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A Black Soybean Seed Coat Extract Prevents Obesity and Glucose Intolerance by Up-regulating Uncoupling Proteins and Down-regulating Inflammatory Cytokines in High-Fat Diet-Fed Mice

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ABSTRACT: Black soybean seed coat extract (BE) is a polyphenol-rich food material consisting of 9.2% cyanidin 3-glucoside, 6.2% catechins, 39.8% procyanidins, and others. This study demonstrated that BE ameliorated obesity and glucose intolerance by up-regulating uncoupling proteins (UCPs) and down-regulating inflammatory cytokines in C57BL/6 mice fed a control or high-fat diet containing BE for 14 weeks. BE suppressed fat accumulation in mesenteric adipose tissue, reduced the plasma glucose level, and enhanced insulin sensitivity in the high-fat diet-fed mice. The gene and protein expression levels of UCP-1 in brown adipose tissue and UCP-2 in white adipose tissue were up-regulated by BE. Moreover, the gene expression levels of major inflammatory cytokines, tumor necrosis factor-α and monocyte chemoattractant protein-1 were remarkably decreased by BE in white adipose tissue. BE is a beneficial food material for the prevention of obesity and diabetes by enhancing energy expenditure and suppressing inflammation.

KEYWORDS: black soybean seed coat extract, obesity, glucose intolerance, UCP-1, adipocytokine

INTRODUCTION

The rate of obesity has grown considerably and become a serious problem in recent decades. Obesity is a state of excessive fat accumulation in the body, especially in abdominal adipose tissue, and is closely linked to metabolic disorders, which include diabetes, cardiovascular disease, nonalcoholic fatty liver disease, dyslipidemia, and other health problems.[1,2] Obesity results from an imbalance between energy uptake and expenditure, with excess intake of dietary fat one of the most important factors in its development. The amount of dietary fat has an almost linear relationship with body weight and glucose tolerance.[3]

Obesity and diabetes are closely related to the development of insulin resistance, a condition in which the level of insulin needed to achieve a normal metabolic response is higher than usual. As the ability of insulin is known to stimulate the utilization of glucose in muscle and adipose tissue and to inhibit hepatic gluconeogenesis, insulin resistance is considered to be a phenotype of type 2 diabetes with the disruption of insulin signaling. Tyrosine phosphorylation of insulin receptor substrate (IRS) is impaired in most cases of insulin resistance.[4,5] Inhibition of this tyrosine phosphorylation is caused by nonesterified fatty acids (NEFA) and inflammatory responses involving c-Jun amino-terminal kinase (JNK) and inhibitor of nuclear factor-κB kinase (IKK), which activate serine kinase.[5−7] These are considered the key factors in the disruption of insulin signaling.

Obesity is accompanied by systemic inflammatory responses, characterized by abnormal cytokine production and the activation of inflammatory signaling pathways. In obese individuals, increased numbers of infiltrating macrophages have been observed in white adipose tissue[8] and recruited macrophages release inflammatory cytokines and activate inflammatory pathways.[9,10] For example, the expression of inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and monocyte chemoattractant protein-1 (MCP-1), plasminogen activator inhibitor-1, and interleukin-6 (IL-6) is up-regulated in the white adipose tissue of obese mice.[8,11] These cytokines are related to the disruption of insulin's actions and the development of insulin resistance.[10,12,13]

In the past two decades, uncoupling proteins (UCPs) have attracted considerable interest in experiments on energy metabolism and obesity.[14,15] Among the members of the UCP family, UCP-1 plays an important role in energy expenditure by fat oxidation and following heat generation in brown adipose tissue. UCP-2 and UCP-3 are also involved in energy metabolism and obesity.[16,17]

Black soybean (*Glycine max*), a type of soybean with a black seed coat, has been widely used as a nutritionally rich food in Asia. Black soybean, called "Kokuzui" in Japan, has been utilized as a traditional herbal medicine for the prevention of diabetes, aiding liver and kidney functions, and enhancing diuretic actions. Black soybean seed coat, different from yellow soybean seed coat, is abundant in polyphenols.[18,19] The black soybean seed coat extract (BE) used in this study contained 9.2% cyanidin 3-glucoside, 6.2% epicatechin, and 39.8% procyanidins. Anthocyanins

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Table 1. Polyphenols in BE

composition	% (w/w)	composition	% (w/w)
epicatechin (monomers)[a]	6.2	total flavanol[c]	45.9
total procyanidin[b]	39.7	cyanidin 3-glucoside[a]	9.2
dimers[a]	6.1	total polyphenol[d]	67.0
trimers[a]	3.4		
tetramers[a]	0.5		

[a] Levels of epicatechin, procyanidins (dimers, trimers, and tetramers), and cyanidin 3-glucoside were quantified using a HPLC system. [b] The total amount of procyanidin was calculated by subtracting the amount of epicatechin from the total amount of flavanol. [c] The total amount of flavanol was determined by the vanillin−HCl assay. [d] The total amount of polyphenol was determined by the Folin−Denis method.

Table 2. Compositions of the Control and High-Fat Diets

	g/100 g diet	
ingredient	control	high fat
casein	14	14
L-cysteine	0.2	0.2
cornstarch	46.6	16.6
dextrin	15.5	15.5
sucrose	10	10
soybean oil	4	4
cellulose	5	5
mineral mixture	3.5	3.5
vitamin mixture	1	1
choline bitartate	0.3	0.3
tert-butylhydroxyquinone	0.0008	0.0008
lard	0	30
energy density	348 kcal/100 g diet	518 kcal/100 g diet

and procyanidins are reported to play an important role in the prevention of oxidative damage and have a broad range of effects including antiatherosclerotic, anticarcinogenic, and anti-inflammatory properties.[20−23] Moreover, BE prevented streptozotocin-induced type 1 diabetes in rats[24] and reduced the risk of coronary disease by inhibiting the expression of VCAM-1 in vitro.[25] However, effects of BE on high-fat diet-induced obesity and type 2 diabetes are not clear. C57BL/6 mice are commonly used to study obesity, because they are lean when fed a low-fat diet but show characteristics of obesity such as increased body fat mass, hyperglycemia, and hyperinsulinemia when fed a high-fat diet.[26,27] In the present study, we examined the effect of BE on obesity and glucose intolerance in C57BL/6 mice fed a high-fat diet containing 30% (w/w) lard for 14 weeks and analyzed the gene and protein expression levels of UCPs and the gene expression levels of inflammatory cytokines in adipose tissue.

MATERIALS AND METHODS

Materials. The BE (Chrono-Care, Fujicco Co., Ltd., Kobe, Japan) was prepared as follows: black soybeans (local variety) were harvested in autumn 2006 in the Inner Mongolia region of China. The extracts of black soybean hulls obtained with acidic water and ethanol were purified using absorbent resin and powdered by spray-drying. The polyphenol content of BE is shown in Table 1.

Animal Treatment. All of the experiments with animals were approved by the Institutional Animal Care and Use Committee (Permission 21-07-02) and carried out according to the Guidelines of Animal Experimentation of Kobe University. Male C57BL/6 mice (4 weeks old, $n = 48$) were obtained from Japan SLC (Shizuoka, Japan) and maintained at 22 ± 3 °C under an automatic lighting schedule (9:00 a.m.−9:00 p.m.). The mice had free access to distilled water and an AIM-93 M laboratory purified diet (Oriental Yeast, Tokyo, Japan) and were acclimatized for 7 days prior to the experiments. They were then randomly divided into eight groups of six each and fed a control (AIN-93M) or high-fat diet containing 30% (w/w) lard for 14 weeks. The compositions of diets and energy densities are described in Table 2. BE was added to these diets at 0% (C-0 and HF-0 groups), 0.2% (C-0.2 and HF-0.2 groups), 1% (C-1 and HF-1 groups), or 2% (C-2 and HF-2 groups) (w/w). Measurements of food and water intake and replacement of the diets were carried out once every 2 days, and total food intake throughout the experimental period was calculated. Body weight was measured weekly. At the end of the experiment, the mice were sacrificed, and mucosa of the small intestine and adipose tissues (subcutaneous, epididymal, mesenteric, and retroperitoneal adipose tissues) were collected. They were washed with 1.15% (w/v) KCl, weighed, immediately frozen using liquid nitrogen, and kept at −80 °C until use.

Oral Glucose Torelance Test (OGTT). The OGTT was carried out in week 12. The mice were fasted for 12 h and then administered a glucose solution (2 g/kg body weight) orally. Blood samples were collected in heparinized tubes from a tail vein 0, 15, 30, 60, and 120 min after administration. Plasma was obtained by centrifugation at 9600*g* for 10 min at 4 °C. The plasma glucose level was measured using a commercial assay kit according to the manufacturer's instructions (Glucose CII-test, Wako Pure Chemical, Osaka, Japan).

Measurements of Plasma Parameters and Adipocytokines. After 14 weeks of feeding, the mice were fasted for 12 h and sacrificed by collecting blood from cardiac puncture using a heparinized syringe under anesthesia with sodium pentobarbital. The plasma was obtained as described above and stored at −80 °C. Plasma triacylglycerol, total cholesterol, NEFA, and glucose levels were measured using commercial assay kits according to the instructions (Triglyceride-E test, Cholesterol-E test, NEFA-C test, and Glucose CII-test, respectively, Wako Pure Chemical). Plasma insulin, adiponectin, and leptin levels were measured by ELISA using commercial assay kits according to the manufacturer's instructions (mouse insulin ELISA kit, Shibayagi, Gunma, Japan; mouse/rat adiponectin ELISA kit, Otsuka Pharmaceutical, Tokyo, Japan; and mouse leptin ELISA kit, Morinaga, Yokohama, Japan, respectively). The homeostasis model assessment of insulin resistance (HOMA-IR) index was calculated using relationships between plasma glucose and insulin levels according to the following formula:[28] HOMA-IR = fasting glucose (mg/dL) × fasting insulin (μU/mL) /405.

Isolation of Total RNA. An aliquot of 50 mg of adipose tissue or muscle was placed in a microtube with 1 mL of TRIzol Reagent (Invitrogen, Carlsbad, CA) and homogenized with a hand microtube homogenizer. The homogenate was incubated for 5 min at room temperature and centrifuged at 12000*g* for 15 min at 4 °C. The supernatant containing RNA was mixed with 200 μL of chloroform and incubated at room temperature for 10 min. Then the mixture was centrifuged at 12000*g* for 15 min at 4 °C. The aqueous phase was transferred to another microtube. To precipitate the RNA, 500 μL of isopropyl alcohol was mixed and incubated for 10 min at room temperature. The mixture was centrifuged at 12000*g* for 10 min at 4 °C. The supernatant was discarded, and the RNA pellet was dried at room temperature. The pellet was dissolved in 30 μL of deionized−distilled water and incubated for 10 min at 60 °C. After ethanol precipitation of final eluate (60 μL), total RNA was redissolved in 30 μL of deionized−distilled water. The quality and concentration of total RNA were measured using spectrophotometry.

Analysis of mRNA by Real-Time Quantitative PCR. The RNA samples were purified by digesting the residual DNA using DNase I (Invitrogen) according to the manufacturer's instructions. The DNase-treated RNA (5.0 μL) was reverse transcribed to cDNA in a reaction mixture (final volume, 20 μL) using Superscript III (Invitrogen). cDNA was subjected to a quantitative real-time PCR amplification using SYBR Green premix Taq (Takara Bio, Shiga, Japan). The forward and reverse primers are listed in Table 3. Reactions were run in a real-time PCR system (Light Cycler, Roche, Basel, Switzerland; or TaKaRa PCR Thermal Cycler Dice, Takara Bio). Relative gene expression levels were calculated by the comparative CT method,[29] using the expression of the GAPDH gene as an internal control. The results were expressed as a fold-increase relative to the expression levels in mice of the C-0 group.

Table 3. Primer Sequences Used for Real-Time PCR Amplification

gene	5′−3′ primer sequence	product size (bp)
UCP-1	F: CTGCACTGGCACTACCTAGC	108
	R: AAAGGACTCAGCCCTGAAGA	
UCP-2	F: CCAACAGCCACTGTGAAGTT	124
	R: GCACTAGCCCTTGACTCTCC	
UCP-3	F: CTGGGAACTGGAGGAGAGAG	119
	R: GCATTTCTTGTGATGTTGGG	
Fas	F: GGGTTCTAGCCAGCAGAGTC	114
	R: TCAGCCACTTGAGTGTCCTC	
CPT-1	F: AGAGAAGCCTGCCAGTTTGT	144
	R: AAAGAGGTGACGGTCAATCC	
TNF-α	F: GCCTCTTCTCATTCCTGCTT	203
	R: CACTTGGTGGTTTGCTACGA	
MCP-1	F: TCTCTCTTCCTCCACCACCA	206
	R: CTTCTTTGGGACACCTGCTG	
IL-6	F: TTCCATCCAGTTGCCTTCTT	277
	R: GGTAGCATCCATCATTTCTTTG	
GAPDH	F: TGTGTCCGTCGTGGATCTGA	150
	R: TTGCTGTTGAAGTCGCAGGAG	

Western Blot Analysis. Preparation of the cell lysate fraction and Western blotting were performed according to our previous paper.[30] The following primary antibodies were used: anti-UCP-1 (Santa Cruz Biotechnology Inc., CA); anti-UCP-2 (BioLegend Inc., San Diego, CA); anti-UCP-3 (Abcam Plc., Cambridge, U.K.); and anti-β-actin (Sigma-Aldrich, St. Louis, MO). The density of specific bands was determined using Image J image analysis software.

Statistical Analysis. Data are presented as the mean ± SE. The statistical significance of experimental observations was determined by using the Tukey−Kramer multiple-comparison test with the level of significance set at $p < 0.05$.

RESULTS

Body Weight, Adipose Tissue Weights, And Energy Intake. The body weight of the mice at week 14 was significantly higher in the HF-0 group than in C-0 group, and 1% BE significantly lowered the high-fat diet-induced increase (Table 4). Body weight was significantly lowered in the HF-1 and HF-2 groups compared with the HF-0 group from week 11

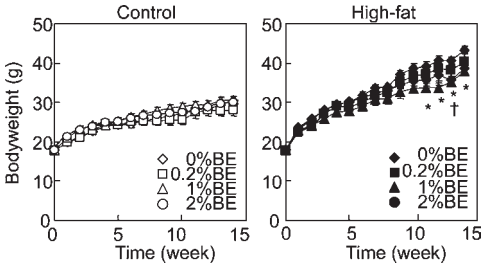

Figure 1. Changes in body weight of mice fed the control and high-fat diets containing BE for 14 weeks. Solid symbols represent high-fat diet-fed groups, whereas open symbols represent control diet-fed groups. Values are the mean ± SE ($n = 6$). Significant differences between the 0% BE group and 1% (asterisks) or 2% (dagger) group are shown ($p < 0.05$, Tukey−Kramer multiple-comparison test).

Table 4. Effects of BE on Body Weight, Total Energy Intake, and Adipose Tissue Weights of Mice Fed Control and High-Fat Diets for 14 Weeks[a]

	control diet				high-fat diet			
	0% BE	0.2% BE	1% BE	2% BE	0% BE	0.2% BE	1% BE	2% BE
body wt (g)	29.1 ± 1.0 a	27.3 ± 1.4 a	29.3 ± 0.9 a	28.0 ± 0.6 a	42.0 ± 1.0 b	39.1 ± 1.83 bc	36.6 ± 1.4 c	38.2 ± 1.1 b
total energy intake (kcal/head)	1132	989	1121	1065	1371	1484	1304	1358
white adipose tissue wt (g/100 g body wt)								
total	10.3 ± 1.14 a	10.9 ± 1.19 a	7.72 ± 0.86 a	6.63 ± 1.07 a	23.4 ± 1.28 b	19.3 ± 2.21 b	20.1 ± 1.95 b	18.6 ± 1.47 b
epididymal	2.50 ± 0.28 a	2.33 ± 0.39 a	2.08 ± 0.19 a	1.74 ± 0.22 a	5.55 ± 0.48 b	4.65 ± 0.42 b	4.99 ± 0.37 b	4.75 ± 0.25 b
mesenteric	1.45 ± 0.17 abc	1.37 ± 0.16 abc	1.01 ± 0.15 ab	0.97 ± 0.20 a	3.31 ± 0.18 d	2.39 ± 0.39 de	2.06 ± 0.19 ce	1.97 ± 0.13 bce
retroperitoneal	1.82 ± 0.20 a	1.74 ± 0.22 a	1.29 ± 0.15 a	1.16 ± 0.18 a	3.94 ± 0.34 b	3.38 ± 0.41 b	3.37 ± 0.24 b	3.1 ± 0.15 b
subcutaneous	4.45 ± 0.55 a	5.48 ± 0.48 ab	3.34 ± 0.43 a	2.76 ± 0.51 a	10.6 ± 0.88 c	8.87 ± 1.16 bc	9.63 ± 1.31 c	8.74 ± 1.02 bc

[a] Mice were fed the control or high-fat diet containing BE for 14 weeks. At the end of the experiment, body weight and adipose tissue weights were measured after a 12 h fast. Values are the mean ± SE ($n = 6$). Values without a common letter in a row differ significantly ($p < 0.05$) by the Tukey−Kramer multiple-comparison test.

to 14 and at week 13, respectively (Figure 1). The weight of all white adipose tissue (epididymal, mesenteric, retroperitoneal, and subcutaneous adipose tissue) was greater in the HF-0 group than in C-0 group. BE suppressed the increase in adipose tissue weight, particularly in mesenteric adipose tissue weight, which in the HF-1 and HF-2 groups was significantly lowered 38 and 40%, respectively, compared with the HF-0 group. In the groups given the control diet, BE did not affect body weight or adipose tissue weights. Total energy intake was higher in groups on the high-fat diet than on the control diet, but BE did not affect food intake (Table 4). These results suggest that the reduction in body weight and white adipose tissue weight observed in the BE-fed groups was not due to the reduction in energy intake. BE has the potential to reduce body weight and white adipose weight gain induced by the high-fat diet.

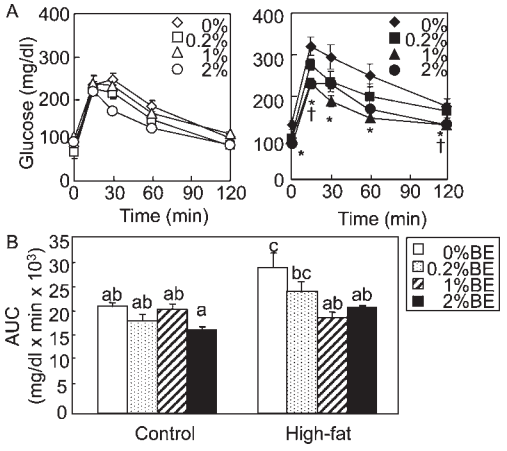

Figure 2. Oral glucose tolerance test (OGTT) in mice fed the control or high-fat diet containing BE at week 12. (A) Fasting plasma glucose levels after the oral administration of glucose (2.0 g/kg BW). Solid symbols represent the high-fat diet-fed groups, whereas open symbols represent the control diet-fed groups. Values are the mean ± SE ($n = 6$). Significant differences between the 0% group and 1% (asterisks) or 2% (daggers) group are shown ($p < 0.05$ by theTukey−Kramer multiple-comparison test). (B) Area under the curve (AUC) from the values in panel A. Values are the mean ± SE ($n = 6$). Values without a common letter differ significantly among groups ($p < 0.05$, Tukey−Kramer multiple-comparison test).

Effects of BE on Glucose Metabolism. An OGTT was performed at week 12 (Figure 2). Plasma glucose levels were significantly higher in the HF-0 group than in C-0 group 0, 15, and 120 min after glucose loading. Supplementation of the high-fat diet with 1% BE improved glucose tolerance at all time points. Plasma glucose levels were significantly lower in the HF-2 group than in HF-0 group 0, 15, and 120 min after glucose loading. In groups on the control diet, there was no significant change in the plasma glucose levels throughout the OGTT. The area under the curve (AUC) was significantly (37%) higher for the HF-0 group than in the C-0 group. The AUC was significantly (34 and 28%, respectively) lower in the HF-1 and HF-2 groups than in HF-0 group (Figure 2B).

At the end of the experiment, plasma glucose and insulin levels were significantly increased in the HF-0 group compared with the C-0 group. The plasma glucose level in the HF-1 group was reduced to the control level (Table 5). BE completely normalized plasma insulin levels in the high-fat diet-fed groups. Even in the HF-0.2 group, insulin levels were significantly lower than in the HF-0 group. The HOMA-IR index was also significantly higher in the HF-0 group than in C-0 group, and supplementation of the high-fat diet with BE reduced the index to values equivalent to those in the control diet-fed groups (Table 5). Inhibiting α-glucosidase activity in the small intestine is an effective means of preventing hyperglycemia.[31] Thus, levels of maltase and sucrase activity in the small intestine were measured (data not shown). BE did not affect these activities in groups fed either the high-fat or control diet.

Effects of BE on Plasma Lipid Levels. Total cholesterol, NEFA, and triglyceride levels were measured (Table 5). Total cholesterol levels were significantly increased in the HF-0 group compared with the C-0 group. BE significantly lowered plasma cholesterol levels to almost the same levels as in the control diet-fed groups. BE did not affect plasma NEFA and triglyceride levels in both the control and high-fat diet-fed groups.

Effects of BE on Plasma Adipocytokine Levels. Leptin and adiponectin are major adipocytokines secreted from adipose tissues and are associated with maintaining glucose homeostasis.[32] Obesity causes an elevation of the circulating leptin

Table 5. Effects of BE on Levels of Plasma Glucose, Insulin, Lipids, and Adipocytokines[a]

	control diet				high-fat diet			
	0% BE	0.2% BE	1% BE	2% BE	0% BE	0.2% BE	1% BE	2% BE
glucose (mg/dL)	124 ± 9 a	142 ± 16 ab	129 ± 7 ab	129 ± 7 ab	236 ± 12 c	202 ± 15 cd	176 ± 9 bd	201 ± 10 cd
insulin (ng/mL)	2.07 ± 0.09 a	2.70 ± 0.36 a	1.73 ± 0.14 a	2.02 ± 0.10 a	7.83 ± 1.93 b	3.96 ± 0.78 a	3.33 ± 0.48 a	3.55 ± 0.37 a
HOMA-IR	17.6 ± 1.4 a	24.9 ± 1.8 a	14.6 ± 1.9 a	16.4 ± 1.1 a	113.2 ± 30.3 b	57.6 ± 12.9 a	46.4 ± 8.8 a	56.5 ± 6.1 a
total cholesterol (mg/dL)	100 ± 7 a	103 ± 3 a	104 ± 8 a	99 ± 3 a	175 ± 13 b	124 ± 9 a	131 ± 6 a	127 ± 7 a
NEFA (mequiv/L)	0.83 ± 0.03 a	0.77 ± 0.08 ab	0.65 ± 0.05 ab	0.65 ± 0.04 ab	0.69 ± 0.06 ab	0.62 ± 0.03 ab	0.70 ± 0.06 ab	0.55 ± 0.06 b
triglyceride (mg/dL)	83 ± 4	60 ± 5	73 ± 7	63 ± 3	70 ± 4	61 ± 1	69 ± 4	58 ± 3
leptin (ng/mL)	3.88 ± 1.25 a	4.18 ± 1.12 a	3.42 ± 0.8 a	3.10 ± 0.38 a	52.46 ± 6.26 b	37.5 ± 7.2 bc	25.5 ± 5.81 cd	14.4 ± 2.2 ad
leptin index	0.39 ± 0.15 a	0.46 ± 0.10 a	0.43 ± 0.20 a	0.46 ± 0.11 a	1.87 ± 0.29 bc	1.28 ± 0.60 bc	1.39 ± 0.47 ac	0.74 ± 0.04 a
adiponectin (μg/mL)	11.4 ± 0.8 ab	13.4 ± 0.4 a	9.6 ± 0.5 a	9.6 ± 0.2 a	12.9 ± 0.4 ab	11.2 ± 0.4 ab	10.8 ± 0.3 ab	10.1 ± 1.1 b
adiponectin index	1.12 ± 0.04 ab	1.07 ± 0.04 ab	1.31 ± 0.16 b	1.56 ± 0.24 b	0.46 ± 0.04 c	0.79 ± 0.05 ac	0.67 ± 0.08 ac	0.62 ± 0.11 ac

[a] Mice were fed the control or high-fat diet containing BE for 14 weeks. At the end of the experiment, levels of plasma glucose, insulin, lipids, and adipocytokines were measured after a 12 h fast. Leptin and adiponectin indices were calculated by dividing each plasma level by relative total white adipose tissue weight (% of body weight) in this table. Values are the mean ± SE ($n = 6$). Values without a common letter in a row differ significantly ($p < 0.05$) by the Tukey−Kramer multiple-comparison test.

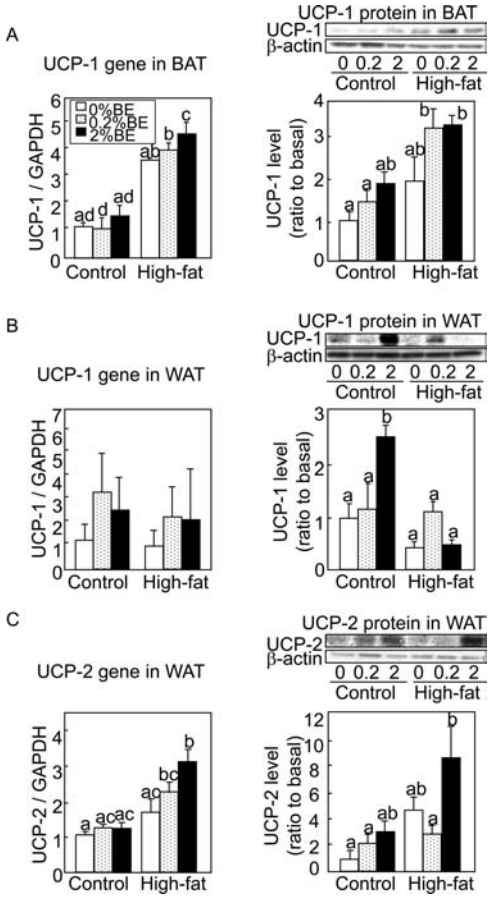

Figure 3. Gene and protein expression levels of UCP-1 amd UCP-2 in brown and white adipose tissues. The gene and protein expression levels of UCP-1 in brown (A, BAT) and subcutaneous adipose tissues (B, WAT) and UCP-2 in WAT (C) are shown by histograms. Open, hatched, and black bars represent BE at 0, 0.2, and 2%, respectively. mRNA and protein levels were normalized for the expression of GAPDH and β-actin, respectively, and the expression in the BE at 0% was assumed to be 1. Representative protein blots are shown on the histograms. Values are the mean ± SE (n = 6). Values without a common letter differ significantly among groups (p < 0.05, Tukey–Kramer multiple-comparison test).

levels and a decrease in circulating adiponectin levels.[32] In our previous study, we defined a secretion index of adipocytokines to normalize their levels under the different body and white adipose tissue weight conditions.[33] In the present study, the plasma leptin level in the HF-0 group was 14-fold that in the C-0 group (Table 5). BE suppressed the high-fat diet-induced increase in leptin in a dose-dependent manner. BE also suppressed the leptin index in high-fat diet groups. The adiponectin index was significantly reduced by intake of the high-fat diet, although plasma levels were not reduced. The reduced adiponectin index tended to increase in the 0.2 and 1% BE groups (p < 0.1).

Effects of BE on the Expression of UCPs in Adipose Tissue and Muscle. UCPs are a proton transporter family located in the mitochondrial inner membrane and play an role in the regulation of energy metabolism and thermogenesis.[16,17,34] The gene and protein expression levels of UCP-1, UCP-2, and UCP-3 were investigated in brown and subcutaneous adipose tissue and skeletal muscle of 0, 0.2, and 2% BE groups (Figure 3). BE increased the gene expression of UCP-1 in brown adipose

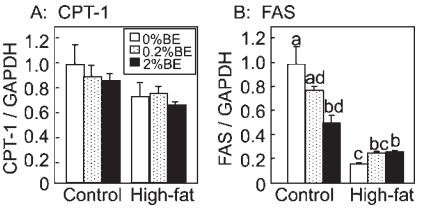

Figure 4. Gene expression levels of CPT-1 and Fas in WAT. The gene expression levels of CPT-1 (A) and FAS (B) in subcutaneous adipose tissues are shown by histograms. Open, hatched, and black bars represent BE at 0, 0.2, and 2%, respectively. mRNA level was normalized for the expression of GAPDH, and the expression in the BE at 0% was assumed to be 1. Values are the mean ± SE (n = 6). Values without a common letter differ significantly among groups (p < 0.05, Tukey–Kramer multiple-comparison test).

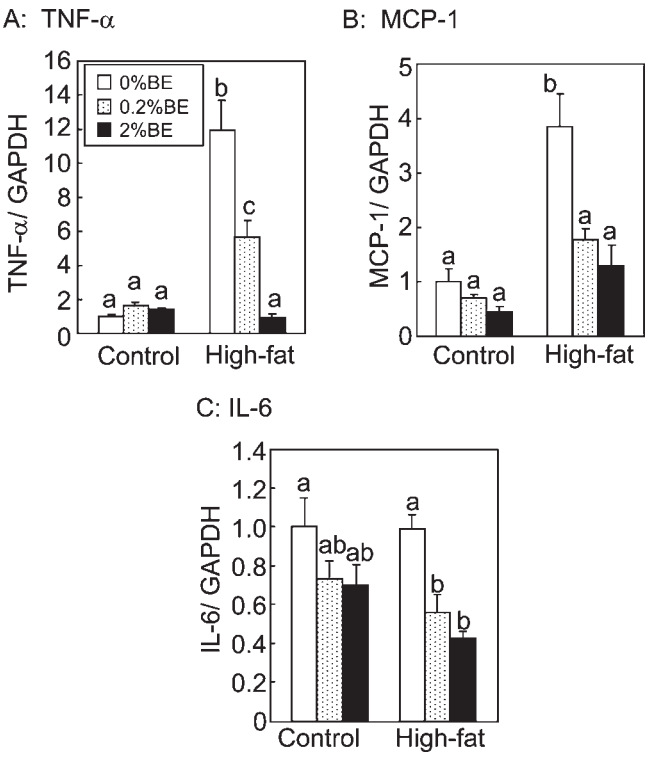

Figure 5. Gene expression levels of TNF-α (A), MCP-1 (B), and IL-6 (C) in mesenteric adipose tissue. The gene expression levels of TNF-α (A), MCP-1 (B), and IL-6 (C) in mesenteric adipose tissues are shown by histograms. Open, hatched, and black bars represent BE at 0, 0.2, and 2%, respectively. mRNA level was normalized for the expression of GAPDH, and the expression in the BE at 0% was assumed to be 1. Values are the mean ± SE (n = 6). Values without a common letter differ significantly among groups (p < 0.05, Tukey–Kramer multiple-comparison test).

tissue in high-fat diet-fed mice (Figure 3A). A significant increase was observed in the HF-2 group compared with the HF-0 group. A similar tendency was observed in the protein expression of UCP-1 (p < 0.1) in brown adipose tissue. A recent paper demonstrated that UCP-1 was expressed in white adipose tissue.[35] We confirmed the expression of UCP-1 in white adipose tissue, but BE did not increase UCP-1 gene expression in either the control or high-fat diet-fed group (Figure 3B). However, the protein expression of UCP-1 was different from the gene expression; that

is, the C-2 group was significantly increased compared with other groups.

In the case of UCP-2 in white adipose tissue, BE significantly increased its gene expression in the HF-2 group compared with the HF-0 group (Figure 3C). The same trend was observed in protein expression. In the control diet-fed groups, BE affected neither the gene nor protein expression of UCP-2. In the muscle, the gene expression levels of both UCP-2 and UCP-3 did not differ significantly in the groups (data not shown).

We further investigated the gene expressions of lipogenesis and lipolysis in white adipose tissue. Expression of carnitine palmitoyltransferase I (CPT-1) did not differ among the groups (Figure 4A). On the other hand, expression of fatty acid synthase (FAS) was significantly decreased in the C-2 group compared with the C-0 group, but its expression in the high-fat diet-fed groups remained unchanged (Figure 4B).

Suppressive Effects of BE on Inflammatory Cytokine Expression in Adipose Tissue. Adipose tissue is an endocrine tissue that secretes adipocytokines such as hormones and inflammatory cytokines.[8−11] Obesity and type 2 diabetes are accompanied by chronic inflammation during which the production of inflammatory cytokines is up-regulated.[8−11] Thus, the gene expressions of TNF-α, MCP-1, and IL-6 were performed in mesenteric adipose tissue of 0, 0.2, and 2% BE groups (Figure 5). The gene expressions of TNF-α and MCP-1 in the HF-0 group increased 12- and 3.9-fold, respectively, compared with those in the C-0 group. BE reduced the increased expression of these cytokines in a dose-dependent manner. The TNF-α and MCP-1 levels in the HF-2 group were 93 and 66% lower, respectively, than those in the HF-0 group. BE also significantly reduced IL-6 mRNA levels in the high-fat diet-fed groups, although IL-6 expression was not up-regulated by the high-fat diet.

DISCUSSION

Obesity is the result of energy imbalance, and it can develop when energy intake exceeds energy expenditure. Along with it, obesity is closely associated with a chronic inflammation characterized by abnormal levels of cytokine production, increased synthesis of acute-phase reactants, and the activation of inflammatory signaling pathways. Systemic chronic inflammation has been proposed to have a key role in the pathogenesis of insulin resistance.[5,32] In this study, we demonstrated the preventive effects of BE on obesity and glucose intolerance through the up-regulation of UCPs and the down-regulation of inflammatory cytokine expression in adipose tissue.

BE suppressed increases in adipose tissue mass, especially mesenteric adipose tissue, without affecting food intake (Table 4). To clarify this phenomenon, we focused on the expression of UCPs. It is known that UCPs are a proton transporter family located in the mitochondrial inner membrane and have attracted considerable interest in research on energy metabolism and obesity.[16,17,34,35] To date, five molecules (UCP-1−UCP-5) have been identified as members of the UCP family. Among the members of the UCP family, UCP-1 is involved in thermogenesis by uncoupling respiration from ATP synthesis through short-circuiting of the inward proton flow.[36] UCP-2 and UCP-3 are also involved in energy metabolism and obesity, although their physiological functions are still debatable.[16,17] In the present study, we found BE increased the expression of UCP-1 and UCP-2 in brown and white adipose tissues, respectively (Figure 3). As to the white adipose tissue, we selected and used subcutaneous adipose tissue, because this tissue is the largest among white adipose tissues and will contribute to energy expenditure mainly. It was noteworthy that BE has potency in increasing UCP-1 expression in white adipose tissue. A recent paper showed overexpression of perilipin, which is the predominant protein present on the surface of lipid droplets in fat cells of white and brown adipose tissues,[37] reduced lipid droplet size in white adipose tissue with brown adipose tissue-like phenotype possessing UCP-1 expression.[35] However, there is little information about food component or material induces UCP-1 expression in white adipose tissue. This is the first report that a polyphenol-rich food material has a possibility to induce UCP-1 in white adipose tissue, though underlying mechanism is unclear yet. As to the expression of UCP-2, caffeine and epigallocatechin gallate are reported to induce the UCP-2 gene in brown and white adipose tissue of rodents,[38,16] but their mechanisms are also unclear. Further study is needed to clarify molecular mechanism by which BE and its components induce UCP-1 expression in white adipose tissue.

Mesenteric adipose tissue is visceral adipose tissue and more metabolically active than subcutaneous adipose tissue. The production of inflammatory cytokines is greater in visceral adipose tissue than in subcutaneous adipose tissue.[39−41] The inflammatory cytokines in visceral adipose tissue contribute to the disruption of insulin signaling.

The results of the present study showed that BE significantly reduced the gene expression of TNF-α, MCP-1, and IL-6 in mesenteric adipose tissue (Figure 5). These findings indicate that BE has the potential to ameliorate the inflammatory responses accompanying obesity. The inhibitory effect of BE on the accumulation of fat probably contributed to the decrease in the production of inflammatory cytokines and subsequently led to the prevention of glucose intolerance.

Among inflammatory cytokines, MCP-1 is responsible for the infiltration of macrophages into adipose tissue.[13,42] Numbers of macrophages in white adipose tissue are increased in an obese state, and the macrophages participate in inflammatory pathways that are activated in adipose tissues of obese individuals.[10,13] Moreover, TNF-α, MCP-1, and IL-6 are related to the disruption of insulin signaling.[5,32,41−43] The suppression of these cytokines by BE probably contributed to the improvement in glucose intolerance. IRS-1 is phosphorylated at serine residues by various kinases such as JNK, IKK, and conventional protein kinase C (PKC), and this phosphorylation inhibits downstream signal transduction and subsequently affects the actions of insulin.[5,32] TNF-α activates these kinases, resulting in a decrease in insulin signaling. MCP-1 also has the ability to activate NF-κB via a PKC-dependent mechanism[44] and to inhibit the insulin signaling pathway.[42] Because BE suppressed both TNF-α and MCP-1, it possibly prevents insulin resistance by inhibiting the activation of NF-κB. Furthermore, IL-6 is related to the pathogenesis of insulin resistance, because it activates suppressors of cytokine signaling (SOCS)-1 and SOCS-3, which inhibit the tyrosine phosphorylation of IRS-1 and induce ubiquitin-mediated degradation of IRS.[32,43] The reduction in these inflammatory cytokines caused by BE probably suppresses the disruption to insulin signaling and ameliorates glucose intolerance. Indeed, it has been reported that BE prevented streptozotocin-induced diabetes in rats and protected against apoptosis in the pancreas.[24] Streptozotocin-treated animals have been used as a model of insulin-dependent type 1 diabetes,[45] and type 1 diabetes is involved in the inflammation in the pancreas.[46] BE may suppress the production of inflammatory cytokines such as TNF-α and MCP-1 in streptozotocin-treated rats. Further study is needed

to clarify the molecular mechanism by which BE suppresses inflammatory signaling and also alters insulin signaling.

The BE used in this study contained 9.2% cyanidin 3-glucoside, 6.2% catechins, and 39.8% procyanidins. These compounds probably contribute to the preventive effects of BE on obesity, glucose intolerance, inflammatory, and energy expenditure through the UCP family. Procyanidins and cyanidin 3-glucoside have been reported to suppress inflammatory cytokines. Procyanidins in grape seed inhibited the NF-κB signaling pathway in RAW 264.7 cells,[47] and cyanidin 3-glucoside down-regulated the expression of plasminogen activator inhibitor-1 and IL-6 in human adipocytes[48] and TNF-α and MCP-1 in KK-A^{y} mice.[49] These findings suggest that components of BE and their metabolites reduce the levels of inflammatory cytokines in visceral adipose tissue as a consequence of the prevention of fat accumulation and the modulation of cytokine production. Moreover, a procyanidin-rich extract from grape seed induced lipolysis through the expression of peroxisome proliferator-activated receptor-γ and cAMP-dependent protein kinase in 3T3-L1 adipocytes.[50] Identification of the active component(s) in BE is an important issue to be clarified in future. We assume that cyanidin 3-glucoside and procyanidins possess beneficial actions independently against obesity, glucose intolerance, inflammatory, and up-regulation of UCPs. We are planning experiments to obtain evidence for our assumption. An increase in lipolysis results in leanness and promotes fatty acid oxidation directly within adipocytes, suggesting that the activation of lipolysis to be a promising therapeutic target for the treatment of obesity.[51] Although our results did not alter the expression of CPT-1 (Figure 4), it should clarify the activity of CPT-1 in future.

BE prevented hypercholesterolemia (Table 4), which is one of the major causes of cardiovascular diseases including atherosclerosis. Previous papers showed that anthocyanin-rich plants and procyanidin-rich extracts from plants can reduce cholesterol levels.[52−54] Moreover, it has been reported that cyanidin 3-glucoside induced cholesterol efflux from macrophages and mediated their cellular cholesterol lowering and anti-inflammatory effects through activation of the liver orphan receptor, LXR, and cholesterol transporter, ABCA1.[55] Procyanidin also has been shown to reduce cholesterol levels in foam cells through the regulation of inflammatory-related gene expression.[56] Cyanidin 3-glucoside and procyanidins in BE probably contributed to the prevention of high-fat diet-induced hypercholesterolemia. Suppression of the inflammatory state by BE may lead to the reduction of the risk of cardiovascular diseases and atherosclerosis.

In conclusion, BE decreased body weight gain and white adipose tissue weights and normalized glucose tolerance in C57BL/6 mice fed a high-fat diet. Dietary BE enhanced energy expenditure through up-regulating the UCPs expression and suppressed the obesity-caused inflammatory state through down-regulation of the inflammatory-related cytokines TNF-α and MCP-1. These effects of BE may contribute to improvements in insulin sensitivity and glucose intolerance. Our results indicate that BE has the potential to reduce the risk of obesity and diabetes.

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ABBREVIATIONS USED

AUC, area under the curve; BE, black soybean seed coat extract; HF, high fat; IL-6, interleukin-6; MCP-1, monocyte chemoattractant protein-1; NEFA, nonesterified fatty acid; OGTT, oral glucose tolerance test; HOMA-IR, homeostasis model assessment of insulin resistance; TNF-α, tumor necrosis factor-α; UCPs, uncoupling proteins; CPT-1, carnitine palmitoyltransferase I; FAS, fatty acid synthase; BAT, brown adipose tissue; WAT, white adipose tissue.

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