Anthocyanins Fail to Suppress Transformation of Aryl Hydrocarbon Receptor Induced by Dioxin

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Dioxins induce adverse effects through transformation of the cytosolic aryl hydrocarbon receptor (AhR). Our previous study found that flavones and flavonols at dietary levels suppress AhR transformation. In the present study, we investigated whether 20 anthocyanins dissolved in trifluoroacetic acid (TFA)–MeOH suppressed AhR transformation in a cell-free system and in Hepa-1c1c7 cells. Although four compounds at 50 μM suppressed 0.1 nM 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-induced AhR transformation and their effects were dose-dependent in the cell-free system, they were ineffective at 0.5 μM, which is close to physiological concentration. Moreover, no anthocyan at 50 μM tested here suppressed 0.1 nM TCDD-induced AhR transformation in Hepa-1c1c7 cells. We also confirmed that protocatechuic acid and related compounds, which are possible metabolites of anthocyanins, did not affect the transformation in the cell-free system. It is concluded that anthocyanins are not suitable candidates for protection from dioxin toxicity.

Key words: aryl hydrocarbon receptor (AhR); dioxin; anthocyan; antioxidative activity

Dioxins, environmental contaminants, induce various adverse effects including body weight loss, immunotoxicity, endocrine disruption, cancer promotion, teratogenesis, and lethality through the event that they bind to the cytosolic aryl hydrocarbon receptor (AhR), resulting in its transformation.1-4 Transformed AhR works as a transcriptional factor after translocation into the nucleus and heterodimerization with AhR nuclear translocator (ARNT), and specifically binds to the dioxin responsive element (DRE), which is located on an enhancer region of drug-metabolizing enzymes, including cytochrome P4501A1 (CYP1A1), glutathione S-transferase (GST), NADP(H): quinine-oxidoreductase (NQO) and so on.5-8 In addition, AhR transformation disrupts intracellular signal transduction by changing the phosphorylation state of several regulatory proteins.9

Since AhR transformation is the initial step in various adverse effects of dioxins, inhibition of AhR transformation would protect humans from the toxic effects. Because dioxins enter the body mainly through the diet,10,11 it is necessary to search for food factors that possess antagonistic effects against AhR. Previously, we found that flavones and flavonols at dietary levels suppress AhR transformation in a cell-free system.12 Not only flavones and flavonols but also catechins are confirmed as antagonists of AhR.13-15 In addition, other food factors, viz., lutein,15) curcumin,16) resveratrol,17) and certain vegetable constituents,18) act as antagonists of AhR. These results suggest that possible antagonists of AhR are contained in plant-based diets.

Anthocyanins are members of the flavonoids widely distributed in vegetables and fruits such as grapes, berries, eggplants, and purple corns, and have been reported to exhibit antioxidative, antimutagenic, and anti-carcinogenic activities.19 The bioavailability of anthocyanins has been reported, including their absorption and metabolism in rats and humans.20 After oral intake of cyanidin 3-glucoside (Cy 3-glc), intact Cy 3-glc and/or its metabolite, protocatechuic acid (PCA), was detected in rat plasma.20 Acylated anthocyanins are also absorbed, at least in part, into the body after ingestion in rats and humans.22 On the other hand, 2,4-
Materials and Methods

Materials. TCDD was purchased from AccuStandard (New Haven, CT), and dissolved in dimethyl sulfoxide (DMSO). The anthocyanids used in the present study are summarized in Table 1. Pelargonidin (Pg), Pg 3-glucoside (Pg 3-glc), cyanidin (Cy), Cy 3-glc, Cy 3-rhamno-
sylglucoside (Cy 3-rha-glc), Cy 3,5-diglucoside (Cy 3,5-diglc), delphinidin (Dp), peonidin (Pn), Pn 3-glc, malvidin (Mv), Mv 3-glc, Mv 3,5-diglc, and Mv 3-galactoside (Mv 3-gal) were purchased from Extra-
synthèse (Genay, France). Pg 3-coumaroylglycoside-5-glucoside (Pg 3-C-glc-5-glc) and Pg 3-coumaroylglycu-
side-5-malonylglucoside (Pg 3-C-glc-5-Ma-glc) were isolated from *Hyacinthus orientalis* L. cv. Hollyhock as previously described.24) Cy 3-glucoside-5-cafcoyl-
glycoside (Cy 3-glc-5-Caf-glc), Cy 3-glucoside-5-cafcoylglycoside-3'-cafeoylglycoside (Cy 3-glc-5-Caf-
glc-3'-Caf-glc), Dp 3-glucoside-5-cafcoylglycoside-3'-glucoside (Dp 3-glc-5-Caf-glc-3'-glc) were from *Gentiana* unnamed cultivar, and Dp 3-coumaroylglycoside-5-
malonylglucoside (Dp 3-C-glc-5-Ma-glc) were from *Hyacinthus orientalis* L. cv. Delft Blue, as previously described.24) All anthocyanids were dissolved in 50% methanol containing 5% trifluoroacetic acid (TFA–
MeOH) or DMSO. All other reagents used were of the highest grade available from commercial sources.

High performance liquid chromatography (HPLC) analysis. Since anthocyanins have two absorption peaks, at 270–280 nm and 510–540 nm, we analyzed at both wavelengths to detect anthocyanins. Cy and Cy 3-glc dissolved in TFA–MeOH or DMSO at a concentration of 5 mM were stored for 4 d at −20°C. Each stock solution was diluted with corresponding solvent to 50 μM, and aliquots of 10 μl were immediately injected into the HPLC. Analytical conditions were as follows: Column, Capcell Pak C18, type UG120 (ϕ 4.6 × 250 mm, Shiseido, Tokyo) maintained at 35°C; mobile phase, 10% formic acid/0.1% HCl in methanol (80:20, v/v); flow rate, 1 ml/min; monitoring with Hitachi UV–VIS detector L-7420 at 528 nm for detection of Cy and 520 nm for Cy 3-glc. To analyze the other absorption peak at 270 nm, Cy and Cy 3-glc were separately diluted to 250 μM, and aliquots of 10 μl were immediately injected into the HPLC. Elution was performed using 50 mM phosphate buffer (pH 2.1) as solvent A and 0.1% HCl/methanol as solvent B. The gradient system used was as follows: 0–20 min, 95–40% A; 20–25 min, 40–0% A; 25–35 min, 0% A. Other analytical conditions were the same as described above.

Measurement of antioxidative activity. To examine the antioxidative activity of anthocyanins, the modified 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method25) was used. Briefly, 500 μl of 5 μM anthocyanins was added to a mixture of 500 μl of 0.1 M acetate buffer (pH 5.5) and 1 ml of ethanol. The reaction mixture was incubated for 30 min with 500 μl of 0.48 mM DPPH solution in ethanol, and absorbance (Abs) at 517 nm was measured. For the positive and negative controls, the same volume of ascorbic acid (50 μM) and distilled water was used instead of anthocyan-

![Anthocyanins Used in This Study](image)

**Table 1.** Anthocyanins Used in This Study

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**Abbreviations:** Pg, pelargonidin; Cy, cyanidin; Dp, delphinidin; Pn, peonidin; Mv, malvidin; glc, glucoside; C, coumaroyl; Ma, malonyl; rha, rhamnose; Caf, caffeoyl; m, methyl.
fetal bovine serum (Sigma, St. Louis, MO), 4 mM MEM, Nissui Pharmaceutical, Tokyo) containing 10% fresh medium, and treated with 0.1 nM (final) TCDD.

Transformation of the AhR under the cell-free conditions. Animal treatment in the present study conformed to the “Guidelines for the Care and Use of Experimental Animals, in Rokkodai Campus, Kobe University”. The livers of male Sprague-Dawley rats (six weeks old, 140–170 g, purchased from Japan SLC, Shizuoka) were used to prepare the cytosol fraction, as previously described. The protein concentration was determined by the Bradford method using bovine serum albumin as a standard protein. Cytosol (15 mg protein/ml) was incubated with 0.1 nM (final) TCDD in HEDG buffer (25 mM HEPES–NaOH, pH 7.4, 1.5 mM ethylenediaminetetraacetic acid [EDTA], 1.0 mM dithiothreitol [DTT], 10% glycerol) at 20 °C for 2 h to induce AhR transformation, and with vehicle (10 μl/ml) alone as a negative control. To estimate antagonistic effects, anthocyanins were added to the above mixture 10 min prior to the addition of TCDD.

Culture and treatments of Hepa-1c1c7 cells. Mouse hepatoma cell line Hepa-1c1c7 cells were grown and maintained at 37 °C in α-minimum essential medium (α-MEM, Nissui Pharmaceutical, Tokyo) containing 10% fetal bovine serum (Sigma, St. Louis, MO), 4 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin under a humidified 95% air/5% CO2 condition. The cells were resuspended at a concentration of 2.5 × 105 cells/ml, seeded on 60-mm plastic dishes, and incubated for 48 h. They were washed with phosphate-buffered saline (PBS), incubated in 2 ml of fresh medium, and treated with 0.1 nM (final) TCDD for a further 2 h. To estimate the antagonistic effect, anthocyanins were treated to the cells 10 min prior to the addition of TCDD. The cells were washed twice with ice-cold PBS and harvested with ice-cold harvest buffer (20 mM HEPES–NaOH, pH 7.6, 20% glycerol, 10 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.1% nonidet P-40 [NP-40], 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride [PMSF], 5 μg/ml aprotonin, and 20 μg/ml leupeptin) and centrifuged at 1,000 × g for 10 min at 4 °C after incubation for 20 min at 4°C. The nuclear pellet was obtained by centrifugation at 10,000 × g for 20 min at 4 °C, and stored at −80 °C until use. The protein concentration was determined by the Bradford method.

Determination of transformed AhR by southwestern chemistry-based enzyme-linked immunosorbent assay (SW-ELISA). To determine the transformed AhR, SW-ELISA was carried out as previously described. For SW-ELISA, the reaction mixture consisted of 40 μl of treated cytosol (containing 600 μg protein) and 10 μl of HEDG buffer containing 750 mM KCl (final concentration 150 mM) or 12.5 μl of nuclear extract (containing 12.5 μg protein) and 37.5 μl of HEDG buffer. The reaction mixture was plated into a 96-well microtiter plate (Maxisorp, Nalge Nunc International, Tokyo) coated with anti-FITC antibody (DakoCytomation, Kyoto) as capturing antibody and FITC-labeled DRE probe (5′-GAT CCG GAG TTG CGT GAG AAG AGC CA-3′ and 5′-GAT CTT GCT TTT CTC ACG CAA CTC CG-3′, purchased from Hokkaido System Science, Sapporo). After successive incubation with specific antibody against ARNT (anti-ARNT C-19, Santa Cruz Biotechnology, Santa Cruz, CA), biotinylated secondary antibody (Jackson Immuno Research Lab., West Grove, PA), and peroxidase-conjugated streptavidin (DakoCytomation), bound peroxidase activity was visualized with tetramethylbenzidin (DakoCytomation), and color development was stopped by the addition of 0.5 M sulfuric acid. Transformed AhR was quantified by measuring absorbance at 450 nm using Wallac ARVO sx multilabel counter (Perkin-Elmer Life Sciences, Boston, MA). The suppressive effects of anthocyanins were evaluated by the ratio of transformed AhR, which was compared to those of both the positive control (0.1 nM TCDD) and the negative one (vehicle alone).

Results

Degradation products of cyanidin suppressed AhR transformation

Previously, we found that flavones, flavonols, and lutein suppress AhR transformation in a cell-free system. To evaluate the suppressive effects of these compounds on transformation, DMSO was used as a solvent. Since anthocyanins are sensitive to pH, the suppressive effects of Cy and Cy 3-glc on AhR transformation were examined after they were dissolved in two solvents, TFA–MeOH (< pH 3) and DMSO, for 4 d. As shown in Fig. 1, Cy and Cy 3-glc dissolved in TFA–MeOH did not suppress transformation. Cy dissolved in DMSO also did not suppress transformation on day 0, but the suppressive effect became stronger day by day. On the other hand, the effect of Cy 3-glc dissolved in DMSO on transformation remained unchanged. These results suggest that Cy dissolved in DMSO was degraded during storage even at −20 °C.

To clarify whether degradation products of Cy exist, HPLC analysis was performed every day. The HPLC chromatograms obtained at a wavelength of 528 nm revealed that the content of Cy dissolved in either solvent, TFA–MeOH (Fig. 2A) or DMSO (Fig. 2B), decreased day by day. Even on day 0, the peak area of Cy dissolved in DMSO was smaller than that in TFA–MeOH. The rest content of Cy on day 4 was calculated as a percent of that on day 0, and 64.5% remained in
TFA–MeOH, while only 8.6% remained in DMSO. We also monitored the other absorption peak at 270 nm, and found that the detectable number of peaks of Cy dissolved in DMSO increased day by day, compared with those in TFA–MeOH (Fig. 2C and D). These results suggest that the flavylium skeleton of anthocyanidins easily broke down in DMSO. On the other hand, the HPLC chromatogram of Cy 3-glc dissolved in either solvent remained unchanged at both 520 nm and 270 nm (data not shown). These results indicate that Cy, especially when dissolved in DMSO, is unstable for storage even at −20°C, whereas Cy 3-glc is apparently stable.

Since the degradation products of Cy affected the suppressive effect on AhR transformation, it is suggested that the bioactivities of anthocyanins changed. To confirm the effect of storage period on the bioactivity of anthocyanins, we measured antioxidative activity at the start and end points of the storage period by the DPPH radical scavenging method. On day 4, the antioxidative activity of Cy and Cy 3-glc at 50 μM on AhR transformation was evaluated by SW-ELISA, as described in "Materials and Methods". Data are shown as a percent of transformed AhR induced by 0.1 nM TCDD, and represented as the mean ± SD from triplicate independent experiments. Asterisks indicate significant differences (p < 0.05, Student’s t-test) from the corresponding value on day 0.

Although no significant difference was observed in the content of Cy 3-glc dissolved in DMSO between day 0 and day 4, the antioxidative activity decreased to 45% on day 4. The antioxidative activity of Cy and Cy 3-glc dissolved in TFA–MeOH was 8.8 and 9.3 times as high respectively as that in DMSO on day 0. These results indicate that the stability of Cy and Cy 3-glc in TFA–MeOH is higher than that in DMSO, and that the stability affects bioactivities, including not only AhR transformation but antioxidative activity. Hence, we dissolved anthocyanins in TFA–MeOH just before use in the following experiments.

**Suppressive effect of anthocyanins on AhR transformation in a cell-free system**

Our previous reports show that several flavonoids at dietary levels suppress AhR transformation induced by 0.1 or 1 nM TCDD in the cell-free system using rat liver cytosol fraction. To compare the suppressive effects of anthocyanins with that of other flavonoids, 20 kinds of anthocyanins (50 μM) on AhR transformation induced by 0.1 nM TCDD were measured using the same system as the previous report. Among the compounds tested here, only 4 anthocyanins, Dp 3-glc-5-Caf-glc-3’-Caf-glc,
Pn, Mv, and Mv 3-gal significantly suppressed transformation, with suppression levels of 53, 58, 43, and 46% respectively (Fig. 4), while no anthocyan itself induced AhR transformation (data not shown). The four efficacious compounds suppressed AhR transformation in a dose-dependent manner, and the 50% inhibitory concentration (IC$_{50}$) values of Dp 3-glc-5-Caf-glc-3'-Caf-glc, Pn, Mv, and Mv 3-gal were determined to be 23, 23, 45, and 51 µM respectively (Fig. 5). But no correlation between structure and suppressive effect was observed. These results suggest that the suppressive effect of anthocyanins on AhR transformation is not as strong as that of luteolin, quercetin, or kaempferol, whose IC$_{50}$ values against 0.1 nM TCDD-induced AhR transformation were under 1 µM.$^{15}$

Anthocyanins do not suppress AhR transformation in Hepa-1c1c7 cells

A small portion of ingested anthocyanins can be absorbed in the plasma and liver, and excreted into the urine as a glycosilated form.$^{28,29}$ To investigate whether anthocyanins can suppress AhR transformation in the liver cells, mouse hepatoma cell line Hepa-1c1c7 cells were used. As the results, 50 µM of anthocyan tested here did not significantly suppress AhR transformation induced by 0.1 nM TCDD in Hepa-1c1c7 cells (Fig. 6). Previous studies found that anthocyanins are degraded into protocatechuic acid (PCA) and related compounds under physiological conditions.$^{20,21}$ Hence, we confirmed the suppressive effect of PCA and related compounds, p-hydroxybenzoic acid (HBA), vanillylic acid (VA), 3,4-dimethoxybenzoic acid (DBA), gallic acid (GA), and 3,4,5-trimethoxybenzoic acid (TBA), on AhR transformation in the cell-free system. As shown in Fig. 7, no compound at 50 µM suppressed 0.1 nM TCDD-induced...
AhR transformation. In addition, 3,5-dihydroxybenzoic acid and 2,4-dihydroxybenzoic acid did not show any suppressive effect (data not shown). These results indicate that neither ingested anthocyanins nor their metabolites can suppress AhR transformation under physiological conditions, suggesting that anthocyanins do not affect the AhR.

Discussion

Dioxins, which are environmental contaminants, induce various adverse effects such as body weight loss, immunotoxicity, endocrine disruption, cancer promotion, teratogenesis, and lethality through transformation of AhR.\textsuperscript{1–4} Since they enter the body mainly through diet,\textsuperscript{10,11} it is critical to search for a food factor that is able to suppress AhR transformation. Previous studies have shown that flavonoids,\textsuperscript{12} lutein,\textsuperscript{15} curcumin,\textsuperscript{16} and certain vegetable constituents\textsuperscript{18} act as antagonists of AhR, suggesting that plant-based diets have the potential to protect humans from dioxin toxicity. Our previous study indicates that flavones and flavonols are stronger antagonists than flavanones, catechins, or isoflavones among the subclasses of flavonoids.\textsuperscript{12} It has been reported that AhR favors compounds that have a hydrophobic coplanar structure as the ligands containing antagonists,\textsuperscript{30} whereas lutein,\textsuperscript{15} curcumin,\textsuperscript{16} and resveratrol\textsuperscript{17} act as antagonists of the AhR, although they do not have a coplanar structure. In the present study, we found that anthocyanins and their possible metabolites neither suppress nor induce AhR transformation in the cell-free system and in Hepa-1c1c7 cells, indicating that anthocyanins do not affect the AhR under physiological conditions. Anthocyanins do not have a coplanar structure due to the flavylium cation, and they are immediately degraded under physiological conditions.\textsuperscript{19} These results suggest that anthocyanins do not show enough antagonistic effect because of their structure and susceptibility to degradation.

The HPLC chromatograms revealed that Cy, particularly in DMSO, was degraded day by day into at least five products (Fig. 2). The degradation products of Cy showed the suppressive effect in this study (Fig. 1), although we did not identify the product. It has been reported that anthocyanins are metabolized to PCA and related compounds, including 2,4-dihydroxybenzoic acid and 2,4,6-trihydroxybenzoic acid, under physiological conditions\textsuperscript{20,21} and some of these compounds are methylated by catechol-\textsuperscript{O}-methyltransferase, and exist in the liver. But possible metabolites of anthocyanins did not suppress AhR transformation in the cell-free system (Fig. 7). These results strongly suggest that the efficacious degraded compounds of Cy in DMSO are not physiological metabolites, but chemically degraded artifacts that have not yet been reported. The antioxidative activity of Cy and Cy 3-glc dissolved in DMSO was clearly lower than that in TFA–MeOH before storage (day 0), although their effect on AhR transformation did not change in either solvent (compare Fig. 1 with Fig. 3). It is noteworthy that the antioxidative activity of Cy 3-glc significantly decreased during the storage period at \textdegree{}C for 4 d even in TFA–MeOH (Fig. 3), although neither the HPLC chromatogram nor the effect on AhR transformation changed. Thus, a portion of anthocyanins is gradually degraded even in...
acidic solution at low temperatures.

In the cell-free system, only four of the tested anthocyanins (50 μM) dissolved in TFA–MeOH significantly suppressed AhR transformation in a dose-dependent manner with relatively higher IC₅₀ values (Fig. 4 and Fig. 5) than those of certain flavones and flavonols. Although we cannot explain why they suppressed AhR transformation because of no structural-activity relationship, these efficacious anthocyanins had suppressed AhR transformation because of no structure.

References

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