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Cyanidin-3-glucoside regulates phosphorylation of endothelial nitric oxide synthase

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Abstract Dietary anthocyanins are widely distributed in fruits, vegetables and red wines, and there are several reports mentioning their beneficial effects. Previously we reported that cyanidin-3-glucoside (Cy3G) induced endothelial nitric oxide synthase (eNOS) expression and nitric oxide (NO) production in bovine vascular endothelial cells. In the present study, we show that Cy3G regulates phosphorylation of eNOS and Akt, affects the interaction between eNOS and soluble guanylyl cyclase, and increases cyclic guanosine monophosphate (cGMP) production. Our results suggest that Cy3G enhances vascular eNOS activity, and may help to improve vascular endothelial function.
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Keywords: Dietary polyphenol; Nitric oxide synthase; Endothelium; Signal transduction; Phosphorylation

1. Introduction

Cardiovascular diseases such as atherosclerosis and hypertension are major factors affecting health. The regular drinking of red wine containing phenolic compounds has been reported to reduce the incidence of coronary heart disease in France, a phenomenon known as the "French paradox" [1]. Dietary polyphenols are widely distributed in vegetables, fruits, and beverages such as tea and wine. Several recent studies have demonstrated that polyphenols such as epigallocatechin-3-gallate [2], resveratrol [3], quercetin [4], and delphinidin [5] enhance nitric oxide (NO) output to improve endothelium-dependent vascular relaxation. Anthocyanins are polyphenols and there are several reports mentioning their beneficial effects. For example, cyanidin-3-glucoside (Cy3G) exhibits free radical scavenging activity [6], suppresses inflammation [7], protects against endothelial dysfunction [8] and decreases myocardium damage [9].

The generation of NO by endothelial nitric oxide synthase (eNOS) plays a major role in maintaining cardiovascular homeostasis by governing blood pressure, improving endothelial function, suppressing vascular smooth muscle mitogenesis, and inhibiting leukocyte adhesion and platelet aggregation. Several studies have reported that estrogen, high-density lipoprotein, shear stress, vascular endothelial growth factor, and bradykinin induce the phosphorylation of eNOS at Ser1179 via Akt and ERK1/2 MAP kinases in endothelium-dependent vasomotion [10–16]. On the other hand, agonist stimulation also results in the dephosphorylation of eNOS at Ser116 by calcineurin [17].

In a previous study, we reported that Cy3G induced eNOS expression and NO production [18]. In the present study, we examined whether Cy3G quickly stimulates eNOS activity. The results showed that Cy3G brought about phosphorylation at Ser1179 and dephosphorylation at Ser116 in eNOS, caused formation of the soluble guanylyl cyclase (sGC)/eNOS complex, and increased cyclic guanosine monophosphate (cGMP) production in BAECs.

2. Materials and methods

2.1. Materials

Cyanidin-3-glucoside (Cy3G) was purchased from Extrasynthese (Lyon, France), PD98059 from Sigma (St. Louis, MO), wortmannin from BIOMOL Research Laboratories (Plymouth Meeting, PA), and cyclosporin A from Wako Pure Chemical Industries (Osaka, Japan). Anti-eNOS mouse monoclonal antibody was obtained from Calbiochem (San Diego, CA). Anti-ERK1/2 MAP kinase rabbit antibody and the direct cGMP enzyme immunoassay kit were purchased from Sigma. Anti-Src kinase rabbit polyclonal antibody, anti-phospho-Src (Tyr416) mouse monoclonal antibody, anti-phospho-ERK1/2 MAP kinase (clone 12D4) mouse monoclonal antibody, anti-phospho-serine/threonine mouse monoclonal IgGs mixture, anti-phospho-eNOS (Ser116) rabbit IgG, Akt cDNA (dom. neg.) in the pUSEamp vector and the wild-type Akt cDNA vector were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-Akt1 mouse monoclonal IgG, anti-phospho-Akt (Ser473) rabbit polyclonal IgG, anti-vasodilator-stimulated phosphoprotein (VASP) goat IgG, anti-sGC-β1 rabbit IgG, and protein A/G Plus agarose were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-eNOS (Ser1177) rabbit IgG was obtained from Cell Signaling Technology (Beverly, MA). Anti-activated-VASP (Ser239) mouse IgG was from nanoTools Antikorpertrchnik (Taningen, Germany). Fugene 6 transfection reagent was purchased from Roche Diagnostics (Mannheim, Germany). Opti-MEM I and G418 were purchased from Invitrogen Corp. (Carlsbad, CA). The ECL plus Western Blot Detection System was from Amersham Biosciences Corp. (Piscataway, NJ).

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Abbreviations: BAECs, bovine vascular endothelial cells; cGMP, cyclic guanosine monophosphate; Cy3G, cyanidin-3-glucoside; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; sGC, soluble guanylyl cyclase; VASP, vasodilator-stimulated phosphoprotein

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2.2. Endothelial cell culture

Bovine artery endothelial cells (BAECs) were obtained from the Human Science Research Resources Bank (Osaka, Japan). Cell culture was performed as described previously [18].

2.3. Immunoprecipitation and western blotting

Preparation, immunoprecipitation, and immunoblot analysis of protein extracts were performed as described previously [18,19].

2.4. Transfection

Transfection of the dominant negative Akt cDNA and wild Akt cDNA vector were performed as described previously [18].

2.5. Detection of cyclic GMP activation

Intracellular cyclic GMP activation was measured with ELISA immunoassay kits according to the manufacturer's instructions. In brief, cell lysates and primary antibody were dispensed into designated wells of a 96-well immunoplate and incubated for 2 h at room temperature. After five washes, cGMP-alkaline phosphatase was added to each well and incubated for 1 h. Finally, the microplate was washed and a *p*-nitrophenyl phosphate substrate solution was added to each well. After 30 min, the reaction was terminated with HCl. The absorbance at 405 nm was measured within 20 min.

2.6. Data presentation

Statistical analysis was performed with an ANOVA test when appropriate. The data are expressed as means ± S.E.M. Values of $P < 0.05$ were considered significant.

3. Results

3.1. Cy3G regulated both phosphorylation at Ser1179 and dephosphorylation at Ser116 of eNOS

Following treatment with Cy3G at 0.005–5 μmol/L for 12 min, the eNOS in BAECs showed phosphorylation at Ser1179 and dephosphorylation at Ser116 (Fig. 1A), with a maximal increase (about 2-fold of the control, $n = 3$) of 0.5 μmol/L at 12 min. The effective concentration for the dephosphorylation at Ser116 was 0.5–5 μmol/L (Fig. 1A), with the minimum band density (average of 0.54-fold of the control, $n = 3$) of 0.5 μmol/L of Cy3G at 12 min. On the other hand, after treatment with 0.5 μmol/L of Cy3G the peak of phosphorylation at Ser1179 occurred at 10–15 min. It should be noted that the dephosphorylation at Ser116 was sustained for over 30 min (Fig. 1B).

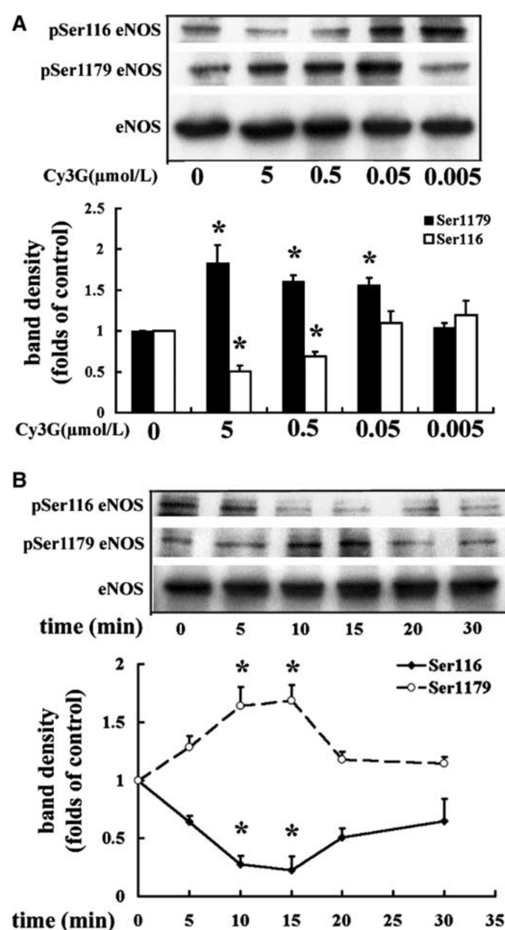

Fig. 1. Cy3G-induced phosphorylation at Ser1179 and dephosphorylation at Ser116 of eNOS in BAECs assayed by Western blotting. (A) BAECs were treated with the indicated concentration for 12 min. (B) BAECs were treated with 0.5 μmol/L of Cy3G for the period indicated. Top: an original bolt. Bottom: results of densitometric analyses. Data are means ± S.E.M. ($n = 3$, *$P < 0.01$).

3.2. Relationship among Src, Akt, and ERK1/2 MAP kinase

In a previous study, it was demonstrated that Cy3G treatment results in rapid phosphorylations of Src and ERK1/2 kinase [18]. In the present study, we tested the role of Akt kinase in mediating the Cy3G-induced phosphorylation of eNOS. Cy3G stimulated the phosphorylation of Akt at Ser473 in a time-dependent manner, with a peak at 5–10 min (Fig. 2A). We next examined the relationships among Src, Akt and ERK1/2 kinase using the Src kinase inhibitor pp2, PI3 kinase inhibitor wortmannin, and MEK inhibitor PD98059. Results showed that pp2 at 20 μmol/L blocked the phosphorylation of Src, Akt, and ERK1/2 kinase (Fig. 2B). PD98059 at 10 μmol/L inhibited the phosphorylation of ERK1/2 MAP kinase and wortmannin at 1μmol/L inhibited the phosphorylation of Akt (Fig. 2B). However, PD98059 did not suppress Akt.

3.3. Role of Src, Akt, ERK1/2, and calcineurin in the Cy3G-induced phosphorylation of eNOS

We also found that pp2, wortmannin, and PD98059 markedly inhibited the phosphorylation at Ser1179 (Fig. 3A), and that the calcineurin inhibitor cyclosporin A at 100 nmol/L also blocked the dephosphorylation at Ser116 (Fig. 3B). Transfection of the dominant-negative Akt cDNA efficiently inhibited the phosphorylation at Ser1179 stimulated by Cy3G. In contrast, transfection of the wild-type Akt cDNA vector increased the level of phosphorylation at Ser1179 stimulated by Cy3G (Fig. 4). Thus, the ERK1/2, Akt, and the upstream Src are necessary for enhanced enzymatic activity of eNOS in response to Cy3G.

3.4. Cy3G affected the association between eNOS and soluble guanylyl cyclase

Soluble guanylyl cyclase (sGC) is an important downstream intracellular target of NO. A recent study reported that the sGC β-subunit interacts directly with heat shock protein 90 (HSP90) and indirectly with eNOS in endothelial cells in response to eNOS-activating agonists [20]. Our results demonstrated that Cy3G caused formation of the eNOS/sGC complex (Fig. 5A), as determined by immunoprecipitation with anti-eNOS antibody and by immunoblotting with anti-sGC-β1 antibody. On pretreatment for 30 min with geldanamycin (10 mg/ml), a specific inhibitor of HSP90 activity

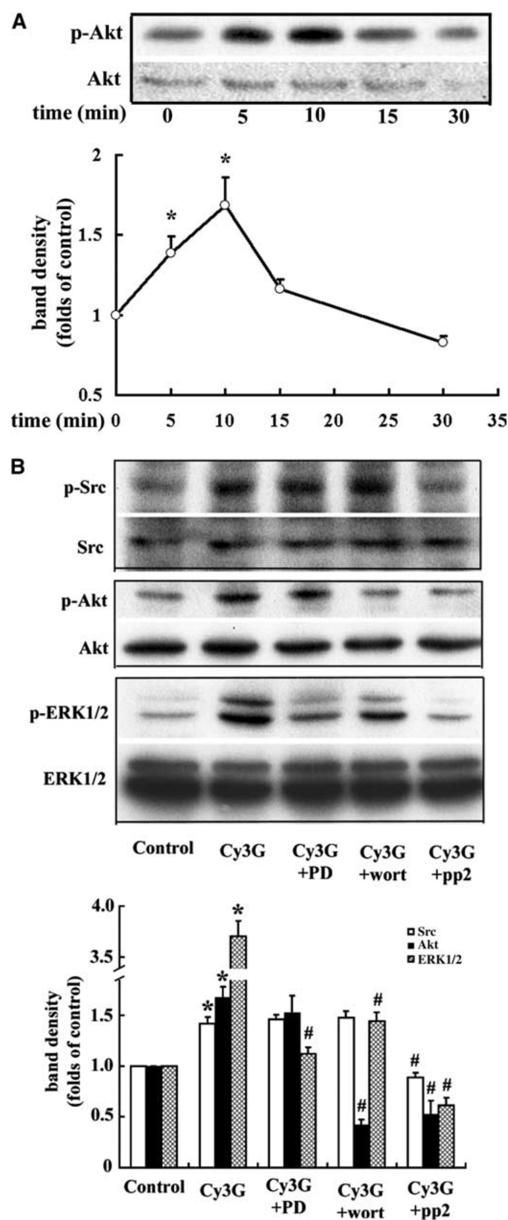

Fig. 2. Interaction among Src, Akt, and ERK1/2. (A) BAECs were treated with 0.5 μmol/L of Cy3G for the period indicated. (B) After pretreatment with pp2 at 20 μmol/L, wortmannin at 1μmol/L, or PD98059 at 10 μmol/L for 30 min, BAECs were stimulated with 0.5 μmol/L of Cy3G. Top: an original bolt. Bottom: results of densitometric analyses. Data are means ± S.E.M. (each $n = 3$, $*P < 0.01$ vs. control; $\#P < 0.01$ vs. Cy3G).

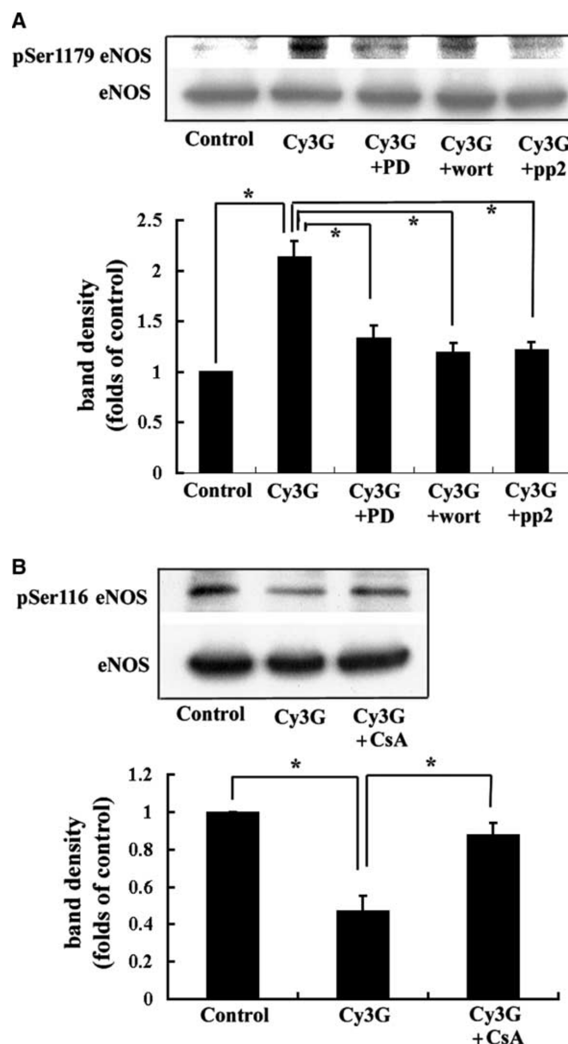

Fig. 3. Inhibitory effect of pp2, wortmannin, PD98059 or cyclosporin A on the phosphorylation at Ser1179 or dephosphorylation at Ser116 in eNOS. (A) After pretreatment with pp2 at 20 μmol/L, wortmannin at 1 μmol/L, or PD98059 at 10 μmol/L for 30 min, BAECs were stimulated with 0.5 μmol/L of Cy3G. (B) After pretreatment with cyclosporin A at 100 nmol/L for 60 min, BAECs were stimulated with 0.5 μmol/L of Cy3G. Top: an original bolt. Bottom: results of densitometric analyses. Data are means ± S.E.M. ($n = 3$, $*P < 0.01$).

[21], this Cy3G-activated process was suppressed (Fig. 5A). Furthermore, Cy3G increased production of cGMP (Fig. 5B) (control: 0.341 ± 0.039 pmol/ml vs. Cy3G: 0.498 ± 0.044 pmol/ml, $n = 4$, $P < 0.05$), however, pretreatment with L-NAME (50 μmol/L) or wortmannin (1 μmol/L) abolished this rise in production (Fig. 5B, each $n = 4$, $P < 0.05$). Cy3G also stimulated phosphorylation at Ser239 of the VASP, a sensitive monitor of defective NO/cGMP signaling [22]. The phosphorylation of VASP occurred 10 min after the treatment with Cy3G (Fig. 5C).

4. Discussion

Several studies have demonstrated that anthocyanins protect against endothelial dysfunction and inhibit endothelial cell apoptosis [6–9,23]. Two studies reported that dietary anthocyanins, such as Cy3G, are directly absorbed in rats and humans, appear in the blood, and are excreted intact in urine [24,25], suggesting that the glucoside form of cyanidin is important. The present study demonstrates that Cy3G had an acute effect on eNOS through non-transcriptional activation, and induced phosphorylation at Ser1179 and dephosphorylation at Ser116 of eNOS in BAECs. After treatment with 0.5 μmol/L of Cy3G for 12 min, phosphorylation at Ser1179 rose up to 2-fold, while dephosphorylation at Ser116 decreased to 54% of the control level (Fig. 1).

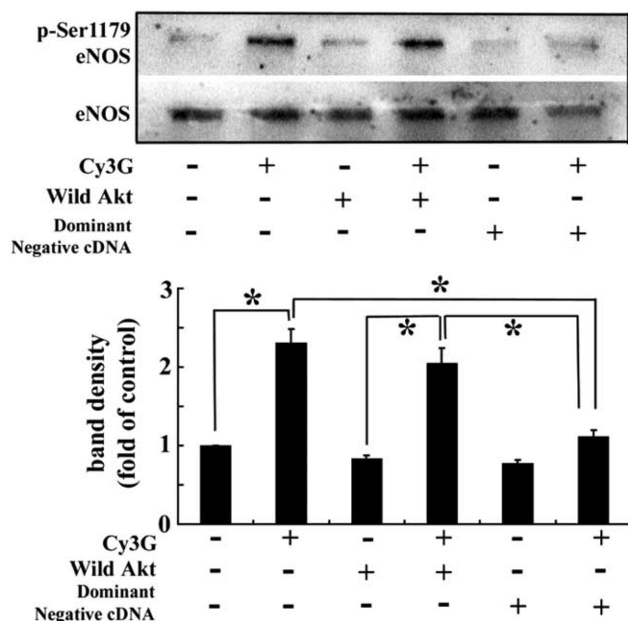

Fig. 4. Dominant negative Akt inhibited the Cy3G-induced phosphorylation of eNOS at Ser1179. Transfections are described in detail under Section 2. The phosphorylation of eNOS at Ser1179 was assayed by Western blotting. Top: an original bolt. Bottom: results of densitometric analyses. Data are means ± S.E.M. ($n = 3$, $^*P < 0.01$).

In the present study and a pervious work [18], we found that Src, Akt, ERK1/2 and calcineurin were involved in the cellular response after Cy3G treatment. Cy3G rapidly caused the phosphorylation of Src (Tyr416), Akt (Ser473), and ERK1/2. The phosphorylation of Akt and ERK1/2 paralleled that of eNOS (Ser1179) (Figs. 1B and 2A). Inhibition of PI3K/Akt and MEK/ERK1/2 prevented phosphorylation of eNOS at Ser1179 by the inhibitors wortmannin and PD98059 (Fig. 3A) or by transfection of the dominant-negative Akt cDNA (Fig. 4), consistent with the well-established role of Akt [26,27] and ERK1/2 [12,14–16] in the phosphorylation of eNOS. Furthermore, the peak of phosphorylation of Src occurred after 5–10 min [18], earlier than that of eNOS at Ser1179. A specific inhibitor of Src (pp2) blocked the phosphorylation of Src, Akt, ERK1/2, and eNOS (Ser1179) (Figs. 2B and 3A), whereas an inhibitor of PI3K, wortmannin, and MEK, PD98059, did not prevent the phosphorylation of Src (Fig. 2B, top panel). These results suggest that Src lies upstream of Akt, ERK1/2, and eNOS. Moreover, wortmannin did not affect ERK1/2 and PD98059 did not change Akt. It seems that these two kinases do not depend on each other, and that there is no crosstalk between the Akt and ERK1/2 signaling pathways, consistent with the findings of Hisamoto et al. [10] and Mineo et al. [13]. Recent studies have shown that Src is involved in the phosphorylation of eNOS during shear stress [14,15], estrogen treatment [11], or exposure to high-density lipoprotein [13].

Bauer et al. [28] indicated that Ser116 of eNOS acts as a negative regulatory site and is important for the response to agonists. Kou et al. [17] reported that the agonist VEGF promotes dephosphorylation of eNOS at Ser116 in endothelial cells. In the present study, we observed that Cy3G treatment also resulted in the dephosphorylation of eNOS at Ser116 (Fig. 1A and B). Cyclosporin A at 100 nmol/L completely blocked this dephosphorylation (Fig. 3B).

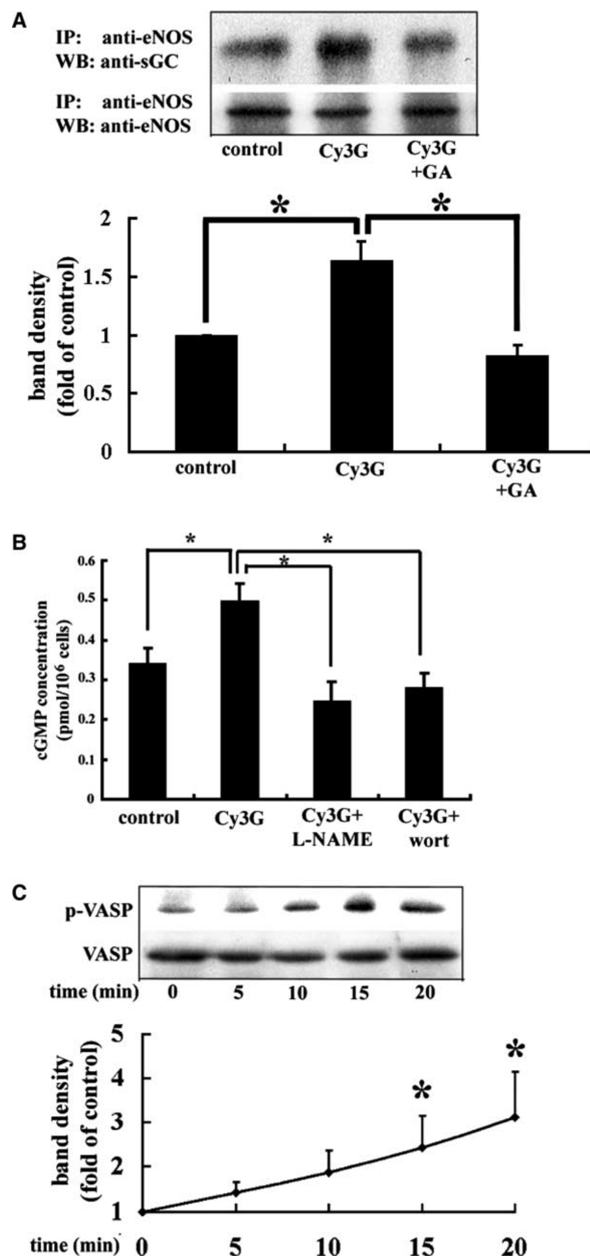

Fig. 5. Cy3G regulated interaction between eNOS and sGC, increased production of cGMP, and induced phosphorylation of VASP. (A) BAECs were pretreated with or without geldanamycin (10 mg/ml) for 30 min and then stimulated with 0.5 μmol/L of Cy3G for 15 min. (B) BAECs were pretreated with or without L-NAME or wortmannin (wort) for 30 min and stimulated with 0.5 μmol/L of Cy3G for 3 h. The cGMP concentration (pmol/10^6 cells/3 h) was measured with an ELISA immunoassay. (C) BAECs were treated with 0.5 μmol/L of Cy3G for the period indicated. Top: an original bolt. Bottom: results of densitometric analyses. Data are means ± S.E.M. ($n = 3$ or 4, $^*P < 0.05$).

The phosphorylation of eNOS at Ser1179 leads to augmented enzyme activity, as evidenced by the regulated association between eNOS and sGC, the increase in production of cGMP, and the phosphorylation induced at Ser239 of VASP after treatment with Cy3G. Recently, sGC was found in caveolae of endothelial cells [29]. Our results showed that Cy3G caused formation of this sGC/eNOS complex. Pretreatment with geldanamycin for 30 min significantly suppressed the

formation (Fig. 5A). Furthermore, Cy3G stimulated phosphorylation at Ser239 of VASP (Fig. 5C) as a sensitive monitor of defective NO/cGMP signaling [22].

We demonstrated that the dietary anthocyanin Cy3G acts as a natural activator of eNOS in endothelial cells by promoting its phosphorylation at Ser1179 and dephosphorylation at Ser116. The results ameliorating endothelial function may in part explain the protective effects of plant-derived flavonoids on cardiovascular diseases.

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