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Upregulation of Endothelial Nitric Oxide Synthase by Cyanidin-3-Glucoside, a Typical Anthocyanin Pigment

Jin-Wen Xu, Katsumi Ikeda, Yukio Yamori

Abstract—Endothelial nitric oxide synthase (eNOS) plays an important role in maintaining blood pressure homeostasis and vascular integrity. Natural dietary flavonoids are thought to protect against cardiovascular diseases by acting as antioxidants and vasodilators. This study examined the effect of cyanidin-3-glucoside (Cy3G), a typical anthocyanin pigment, on eNOS expression. Treatment of bovine artery endothelial cells (BAECs) with Cy3G for 8 hours of enhanced eNOS protein expression in a dose- and time-dependent manner was determined by Western blot analysis. Longer incubation (12, 16, and 24 hours) of BAECs with 0.1 $\mu\text{mol/L}$ of Cy3G caused a further increase in eNOS expression, and subsequently Cy3G also significantly increased nitric oxide output 2-fold (24 hours). Furthermore, Cy3G stimulated the phosphorylation of Src and extracellular signal-regulated kinase 1/2 (ERK1/2) in a time-dependent manner. An Src kinase inhibitor, pp2, and MEK inhibitor, PD98059, blocked the ERK1/2 phosphorylation and eNOS expression. Transfection with dominant-negative Src cDNA also inhibited the eNOS expression stimulated by Cy3G. In addition, stimulation with Cy3G for 30 minutes resulted in a phosphorylation of Sp1 that was blocked by PD98059. Cy3G enhanced the binding activity of the transcription factor Sp1 to the GC box in the proximal eNOS promoter of BAECs, as revealed by chromatin immunoprecipitation assay. The present study demonstrated that Cy3G induced eNOS expression and escalated NO production via an Src-ERK1/2-Sp1 signaling pathway in vascular endothelial cells. Increased eNOS expression may help to ameliorate endothelial dysfunction, harmonize blood pressure, and prevent atherosclerosis as long-term beneficial effects of flavonoids. (*Hypertension*. 2004;44:217-222.)

Key Words: nutrition ■ nitric oxide synthase ■ endothelium ■ signal transduction ■ gene expression

Cardiovascular diseases such as atherosclerosis and hypertension are major factors affecting public health. The regular drinking of red wine containing phenolic compounds has been found to reduce the incidence of coronary heart disease in France, a phenomenon known as the “French paradox.”¹

Anthocyanins are polyphenols, and there are several reports mentioning their beneficial effects. For example, cyanidin-3-glucoside (Cy3G) exhibits free radical scavenging activity; suppresses inflammation; protects against endothelial dysfunction, vascular failure, and myocardium damage; prevents obesity; ameliorates hyperglycemia; and seems to help prevent cardiovascular disease.²⁻⁸

Dietary polyphenols are widely distributed in vegetables, fruits, and beverages such as tea and wine. Recently, studies have shown that polyphenols, such as resveratrol, quercetin, delphinidin, and epigallocatechin-3-gallate, enhance NO output to improve endothelium-dependent vascular relaxation.⁹⁻¹⁵

The generation of NO by endothelial nitric oxide synthase (eNOS) plays a major role in maintaining cardiovascular homeostasis by influencing blood pressure, endothelial dysfunction, vascular smooth muscle mitogenesis, matrix synthesis, leukocyte adhesion, and platelet aggrega-

tion. The expression of eNOS has been shown to be unregulated by resveratrol,^{10,11} shear stress,^{16,17} lysophosphatidylcholine,¹⁸ ceramide,¹⁹ and perinuclear EP₃ receptors,²⁰ among others. In the eNOS promoter, Sp1 binds the GC-rich region and is necessary for basal transcription in endothelial cells.²¹⁻²³ The expression of Sp1 mutants resulted in $\approx 90\%$ reduction of promoter activity in luciferase assays and in DNase I footprinting assays coupled with gel shift assays.^{21,24} There is some evidence that extracellular signal-regulated kinase 1/2 (ERK1/2) mitogen-activated protein (MAP) kinase is involved in signal transduction for eNOS expression.^{18,20,25-26}

Although some studies have indicated that Cy3G, a typical anthocyanin pigment, can stimulate endothelium-dependent vascular relaxation, no study to date has examined the effect of Cy3G on eNOS expression, and subsequently on NO production. Thus, the aim here was to verify the hypothesis that Cy3G induces NO output due to accelerated eNOS expression in vascular endothelial cells. The present study demonstrated that Cy3G induced eNOS expression and activated the Src-ERK1/2-Sp1 signaling pathway in bovine artery endothelial cells.

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Materials and Methods

An extended Methods section is available online at <http://www.hypertensionaha.org>.

Chromatin Immunoprecipitation Assay

Bovine artery endothelial cells (BAECs) were cultured for 6 hours with or without Cy3G (0.1 $\mu\text{mol/L}$) after pretreatment with or without PD98059 (20 $\mu\text{mol/L}$) for 30 minutes. The by chromatin immunoprecipitation (ChIP) assay was performed using an assay kit (Upstate Cell Signaling Solutions) according to the manufacturer's instructions. Briefly, cells were cross-linked with 1% formaldehyde for 10 minutes at room temperature. Glycine was added at a final concentration of 0.125 mol/L to stop cross-linking. After a wash with ice-cold PBS, cells were lysed for 10 minutes (1×10^6 cells/200 μL of SDS lysis buffer). The chromatin was sheared by sonication $4 \times$ for 40 seconds at one-third maximum power with 1 minute cooling on ice between each pulse. Cross-linked chromatin was quantitated to determine the initial amount of DNA present in the different samples (input chromatin). The remaining chromatin fractions were precleared with salmon sperm DNA/protein A agarose for 1 hour and immunoprecipitated with anti-Sp1 antibody (10 μL) overnight at 4°C . Immune complexes were collected with salmon sperm DNA/protein A agarose for 1 hour, washed $5 \times$, and finally eluted in 1% SDS, 0.1 mol/L NaHCO_3 . Sp1-DNA cross-linking was reversed at 65°C overnight and digested with 100 μg of proteinase K at 45°C for 1 hour. DNA was then recovered in DEPC- H_2O for polymerase chain reaction (PCR). PCRs were performed using human eNOS (-170 to -7) promoter-specific primers: 5'-CGGGCGTGGAGCTGAGGCTT-3' and 5'-CCAGCAGAG-CCCTGGCCTT-3'. The PCR products were analyzed by electrophoresis on 3% agarose gels. The optical density of each PCR fragment was estimated (Electronic UV Transilluminator, ULTRA-LUM Inc), and sample-specific PCR fragment density was used to calculate the equivalence point.

Results

Cy3G Induced NO Release and Expression of eNOS Protein

To examine whether Cy3G activates eNOS in BAECs, we first detected NO production. The results showed that Cy3G induced a 2-fold increase in NO production compared with untreated or N^G -nitro-L-arginine methyl ester (L-NAME)-treated BAECs after treatment at 0.1 $\mu\text{mol/L}$ for 24 hours (each $n=5$, $P<0.01$, Figure I available online). Furthermore, Cy3G also increased eNOS expression in BAECs in a dose- and time-dependent manner. The maximal increase in eNOS expression was observed with 0.1 $\mu\text{mol/L}$ Cy3G treatment for 8 hours ($n=3$, $P<0.01$ versus control, Figure 1A). A longer incubation (12 to 24 hours) of cells with 0.1 $\mu\text{mol/L}$ Cy3G caused a further increase in eNOS expression (each $n=3$, $*P<0.01$ or $\#P<0.05$, Figure 1B). Thus, we selected treatment with 0.1 $\mu\text{mol/L}$ Cy3G for 8 hours in subsequent experiments.

Cy3G Increased eNOS Expression by Activating the Src-ERK1/2 Kinase-Signaling Pathway

We next tested whether the Src-ERK1/2 kinase pathway mediates this Cy3G-induced eNOS expression. Cy3G stimulated phosphorylation of Src in a time-dependent manner with a peak at 5 to 10 minutes and, on average, 2-fold the control value (each $n=3$, $*P<0.01$, Figure IIA available online). Similar to Src kinase, Cy3G also increased the phosphorylation of ERK1/2 kinase, with a peak at 10 to 15 minutes (each $n=3$, $*P<0.01$, Figure IIB). To identify whether the Src kinase as upstream signaling mediates the phosphorylation of

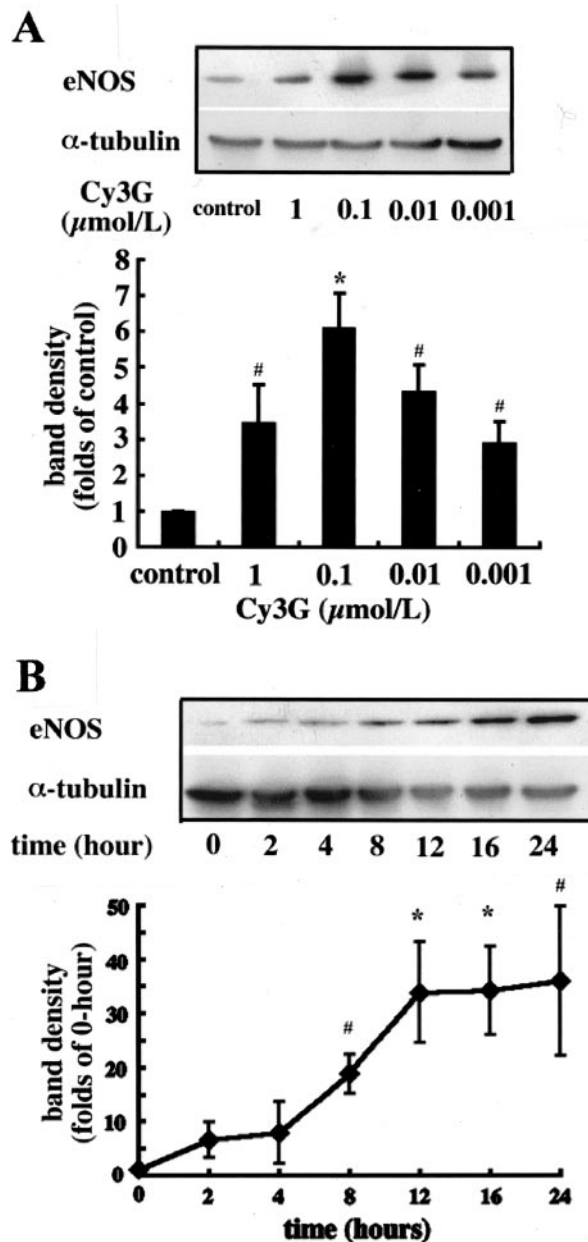


Figure 1. Cy3G-induced eNOS expression in BAECs. Protein expressions were detected by Western blot. BAECs were treated with the concentration, or for the period indicated, or both. Upper part of each figure indicates an original bolt; lower part, results of densitometric analyses. Data are means \pm SEM (each $n=3$, $*P<0.01$ or $\#P<0.05$ vs control).

ERK1/2 kinase, we examined the relationship between Src and ERK1/2 kinase using Src kinase inhibitor, pp2, and MEK inhibitor, PD98059. The results showed that pp2 blocked the phosphorylation of Src and ERK1/2 kinase in a dose-dependent manner (each $n=3$, $*P<0.01$, Figure 2A and 2B), whereas PD98059 also inhibited the phosphorylation of ERK1/2 kinase (each $n=3$, $*P<0.01$ versus Cy3G, Figure 2C). Moreover, both inhibitors, pp2 at 10 $\mu\text{mol/L}$ and PD98059 at 20 $\mu\text{mol/L}$, strongly suppressed eNOS expression induced by Cy3G (each $n=3$, $P<0.01$, Figure 3A and 3B). Furthermore, transfection of a dominant-negative Src cDNA similarly inhibited eNOS expression (each $n=3$,

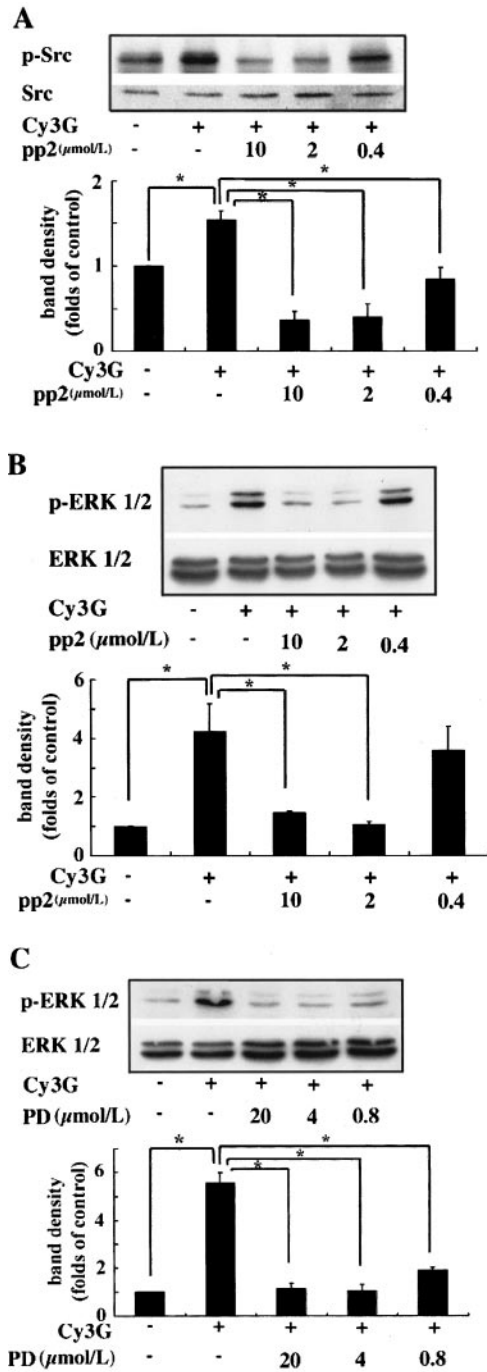


Figure 2. Cy3G activated the Src-ERK1/2 kinase pathway in BAECs. Phosphorylated proteins were detected by Western blotting. BAECs were pretreated with or without pp2 (A and B) or PD98059 (C) at the concentration indicated for 30 minutes before Cy3G-stimulation for 8 minutes (A) or 12 minutes (B and C). Upper part of each figure indicates an original bolt; lower part, results of densitometric analyses. Data are means±SEM (each n=3, *P<0.01).

*P<0.01 or #P<0.05) induced by Cy3G; in contrast, transfection of the empty vector did not inhibit eNOS expression (Figure 4). These results indicate that p60src is activated upstream of ERK1/2 in response to Cy3G and that eNOS expression is mediated at least in part by the Src-ERK1/2 kinase signaling pathway.

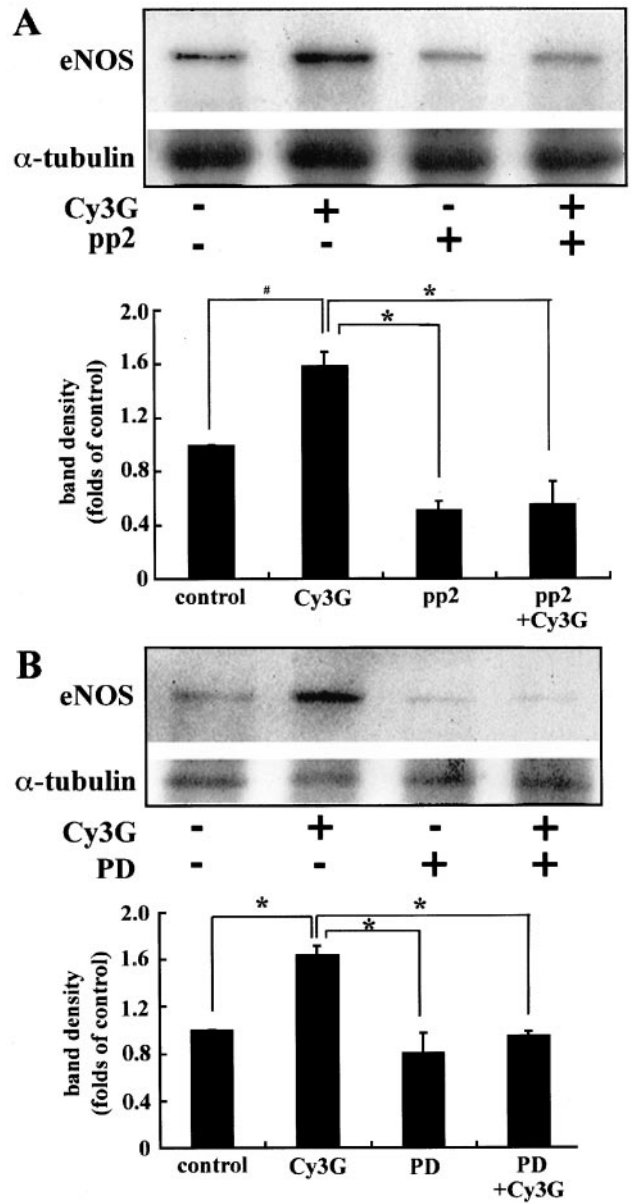


Figure 3. Inhibitory effect of pp2 and PD98059 on Cy3G-induced eNOS expression. After pretreatment with pp2 at 20 μmol/L (A) or PD98059 at 10 μmol/L (B) for 30 minutes, BAECs were coincubated with 0.1 μmol/L of Cy3G for 24 hours. Upper part of each figure indicates an original bolt; lower part, results of densitometric analyses. Data are means±SEM (each n=3, *P<0.01 or #P<0.05).

Role of Sp1 in the Cy3G-Induced eNOS Expression

As shown in Figure 5A, Cy3G caused Sp1 phosphorylation in a time-dependent manner, which was blocked by PD98059 at 20 μmol/L. The average band density of the Cy3G-induced Sp1 phosphorylation at 30 minutes reached 1.7-fold the control level (n=3, P=0.0564, Figure 5A), whereas PD98059 significantly blocked this phosphorylation (n=3, P=0.0385, Figure 5A). In addition, Cy3G also caused a complex to form between ERK1/2 and Sp1, the band density of which reached 2-fold (with ERK1) or 3-fold (with ERK2) the control level (n=2, Figure III available online). To obtain

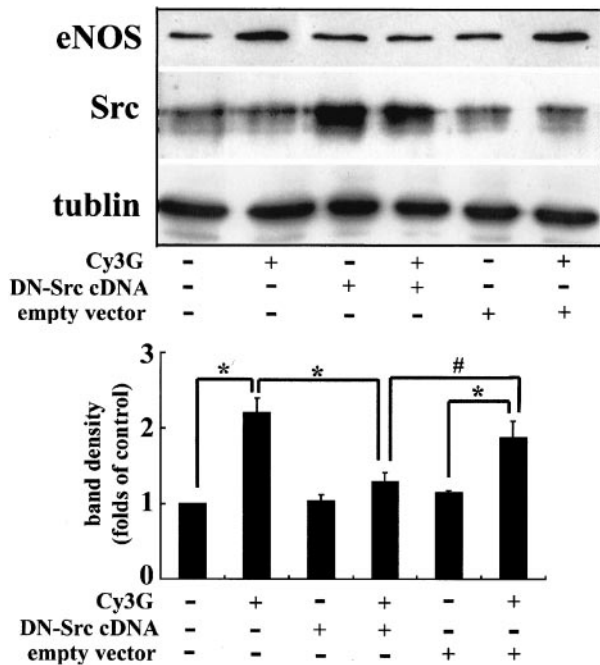


Figure 4. Dominant negative Src kinase inhibited the Cy3G-induced eNOS expression. Transfection of the dominant negative Src kinase cDNA or empty vector is described in detail in the Supplemental Materials and Methods online. Expression of eNOS was assayed by Western blotting. Upper part of figure indicates an original bolt; lower part, results of densitometric analyses. Data are means±SEM (n=3, *P<0.01 or #P<0.05).

direct evidence in vivo of Sp1 binding to the GC box in the proximal eNOS promoter, a ChIP assay was performed in BAECs. PCR primers for the assay were designed to amplify a 163-bp sequence encompassing the proximal GC box in the eNOS promoter.²⁷ As shown in Figure 5B, when incubated with Cy3G, the GC box-containing region of the eNOS promoter bound Sp1 protein in vivo (n=3, P<0.01). Importantly, the binding of Sp1 to the GC box of the eNOS promoter was significantly blocked by PD98059 (n=3, P<0.01 versus Cy3G).

Discussion

In the present study, we showed that Cy3G upregulates eNOS expression in BAECs. The increase in eNOS expression resulted in an enhancement of NO release (Figure 1). The intake of anthocyanins has been estimated to be 180 to 215 mg/d in the United States.²⁸ When 4 healthy elderly women consumed 10.9 mg of anthocyanin per kilogram of weight, the total concentration of anthocyanins in plasma varied from 55.3 to 168.3 nmol/L with an average of 97.4 nmol/L.²⁹ The plasma concentration in a Japanese man was 115 nmol/L on consumption of 3.58 mg of anthocyanins per kilogram of weight.³⁰ The dosage used in the present study was 0.1 μmol/L, close to the plasma concentration in the investigated Japanese man and American women.

Several pieces of evidence from the present study indicate that Src kinase and ERK1/2 kinase activation are essential for eNOS expression induced by Cy3G. (1) Cy3G caused the phosphorylation of Src and ERK1/2 kinase in a time-dependent manner (Figure II). (2) The Src inhibitor pp2 and

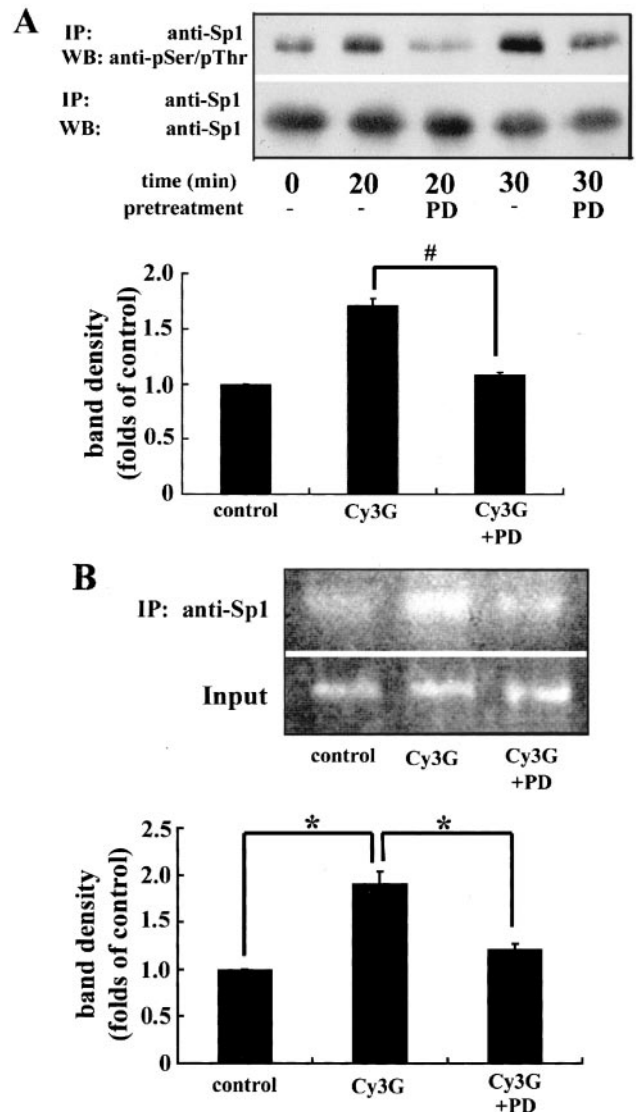


Figure 5. Role of Sp1 in Cy3G-induced eNOS expression. (A) Cy3G induced Sp1 phosphorylation. BAECs were pretreated with or without PD98059 at 10 μmol/L for 30 minutes, and then stimulated with 0.1 μmol/L of Cy3G for the period indicated. Immunoprecipitation and Western blot assay were performed. Upper part of figure indicates an original bolt; lower part, Sp1 phosphorylation at 30 minutes of densitometric analyses. Data are means±SEM (n=3, #P<0.05). (B) Cy3G induced Sp1 binding to the eNOS promoter as detected by chromatin immunoprecipitation (ChIP) Assay in detail is in Materials and Methods. BAECs were incubated with 0.1 μmol/L of Cy3G for 8 hours. Upper part of figure indicates an original density image of PCR fragment; lower part, results of densitometric analyses. Data are means±SEM (n=3, *P<0.01).

MEK inhibitor PD98059 suppressed phosphorylations of Src and ERK1/2 and expression (Figures 2 and 3). (3) A dominant negative Src kinase blocked the expression of eNOS induced by Cy3G (Figure 4). Thus, the responses to Cy3G link the Src-ERK1/2 signal pathway to eNOS expression. Recently, Jalali et al¹⁶ and Davis et al¹⁷ reported that Src kinase is involved in eNOS expression regulated by shear stress, activating p60src-Ras-MAPK signaling pathways. In addition, ERK1/2 kinase is also identified in perinuclear EP₃ receptors-regulated and lysophosphatidylcholine-regulated

eNOS expression pathways.^{18,20} Furthermore, consistent with our results, recent studies indicate that in the eNOS expression and phosphorylation in response to shear stress and high-density lipoprotein, Src kinase equally mediates the phosphorylation and activation of ERK1/2 kinase.^{16,17,31}

We identified that ERK1/2 kinase contributed to the formation of a complex with the transcription factor Sp1 (Figure III) and to Sp1 phosphorylation (Figure 5A). We also examined eNOS promoter activation in vivo using the ChIP assay. The results indicated that the GC box-containing region of the eNOS promoter bound Sp1 protein after treatment with Cy3G, and that PD98059 abrogated this binding (Figure 5B). It is clear that the eNOS promoter has proximal elements containing several putative Sp1 binding sites.^{22–23} The expression of Sp1 mutants resulted in about a 90% reduction of promoter activity in luciferase assays and in DNase I footprinting assays coupled with gel shift assays.^{21,24} Milanini-Mongiati et al³² provided evidence that ERK1/2 kinase directly phosphorylates Sp1 at threonines 453 and 739, both in vitro and in vivo. In addition, ras and ERK 2 also target the GC-element in the gastrin promoter and the DNA binding by Sp1.³³ Furthermore, lysophosphatidylcholine enhances Sp1 binding and Sp1-dependent eNOS promoter activity via the MEK1-ERK1/2 signaling pathway.¹⁸

In the dose–response experiment, the phenomenon that the highest dose (1 $\mu\text{mol/L}$) of Cy3G reduced eNOS expression more than lower doses (0.1 and 0.01 $\mu\text{mol/L}$, Figure 1A) seems to suggest negative feedback in the transcription level modulated by NO. Zhang et al²⁷ indicated that NO down-regulated eNOS expression by decreasing Sp1 binding to a proximal GC box element of eNOS promoter independently of cGMP or NO-mediated transcription factor damage. In TATA-less promoters, such as eNOS, proximal Sp1 binding sites are essential elements in the recruitment, positioning, and stabilization of the transcriptional complex.³⁴ Moreover, the proximal GC box of eNOS, unlike tumor necrosis factor- α promoter in which Sp and AP1 sites were both present, is not flanked by sequences that bind strong transactivating factors.^{27,35} Thus, in eNOS promoter, NO-induced reduction in Sp1 binding would be expected to “turn off” transcription.

In a prior study, we demonstrated that an extract of wine phenolics can ameliorate aortic biomechanical properties in stroke-prone spontaneously hypertensive rats.³⁶ The improvement in aortic biomechanical properties not only involved antioxidant activities but also endothelial NO-dependent vasorelaxation. In the present study and another work,³⁷ we showed that Cy3G, a wine flavonoid, could cause both long-term and short-term enhancement of eNOS activity through gene expression and rapid phosphorylation at Ser1179 of eNOS, the peak of which was 10 to 15 minutes. These beneficial effects may help to prevent cardiovascular diseases such as hypertension and atherosclerosis. Recently, Cy3G has been shown to protect against peroxynitrite-induced endothelial dysfunction and vascular failure.³

Perspectives

Cy3G is a natural dietary polyphenol in wine and various fruits. In the present study, we provide evidence that Cy3G

induces eNOS expression and augments NO release in a time- and dose-dependent manner. We also found that Cy3G stimulated phosphorylation of Src and ERK1/2 in vascular endothelial cells. The phosphorylations of Src and ERK1/2 link to eNOS promoter activation through regulating phosphorylation of transcription factor Sp1 and DNA binding activation. Importantly, 0.1 $\mu\text{mol/L}$, the dosage of Cy3G used in the present study, is close to the plasma concentration in the investigated Japanese men and American women, suggesting that the beneficial effect of Cy3G may help to ameliorate endothelial dysfunction, harmonize blood pressure, and prevent atherosclerosis.

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