Blackcurrant Extract Ameliorates Hyperglycemia in Type 2 Diabetic Mice in Association with Increased Basal Secretion of Glucagon-Like Peptide-1 and Activation of AMP-Activated Protein Kinase

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Summary  Blackcurrants are berries that contain high levels of anthocyanins, particularly delphinidin 3-rutinoside (D3R). Several studies have reported that the consumption of blackcurrant extract (BCE) lowers blood glucose levels and ameliorates glucose tolerance, but the mechanism underlying this effect remains unclear. Glucagon-like peptide-1 (GLP-1) and AMP-activated protein kinase (AMPK) are considered one of the most significant molecular targets for the prevention and treatment of type 2 diabetes. In this study, we showed that dietary BCE significantly reduced blood glucose concentration and improved glucose tolerance in type 2 diabetic mice (KK-A^®). The basal GLP-1 concentration in plasma was significantly increased in the BCE group accompanied by upregulation of prohormone convertase 1/3 (PC1/3), the enzyme that processes intestinal proglucagon. Moreover, the level of phospho-AMPKα protein in skeletal muscle was significantly increased in the BCE group, and this was increase accompanied by significant upregulation of glucose transporter 4 (Glut4) proteins in the plasma membrane of BCE group. In conclusion, dietary BCE significantly reduced blood glucose concentration and improved glucose tolerance in association with increased basal GLP-1 concentration in plasma, upregulation of PC1/3 expression, and translocation of Glut4 to the plasma membrane of skeletal muscle in type 2 diabetic mice; furthermore, these effects were accompanied by activation of AMPK. Our findings demonstrated that D3R-rich BCE may help prevent diabetes and allow the dosages of diabetes drugs to be reduced.

Key Words  delphinidin 3-rutinoside, blackcurrant extract, diabetes, glucagon-like peptide-1, AMP-activated protein kinase

Anthocyanins are plant pigments belonging to the flavonoid family and are found in plants such as berries. Recent intervention and epidemiological studies of berries in humans show that the ingestion of anthocyanins improves insulin sensitivity and decreases the risk of type 2 diabetes (1–3). Blackcurrant (Ribes nigrum L.) is a type of berry cultivated mainly in Europe and New Zealand. The berries contain high levels of anthocyanins, particularly delphinidin 3-rutinoside (D3R). Several studies have reported that the consumption of blackcurrant extract (BCE) lowers blood glucose levels and ameliorates glucose tolerance in mice and rats (4, 5). In addition, BCE drinks decreased postprandial blood glucose concentration in humans (6, 7). However, the mechanism remains unclear.

Glucagon-like peptide-1 (GLP-1) and AMP-activated protein kinase (AMPK) are considered to be among the most significant molecular targets for prevention and treatment of diabetes (8). GLP-1 is an important molecular target in light of the diabetes-preventing and -suppressing effects of dietary factors. GLP-1 is a type of incretin, secreted from enteroendocrine L-cells, that stimulates glucose-dependent insulin secretion and the proliferation of pancreatic β-cells (9–11). There is generally considered to be a close relationship between incretin and diet, and increase of endogenous GLP-1 secretion by dietary factors is one of the important strategies for prevention and treatment of diabetes (8, 12). Similar to GLP-1, AMPK is a serine/threonine kinase expressed in most eukaryotic cells, and the activation of AMPK induces an increase of cellular glucose uptake, which follows the membrane translocation of glucose transporter 4 (Glut4) in the skeletal muscle, and the suppression of gluconeogenesis in the liver, and results in improving hyperglycemia (8).

In our previous studies, we screened many types of anthocyanins to find molecules that stimulate GLP-1 secretion and found that D3R significantly increases secretion in the mouse enteroendocrine L cell line (13, 14). In addition, a single oral administration of D3R-rich BCE significantly improved glucose tolerance by stimulating GLP-1 and insulin secretion, while GLP-1 secretion was stimulated by BCE but not the degradation products (gallic acid or phlorogucinol aldehyde) (14).
Moreover, we previously demonstrated that anthocyanins-rich bilberry extract (which includes many varieties of anthocyanin molecules, but not D3R) or the cyanidin 3-glucoside-rich black soybean seed coat extract significantly reduces blood glucose levels and improves insulin sensitivity via the activation of AMPK in type 2 diabetic mice (15, 16).

Based on these background factors, we hypothesized that molecular mechanism of dietary BCE-mediated improvement of hyperglycemia and glucose tolerance is due to stimulation of basal GLP-1 secretion and activation of AMPK in skeletal muscle and liver. In the present study, we demonstrated that dietary D3R-rich BCE significantly reduced blood glucose concentration and improved glucose tolerance in type 2 diabetic mice. Moreover, the molecular mechanism can be explained as a dietary BCE-stimulated increase in basal GLP-1 concentration in plasma via upregulation of intestinal prohormone convertase 1/3 (PC1/3) expression, and activated AMPK-mediated translocation of Glut4 in the skeletal muscle.

MATERIALS AND METHODS

Chemicals. Commercially available BCE (Blackcurrant Polyphenol Extract 75, Lot No. P36313004) was provided by Just the Berries PD Corporation, USA (Los Angeles, CA). Composition of anthocyanins and polyphenol content in BCE (%) was as follows: total anthocyanins, 45.2 (D3R, 19.3; delphinidin 3-glucoside, 4.6; cyanidin 3-rutinoside, 19.4; cyanidin 3-glucoside, 2.0); and total polyphenol, 82.3. The composition of anthocyanins in BCE was analyzed by HPLC (4). Anti-Glut4, anti-phospho-AMPKα (Thr172), anti-AMPKα and anti-β-actin antibodies were obtained from Cell Signaling Technology (Beverly, MA). Anti-PC1/3 antibody was obtained from Abcam (Tokyo, Japan).

Animal experiments permission. All animal experiments were approved by the Animal Experiment Committee of Chubu University, and the care and treatment of mice was in accordance with their guidelines (Permission Nos. 2610031 and 2910006).

Effect of dietary BCE on hyperglycemia in type 2 diabetic mice (13, 14). Type 2 diabetic mice (male KK-A°, SPF) (17), age 4 wk (n=16) (CLEA Japan, Inc., Tokyo, Japan) were used and maintained at 23±3°C under an automatic lighting schedule (08:00–20:00 light) (15, 16). As in our previous studies (15, 16), the mice were allowed free access to water and a laboratory diet (CE-2, CLEA Japan, Inc.). After 1 wk, the mice were divided into 2 groups (n=8) and assigned to the control (AIN-93G) (18, 19) or BCE diet (control diet supplemented with BCE; 11 g of BCE/kg diet, i.e., 2 g of D3R/kg diet). The compositions of the diets are shown in Table 1. The control and BCE diets were replaced every 2 d to avoid depletion of the anthocyanins. The dose of BCE was based on a preliminary experiment to show that the supplementation level did not affect food intake. Blood samples were collected from the tail vein once a week, and blood glucose concentration was assayed using a Glucose CII-test kit (Wako Pure Chemical Industries, Ltd.) (15, 16).

After 7 wk of feeding, the mice were deprived of food for 13 h, and then blood was collected from the portal vein under anesthesia (isoflurane) and the ileum, skeletal muscle, and liver were removed. The blood was drawn into a syringe containing EDTA disodium salt (final concentration, 1 mg/mL; Dojindo, Kumamoto, Japan), aprotinin (final concentration, 500 kIU/mL; Wako), and dipeptidyl peptidase-IV inhibitor (final concentration, 100 μg; Diprotin A; Peptide Institute, Inc., Osaka, Japan) (20–22). Samples were centrifuged at 1,600 ×g for 15 min at 4°C, and plasma total GLP-1 and insulin concentrations were measured by ELISA (GLP-1 Total ELISA kit, Millipore, St. Charles, MO; Mouse Insulin ELISA Kit, Morinaga Institute of Biological Science, Yokohama, Japan) according to the manufacturer’s instructions (20–22). The removed tissues were immediately frozen using liquid nitrogen and stored at −80°C until use.

Oral glucose tolerance test (OGTT) in type 2 diabetic mice. After 5 wk of feeding, the mice were deprived of food for 13 h, and then glucose solution was administered to mice orally (2 g/kg body weight). Blood samples were collected from the tail vein at set times after glucose administration, and serum glucose concentrations were measured using a Glucose CII-Test (Wako) kit (15, 16).

Measurement of gene expression levels. Isolation of total RNA from the ilea and liver, and quantification of the gene expression level using a real-time PCR system (ABI Prism 7300; Life Technologies, Tokyo, Japan) were performed according to our previous method (15, 16). ID numbers of the TaqMan Gene Expression Assays were as follows: proglucagon, Mm01269055_m1; PC1/3 (gene name: PCSK1), Mm00479023_m1; β-2 microglobulin, Mm00437762_m1; glucose-6-phosphatase (G6Pase), Mm00839363_m1; phosphoenolpyruvate carboxykinase (PEPCK), Mm00440636_m1; β-actin, Mm00607939_m1.

Preparations of immunoblot samples from tissues and immunoblot analysis of various proteins. Preparations of immunoblot samples from tissues and immunoblot analysis were performed according to our previous studies (15, 16, 23, 24). Briefly, the prepared immunoblot samples (50 μg protein) were loaded into the SDS-PAGE

| Table 1. Composition of the experimental diets. |
|-----------------------------------------------|
| Control | BCE |
|---------|-----|
| Casein  | 20.4| 20.4|
| Cornstarch | 32.95| 32.4|
| Sucrose | 32.95| 32.4|
| Corn oil | 5.0| 5.0|
| Cellulose powder | 4.0| 4.0|
| Mineral mixture | 3.5| 3.5|
| Vitamin mixture | 1.0| 1.0|
| Choline chloride | 0.2| 0.2|
| BCE | — | 1.1|
system and the gels were transblotted onto PVDF membranes. Sheets were probed with various antibodies for 16 h at 4ºC, and reacted with horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibody. Immunoreactivity was visualized with Pierce Western Blotting Substrate (Thermo Fisher Scientific, Yokohama, Japan) (15, 16, 23, 24).

**Statistical analysis.** In Fig. 1, the data were analyzed by two-way ANOVA, followed by Student’s t-tests to compare the differences in the blood glucose concentration at each time point. In other cases, the differences among the means of the two groups were analyzed by Student’s t-test. Differences with p values of <0.05 were considered to be significant. Statistical analyses were performed using StatView 5.0 software (SAS Institute, Cary, NC).

**Table 2.** Body weight, food intake, and plasma insulin concentrations in KK-Ay mice fed the control or BCE diet for 7 wk.1

|                          | Control | BCE   |
|--------------------------|---------|-------|
| Initial body weight, g   | 23.6±0.4| 23.8±0.3|
| Final body weight, g     | 40.7±1.4| 39.0±0.5|
| Food intake, (g/7 wk · mouse) | 332.7±13.6| 331.2±16.7|
| Plasma insulin, (ng/mL)  | 2.0±0.6 | 1.7±0.3 |

1 Values are means±SE, n=8.

**RESULTS**

**Dietary BCE ameliorates hyperglycemia and glucose tolerance in type 2 diabetic mice**

First, we investigated whether dietary BCE significantly ameliorates hyperglycemia, and modulates basal GLP-1 concentration in type 2 diabetic mice. Body weight gain and food intake between the control and BCE groups did not differ during the experimental period (Table 2). Plasma insulin concentration did not differ between the groups (Table 2). Serum glucose concentration was significantly reduced in the BCE group.

![Fig. 1. Serum glucose concentration (A), and OGTT (B) of KK-Ay mice fed the control or BCE diet. Values are means±SE, n=7–8. * Significantly different at p<0.05 compared with the control.](image1)

![Fig. 2. Plasma total GLP-1 concentration in the portal vein (A), and mRNA level of proglucagon (B) and PC1/3 (C) and protein level of PC1/3 in the ileum of KK-Ay mice fed the control or BCE diet. (B), (C) The gene expression levels are expressed as fold of the control (=1) after normalization using the β-2 microglobulin gene expression level. (D) The protein levels are expressed as fold of control (=1) after normalization of the expression level of β-action protein. Values are means±SE, n=7–8. * Significantly different at p<0.05 compared with the control.](image2)
Blackcurrant Ameliorates Hyperglycemia via GLP-1 and AMPK

compared with the control group from 2 to 7 wk (Fig. 1A). The serum glucose concentration was significantly reduced in the BCE group at 15 and 60 min after oral glucose administration compared with the control (Fig. 1B).

**Dietary BCE significantly increases basal levels of plasma GLP-1 concentration via PC1/3 expression in type 2 diabetic mice**

The basal GLP-1 concentration was significantly increased in the BCE group compared with the control at the end of the experimental period (7 wk) (Fig. 2A). Proglucagon is cleaved by PC1/3 and GLP-1 is released in intestinal L cells (25); the expression of the processing enzyme is essential for intestinal proglucagon processing and production of GLP-1 (26). The increase in basal GLP-1 concentration in the BCE group may be due to upregulation of the gene expression level of proglucagon (GLP-1 precursor) and/or PC1/3. Although the gene expression level of proglucagon did not differ between the groups (Fig. 2B), the gene expression level of PC1/3 (gene name, PCSK1) and the protein level of PC1/3 in the ileum were significantly elevated in the BCE group compared with the control in type 2 diabetic mice (Fig. 2C and D).
Dietary BCE significantly induces phosphorylation of AMPK and translocation of Glut4 in skeletal muscle

The activation of AMPK stimulates the energy production system and suppresses the synthesis of glucose and fat. AMPK is recognized as a crucial target for the prevention and treatment of type 2 diabetes (8). Activation of AMPK stimulates upregulation of GLUT4 expression or translocation to the plasma membrane (PM) via an insulin-independent mechanism (8). Dietary BCE significantly increased the phosphorylation of AMPKα at Thr172 and the ratio of phosphorylation/total AMPKα in skeletal muscle compared to that in the control (Fig. 3A). GLUT4 protein expression in the PM of the BCE group was significantly higher than that of the control (Fig. 3B); however, the expression level of whole-tissue lysates of skeletal muscle did not differ between the groups.

The effect of dietary BCE on the protein level of AMPK and the gene expression level of gluconeogenesis enzymes in the liver

Both the phosphorylation level and the ratio of phosphorylation/total AMPKα in the liver of the BCE group were significantly higher than those of the control (Fig. 4A). Activation of AMPK in the liver results in downregulation of the expression of rate-limiting gluconeogenesis enzymes (PEPCK and G6Pase) involved in hepatic glucose production. However, the gene expression levels of PEPCK and G6Pase did not differ between the groups (Fig. 4B).

DISCUSSION

Recent studies show that the ingestion of anthocyanin-rich berries improves insulin sensitivity and decreases the risk of type 2 diabetes in humans (1–3). In our previous studies, we found that D3R significantly increases GLP-1 secretion in the mouse enteroendocrine L cell line (I 3), and a single oral administration of D3R-rich BCE (5 mg/kg) significantly improved glucose tolerance by stimulating GLP-1 and insulin secretion in rats (15). Although D3R was not contained in anthocyanin-rich bilberry extract or black soybean seed coat extract, these extracts significantly reduced blood glucose levels and improved insulin sensitivity via the activation of AMPK in type 2 diabetic mice (15, 16). Therefore, it can be expected that the anti-diabetes mechanism of BCE may be clarified from the point of stimulation of GLP-1 secretion and/or AMPK activation.

In the present study, dietary D3R-rich BCE significantly suppressed the serum glucose concentration and improved glucose tolerance, and increased basal GLP-1 concentration in plasma, which is associated with upregulation of PC1/3 expression in type 2 diabetic mice. Because the mice were deprived of food for 13 h, the elevation of the basal GLP-1 concentration in the BCE group may have been caused by the increased spontaneous GLP-1 secretion, not stimulation by the residual chyme in the intestine. These results suggest that chronic dietary intake of BCE modulates the basal GLP-1 concentration in type 2 diabetic mice. Proglucagon is cleaved by PC1/3 and GLP-1 is released in intestinal L cells (24). The expression of PC1/3 is essential for intestinal proglucagon processing and production of GLP-1 (26). Furthermore, a recent study demonstrated that immuno content of GLP-1 in the jejunum could be impaired by the lower mRNA expression of PC1/3 in obese patients with type 2 diabetes (27). Based on our results, dietary BCE does not upregulate proglucagon; however, increases in proglucagon processing via upregulation of PC1/3 expression results in an increase of the plasma basal GLP-1 concentration. Despite this finding, a limitation of this study that needs to be addressed is that the molecular mechanism of PC1/3 upregulation by chronic intake of BCE remains unclear.

Activation of AMPK in skeletal muscle results in a significant increase in GLUT4 protein expression in PM, which enhances glucose uptake into skeletal muscle via an insulin-independent mechanism (8). This enhanced glucose uptake can help ameliorate hyperglycemia in type 2 diabetic mice (8, 15, 16). The suppression of gluconeogenesis via downregulation of the rate-limiting enzymes (PEPCK and G6Pase) is also effective in improving hyperglycemia. It has been demonstrated that activation of hepatic AMPK improves hyperglycemia in diabetic obese mice by downregulating gluconeogenesis enzymes (28). In the present study, dietary BCE-induced activation of AMPK resulted in translocation of GLUT4 in the PM and may have enhanced glucose uptake in skeletal muscle. Although dietary BCE induced the activation of AMPK in the liver, the gene expression levels of gluconeogenic enzymes were unaltered. The exact reason for this remains unclear, but the protein expression levels of these gluconeogenic enzymes and the level of glucose production in the liver should be investigated.

This study raises another question regarding the contribution of degradation products and metabolites of BCE anthocyanins. Recently, various phenolic acids, which are degradation products or metabolites of anthocyanins, have been linked to the health benefits of anthocyanins (29). These phenolic acids were detected as metabolites in humans (30–33). In addition, a recent study showed that feeding mice a diet containing BCE for 8 wk suppressed weight gain and improved glucose metabolism (4). Although this paper did not clarify the molecular mechanism of BCE-mediated hypoglycemia, interestingly these effects were not observed in mice with intestinal flora altered by antibiotics (4). In our previous study, we demonstrated that a single oral administration of BCE induced GLP-1 secretion and this secretion was not due to stimulation by degradation products (gallic acid and phloroglucinol aldehyde) (14). However, in the present study, it remains unclear whether degradation products or metabolites derived from chronic BCE consumption stimulates basal GLP-1 secretion in the intestine, and whether these absorbed degradation products or metabolites contribute to the activation of AMPK in skeletal muscle and the liver. Exploring the link between functional doses of D3R and metabolite concentrations may contribute to elucidating the health benefits of anthocyanins.

In conclusion, dietary D3R-rich BCE significantly
reduced blood glucose concentration and improved glucose tolerance in type 2 diabetic mice. The mechanism may be explained by 1) an increase in the basal GLP-1 concentration in plasma in association with the upregulation of PC1/3 expression, and 2) translocation of Glut4 to the PM of skeletal muscle accompanied by activation of AMPK. These findings demonstrated that D3R-rich BCE may help prevent diabetes and allow the dosages of diabetes drugs to be reduced.

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