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Black soybean seed coat polyphenols promote nitric oxide production in the aorta through glucagon-like peptide-1 secretion from the intestinal cells

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Abstract

Black soybean seed coat polyphenols were reported to possess various bioregulatory functions. However, the effects of black soybean seed coat polyphenols on vascular functions are unknown. Vascular dysfunction caused by aging and vascular stiffness is associated with a risk of cardiovascular disease (CVD), and a reduction in nitric oxide (NO) levels can trigger the onset of CVD. In the present study, we investigated the effect of polyphenol-rich black soybean extract (BE) on vascular functions and the underlying mechanisms involved. The oral administration of BE at 50 mg/kg body weight to Wistar rats increased NO levels as determined by eNOS phosphorylation. The administration of BE also increased GLP-1 and cAMP levels. Furthermore, the effects of BE were inhibited in the presence of a GLP-1 receptor antagonist. This suggests that GLP-1 is strongly involved in the underlying mechanism of NO production *in vivo*. In conclusion, BE contributes to the improvement of vascular function by promoting NO production. Regarding the putative underlying mechanism, GLP-1 secreted from intestinal cells by the polyphenols in BE activates eNOS in vascular endothelial cells.

Keywords

Black soybean seed coat polyphenols; NO; eNOS; GLP-1; vascular function

Introduction

Vascular function is important to the pathogenesis of cardiovascular diseases (CVD).[1] Vascular dysfunction caused by aging and vascular stiffness is associated with a risk of CVD. In addition, injurious stimuli such as oxidative stress, inflammation, diabetes, and obesity result in the dysfunction of vascular endothelial cells.[2-4] Because vascular dysfunction is recoverable, it is important to detect vascular dysfunction as early as possible and improve it.

Nitric oxide (NO) regulates vascular functions by inducing vasodilation and inhibiting platelet aggregation in blood vessels.[5,6] A reduction of NO levels can trigger the onset of CVD. Therefore, increasing NO production in the vascular endothelium might prevent CVD and improve vascular function. NO is produced by endothelial nitric oxide synthase (eNOS) in vascular endothelial cells. eNOS activation is regulated by several molecular mechanisms including Ca^{2+}/calmodulin binding,[5,7] cAMP-dependent protein kinase, AMP-activated protein kinase[5] and Akt.[5,8] Of note, Akt promotes the phosphorylation of eNOS at Ser1177 residues in response to various stimuli including insulin.[8]

Glucagon-like peptide-1 (GLP-1) is an incretin hormone secreted from intestinal L cells.[9] GLP-1 secretion is dependent on food intake and promoted insulin secretion in pancreatic β cells.[9] In addition, GLP-1 induced endothelium-dependent vasodilation.[10,11] It was reported that GLP-1 affected vascular endothelial cells and increased eNOS phosphorylation and

72 subsequent NO production via the cAMP/PKA pathway *in vitro*.[12,13]

73 Black soybean is a nutrient-rich food that contains abundant
74 polyphenols in its seed coat and grain, and is widely eaten in Eastern Asian
75 countries. It contains abundant anthocyanins and flavan-3-ols including
76 epicatechin, procyanidin B2, procyanidin C1, and cinnamtannin A2 in its seed
77 coat, in contrast to yellow soybean.[14,15] Previous studies reported that
78 polyphenols contained in the black soybean seed coat had beneficial
79 physiological effects, such as antioxidant,[16] anti-obesity and anti-diabetic
80 activities.[17] In addition, a previous study has been reported that mean blood
81 pressure decreased significantly following oral administration of flavan 3-ols
82 extracted from cocoa for 2 weeks in normal rats.[18] Thus, in the present study,
83 we conducted a single-dose study of BE containing abundant flavan3-ol, and
84 tried to clarify the effect and underlying mechanism of BE on NO production
85 as one of the markers for the vascular function. Previously, we reported that
86 cinnamtannin A2, a tetrameric procyanidin, increased GLP-1 secretion in
87 mice.[19] Therefore, we hypothesized that polyphenols contained in the black
88 soybean seed coat increase NO production in vascular endothelial cells
89 through GLP-1 secretion from intestinal L cells. In the present study, we
90 investigated the effects of black soybean seed coat polyphenols on vascular
91 function by measuring NO levels. Furthermore, we explored the underlying
92 mechanisms involved including the GLP-1 related pathway.

93

94 **Materials and methods**

95 **Materials**

Black soybean seed coat extract (BE) is a product of Fujicco Co., Ltd. (Kobe, Japan). BE 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.[21] BE consisted of 6.2% epicatechin, 39.7% procyanidin (6.1% procyanidin B2, 3.4% procyanidin C1, and 0.5% cinnamtannin A2), 9.2% cyanidin 3-glucoside and others, in particular non-specified polyphenols including highly-polymerized procyanidins (degree of polymerization ≥ 5). Total amount of polyphenols in BE was 67.0% by the Folin–Denis method[22]. Exendin9-39 and exendin-4 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against p-eNOS (Ser1177), p-Akt (Ser473), p-Akt (Thr308), Akt, and β-actin were from Cell Signaling Technology (Danvers, MA, USA). Antibodies against eNOS were from Santa Cruz Biotechnology (Dallas, TX, USA). All other reagents used were of the highest grade available from commercial sources.

Animal treatment

All animal experiments were approved by the Institutional Animal Care and Use Committee (Permission number 27-05-09) and carried out according to the guidelines for animal experiments at Kobe University. Male Wistar rats aged 5 weeks (Japan SLC, Inc., Shizuoka, Japan) were maintained at 22 ± 3°C under a 12:12-h light/dark cycle. Rats were acclimatized for 7 days with free access to an AIM-93M laboratory purified diet (Research Diets, New Brunswick, NJ, USA) and tap water. They were used for the following experiments.

Experiment 1: Wistar rats were randomly divided into four groups (n=4). BE was dissolved in distilled water and orally administrated to rats at 0, 10, 20 or 50 mg/kg body weight. The rats were euthanized under anesthesia using sodium pentobarbital (1.62 mg/head, intraperitoneal injection) and sevoflurane, and their aortas were collected 60 min after the BE administration.

Experiment 2: Wistar rats were randomly divided into six groups (n=3-5) and orally administrated BE at 50 mg/kg body weight. The rats were euthanized under anesthesia as for Experiment 1 and their aortas were collected 0, 7.5, 15, 30, 60 or 120 min after BE administration.

Experiment 3: Wistar rats were randomly divided into three groups (n=4) and intravenously injected with a GLP-1 receptor agonist (exendin-4) at 5 nmol/kg body weight, according to a previous study.[23] The rats were euthanized under anesthesia as for Experiment 1 and their aortas were collected 0, 7.5 or 60 min after the injection of exendin-4.

Experiment 4: Wistar rats were randomly divided into three groups (n=5) and intraperitoneally injected with a GLP-1 receptor antagonist (exendin9-39) at 200 nmol/kg body weight according to a previous study.[24] Subsequently, the rats were orally administrated BE at 50 mg/kg body weight 30 min after the injection of exendin9-39. The rats were euthanized under anesthesia as for Experiment 1 and their aortas were collected 0, 7.5 or 60 min after BE administration. Tissues were immediately frozen and stored at −80°C until used.

Measurements of NO_2 and NO_3 in aortas

Aortic tissue was homogenized with PBS using a hand-held microtube homogenizer. The homogenate was centrifuged at 10,000 ×*g* for 20 min at 4°C. The obtained supernatant was applied to an ultrafilter membrane at 7,000 ×*g* for 30 min at 4°C to remove hemoglobin and proteins, and the sum of NO_2 and NO_3 concentrations was determined using a NO_2/NO_3 Assay kit-FX (Fluorometric) 2,3-diaminonaphthalene kit (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. Protein concentrations in each sample were quantified by Lowry's method.[25] Data are expressed as the sum of NO_2 and NO_3 concentrations per mg of protein.

Western blotting analysis

Aortic tissue was homogenized with radio-immunoprecipitation assay (RIPA) 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 (DTT), protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 5 μg/ml aprotinin) and phosphatase inhibitors (10 mM NaF and 1 mM Na_3VO_4) using a hand-held microtube homogenizer, and then left on ice for 1 h with occasional mixing. The homogenate was centrifuged at 12,000 ×*g* for 20 min at 4°C. The obtained supernatant was used as the whole protein lysate. The detection of each target protein was performed by western blotting using SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After SDS-PAGE, the separated proteins in the gels were transferred onto a polyvinylidene fluoride membrane that was incubated with blocking One (Nacalai Tesque, Kyoto,

Japan) for 1 h at room temperature. Then, the membrane was 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. The protein bands were visualized using Immuno Star® LD (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) and detected by Light-Capture II (ATTO, Tokyo, Japan). The density of a specific band was determined using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Measurement of GLP-1 in plasma

GLP-1 concentrations in plasma were measured using a LBIS GLP-1 (active) ELISA Kit (Fujifilm Wako Pure Chemical Corporation) according to the manufacturer's instructions.

Measurement of cAMP in aortas

Measurements of cAMP in aortas were performed according to a previous method.[26] Briefly, aortic tissue was homogenized with 0.4 M perchloric acid using a hand-held microtube homogenizer. The homogenate was centrifuged at 20,000 ×*g* for 10 min at 4°C, and the supernatant was neutralized by 1 M sodium acetate (pH 8.0). cAMP was analyzed using a triple quadrupole mass spectrometer (LCMS-8040, Shimadzu Corp., Kyoto, Japan) equipped with a column (L-column 2 ODS, 1.5 × 150 mm; Tokyo Metropolitan Institute for Chemical Evaluation) according to a previous method.[26] Protein concentration was determined using Lowry's method.[25] Data are expressed as the cAMP concentration per mg of protein.

Statistical analysis

Data are represented as the mean ± SE. The statistical significance of experimental observations was determined using Dunnett's multiple comparison test with the level of significance set at $p<0.05$.

Results

Effects of BE on NO production and eNOS phosphorylation in the aorta

First, we investigated whether BE promotes NO production and eNOS phosphorylation in the aorta. When the dose-dependent action of NO production by BE administration was examined (Experiment 1), BE increased NO production in the aorta dose-dependently (Fig. 1A). Of note, BE at 50 mg/kg body weight significantly increased NO production in the aorta. Regarding the upstream events, BE at 50 mg/kg body weight also increased eNOS phosphorylation in the aorta (Fig. 1B). This suggested that BE increased NO production through eNOS phosphorylation in the aorta. On the basis of these results, we used BE at 50 mg/kg body weight in the following experiments.

Fig. 1

In Experiment 2, we examined the induction of NO by BE at different timepoints, and found that BE significantly increased NO production in the aorta at 60 and 120 min after BE administration (Fig. 2A). Furthermore, eNOS phosphorylation was increased at 7.5 and 60 minutes after BE administration (Fig. 2B). These results indicated that BE has a biphasic action, i.e., early and late responses were observed, and an increase in NO production occurred about 60 min after eNOS phosphorylation.

Fig. 2

Involvement of the GLP-1/cAMP pathway in NO production

Next, we investigated whether the GLP-1/cAMP pathway was involved in the increased NO production by BE (Experiment 2). BE significantly increased GLP-1 levels in the plasma at 7.5 and 60 min after its administration (Fig. 3A) similar to that for eNOS phosphorylation. BE also increased the cAMP level in the aorta at 15 and 60 min after its administration (Fig. 3B). These results suggested that the GLP-1/cAMP pathway was involved in promoting eNOS phosphorylation after BE administration. Furthermore, as shown in Fig. 4, a GLP-1 agonist (exendin-4) significantly increased NO production in the aorta after 60 min (Experiment 3) similar to that induced by BE (Fig. 2A). This suggested that NO production induced by polyphenols in BE was, at least in part, exerted through GLP-1 secretion from intestinal cells. Of note, BE did not change the phosphorylation level of Akt in Experiment 2 (Fig. 5), although Akt is involved in the insulin-induced phosphorylation of eNOS.[8]

Fig. 3

Fig. 4

Fig. 5

Effects of a GLP-1 receptor antagonist on BE-induced NO production

To confirm whether BE-induced NO production involved the GLP-1/cAMP/eNOS pathway, we used exendin9-39 as a GLP-1 receptor antagonist (Experiment 4). Exendin9-39 significantly decreased the cAMP levels in the aorta (Fig. 6A) and inhibited BE-induced eNOS phosphorylation and NO production (Fig. 6B and C). This suggested that GLP-1 secreted from intestinal cells *in vivo* induced by polyphenols in BE is involved in the underlying mechanism of NO production.

Fig. 6

Discussion

240 Lifestyle disorders and oxidative stress cause vascular dysfunction,
241 which is significantly associated with CVD.[1] NO produced by eNOS in
242 vascular endothelial cells regulate vascular functions through vasodilation
243 and the inhibition of platelet aggregation in blood vessels.[5,6] Black soybean
244 contains abundant polyphenols in its seed coat that have various bioregulatory
245 functions. We previously reported that procyanidins, particularly
246 cinnamtannin A2, promoted GLP-1 secretion in mice.[19,27] Thus, cinnamtannin
247 A2 is a strong candidate for the effective compound in BE. GLP-1 is an
248 incretin hormone and is an upstream factor of NO production.[14-17] In this study,
249 we demonstrated that BE increased NO production in the aortas of rats (Fig.
250 1 and 2). Regarding the underlying mechanism of BE-induced NO production,
251 BE promoted the phosphorylation of eNOS in vascular endothelial cells
252 through GLP-1 secretion from intestinal cells (Fig. 1, 2, and 3). We confirmed
253 that a GLP-1 receptor antagonist (exendin9-39) inhibited BE-induced NO
254 production and eNOS phosphorylation (Fig. 6). To the best of our knowledge,
255 this is the first report that food components increase NO production in the
256 aorta through GLP-1 secretion from intestinal cells.

257 Because NO is strongly associated with the regulation of vascular
258 function,[5,6] we focused on NO production in the aorta to investigate whether
259 BE improves vascular function. NO produced by eNOS in vascular
260 endothelial cells rapidly diffuse into vascular smooth muscle cells to induce
261 muscle relaxation though the activation of sGC and cGMP.[28,29] A previous
262 study reported that the blood pressure in eNOS knockout mice was higher
263 than that of normal mice.[30] In addition, polyphenol-rich cacao powder

reduced the blood pressure in spontaneous hypertensive rats through NO production.[31] These previous results strongly support our current findings. We hypothesized that BE induces vasodilation in the aorta through NO production. BE increased NO production through eNOS phosphorylation in the aorta (Fig. 1 and 2); however, we did not address whether BE improved vascular function in this study. Future studies should investigate direct evidence for the effects of BE on vascular functions such as improved blood pressure.

In the present study, we found that BE induced eNOS phosphorylation through GLP-1 secretion as an upstream factor, although eNOS phosphorylation is regulated by several mechanisms.[5,7-9] BE had a biphasic action on activation of the GLP-1/cAMP/eNOS pathway (Fig. 1, 2 and 3). Why BE possesses this unique biphasic action might be explained by GLP-1 secretion being regulated by two different mechanisms. A previous study reported two mechanisms of GLP-1 secretion: i) the direct action of food factors on intestinal L cells in the lower digestive tract; and ii) the indirect action of food factors on intestinal L cells via vagal signaling from the upper digestive tract.[32] In the latter case, the secretion of GLP-1 into the plasma occurred rapidly. In the current study, cAMP levels and eNOS phosphorylation increase in response to GLP-1 secretion after BE administration with the biphasic manners (Fig. 2B and 3), though statistical significant increase was not obtained on eNOS phosphorylation at 7.5 min and cAMP level at 60 min, may be due to the individual differences in the responsibility of animals. To increase the NO production, it may need 50-60

min after the increase in the GLP-1 secretion, cAMP level, and eNOS phosphorylation (Fig. 2B and 3), i.e. the first and second increases in these upstream events may lead to an increase of NO production 60 min and 120 min after the administration of BE (Fig 2A). Moreover, a GLP-1 receptor antagonist, exendin9-39, inhibited the action of BE (Fig. 6), indicating that exendin9-39 blocked GLP-1-related pathways. Therefore, BE may also affect vagal signaling from the upper digestive tract indirectly and L cells in the lower digestive tract directly to activate the GLP-1/cAMP/eNOS pathway.

In this study, we did not address the detailed molecular mechanisms by which BE directly and indirectly increased GLP-1 secretion. A previous study reported that polyphenols increased GLP-1 secretion both *in vitro* and *in vivo*,[33] but the underlying mechanism of GLP-1 secretion remains poorly understood. Recently, it was reported that curcumin and delphinidin 3-rutinoside increased GLP-1 production via the Ca^{2+}/calmodulin-dependent kinase II pathway *in vitro*.[34,35] In addition, another report suggested that polyphenols increased GLP-1 secretion via hormonal factors or microbiota.[36] Further study is needed to clarify the molecular mechanism involved in the effects of polyphenols in BE on GLP-1 production.

Akt activates eNOS[5,8] and previous studies reported that procyanidins increased NO production via the Akt/eNOS pathway *in vitro*.[37-39] We have previously reported that (−)-epicatechin and procyanidins in BE were absorbed from the intestinal tract and appeared in the plasma after the oral administration of BE to mice.[21] Thus, we expect that some polyphenols in BE

312 are absorbed and activate the Akt/eNOS pathway directly. However, contrary
313 to our expectations, BE did not increase the phosphorylation levels of Akt
314 (Fig. 5). This suggested that GLP-1 secreted by non-absorbable polyphenols
315 in BE mainly contributes to the promotion of NO production *in vivo*. However,
316 there remains a possibility that certain absorbable polyphenols in BE act on
317 vascular endothelial cells directly after their absorption from the intestinal
318 tract. It needs further study to clarify this issue in future. In addition, BE
319 contains 33% of unknown ingredients except for polyphenols, but their
320 chemical characteristics were unclear yet. Therefore, it remains possibility
321 that these unknown ingredients are involved in the NO production.

322 In conclusion, BE promotes NO production in the aorta of rats.
323 Regarding the putative underlying mechanism, GLP-1 secreted from
324 intestinal cells by polyphenols in BE activated eNOS in vascular endothelial
325 cells. Our findings show that polyphenols in the black soybean seed coat may
326 prevent CVD by improving vascular function.

327

328 **Abbreviations used**

329 CVD; cardiovascular diseases, NO; nitric oxide, BE; black soybean extract,
330 GLP-1; glucagon-like peptide-1, cAMP; cyclic adenosine monophosphate,
331 eNOS; endothelial nitric oxide synthase, Akt; phosphoinositide 3-kinase,
332 PKA; protein kinase A, sGC; soluble guanylate cyclase, cGMP; cyclic
333 guanosine monophosphate

334

335 **Conflicts of interest**

Fujicco Co. Ltd. partly funded the investigations described in the current manuscript. F.N., T.M., and T.S. are employees of Fujicco Co. Ltd. They contributed to the preparation of extract (BE) and the study design.

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492 **Figure legends**

493 **Figure 1. Dose-dependent changes in NO production and eNOS**
494 **phosphorylation by BE in the aortas of rats.**

495 Wistar rats were orally administrated BE at 0, 10, 20, 50 mg/kg body weight.
496 NO production (A) and eNOS phosphorylation (B) in aortas were measured
497 60 min after BE administration. Values are the mean ± SE (n=4), *$p < 0.05$
498 vs 0 mg/kg B.W. (Dunnett's multiple comparison test).

499

500 **Figure 2. Time-dependent changes in NO production and eNOS**
501 **phosphorylation by BE in the aortas of rats.**

502 Wistar rats were orally administrated BE at 50 mg/kg body weight. NO
503 production (A) and eNOS phosphorylation (B) in aortas were measured at 0,
504 7.5, 15, 30, 60 and 120 min after BE administration. Values are the mean ±
505 SE (n=5), *$p < 0.05$ vs 0 min (Dunnett's multiple comparison test).

506

507 **Figure 3. Changes in GLP-1 levels in the plasma and cAMP levels in the**
508 **aortas of rats after BE administration.**

509 Wistar rats were orally administrated BE at 50 mg/kg body weight. GLP-1
510 levels in the plasma (A) and cAMP levels in aortas (B) were measured at 0,
511 7.5, 15, 30, 60 and 120 min after BE administration. Values are the mean ±
512 SE (n=3-5), *$p < 0.05$ vs 0 min (Dunnett's multiple comparison test).

513

514 **Figure 4. A GLP-1 receptor agonist, exendin-4, increases NO production**
515 **in rat aortas.**

Wistar rats were intravenously injected with a GLP-1 receptor agonist (exendin-4) at 5 nmol/kg body weight. NO production in aortas was measured at 0, 7.5 and 60 min after injection. Values are the mean ± SE (n=4), *$p < 0.05$ vs 0 min (Dunnett's multiple comparison test).

Figure 5. Changes in the phosphorylation levels of Akt by BE in the aortas of rats.

Wistar rats were orally administrated with BE at 50 mg/kg body weight. Phosphorylation levels of Akt in aortas were measured at 0, 7.5, 15, 30, 60 or 120 min after BE administration. Values are the mean ± SE (n=5), Statistical significance was estimated by Dunnett's multiple comparison test.

Figure 6. Changes in cAMP levels, NO production and eNOS phosphorylation by BE after pretreatment with a GLP-1 receptor antagonist, exendin 9-39, in rat aortas.

Wistar rats were orally administrated BE at 50 mg/kg body weight 30 min after the intraperitoneal injection of a GLP-1 receptor antagonist (exendin9-39) at 200 nmol/kg body weight. cAMP levels (A), eNOS phosphorylation (B) and NO production (C) in aortas were measured at 0, 7.5 and 60 min after BE administration. Values are the mean ± SE (n=5), *$p < 0.05$ vs 0 min (Dunnett's multiple comparison test).

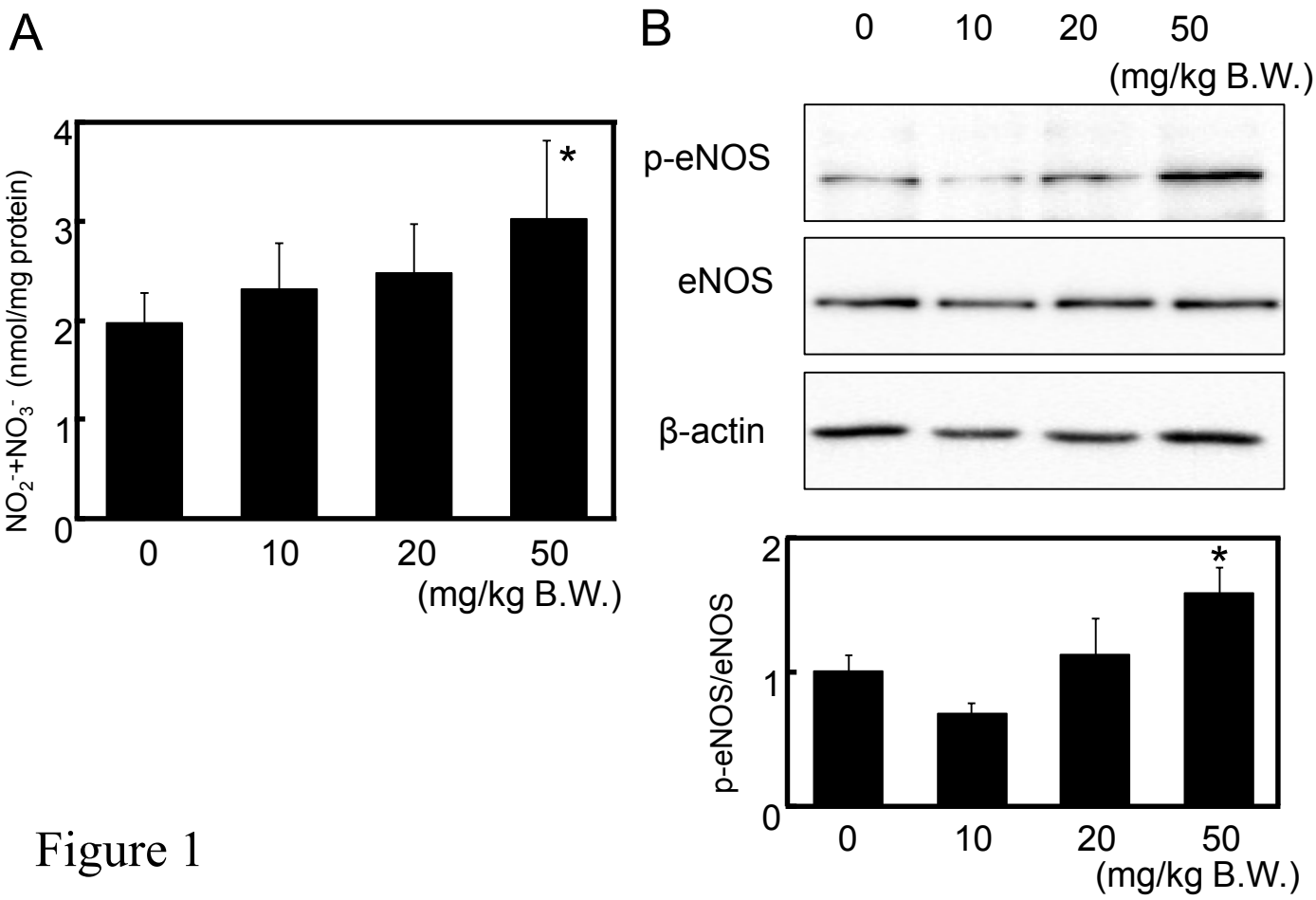

A
$NO_2^-+NO_3^-$ (nmol/mg protein)
0
1
2
3
4
*
0
10
20
50
(mg/kg B.W.)
B
0
10
20
50
(mg/kg B.W.)
p-eNOS
eNOS
β-actin
p-eNOS/eNOS
0
1
2
*
0
10
20
50
(mg/kg B.W.)

Figure 1

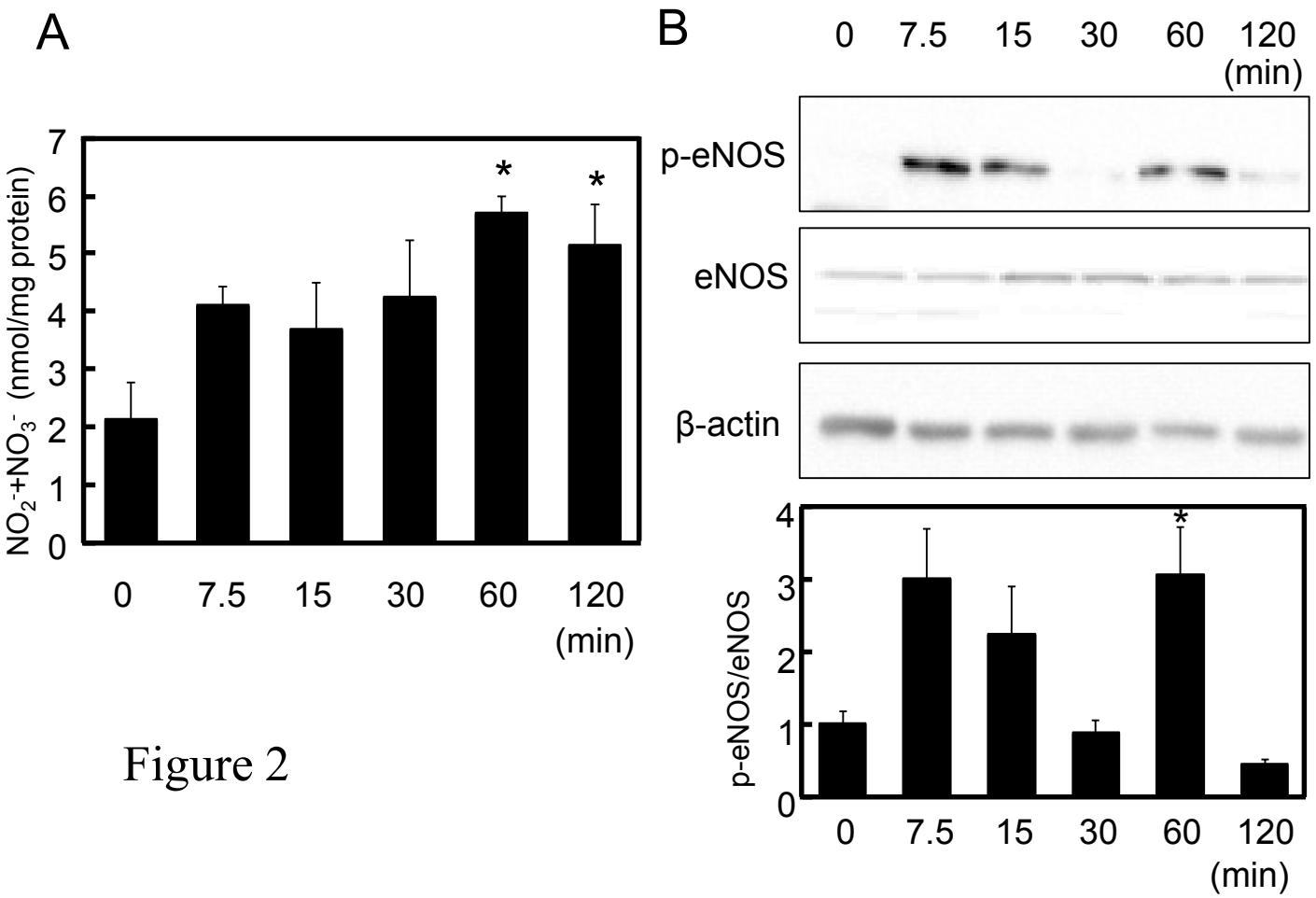

A
B
0 7.5 15 30 60 120
(min)
p-eNOS
eNOS
β-actin
$NO_2^-+NO_3^-$ (nmol/mg protein)
p-eNOS/eNOS
*
*
*
0 7.5 15 30 60 120
(min)

Figure 2

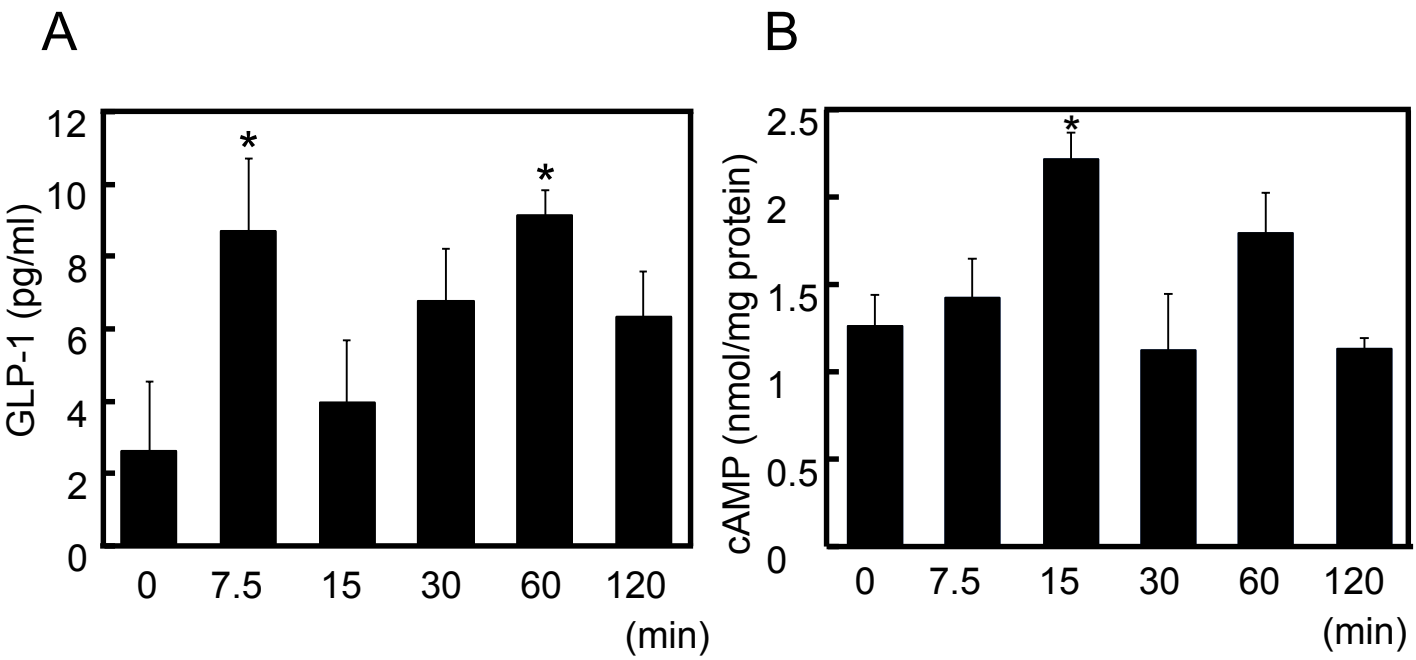

A
B
GLP-1 (pg/ml)
12
10
8
6
4
2
0
*
*
0
7.5
15
30
60
120
(min)
cAMP (nmol/mg protein)
2.5
2
1.5
1
0.5
0
*
0
7.5
15
30
60
120
(min)

Figure 3

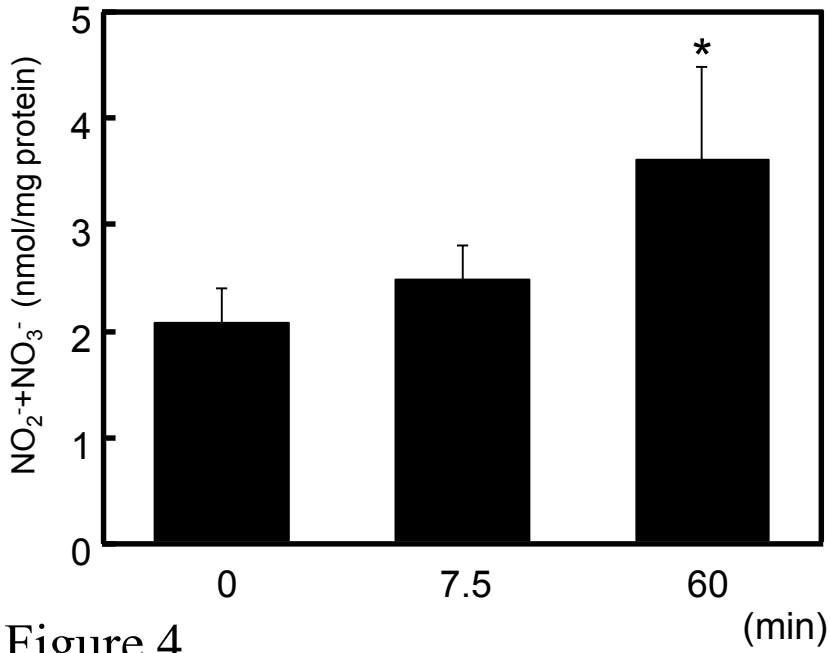
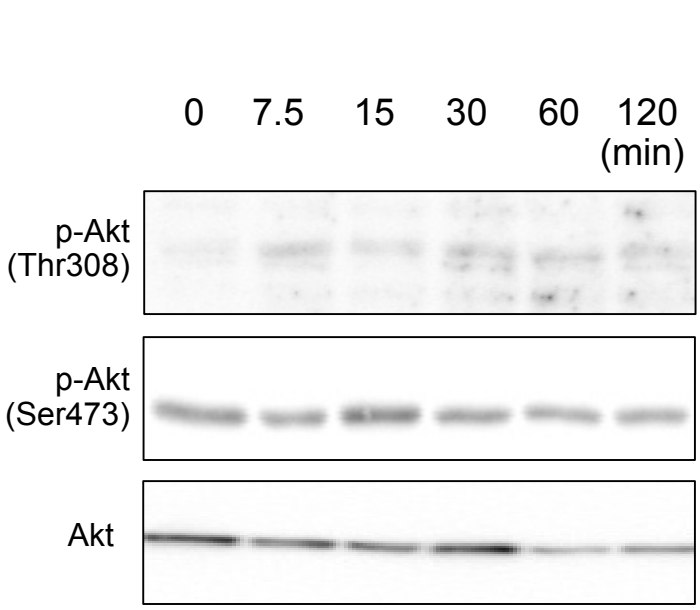


Figure 4


0
7.5
15
30
60
120
(min)
p-Akt
(Thr308)
p-Akt
(Ser473)
Akt

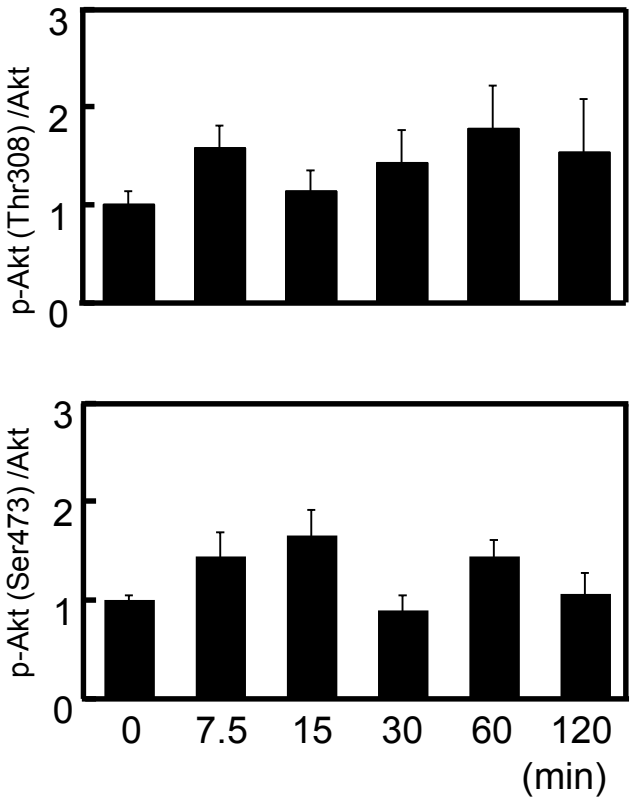

3
2
1
0
p-Akt (Thr308) /Akt
p-Akt (Ser473) /Akt
0
7.5
15
30
60
120
(min)

Figure 5

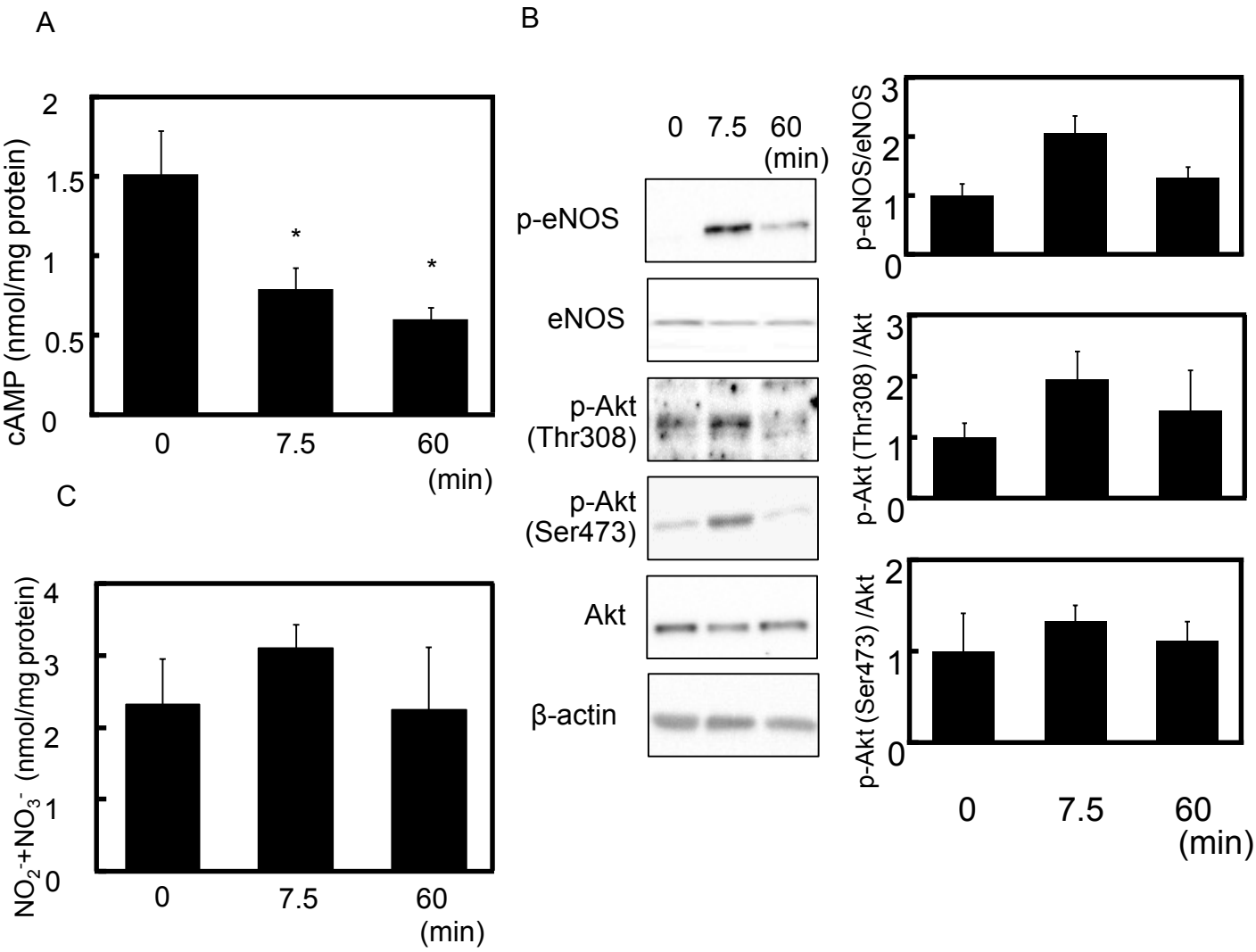

A
cAMP (nmol/mg protein)
2
1.5
1
0.5
0
*
*
0
7.5
60
(min)
B
0 7.5 60
(min)
p-eNOS
eNOS
p-Akt
(Thr308)
p-Akt
(Ser473)
Akt
β-actin
p-eNOS/eNOS
p-Akt (Thr308) /Akt
p-Akt (Ser473) /Akt
3
2
1
0
0
7.5
60
(min)
C
NO2-+NO3- (nmol/mg protein)
4
3
2
1
0
0
7.5
60
(min)

Figure 6