment.

Licorice was extracted with 10 times of boiling distilled water for 4 h (licorice hot water extract, LHW) and 70% ethanol overnight at room temperature (licorice ethanol extract, LET). The extracted solutions were concentrated at 50 °C using rotary vacuum evaporator (BUCHI, Flawil, Switzerland) and lyophilized. The yield of extraction was 16.3% (w/w) and 10.9% (w/w), respectively.

The Supercritical Extraction System (ILSHIN Autoclave Co., Daejeon, Korea) with a 300 mL extraction cell was used to perform supercritical fluid extraction of licorice (LSC). The extraction cell was filled with 154 g of dried licorice. The extraction was conducted at extraction times of 1 h, extraction pressures of 30 MPa, temperature of 40 °C and CO_2 flow rates of 150 g/min. The yield of extraction was 3.57% (w/w).

All licorice extracts were kept at −20 °C in the dark until use.

2.2. Chemicals

Glycyrrhizic acid ammonium salt, isoliquiritigenin, and Oil red O were purchased from Sigma–Aldrich (St. Louis, MO). Glabridin was obtained from Wako Chemicals (Osaka, Japan). Anti-sterol regulatory element-binding protein-1c (SREBP-1c, sc-366), and horseradish peroxidase (HRP)-linked anti-rabbit IgG (sc-2357) were supplied by Santa Cruz Biotechnology (Santa Cruz, CA). Anti-CCAAT enhancer binding protein alpha (C/EBPα, #2843), anti- peroxisome proliferator-activated receptor gamma (PPAR γ, #2443), anti-fatty acid synthase (FAS, #3180), and anti-β-actin (#4967) were obtained from Cell Signaling Technology (Danvers, MA).

2.3. HPLC analysis of phenolic compounds

All chromatographic analyses were performed using high-performance liquid chromatography (HPLC) apparatus equipped with a PU-2089 pump, auto sampler, and an ultraviolet and refractive index detector (Jasco, Tokyo, Japan).

The LHW, LET, and LSC were dried, dissolved in HPLC-grade methanol, filtered through a 0.45 μm membrane filter, and degassed in an ultrasonic bath. HPLC was equipped with a XTerra RP C18 column (250 × 4.6 mm, 5 μm; Waters, Milford) set to 252 nm and 40 °C. Mobile phases consisted of 0.2% acetic acid in 10% methanol (A), 0.2% acetic acid in 70% methanol (B), and 100% methanol (C) at a flow rate 1.0 mL/min. The gradient system was initially 100% (A) for 12 min, 100% (B) for 17 min, back to 100% (A) for 1 min, to 100% (C) for 7 min, and finally to 100% (C) for 3 min for a total duration of 40 min.

The standard solutions of isoliquritigenin (ISL), glycyrrhizic acid (GA), and glabridin (GB) were prepared in the same mobile phase for HPLC analysis.

2.4. Anti-adipogenesis assay

Mouse 3T3-L1 fibroblasts (ATCC, Manassas, VA) were grown at 37 °C in a humidified atmosphere of 5% CO_2 and were maintained in growth medium (GM) of DMEM containing 25 mM glucose, 10% calf serum (CS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine. Cells were plated at a density that allowed them to reach confluence in 3 days. At this point (day 0), cells were switched to differentiation medium (DM) of DMEM supplemented with 10% fetal bovine serum (FBS) with MDI (0.25 μM dexamethasone, 0.25 mM 1-methyl-3-(2-methylpropyl)-7H-purine-2,6-dione [IBMX], and 1 μg/mL insulin) in the presence of licorice extracts or licorice compounds to examine the effect on adipogenic differentiation. On day 3, the dexamethasone and IBMX were removed, and cells were cultured in insulin-containing cell medium for an additional 2 days, followed by an additional 4 days in 10% FBS/DMEM medium, at which time (day 8) 90% of the control cells were mature adipocytes with accumulated fat droplets. For Oil red O staining, the cells were washed twice with ice-cold PBS, fixed with 10% formalin at room temperature for 1 h, and stained with 0.2% Oil red O in isopropanol for 10 min. Cells were then washed with 60% isopropanol and water. Images were collected using an Olympus (Tokyo, Japan) microscope. Stained oil droplets were dissolved in 100% isopropanol and quantified using a spectrophotometer at 500 nm. For measurement of cell viability, we used Cell Counting Kit-8 (Dojindo Molecular Technology Inc., Kumamoto, Japan).

2.5. Western blot analysis

Cells were lysed in RIPA buffer containing 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris–HCl (pH 7.4), 50 mM glycerophosphate, 20 mM NaF, 20 mM EGTA, 1 mM DTT, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitors. Total proteins (20 μg) were separated by 12% SDS–polyacrylamide gel electrophoresis, transferred to a polyvinylidene fluoride membrane, and then hybridized with primary antibodies (diluted 1:1000) overnight at 4 °C. After incubation with HRP-conjugated secondary antibody (diluted 1:2000) for 1 h at room temperature, the immunoreactive proteins were visualized with the WEST-one Western Blot Detection System (iNtRON Biotechnology, Seoungnam, Korea).

2.6. Animal and diets

Five-week-old male C57BL/6J mice were obtained from Orient Bio Inc. (Seoungnam, Korea). After 1 week of acclimation, the mice were divided into four diet groups ($n = 10$ per group): normal control (NC, 10% of total calories from fat), high-fat diet (HF, 45% of total calories from fat), high-fat diet supplemented with 0.1 and 0.25 (w/w)% LSC-fed groups. After 8 weeks on the diet, mice were sacrificed following a 12 h fast. Food intake was measured weekly. Oxygen consumption rates (VO_2) for 12 h (between 18:00 and 06:00) were measured in Oxylet System (Panlab S.L.U, Spain) after 8 weeks fed with experimental diets. From the change in oxygen, VO_2 was calculated as oxygen consumption per hour normalized to body weight (ml/kg/hour). All animal studies were conducted in accordance with a protocol approved by the Institutional Animal Care and Use Committee of the Korea Food Research Institute.

2.7. Blood chemistry

Plasma total cholesterol, triglyceride, and glucose levels were determined using a commercial kit (Asan Pharmaceutical, Seoul, Korea). The plasma leptin concentration was measured with an ELISA assay kit (R&D systems, Minneapolis, MN).

2.8. Histological analysis

For histological analysis, perirenal adipose tissue and livers were fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. The stained areas were observed using a light microscope (Olympus, Tokyo, Japan) with a magnifying power of ×200.

2.9. Quantitative real-time PCR analysis

Livers were excised, snap-frozen immediately and stored at −80 °C until use. Total RNA was extracted from 20–30 mg of each liver tissue with a NucleoSpin RNA II kit (Macherey–Nagel GmbH & Co. KG, Duren, Germany) according to manufacturer's protocol. The kit contains DNase for removal of contaminating DNA. Quantification of RNA in the extracted samples was done by NanoDrop (Thermo Scientific, Wilmington, DE). RNA purity was assessed by measuring the A260/A280 ratio. A value of 1.9–2.1 was used for experiment.

One micro gram of total RNA was used for cDNA synthesis with Maxime RT-PCR PreMix Kit (Intron, Seongnam, Korea) as per the manufacturer's instructions. Total reaction volume was 20 μL. The primers and reaction conditions are shown in Table S1. qPCR was conducted on an StepOnePlus Real-time PCR system (Applied Biosystems, Foster City, CA) using SYBR® Green Realtime PCR Master Mix (TOYOBO, Osaka, Japan) with a 95 °C pre-denaturation for 5 min followed by 40 cycles of 95 °C