**Regular Article**

**Grape Extract from Chardonnay Seeds Restores Deoxycorticosterone Acetate–Salt-Induced Endothelial Dysfunction and Hypertension in Rats**

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Grape extract (GE), which contains various polyphenolic compounds, exerts protective effects against lifestyle-related diseases, such as diabetes and hypertension. We pharmacologically investigated whether dietary supplements with an extract from Chardonnay exerted antihypertensive effects in deoxycorticosterone acetate (DOCA)–salt-induced hypertensive rats. GE increased nitric oxide (NO) production by activating the phosphatidylinositol 3-kinase (PI3K)/Akt pathway in cultured endothelial cells and induced vasorelaxation in the aorta and mesenteric artery via the same pathway. The development and progression of hypertension by the DOCA–salt treatment was significantly inhibited in GE-fed rats. Reduced vasoreactive responses to acetylcholine in the aorta of DOCA–salt rats were significantly ameliorated by the GE diet. Dietary GE supplements slightly diminished vascular superoxide anion production induced by the DOCA–salt treatment. On the other hand, dietary GE supplements had no effect on the progression of hypertension in rats in which NO synthase was pharmacologically and chronically suppressed. In addition, the oral administration of GE for 5 d in healthy rats enhanced endothelial NO synthase (eNOS) gene expression and vascular reactivity to acetylcholine in the aorta. Thus, GE has endothelium-dependent vasorelaxant properties that are mediated by the activation of endothelial NO synthase via the PI3K/Akt pathway, and this mechanism is conducive to the antihypertensive effects of GE observed in DOCA–salt-treated rats.

**Key words** grape extract; nitric oxide; deoxycorticosterone acetate (DOCA)–salt; antihypertensive effect

**INTRODUCTION**

Grape extract (GE) mainly comprises oligomeric proanthocyanidins (OPCs) and contains several polyphenols. Dietary GE supplements have been shown to exert various potentially protective effects against lifestyle-related diseases, such as diabetes, atherosclerosis, and hypertension. The precise mechanisms underlying the protective effects of GE against these diseases remain unclear; however, several in vitro studies have indicated that compounds derived from grapes, for example, grape seed extract, grape juice, and red wine polyphenols, cause endothelium-dependent vasorelaxation. Thus, GE supplements may exert favorable and prophylactic effects against the development of cardiovascular diseases.

Endothelium-derived nitric oxide (NO) synthesized by endothelial NO synthase (eNOS) diffuses into smooth muscle cells and stimulates soluble guanylate cyclase (sGC), which leads to an increase in cyclic GMP (cGMP) production and smooth muscle relaxation. Endothelial NO production and its bioavailability have been shown to play an important role in the regulation of blood pressure. On the other hand, previous findings have suggested that excessive levels of superoxide anion (O$_2^-$) produced in vascular cells interact with NO to induce abnormalities in vascular tone regulation, resulting in the development of hypertension. Furthermore, the development of hypertension and altered endothelium-dependent vascular relaxation were both attenuated when experimental hypertensive rats were treated with the membrane-targeted forms of superoxide dismutase. Therefore, the suppression of oxidative stress may ameliorate NO bioavailability and prevent the development of hypertension and end-organ damage.

We previously reported the antihypertensive effects of the French maritime pine bark extract, flavangenol (also referred to as pycnogenol), which also consists of a complex mixture of OPCs. The daily administration of flavangenol suppressed the development of deoxycorticosterone acetate (DOCA)–salt-induced hypertension, but not N^\text{G}-nitro-L-arginine (NOARG)-induced hypertension. Furthermore, an in vitro experiment showed that flavangenol exerted endothelium-dependent vasorelaxant effects and that a non-selective NOS inhibitor completely inhibited this vasorelaxation. Thus, the antihypertensive effects of OPCs appear to be closely related to endothelial NO production; however, the degree of polymerization that exerts the most beneficial effects remains unclear.

Polyphenolic content of white wine is known to be less than 1/10 of red wine. In addition, the content of resveratrol in white wine is very small compared to red wine. This is one of the reasons why white wine is given much less attention than red wine in the literature. Indeed, the effect of white wine against cardiovascular diseases has not been well known. Therefore, we aimed to prepare extracts from Chardonnay, a grape cultivar for white wine, and to examine the effect of GE intake on hypertension. The main constituents of GE used in the present study are OPCs, which account for approximately 70% of all components. OPCs contained in GE mostly consist of dimer and oligomer chains of catechin and/or epicatechin. In addition, GE contains several polyphenols, such as monomer catechin and taxifolin, and other condensed tannins; however, the amounts of these components are very small. In this work, we showed the pharmacological effects of dietary

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GE supplements on DOCA–salt-induced hypertension in rats.

MATERIALS AND METHODS

Materials A proanthocyanidin-rich extract was prepared from grape seeds and skin (Chardonnay), and the contents of proanthocyanidins and monomeric flavanols were assessed according to previously reported methods. In brief, grape seeds and skin were extracted with 10% aqueous ethanol at 80°C for 1 h. The 10% aqueous ethanol extract was then chromatographed on DIAION™ HP20. The 70% aqueous ethanol elute was concentrated and then freeze-dried to obtain a proanthocyanidin-rich extract. The extract consisted of 7% epicatechin, 12% catechin, and 73% proanthocyanidins. All other reagents were of the highest grade available.

Cell Culture The human umbilical vein cell line, EA.hy926 endothelial cells (kindly gifted by Prof. Cora-Jean S. Edgell, Department of pathology and laboratory medicine, University of North Carolina, U.S.A.) were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with high glucose (4.5 g/L), 10% fetal calf serum, 100 U/mL of penicillin, and 100 µg/mL streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO₂. All cell cultures were used from passages 266 to 281. For all experiments, EA.hy926 cells were grown to confluence (approximately 3.8 × 10⁶ cells/cm²) then made quiescent by incubation with serum-free DMEM overnight.

Western Blot Analysis An equal amount of extracted protein was fractionated using a sodium dodecyl sulfate (SDS)-polyacrylamide gel (7.5%) and transferred to nitrocellulose membranes (GE Healthcare Life Sciences, U.K.). After blocking with Blocking One-P (Nacalai Tesque, Inc., Kyoto, Japan) or 5% non-fat dry milk, membranes were incubated overnight with primary antibodies against actin, eNOS, Akt, phosphorylated-eNOSSer1177 (p-eNOS), and phosphorylated-AktSer473 (p-Akt) at 4°C, and then incubated with secondary antibodies for 1 h. Blots were developed with Pierce™ ECL Western blotting Substrate (Thermo Fisher Scientific, U.S.A.) and quantified using Quantity One software (Bio-Rad Laboratories, U.S.A.).

NO Metabolite Measurement The concentration of the NO metabolite (NOx: nitrite (NO₂) + nitrate (NO₃)) in the culture medium was measured using an ENO-20 NOX Analyzer (Eicom, Kyoto, Japan) and standard solutions (NO-STD, Eicom).

Animals Male Sprague-Dawley rats (SLC, Inc., Hamamatsu, Japan) were used in experiments. Rats were housed with free access to food (NMF; Oriental Yeast Co., Ltd., Tokyo, Japan) and water ad libitum. All experiments involving animals were approved by the Experimental Animal Committee at Osaka University of Pharmaceutical Sciences.

GE-Induced Vasodilator Response in Aortic Rings The thoracic aortas of rats (8 weeks old) were prepared as described previously. Approximately 2-mm aortic segments of the thoracic aorta were suspended in organ chambers containing 10 mL of Krebs–Ringer bicarbonate solution (118.5 mmol/L NaCl, 4.7 mmol/L KCl, 2.5 mmol/L CaCl₂, 1.2 mmol/L KH₂PO₄, 1.2 mmol/L MgSO₄, 25 mmol/L NaHCO₃, and 10 mmol/L glucose) under a resting tension of 1.5 g at 37°C and gassed with 95% O₂, 5% CO₂. Contractions and relaxations were measured as changes in isometric tension by a force transducer (TB-612T, Nihon Kohden, Osaka, Japan) coupled to a polygraph and an equilibration period was allowed before the start of the experiments. Relaxant responses to GE (0.05 to 1 µg/mL) were evaluated using vessels contracted with 10⁻⁶ mol/L 1-phenylephrine (Phe). To investigate the mechanisms involved in the vasorelaxant effects of GE, aortic rings were pretreated with the phosphatidylinositol 3-kinase (PI3K) inhibitor wortmannin (0.1 µM), soluble guanylate cyclase (sGC) inhibitor 1H-[1,2,4]oxadiazolo-[4,3-a]-quinoxaline-1-one (ODQ; 10 µM), or NOS inhibitor N-[3-aminomethyl]-nitro-L-arginine methyl ester (L-NAME; 0.1 mM) for 15 min before the addition of Phe. The endothelium was removed by gently rubbing the intimal surface with a cotton ball. The removal of the endothelium was confirmed by the disappearance of or a reduction in the relaxant response to acetylcholine (ACH) and by sodium nitroprusside (SNP, at 1 µM)-induced strong relaxation (at least 90%). The vasodilator responses to GE were expressed as a percentage of the response to Phe in each tissue.

Mesenteric Artery Preparation The mesenteric artery of rats (8 weeks old) was prepared as described previously. Rats were anesthetized, the abdomen was opened, and the superior mesenteric artery was cannulated and flushed gently with Krebs–Ringer bicarbonate solution to eliminate blood from the vascular bed. After removal of the entire intestine and associated vascular bed, the mesenteric vascular bed was separated from the intestine by cutting close to the intestinal wall. Only five main arterial branches from the superior mesenteric trunk running to the terminal ileum were perfused. All other branches of the superior mesenteric artery were tied off. The isolated mesenteric vascular bed was placed on a water-jacketed tray maintained at 37–38°C and perfused with Krebs–Ringer bicarbonate solution at a constant flow rate of 4 mL/min with a peristaltic pump in the closed system. The perfusate was constantly bubbled with a mixture of 95% O₂ and 5% CO₂ to adjust the pH of the perfusate to 7.4 and for purposes of oxygenation. Changes in the perfusion pressure were measured at a point close to the mesentery by means of a pressure transducer and were recorded on a polygraph system (RM6000G, Nihon Kohden). The vasomotor responses of the preparation to 10⁻⁶ mol/L Phe were repeated after an equilibration period of 30 min until reproducible responses were obtained, and the preparation was subsequently treated with 10⁻⁶ mol/L ACH to test the viability of the tissue. The endothelium was considered functional when the relaxation of precontracted vessels to ACh was at least 90%. Relaxant responses to GE (0.3, 1, and 3 µg/mL) were evaluated after pre-contraction with 10⁻² mol/L Phe. The vasodilator responses to GE were expressed as a percentage of the response to Phe in each tissue. To remove the endothelium, the detergent sodium deoxycholate (SD, 1.8 mg/mL in perfusate) was infused into the preparation for 40 s. The successful removal of the endothelium was confirmed by the lack of a vasorelaxant response to ACH.

DOCA–Salt Treatment in Rats Rats (6 weeks old) were used in experiments. The right kidney was excised via a right flank incision under anesthesia. After a 1-week postsurgical recovery period, rats were then divided into a sham-operated group and DOCA–salt group. The latter was further divided into three groups (normal diet group, 0.01 or 0.03% GE-containing diet group). The sham-operated group was given...
tap water and a normal diet ad libitum. The DOCA–salt group were administered DOCA (12 mg/kg, subcutaneous injection twice weekly) and 1% NaCl drinking water. The GE-containing diet (0.01 or 0.03% (w/w) in NMF) was provided from the start of the DOCA–salt treatment. Systolic blood pressure (SBP) was measured weekly using the non-invasive tail cuff method and a pneumatic pulse transducer (BP-98A, Softron, Tokyo, Japan). The thoracic aortas were removed 5 weeks after the beginning of the DOCA–salt treatment and were then used to determine aortic \( \text{O}_2^- \) production and in isometric tension studies.

**ACh-Induced Vasodilator Responses in Aortic Rings**

The thoracic aortas of sham and DOCA–salt hypertensive rats were prepared as previously reported.\(^1\)\(^3\) Approximately 2-mm segments of the thoracic aorta were suspended in an organ bath in the same manner as described above. Aortic rings were precontracted with \( 10^{-6} \) mol/L Phe. After a plateau had been attained, the rings were exposed to ACh to construct dose–response curves.

**Measurement of \( \text{O}_2^- \) Production**

\( \text{O}_2^- \) production was measured using a lucigenin-enhanced chemiluminescence assay and luminometer (Sirius-2, Funakoshi, Tokyo, Japan) as reported previously.\(^1\)\(^3\) Three 2-mm aortic segments were placed in test tubes containing modified Krebs–N-(2-hydroxyethyl)piperazine-N\(^{-2}\)-ethanesulfonic acid (HEPES) buffer [pH 7.4, 99.01 mmol/L NaCl, 4.69 mmol/L KCl, 1.87 mmol/L CaCl\(_2\), 1.2 mmol/L MgSO\(_4\), 1.03 mmol/L K\(_2\)HPO\(_4\), 25 mmol/L N\(^{-2}\)-hydroxyethylpiperazine-N\(^{-2}\)-ethanesulfonic acid sodium salt (Na-HEPES), and 11.1 mmol/L glucose] and allowed to equilibrate in the dark for 15 min at 37°C before measurements were taken. Lucigenin (5 \( \mu \)mol/L) was added to the tube after 30 s. The relative light unit (RLU) was integrated every 3 s for 15 min and averaged. Background counts were determined from identically treated vessel-free readings and subtracted from the vessel readings. \( \text{O}_2^- \) production levels in the aorta were expressed as RLU/min/dry tissue weight (mg).

**Chronic NOS Inhibition in Rats**

Seven-week old rats were administered the non-selective NOS inhibitor \( \text{A}^{\text{NO}} \)-nitro-L-arginine (NOARG, 2.74 mM) in drinking water for 3 weeks in order to inhibit NOS. Rats were separated into three groups: vehicle (distilled water) + normal diet group; NOARG + normal diet group; and NOARG + 0.03% GE-containing diet group. The GE-containing diet was fed from the start of the NOARG treatment. SBP was measured using the non-invasive tail cuff method.

**Short-Term Oral Administration in Healthy Rats**

Rats (7 weeks old) were gavaged with vehicle or GE (500 mg/kg) every day for 5 d. Rats were sacrificed 4 h after the last treatment, and the thoracic aortas were then used in isometric tension studies as described above and the measurement of aortic eNOS mRNA expression. Total RNA was isolated from thoracic aortas using the RNeasy Mini Kit (Qiagen). cDNA was synthesized from total RNA using the High-Capacity cDNA reverse transcription kit (Applied Biosystems), and PCR amplification was performed. Relative quantitation to 18S ribosomal RNA (rRNA) was assessed using the comparative cycle threshold (CT) method.

**Statistical Analysis**

Data represent the mean ± standard error of the mean (S.E.M.) (\( n = 8 \)). **\( p < 0.01 \) significantly different from no addition.

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**Fig. 1. Effects of GE on Akt and eNOS Protein Expression in EA.hy926 Cells**

Quiescent EA.hy926 cells were treated with the indicated concentrations of GE for 10 min. Cells were then lysed, and the cell lysate (50 \( \mu \)g protein) was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) followed by a Western blot analysis with specific antibodies against Akt, eNOS, phosphorylated-Akt (p-Akt) and -eNOS (p-eNOS), and actin, respectively. The expression levels of each protein were quantified by a densitometric analysis. Each column and bar represents the mean ± standard error of the mean (S.E.M.) (\( n = 8 \)). **\( p < 0.01 \) significantly different from no addition.
error of the mean (S.E.M.). The unpaired Student’s t-test was used for two-group comparisons and a one-way ANOVA followed by Dunnett’s or Bonferroni’s tests for multiple comparisons. Statistical analyses on differences in ACh-induced vasorelaxation between vehicle- and GE-treated rats were performed using a two-way repeated ANOVA (Fig. 8). p Values of less than 0.05 were considered to be significant.

RESULTS

Effects of GE on NO Production in Cultured Endothelial Cells
GE (5 to 100 μg/mL) had no effect on Akt or eNOS protein expression, but markedly increased p-Akt and p-eNOS protein expression in a dose-dependent manner (Fig. 1). The PI3K inhibitor wortmannin (1 μM) almost completely suppressed increases in GE-induced p-Akt and p-eNOS protein expression (Fig. 2). The results obtained also confirmed that another selective PI3K inhibitor LY 294, 002 (10 μM) exerted similar inhibitory effects (data not shown). Furthermore, NOx concentrations in the culture medium significantly increased 10 min after the addition of GE (Fig. 3).

Effects of GE on the Rat Aorta and Mesenteric Artery
GE itself produced dose-dependent potent vasorelaxation in rat aortic rings (Fig. 4A). These vasorelaxant responses were almost completely abolished by the pretreatment with wortmannin. GE-induced vasorelaxation was also not observed following the removal of the endothelium or with the combination of the NOS inhibitor l-NAME or sGC inhibitor ODQ (Fig. 4B). These results suggested that GE-induced vasorelaxation was mediated by the activation of eNOS and subsequent production of cGMP. When the effects of various receptor antagonists on GE-induced vasorelaxation were evaluated, the estrogen receptor antagonist fulvestrant (96.7 ± 1.3% at 30 μM), histamine receptor antagonist pyrilamine (98.1 ± 1.6% at 10 μM), β-adrenergic receptor antagonist propranolol

Fig. 2. Effects of Wortmannin on Akt and eNOS Protein Expression in EA.hy926 Cells Treated with GE
Quiescent EA.hy926 cells were pretreated with wortmannin (1 μM) for 1 h and then incubated with or without the indicated concentrations of GE for 10 min. Cells were then lysed, and the cell lysate (50 μg protein) was subjected to SDS-PAGE followed by a Western blot analysis with specific antibodies against Akt, eNOS, phosphorylated-Akt (p-Akt) and -eNOS (p-eNOS), and actin, respectively. The expression levels of each protein were quantified by a densitometric analysis. Results represent the mean ± S.E.M. (n = 7). ** p < 0.01 significantly different from no addition. † p < 0.05, ‡ p < 0.01 significantly different from each concentration of GE alone.

Fig. 3. Effects of GE on NO Release from EA.hy926 Cells
Quiescent EA.hy926 cells were treated with the indicated concentrations of GE for 10 min, and the concentration of NOx (NO2− + NO3−) in the culture medium was then measured (n = 5–6). Results represent the mean ± S.E.M. * p < 0.05, ** p < 0.01 significantly different from no addition.
3.5% at 10 µM) and muscarinic receptor antagonist atropine (93.7 ± 2.7% at 1 µM) did not significantly modify vasorelaxant responses to GE. GE (0.3 to 3 µg/mL) also exerted potent vasorelaxant effects in the mesenteric artery (Fig. 4C). GE-induced vasorelaxation disappeared following the removal of the endothelium (data not shown).

Effects of GE Feeding on DOCA–Salt-Induced Hypertension

As shown in Table 1, no significant changes were observed in body weight among the groups. There were also no significant differences in the daily intake of drinking water (1% NaCl) between the normal diet group and GE diet-fed DOCA–salt groups. Food intake was similar among the four groups. No significant differences were observed in SBP in each group at the beginning of the experiment. The treatment with DOCA and salt in the normal diet group gradually and continuously elevated blood pressure over that in the sham group (95.8 ± 1.5% at 10 µM), and muscarinic receptor antagonist atropine (90.7 ± 2.7% at 1 µM) did not significantly modify vasorelaxant responses to GE. GE (0.3 to 3 µg/mL) also exerted potent vasorelaxant effects in the mesenteric artery (Fig. 4C). GE-induced vasorelaxation disappeared following the removal of the endothelium (data not shown).

Table 1. Effects of GE on Body Weight, Food Intake, and Water Intake in DOCA–Salt Hypertensive Rats

| Experimental group                  | n  | Body weight (g) | Food intake (g/d) | Water intake (mL/d) |
|------------------------------------|----|----------------|-------------------|--------------------|
| Sham                              | 10 | 384.7 ± 5.6     | 21.4 ± 0.5        | 38.4 ± 1.1         |
| DOCA–salt + the normal diet       | 11 | 348.5 ± 10.7    | 17.8 ± 0.8        | 98.9 ± 13.3**      |
| DOCA–salt + GE 0.01%              | 8  | 351.9 ± 9.2     | 17.1 ± 1.4        | 76.9 ± 12.7*       |
| DOCA–salt + GE 0.03%              | 8  | 369.3 ± 7.6     | 20.7 ± 1.2        | 81.9 ± 2.9**       |

The values shown for food and water intakes represent average daily food/water consumption in each metabolic cage from 4 to 5 weeks. Each value represents the mean ± S.E.M. *p < 0.05, **p < 0.01 vs. sham.
group throughout the 5-week period (Fig. 5A). On the other hand, this elevation was significantly suppressed by dietary GE supplements. The cumulative addition of ACh (10^{-9} to 10^{-5}M) induced concentration-dependent relaxation in all aortic rings (with an endothelium) precontracted with Phe (Fig. 5B). The responses to ACh in the DOCA–salt normal diet group were markedly weaker than those in the sham group. GE feeding significantly ameliorated the decreased responses to ACh. Furthermore, aortic O_2^- production in the DOCA–salt normal diet group was slightly higher than that in the sham group. In the DOCA–salt GE diet group, O_2^- production levels were similar to those in the sham group (Fig. 6).

**Effects of GE Feeding on NOARG-Induced Hypertension**  The administration of NOARG rapidly and significantly increased SBP on day 2 of the experimental period in normal diet-fed rats (Fig. 7) and the elevation in blood pressure was retained throughout the 3 weeks. Changes in SBP in NOARG-treated GE-fed rats were similar to those in normal diet-fed rats. No significant changes were observed in blood pressure in the vehicle group fed the normal diet throughout the experimental period.

**Effects of Oral GE Administration in Normotensive Rats**

![Graph showing effects of oral GE administration in normotensive rats](image)

Fig. 6. Effects of GE on Vascular O_2^- Production in DOCA–Salt Hypertensive Rats

Results represent the mean ± S.E.M. (n = 7–11).

![Graph showing effects of oral GE administration in normotensive rats](image)

Fig. 7. Effects of GE on Systolic Blood Pressure in Chronic NOS Inhibitor-Induced Hypertensive Rats

NOARG (2.74 mM) was dissolved in drinking water. ○, sham (n = 4); □, NOARG + the normal diet (n = 3); ▪, NOARG + GE 0.03% (n = 3). Results represent the mean ± S.E.M. *p < 0.05, **p < 0.01 significantly different from sham.

![Graph showing effects of oral GE administration in normotensive rats](image)

Fig. 8. Effects of the Oral GE Administration in Normotensive Rats

(A) Effects of GE on eNOS mRNA expression in an isolated aorta from male Sprague-Dawley rats. (B) Endothelium-dependent vasorelaxation of aortic rings to ACh (10^{-9} to 10^{-5} mol/L). ○, control (n = 6); ▪, GE (n = 5). Results represent the mean ± S.E.M. *p < 0.05 significantly different from the control.
increases in NOx concentrations in the culture medium were observed 10 min after the addition of GE. Based on these results, GE activates eNOS in endothelial cells via phosphorylation, resulting in enhanced NO production. eNOS activity is dependent on the phosphorylation and dephosphorylation of serine and threonine residues at various sites. Since Ser-1177, Ser-116, Ser-617, Ser-635, and Thr-497 residues are listed as sites that undergo phosphorylation and dephosphorylation, we investigated the phosphorylation of the Ser-1177 residue, which is considered to be the most important. Akt has been shown to activate eNOS by phosphorylating the Ser-1177 residue of eNOS and promoting NO production. Thus, the PI3K/Akt pathway is primarily responsible for the activation of eNOS. Polyphenol components, such as red wine polyphenols and epigallocatechin gallate, induce the activation of eNOS via the PI3K/Akt pathway and induce endothelium-dependent vasorelaxation. In the present study, GE caused rapid and marked increases in p-Akt protein levels. Furthermore, the increases observed in p-Akt and the p-eNOS protein by GE almost completely disappeared with the pretreatment with the PI3K inhibitor wortmannin or LY-294,002. Collectively, these results demonstrated that GE mainly promotes the phosphorylation of eNOS via the PI3K/Akt pathway.

Previous studies indicated that GE and grape juice caused endothelium-dependent vasorelaxation; however, the underlying mechanisms remain controversial. The present study showed that GE produced endothelium-dependent vasorelaxation via the eNOS/NO/sGC/cGMP pathway. Naturally occurring components, such as naringenin, apigenin, daidzein, and genistein, which are phytostrogens, act on the estrogen receptor on vascular endothelial cells and cause NO-dependent vasorelaxation by activating eNOS via the PI3K/Akt pathway. Therefore, the GE-induced vasorelaxant mechanism may be mediated by the stimulation of a receptor that enhances eNOS activity and NO-dependent vasorelaxation. However, in our experiments, fulvestrant, pyrilamine, propranolol, and atropine failed to affect vasorelaxant responses to GE. Thus, it remains unclear how GE activates the PI3K/Akt pathway. On the other hand, Mendes et al. reported that procyanidin from grape seeds acted via the initial release of nucleotides, which, in turn, may activate the P2Y1 and/or P2Y2 purinergic receptors of endothelial cells, triggering the synthesis and release of NO followed by vasorelaxation. The stimulation of P2Y receptors is known to induce Ca++ responses and increase NO production via the phosphorylation of eNOS at Ser¹¹⁷⁷. Therefore, we hypothesized that GE may stimulate the eNOS/NO/sGC signaling pathway and its upstream mechanism may be related to P2Y receptors. Although we found that purinergic P2Y receptor antagonists, such as reactive blue 2 and pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS), significantly suppressed GE-induced vasorelaxation (Sato et al., unpublished observations), further detailed studies are needed in the future to elucidate the upstream signal transduction mechanisms by which GE activates the PI3K/Akt pathway.

Vascular hyposresponsiveness to vasodilatory substances has been reported under hypertensive conditions, and this has been closely related to the pathogenesis of hypertension. The biosynthesis and release of NO and its vasodilating effects are reduced in DOCA–salt-induced hypertensive rats, and this endothelial dysfunction accelerates blood pressure elevations and tissue damage. In the present study, the GE treatment also prevented endothelial dysfunction in rats administered the DOCA–salt. Vascular reactivity to ACh in DOCA–salt hypertensive rats was significantly weaker than that in sham rats, and dietary GE supplements significantly ameliorated this hyporeactivity. This improvement in vascular reactivity to ACh may be related to the effects of GE on eNOS protein such as the increase in eNOS protein expression and/or the inhibition of eNOS uncoupling. Because the ACh-induced eNOS activation is dependent on not the PI3K/Akt pathway but Ca^{++}/calmodulin. In fact, other in vitro studies clearly demonstrated that GE-derived polyphenols enhanced eNOS expression and NO production; however, limited information is currently available on the in vivo effects of GE-derived polyphenols on NO biosynthesis and its bioavailability. We also failed to measure plasma NOx concentrations in GE-fed rats and directly show that enhancements in NO production are responsible for GE-induced NO production. However, dietary GE supplements had no effect on the progression of hypertension in NOARG-induced hypertensive rats, which suggested that GE did not exert antihypertensive effects, at least under pathological conditions in which NO activity is suppressed. Furthermore, we found that the oral administration of GE increased eNOS mRNA expression in vascular tissue and enhanced dose-dependent vasodilator responses to ACh, but not those to SNP, in normotensive rat aortas, suggesting that GE enhances endothelium-dependent vasodilator responses by up-regulating eNOS expression. Therefore, the antihypertensive effects of GE observed in DOCA–salt hypertensive rats appear to be due to the activation of eNOS. The present results clearly demonstrate the beneficial effects of GE at the in vivo level; however, further studies are needed to identify whether pathological improvements by GE in DOCA–salt-induced hypertension are due to non-genomic eNOS activation or increase in genomic eNOS gene expression and subsequent increase in protein expression. In vascular tissues of hypertensive patients and animal models, endothelium-dependent vasorelaxation is attenuated, while vascular responsiveness to contracting factors is enhanced. Reduction of the endothelium-dependent vasorelaxation is caused by an increase in endothelin-derived contraction factors such as endothelin-1 (ET-1) and thromboxane A₂ and a decrease in endothelin-derived relaxation factor and endothelin-derived hyperpolarizing factor (EDHF). Consequently, peripheral vascular resistance is increased, followed by the elevation of blood pressure. Hypertension is also associated with increased peripheral vascular resistance to blood flow. In DOCA–salt hypertension, resistance arteries present with morphological changes within 2 weeks of elevations in blood pressure, including narrowing of the lumen and external diameters, consistent with remodeling, and growth of the vascular wall that manifests as increases in media width and cross-sectional area. In the present study, we observed GE-induced potent vasorelaxant effects in resistance arteries and thoracic aortas, suggesting that GE has vasorelaxant properties in resistant arteries that may contribute to its antihypertensive activity. On the other hand, Kaufeld et al. demonstrated that NO and EDHF are closely related to vasorelaxant effects by procyanidins. In addition, EDHF-mediated dilation of mesenteric arteries is impaired in DOCA–salt-induced hypertensive rats. Thus, further studies are needed to clarify
the involvement of EDHF in GE-induced vascular relaxation and hypotensive action in DOCA–salt rats.

We previously reported that ET-1 production significantly increased in the vascular walls and kidneys of DOCA–salt hypertensive rats, and the overproduction of ET-1 in these organs was closely involved in the onset and progression of hypertension in this model animal.35,36 We also demonstrated that the combination of DOCA–salt and a NOS inhibitor strongly promoted the development of severe renal dysfunction and tissue injury.37 ET-1 mRNA expression was also stronger in the kidney in these animals than in rats treated with DOCA–salt alone. Thus, reduced NO production rapidly deteriorates endothelial function and this is followed by further increases in blood pressure and the aggravation of renal function in DOCA–salt hypertensive rats. On the other hand, we have more recently reported the inhibitory effects of GE on ET-1 production in cultured endothelial cells.38 In the present study, although it remains unclear whether GE affects ET-1 production in the vasculature and kidneys, the antihypertensive effects of GE may be partly responsible for the reduction observed in ET-1 production.

Treatments with the mineralocorticoid DOCA and salt for several weeks produce severe hypertension, which is, at least partly, associated with an increase in O2 production in vascular tissues.39 In the present study, we demonstrated that dietary GE supplements slightly diminished vascular O2 production induced by the DOCA–salt treatment. A previous study reported that aldosterone induced O2 generation via the mineralocorticoid receptor-mediated activation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in vascular endothelial cells,40 and this enhancement in vascular O2 production has been shown to play a role in the development and progression of vascular injury, which is one of the most important factors for blood pressure elevations in this hypertensive model.41 Additionally, in a state where O2 production is remarkably increased such as hypertension, a decrease in NO bioavailability causes endothelial dysfunction, resulting in further deterioration of the pathological condition. Therefore, antioxidant drugs may be an effective tool for preventing endothelial dysfunction seen in hypertensive patients. Indeed, a typical example is that statins, which are widely used for dyslipidemia, increase NO bioavailability through an antioxidant action and restore endothelial dysfunction.42 On the other hand, we have reported that GE exerts renoprotective effects against ischemia/reperfusion-induced acute kidney injury (AKI) in mice and that its action is closely related to GE-induced NO production in renal tissues.43 In AKI, the enhancement of O2 production in renal and vascular tissues in the early stage after reperfusion is associated with subsequent renal dysfunction and tissue damage. It has also been shown that various drugs with antioxidant activity provide a beneficial effect on ischemic AKI.44,45 Although whether or not GE directly or indirectly suppresses O2 production cannot be determined from the present study, it is quite possible that the antioxidative action of GE contributes to its antihypertensive properties.

In conclusion, dietary GE supplements exerted antihypertensive effects in DOCA–salt-treated hypertensive rats. The potent vasodilator effects of GE itself appear to be closely related to its antihypertensive properties. Moreover, the above vasodilation was based on the PI3K/Akt/eNOS signaling pathway.

Conflict of Interest The authors declare no conflict of interest.

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