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Cyanidin-3-glucoside derived from black soybeans ameliorate type 2 diabetes through the induction of differentiation of preadipocytes into smaller and insulin-sensitive adipocytes

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Abstract

Black soybean is a health food has been reported to have antidiabetes effect. The onset of diabetes is closely associated with adipocyte differentiation, and at present, the effect of black soybean on adipocyte differentiation is unknown. Here, we investigated the antidiabetes effect of black soybean, and its anthocyanin cyanidin-3-glucoside (Cy3G), on adipocyte differentiation. Orally administered black soybean seed coat extract (BSSCE) reduced the body and white adipose tissue (WAT) weight of db/db mice accompanied by a decrease in the size of adipocytes in WAT. Furthermore, 3T3-Ll cells treated with BSSCE and Cy3G were observed to differentiate into smaller adipocytes which correlated with increased *PPARγ* and *C/EBPα* gene expressions, increased adiponectin secretion, decreased tumor necrosis factor-α secretion, activation of insulin signalling and increased glucose uptake. C2C12 myotubes cultured with conditioned medium, obtained from 3T3-L1 adipocyte cultures treated with Cy3G, also showed significantly increased expression of *PGC-1α*, *SIRT1* and *UCP-3* genes. Here we report that BSSCE, as well as its active compound Cy3G, has antidiabetes effects on db/db mice by promoting adipocyte differentiation. This notion is supported by BSSCE and Cy3G inducing the differentiation of 3T3-L1 preadipocytes into smaller, insulin-sensitive adipocytes, and it induced the activation of skeletal muscle metabolism. This is the first report on the modulation effect of Cy3G on adipocyte differentiation.

Keywords: Type 2 diabetes; Insulin sensitivity; Black soybean seed coat; Cyanidin-3-glucoside; Adipocyte differentiation

1. Introduction

Type 2 diabetes mellitus (T2DM) is a serious metabolic disorder, comprising 75%–80% of all diabetes cases, and the number of patients is increasing every year around the world. T2DM raises various disease onset risks, such as cardiac infarction, atherosclerosis, cancer and neurological disease [1,2]. Insulin resistance, a physiological condition in which cells fail to respond to the normal actions of insulin, is the main cause of T2DM. Insulin resistance lowers glucose uptake in adipocytes and muscles, leading to increased blood glucose levels [2]. It has also been reported that subjects with insulin resistance have decreased mitochondrial content and the activity of electron transporter chain in skeletal muscle [3]. The improvement of insulin resistance is therefore necessary for treatment or prevention of T2DM [2].

Adipocytes regulate energy balance and glucose homeostasis through the secretion of adipokines [4,5]. Obesity can cause adipocytes to undergo hypertrophy which then lead to increased secretion of inflammatory adipocytokines such as tumor necrosis factor (TNF-α), interleukin-6 (IL-6), monocyte chemotactic protein-1 (MCP-1) and circulating free fatty acids (FFAs), all of which reduce insulin signaling, inhibiting insulin-stimulated tyrosine phosphorylation and decreasing glucose uptake [4–7]. In addition, hypertrophic adipocytes present decreased secretion of adiponectin, a hormone that regulates energy expenditure, glucose homeostasis and insulin sensitivity [4,6], and increasing adiponectin levels is an effective method for improving insulin resistance. *In vivo*, diabetes mice models, which show increased insulin sensitivity after oral administration of adiponectin [8]. In addition, adiponectin increases mitochondrial biogenesis and oxidative capacity in skeletal muscle by the increase of peroxisome proliferator-activated receptor γ coactivatorl α (PGC-1α) expression and activity through adenosine-monophosphate-activated protein kinase (AMPK) activation [9]. Since smaller adipocytes secrete more adiponectin and are more sensitive to insulin, it seems that increasing the number of small adipocytes might be a good strategy to improve insulin resistance [10–12].

Thiazolidinediones (TZDs), the medicine used for the treatment of T2DM, improve insulin resistance by increasing the number of small adipocytes. TZDs functions as a peroxisome proliferator-activated receptor γ (PPARγ) agonist, causes PPARγ transcription activation and

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promotes adipocytes differentiation [11–14]. Although TZD has therapeutic effects against diabetes, treatment with TZD has adverse effects to the body such as weight gain and edema [15]. Therefore, it is desirable to find alternative treatments for prevention or treatment of T2DM with milder or no side effects. In this regard, polyphenols, which are plant-derived compounds with anticancer, anti-inflammatory, antioxidative, antiobesity and antidiabetes effects [16,17], have been reported to improve insulin resistance by promoting adipocyte differentiation. For example, nobiletin [18], sakuranetin [19] and magnolol [20] promote adipocyte differentiation by increasing PPARγ and CCAAT/enhancer binding protein α (C/EBPα) expressions, two transcriptional master regulators of adipocytes differentiation [21]. The induction of differentiation of preadipocytes into small adipocytes by plant-derived polyphenols, as an effective therapy in the prevention or treatment of T2DM, is desired because of the low side effects compared to treatment with TZDs.

Black soybean [*Glycine max* (L.) Merr], called "Kuromame" in Japan, is one of the soybean species which contain high amount of anthocyanins and has higher antioxidative effect compared to yellow soybeans [22]. Black soybean is widely recognized as nutritionally rich food and folk medicine [23]. In Japan, black soybeans extract is traditionally used as black soybean tea (called "Kuromame-cha") and is believed to be beneficial for inflammatory- or obesity-related diseases. Recently, soybean seed coat has gained attention because of its good effects on healthy, and this is because it is abundant in anthocyanins, such as cyanidin-3-glucoside (Cy3G) and petunidin-3-glucoside and delphinidin-3-glucoside [23,24], and has high antioxidative [22], anti-inflammatory [25] and antiobesity effects [26]. Black soybean seed coat extract (BSSCE) also ameliorates serum glucose concentration and insulin sensitivity in diabetes model mice [27] and has a potential therapeutic effect against T2DM. The onset of T2DM and insulin resistance is closely associated with adipocyte differentiation [4,6]. However, the effect of BSSCE on adipocyte differentiation is not fully understood, so here we investigated the preventive effect of BSSCE on T2DM, focusing on its effects on adipocyte differentiation.

2. Materials and methods

2.1. Preparation of BSSCE and Cy3G

Black soybean (*Hitachi oguro*) was provided by Daigo town (Ibaraki, Japan). Its seed coats (1 g) were extracted with water (10 ml) at 105°C for 20 min. Cy3G derived from black soybean was purchased from Fujicco Co., Ltd. (Kobe, Japan). Black soybean extract and Cy3G were stored at −80°C until use.

2.2. Animals

Five-week-old diabetic model BKS.Cg-*Dock7*^{m} +/+ *Lepr*^{db}/J male mice (db/db mice) and lean mice were obtained from Charles River Laboratories (Yokohama, Japan). The mice were housed at one mouse per cage and were maintained under a 12-h light/dark cycle and had free access to water and food. After 1 week of acclimatization, db/db mice were divided into two groups: group 1 was orally administered with 30 mg/kg of BSSCE every day (n=8 mice), while group 2 was given only water (n=8 mice). Lean mice were also divided into two groups: group 1 was orally administered with 30 mg/kg of BSSCE every day (n=5 mice), while group 2 was given only water (n=5 mice). The body weight of mice was measured daily, and food intake was measured every week. After 30 days, mice were subjected to fasting for 20 h before sacrificed. The white adipose tissue (WAT) samples were collected from epididymis, perinephric, retroperitoneum and intestinal membrane, weighed and kept at −80°C until use. All the procedures used in this study were approved by the International Animal Care and Use Committee and in compliance with the Guide for the Care and Use of Laboratory Animals of the University of Tsukuba.

2.3. Histological analysis of WAT of db/db mice

WAT were fixed in 10% formalin at room temperature, frozen soaked for 2 h in 10% sucrose solution and embedded with O.C.T compound ESC21 and Cryomold 3 (SAKURA Finetek JAPAN Co., Ltd., Tokyo, Japan) by dry ice/hexane immersion. The frozen block of samples were cut into 6-μm sections in −35°C using Cryostats (CM1850; Leica, Tokyo, Japan), and photographs of the tissue sample cross-sections were taken using a microscope (DFC290 HD; Leica, Tokyo, Japan).

2.4. Cell culture and induction of cell differentiation

Mouse 3T3-L1 cells (Riken, Tsukuba, Japan) and mouse C2C12 cells (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma, Tokyo, Japan) supplemented with 10% fetal bovine serum (Bio West, Kansas City, MO, USA) and 1% penicillin (5000 μg/ml)–streptomycin (5000 IU/ml) (Lonza, Tokyo, Japan) at 37°C in a humidified atmosphere of 5% CO_2.

Adipocyte differentiation assay and Oil Red O staining were performed using Adipogenesis Assay kit (Cayman Chemical Co., Ann Arbor, MI, USA) following the manufacturer's instructions. Briefly, 3T3-L1 cells were seeded, allowed to reach confluence (day 0), treated with differentiation medium (DMEM containing dexamethazone, 3-isobuthyl-1-methylxanthine and insulin) for 72 h and transferred to DMEM containing insulin that was changed every 2 days until more than 80% of cells were differentiated (day 8). BSSCE and Cy3G were added to the medium during differentiation (8 days) at the indicated concentrations.

For the differentiation of myoblasts into myotubes, C2C12 cells were cultured at confluence and transferred to DMEM containing 2% horse serum (Gibco, Grand Island, NY, USA) for 5 days. The medium was changed every 2 days. After differentiation, C2C12 myotubes were treated with conditioned medium, which is the Cy3G-treated 3T3-L1 culture medium, or control (untreated with Cy3G) for 24 h.

2.5. Measurement of adiponectin and TNF-α secretion in 3T3-L1 adipocytes

The quantification of the adiponectin and TNF-α concentrations in 3T3-L1 adipocytes culture medium was performed using adiponectin enzyme-linked immunosorbent assay (ELISA) kit (Otsuka Pharma., Co., Ltd., Tokyo, Japan) and TNF-α ELISA kit (R&D Systems, Minneapolis, MN, USA), respectively, according to manufacturers' instructions.

2.6. Gene expression analysis

Total RNA was isolated from 3T3-L1 adipocytes and C2C12 myotubes using ISOGEN (NipponGene, Tokyo, Japan) following the manufacturer's instructions. Reverse transcription reactions were carried out with Superscript III reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA) using 1 μg of total RNA. For the quantification of mRNA, TaqMan real-time polymerase chain reaction (PCR) amplification reactions were performed using the Applied Biosystems 7500 Fast Real-Time System (Applied Biosystems, Foster City, CA, USA). All primers and TaqMan Universal PCR Master mix were obtained from Applied Biosystems. Specific primers *β-actin* (Mm00607939_s1), *PPARγ* (Mm01184322_m1), *C/EBPα* (Mm00514283_s1), *GLUT4* (Mm00436615_m1), *PGC-1α* (Mm01208835_m1), *SIRT1* (Mm00490758_m1) and *UCP-3* (Mm00494077_m1) were used. Gene expression levels were normalized to the *β-actin* expression level.

2.7. Triglyceride (TG) accumulation and glycerol-3-phosphate dehydrogenase (GPDH) activity in 3T3-L1 adipocytes

After the 3T3-L1 preadipocytes have differentiated, cell lysates were collected, and TG content was determined using the Triglyceride E-test Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan) according to the manufacturer's procedures. Lysates (50 μl) were centrifuged at 3750 rpm for 30 min at 4°C, and the supernatants were assayed for GPDH activity using the GPDH activity kit (Takara Bio, Tokyo, Japan) according to the manufacturer's procedures. The TG concentration and GPDH activity in the cells were calculated using the cell's protein concentration which was measured using 2-D quant kit (GE Healthcare UK Ltd., Buckinghamshire, England).

2.8. Western blotting of insulin signaling proteins in 3T3-L1 adipocytes

Differentiated 3T3-L1 cells were transferred to serum-free DMEM (3 h) and treated with 100 nM insulin (10 min). Total proteins were extracted using radio-immunoprecipitation assay (RIPA) buffer (Sigma, Tokyo, Japan) according to the manufacturer's instructions. Protein samples (20 μg) were used for 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and then they were transferred to polyvinylidene difluoride membrane. The membrane was incubated with primary antibody at 4°C overnight. The protein expression was determined by incubating the membrane in secondary antibodies IRDye 800CW Donkey antirabbit IgG (LI-COR, Inc., Lincoln, NE, USA) at room temperature for 1 h. The detection of signal was performed using OdysseyFc Imaging System (LI-COR, Inc., Lincoln, NE, USA). All protein quantifications were normalized to the β-actin expression. All primary antibodies [β-actin, Akt, phospho-Akt (Ser473), phospho-insulin receptor β (Tyr1146), insulin receptor β] were purchased from Cell Signaling Technology (Hertfordshire, UK).

2.9. Assay for glucose uptake in 3T3-L1 adipocytes

Glucose uptake was measured in differentiated 3T3-L1 cells using 2-Deoxyglucose Uptake Measurement Kit (Cosmo Bio, Tokyo, Japan) according to the manufacturer's instructions.