Anthocyanin Cyanidin-3-Glucoside Attenuates Platelet Granule Release in Mice Fed High-Fat Diets

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Summary Platelet granule release is considered an important target for preventing and treating cardiovascular diseases (CVDs). Cyanidin-3-glucoside (Cy-3-g) is a predominant bioactive anthocyanin compound in many edible plants and has been reported to be protective against CVDs by attenuating platelet dysfunction. However, direct evidence of the action of Cy-3-g on platelet granule secretion in purified platelets from in vivo assays is still poor. In the present study, we demonstrated that dietary supplementation of purified Cy-3-g reduces serum lipid levels and facilitates down-regulation of the platelet granule release of substances such as P-selectin, CD40L, 5-HT, RANTES and TGF-β1 in gel-filtered platelets, in addition to attenuating serum PF4 and β-TG levels in mice fed high-fat diets. These results provide evidence that Cy-3-g protects against thrombosis and CVDs by inhibiting purified platelet granule release in vivo.

Key Words anthocyanins, platelet, granule release, cardiovascular diseases

Platelets are small anucleate cells in the blood that play important roles in hemostasis, thrombosis, inflammation, and cardiovascular diseases (CVDs) (1, 2), which represent a major source of morbidity and mortality worldwide (3, 4). Platelets are replete with secretory granules, which are critical to normal platelet function. Among the three types of platelet secretory granules (α-granules, dense granules, and lysosomes), α-granules are the most abundant (5). Platelets release coagulation factors, adhesion molecules, growth factors, and chemokines from α-granules, including substances such as P-selectin, β-thromboglobulin (β-TG), CD40L, and the Regulated on Activation, Normal T cell Expressed and Secreted (RANTES) protein, which promotes platelet adhesion/aggregation and inflammation (6, 7). Dense granules contain calcium, pyrophosphate, and soluble platelet agonists, such as serotonin, adenosine triphosphate (ATP), and adenosine diphosphate (ADP) at high concentrations (8). Lysosomes contain hydrolases and express CD63 and LAMP-2 on their membrane (9). Platelet granule secretion is enhanced in response to certain platelet agonists, such as ADP, collagen and thrombin, and in some CVDs, such as hypercholesterolemia, which is considered one of the main determinants of a prothrombotic state in CVDs (10, 11). Hence, down-regulation of platelet granule release is considered to represent a feasible approach for preventing and treating CVDs (11).

Many studies have shown that diet is a major modifiable risk factor for CVDs. Intake of plants rich in phytochemicals is negatively associated with CVDs (12). Anthocyanins, one of the best-studied types of polyphenols, are abundant in various fruits and vegetables, such as black rice, grapes, berries, and red cabbage. Cyanidin-3-glucoside (Cy-3-g) is one of the main components of anthocyanins. Many studies have demonstrated that anthocyanins exhibit a series of benefits in various chronic diseases, such as dyslipidemia, obesity, and atherosclerosis, through their anti-oxidative and anti-inflammatory properties (13). However, it is difficult to explain the biological mechanism of anthocyanins because food extracts usually contain an enormous variety of bioactive compounds. Our previous studies have shown that the anthocyanin delphinidin-3-glucoside (Dp-3-g, another component of anthocyanins) can inhibit platelet activation and aggregation in vitro (11, 12, 14). We have also reported that capsules containing an anthocyanin mixture can inhibit platelet granule secretion in the plasma of patients with hypercholesterolaemia (11). However, the underlying biological effects and mechanisms of action of the attenuation of platelet granule release by Cy-3-g, which is the most common anthocyanin with a glucoside, have not been well elucidated in vivo (15). The objective of this study was to...
determine the effects of the anthocyanin Cy-3-g, purified from black rice, on gel-filtered platelets in mice fed high-fat diets.

**MATERIALS AND METHODS**

**Cy-3-g extraction and analyses.** Extraction and purification of Cy-3-g from black rice was performed as described previously (16). Briefly, highly purified Cy-3-g was isolated from black rice using middle-pressure liquid chromatography (MPLC). Characterization and quantification of Cy-3-g were performed using high-performance liquid chromatography (HPLC) (250×4.6 mm i.d., 5 μm ZORBAX SB-C18 column, Waters, Milford, MA), followed by liquid chromatography–mass spectrometry (LC-MS) analysis.

**Reagents and antibodies.** FITC-conjugated anti-mouse CD62P monoclonal antibody and PE-conjugated anti-mouse CD40L monoclonal antibody were purchased from BD Biosciences (Franklin Lakes, NJ). Kits for immunohistochemistry, such as 5-hydroxytryptamine (5-HT) and β-TG ELISA kits, were purchased from Shanghai Lanji Biotechnology Co. (Shanghai, China). Transforming growth factor-β1 (TGF-β1) and RANTES were purchased from eBioscience Co. (San Diego, CA), and kits for platelet factor 4 (PF4) were purchased from R&D Systems Inc. (Minneapolis, MN). ATP kits were purchased from Chrono-Log (Minneapolis, MN). LDL-C ELISA kits were purchased from Clon-Cloud Corp. (Buckingham, UK). Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich Chemicals (St. Louis, MO). All of the other chemicals used in this study were of analytical grade and were purchased from Sigma-Aldrich (Minneapolis, MN) unless otherwise noted.

**Animals and diets.** All animal procedures were approved by the Animal Care and Protection Committee of Sun Yat-Sen University (No. 2015021). Male C57BL/6j mice, aged 8–9 wk and weighing 20–25 g, were obtained from the animal center of Guangdong, P. R. China. The mice were housed at 22˚C under a 12 h light-dark cycle with free access to food and water. After 2 wk of adaptation, the animals were randomly divided into three groups (n = 20 for each group) and fed one of the following diets for 12 wk: a normal diet (control group), a high-fat diet (HFD group), or a HFD supplemented with Cy-3-g at 1,000 mg/kg diet (equal to 120 mg/kg bw/d) (HFD+Cy-3-g group). Cy-3-g is nontoxic, and a moderate dose was adopted (1,000 mg/kg diet) (17) and the feeding period for the present study according to previous studies in vivo in which Cy-3-g supplementation at 1,000 mg/kg diet was found to significantly improve obesity and triglyceride metabolism in KK-Ay mice (18). The compositions of the diets were analyzed chemically and prepared as previously described (Table 1). The mice were weighed every week during the experiment. At the end of the experiment, all mice were fasted overnight and sacrificed, and their blood was collected for serum biochemistry analysis and stored at −20˚C until analysis.

**Determination of the serum lipid profile.** Serum triglyceride (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) levels were determined in accordance with the instructions of the manufacturer (Cloud-Clone Corp.). Briefly, avidin conjugated to horseradish peroxidase (HRP) was added to each microplate well, followed by incubation. The enzyme-substrate reaction was terminated by the addition of a sulfuric acid solution, and the color change was measured spectrophotometrically at a wavelength of 450 nm. The concentration of low-density lipoprotein (LDL) in the samples was then determined by comparing the O.D. of the samples with the standard curve.

**Platelet preparation.** Blood was collected in a tube containing sodium citrate. Platelet-rich plasma (PRP) was obtained from blood samples via centrifugation at 1,250 rpm for 10 min at room temperature. Platelets were then isolated from citrated PRP using a Sephadex 2B column in PIPES buffer (5 mM PIPES, 1.37 mM NaCl, 4 mM KCl, 0.1% (wt/vol) glucose, pH 7.0), and gel-filtered platelets were then prepared as previously described (19, 20). This method can remove at least 99.85% of plasma proteins from PRP without modifying platelet structure, function, or contents (21).

**Assessments of platelet P-selectin and CD40L levels.** To measure the expression of P-selectin and CD40L on the platelet surface, gel-filtered platelets were incubated with a FITC-conjugated anti-mouse CD62P antibody and a PE-conjugated CD40L antibody for 30 min without light at room temperature. Then, 1 mm CaCl₂ and 0.5 U/mL thrombin were added and sufficiently mixed for 1 min at room temperature, after which 500 μL of paraformaldehyde was added, resulting in a final concentration of 1%. The cells were subsequently analyzed using a calibrated FACScalibur flow cytometer (BD Biosciences).

**Platelet ATP release assay.** The gel-filtered platelet

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| Ingredient | Content per kilogram of feed |
|------------|-----------------------------|
|            | Control | HFD | HFD+Cy-3-g |
| Energy, kJ | 3,960   | 4,679.5 | 4,679.5 |
| Cy-3-g, g  | 0       | 0   | 1         |
| Cornstarch, g | 397.5 | 150 | 150      |
| Dextrinized cornstarch, g | 132 | 0 | 0 |
| Sucrose, g | 100     | 329.5 | 392.5 |
| Casein, g  | 200     | 195 | 195       |
| l-cystine, g | 3    | 3   | 3         |
| Soybean oil, g | 70  | 0   | 0         |
| Cholesterol, g | 0   | 12.5 | 12.5 |
| Lard, g    | 0       | 210 | 210       |
| Fiber, g   | 50      | 50  | 50        |
| Mineral mix, g | 35  | 35  | 35        |
| Vitamin mix, g | 10  | 10  | 10        |
| Choline bitartrate, g | 2.5 | 0  | 0 |

1 Control group fed a normal diet; HFD group fed a high-cholesterol diet; HFD+Cy-3-g group fed an HFD diet supplemented with Cy-3-g.
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suspension (2.5–3.0 \times 10^8 \text{ platelets/mL}) was stimulated with 0.5 U/mL of thrombin and 1 mM Ca^{2+} for 10 min, followed by centrifugation at 12,000 rpm for 15 min at 4°C. The cell-free supernatant was subsequently transferred to another tube, and ATP release was determined with a Chronolog Lumi-aggregometer (Chrono-Log). Briefly, the luciferin-luciferase reagent was added directly to the platelet suspensions, which were then continually stirred (1,000 rpm) at 37°C. Thrombin (0.5 U/mL) was added to activate the platelets in the presence of 1 mM Ca^{2+}, and real-time ATP secretion was recorded.

Determination of RANTES, TGF-β1, and 5-HT levels. Anti-coagulated blood was immediately centrifuged at 3,000 rpm for 20 min at 4°C, and the supernatant was collected. Serum levels of PF4 and β-TG were measured with an ELISA kit as previously described (11).

Statistical analysis. The results were expressed as the means±SD. Data were analyzed via one-way ANOVA coupled with the Bonferroni multiple comparison test. Differences were considered significant at \( p < 0.05 \). The SPSS 16.0 statistical package was used for statistical analysis, and GraphPad Prism 6.0 software was employed to create graphs.

RESULTS

Cy-3-g extraction and analyses

Cy-3-g was identified by both its retention time and mass profile, based on comparison with authenticated standards. Cy-3-g was analyzed using the HPLC-PAD
method, and the obtained purity was approximately 96.0% (Supplemental Online Materials, Fig. S1 and S2).

Body weight and food intake

After 12 wk of intervention, the body weight of mice in the HFD and HFD + Cy-3-g groups was significantly greater than in the control group \((p < 0.05)\) (Table 2), but no significant difference was observed between the HFD and HFD + Cy-3-g groups. The food intake was not significantly different among the three groups during the experimental period (Supplemental Online Materials, Table S1).

Cy-3-g significantly decreased serum lipid levels

Compared with the HFD group, TC and LDL-C levels were reduced in the HFD + Cy-3-g group \((p < 0.05)\), as shown in Fig. 1A and C, and the serum HDL-C level in the HFD + Cy-3-g group was significantly increased \((p < 0.05)\) compared with the HFD group. The serum TC and TG levels of the HFD and HFD + Cy-3-g groups were significantly increased compared with the control group \((p < 0.05)\) (Fig. 1A and B).

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**Fig. 2.** Cy-3-g significantly inhibited P-selectin and CD40L expression in gel-filtered platelets. (A) P-selectin expression in gel-filtered platelet suspension. (B) CD40L expression in gel-filtered platelet suspension. The presented values are the means ± SD, \(n = 10\) per group. * \(p < 0.05\) versus the control group; # \(p < 0.05\) versus the HFD group.

**Fig. 3.** Cy-3-g decreased ATP release in gel-filtered platelets. ATP levels were measured in the gel-filtered platelets of mice fed a normal diet (control), a high-fat diet (HFD) or the HFD supplemented with an anthocyanin extract from mulberries (HFD + Cy-3-g) at 12 wk. Values represent the means ± SD, \(n = 10\) per group. * \(p < 0.05\) versus the control group; # \(p < 0.05\) versus the HFD group.

**Fig. 4.** Cy-3-g decreased granular release in gel-filtered platelets. (A) The level of 5-HT release was assessed using an ELISA kit. (B) RANTES concentrations were determined via ELISA. (C) TGF-β1 concentrations were determined via ELISA. The presented values are the means ± SD, \(n = 10–12\) per group. * \(p < 0.05\) versus the control group; # \(p < 0.05\) versus the HFD group.
Cy-3-g significantly decreased serum PF4 and β-TG levels. Serum PF4 and β-TG levels were measured via ELISA. The presented values are the means±SD, n=10 per group. *p<0.05 versus the control group; #p<0.05 versus the HFD group.

Fig. 5. Cy-3-g decreased serum PF4 and β-TG levels. Serum PF4 and β-TG levels were measured via ELISA. The presented values are the means±SD, n=10 per group. *p<0.05 versus the control group; #p<0.05 versus the HFD group.

Cy-3-g significantly inhibited the expression of P-selectin and CD40L in gel-filtered platelets.

The expression levels of P-selectin and CD40L in the HFD+Cy-3-g and HFD groups were significantly higher than in the control group (Fig. 2; the flow cytometry results are shown in Supplemental Online Materials, Fig. S3). However, Cy-3-g supplementation significantly decreased the expression levels of P-selectin and CD40L in gel-filtered platelets by 18.3% (p<0.05) and 23.9% (p<0.05), respectively, compared with the HFD group (Fig. 2B).

Cy-3-g significantly decreased ATP release in gel-filtered platelets.

Compared with the control group, ATP release in the HFD and HFD+Cy-3-g groups was increased (p<0.05) (Fig. 3). However, Cy-3-g significantly decreased ATP secretion by 20.7% in gel-filtered platelets compared with the HFD group (p<0.05).

Cy-3-g significantly inhibited 5-HT, RANTES, and TGF-β1 release in gel-filtered platelets.

The levels of 5-HT, RANTES, and TGF-β1 in the HFD and HFD+Cy-3-g groups were higher than in the control group (p<0.05) (Fig. 4). However, compared with the HFD group, Cy-3-g significantly decreased the expression of 5-HT by 29.1% (p<0.05) (Fig. 4A), RANTES by 43.4% (p<0.05) (Fig. 4B), and TGF-β1 by 49.3% (p<0.05) in the HFD+Cy-3-g group (Fig. 4C).

Cy-3-g significantly decreased serum PF4 and β-TG levels.

Compared with the control group, the serum levels of PF4 and β-TG were significantly increased in the HFD and HFD+Cy-3-g groups (p<0.05). Compared with the HFD group, Cy-3-g significantly decreased the serum levels of PF4 and β-TG by 31.5% (p<0.05) (Fig. 5A) and 38.0% (p<0.05) (Fig. 5B), respectively, in the HFD+Cy-3-g group.

DISCUSSION

Many studies have demonstrated that phytochemicals display beneficial effects for cardiovascular protection (22). Recent work has shown that phytochemicals exert protective effects on platelet function, such as the inhibition of calcium influx by trans-resveratrol in thrombin-stimulated human platelets (23) and the inhibition of platelet activation and thrombus formation by ginsenoside-Rp1 (24). Cy-3-g is one of the most common anthocyanin monomers with a glycoside. It is well known that foods such as berries, grapes, and black rice are rich in anthocyanins. The anthocyanin used in the present study was extracted from black rice. Our results demonstrated that Cy-3-g from black rice was capable of attenuating lipid profiles and down-regulating platelet granule release in mice fed high-fat diets. To the best of our knowledge, this work provides the first evidence that Cy-3-g can directly attenuate granule release in gel-filtered platelets in mice in vivo.

It is well established that high blood cholesterol can result in platelet hyperactivity and impair platelet function (25). High cholesterol levels can increase platelet sensitivity and enhance reactive oxygen species (ROS) formation in the platelet membrane, which increases platelet aggregation and is responsible for the role of platelets in CVDs (26, 27). Several studies have indicated that dietary anthocyanins exert an anti-thrombotic effect by regulating TG synthesis or lipid hydrolysis (28, 29). The present study showed that Cy-3-g significantly decreased serum TC and LDL-C levels in mice fed high-fat diets. The observed effects of the anthocyanin Cy-3-g in modulating lipid profiles were consistent with previous findings (28).

We previously reported the effects of Cy-3-g on the expression of P-selectin and CD40L on the platelet surface in gel-filtered platelets from hypercholesterolemic patients in vitro, in addition to the effects on 5-HT levels and ATP release; the same reductions were demonstrated in vivo for the first time in the present study. Because circulating 5-HT, RANTES and TGF-β1 are secreted from several cell types, such as platelets, endothelial cells, smooth muscle cells, activated T cells, and macrophages (30), their levels in purified platelets will be higher and more effective for demonstrating the direct effects of Cy-3-g on platelet granule release. This may be one of the reasons that TGF-β1 release from platelets was significantly decreased by Cy-3-g in mice fed high-fat diets for 12 wk in the present study, although we previously found that a purified anthocyanin mixture produced a minor reduction in TGF-β1 levels after 24 wk of intervention in hypercholesterolemic patients (p>0.05).

Interestingly, serum PF4 levels were also significantly
increased by Cy-3-g in vivo in the mouse model compared with the minor reduction observed in hypercholesterolemic patients (p>0.05), despite the fact that a significant decrease was found in vitro. The biosynthesis of PF4 was previously thought to be almost exclusively limited to megakaryocytes, from which mature circulating platelets are derived. However, low levels of PF4 have recently been reported to be synthesized in activated human monocytes as well, although the specific amount of PF4 as well as its relative level compared with that in platelets and platelet releasates and its biological role are uncertain (31). The multiple origins of PF4 may help to explain why PF4 showed a greater decrease in the present study as discussed above. Recent reports have shown that PF4 promotes clot formation only over a narrow range of concentrations, and both higher and lower PF4 concentrations may interfere with optimal clot formation (32). The PF4 concentrations detected in our study clearly differ from those found in the previous human study; this difference may be due to the different species involved or differences in the responses to Cy-3-g in the digestion, absorption rates or metabolic transformation in the gastrointestinal tract (33). The feeding time and dose of anthocyanins also differed between the animal models and previous human studies. Therefore, the potential biological roles of PF4 defined in mice in vivo need to be tested further, and whether these observations are of biologic relevance in humans must be assessed in future work.

Our results indicating decreasing effects on platelet granule release are consistent with previous in vivo studies using other polyphenolic substances (34, 35) and provide additional evidence of the mechanisms underlying the beneficial effects of anthocyanins. Several studies have demonstrated that polyphenolic substances may modulate platelet adhesion by interfering with arachidonic acid metabolism (36) or exerting anti-oxidative stress effects (37). Our previous work suggested that PI3K/Akt signaling mediates platelet granule secretion primarily by activating the eNOS/NO-cyclic GMP pathway and subsequently activating MAPK in vitro (38, 39), which should be confirmed in further in vivo studies.

Most studies have indicated that the bioavailability of anthocyanins is limited, with less than 1% of the ingested amount reaching the plasma (40). However, a study in humans using 13C5-labeled anthocyanin showed that the relative bioavailability was 12.38±1.18% (17). Furthermore, anthocyanins can be absorbed into the circulation in their intact form in small amounts, while substantial amounts of anthocyanins enter the colon and undergo extensive metabolic transformation by the gut microbiota, producing a wide range of aromatic and phenolic compounds (33). Several in vivo studies have shown that some of these compounds, such as protocathechuic acid (PCA), can be detected in the systemic circulation of rats and humans after consumption of the parent compounds (41). Notably, gut microbiota metabolites of anthocyanin may also contribute to the benefits of anthocyanin supplementation in vivo. Further studies are needed to clarify the functions and mechanisms of the metabolism of anthocyanins and their metabolic compounds in relation to their effects on platelets.

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Supporting Information

Supplemental Online Material is available on J-STAGE.

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