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A major daidzin metabolite 7,8,4′-trihydroxyisoflavone found in the plasma of soybean extract-fed rats attenuates monocyte-endothelial cell adhesion

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ABSTRACT

Among many functional foods and their phytochemicals, ingestion of soybean (*Glycine max*) is highly correlated to reduced risk of cardiovascular diseases. Validation of potential health benefits of functional foods requires information about the bioavailability and metabolism of bioactive compounds. In this context, several phase I and II metabolites of isoflavones were target-analyzed in the plasma of rats acutely supplemented with soybean embryo extract. A daidzein metabolite, 7,8,4′-trihydroxyisoflavone (7,8,4′-THI), was found to have the highest average area under curve value (574.3 ± 112.8). Therefore, its potential prevention effect on atherosclerosis was investigated using monocyte-endothelial cell adhesion assay. Different from its precursor daidzein or daidzin, 7,8,4′-THI attenuated adhesion of THP-1 monocytes to tumor necrosis factor-alpha (TNF-α) stimulated human umbilical vein endothelial cells (HUVECs). In addition, 7,8,4′-THI significantly downregulated TNF-α stimulated the expression of vascular cell adhesion molecule-1 and monocyte chemotactic protein-1 and phosphorylation of IκB kinase and IκBα involved in the initiation of atherosclerosis in HUVECs. Therefore, 7,8,4′-THI, a highly bioavailable hydroxylated isoflavone metabolite, has potential anti-atherosclerotic effect via inhibiting monocyte-endothelial adhesion.

1. Introduction

An increasing number of people are interested in maintaining a state of well-being, improving health, and reducing the risk of chronic diseases by consuming healthy foods. Such food is often called 'Functional Food' (Granato, Branco, Nazzaro, Cruz, & Faria, 2010). However, most previous studies on biological properties of functional foods and their bioactive phenolic compounds were conducted in their native forms. Validation of the potential health benefits of functional foods requires information about the bioavailability and metabolism of bioactive compounds because native compounds found in plants are recognized as xenobiotics in the human body. They go through structural changes via several metabolic reactions, such as phase I and II metabolisms (Cassidy & Minihane, 2017). Most dietary flavonoids are first subjected to enzymatic hydrolysis since their glycoside conjugates are too hydrophilic to be absorbed by the small intestine (Chen, Zheng, Li, & Jiang, 2014). In enterocytes, absorbed aglycones will mainly undergo phase II reactions, such as methylation, sulfation, and glucuronidation involving catechol-*O*-methyltransferases, sulfotransferases and uridine-5′-diphosphate glucuronosyltransferases, respectively (Chen et al., 2014). Absorbed conjugates or aglycones are then transported to the liver via the portal vein for further phase II metabolism (Cassidy & Minihane, 2017; Chen et al., 2014). In addition to phase II metabolism, hydrolyzed flavonoids after absorption will undergo phase I metabolism, such as hydroxylation and demethylation involving cytochrome P450 (Cassidy & Minihane, 2017; Chen et al., 2014). Some

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metabolites can be effluxed back to the intestinal lumen directly or through bile (Cassidy & Minihane, 2017; Chen et al., 2014). Flavonoids that cannot be absorbed in the small intestine and effluxed metabolites will reach the large intestine where they are extensively metabolized by gut microbiota (Cassidy & Minihane, 2017; Chen et al., 2014).

Among many functional foods and their phytochemicals, consumption of soybean (*Glycine max*), especially its two functional constituents (protein and isoflavones), is highly correlated with a reduced risk of cardiovascular diseases (CVDs) (Zhan & Ho, 2005). However, it is currently unclear which isoflavone is highly bioavailable in our body after soybean intake to exert the bioactivity for CVD prevention. For many years, research in metabolomics of isoflavones has been focused on aglycones, mostly genistein and daidzein, and their reductive metabolites, such as equol (Manach, Williamson, Morand, Scalbert, & Remesy, 2005). In this study, several phase I and II metabolites of isoflavones in addition to aglycones were target-analyzed in the plasma of rats fed with soybean embryo (SE) extract and the potential anti-atherosclerotic effect of major circulating metabolite was compared to that of its precursors.

2. Materials and methods

2.1. Chemicals and reagents

7,8,4′-Trihydroxyisoflavone (7,8,4′-THI) was purchased from Indofine Chemical Company (Hillsborough, NJ, USA). Isoflavone standards (daidzin, daidzein, genistein, and coumestrol), medium199 (M199), hydrocortisone, fetal bovine serum (FBS), and calcein AM dye were purchased from Sigma-Aldrich (St. Louis, MO, USA). RPMI 1640 medium was purchased from Welgene (Daegu, Republic of Korea). l-glutamine, basic fibroblast growth factor (bFGF), and recombinant human epidermal growth factor (hEGF) were purchased from Gibco (Grand Island, NY, USA). Penicillin (10,000 units/ml)-streptomycin (10,000 μg/ml) (P/S) was purchased from Corning (Corning, NY, USA). Tumor necrosis factor-alpha (TNF-α) was purchased from PeproTech Korea (Seoul, Republic of Korea). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium salt (MTT) solution was purchased from USB Corporation (Cleveland, OH, USA). Dimethylsulfoxide (DMSO) was purchased from Duksan Pure Chemicals (Ansan, Republic of Korea). Antibodies against vascular cell adhesion molecule-1 (VCAM-1) and β-actin were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Phosphorylated and basal IκB kinase (IKK) and IκBα were purchased from Cell Signaling Biotechnology (Danvers, MA, USA). Liquid chromatography grade solvents such as acetone and acetonitrile were purchased from EMD Millipore Chemicals (Billerica, MA, USA). Glacial acetic acid, formic acid, and phosphoric acid were obtained from Anachemia (Lachine, QC, Canada). All other analytical chemicals were purchased from Merck KGaA (Darmstad, Germany).

2.2. Soybean sample preparations

Soybean embryo (SE) (*Glycine max*, registration number: 01-0003-2013-3) was provided by the Rural Development Administration, Republic of Korea. SE was cut into 3 mm in thickness and extracted with 70% food-grade ethanol (1:10 w/v) at 75 °C and 1.2 bar for 3 h. The extract was filtrated (100 mesh filter), centrifuged at 3000 × *g* for 30 min, and evaporated (60 °C, 0.08 bar, 3 h) before freeze-drying. Dried powders were stored at −20 °C until further analyses. Isoflavone compositions (Table 1) of SE extract were analyzed by UHPLC-PDA, as described previously (Lee et al., 2017).

2.3. Animal study and plasma preparation

Twenty male Wistar rats (Charles River, St. Constant, QC, Canada) were placed in temperature- and humidity-controlled rooms (21 ± 2 °C, 35–40%) with a daily light-dark cycle of 12 h–12 h. Animal facilities met the guidelines of the Canadian Council on Animal Care. Protocols were approved by the Animal Care Committee of Laval University (reference CPAUL 2015-137-1). Animals were acclimated to their environment for a minimum of 5 days. They were provided *ad libitum* access to nonpurified rodent diet (rodent chow no. 2918, Harlan Teklad, Madison, WI, USA). All animals had continuous access to tap water. After the acclimation period, rats were fed a soybean excluded diet (AIN-76A, Envigo Teklad Diets, Madison, WI, USA) for 3 days. The day before the test, rats were fasted for 12 h and randomly allocated to two groups (10 rats per group). The SE group was administered a single dose of SE extract with an average intake of 13.39 mg/kg body weight (BW) of isoflavones (Table 1) while the control group was only administered vehicle (water) by intragastric gavage. Blood samples were collected from the saphenous vein using EDTA-containing syringes (Sarstedt Microvettes) at time 0 (pre-ingestion) and at 30, 60, 120, and 240 min post-ingestion. Plasma samples were obtained by centrifugation (1100 × *g*, 10 min at 4 °C) and stored at −80 °C until analysis.

Isoflavone derivatives were extracted from plasma as previously described by Dudonne et al. (2014) with slight modifications. Briefly, Waters OASIS HLB micro-elution plates 2 mg–30 mm were preconditioned using 250 μl of methanol and 250 μl of 0.2% acetic acid. Plasma samples were mixed with 4% phosphoric acid in ultrapure water (v/v) to disrupt phenol-protein binding before loading into plates. Loaded plates were washed with 200 μl of ultrapure water and 200 μl of 0.2% acetic acid. Retained phenolic compounds were eluted with 75 μl of acetone/ultrapure water/acetic acid solution (70/29.5/0.5, v/v/v). Eluted phenolic metabolites were then directly analyzed by UHPLC–MS/MS.

2.4. Targeted analysis of isoflavone metabolites in plasma

Isoflavone metabolites were analyzed by reverse-phase UHPLC coupled to tandem mass spectrometry. Separation was achieved at 45 °C with a Waters Acquity HSS T3 column (2.1 mm × 100 mm, 1.8 μm) at a flow rate of 0.45 ml/min and an injection volume of 2.5 μl. Gradient elution was performed with a mobile phase consisted of 0.2% acetic acid in ultrapure water and acetonitrile (solvent A and B, respectively) using the following conditions: 0–0.5 min, 5% B; 0.5–1 min, 5–25% B; 1–3 min, 25–45% B; 3–4.5 min, 45–55% B; 4.5–4.7 min, 55–100% B. MS/MS analyses were carried out in negative mode using electrospray source parameters as follows: electrospray capillary voltage, 1.3 kV; source temperature, 150 °C; desolvation temperature, 400 °C; cone and desolvation gas flows, 50 and 800 L/h, respectively. Data were acquired at Multiple Reaction Monitoring (MRM) mode to track specific parent-product ion transition of the following compounds: genistein, daidzein, glycitein, 5,7,3′4′-tetrahidroxyisoflavone, 6,7,4′-trihydroxyisoflavone (THI), 7,3′,4-THI, 7,8,4′-THI, equol, equol glucuronide, equol sulfate, equol disulfate and coumestrol. Cone voltage and collision energy were optimized for each compound.

The identification of metabolites was validated by comparing their retention times and molecular ions with those of available standards or with fragmentation information described in literatures otherwise. External quantification was achieved using calibration curves constructed with available standards. Parent/product ion pairs (MRM transitions) and standards used for the quantification of identified metabolites are summarized in Table 2.

2.5. Cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Walkersville, MD, USA). To culture HUVECs, M199 with 25 mM HEPES containing 3 growth factors (1 ng/ml hydrocortisone, 1 ng/ml hEGF, and 2 ng/ml bFGF), 1% (v/v) P/S, 2 mM l-glutamine, and 10% FBS (Gibco) was used. Usage of HUVECs was limited to passage numbers between 7 and 12. For culturing THP-1 monocyte-like leukemia derived cells (the Korean Cell Line Bank), RPMI1640