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RESEARCH NOTE

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Anti-Proliferative and Anti-Carcinogenic Enzyme-inducing Activities of Delphinidin in Hepatoma Cells

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Abstract Delphinidin, an aglycone form of anthocyanins, was demonstrated to have anti-carcinogenic potential. The compound at 50 μg/mL caused a significant increase of quinone reductase activity, an anti-carcinogenic marker enzyme, in mouse hepatoma cell lines (Hepa1c1c7 and BPRc1). Delphinidin enhanced the expression of other detoxifying or antioxidant enzymes including glutathione *S*-transferase, gamma-glutamylcysteine synthetase, heme oxygenase 1, and glutathione reductase. It suppressed the proliferation of murine hepatoma cells in a dose-dependent manner, with approximately IC_{50} of 70 μg/mL. These results suggest that delphinidin might be useful for cancer prevention.

Keywords: delphinidin, antioxidant, cancer prevention, quinone reductase

Introduction

Anthocyanins, the dietary phenolic plant metabolites belonging to the flavonoid family, are the soluble pigment-glycosides which have the black, blue, and purple colors, depending on pH (1, 2). A large number of researches suggested that anthocyanins have health-promoting benefits including anti-oxidative and anti-inflammatory effects. They are also known to improve the immunity, prevent several chronic diseases and delay aging. Black soybean that has been used as folk medicine as well as food ingredient in Korea is one of the richest sources of anthocyanins (2).

It has been reported that the contents of delphinidin-3-glucoside, cyanidin-3-glucoside, petunidin-3-glucoside, and total anthocyanins in seed coats of 10 varieties of black soybean were found in the ranges of 0-3.71, 0.94-15.98, 0-1.41, and 1.58-20.18 mg/g, respectively (3). The contents of anthocyanins per 100 g black soybean are 10-fold higher than the whole anti-oxidative contents present in the orange of the same weight and similar to the grape, apple, and cranberry (4). These suggest that black soybean coat could be an abundant source of anthocyanins for food pigments endowed with multiple functions, such as antioxidant properties, anti-carcinogenic and immunostimulating effects (3).

Exposure of cells to xenobiotics and antioxidants leads to the induction of a battery of genes that include detoxification and/or antioxidant enzymes. The induced enzymes and proteins provide critical protection against oxidative stress. The antioxidant enzymes that are induced include NAD(P)H:quinone oxidoreductase (NQO or QR, EC1.6.99.2), glutathione *S*-transferase (GST, EC2.5.1.18), heme oxygenase 1 (HO-1, EC 1.14.99.3), gamma-glutamylcysteine synthetase (γ-GCS, EC 6.3.2.2) and others. QR is a flavoprotein that competes with cytochrome P450 reductase and catalyzes two-electron reduction and detoxification of quinones and other redox cycling compounds (5). The enzyme is widely distributed in human tissues and induced by a plethora of chemically diverse inducers that activates the regulatory antioxidant response element (ARE) commonly present in the antioxidant enzyme genes (6). Furthermore, it is well established that most of QR inducers exert anti-carcinogenic activity via up-regulation of phase 2 enzymes involved in carcinogen metabolism. In this study we hypothesized that delphinidin, one of anthocyanidins with antioxidant activity, would induce phase 2 enzymes via ARE-mediated pathway.

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Materials and Methods

Reagents and chemicals Reagents for cell culture medium including alpha-modified minimum essential medium (α-MEM) were obtained from Gibco-BRL (Rockville, MD, USA), fetal bovine serum (FBS) from Hyclone (Logan, UT, USA) and 0.25% trypsin from Gibco-BRL. Delphinidin and cyanidin were from Indofine Chemical Inc. (Hillsborough, NJ, USA). Other reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and Merck (Darmastadt, Germany). Reagents for gel electrophoresis and Western blotting were purchased from Bio-Rad (Hercules, CA, USA). Water used for all procedures was purified by a water purification system, Milli-Q, with a resistance of 18.2 MΩ. Primary antibodies and horse radish peroxidase (HRP)-conjugated secondary antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), Supersignal (Pierce, Rockford, IL, USA) was used as the chemiluminescent substrate.

Preparation of crude anthocyanins from black soybean seed coat The seed coat of black soybeans was peeled

manually after heating at 105°C for 2 hr. The separated seed coats were extracted 3 times with 1,000 mL of 1% HCl-40% CH_3OH at 4°C for 24 hr. The combined extracts were filtered and concentrated at 30°C *in vacuo* (3).

Cell lines and cell culture Mouse hepatoma Hepa1c1c7 and its mutant BPRc1 cells were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured in α-MEM containing 10% FBS and grown at 37°C under a 5% CO_2-95% air atmosphere.

Anti-proliferative activity assay Cytotoxicity of samples were evaluated by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (7). Briefly, mouse hepatoma cells (5×10^3 cells/well) were inoculated into 96-well plates (Nunc, Rochester, NY, USA) cultured in fresh α-MEM containing 10% FBS for 4 hr, followed by sample addition. After cells were incubated for 72 hr, MTT working solution prepared by dilution of MTT stock solution (5 mg/mL) by 5-fold was added to each well (50 μL/well). The plate was incubated at 37°C for 4 hr. The wells were then drained completely, and 150 μL of dimethyl sulfoxide (DMSO) was added to each well to dissolve the crystalline formazan product. Absorbance in each well was then measured at 490 nm using 96-well plate reader (Model 550; Bio-Rad). The cytotoxicity of sample was expressed as relative absorbance of sample to the untreated or vehicle treated, yielding a relative measure of remaining viable, metabolically active cell after exposure.

Quinone reductase activity assay Hepa1c1c7 and its mutant (BPRc1) cells were plated at a density of 3×10^5 or 5×10^5 cells per plate (Nunc) in 10 mL α-MEM supplemented with 10% FBS. The cell culture was performed in a humidified incubator in 5% CO_2 at 37°C. Cells were cultured for 48 hr, followed by exposure to various concentrations of the sample for another 24 hr. QR activity was measured according to the method described by Benson *et al.* (8). Briefly, cells were plated, grown, and exposed to different concentrations of samples for 24 hr before being harvested. The cells were washed with ice-cold 0.15 M KCl-10 mM potassium phosphate (pH 7.4), removed from the plates by scraping with a rubber policeman, and disrupted for 5 sec using an ultrasonic cell disrupter (50 W; Kontes, Vineland, NJ, USA). Cell homogenates were centrifuged at 10,500×g for 5 min in a microcentrifuge (VS-15000CFN11; Vision, Seoul, Korea). QR activity was assayed by measuring the rate of reduction of 2,6-dichlorophenolindophenol at 600 nm in the assay system containing 25 mM Tris-HCl (pH 7.4), 0.7 mg crystalline bovine serum albumin at pH 7.4, 0.01% Tween 20, 5 μM FAD, 0.2 mM NADH, 0 or 10 μM dicoumarol, and 200 μL cell extract in a final volume of 3.0 mL. The QR induction was expressed as nmoles of 2,6-dichlorophenolindophenol reduced/min/mg protein.

Western blot Hepa1c1c7 cells exposed to delphinidin (0-100 μM) for 24 hr were lysed in SDS sample buffer (62.5 mM Tris-HCl pH 6.8, 2.3% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.005% bromophenol blue). Western blot was performed using primary antibodies (Santa Cruz Inc., Santa Cruz, CA, USA) against QR, γ-GCS, HO-1, and GST. Proteins separated on 12% polyacrylamide gels were transferred onto polyvinylidinedifluoride (PVDF) membrane with transfer buffer (48 mM Tris, 39 mM glycine, 20% methanol, pH 9.2) using a wet (Trans-Blot Electrophoretic Transfer Cell; Bio-rad) blotting unit. The blots were blocked in 5% skim milk in 0.1% Tween 20 in TBS (TBS/T) at room temperature for 1 hr or at 4°C overnight, and incubated in dilution buffer (3% skim milk in TBS/T) with a primary antibody for 1 hr. After washing 5 times with TBS/T, the blots were incubated in dilution buffer (3% skim milk in TBS/T) with secondary antibody for 1 hr, followed by washing 8 times with TBS/T. Washed blots were incubated with Supersignal (West pico chemiluminescent substrate; Pierce) and exposed to a film. Protein concentrations were measured using a modified Lowry procedure with BSA as standard (9).

Assay of reporter gene activity HepG2-C8 cells were plated in 6-well plates at a density of 10^5 cells/well (10). After overnight incubation, cells were cultured in fresh F-12 containing 0.5% FBS for 12 hr before sample treatment. The luciferase activity was determined according to the protocol provided by the manufacturer (Promega Corp., Madison, WI, USA). Briefly, after sample treatment, cells were washed twice with ice-cold PBS and harvested in reporter lysis buffer. The homogenates were centrifuged at 12,000×g for 2 min at 4°C. A 20 μL supernatant was assayed for luciferase activity using Victor 3 Multilabel counter (Perkin Elmer, Wellesley, MA, USA). Luciferase activity was normalized against protein concentration.

Radical scavenging activity Free radical scavenging activity of anthocyanidins, was determined using the DPPH method (11). An aliquot (0.5 mL) of ethanol solution containing different amounts of compounds (1, 2, 5, and 10 uM) was added to 3 mL of daily prepared ethanol DPPH solution (0.1 mM). The optical density change at 517 nm was measured after 30 min incubation in dark. The antioxidant assay was carried in triplicate and the readings were averaged. The scavenging activity was measured as the decrease in absorbance of the samples versus DPPH standard solution.

Statistical analysis Data are presented as means±SD. Differences among means were tested by analysis of variance, followed by Duncan's multiple range test ($p< 0.05$).

Results and Discussion

The growth inhibitory activities of 40% ethanol extract of black soybean coat, cyanidin, and delphinidin were investigated in mouse hepatoma hepa1c1c7 cells and its mutant BPRc1 cells. Delphinidin showed relatively strong cytotoxicity against the cells, with IC_{50} of approximately 70 μg/mL (Fig. 1). However, black soybean coat extract and cyanidin did not inhibit cell growth in the range of 3-100 μg/mL. This result is consistent with the report by Yeh and Yen (12) who observed the apoptotic activity of

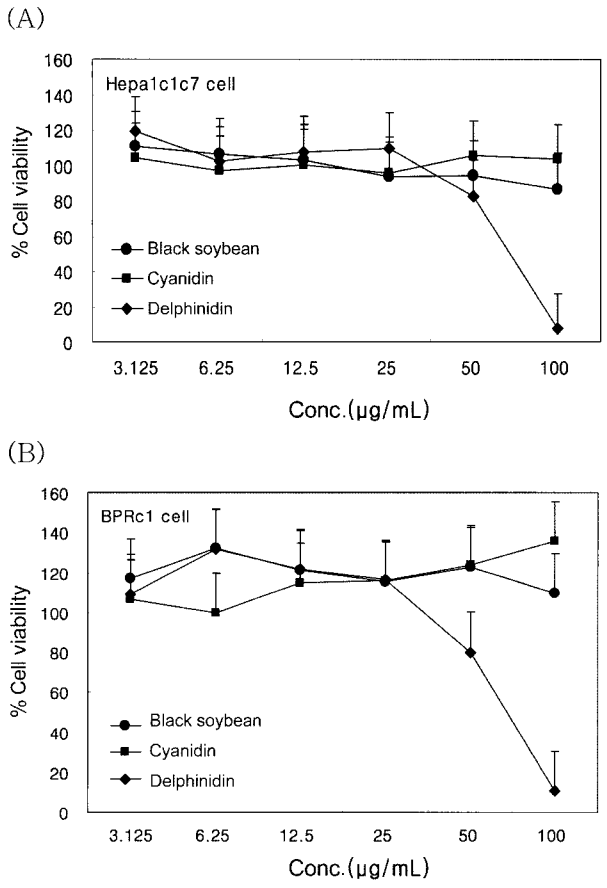

Fig. 1. Anti-proliferative effect of 40% ethanol extract of black soybean coat and anthocyanidins. Hepa1c1c7 (A) and BPRc1 (B) cells were plated at the concentration of 3×10^3 and 5×10^3 cells per well in the 96-well plate, respectively. Results are expressed as the percent of cell viability of control (0.1% HCl in MeOH). Data are means of triplicate assays±SD.

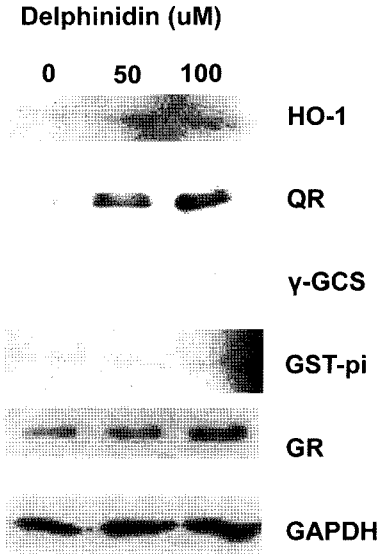

Fig. 2. Increased expression of phase 2 enzymes by delphinidin in Hepa1c1c7 cells. Hepa1c1c7 cells were plated at the concentration of 3×10^5 cells per plate. HO1, Heme oxygenase 1; QR, quinone reductase; γ-GCS, gamma glutamylcysteine synthetase; GST-pi, glutathione s-transferase; GR, glutathione reductase; GAPDH, glyceraldehyde phosphate dehydrogease as a positive control.

delphinidin in human hepatoma HepG2 cells. Meanwhile, cyanidin-3-glucoside, abundantly existing in blackberry, has been reported to cause growth inhibition of various cancer cells such as lung carcinoma, mammary tumor, and leukemia cells (12, 13).

To examine the effect of seed coat extract and anthocyanidins on QR activity, Hepa1c1c7 and BPRc1 cells were exposed to 40%(v/v) ethanol extract (50 μg/mL) of seed coat and anthocyanidins (50 μg/mL) for 24 hr. As shown in Table 1, delphinidin showed the highest QR induction activity in both Hepa1c1c7 and BPRc1 cells. Also 40% ethanol extract of seed coat was shown to significantly induce QR activity in Hepa1c1c7 cell, although it did not affect QR activity in BPRc1 cells lacking arylhydrocarbon receptor nuclear translocator (ARNT). Although soy components responsible for QR induction are not clearly understood, anthocyanins and maybe isoflavones appear to be the most plausible candidates (14-17). A common feature of QR inducers will be the presence of electrophilic or Michael reaction acceptor on chemical structure (6). The structural difference between cyanidin and delphinidin is that delphinidin has one more hydroxyl group than cyanidin. As shown in Table 1, QR was induced by delphinidin but not by cyanidin. Therefore, the hydroxyl group in anthocyanins seems significantly contribute to induction of the phase 2 detoxifying enzymes. Delphinidin induced the expression of not only QR but other antioxidant enzymes including GST, GR, γ-GCS, and HO-1 in a dose-dependent

Table 1. QR induction activity of 40%(v/v) ethanol extract of black soybean coat and anthocyanidins in Hepa1c1c7 and BPRc1 cells[1)]

Cell line	Vehicle, 20 μL/plate			Sample, 50 μg/mL			
	DMSO	40% EtOH	MeOH	Soybean coat extract[2)]	Delphinidin[3)]	Cyanidin[3)]	*t*-BHQ[4)]
Hepa1c1c7	185.0±14.9[a]	214.2±25.9[ab]	174.8±33.8[a]	277.9±59.8[bc]	272.2±29.4[c]	203.9±33.1[ab]	470.0±19.7[d]
BPRc1	102.3±16.6[a]	117.5±22.1[a]	151.9±51.8[a]	135.9±17.2[a]	192.2±39.2[b]	108.7±9.1[a]	248.1±52.6[c]

[1)]Data are means of triplicate measurements±SD. The different letters denote statistically significant difference, $p<0.05$.
[2)]Dissolved in 40% EtOH.
[3)]Dissolved in MeOH.
[4)]*t*-BHQ (20 μM dissolved in DMSO) was used as a positive control.

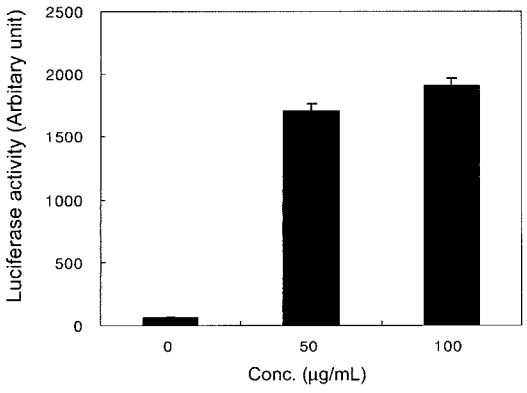

Fig. 3. Induction of luciferase expression by delphinidin in HepG2-C8 cells.

manner (Fig. 2). It has been known that many antioxidants and dietary components can protect humans and animals against insults from environmental toxins and carcinogens through the enhancement of detoxifying enzymes, which in turn decrease the chemical reactivity of carcinogens and their metabolites through conjugation, reduction, and hydrolysis to facilitate their elimination. Genetic analysis has revealed that the basal and inducible levels of GST gene expression are regulated mainly by a *cis*-acting enhancer sequence known as antioxidant response element (ARE). Subsequent studies identified the ARE sequence in the promoter regions of QR, HO-1, and γ-GCS, thereby establishing a central role for ARE in cellular defense and detoxifying enzyme system regulation (18). More specifically, the ARE consensus sequence is recognized by NF-E2-related factor 2 (Nrf2), which binds to the ARE as a heterodimer with a small Maf protein, leading to transcriptional activation of the genes. Current evidence suggests a model in which Nrf2 interact with a cytoplasmic repressor protein, designated Kelch-like ECH-associated protein 1 (Keap1), which is associated with actin cytoskeleton. In response to oxidative stress or electrophiles, Nrf2 becomes dissociated from Keap1 and is migrated into nucleus, where it enhances gene transcription by binding the ARE in the promoter region of target genes (18-20). Delphinidin at higher 50 μg/mL caused more than 100-fold induction of luciferase activity in HepG2-C8 cells (9) which was generated by transfection of HepG2 cells with plasmid carrying the ARE sequence and luciferase reporter gene (Fig. 3). Furthermore, delphinidin showed 4 times higher radical scavenging activity than α-tocopherol as evaluated by DPPH (data not shown). These data confirmed the anti-oxidative potential of delphinidin. Accordingly, delphinidin appeared to induce phase 2 enzymes including QR, GST, γ-GCS, GR, and HO-1 through ARE-mediated pathway. Black soybean coat extract also showed a potential to induce QR although their QR-inducing potential were lower than that of *tert*-butylhydroquinone (*t*-BHQ, 20 μM), a well-known QR inducer (Table 1). In conclusion, there is a good possibility that delphinidin, one of the major anthocyanins found in various food ingredients such as black soybean, exert anti-carcinogenic action via induction of phase 2 detoxifying enzymes and/or direct suppression of tumor cell proliferation.

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