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Inhibitory Effects of Polyphenol-Rich Fraction Extracted from *Rubus coreanum* M on Thoracic Aortic Contractility of Spontaneously Hypertensive Rats

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Abstract

The purpose of the present study was to investigate whether polyphenol-rich fraction extracted from fruit wine of *Rubus coreanum* M (PCRC) can affect the contractility of the thoacic aortic strips isolated from spontaneously hypertensive rats (SHRs), and to clarify its mechanism of action. PCRC (200-800 μg/ml) concentration-depenedently blocked phenylephrine (10 μM)-induced contractile responses of the isolated aortic strips of SHRs. PCRC (400 μg/ml), added in to bath medium, also depressed the contractile active tension evoked by both phenylephrine (3 and 10 μM) and high potassium (25 and 56 mM). In the simultaneous presence of PCRC (400 μg/ml) and L-NAME (a selective inhibitor of NO synthase, 300 μM), the contractile responses evoked by phenylephrine and high K^+ were recovered to considerable level of the corresponding control contractility compared with those effects of PCRC-treatment alone. However, in the simultaneous presence of indomethacin (10 μM, a selective cyclooxygenase inhibitor) and PCRC (400 μg/ml), they were not affected. In the endothelium-denuded aortic strips by CHAPS-treatment, PCRC did not affect the contractile responses induced by phenylephrine or high potassium. Interestingly, PCRC (1.0, 3.0 and 10.0 mg/kg/30 min, i.v., respectively) dose-dependently suppressed norepiphrine-induced vasopressor responses in anesthetized SHRs. Collectively, we concluded that PCRC causes vasorelaxation in the thoracic aortic strips with intact endothelium of SHRs at least partly by the increased NO production through the activation of NO synthase of vascular endothelium, but not through the activation of cyclooxygenase.These results suggest that PCRC might be helpful to prevent or alleviate cardiovascular diseases, including hypertension.

Key Words: PCRC, Vasorelaxation, NO production, NO Synthase, Thoracic aorta, SHRs

INTRODUCTION

The fruit of *Rubus coreanum* M has been presently used in treating the disease of the aged, spermatorrhea and impotence in oriental medicine. It is also the principal products of Gochang county, Chonbuk province, Korea, where is famous for wine brewed from the fruit of *Rubus coreanum* M (Bokboonja liquor). So far this fruit has been found to possess several polyphenolic compounds, such as (-)-epicatechin, (+)-catechin, proanthocyanidin, etc. Ethanol extract of fruit of *Rubus coreanum* showed the antioxidative activity with inhibitory effects on linoleic acid oxidation and LDL oxidation (Lee and Do, 2000). Cho (2005) found that total phenol content of extract from *Rubus coreanum* M was contained highly in hot-water extract than other extracts. These extracts elicited antioxidant protection as well as inhibitory activities on xanthine oxidase, pancreatin, α-amylase, and angiotensin converting enzyme (Cho, 2005). Recently, it has been demonstrated that polyphenol compounds (PCRC), isolated from Bokboonja liquor, inhibits the CA secretory responses evoked by cholinergic (both muscarinic and nicotinic) stimulation as well as by direct membrane-depolarization from the isolated perfused adrenal gland of the normotensive rats (Kee and Lim, 2007) and spontaneously hypertensive rats (Yu *et al.*, 2009). It seems that this inhibitory effect of PCRC is exerted by inhibiting both the Ca^{2+} influx into the rat adrenal medullary chromaffin cells and the uptake of Ca^{2+} into the cytoplasmic calcium store partly through the increased NO production due to the activation of nitric oxide synthase (Kee and Lim, 2007; Yu *et al.*, 2009).

Generally, the presence of polyphenolic compounds is

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widespread among plants and plant products (Formica and Regelson, 1995; Zenebe and Pecháňová, 2002). Several epidemiological studies have shown that consumption of foods rich in polyphenolic compounds is associated with lower incidence of cardiovascular disease. It was hypothesized that the cardioprotective effect of polyphenols results from their ability to protect low-density lipoprotein from oxidation, to prevent platelet aggregation and leukocyte adhesion, and to promote relaxation of vascular smooth muscle (Keli *et al.*, 1996; Hertog *et al.*, 1997). Polyphenols also act on other targets involved in the metabolism of mammalian cells, including nitric oxide (NO), which by itself regulates hemostasis (Palmer *et al.*, 1987), thrombus development (Radomski *et al.*, 1987) and vascular tone (Moncada *et al.*, 1991; Zenebe *et al.*, 2003). The properties of NO may therefore explain, at least in part, the beneficial effects of plant polyphenols. Several authors have reported that extracts from grapes and wine induce endothelium-dependent relaxation via enhanced generation and/or increased biological activity of NO leading to the elevation of cGMP levels (Fitzpatrick *et al.*, 1993; Flesch *et al.*, 1998). The critical step for the activation of NO synthase in endothelial cells is the increase in Ca^{2+} concentration leading to the production of NO and the subsequent endothelium-dependent vasorelaxation (Andriambeloson *et al.*, 1999). The biological activity of NO can be effectively increased by the scavengers of oxygen-free radicals (Bouloumié *et al.*, 1997).

Thus, there are so far many reports about pharmacological effects of polyphenolic compound isolated from red grape wine on cardiovascular system. However, there have been a few reports on the effects of polyphenol-rich fraction extracted from Bokboonja liquor (PCRC), especially on cardiovascular system. Therefore, the purpose of the present study was to examine whether PCRC affects the contractile responses of the aortic strips isolated from spontaneously hypertensive rats (SHRs), blood pressure, and to clarify its mechanism of action.

MATERIALS AND METHODS

Experimental procedure

Mature male spontaneously hypertensive rats (purchased from DAMOOL SCIENCE, International Customer Service, Seoul, Korea), weighing 200 to 300 g, were used in the experiment. The animals were housed individually in separate cages, and food (Cheil Animal Chow) and tap water were allowed *ad libitum* for at least a week to adapt to experimental circumstances. On the day of experiment, a rat was anesthetized with thiopental sodium (50 mg/kg) intraperitoneally, and tied in supine position on fixing panel.

Isolation of thoracic aortic strips: The thorax was opened by a midline incision, and the heart and surrounding area were exposed by placing three hook retractors. The heart and portion of the lung were not removed, but pushed over to the right side and covered by saline-soaked gauge pads in order to obtain enough working space for isolating thoracic aortic vessel. The aorta was isolated from the proximal part of the heart to the vicinity of liver and immediately immersed in cold Krebs solution. The blood within the aorta was rapidly removed. The aorta was cut into the ring of 4-5 mm length.

Preparation for measurement of arterial pressure: The animal was tied in supine position on fixing panel to insert a T-formed cannula into the tachea for securing free air passage. The rectal temperature was maintained at 37-38°C by a thermostatically controlling blanket and heating lamp throughout the course of the experiment.

Recording of mechanical activity

The ring segment of thoracic aorta was mounted in a muscle bath by sliding the ring over two parallel stainless-steel hooks (0.15 mm in diameter). The lower hook was fixed on bottom of the bath and the upper was connected to isometric transducer (Grass FT. 03). The signal from the transducer was displayed on a polygraph (Grass Instruments Model 79). The volume of bath was 25 ml and the bath solution was saturated with 95% O_2 and 5% CO_2 at 37°C. The composition (mM) of Krebs was: NaCl, 118.4; KCl, 4.7; $CaCl_2$, 2.5; $MgCl_2$, 1.18; $NaHCO_3$, 25; KH_2PO_4, 1.2; glucose, 11.7. The final pH of the solution was maintained at 7.4-7.5. During equilibration period of 2 hours, the resting tension was adjusted to 0.5 g. After the equilibration period, the ring was challenged with 35 mM KCl two times, and if it responded with contraction, the proper experiment was started. Vasoconstrictors were administered into the bath in order to obtain dose-response curves. In the subsequent experiments, under the presence of PCRC, some vasoconstrictors were administered, respectively. The data were expressed as % of the control tension.

Measurement of blood pressure

In order to observe the change of arterial pressure, one of the common carotid arteries or of the femoral arteries was catheterized with polyethylene tubing [outside diameter (o.d): 0.5 mm]. The tubing was connected to a pressure transducer (Gould Co., U.S.A.) and pulse of mean arterial blood pressure was recorded on a biological polygraph (Grass Co., U.S.A.) continuously. The chart speed was adjusted to 2 cm per minute. The artery tubing was filled with heparin solution (400 I.U.) to prevent the blood coagulation during the experiment. Another cannulation with polyethylene tubing (o.d.: 0.3 mm) was made into a femoral vein for the administration of drugs and supplemental anesthetic agents as needed to maintain light surgical anesthesia. Each rat was left undisturbed for at least 30 minutes after completion of the operative procedures to permit cardiovascular parameters to be stabilized and drugs under investigation were administered at intervals of 60 minutes.

Removal of endothelium

A solution containing 0.4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate (CHAPS) was perfused into the aortic lumen for 30 s to remove the endothelium (Moore *et al.*, 1990), followed by washout with the drug-free solution. The effect of CHAPS was confirmed by the absence of a flow increase due to 10^{-6} M acetylcholine and the presence of a response to 10^{-6} M sodium nitroprusside before the experiments were started. The vasoconstrictor-induced response of non-treated (control) and CHAPS-treated preparations was compared in parallel.

Isolation of polyphenolic compounds

Polyphenolic compounds were prepared as described by Caderni *et al*. (2000), using adsorption chromatography from a 1-year old wine brewed from the fruit of *Rubus coreanum* M at the Research Institute of Bokboonja, Gochang County, Cheollabukdo Province, Korea, as follows (Fig. 1): alcohol

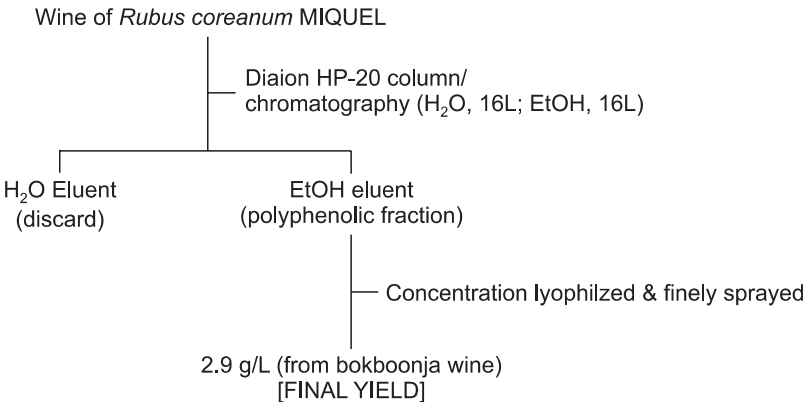

Fig. 1. Preparation of polyphenol-rich fraction extracted from fruit wine of *Rubus coreanum* M (PCRC).

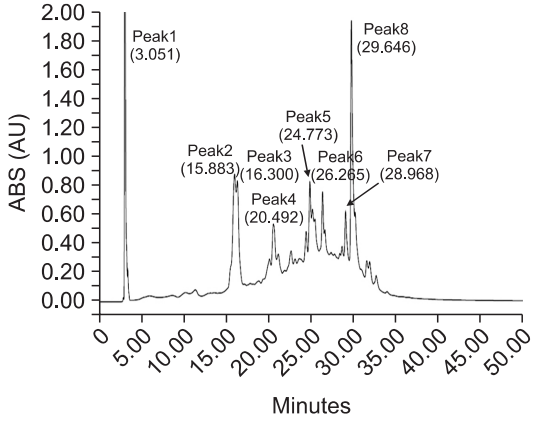

Fig. 2. HPLC chromatograms of polyphenol-rich fraction of *Rubus coreanum* M. PCRC, dissolved in methanol, was separated on a RP-C18 column [Capcell pak UG 120 column, 4.6×150 mm, 5 μm, Shiseido, Japan] by HPLC equipped with a Waters 600E solvent delivery system and a Waters 2487 UV detector (190-400 nm). The flow rate was 1.0 ml/min and the detection wavelength was set at 254 nm. The solvent gradient for HPLC was a mixture of 0.1% formic acid (solvent A) in MeOH (solvent B): 2-10% B from 0 to 5 min, 10-30% B from 5 to 15 min, 30-60% A from 15 to 20 min, 60% B from 20 to 45, and 100% B from 45 to 55 min.

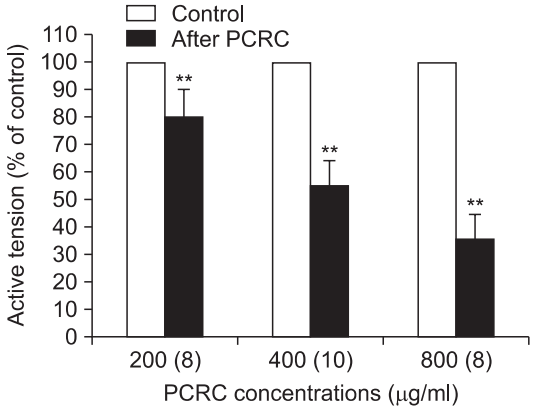

Fig. 3. Dose-dependent inhibitory effects of polyphenol-rich fraction extracted from fruit wine of *Rubus coreanum* M (PCRC) on phenylephrine (PE)-induced contractile responses in the isolated aortic strips of spontaneously hypertensive rats (SHRs). The contractile responses were induced by adding 10 μM of PE at 120 min interval after adaptation with normal Krebs solution for two hours prior to initiation of the experimental protocol. "Control" and "After" denote active tension induced evoked by PE before (Control) and after adding 200, 400 and 800 μg/ml of PCRC for 20 min. Numeral in the parenthesis indicates number of aortic strips isolated from SHRs. Vertical bars represent the standard error of the mean (S.E.M). Ordinate: the active tension (% of control [PE, 10 μM]). Abscissa: Concentrations of PCRC (μg/ml). Statistical difference was obtained by comparing the control with the PCRC-pretreated group. ***p*<0.01.

was eliminated by distillation, and the remaining solution was deposited on a Diaion HP-20 column (Mitsubish Chemical Industries, Japan). After rinsing with water to remove sugars and organic acids, the phenolic pool of chemicals present in wine was eluted with 100% ethanol in water, concentrated by vacuum, evaporation and atomized, lyophilized by freezing dryer (Coldvac -80, Hanil R & D, Korea). About 2.9 g PCRC was obtained from 1 L Bokboonja wine. This indicates that the content of PCRC is higher in Bokboonja wine than red wine (2.1 g/1 L). The working solution of this PCRC was prepared by dissolving in 0.9% NaCl solution on the day of each experiment and filtered before administration.

As shown in Fig. 2, the polyphenol-rich fraction, dissolved in methanol, was separated on a RP-C18 column [Capcell pak UG 120 column, 4.6×150 mm, 5 μm, Shiseido, Japan] by HPLC equipped with a Waters 600E solvent delivery system and a Waters 2487 UV detector (190-400 nm). The flow rate was 1.0 ml/min and the detection wavelength was set at 254 nm. The solvent gradient for HPLC was a mixture of 0.1% formic acid (solvent A) in MeOH (solvent B): 2-10% B from 0 to 5 min, 10-30% B from 5 to 15 min, 30-60% A from 15 to 20 min, 60% B from 20 to 45, and 100% B from 45 to 55 min.

Statistical analysis

The statistical significance between groups was determined by the Student's *t*- and ANOVA- tests. A *p*-value of less than 0.05 was considered to represent statistically significant changes unless specifically noted in the text. Values given in the text refer to means and the standard errors of the mean (S.E.M.). The statistical analysis of the experimental results was made by computer program described by Tallarida and Murray (1987).

Drugs and their sources

The following drugs were used: polyphenol-rich fraction extracted from the fruit of *Rubus coreanum* M (PCRC), phen-

ylephrine hydrochloride, potassium chloride, Indomethacin, N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME), acetylcholine chloride, 3-[(3-cholamidopropyl) dimethylammonio]-1 -propane sulfonate (CHAPS), and norepinephrine bitartrate (Sigma Chemical Co., U. S. A.), thiopental sodium and heparin sodium (Daehan Choongwae Pharm. Co., Korea). Drugs were dissolved in distilled water (stock) and added to the normal Krebs or saline solution as required. Concentrations of all drugs used are expressed in terms of molar base and gram.

RESULTS

Effects of polyphenols isolated from PCRC on contractile responses induced by phenylephrine and high K^+ in the thoracic aortic strips of SHRs

The resting (basal) tension from the isolated aortic strips of SHRs with intact endothelium reaches a steady state after the perfusion with oxygenated Krebs-bicarbonate solution for 90 min before the experimental protocol is initiated. The resting tension was adjusted to 0.5 g. The effects of PCRC on phenylephrine- as well as high potassium-induced contractile responses in the aorta of SHRs with intact endothelium were examined. In the present study, PCRC itself did not produce any effect on the resting tension in the aortic strips with intact endothelium isolated from the SHRs (data not shown). To establish dose-response curve of the inhibitory effects of PCRC on phenylephrine (10^{-6} M)-induced contractile responses, in the presence of PCRC at 200, 400 and 800 μg/ml, 5 min before addition of phenylephrine, the contractile responses of phenylephrine (10^{-6} M) were dose-dependently reduced to 80 ± 10% (p<0.01, n=8), 55 ± 9% (p<0.01, n=10) and 36 ± 7% (p<0.01, n=8) of the corresponding control response, respectively (Fig. 3). In all subsequent experiments, a single dose of PCRC (400 μg/ml) was used.

When 3×10^{-6} M and 10^{-5} M of phenylephrine concentrations were administered into the bath, their active tensions amounted to 1.7 ± 0.2 g and 2.9 ± 0.3 g from the resting tension level, respectively. However, in the presence of PCRC (400 μg/ml), their active tensions were reduced to 50 ± 10% (p<0.01, n=15) and 55 ± 11% (p<0.01, n=15) of the control contractile responses, respectively (Fig. 4).

High K^+ exerts two distinct effects on cells: (1) depolarization of cell membrane, and (2) depolarization- induced influx of calcium via voltage-dependent calcium channels (Wada *et al.*, 1985). When added through the bath, high potassium at the concentrations of 2.5×10^{-2} M and 5.6×10^{-2} M, which is a membrane-depolarizing agent, caused an increase in aortic contraction. As shown in Fig. 5, high potassium (2.5×10^{-2} M and 5.6×10^{-2} M)-induced contractile responses after pre-loading with 400 μg/ml of PCRC 5 min before high potassium were 43 ± 12% (p<0.01, n=10) and 58 ± 8% (p<0.01, n=10) of their corresponding control responses (0.7 ± 0.1 g and 1.2 ± 0.1 g), respectively.

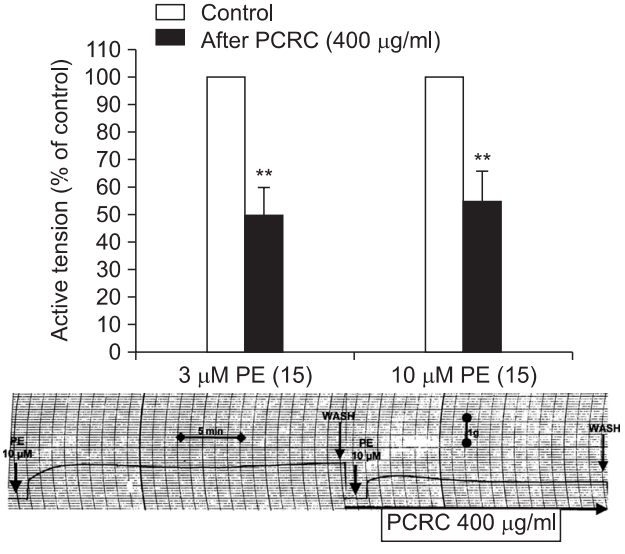

Fig. 4. Upper: Influence of PCRC on phenylephrine (PE)-induced contractile responses in the isolated aortic strips of spontaneously hypertensive rats (SHRs). The contractile responses were induced by adding 3 and 10 μM of PE at 120 min interval after adaptation with normal Krebs solution for two hours prior to initiation of the experimental protocol, respectively. Other legends are the same as in Fig. 3. **p<0.01. Lower: The typical tracing showing the inhibitory effect of PCRC on phenylephrine (PE)-induced contractile response in the aortic strip OF the SHR. Left: PE-induced contractile response (Control). Right: PE-induced contractile response in the presence of PCRC (400 μg/ml). At arrow mark, the indicated dose (10^{-5} M) of phenylephrine was added to the bath. The chart speed was 5 mm/min.

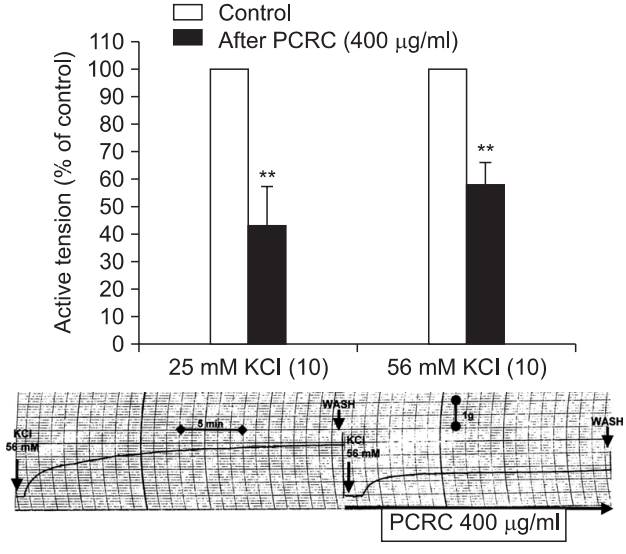

Fig. 5. Upper: Influence of PCRC on high potassium (KCl)-induced contractile responses in the isolated aortic strips of SHRs. The contractile responses were induced by adding 25 and 56 mM of KCl at 120 min interval after adaptation with normal Krebs solution for two hours prior to initiation of the experimental protocol, respectively. Other legends are the same as in Fig. 3. **p<0.01. Lower: The typical tracing showing the inhibitory effect of PCRC on high potassium (KCl)-induced contractile response in the aortic strip. Of SHR. Left: KCl-induced contractile response (Control). Right: KCl-induced contractile response in the presence of PCRC (400 μg/ml). At arrow mark, the indicated dose of KCl (56 mM) was added to the bath. The chart speed was 5 mm/min.

Influence of PCRC plus L-NAME on PCRC-induced inhibition to the contractile responses evoked by phenylephrine (PE) and high potassium (KCl) in the isolated aortic strips of SHRs

In previous study, it has been demonstrated that PCRC inhibits the CA secretion evoked by cholinergic stimulation and direct membrane-depolarization from the perfused rat adrenal medulla, which was blocked in the presence of L-NAME, a NO synthase inhibitor (Yu *et al*., 2009). These results suggest that PCRC can inhibit the CA release at least partly through the activation of nNOS in the adrenal medulla of SHRs. Therefore, in the presence of L-NAME, it was likely interesting to compare the effects of PCRC on the contractile responses induced by high potassium and phenylephrine.

In the simultaneous presence of PCRC (400 μg/ml) and L-NAME (300 μM), the aortic contractile response evoked by phenylephrine (10^{-5} M) was 111 ± 11% (p<0.01, n=9) of the control in comparison with the inhibitory response of PCRC-treatment alone (55 ± 11%) from the resting tension level as shown in Fig. 6. High potassium (5.6×10^{-2} M)-induced contractile response in the simultaneous presence of PCRC (400 μg/ml) and L-NAME (300 μM) was recovered to 92 ± 8% (p<0.01, n=7) of the corresponding control compared with the inhibitory response of PCRC-treatment alone (58±8%) from the resting tension level (Fig. 6).

Influence of PCRC plus indomethacin on PCRC-induced inhibition to the contractile responses evoked by phenylephrine (PE) and high potassium (KCl) in the isolated aortic strips of SHRs

Previously, procyanidins, oligomers of 3-flavanols (a subclass of flavonoids), induced concentration-dependent and

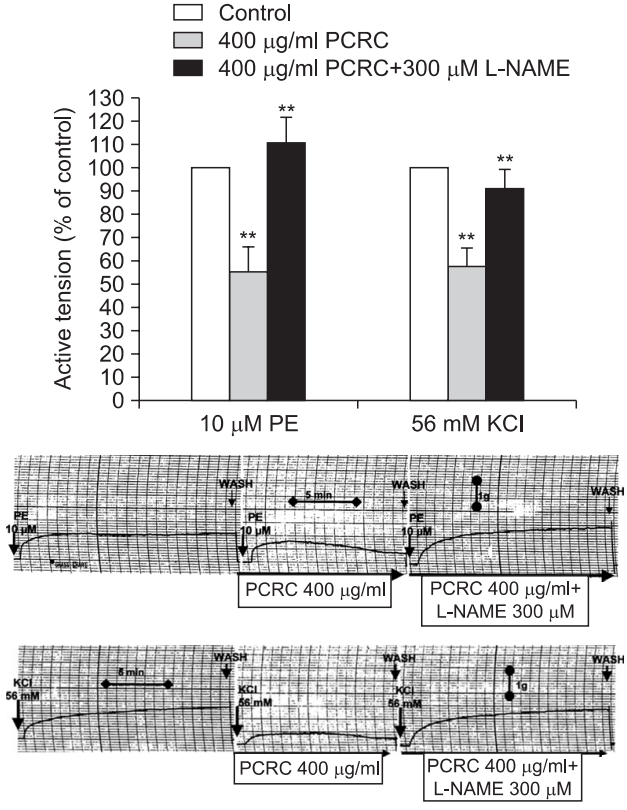

Fig. 6. Upper: Influence of PCRC plus L-NAME on PCRC-induced vasodilation to the contractile responses evoked by phenylephrine (PE) and high potassium (KCl) in the isolated aortic strips of SHRs. The contractile responses were induced by adding 10 μM PE and 56 mM KCl after adaptation with normal Krebs solution for two hours prior to initiation of the experimental protocol, respectively. Other legends are the same as in Fig. 3. **p<0.01. Lower: The typical tracing showing the effect of PCRC plus L-NAME on PCRC-evoked inhibition to phenylephrine (PE, middle)- or high potassium (KCl, bottom)-induced contractile responses in the aortic strip of the SHR. Left: PE- or KCl-induced contractile responses (Control). Center: PE- or KCl-induced contractile responses in the presence of PCRC (400 μg/ml). Right: PE- or KCl-induced contractile responses in the presence of PCRC (400 μg/ml) plus L-NAME (300 μM). At arrow mark, the indicated doses of PE (10 μM) or KCl (56 mM) were added to the bath. The chart speed was 5 mm/min.

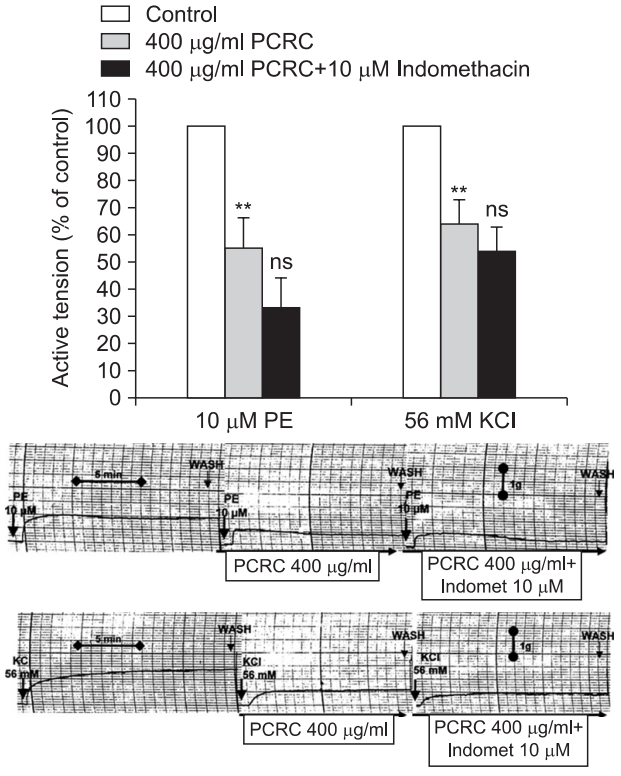

Fig. 7. Upper: Influence of PCRC plus indomethacin on PCRC-induced vasodilation to the contractile responses evoked by phenylephrine (PE) and high potassium (KCl) in the isolated aortic strips of SHRs. The contractile responses were induced by adding 10 μM PE and 56 mM KCl after adaptation with normal Krebs solution for two hours prior to initiation of the experimental protocol, respectively. Other legends are the same as in Fig. 3. **p<0.01. Lower: The typical tracing showing the effect of PCRC plus indomethacin (INDOMET) on PCRC-evoked inhibition to phenylephrine (PE, middle)- or high potassium (KCl, bottom)-induced contractile responses in the aortic strip of the SHR. Left: PE- or KCl-induced contractile responses (Control). Center: PE- or KCl-induced contractile responses in the presence of PCRC (400 μg/ml). Right: PE- or KCl-induced contractile responses in the presence of PCRC (400 μg/ml) plus INDOMET (10 μM). At arrow mark, the indicated doses of PE (10 μM) or KCl (56 mM) were added to the bath. The chart speed was 5 mm/min.

endothelium-dependent relaxation in isolated human internal mammary artery, with a maximal vasorelaxant effect at 50 μM (Aldini *et al.*, 2003). This effect was significantly reduced (by almost 50%) following preincubation of arterial rings with indomethacin, a cyclooxygenase inhibitor, indicating the involvement of a prostanoid (Aldini *et al.*, 2003). Therefore, in the presence of indomethacin, it was likely interesting to compare the effects of PCRC on the contractile responses induced by phenylephrine and high potassium.

In the simultaneous presence of PCRC (400 μg/ml) and indomethacin (10 μM), the aortic contractile response evoked by phenylephrine (10^{-5} M) was not affected by relaxant effect of 33 ± 11% (ns, n=6) of the control in comparison with the inhibitory response of PCRC-treatment alone (55 ± 11%) from the resting tension level as shown in Fig. 7. High potassium (5.6×10^{-2} M)-induced contractile response in the simultaneous presence of PCRC (400 μg/ml) and indomethacin (10 μM) was also not affected by the inhibitory effect of 54±9% (ns, n=7) of the corresponding control compared with the inhibitory response of PCRC-treatment alone (64 ± 9%) from the resting tension level (Fig. 7).

Influence of PCRC plus CHAPS on contractile responses induced by phenylephrine (PE) and high potassium (KCl) in the isolated aortic strips of SHRs

As shown in Fig. 6, PCRC-induced vasorelaxation was markedly blocked in the presence of L-NAME, a NO synthase inhibitor. Therefore, it is likely interesting to examine the effects of CHAPS, a detergent which suppresses endothelial function (Moore *et al.*, 1990), on PCRC-induced inhibitory responses to the contractile active tension evoked by high potassium and phenylephrine.

In the presence of PCRC (400 μg/ml) after pretreatment with 0.4% CHAPS, the aortic contractile response evoked by phenylephrine (10^{-5} M) elicted 103 ± 4% (ns, n=10) of the control in comparison with the corresponding control response (100%) from the resting tension level as shown in Fig. 8. High potassium (5.6×10^{-2} M)-induced contractile response in the simultaneous presence of PCRC (400 μg/ml) after pretreatment with CHAPS elicted 100 ± 17% (ns, n=9) of the control in comparison with the corresponding control response (100%) from the resting tension level (Fig. 8).

Influence of intravenous PCRC on norepinephrine (NE)-evoked pressor responses in the anesthetized SHRs

Since PCRC greatly inhibited phenylephrine-induced contractile response of the aortic strip of the SHR, as shown in Fig. 4, 5, it suggests that PCRC might cause hypotension through the blockade of peripheral adrenergic α-receptors. It is also of interest to examine the effect of PCRC on norepinephrine-evoked pressor responses. When cardiovascular parameters were stabilized for 30 min before the experimental protocols were initiated, the administration of physiological saline solution in a volume of 0.2 ml into a femoral vein did not cause any changes in arterial blood pressure. Then, it was tried to test the effect of PCRC on norepenephrine-induced hypertensive responses in the anesthetized SHRs.

In 10 SHRs, as shown in Fig. 9 (Upper), norepinephrine at doses of 0, 3, 1.0 and 3.0 μg/kg caused dose-dependent pressor responses of 14 ± 1 mmHg, 22 ± 2 mmHg and 32 ± 2 mmHg from the original baseline (183 ± 11 mmHg), respectively. After infusion of PCRC with a rate of 1 mg/kg/30 min,

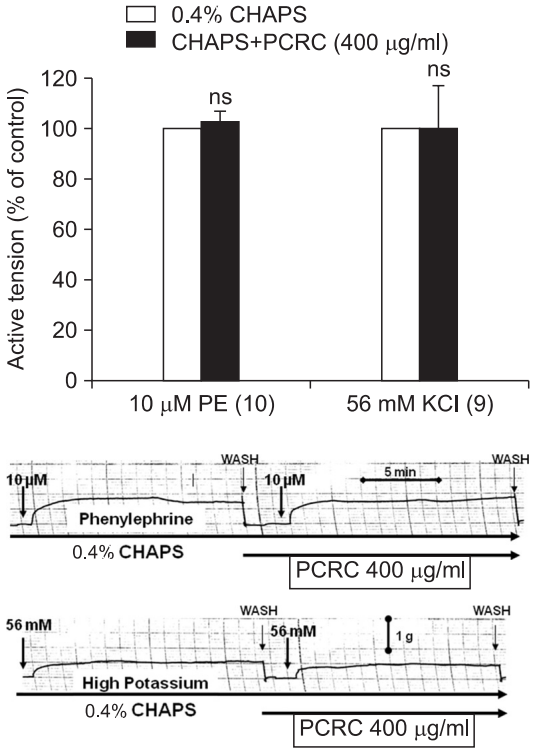

Fig. 8. Upper: Influence of CHAPS on PCRC-induced inhibition to contractile responses induced by phenylephrine (PE) and high potassium (KCl) in the isolated aortic strips of SHRs. The contractile responses were induced by adding 10 M PE and 56 mM KCl at 120 min interval after adaptation with normal Krebs solution for two hours prior to initiation of the experimental protocol, respectively. Statistical difference was obtained by comparing the control (% of control) with the PCRC-treated group after pretreatment of 0.4% CHAPS. Other legends are the same as in Fig. 3. ns: Statistically not significant. Middle: The representative tracing of CHAPS effect on PCRC-induced inhibition to phenylephrine-evoked contractile responses in the isolated aortic strips of SHRs. At arrow marks, phenylephrine (10 μM) was added into CHAPS-pretreated aortic strips. Lower: The representative tracing of CHAPS effect on PCRC-induced inhibition to high potassium-evoked contractile responses in the isolated aortic strips of SHRs. At arrow marks, high potassium (56 mM) was added into CHAPS-pretreated aortic strips. The chart speed was 5 mm/min.

hypertensive responses of norepinephrine were not altered compared to the corresponding controls (Fig. 9-Upper). However, after increasing the dose of PCRC to 3 mg/kg/30 min, norepinephrine-evoked hypertensive responses at doses of 0, 3, 1.0 and 3.0 μg/kg were significantly inhibited to 67 ± 7% (p<0.01), 70 ± 4% (p<0.01) and 78 ± 6% (p<0.01) of control responses at the above same doses, respectively, as shown in Fig. 9 (Upper and Lower). Also, in the presence of larger dose of PCRC (10 mg/kg/30 min), they were greatly inhibited to 58 ± 8% (p<0.01), 61 ± 6% (p<0.01) and 62 ± 7% (p<0.01) of control responses at the above same doses, respectively (Fig. 9).

DISCUSSION

The present experimental results demonstrate that PCRC causes vasorelaxation in the isolated aortic strips of SHRs at least partly by the increased NO production through the activation of NO synthase of vascular endothelium, but not

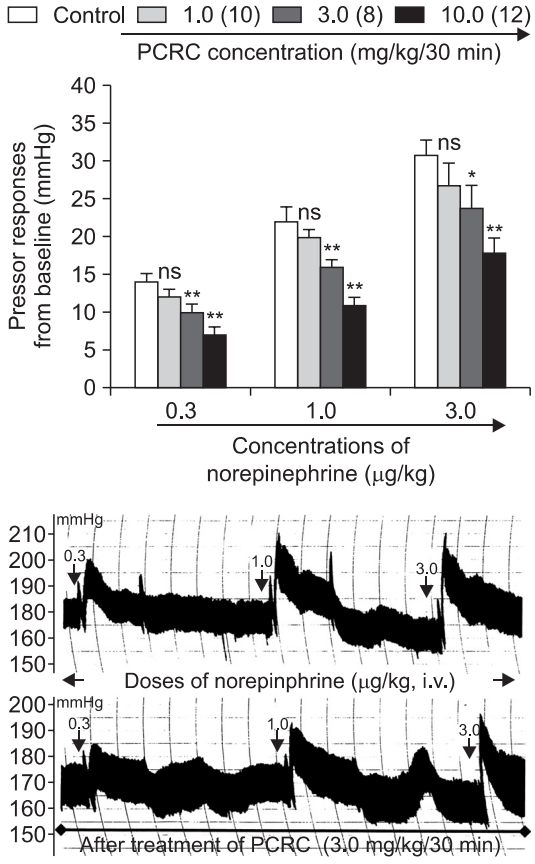

Fig. 9. Upper: Influence of intravenous PCRC on norepinephrine (NE)-evoked pressor responses in anesthetized SHRs. PCRC (1.0, 3.0 and 10.0 mg/kg/30 min, respectively) was given intravenously after obtaining the corresponding control responses of intravenous norepinephrine (0.3, 1.0 and 3.0 μg/kg). Ordinate: changes of arterial blood pressure in mmHg from 8 rats. Abscissa: intravenous doses of NE in μg/kg. Vertical bars on each column indicate standard error of mean (S.E.M.). There was statistically significant difference in changes of NE-evoked arterial pressor responses from pre-injection level before and after pretreatment of PCRC (1.0, 3.0 and 10.0 μg/kg/30 min). **p*<0.05, ***p*<0.01. ns: Statistically not significant. Lower: The representative tracing of PCRC effect on intravenous norepinephrine (NE)-induced pressor responses in an anesthetized SHR. At arrow marks, the indicated doses (0.3, 1.0 and 3.0 μg/kg) of NE were administered into a femoral vein. Upper tracing: NE-induced hypertensive responses in a non-treated rat. Lower tracing: NE-induced hypertensive responses in a PCRC-pretreated rat. PCRC was infused into a femoral vein with a rate of 3 mg/kg/30 min. Arterial blood pressure was expressed in mmHg. The chart speed was 10 mm/min.

through the activation of cyclooxygenase. In support of this idea, previousy, it has been demonstrated that PCRC inhibits the CA secretory responses evoked by stimulation of cholinergic (both muscarinic and nicotinic) receptors as well as by direct membrane-depolarization from the isolated perfused adrenal gland of the normotensive rats (Kee and Lim, 2007) and spontaneously hypertensive rats (Lim and Hong, 2007). It seems that this inhibitory effect of PCRC is exerted by inhibiting both the Ca^{2+} influx into the rat adrenal medullary chromaffin cells and the uptake of Ca^{2+} into the cytoplasmic calcium store partly through the increased NO production due to the activation of nitric oxide synthase (Kee and Lim, 2007; Yu *et al.*, 2009). In the present study, PCRC elicited a concentration-dependent inhibition in phenylehrine-induced contractile responses of aortic rings of SHRs with functional endothelium. This effect was greatly abolished in the absence of functional endothelium by treatment with CHAPS, which is a detergent for removal of endothelium, indicating that the vasodilator effect of PCRC is dependent on endothelium-derived relaxing factors. To evaluate the participation of NO in the vasorelaxant activity of PCRC, aortic rings were treated with L-NAME, a classical NO synthase inhibitor. In the present experimental condition, the PCRC-induced vasodilatation was markedly blocked, as similarly observed in endothelium-denuded aortic rings by CHAPS, suggesting that NO is the main endothelium-derived relaxing factor involved in PCRC activity. The present results are fully in accordance with previous those findings obtained from red wines and grapes. Previously, it has been reported that red wines and grapes exhibit endothelium-dependent relaxation of blood vessels via enhanced generation and/or increased biological activity of NO, leading to the elevation of cGMP levels (Fitzpatrick *et al.*, 1993; Fitzpatrick *et al.*, 1995; Fitzpatrick *et al.*, 2000; Zenebe *et al.*, 2003). *In vivo* the polyphenol compounds of red wine (PCRW) were shown to reduce blood pressure in normotensive and hypertensive rats (Mizutani *et al.*, 1999; Diebolt *et al.*, 2001; Bernátová *et al.*, 2002). The administration of purple grape juice improved the endothelium dependent, flow-mediated vasodilation in coronary artery disease patients with impaired endothelial function (Stein *et al.*, 1999). A correlation between the phenolic content with vasodilatatory effect was later confirmed by Burns and his colleagues (2000). While the antioxidant activity was associated with different classes of phenols (gallic acid, resveratrol and catechins), vasodilatation activity was correlated only with the total content of anthocyanosides (ACs) (Burns *et al.*, 2000). PCRW enhanced NO synthesis and cGMP accumulation only in the presence of functional endothelium. In denuded aortic rings, PCRW concentration 103-fold higher was necessary to induce relaxation (Corder *et al.*, 2001; Ndiaye *et al.*, 2003). Besides NO, red wine affected the formation of other mediators of vascular tone, such as endothelium-derived hyperpolarizing factor (Ndiaye *et al.*, 2003) and prostacyclin (Derek *et al.*, 1997). The mechanisms underlining NO-dependent vasorelaxation caused by PCRW were investigated (Andriambeloson *et al.*, 1999; Martin *et al.*, 2002; Zenebe *et al.*, 2003). It has also been that Provinol elicited endothelium-dependent relaxation of rat femoral artery by the Ca^{2+}-induced increase of NO synthase activity and by protecting NO from degradation (Zenebe *et al.*, 2003). Recently, Yu and his colleagues (2008) have found that PCRW inhibits the CA secretory responses evoked by stimulation of cholinergic (both muscarinic and nicotinic) receptors as well as by direct membrane-depolarization from the isolated perfused adrenal gland of the normotensive rats. It seems that this inhibitory effect of PCRW is mediated by blocking the influx of both ions through Na^+ and Ca^{2+} channels into the rat adrenomedullary chromaffin cells as well as by inhibiting the release of Ca^{2+} from the cytoplasmic calcium store, which are due at least partly to the increased NO production through the activation of nitric oxide synthase.

Consumption of wine polyphenol-, quercetin- or catechin-enriched diets increased aortic NO production in rats (Benito *et al.*, 2002). Intragastric administration of resveratrol (3 mg/kg/day), red wine (4 ml/kg/day) or even dealcoholized red wine (4 ml/kg/day) for 12 weeks to hypercholesterolemic rab-

bits improved the endothelial function, reduced plasma endothelin-1 levels and induced a significant elevation in NO levels (Zou *et al.*, 2003). The endothelium-dependent vasodilation was also improved after acute intake of 500 ml of red wine or red wine without alcohol in men, as determined by ultrasonography of the brachial artery (Hashimoto *et al.*, 2001). Endothelium-derived NO plays an important role in the control of vascular homeostasis. NO modulates the vascular tone, the growth of vascular smooth muscle cells, and decreases platelet adhesion and aggregation. It also decreases the adherence of other blood components (Moncada *et al.*, 1991; Scott-Burden and Vanhoutte, 1994). A decrease in NO production or bioavailability is closely associated with endothelial dysfunction or injury, which is an important factor in pathologies such as atherosclerosis, restenosis and hypertension (Landmesser and Drexler, 2007). PCRW and a grape skin extract also reduced blood pressure in males in several models of experimental hypertension (Bernatova *et al.*, 2002; Soares de Moura *et al.*, 2002; Pechanova *et al.*, 2004; Sarr *et al.*, 2006; Jiménez *et al.*, 2007), which was related to a combination of vasodilator and antioxidant actions. Pechanova and his colleagues (2004) also provided evidence that Provinols partially prevents L-NAME-induced hypertension, cardiovascular remodeling and vascular dysfunction via the increase of NO-synthase activity and prevention of oxidative stress. Thus, in view of the beneficial effects of plant polyphenols, the present results of PCRC should shed light on the fact that the unique components of PCRC may contribte to the treatment or prevention of hypertension through their complex influence on the NO balance in the cardiovascular system.

Generally, it is well known that potassium chloride (KCl) opens voltage-dependent calcium channels by depolarizing the cell membrane of vascular smooth muscle, resulting in increased influx of extracellualr Ca^{2+} (Bolton, 1979; Schwartz and Taira, 1983; Dube *et al.*, 1985; 1988). Kim and his colleagues (1989) have shown that the contractile responses of vascular smooth muscle induced by $CaCl_2$ and KCl may result most likely from the increased influx of extracellular Ca^{2+} through the voltage-dependent calcium channels (VDCCs). VDCCs are activated by depolarization of the plasma membrane when the extracellular K^+ concentration is increased. In the present work, incubation with PCRC inhibited KCl concentration-dependent contractile response in thoracic aortic strips of SHRs. This result is consistent with the effect of 17-β estradiol on a large elastic aorta as in previous report (Li *et al.*, 2002; 2006) and is also supported by another study (Nevala *et al.*, 1998). These findings suggest that PCRC may have Ca^{2+} antagonistic properties and can inhibit extracellular Ca^{2+} influx through VDCCs, which are similar to those of 17-β estradiol or resveratrol. Previously, the mechanism of potassium-induced vasoconstriction has been shown to be through the Ca^{2+} influx by the opening of the voltage-dependent calcium channels (Ryman *et al.*, 1989; Spedding and Paoletti, 1992). Voltage-dependent calcium channel blockers such as nifedipine or verapamil have been reported to attenuate potassium-induced vasoconstriction (Cortijo *et al.*, 1986; Triggle *et al.*, 1989). The contractile activity of vascular smooth muscle cells is mainly regulated by control over the cytoplasmic calcium concentration and both intracellular and extracellular calcium pools (Johns *et al.*, 1987; Triggle *et al.*, 1989). Based on these findings, the present results showed that PCRC can inhibit high K^+-evoked contractile responses, indicating that PCRC may block the VDCCs in aortic smooth muscle cells.

In the present work, PCRC inhibited the norepinephrine-induced pressor responses as well as phenylephrine-evoked contractile responses in thoracic aortic strips isolated from SHRs. These results suggest that PCRC may elicit the antagonistic activity of adrenergic α_1-receptors.

In general, among drugs which interfere with peripheral sympathetic function, adrenergic α-receptor blocking agents alone cause reversal of the epinephrine pressor response (Constantine *et al.*, 1973). When epinephrine is administered to untreated animals, its α-agonist properties predominate, resulting in a rise in mean arterial pressure. However, in the presence of adrenergic α-receptor blockade, the peripheral β_2-agonist properties of epinephrine predominate and a fall in arterial pressure or reversal of the pressor response is observed. In contrast, the pressor responses to norepinephrine are impaired by adrenergic α-receptor blockade, but are not reversed (Freis *et al.*, 1951) as this agent processes little β_2-agonist activity (Ablad *et al.*, 1975). Based on these findings, our results that PCRC strongly depressed phenylephrine-evoked contractile response and norepinephrine-induced hypertensive response suggest the possibility that the vasorelaxant activity of PCRC may be medated through the adrenergic α-receptor blockade.

On the other hand, PCRC-induced vasodilatation was not significantly modified in aortic rings treated with indomethacin, a cyclooxygenase inhibitor, at a concentration which inhibited contraction by arachidonic acid. This finding demonstrates that prostanoids are probably not involved in vasodilatation induced by PCRC. In support of idea, indomethacin inhibits the synthesis of prostaglandins and markedly decreases the vascular relaxation induced by arachidonic acid in sheep isolated coronary artery (Cornish *et al.*, 1983) and in newborn pigs (Leffler *et al.*, 1993). However, in the present study, indomethacin did not affect PCRC-induced relaxation in endothelium-intact aortic strips. This result indicates that the release of vasodilator prostanoids is not involved in PCRC-induced relaxation in thoracic aortic strips isolated from SHRs. This finding is also in agreement with the report that incubation with indomethacin did not inhibit the concentration-dependent vasorelaxation induced by resveratrol in porcine coronary rings with endothelium (Li *et al.*, 2006).

Based on all these results, many studies strongly support the view that polyphenol-rich diet, such as Bokbooja (*Rubus coreanum* M) and red wine, can improve endothelial function, and that the mechanisms of this beneficial effect found in above discussed *in vitro* studies (especially increased NO) might be involved *in vivo*, both in patients and in animals.

In conclusion, the present study provides conclusive data showing for the first time that PCRC elicits the endothelium- and NO-dependent vasorelaxation, which are due to PCRC that may augment eNOS activity and thus facilitates endothelial NO output, and suggesting that PCRC might be helpful in treating or alleviating cardiovascular diseases, such as hypertension and angina pectoris. It also seems that the identification of the responsible constituents should be helpful in the design of strategies to prevent or to improve cardiovascular diseases.

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