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Foenumoside B from *Lysimachia foenum-graecum* inhibits adipocyte differentiation and obesity induced by high-fat diet

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ABSTRACT

We have previously reported anti-obesity effects of *Lysimachia foenum-graecum* in high-fat diet (HFD)-induced obesity model. Here we isolated a triterpene saponin foenumoside B as an active component of *L. foenum-graecum*. Foenumoside B blocked the differentiation of 3T3-L1 preadipocytes in a dose-dependent manner with an IC50 of 0.2 μg/ml in adipogenesis assay and suppressed the induction of PPARγ, the master regulator of adipogenesis. Foenumoside B induced the activation of AMP-activated protein kinase (AMPK), and modulated the expression of genes involved in lipid metabolism towards lipid breakdown in differentiated adipocytes. In mouse model, oral administration of foenumoside B (10 mg/kg/day for 6 weeks) reduced HFD-induced body weight gain significantly without affecting food intake. Treatment of foenumoside B suppressed lipid accumulation in white adipose tissues and the liver, and lowered blood levels of glucose, triglycerides, ALT, and AST in HFD-induced obese mice. Consistent with the *in vitro* results, foenumoside B activated AMPK signaling, suppressed the expression of lipogenic genes, and enhanced the expression of lipolytic genes *in vivo*. Foenumoside B also blocked HFD-induced proinflammatory cytokine production in adipose tissue, suggesting its protective role against insulin resistance. Taken together, these findings demonstrate that foenumoside B represents the anti-obesity effects of *L. foenum-graecum*, and suggest therapeutic potential of foenumoside B in obesity and obesity-related metabolic diseases.

1. Introduction

Obesity arises from chronic caloric overconsumption that leads to adipocyte overgrowth and excess fat deposition in adipose tissue [1]. In mild obesity, excess energy is stored in the cytoplasm of adipocytes in the form of triglycerides (TGs) through lipogenesis, resulting in increased adipocyte size. More severe obesity or childhood obesity involves adipocyte overgrowth through preadipocyte differentiation, resulting in an increased adipocyte number.

Adipogenesis is a differentiation process by which undifferentiated preadipocytes are converted to fully differentiated adipocytes, regulated by a highly organized cascade of transcription factors such as members of the peroxisome proliferator-activated receptor γ (PPARγ), the CCAAT/enhancer binding proteins (C/EBPs), and the sterol regulatory element binding protein 1c (SREBP1c) [2]. PPARγ and C/EBPα are major transcription factors of adipogenesis and activate the expression of adipocyte markers such as fatty acid synthase (FAS), strearoyl-CoA desaturase-1 (SCD-1), and lipoprotein lipase. Differentiated adipocytes store TGs through lipogenesis, with the involvement of various enzymes such as FAS and SCD-1. AMP-activated protein kinase (AMPK) stimulates the pathways that increase energy production such as glucose transport and fatty acid oxidation, and switches off the pathways that consume energy such as lipogenesis, protein synthesis, and gluconeogenesis in response to cellular energy status and becomes a therapeutic target for metabolic disorders [3].

Obesity has become a global health problem due to its association with various metabolic disorders such as type 2 diabetes, cardiovascular diseases, and non-alcoholic fatty liver disease (NAFLD) [1]. Excessive fat accumulation in non-adipose tissues causes cell dysfunction or cell death and is involved in the development of insulin resistance in the muscle and liver, and functional losses in the pancreas, heart, and liver [4]. Despite the importance of controlling obesity, available anti-obesity medication is limited. Conventional drugs used in the treatment of obesity either restrict fat intake by suppressing lipid breakdown in the intestine (orlistat) or reduce appetite by modulating the central nervous system (sibutramine and rimonabant) [5]. Though effective, the use of

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appetite-controlling drugs has been suspended due to serious cardiac and psychiatric side effects. Due to side-effects, attention has been focused on developing drugs that directly modulate energy metabolism without affecting the central nervous system, and natural compounds including berberine, resveratrol, and curcumin are known to modulate obesity either through increasing energy expenditure or inhibiting adipocyte differentiation [6–8].

Lysimachia foenum-graecum, an herbal plant, has been used as a traditional oriental medicine and reported to have anti-oxidant and anti-inflammatory effects [9]. We recently showed that *L. foenum-graecum* extract inhibits adipocyte differentiation *in vitro* and reduces high-fat diet (HFD)-induced weight gain in mouse obesity model by modulating lipid metabolism [10]. Previous phytochemical analyses have demonstrated the presence of various triterpene saponins including foenumoside A–E and lysimachiagenoside A–F in *L. foenum-graecum* extract [9,11], but their anti-obesity effects remain to be studied. Here we isolated the anti-obesity component of *L. foenum-graecum* and verified its activity in 3T3-L1 adipogenesis assay and HFD-induced mouse obesity model.

2. Materials and methods

2.1. Purification and identification of the active anti-adipogenic constituent

Schematic diagram of the isolation of foenumoside B is shown in Supplementary Fig. 1. Ethanol extract of *L. foenum-graecum* was obtained as described previously [10]. The extract was dissolved in water and sequentially partitioned with hexane, chloroform, ethyl acetate, water-saturated butanol and water. Solvents were evaporated at 60 °C and each fraction was dried completely by freeze drying. At each purification step, active fractions were identified by 3T3-L1 adipocyte differentiation assay and further separated by subsequent column purification. The water-saturated butanol fraction was loaded onto Diaion HP20 column (Mitsubishi, Tokyo, Japan), and eluted with a water–methanol gradient (3:7–1:10) to yield fractions I–IV. Fraction III was subjected to Flash 40+M C18-HS MPLC (Biotage, Uppsala, Sweden) and eluted with a water–methanol gradient (3:7–1:10). Fraction III was further separated with Eclipse XDB-C18 HPLC (Agilent, California, USA) using a mobile phase of water and methanol (3:7) at a flow rate of 1 ml/min and detection with a UV detector at 220 nm. ^{1}H and ^{13}C NMR spectra were recorded on an ECA 600 spectrometer (JEOL, Tokyo, Japan) at 600 and 150 MHz, respectively, using tetramethylsilane as an internal standard. HMBC, HMQC, ROESY, DEPT135 and COSY spectra were acquired using the standard JEOL software. Mass spectra were obtained on a JEOL GMX AX505WA spectrometer (Tokyo, Japan).

2.2. Cell culture and Nile Red staining

3T3-L1 preadipocytes were maintained in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum at 37 °C with 10% CO_2. Differentiation of 3T3-L1 cells was induced as described previously [12]. Differentiated 3T3-L1 adipocytes were fixed with 3.7% formaldehyde in PBS for 1 h, washed with PBS twice, and stained with 0.5 μg/mL Nile Red (Sigma–Aldrich) for 10 min. The fluorescence intensity was measured by an EnVision Multilabel Plate Reader (Perkin-Elmer, Wellesley, MA) with an excitation at 485 nm and emission at 535 nm. Cells were subsequently stained with Hoechst 33342 (Invitrogen, Carlsbad, CA) and photographed using an IN Cell Analyzer 1000 (GE Healthcare, Buckinghamshire, UK).

2.3. Animal experiment

All experimental procedures were approved by the Seoul National University Animal Experiment Ethics Committee. Seven-week-old male C57BL/6 mice were obtained from Central Laboratory Animals (Seoul, Korea) and housed in a controlled environment (22 ± 2 °C, 55 ± 5% relative humidity, 12 h light–dark cycle). After acclimation for 1 week, 8 mice were randomly assigned to one of the three treatment groups with equal mean body weight between groups. Mice in a normal diet group (ND + vehicle) were fed a standard chow diet and mice in high-fat diet (HFD) groups were fed a HFD (60% of calories derived from fat; Research Diets Inc., New Brunswick, NJ). Foenumoside B was dissolved in 0.5% methylcellulose, and administrated by oral gavage (10 mg/kg/day) for 6 weeks with HFD. Mice in vehicle groups were given an equal volume of 0.5% methylcellulose. Food intake was monitored weekly and body weight was measured twice per week. At the end of the experiment, mice were sacrificed after 16 h fasting. Blood was collected from the abdominal vena cava of every mouse and white adipose tissues and livers were removed, weighed, and frozen in liquid nitrogen.

2.4. Blood and histological analysis

Blood was immediately mixed with EDTA, and plasma was isolated by centrifugation at 3000 rpm for 20 min. Plasma levels of glucose, triglycerides, alanine aminotransferase, and aspirate aminotransferase were analyzed on an Olympus AU400 Chemistry Auto Analyzer (Olympus, Japan). The epididymal adipose and liver tissues were fixed with 4% paraformaldehyde, embedded in optimal cutting temperature compound (Bright Instruments, Huntingdon, UK), and frozen with cryospray (MEDITE, Germany). Fifty micrometer sections were obtained with a cryostat microtome and stained with hematoxylin and eosin or Oil-red O solution. Images were acquired by EVOS microscope (Advanced Microscopy Group, WA, USA).

2.5. Quantitative real-time RT-PCR

Total RNA was isolated from differentiated 3T3-L1 and epididymal adipose tissue using Trizol reagent (Invitrogen). Equal amounts of total RNA (2 μg) were used for cDNA synthesis with SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). Quantitative real-time PCR was performed on iCycler iQ real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA) with iQ™ SYBR Green Supermix (Bio-Rad). Relative values of mRNA were normalized with the level of cyclophilin mRNA. Primers were designed as described previously [10].

2.6. Western blot analysis

3T3-L1 adipocytes and epididymal adipose tissues were lysed with TGN buffer (50 mM Tris–Cl (pH 7.5), 150 mM NaCl, 1% Tween 20, 0.2% NP-40). Equal amounts of extracted proteins (60 μg/lane) were separated on SDS–polyacrylamide gels and transferred to nitrocellulose membranes (GE Healthcare). Blots were blocked with 5% BSA in Tris-buffered saline containing 0.1% Tween 20 (TBST) at room temperature for 30 min, followed by overnight incubation with primary antibodies at 4 °C. Antibodies against phospho-AMPKα (Thr172), AMPK, phospho-ACC (Ser79), and ACC were obtained from Cell Signaling (Denver, NY), and antibodies against C/EBPα and PPARγ were from Santa Cruz. After incubating with secondary antibodies for 1 h, luminescent signals were detected using SuperSignal West Dura Extended Duration substrate (Thermo Fisher Scientific, Waltham, MA) with a LAS-3000 image analyzer (FujiFilm, Tokyo, Japan).