Black Soybean Seed Coat Extract Prevents Hydrogen Peroxide-Mediated Cell Death via Extracellular Signal-Related KinaseSignalling in HepG2 Cells

Naoto HASHIMOTO1,2, Tomoyuki OKI1, Kazunori SASAKI1, Ikuo SUDA1 and Shigenori OKUNO1

1 Crop and Agribusiness Research Division, NARO Kyushu Okinawa Agricultural Research Center, Saga 842-8556, Japan
2 Upland Farming Resource Research Division, NARO Hokkaido Agricultural Research Center, Minami 9-4, Shinsei, Kasai, Hokkaido 082-0071, Japan
(Received August 20, 2014)

Summary Oxidative stress reduces cell viability and contributes to disease processes. Flavonoids including anthocyanins and proanthocyanidins reportedly induce intracellular antioxidant defence systems. Thus, in this study, we examined the antioxidant effects of a commercial extract from black soybean seed coats (BE), which are rich in anthocyanin and proanthocyanidin, and investigated the associated intracellular mechanisms in HepG2 cells. HepG2 cells treated with hydrogen peroxide (HPO) showed 60% viability, whereas pretreatment with BE-containing media for 2 h ameliorated HPO-mediated cell death by up to 90%. Pretreatment with BE for 2 h partially blocked HPO-mediated activation of ERK in HepG2 cells, and that for 1 h led to a 20% increase in intracellular total protein phosphatase (PP) activity, which is known to deactivate protein kinases. These results indicate that BE prevents HPO-mediated cell damage by inhibiting ERK signalling, potentially via PPs.

Key Words black soybean coat extract, ERK, HepG2, protein phosphatase

Black soybeans have black seed coats that are abundant in flavonoids such as anthocyanin and proanthocyanidin (PAC) (1, 2), indicating that black soybean can provide these flavonoids in addition to components of the common soybean. Revelation of the physiological functions of flavonoids of black soybean may contribute to consumption of black soybean, an increase in the income of farm producers, and a consequent stabilization of the agricultural industry. These flavonoids are minor nutrients but considered potent natural scavengers of reactive oxygen species (ROS) (2) and have been shown to reduce the risk of liver injury (3), improve blood pressure (4) and ameliorate metabolic abnormalities (5, 6), which are often related to oxidative stress. Additionally, anthocyanin from black soybean prevented oxidative stress in neurocytes in vitro (7, 8). ROS are reportedly involved in many diseases and stimulate mitogen-activated protein kinase (MAPK) pathways (7, 9, 10) including extracellular signal-related kinase (ERK), p38 and c-jun N-terminal kinase (JNK). Signals through these pathways are transmitted by sequential protein phosphorylation reactions and are blocked by dephosphorylation by protein phosphatases (PPs) (11–13). While activation of MAPKs can lead to cell death (7), MAPKs have been shown to be involved in anti-oxidant defence systems (14), cellular survival (6) and proliferation (15).

In our previous studies, anthocyanin fractions from coloured potatoes induced superoxide dismutase-2 mRNA expression via the ERK 1/2 pathway in HepG2 human hepatoma cells (16), and in other studies, flakes of coloured potatoes prevented hepatic injury following galactosamine injections (3). However, the mechanisms by which black soybean seed coat extract (BE) defends against hepatic injury remain poorly characterised. In this study, we first confirmed the antioxidant activity of BE and then investigated the relationships between the antioxidant effects of BE and activities of MAPKs and PPs in HepG2 cells.

Materials and Methods Primary hepatocytes may be suitable for a liver model to speculate about antioxidant mechanisms in the liver (17), in which parts of liver functions reportedly decrease gradually (18), and, in view of animal welfare, sacrifice of animals to obtain primary hepatocytes should be replaced by other models if the models do not have crucial defects. As a model of mammalian hepatocytes, therefore, HepG2 human hepatoma cells were used in this study because HepG2 cells stably express MAPK and related proteins and show responses to oxidative stress similarly to those of in vivo studies despite the lack of some liver functions (6, 10, 19). Cells were grown in DMEM (Life Technologies Japan Co., Tokyo, Japan) supplemented with 10% foetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C in an atmosphere of 5% CO2. Before the experiments, HepG2 cells were trypsinised, seeded onto plates at a density of 5×104 cells/cm2 and incubated for >16 h in culture medium. Cells were pre-treated in...
DMEM containing 50 μg/mL Kuraromarin-10® (Functional Material Laboratory Co., Ltd., Tokyo, Japan), a preparation of anthocyanin and procyanidin from black soybean seed coat, as BE. After treatments with BE-containing medium, cells were washed twice with adequate volumes of phosphate-buffered saline and were then treated with HPO-containing DMEM for up to 24 h.

The total contents of anthocyanin and PAC in BE were measured using pH differential (20) and vanillin-sulphate methods (21), respectively (total anthocyanin and PAC contents in the BE powder in this study were 67 mg-cyanidin 3-glucoside equivalents/g and 406 mg-(+)-catechin equivalents/g, respectively). PAC compositions of the BE powder were determined using HPLC analyses as follows: Extracts were eluted through a Discovery HS PEG column (250×4.6 mm, 5 μm; Sigma-Aldrich Japan, Co., LLC., Tokyo, Japan) with 0.1% formic acid (mobile phase A) and acetonitrile (mobile phase B) under a linear gradient from 0% B at 0 min to 50% B at 50 min at a flow rate of 1.0 mL/min and a column temperature of 35°C. Fluorescence of flavonoids was detected at excitation and emission wavelengths of 276 and 316 nm, respectively. Individual contents of catechin, epicatechin, procyanidin B2, and procyanidin C1 in the BE powder were 9, 92, 115, and 57 mg/g, respectively.

Cell viability was estimated using MTT assays. After treatment with HPO for 24 h, MTT solution was added to the media to a final concentration of 227 μg/mL and cells were incubated for 1 h at 37°C in a CO2 incubator. Subsequently, media was discarded, cells were washed once in PBS and formazan contents were measured in dimethylsulfoxide at an absorbance of 550 nm.

Immunoblotting experiments were performed as previously described (22). In brief, cell lysis buffer was added to treated cells and lysates were sonicated and centrifuged at 11,000 ×g for 5 min at 4°C and supernatants were collected and boiled with sample buffer for 10 min prior to immunoblotting. Proteins were visualized using Western Blot Quant HRP Substrate (Takara Bio Inc., Tokyo, Japan) according to the manufacturer’s instructions. Antibodies against MAP kinase 1/2 (ERK 1/2) and phospho MAP kinase 1/2 (p-ERK) were purchased from Upstate Inc. (Charlottesville, VA). Antibodies specific for p-JNK and JNK 1/3 were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

Intracellular PP activity was then assayed using colorimetric Sensosbyte pNPP Protein Phosphatase Assay kits (AnaSpec Inc., Fremont, CA) according to the manufacturer’s instructions. Treated HepG2 cells were lysed in 2-phospho-glyceride- and orthovanadate-free cell lysis buffer and were sonicated. Lysates were then centrifuged at 11,000 ×g for 5 min at 4°C, and supernatants were collected and assayed for PP activity and protein concentrations using Pierce BCA protein assay kits (Thermo Fisher Scientific Inc., Waltham, MA) according to the manufacturer’s instructions.

Band intensities were quantified using Image J version 1.37 software. Relative abundances (activities) of p-ERK 1/2 and p-JNK proteins were normalized to those of ERK 1/2 and JNK, respectively. Activities of PP were expressed relative to protein concentrations of cell lysates. Values were expressed as mean ± standard errors of the mean (SEM) of 6 or 8 replicates, and were expressed relative to vehicle treated controls. Two-way (or partly one-way) ANOVA was performed to assess differences between treatments, followed by Tukey’s HSD multiple comparison test using the EZR software (Saitama Medical Center, Jichi Medical University, Saitama, Japan) which is a graphical user interface for R (The R Foundation for Statistical Computing, version 2.13.0) (23). Differences between treatment groups were considered significant when p<0.05.

Fig. 1. Cell viability after pretreatment with black soybean seed coat extract (BE). HepG2 cells were pretreated with or without BE (50 μg/mL) for 2 h. Cells were then washed twice in PBS and treated with hydrogen peroxide (HPO, 500 μM) for 24 h and cell viability was assessed using MTT assays. Data are expressed as the mean ± SEM of 8 samples. Significant differences are indicated by differing letters (p<0.05).

Results and Discussion

In the present study, we showed that the BE prevented a decrease in cell viability from HPO treatment, in which the ERK 1/2 pathway may be involved in HepG2 cells.

Cell viability after treatments with BE and/or HPO are shown in Fig. 1. In the absence of HPO, cell viability did not differ significantly between BE-pretreated and untreated cells (1.00±0.06 without BE and 1.05±0.05 with BE). However, in the presence of HPO, viability of BE-pretreated cells was significantly higher than that in the absence of BE pretreatment (0.87±0.08 with BE and 0.61±0.05 without BE). These results indicate that BE prevents HPO-mediated cell death. BE is abundant in anthocyanins and PACs, which are considered natural scavengers of reactive oxygen species (2). However, BE-treated cells were washed thoroughly prior to exposure to HPO. The antioxidant activity of BE against HPO treatment may reflect a cellular defence system rather than direct scavenging of HPO by BE in this study. The preparation of BE in this study seems to contain not only flavonoids but also a diluent. This diluent, but not the flavonoids, was possibly involved in antioxidant activity.

In a preliminary study, however, an anthocyanin-rich fraction separated from BE by a column chromatography was assayed with a cell viability test to determine the influence of the diluent on the antioxidant system, and we found the anthocyanin-rich fraction effective (data not shown). These data did not contradict those of...
our previous studies (3, 16). Meanwhile, contents and compositions of anthocyanins and PACs in BE will differ depending on year, area, race, and other factors, suggesting that repeatability and reproducibility should be assessed in further studies.

Previous reports demonstrate oxidative stress signaling via the MAPK pathway (7, 9, 12). In our preliminary experiment, we found that inhibition of the ERK pathway, but not the p38 pathway, ameliorated cell viability reduced by HPO in HepG2 (data not shown). These results are in agreement with previous studies (7, 14) and suggest that the ERK pathway but not the p38 pathway is associated with reduced cell viability under conditions of oxidative stress. Thus, we determined phosphorylation levels of ERK 1/2 and JNK after treatments with BE and/or HPO. As shown in Fig. 2a, in the absence of HPO, ERK 1/2 phosphorylation levels did not differ between BE- and vehicle-pretreated cells (0.88±0.10 and 1.00±0.11, respectively). In contrast, after treatment with HPO for 1 h, phosphorylated ERK levels were lower in BE-pretreated cells (3.59±0.14) than in vehicle-pretreated cells (5.19±0.27). Phosphorylation levels of JNK did not differ significantly between treatment conditions (Fig. 2b). These results indicate that oxidative stress excessively activates ERK but not JNK in HepG2 cells and that pretreatment with BE partly prevents excessive activation of ERK (Fig. 2). However, the MAPK activities at one time point cannot completely explain the results in Fig. 1.

To investigate further mechanisms, we determined time-dependent intracellular activities of PPs after treatment with BE because PPs deactivate MAPKs in cells (11, 13). As shown in Table 1, PP activity in BE-treated cells was higher at −2 h (or 1 h after BE treatment) than at −2 h (start of this experiment). At −1 h, PP activity was 13% higher in BE-treated cells compared with the vehicle-treated cells. These results suggest that PPs were induced by BE treatment within 1 h in HepG2 cells. In our previous study, an anthocyanin fraction from purple potato activated ERK within 15 min in HepG2 cells (16). Although PPs are known to silence MAPK signalling, activated ERK reportedly induces and activates PPs (13, 24). Hence, BE may activate PPs via the ERK pathway and may suppress excess activation of ERK by HPO.

Similar to a previous study (25), PP activity was gradually decreased after HPO treatment and was significantly lower than that with vehicle treatment at 2 h (Table 1).

Table 1. Changes in relative activities of protein phosphatase in HepG2 cells.

| Time | BE (−) | BE (+) |
|------|-------|-------|
|      | HPO (−) | HPO (+) | HPO (−) | HPO (+) |
| −2 h | 1.00±0.05 | — | — | — |
| −1 h | 1.06±0.05 | — | 1.19±0.01* | — |
| 0 h  | 1.12±0.05 | 0.95±0.03 | 1.03±0.05 | — |
| 1 h  | 1.06±0.02 | 0.76±0.02† | 1.12±0.02 | 0.92±0.01† |
| 2 h  | 0.94±0.03 | — | 0.93±0.03 | 0.78±0.02† |

Cells were pretreated with black soybean seed coat extract (BE, 50 µg/mL) for 2 h (−2−0 h time points) and then treated with hydrogen peroxide (HPO, 500 µM) for 2 h (0−2 h time points). Data were normalized to protein concentrations, are expressed relative to the −2 h control and are presented as means±SEM of 6 replicates.

*†Significant difference from the value of BE(−) treatment and from the value of HPO(−) treatment within the same time point, respectively (p<0.05), which was assessed by one-way ANOVA at −1 h and 0 h and two-way ANOVA at 1 h and 2 h, followed by Tukey’s HSD multiple comparison test.
suggested a time-dependent increase in MAPK activities. However, reduction of PP activity at 1 h after HPO treatment may not completely explain ERK activation in Fig. 2a because BE pretreatment did not affect PP activity in HPO-treated cells at 1 h (Table 1). Previous studies have demonstrated that protein kinases such as MAPK kinase or MAPK kinase kinase are located upstream of ERK (13). In HepG2 cells, BE treatment activated PP within 1 h, which may inactivate upstream and downstream protein kinases of ERK prior to oxidative stress, suppressing subsequent HPO-mediated ERK signalling. This hypothesis can also explain unchanging JNK activity at 1 h (Fig. 2b) because HPO treatment did not seem to stimulate JNK signalling. Therefore, the induction of PP activity by BE at −1 h (Table 1) may be central to its ability and reproducibility of the effects of BE should be also assessed.

In conclusion, BE ameliorated cell death induced by oxidative stress, which may be due to passage through ERK signalling. Subsequent experiments indicated that PP activities are possibly involved during the early stages of BE treatment. However, relationships between ERK and PP activities remain unclear and the associated mechanisms will be elucidated in future studies. Repeatability and reproducibility of the effects of BE should be also assessed.

Acknowledgments
This work was partly supported by JSPS Kakenhi Grant Number 25450196 and grants-in-aid from The Ministry of Agriculture, Forestry and Fisheries of Japan. The authors would like to thank Enago (www.enago.jp) for the English language review.

Conflict of Interest
The authors declare that they have no conflict of interest.

REFERENCES
1) Ito C, Oki T, Yoshida T, Nanba F, Yamada K, Toda T. 2013. Characterisation of proanthocyanidins from black soybeans: isolation and characterisation of proanthocyanidin oligomers from black soybean seed coats. Food Chem 141: 2507–2512.
2) Jeng TL, Shih YJ, Wu MT, Sung JM 2010. Comparisons of flavonoids and anti-oxidative activities in seed coat, embryonic axis and cotyledon of black soybeans. Food Chem 123: 1112–1116.
3) Han KH, Hashimoto N, Shimada K, Sekikawa M, Noda T, Yamauchi H, Hashimoto M, Chiji H, Topping DL, Fukushima M. 2006. Hepatoprotective effects of purple potato extract against D-galactosamine-induced liver injury in rats. Biosci Biotechnol Biochem 70: 1432–1437.
4) Shindo M, Kasai T, Abe A, Kondo Y. 2007. Effects of dietary administration of plant-derived anthocyanin-rich colors to spontaneously hypertensive rats. J Nutr Sci Vitaminol 53: 90–93.
5) Sun CD, Zhang B, Zhang JK, Xu CJ, Wu YL, Li X, Chen KS. 2012. Cyanidin-3-glucoside-rich extract from Chinese bayberry fruit protects pancreatic β cells and ameliorates hyperglycemia in streptozotocin-induced diabetic mice. J Med Food 15: 288–298.
6) Lee SE, Yang H, Jeong SI, Jin YH, Park CS, Park YS. 2012. Induction of heme oxygenase-1 inhibits cell death in crotonaldehyde-stimulated HepG2 cells via the PKC-β/p38-Nrf2 pathway. PLoS One 7: e41676.
7) Kim SM, Chung MJ, Hu TJ, Choi HN, Jang SJ, Kim SO, Chun MH, Do SJ, Choo YK, Park YI. 2012. Neuroprotective effects of black soybean anthocyanins via inactivation of ASK1-JNK/p38 pathways and mobilization of cellular sialic acids. Life Sci 90: 874–882.
8) Bhuiyan ML, Kim JY, Ha TJ, Kim SY, Cho KO. 2012. Anthocyanins extracted from black soybean seed coat protect primary cortical neurons against in vitro ischemia. Biol Pharm Bull 35: 999–1008.
9) Poli G, Biasi F, Leonardi Z. 2013. Oxysterols in the pathogenesis of major chronic diseases. Redox Biol 1: 125–130.
10) Bak MJ, Jun M, Jeong WS. 2012. Antioxidant and hepatoprotective effects of the red ginseng essential oil in H2O2-treated HepG2 cells and CCL4-treated mice. Int J Mol Sci 13: 2314–2330.
11) Shah S, King EM, Chandrasekhar A, Newton R. 2014. Roles for the mitogen-activated protein kinase (MAPK) phosphatase, DUSP1, in feedback control of inflammatory gene expression and repression by dexamethasone. J Biol Chem 289: 13667–13679.
12) Ng IH, Yeap YY, Ong LS, Jans DA, Bogoyevitch MA. 2014. Oxidative stress impairs multiple regulatory events to drive persistent cytokine-stimulated STAT3 phosphorylation. Biochim Biophys Acta 1843: 483–494.
13) Wancket LM, Frazier WJ, Liu Y. 2012. Mitogen-activated protein kinase phosphatase (MKP)-1 in immunity, physiology, and disease. Life Sci 90: 237–248.
14) Hwang YP, Choi JH, Choi JM, Chung YC, Jeong HG. 2011. Protective mechanisms of anthocyanins from purple sweet potato against tert-butyl hydroperoxide-induced hepatotoxicity. Food Chem Toxicol 49: 2081–2089.
15) Xiao J, Wang J, Xing F, Han T, Jiao R, Liang EC, Fung ML, So KE, Tiope GL. 2014. Zeaxanthin dipalmitate therapeutically improves hepatic functions in an alcoholic fatty liver disease model through modulating MAPK pathway. PLoS One 9: e95214.
16) Hashimoto N, Noda T, Kim SJ, Yamauchi H, Takigawa T, Matsuura-Endo C, Suzuki T, Han KH, Fukushima M. 2010. Colored potato extracts induce superoxide dismutase-2 mRNA via ERK1/2 pathway in HepG2 cells. Plant Foods Hum Nutr 65: 266–270.
17) Essid E, Dernawi Y, Petzinger E. 2012. Apoptosis induction by OTA and TNF-α in cultured primary rat hepatocytes and prevention by silibinin. Toxicol 4: 1139–1156.
18) Brandon EE, Raap CD, Meijerman I, Beijnen JH, Schellens JH. 2003. An update on in vitro test methods in human hepatic drug biotransformation research: pros and cons. Toxicol Appl Pharmacol 189: 233–246.
19) Alia M, Ramos S, Mateos R, Granado-Serrano AB, Bravo L, Goya L. 2006. Quercetin protects human hepatoma HepG2 against oxidative stress induced by tert-butyl hydroperoxide. Toxicol Appl Pharmacol 212: 110–118.
20) Lee J, Durst RW, Wrolstad RE. 2005. Determination of total monomeric anthocyanin pigment content of fruit juices, beverages, natural colorants, and wines by the pH differential method: collaborative study. J AOAC Int 88: 1269–1278.
21) Oki T, Masuda M, Kobayashi M, Nishiba Y, Furuta S,
Suda I, Sato T. 2002. Polymeric procyanidins as radical-scavenging components in red-hulled rice. *J Agric Food Chem* **50**: 7524–7529.

Hashimoto N, Hara H. 2004. Dietary branched-chain amino acids suppress the expression of pancreatic amylase mRNA in rats. *Biosci Biotechnol Biochem* **68**: 1067–1072.

Kanda Y. 2013. Investigation of the freely available easy-to-use software ‘EZR’ for medical statistics. *Bone Marrow Transplant* **48**: 452–458.

Ayroldi E, Cannarile L, Miglioriti G, Nocentini G, Delfino DV, Riccardi C. 2012. Mechanisms of the anti-inflammatory effects of glucocorticoids: genomic and non-genomic interference with MAPK signaling pathways. *FASEB J* **26**: 4805–4820.

Antony R, Lukiw WJ, Buzan NG. 2010. Neuroprotectin D1 induces dephosphorylation of Bcl-xL in a PP2A-dependent manner during oxidative stress and promotes retinal pigment epithelial cell survival. *J Biol Chem* **285**: 18301–18308.