Anthocyanin Administration Elevates Plasma Homocysteine in Rats

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Summary The data accumulated from epidemiological studies suggests that individuals with elevated blood levels of homocysteine have an increased risk of cardiovascular disease. However, little is known of the food factor that may affect the homocysteine status, except for folate and B-vitamins. Here, we tested the effect of dietary phenolics (i.e., anthocyanin of food colorant) administration on plasma homocysteine concentration in a rat study, since a profound effect on the methionine metabolism was speculated from the 3',4'-catechol skeletal structure of anthocyanin. Female Sprague-Dawley rats (body weight 100 g) orally ingested a single dose of anthocyanin mixture (total 100 mg) composed of cyanidin-3-glucoside (50 mg), cyanidin-3-sambubioside (48 mg), and cyanidin-3,5-diglucoside (2 mg). The total homocysteine in the plasma collected 90–240 min after anthocyanin intake was 1.4 to 1.8-fold (5.2–6.7 μmol/L) higher than the basal homocysteine level (3.7 μmol/L). In the liver and kidney, anthocyanin significantly affects sulfur amino acid (S-adenosylmethionine, SAM, and S-adenosylhomocysteine, SAH) levels, both of which are precursors of plasma homocysteine, and the SAH/SAM ratio showed a significant increase in the liver and kidney. Accordingly, these results suggest that dietary anthocyanin stimulates homocysteine synthesis from SAH in the liver and kidney, and the homocysteine yielded transfers into the blood stream. The intake of anthocyanin and its structural homologues may have an effect on the metabolic regulation of sulfur amino acids and possibly increase the risk of vascular disease in humans.

Key Words homocysteine, anthocyanin, plasma, rats

Anthocyanins are the largest group of water-soluble pigments in the plant kingdom. The common naturally occurring anthocyanins are glycoside or sambubioside derivatives of the 2-phenylbenzophenyl (flavilium) structure (Fig. 1). They are widely distributed in the human diet through crops, beans, fruits, vegetables, and red wines. Anthocyanins are also used as food colorants in several food products. Thus, humans ingest considerable amounts of anthocyanins from plant-based diets and food products on a daily basis (1, 2).

During the past decade, interest has grown in anthocyanins regarding their antioxidant activity (3–5), which is postulated to relate to anti-atherosclerotic (6, 7), anti-carcinogenic (8, 9), and anti-inflammatory effects (10, 11). Because a great deal of attention has been focused on the bioavailability of anthocyanin, studies of the absorption and metabolism of the compounds are required. For this purpose, we have previously investigated the metabolic fate of anthocyanins in rats and humans (12). The study clearly demonstrated that anthocyanins are incorporated as keeping their intact forms into blood plasma from the digestive tract, while the bioavailability (i.e., absorptivity) of anthocyanins seems to be rather lower than that of other polyphenols (i.e., tea catechins) in humans. In addition, we were aware that a large part of the anthocyanins transported to the liver were converted to 3'-O-methylated forms, where S-adenosylmethionine (SAM) would serve as the methyl donor and S-adenosylhomocysteine (SAH) might be produced as a byproduct as shown in Fig. 2. Hence, the possibility exists that anthocyanin intake may predispose an individual to impaired sulfur amino acid regulation and lead to the increased production of homocysteine from SAH (Fig. 2). This implies that an excessive intake of anthocyanin may be harmful by increasing the homocysteine status, primarily because of the realization that an elevated blood level of homocysteine is a risk factor for atherothrombotic vascular disease (13–15). Supposedly, moderate intake of anthocyanins is beneficial due to their antioxidant function (3–5), but the effect of excessive intake has never been understood. In addition, the interaction of homocysteine with food factors such as anthocyanin has not been identified. In this study, we tested this hypothesis in a rat study together with a study of the anthocyanin metabolism, and discussed the possible physiological effect of dietary food phenolics.

MATERIALS AND METHODS

Chemicals. Anthocyanin extract, prepared from elderberry, was donated by Lion Co. (Tokyo, Japan). In the extract, three kinds of anthocyanins (cyanidin-3-glucoside, cyanidin-3-sambubioside, and cyanidin-3,5-diglu-
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Fig. 1. Chemical structures of anthocyanin.

Fig. 2. Proposed pathway for the methylation of an anthocyanin and production of homocysteine. An orally administered anthocyanin is converted to 3'-O-methylated form by catechol O-methyltransferase (COMT). During this O-methylation, S-adenosylmethionine (SAM) serves as the methyl donor, resulting in the conversion of SAM to homocysteine via S-adenosylhomocysteine (SAH).

coside; Fig. 1) were isolated with high-performance liquid chromatography (HPLC) and characterized on the basis of one- and two-dimensional nuclear magnetic resonance spectroscopy and electrospray mass spectrometry (16, 17). These isolated individual anthocyanins and peonidin-3-glucoside (3'-O-methylated cyanidin-3-glucoside; Funakoshi, Tokyo, Japan) were used as standards. S-Adenosylmethionine (SAM), S-adenosylhomocysteine (SAH) and homocysteine were obtained from Sigma (St. Louis, MO, USA). A thiol-specific fluorogenic reagent, ammonium 7-fluoro-2,1,3-benzoxadiazole-4-sulphonate (SBD-F), was obtained from Dojindo Laboratories (Kumamoto, Japan). All the other reagents used were of analytical grade.

Anthocyanin. The anthocyanin extract was dissolved in 2 mL of distilled water. The solution contained a total of 100 mg anthocyanin (cyanidin-3-glucoside, 50 mg; cyanidin-3-sambubioside, 48 mg; and cyanidin-3,5-diglucoside, 2 mg). This 2 mL anthocyanin solution was orally administered in a single dose to each rat by stomach tube.

Animals and diets. This study was conducted in conformity with the policies and procedures detailed in the Guide for the Care and Use of Laboratory Animals (18). Four-week-old female Sprague-Dawley rats were obtained from Japan SLC Inc. (Hamamatsu, Japan) and housed in stainless-steel wire-mesh cages in a room kept at 23±1°C with a 12 h light : dark cycle. After acclimatization with F-2 Standard Rodent Chow (Funabashi Farm Co., Funabashi, Japan) and distilled water (free access) for 1 wk, each rat (100 g) was administered a single dose of anthocyanin solution (100 mg anthocyanin/rat) via stomach tube. Before and 30–240 min after administration, rats were anesthetized with ether and blood was collected into a EDTA-treated blood collection tube. Plasma was prepared from blood by centrifugation at 1,000×g for 15 min at 4°C. Plasma was then stored at −80°C until homocysteine analysis. Immediately after blood collection, the liver and kidney were perfused in situ with ice-cold saline, and then removed and stored at −80°C until analysis. Total homocysteine, anthocyanin, SAM, and SAH concentrations were determined by validated methods using HPLC, as described below.

Analytical methods. Anthocyanins in plasma, liver and kidney were extracted by the solid-phase extraction procedure and determined by UV-HPLC as previously reported (12). The chemical structures of anthocyanins and their metabolites (3'-O-methylated anthocyanins) in biological samples were confirmed by HPLC with online mass spectrometry.

Plasma total homocysteine concentration was determined as follows (19): plasma (0.3 mL) was treated with 30 μL of a 0.4 mmol/L solution of tri-n-butylphosphine in dimethylformamide. The solution was incubated at 4°C for 30 min, and then mixed with 0.3 mL of 0.6 mmol/L trichloroacetic acid (containing 1 mmol/L EDTA). After centrifugation at 10,000×g for 10 min, 100 μL of the supernatant was added to a mixture of 20 μL 1.6 mol/L sodium hydroxide, 250 μL of 130 mmol/L borate buffer (containing 4 mmol/L of EDTA; pH 9.5) and 100 μL 4.3 mmol/L SBD-F. The mixture was incubated for 1 h at 60°C, and a 20 μL of the aliquot was subjected to a fluorescence-HPLC system (Japan Spectroscopic Co., Tokyo, Japan). Separation was carried out at ambient temperature with an analytical column, ODS-pack (4.6×250 mm: Shodex Co., Tokyo, Japan). A mixture of water and acetonitrile (96 : 4, v/v; containing 0.1 mmol/L potassium dihydrogenphos-
phate; pH 2.1) was used as a mobile phase with a flow rate of 1.2 mL/min. Fluorescence intensities were measured with excitation at 385 nm and emission at 515 nm.

SAM and SAH were measured by the UV-HPLC method (20). In short, tissues (500 mg) were homogenized in 5 and 2.5 mL of 0.4 mol/L perchloric acid for liver and kidney, respectively, with a teflon-glass homogenizer under ice-cold conditions. These tissue homogenates were centrifuged at 10,000×g for 20 min. An aliquot of the supernatant (50 µL) was directly applied to UV-HPLC at 254 nm detection. An ODS column (YMC ODS-H80, 4.6×250 mm; YMC Co., Tokyo, Japan) was used with a mixture of water and methanol (82:18, v/v; containing 40 mmol/L sodium dihydrogenphosphate and 8 mmol/L 1-heptanesulfonic acid; pH 3.0) as column eluant at a flow rate of 1.0 mL/min.

Statistical analysis. All data are expressed as means±SD. Statistical comparisons were made with Student’s t-test using the StatView Ver. 4.5 statistical package for Macintosh (Abacus Concepts Inc., Berkeley, CA, USA). Differences were considered significant at p<0.05.

RESULTS AND DISCUSSION

In the present study, rats (100 g) received a total of 100 mg anthocyanin. Like this study, the dose (approximately 10–100 mg/100 g rat) is frequently used for rat studies to evaluate the bioavailability of anthocyanin in vivo. This dose is, however, higher than the daily intake of anthocyanin by humans (180–215 mg/d) (1).

When a single 100 mg dose of anthocyanin (50 mg cyanidin-3-glucoside, 48 mg cyanidin-3-sambubioside, and 2 mg cyanidin-3,5-diglucoside) was administered to rats, anthocyanins rapidly appeared as their unchanged glycated forms in the plasma (Fig. 3). The plasma anthocyanin level reached a maximum at 30 min after oral intake and then decreased (Fig. 4). This absorption profile was identical to that we previously observed (12). Therefore, orally administered anthocyanins are absorbed rapidly as intact forms from intestine, as previously confirmed by us (12) and other researchers (21–23). On the other hand, it has been speculated that bacterial digestion of the glycosidic linkage of flavonoids by the gastrointestinal system would occur before absorption (24). Since aglycone (cyanidin) of anthocyanins was not detected in the plasma (Fig. 3), the flavilium cation structure of anthocyanins would be metabolically stable against bacterial hydrolysis, unlike in the case of other flavonoids lacking the cation group.

In this study, in contrast to plasma, anthocyanins as intact forms (unmetabolized anthocyanins) were observed at trace levels in the liver and kidney, and they were present predominantly as methylated forms (Fig. 5). Thus, some orally administered anthocyanins are metabolized to methylated forms in the liver. The other anthocyanins, which are an escape for methylation in the liver, can be incorporated as intact forms into the blood stream from the liver, and are then transported to the kidneys and several tissues. In the kidney, anthocyanins are methylated and finally excreted into urine.

It is well known that almost all types of flavonoids are enzymatically metabolized into glucuronide and sulfate conjugates as well as methylated forms in vivo (12, 24–27). For conjugation reactions, glucuronosyl transferase and sulfotransferase are responsible enzymes. These enzymes act upon flavonoids to increase their water solubility, thereby facilitating the removal of flavonoids from the body. However, in the present study, it is noteworthy that anthocyanins were present predominantly as methylated forms in the liver and kidney (Fig. 5), and that conjugates such as sulfate and glucuronide of anthocyanins were not actually detected in both the plasma and these tissue organs. Anthocyanin is basically a highly water-soluble compound, which would diminish the necessity of glucuronidation and...
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Fig. 5. Time course of changes in the concentrations of methylated anthocyanins in the liver and kidneys. Bar represents the sum of concentrations of methylated anthocyanins (3’-O-methylated cyanidin-3-glucoside, 3’-O-methylated cyanidin-3-sambubioside, and 3’-O-methylated cyanidin-3,5-diglucoside). Means±SD; n=5.

Fig. 6. HPLC chromatograms of rat plasma homocysteine before and 240 min after oral administration of anthocyanin.

Fig. 7. Effect of orally administered anthocyanin on plasma total homocysteine concentrations. Means±SD; n=5. *# Significantly different from baseline (* p<0.05; # p<0.005).

sulfation of anthocyanin in vivo. Hence, we proposed that anthocyanin is a unique and rare flavonoid from the point of view that it undergoes metabolism solely by methylation. This character implies that excess anthocyanin intake may accelerate the methylation of anthocyanin to an extreme degree, which could cause unexpected side effects and adverse reactions. As shown in Fig. 5, the concentration of methylated anthocyanins in the liver and kidneys was dramatically increased after the administration of anthocyanin. The methylated anthocyanin concentration had already reached the maximum at 30 min after intake and then gradually decreased (Fig. 5). Since the 3’-hydroxyl of anthocyanin was selectively methylated (data not shown), we can conclude that catechol O-methyltransferase (COMT) catalyzed the modification. COMT requires Mg²⁺ as a cofactor and as methyl donor SAM, which was synthesized from adenosintriphosphate and methionine (28). Therefore, we assume that the O-methylation of anthocyanin to 3’-O-methylated anthocyanin associates with the conversion of SAM to SAH, which causes subsequent homocysteine production (Fig. 2). Recently, elevated homocysteine levels are proposed to be a risk factor for vascular disease (13–15). Risk factors associated with increased homocysteine include age, sex, smoking, blood pressure, and cholesterol, and genetically inborn errors of homocysteine enzyme metabolism (29–31). However, the interaction of homocysteine with anthocyanin has never been reported. Hence, we secondly determined plasma homocysteine and sulfur amino acids (SAM and SAH) levels in the liver and kidneys.

After anthocyanin administration to rats, the proposed increase of plasma homocysteine was documented by HPLC analysis (Fig. 6). The mean homocysteine concentration started to increase at 90 min after anthocyanin intake and continued to rise throughout the 240-min observation period (Fig. 7). Compared to basal homocysteine levels (3.7 μmol/L), plasma concentrations 240 min after administration were 1.8-fold higher. On the other hand, the molar ratio of SAH to SAM increased during the period from 60–90 min after intake (Figs. 8 and 9). The results indicated that the methylation of anthocyanin causes a decrease in SAM and increase in SAH. In the following period (90–240 min), when the plasma homocysteine elevation was confirmed, the SAH/SAM ratio began to attenuate toward basal values (Fig. 9), suggesting SAH hydrolysis into homocysteine and adenosine. Consequently, when anthocyanin is administered, homocysteine production is enhanced as shown in Fig. 2. This leads to its export from organs into the blood, causing hyperhomocysteinemia. If rats were given anthocyanin repeatedly, the SAH/SAM ratio may be maintained at a higher rate,
causing a greater elevation of plasma homocysteine.

To date, there have been several reports on the association of homocysteine with drinks, food factors, and drugs. Recently, van der Gaag and coworkers (32) observed that, in nonalcoholic subjects, drinking red wine increased serum homocysteine levels by nearly 10%. They noted that such an increase coincides with a 10–20% increase in cardiovascular disease risk. The biochemical mechanism responsible remains unknown (32), but our present results (Figs. 6 and 7) suggest that anthocyanin, a major polyphenol in red wine, may be partly responsible for the higher homocysteine concentrations observed in wine drinkers. Most recently, Olthof et al. (33) have reported that human homocysteine concentrations 4–5 h after administration of chlorogenic acid (2 g) were 12% higher than that after administration of a placebo. Chlorogenic acid is a major polyphenol in coffee. Yasui et al. (34) found that the levels of homocysteine were elevated by 60% in levodopa (L-DOPA)-treated patients with Parkinson’s disease, with the most marked elevation occurring in patients with the MTHFR C677T genotype. Levodopa, anthocyanin, and chlorogenic acid each contain a catechol structure, which is a substrate for COMT. We suspect that extreme O-methylation of the polyphenol bearing catechol structure leads to decreases in SAM and increases in SAH, resulting in hyperhomocysteinemia.

Fig. 8. HPLC chromatograms of S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) in rat liver and kidneys before and 90 min after oral administration of anthocyanin.

Two major hypotheses have been proposed to explain how homocysteine induces its harmful effects. First, it can damage endothelial cells lining the vasculature, thus allowing plaque formation (35), while simultaneously interfering with the vasodilatory effect of endothelial derived nitric oxide (36). Additionally, homocysteine has been found to promote vascular smooth muscle cells hypertrophy (37).

In conclusion, we have demonstrated here, for the first time, that anthocyanin is responsible for the elevation of homocysteine levels. Thus, high doses of anthocyanin may increase vascular risk for humans. For polyphenols other than anthocyanin, quercetin was reported to inhibit the COMT-catalyzed methylation of catecholestrogens, thereby enhancing estradiol induction of the kidney tumors in hamsters (38). We propose that a moderate intake of polyphenols may be beneficial due to their antioxidant function, but that excessive intake may be harmful. Currently, various supplements that boast high amounts of polyphenols are likely to be consumed in Japan; however, their effects in humans are unknown. Further investigations are necessary to clarify the association of excess intake of polyphenols, homocysteine concentrations, and the risk of cardiovascular disease.

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