



Black soybean seed coat polyphenols promote nitric oxide production in the aorta through the Akt/eNOS pathway.

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ABSTRACT

Background: Black soybean seed coat contains abundant flavan-3-ols and possesses various bioregulatory functions. Nitric oxide (NO) is produced by endothelial nitric oxide synthase (eNOS) in vascular endothelial cells and regulates the vascular function through vasodilation and the inhibition of platelet aggregation in blood vessels. It has been reported that flavan-3-ols increase NO production, but almost previous reports used high concentration of flavan-3-ols. In the present study, we investigated the effect of flavan-3-ols-rich black soybean seed coat extract (BE) on NO production at low concentration that is close to the concentration after permeation through Caco-2 cells monolayer.

Methods: Human umbilical vein endothelial cells (HUVEC) were incubated with BE, and then NO production in the medium and eNOS phosphorylation in the cells were examined. Intestinal epithelial Caco-2 cells on the upper side of transwell filter were co-cultured with HUVEC on the basolateral compartment of the transwell apparatus. BE was added from the upper side, and the basolateral medium was collected to measure the concentration of NO and the content of

flavan-3-ols. Furthermore, HUVEC was incubated with each flavan-3-ol in order to specify the most effective compound in BE.

Results: BE significantly increased NO production in the medium of HUVEC. When polyphenols in BE were removed from the basolateral medium by extracting with ethyl acetate, increased NO production from HUVEC was not observed. Along with that, BE increased phosphorylation of eNOS and Akt in HUVEC. A part of flavan-3-ols in BE was permeated through intestinal epithelial cells. Among the permeated flavan-3-ols, procyanidin C1 had the strongest effect on the NO production in HUVEC at the concentration that permeated Caco-2 cells monolayer. Procyanidin C1 (0.05 μ M) also induced phosphorylation of eNOS and Akt in HUVEC without affecting the cAMP level.

Conclusion: A part of flavan-3-ols in BE were directly promoted NO production through the Akt/eNOS pathway in vascular endothelial cells. These findings suggest that flavan-3-ols in the black soybean seed coat may contribute to improve the vascular function.

Keywords: Black soybean seed coat polyphenols; NO; eNOS; Akt; vascular endothelial cells

BACKGROUND

Vascular endothelial cells play an important role in maintaining vascular homeostasis. It is known that diabetes, obesity, oxidative stress cause a damage to vascular endothelial cells and lead to the vascular endothelial dysfunction [1, 2, 3, 4]. Moreover, aging and vascular stiffness are the risk factors of cardiovascular diseases (CVD) through the vascular dysfunction. It is, therefore, important to search for the food factors that can prevent and ameliorate the vascular function.

Vascular homeostasis is regulated mainly by the balance of paracrine factors including nitric oxide (NO) [5, 6]. NO is produced by inducible nitric oxide synthase (iNOS), neuronal nitric oxide synthase (nNOS), and endothelial nitric oxide synthase (eNOS). nNOS and eNOS are constitutively expressed mainly in the nerve cells and the vascular endothelial cells, respectively, iNOS is an inducible isoform upon inflammatory stimuli and, in turn, overproduced NO causes oxidative stress, oncogenesis, inflammation and so on [7]. On the other hand, produced

NO by eNOS in the vascular endothelial cells contributes to regulate the vascular function. eNOS activation is regulated by several molecular mechanisms including Ca^{2+} /calmodulin binding [5, 8], cAMP-dependent protein kinase, AMP-activated protein kinase (AMPK) [5] and Akt [5, 8]. Particularly, Akt promotes the phosphorylation of eNOS at Ser1177 residues in response to various stimuli including insulin [9].

Black soybean contains abundant flavan-3-ols and anthocyanins including epicatechin, procyanidin B2, procyanidin C1, cinnamtannin A2 and other polymer procyanidins in its seed coat [10, 11]. It has been reported that these flavan-3-ols in black soybean seed coat have health beneficial effects, such as antioxidant [12], anti-obesity and anti-diabetic activities [13]. Recently, we have reported that an intake of roasted black soybean improved vascular function through increasing NO production in human [14]. Moreover, we have found that black soybean seed coat extract (BE) increased NO production accompanied by GLP-1 secretion from the intestine of rat

[15]. Although bioavailability of flavan-3-ols is poor, it has been reported that (–)-epicatechin and the slight amounts of oligomer procyanidins such as procyanidin B2, procyanidin C1 and cynamtannin A2 in BE were absorbed from the intestinal tracts and appeared in the plasma and various tissues after the oral administration of BE to mice [16]. Thus, it is expected that certain flavan-3-ols in BE may promote NO production directly in vascular endothelial cells after absorption from the intestinal tracts. Other reports also demonstrate that flavan-3-ols increase NO production via both the Akt/eNOS pathway and Ca²⁺ dependent pathway *in vitro* [17-19]. However, it is unclear whether BE promote NO production at the low concentration that is close to plasma or tissue concentration after their absorption. In this study, therefore, we investigated the effect of BE and its flavan-3-ols on NO production in human umbilical vein endothelial cell (HUVEC) and its underlying mechanism at the low concentration that is close to the concentration after permeation through Caco-2 cells monolayer.

METHODS

Materials: BE, a product of Fujicco Co., Ltd. (Kobe, Japan), was prepared by extraction with acidic water and ethanol according to the previous method [20], and its polyphenol composition was measured by a high-performance liquid-chromatography [16]. BE consisted of 6.2% epicatechin, 39.7% procyanidin (6.1% procyanidin B2, 3.4% procyanidin C1, and 0.5% cynamtannin A2), 9.2% cyanidin 3-glucoside and unidentified polyphenols including highly-polymerized procyanidins (degree of polymerization ≥ 5). Total amount of polyphenols in BE was 67.0% by the Folin–Denis method [21].

LY294002 was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Antibodies against p-

eNOS (Ser1177), p-Akt (Ser473), p-Akt (Thr308), Akt, and β-actin were from Cell Signaling Technology (Danvers, MA, USA). An antibody against eNOS was from Santa Cruz Biotechnology (Dallas, TX, USA). All other reagents used were of the highest grade available from commercial sources.

Cell culture: HUVEC was obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan) and grown in Ham's F-12K (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) supplemented with 20% (v/v) fetal bovine serum (Biowest, Nuaille, France), 50 µg/ml endothelial cell growth supplement from bovine neural tissue (Sigma-Aldrich, St. Louis, MO, USA) and 100 µg/ml heparin sodium under a humidified atmosphere of 95% (v/v) air and 5% (v/v) CO₂ at 37 °C.

Intestinal epithelial Caco-2 cell lineage was obtained from the American Type Culture Collection (Manassas, VA, USA) and grown in Dulbecco's modified Eagle's medium (Nissui Pharmaceutical, Tokyo, Japan) containing high glucose (4.5 g/L glucose), 10% (v/v) fetal bovine serum, 4 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin under a humidified atmosphere of 95% (v/v) air and 5% (v/v) CO₂ at 37 °C.

Measurement of NO production in the cell culture

medium: As an indicator of NO production, nitrite was determined in the cell culture medium using the Griess method [22]. Briefly, HUVEC was cultured in a 24-well plate to reach confluence. The cells were pretreated with or without 10 µM PI3K inhibitor (LY294002) for 1h and then either BE, (–)-epicatechin, procyanidin B2, procyanidin C1 and cynamtannin A2 was added to each well for 1h. For the positive and negative controls, 100 nM insulin and

DMSO, respectively, were used. The cell culture medium was collected, and aliquot of 150 μ l of it was mixed with 75 μ l of 1% sulfanilamide in 5% phosphoric acid, and an equal volume of 0.1% *N*- (1-naphthyl) ethylenediamine dihydrochloride in distilled water. The absorbances at 535 nm were measured using a Wallac 1420 ARVO Sx microplate reader (Perkin Elmer Inc., Waltham, MA, USA). A calibration curve was prepared using a 0-100 μ M sodium nitrite solution as a standard.

Western blotting analysis: HUVEC was cultured on a 35-mm dish to reach confluence, the cells were treated with either BE, (-)-epicatechin, procyanidin B2, procyanidin C1 and cynnamtannin A2 at the indicated concentrations in each figure. Insulin at 100 nM and DMSO were used as the positive and negative controls, respectively. The cells were washed with phosphate-buffered saline (PBS) and lysed with radio-immunoprecipitation assay buffer [10 mM Tris-HCl pH 8.0, 1% Nonidet P-40, 150 mM NaCl, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS)] containing 0.5 mM dithiothreitol protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 5 μ g/ml leupeptin, and 5 μ g/ml aprotinin) and phosphatase inhibitors (10 mM NaF and 1 mM Na_3VO_4) and homogenized using a hand-held microtube homogenizer. Homogenate was left on ice for 1 h with occasional mixing and centrifuged at 12,000 $\times g$ for 20 min at 4°C. Obtained supernatant was used as cell lysate. Detection of each target protein was performed by western blotting. After SDS-polyacrylamide gel electrophoresis, the separated proteins in the gel were transferred onto a polyvinylidene fluoride membrane. The membrane was incubated with Blocking One (Nacalai Tesque, Kyoto, Japan) for 1 h at room temperature. Then, the membrane was further incubated with the primary antibody overnight at

4°C, followed by incubation with the corresponding horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Primary and secondary antibodies were diluted in the blocking solution (Blocking One: TBST = 1:19) at ratio of 1:5,000 and 1:50,000, respectively. The protein bands were visualized using Immuno Star[®] LD (FUJIFILM Wako Pure Chemical Corporation,) and detected by Light-Capture II (ATTO, Tokyo, Japan). The density of a specific band was quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

A co-culture system of Caco-2 cells and HUVEC: Caco-2 cells were cultured on a 24-well culture insert of transwell apparatus (BD Biosciences, Erembodegem, Belgium) at a density of 6×10^4 cells per insert, and the cells were used for the experiment on the 15th-19th day. The transepithelial electrical resistance (TER) value of the monolayers was measured using a Millicell-ERS instrument (Millipore Corp., Bedford, MA, USA). After confirmation of differentiation by the TER value (higher than 600 Ω cm^2), the Caco-2 cells were co-cultured with HUVEC on the basolateral side of the transwell apparatus. BE at 0.1, 1 or 10 mg/ml was added to the apical side and incubated for 24 h, and the cell culture medium from the basolateral side was collected to measure nitrite concentration and composition of flavan-3-ols.

Influence of secretory factor from Caco-2 cell for the NO production: BE at 10 mg/ml was added to Caco-2 cells on the apical side of transwell apparatus for 24h. Then, the medium from the basolateral side was collected. The polyphenols in the permeated medium was removed by ethyl acetate extraction. After remove the ethyl acetate

layer, bottom layer was collected and evaporated to dryness using the centrifugal separator. Residue was dissolved by water, obtained solution was treated to HUVEC for 1hr and subjected the Griess method to measure the NO concentration.

HPLC analysis: To determine the levels of flavan-3-ols permeated through Caco-2 cells, the collected medium from the basolateral side of the transwell apparatus was analyzed by HPLC with or without deconjugation reaction using glucuronidase and sulfatase [16]. HPLC was performed according to a previous method [23] using a SHIMADZU LabSolutions system (SHIMADZU, Kyoto, Japan) with SPD-M20A diode array detector, equipped with a column [Cadenza CL-C18 column (ϕ 250 mm \times 4.6 mm, 3 μ m, Imtakt, Kyoto, Japan)]. HPLC separation was done with a gradient system using 0.1% formic acid solution as mobile phase A and acetonitrile as mobile phase B. Separation of procyanidins was achieved using a linear gradient from 5-15% B over 0-45 minutes; 15-80% B over 45-50 minutes; 80% B over 50-53 minutes; and 5% B over 53-70 minutes. The flow rate was set at 0.7 ml/min [23].

Measurement of intracellular Ca^{2+} levels: HUVEC was cultured in a 12-well plate with coverslip up to reach confluence. The cells were washed three times with PBS, and incubated in a recording medium (20 mM HEPES, 115 mM NaCl, 5.4 mM KCl, 13.8 mM glucose, 0.8 mM $MgCl_2$ and 1.8 mM $CaCl_2$) containing 5 μ M Fura2-AM (Dojindo, Kumamoto, Japan), 0.04% Pluronic F-127 and 1.25 mM probenecid for 1 h at 37 °C. The medium was removed and the cells were washed tree times with PBS. Then, the PBS was removed and replaced with the recording medium containing 1.25 mM probenecid. After treatment with 0.05

or 50 μ M procyanidin C1 or A23187 (Calcium Ionophore) as a positive control for 10 min, the medium was removed and the cells were washed three times with PBS. The cells were immobilized with 4% paraformaldehyde solution for 20 min, and washed three times with PBS. Coverslips were mounted on microscope slide by a mounting solution (glycerol containing 1% 1,4-diazabicyclo[2,2,2]-octane and 20 mM Tris-HCl, pH 7.5). The fluorescence of Fura2-AM was measured with an excitation wavelength at 340/380 nm and emission one at 510 nm using an FSX100 microscope (Olympus, Tokyo, Japan).

Statistical analysis: Data are represented as the mean \pm SE. The statistical significance of experimental observations was determined using Dunnett's multiple comparison test (Fig. 1, 2, 3, and 4) and Tukey's HSD test (Fig. 5). The level of significance was set at $p < 0.05$.

RESULTS

Effects of BE on NO production and eNOS phosphorylation in HUVEC:

In order to determine whether BE promotes NO production in HUVEC, the cells were treated with BE (0.01-100 μ g/ml) for 1 h and measure nitrite concentration in the culture medium to estimate the NO production. As shown in Fig. 1A, 1-100 μ g/ml BE significantly increased NO production from HUVEC. To investigate the upstream factors of NO production, HUVEC was treated with BE (0.01-100 μ g/ml) for 30 min and western blotting analysis was performed. BE 1-100 μ g/ml significantly increased phosphorylation of Akt at Thr308 and eNOS in HUVEC (Fig. 1B and C). Phosphorylation of Akt at Ser473 was also significantly increased by only BE at 100 μ g/ml. These results suggested that BE could increase NO production via the Akt/eNOS pathway in HUVEC.

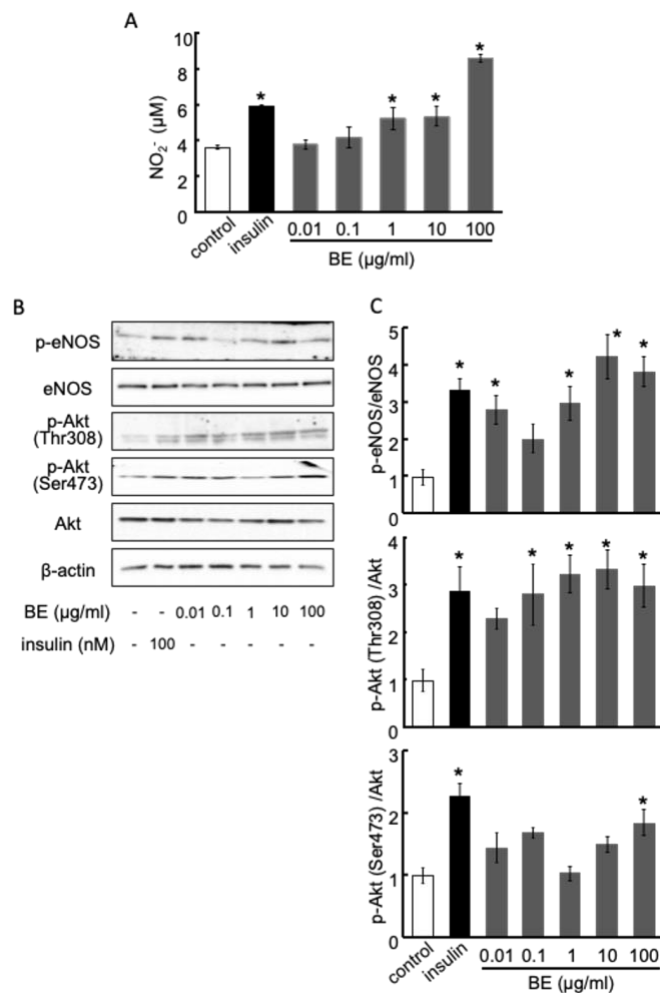


Figure 1. Effect of BE on NO production and its signaling pathways in HUVEC. HUVEC was treated with 0.01–100 µg/ml BE or 100 nM insulin as a positive control. A; NO concentrations in the medium were determined by Griess method. Values are the mean ± SE (n=4). B and C; The phosphorylation level of eNOS and Akt were determined by western blot analysis. Each panel shows a typical result (B). The density of each phosphorylation and expression level was determined using Image J image analysis software (C). Values are the mean ± SE (n=3). **p* < 0.05 vs control (Dunnett’s multiple comparison test).

Effect of permeated flavan-3-ols in BE on NO production in a co-culture system consisting of Caco-2 cells and HUVEC:

It is known that polyphenols in BE are slightly but significantly absorbable [16]. To take account of their low bioavailability, the effect of BE on NO production was measured in a co-culture system consisting of Caco-2 cells and HUVEC. BE (0.1-10 mg/ml) was added to the apical side of the transwell apparatus and incubated for 24 h, and then NO production in the cell culture medium from the basolateral side was measured. As shown in Fig. 2A, when 10 mg/ml BE was added to Caco-2 cells on the apical side

of the apparatus, a significant increase in NO production was observed in the basolateral medium of HUVEC. This result suggested that flavan-3-ols permeated through monolayer of Caco-2 cells, or certain substance(s) secreted from the Caco-2 cells could contribute to increase the NO production from HUVEC. When flavan-3-ols were removed from the basolateral medium by extracting with ethyl acetate, increased NO production from HUVEC was canceled (Fig 2C). This result suggested that permeated flavan-3-ols through Caco-2 cells directly increased NO production from HUVEC. To confirm this phenomenon,

permeated flavan-3-ols through Caco2 cells were determined by HPLC after treatment with BE at 10 mg/ml to Caco-2 cells in the apical side for 24 h (Table 1). About 3% of each flavan-3-ol was detected as an aglycone form under our experimental conditions, estimating that about 0.3 mg/ml of BE was existed in the basolateral medium of

the co-culture system. It was confirmed that a significant increase in the NO production was observed after direct treatment with 0.3 mg/ml of BE to HUVEC with the same extent as 10 mg/ml BE-increased NO production in the co-culture system (Fig. 2B)

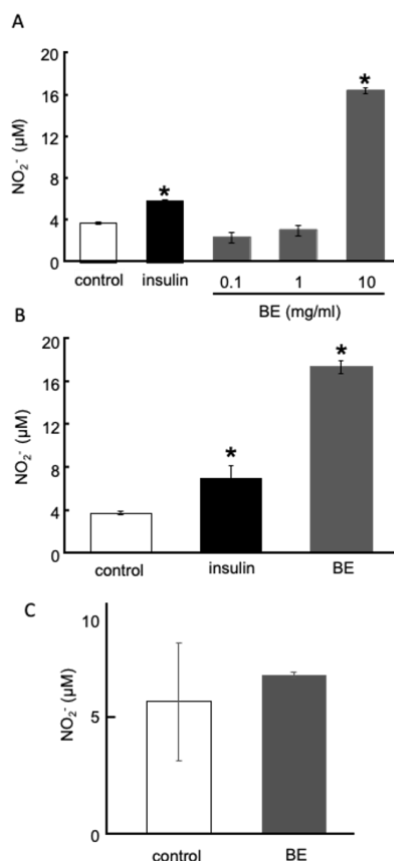


Figure 2. BE increased NO production in a co-culture system of Caco-2 cells and HUVEC. A; Caco-2 cells were cultured on cell culture inserts and co-cultured with HUVEC on the basolateral side of the transwell apparatus. BE was added to the apical side and incubated for 24 h, and the cell culture medium from the basolateral side was collected to measure nitrite concentration. B; HUVEC was directly treated with 300 µg/ml BE, which was almost equivalent to the concentration permeated through caco-2 cells or 100 nM insulin as a positive control for 1 h. C; Caco-2 cells were cultured on cell culture inserts. BE was added to the apical side and incubated for 24 h, and the cell culture medium from the basolateral side was collected. The polyphenols in permeated medium was removed and treated to the HUVEC for 1h. NO concentration in the medium was determined by Griess method. Values are the mean ± SE (n=3), *p < 0.05 vs control (Dunnett’s multiple comparison test).

Table. 1 Concentration of flavan-3-ols after permeation through Caco-2 cells.

Flavan-3-ol (nM)	BE 0.1 mg/ml		BE 1 mg/ml		BE 10 mg/ml	
	aglycon	aglycon + conjugate	Aglycon	aglycon + conjugate	aglycon	aglycon + conjugate
(-)-epicatechin	N.D.	0.66±0.15	2.37±0.52	2.49±0.07	67.6±6.3	64.8±7.7
procyanidin B2	N.D.	N.D.	0.58±0.11	0.59±0.01	24.0±2.4	23.0±2.5
procyanidin C1	N.D.	N.D.	0.18±0.02	0.17±0.01	6.67±0.74	6.14±0.84
cinnamtannin A2	N.D.	N.D.	N.D.	N.D.	2.84±0.50	2.42±0.34

N.D.: not detected

Effects of flavan-3-ols in BE on NO production and eNOS phosphorylation in HUVEC:

To specify the active compound in BE, HUVEC was treated with (–)-epicatechin, procyanidin B2, procyanidin C1 or cinnamtannin A2 for 1 h and measured NO production in the cell culture medium. Based on the previous *in vivo* results [16], the concentration of each flavan-3-ol in BE was set to the concentration that is possible to exist in the plasma of mice. As shown in Fig. 3A, 0.5 μM (–)-epicatechin, 0.1 μM procyanidin B2 and 0.05 μM procyanidin C1 significantly increased NO production in the culture medium. On the other hand, 0.025 μM cinnamtannin A2 did not increase NO production significantly. To investigate the upstream factors of NO production in HUVEC, the cells were treated

with each flavan-3-ol for 30 min and western blotting analysis was performed. As shown in Fig. 3B and C, 0.5 μM (–)-epicatechin, 0.1 μM procyanidin B2 and 0.05 μM procyanidin C1 significantly increased phosphorylation of eNOS in HUVEC. In addition, 0.1 μM procyanidin B2 and 0.05 μM procyanidin C1 significantly increased phosphorylation of Akt at both of Thr308 and Ser473. Although significant difference was not observed, 0.5 μM (–)-epicatechin also tended to increase phosphorylation of Akt at Thr308 and Ser473. These results suggested that (–)-epicatechin, procyanidin B2 and procyanidin C1 had an ability to increase NO production via the Akt/eNOS pathway in HUVEC at low concentrations that are close to the plasma concentrations in mice after intake of BE [16].

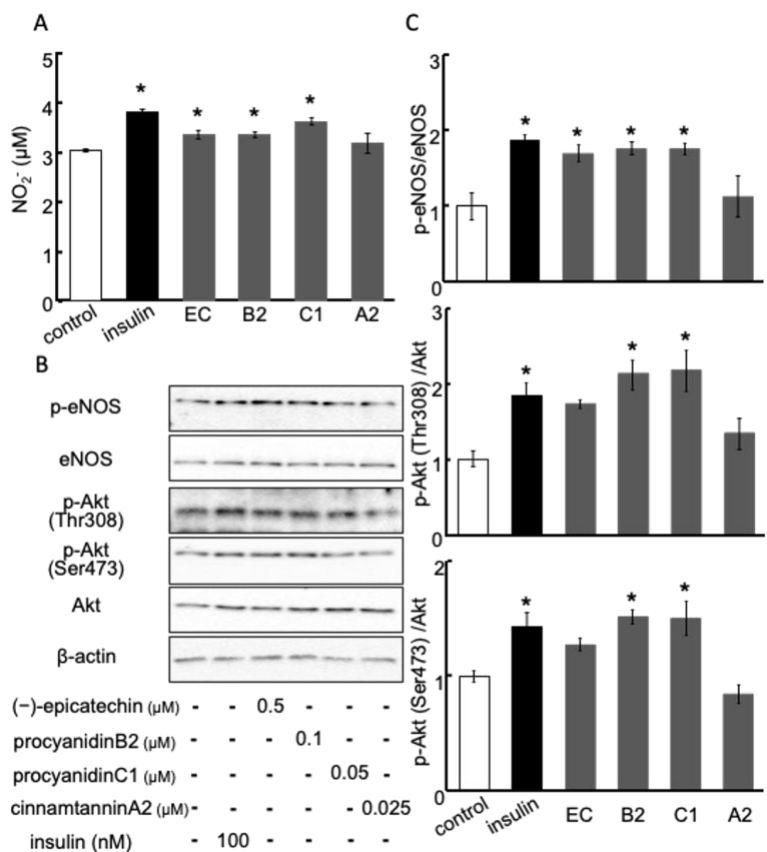


Figure 3. Flavan-3-ols contained in BE increased NO production through Akt/eNOS signaling pathway. HUVEC was treated with 0.5 μM (–)-epicatechin, 0.1 μM procyanidinB2, 0.05 μM procyanidinC1 and 0.025 μM cinnamtannin A2 or 100 nM insulin as a positive control. A; NO concentration in the medium was determined by Griess method. B and C; The phosphorylation level of eNOS and Akt were determined by western blot analysis. Each panel shows a typical result (B). The density of each phosphorylation and expression level was determined using Image J image analysis software (C). Values are the mean ± SE (n=3), *p < 0.05 vs control (Dunnett’s multiple comparison test).

To compare the effect of each flavan-3-ol in BE, HUVEC was treated with (-)-epicatechin, procyanidin B2, procyanidin C1 or cinnamtannin A2 at the same concentration (0.05 μM), and measured NO production and phosphorylation of Akt and eNOS. As a result, procyanidin C1, but not (-)-epicatechin, procyanidin B2 and cinnamtannin A2, significantly increased NO production from HUVEC via the Akt/eNOS pathway (Fig. 4). Moreover, pretreatment with LY294002 canceled procyanidin C1-increased NO production (Fig. 5). It is reported that Ca²⁺-dependent pathway is also involved in the NO production. Thus, HUVEC was treated with 0.05 or 50 μM procyanidin

C1 after preincubation with 5 μM Fura2-AM, and measured intracellular Ca²⁺ level using fluorescent microscopy. As shown in Fig. 6, 0.05 μM procyanidin C1 did not increase intracellular Ca²⁺ level, although 50 μM procyanidin C1 increased it. Thus, procyanidin C1 increased mainly through the Akt/eNOS pathway, but not through Ca²⁺-dependent pathway, at the low concentration. From these results, it is suggested that procyanidin C1 was a main active compound in BE for promotion of the NO production via the Akt/eNOS pathway at the low concentration that close to the plasma concentration in mice after intake BE.

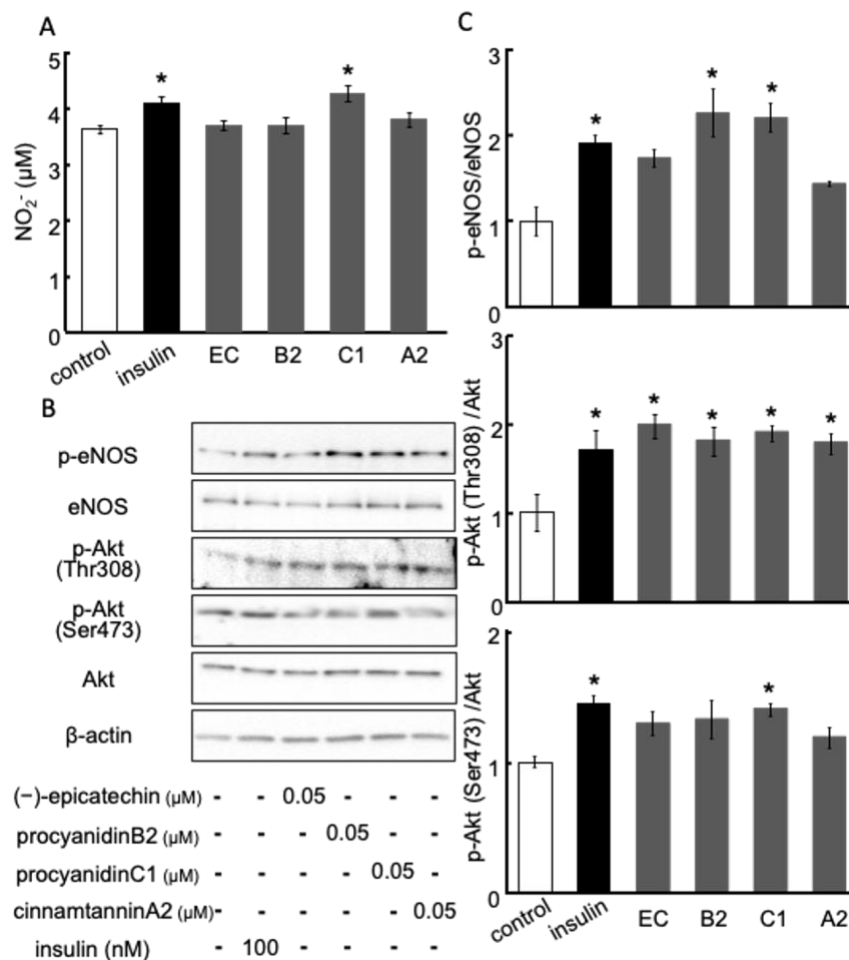


Figure 4. Procyanidin C1 increased NO production thorough the Akt/eNOS signaling pathway. HUVEC was treated with (-)-epicatechin, procyanidinB2, procyanidinC1 and cynnamtannin A2 at 0.05 μM or 100 nM insulin as a positive control. A; NO concentration in the medium was determined by Griess method. The phosphorylation level of eNOS and Akt were determined by western blot analysis. B and C; Each panel shows a typical result (B). The density of each phosphorylation and expression level was determined using Image J image analysis software (C). Values are the mean ± SE (n=3), *p < 0.05 vs control (Dunnett’s multiple comparison test).

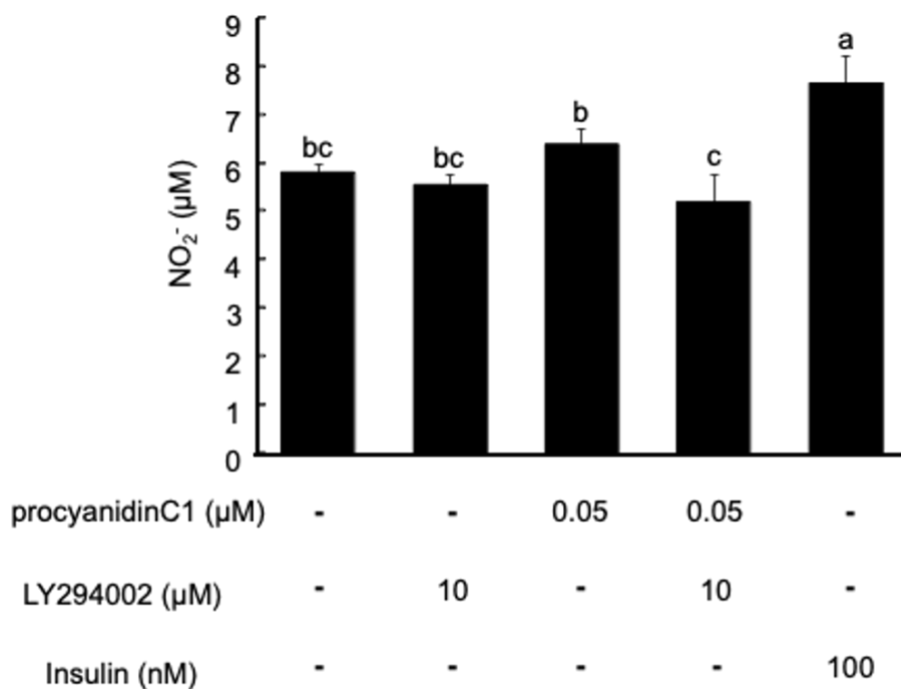


Fig. 5

Figure 5. Procyanidin C1-induced NO production was canceled by a PI3K inhibitor. HUVEC was pretreated with or without 10 µM PI3K inhibitor (LY294002) for 1h and then 0.05 µM procyanidinC1 or 100 nM insulin as a positive control were treated to the cells. NO concentration in the medium was determined by Griess method. Values are the mean ± SE (n=3). Means with different letters differ significantly ($p < 0.05$; Tukey's HSD test).

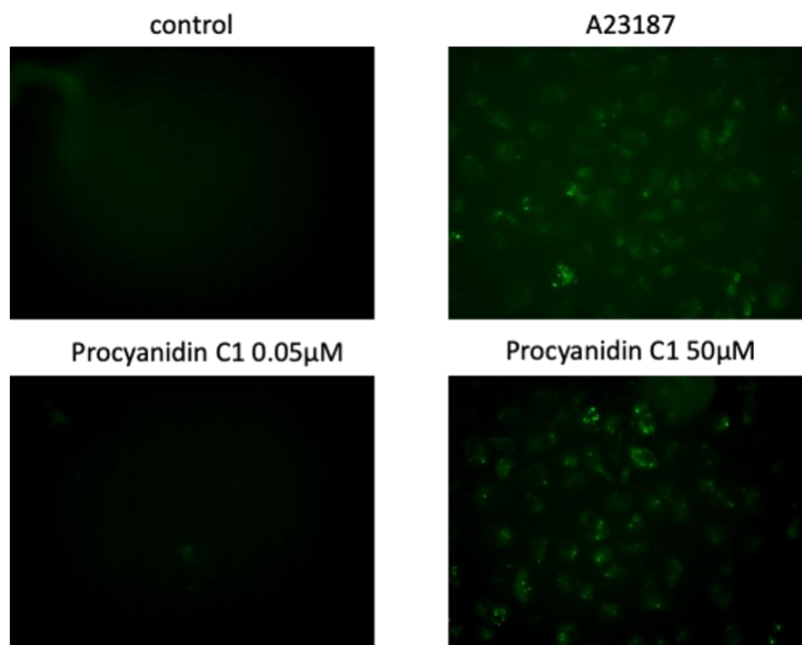


Figure 6. The effect of procyanidin C1 on intracellular Ca²⁺ levels. HUVEC was incubated with 0.05 or 50 µM procyanidin C1 or A23187 (Calcium Ionophore) as a positive control for 10 min, after preincubation with Fura2-AM. The intracellular Ca²⁺ level was then estimated by a fluorescence microscope.

DISCUSSION

Vascular dysfunction, which is caused by aging and vascular stiffness, is associated with a risk of CVD [1], and a reduction in the NO level can be a trigger of the onset of CVD. Black soybean seed coat contains abundant flavan-3-ols and these compounds have been reported to increase NO production in vascular endothelial cells [17-19]. However, these previous reports used flavan-3-ols at high concentrations. In this study, we demonstrated that BE directly increased NO production via the Akt/eNOS pathway in HUVEC at the low concentration (Fig. 3 and 4). In addition, procyanidin C1 was the most effective compound in BE to increase the NO production at the low concentration (Fig. 4). To the best of our knowledge, this is the first report demonstrating flavan-3-ols, in particular procyanidin C1, increase NO production in HUVEC at the concentration that close to the plasma concentration in mice after intake of flavan-3-ols-rich food materials.

In this study, about 3% of flavan-3-ols in BE was permeated through the intestinal epithelial cells (Table 1) and promoted the NO production in HUVEC (Fig. 2). The passive diffusion across the intestinal epithelium can occur either through the transcellular route or the paracellular route [24]. It has been reported that highly hydrophilic compounds prefer the paracellular route and enter the body through tight junctions [24]. Since flavan-3-ols containing in BE are hydrophilic, they would absorbed through the paracellular route. Previous study has been reported that flavan-3-ols in cocoa powder extract affects the function of tight junction and are permeated through intestinal epithelial cells by the paracellular route [25]. In the present study, permeated sum of flavan-3-ols reached about 100 nM after treatment with 10 mg/ml of BE to Caco-2 cells. Recently, we have reported that about 100 nM

flavan-3-ols are detected in the human plasma after the intake of 30 g/day black soybean for 8 weeks [14]. Moreover, almost the same concentration of flavan-3-ols was detected as the aglycone form after orally administered 250 mg/kg B.W. of BE in ICR mice. Therefore, used concentration of BE in the present study was relevant to detected concentration of BE-derived flavan-3-ols in both human and animal experiments.

Among the permeated flavan-3-ols in the co-culture system, procyanidin C1 was the most effective compound in BE for the NO production through the Akt/eNOS pathway, but not through Ca²⁺-dependent pathway, and a minimum effective concentration of it was 0.05 μM (Fig. 6). Similarly, previous study was reported that 50 μM procyanidin C1 activated eNOS mainly through Ca²⁺-dependent pathway, and its effect was stronger than (-)-epicatechin and procyanidin B2 [17]. However, such a high concentration of procyanidin C1 is difficult to exist in the body after oral intake of flavan-3-ol-rich food materials or procyanidin C1 itself. From our result and previous one, at least, we can safely speculate that procyanidin C1 is the most effective compound among flavan-3-ols. It is, however, unclear that procyanidin C1-specific target molecule that lead to activate signaling pathway(s) of the NO production. We assume that the core structure of procyanidins (polymerized 2-phenyl-3,4-dihydro-2H-chromen-3-ol) interacts with a target molecule for Akt, such as insulin-like growth factor-1 receptor (IGF-1R) [26] or epidermal growth factor receptor (EGFR) [27]. Previous study reported that procyanidins from *Cinnamomi Cortex* activated IGF-1R *in vitro* [28]. Another study reported that oligomer procyanidins from grape seed activated EGFR *in vitro* and *in vivo* [29]. Further study is needed to search for the target molecule of procyanidin C1 and its underlying molecular

mechanism in future.

In this study, HUVEC was treated with flavan-3-ols in BE at a concentration that can exist as the aglycon form in the plasma [16], because we detected only the aglycon form of them in basolateral medium from the co-culture system. However, it has been reported that flavan-3-ols in BE exist mainly as the conjugate forms in the plasma [16]. Thus, future study should clarify whether the conjugated forms of flavan-3-ols increase the NO production in vascular endothelial cells.

CONCLUSION

In conclusion, a part of flavan-3-ols in BE directly promotes the NO production from vascular endothelial cells through the Akt/eNOS pathway at the low concentration that close to the plasma concentration in mice after intake BE. In addition, procyanidin C1 is the most effective compound among flavan-3-ols in BE to promote the NO production. Our findings provide the possibility that flavan-3-ols in the black soybean seed coat may prevent CVD by improving vascular function.

List of abbreviations used: NO; nitric oxide, eNOS; endothelial nitric oxide synthase, BE; black soybean seed coat extract, CVD; cardiovascular diseases, Akt; phosphoinositide 3-kinase, PI3K; phosphoinositide 3-kinase, PBS; phosphate-buffered saline

Competing interests: There is no competing interest in this study.

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Yamashita Y, and Ashida H. wrote the manuscript. All authors have read and approved the final version of the manuscript.

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