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Isoflavone-enriched soybean (*Glycine* max) leaves prevents ovariectomy-induced obesity by enhancing fatty acid oxidation

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ABSTRACT

Dietary isoflavones have been gaining increased interest in the field of functional foods due to their ability to ameliorate several postmenopausal symptoms. Using ovariectomized (OVX) rats supplemented with isoflavone-enriched soybean leaves (IESLs), we investigated the effect of dietary isoflavones on menopausal-associated obesity. Oral administration of IESLs significantly reduced OVX-induced weight gain and visceral fat mass. Additionally, IESLs significantly ameliorated OVX-induced hepatic steatosis as reflected by decreased triacylglycerol accumulation in the liver and decreased serum alanine transaminase and aspartate aminotransferase levels. These anti-obesity effects of IESLs were confirmed to be due to enhanced fatty acid oxidation via up-regulation of peroxisome proliferator activated receptor alpha, peroxisomal acyl-coenzyme A oxidase 1, and carnitine palmitoyltransferase 1a mRNA expression. These results demonstrate that IESLs may be an effective functional food-based approach to protect against menopause-associated obesity.

1. Introduction

Menopause, a normal physiological event experienced by women, is characterized by the depletion of ovarian follicular function and a corresponding reduction in estrogen levels (Mastorakos, Valsamakis, Paltoglou, & Creatsas, 2010). Menopause-associated estrogen loss may lead to obesity and related metabolic pathologies, including insulin resistance, type 2 diabetes, and cardiovascular disease (Iwamoto et al., 2016; Rogers, Perfield, Strissel, Obin, & Greenberg, 2009). Most women experience menopause at about 50 years of age, and owing to increased life expectancy, one-third to one-half of a women's life is spent in post-menopause (Yim et al., 2015). Therefore, strategies toward the prevention and treatment of menopause-associated obesity are essential for improving quality of life for postmenopausal women. Hormone replacement therapy (HRT) is regarded as the first-line and most effective conventional treatment for most menopausal symptoms; however, serious side effects associated with HRT include thrombosis, hypertension, atherosclerosis, and an increased risk of breast cancer (Lee et al., 2016; Peng, Adams, Sibbritt, & Frawley, 2014). Therefore, there is an urgent need for the development of new preventive and treatment approaches that can effectively protect against menopause-associated obesity.

Soy isoflavones are well recognized as natural active phytoestrogens, and due to their health benefits and general safety, are widely accepted as natural alternatives to HRT or as HRT supplements (Belcher & Zsarnovszky, 2001). Isoflavones are distinct secondary metabolites produced predominately in leguminous plants and are structurally similar to 17-β-estradiol, which binds estrogen receptors to produce estrogenic effects (Khalil, 2013; Yuk et al., 2016). Several studies in rodents have demonstrated that dietary isoflavone intake decreases body weight and adipose tissue, and in ovariectomized rats, dietary isoflavones and can prevent weight gain and increased serum lipid levels (Kurrat et al., 2015; Uesugi, Toda, Tsuji, & ISHIDA, 2001). Epidemiologic evidence supports a lower prevalence of obesity and related disease in Asian countries, which might be due to high consumption of isoflavone-containing soy (Adlercreutz & Mazur, 1997; Llaneza et al., 2011). Many efforts have been made to increase isoflavone levels in soybeans by breeding with recombinant inbred lines with high, intermediate, and low isoflavone levels (Primomo, Poysa, Ablett, Jackson, & Rajcan, 2005). Recombinant inbred lines are produced by manipulating the isoflavone synthase gene (Jung et al., 2000) or by using elicitors such as lipo-chitooligosaccharides, chitosan, actinomycetes spores, and yeast extract (Tawaha, Seguin, Smith, & Beaulieu, 2005). However, these approaches have limited capability to increase isoflavones in soybeans. We previously isoflavone-enriched

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soybean leaves (IESLs) by treating ordinary soybean plants (*Glycine* max). with ethylene or the ethylene donor Ethephon. Total isoflavone levels reached 13,584 μg/g, and were comprised of daidzin, genistin, malonyl-daidzin, malonyl-genistin, daidzein, and genistein (Yuk et al., 2016). We hypothesize, therefore, that IESLs represent a potential alternative treatment against menopause-associated obesity.

In rats, surgical removal of the ovaries, or bilateral ovariectomy, leads to estrogen deficiency and mimics what is observed during menopause in humans, including menopause-associated obesity. Here, we investigated the ability of IESLs to prevent obesity in ovariectomized rats, and to clarify the molecular mechanism of action of obesity prevention.

2. Materials and methods

2.1. Materials

Soybean plants (*Glycine* max), were obtained from the National Institute of Crop Science (NICS), Miryang, Republic of Korea. Standard isoflavones (daidzin, genistin, daidzein, genistein, malonyldaidzin, and malonylgenistin) were purchased from Sigma-Aldrich Corporation (Saint Louis, MO, USA). HPLC-grade water and acetonitrile were purchased from Fisher Scientific (Fairlawn, NJ, USA). All other reagents were analytical grade.

2.2. Preparation of IESLs

IESLs were produced by treatment with ethylene or the ethylene donor Ethephon according to the method of Yuk et al. (2016). Briefly, soybeans were cultivated in a greenhouse over a period of about 60 days until the plant reached a maximum growth stage (R3), at which pod development occurs. At this stage, 250 μg/mL of Ethephon was applied twice to each soy plant every 24 h until the solution started dripping. After 96 h, plant leaves from each plant were harvested and cut into small pieces using a laboratory blade cutter and then dried at 35 °C. The dried leaves were mixed with water at a ratio of 1:10 and extracted at 100 °C for 6 h. Aqueous extracts of IESLs were adjusted to a final isoflavone concentration of 500 μg/mL and these extracts were used in downstream experiments.

2.3. Quantification of isoflavones in IESLs

We quantified isoflavones in the IESLs using the method described by Lee, Lee et al. (2013). Briefly, the sample was filtered with a 0.45 μm syringe filter and for analysis via an HPLC equipped with a LiChrospher 100 RP C18 (4.6 × 150 mm, 5 μm, Waters Corp., USA). A total of 20 μL of sample were injected and gradient elution was performed using a two solvents mixture: a 0.1% glacial acetic acid solution (solvent A) and 100% acetonitrile (solvent B). Isoflavones were eluted with the following gradient: 0–20 min, 10% solvent B; 30 min, 20% solvent B; 40 min, 25% solvent B; 50 min, 35% solvent B. The flow rate, oven temperature, and detection wavelength were 1 mL/min, 30 °C, and 254 nm, respectively.

2.4. Animals

Six-month-old female Sprague Dawley (SD) rats were purchased from Central Lab Animal Inc. (Seoul, Republic of Korea). After an adaptation period of one week, a bilateral ovariectomy was performed. A postsurgical period of three months was used to obtain significant depletion of estrogen levels and subsequent obesity; at this point, the rats were divided into four groups as follows: non-ovariectomized rats receiving only the water vehicle (NM; n = 5), ovariectomized rats receiving only the water vehicle (OVX; n = 6), ovariectomized rats treated with 6.25 mg isoflavones/kg/day (low dose; OVX/L; n = 9), and ovariectomized rats treated with 18.8 mg isoflavones/kg/day (high dose; OVX/H; n = 9). IESLs were orally administered daily for a period of three months. All animals were fed with a modified calcium deficient AIN-76A purified rodent diet (New Brunswick, NJ, USA, Research Diets). Animals were housed individually and maintained in a temperature controlled environment with a 12-h light/dark cycle during the entire experimental period of six months. Body mass was measured weekly. At the end of the experimental period, animals were sacrificed. Blood was collected from the hearts using a 5 mL syringe and was drawn into a 5 mL VACUETTE tube (Greiner Bio-one, Thailand). After centrifugation at 3000 rpm for 10 min, serum was stored at −80 °C until analyzed. Mesenteric and perirenal white adipose tissues (WAT) and the liver were removed and weighed. Portions of the liver and mesenteric and perirenal WAT were immersed in 4% neutralized buffered paraformaldehyde for histological inspection; other portions were frozen immediately in liquid nitrogen and stored at −80 °C for downstream mRNA analysis. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Gyeongsang National University (Jinju, Republic of Korea), and the overall experimental study design is illustrated in Fig. S1.

2.5. Liver and WAT hematoxylin and eosin (H&E) staining

Fixed liver and mesenteric and perirenal WAT tissues were dehydrated using a graded series of ethanol (70%, 80%, 90%, 95%, and 100%) for 2 h, washed twice in xylene for 2 h, and impregnated twice with molten paraffin wax for 2 h, using a Leica tissue processor (Leica TP 1020, Germany). The embedded tissues were sectioned at 5 μm with a microtome (Leica RM2235, Germany) and subjected to the H&E staining. All of the stained tissue sections were imaged with a virtual slide microscope (VS120 S1, Olympus BX61VST, Hamburg, Germany) at 20× magnification using OlyVia software.

2.6. Liver Oil Red O staining

Fixed liver tissue was cryoprotected in 30% sucrose and embedded in Tissue-Tek optimal cutting temperature (OCT) compound (Sakura Fineteck USA, Inc., Torrance, CA, USA). The embedded tissues were then cryosectioned at 10 μm using a cryostat (Leica CM 1950, Germany) and Oil Red O stained using a commercially available Oil Red O kit (Abcam #ab 150678). All stained tissue sections were imaged with a virtual slide microscope (VS120 S1, Olympus BX61VST, Hamburg, Germany) at 20× magnification using OlyVia software.

2.7. Quantification of serum cholesterol, aspartate aminotransferase, and alanine transferase levels

Serum total cholesterol (TC), low density lipoprotein-cholesterol (LDL-C), aspartate aminotransferase (AST), and alanine transaminase (ALT) levels were quantified using a Roche Modular Analytics P module (Roche Diagnostics, Germany) at the Green Cross Reference Laboratory (Seoul, Republic of Korea).

2.8. Liver RNA extraction and analysis by qPCR

Total liver RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and the concentration and purity were measured using a microplate reader (Infinite M200, Tecan). Following the manufacturer's instructions, cDNA was then synthesized using a M-MLV reverse transcriptase kit (Enzynomics, Daejeon, Korea). Briefly, reactions were initiated at 4 °C and held for 5 min and then incubated at 37 °C for 60 min, followed by a 10-min incubation at 72 °C.

Next, mRNA levels of peroxisome proliferator activated receptor alpha (*PPARα*), peroxisomal acyl-coenzyme A oxidase 1 (*ACOX1*), carnitine palmitoyltransferase 1 a (*CPT1a*), lipoprotein lipase (*LPL*), and adipocyte fatty acid binding protein (*aP2*) genes were quantified via qPCR using the ToprealTM qPCR 2× PreMix PCR kit (Enzynomics,