Modulation of vascular reactivity in normal, hypertensive and diabetic rat aortae by a non-antioxidant flavonoid

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Modulation of vascular reactivity in normal, hypertensive and diabetic rat aortae by a non-antioxidant flavonoid

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Abstract

In this study, we report the effects of a non-antioxidant flavonoid flavone on vascular reactivity in Wistar–Kyoto (WKY) rat isolated aortae. Whether flavone directly modulates vascular reactivity in spontaneously hypertensive rat (SHR) and streptozotocin-induced diabetic-WKY rat isolated aortae was also determined. Thoracic aortic rings were mounted in organ chambers and exposed to various drug treatments in the presence of flavone (10 μM) or its vehicle (DMSO), which served as control. Pretreatment with flavone enhanced relaxant effects to endothelium-dependent vasodilator acetylcholine (ACh) and attenuated contractile effects to α1-receptor agonist phenylephrine (PE) in WKY aortae compared to those observed in control aortic rings. Flavone had no effect on relaxations to ACh in WKY aortae incubated with either l-NAME or methylene blue, but enhanced relaxations to ACh in WKY aortae incubated with indomethacin or partially depolarized with KCl. Relaxations to ACh are totally abolished in both control or flavone pretreated endothelium-denuded WKY aortae. Flavone attenuated the inhibition by β-NADH of ACh-induced relaxation in WKY aortae, but it had no significant effect on the transient contractions induced by β-NADH nor the pyrogallol-induced abolishment of ACh-induced relaxation in WKY aortae. Flavone enhanced endothelium-independent relaxation to sodium nitroprusside (SNP) in both endothelium-intact and -denuded WKY aortae. Flavone enhanced relaxation to ACh and SNP as well as attenuated contractile effects to PE in SHR and diabetic aortae, a finding similar to that observed in normal WKY aortae. From these results, we conclude that flavone modulates vascular reactivity in normal as well as hypertensive and diabetic aortae. These effects of flavone results probably through enhanced bioactivity of nitric oxide released from the endothelium.

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1. Introduction

The vascular endothelium plays a crucial role in the regulation and maintenance of vascular tone. Endothelial dysfunction, generally indicated as a reduction of endothelium-mediated vascular relaxation, has been reported in hypertensive and diabetic arteries [1,2]. Decreased production and/or deactivation by superoxide anions of endothelium-derived nitric oxide (EDNO), in either case reduction in the bioavailability of EDNO, has been predominantly implicated in the etiology of endothelial dysfunction in hypertensive and diabetic vessels [1,2]. Thus, pharmacological agents capable of enhancing the bioavailability of EDNO in the vessel wall may result in better vascular health in hypertension and diabetes. One class of such compounds garnering much attention in recent times is the flavonoids, which are polyphenolic compounds widely found in plant-based human diets such as fruits and vegetables.

Several studies including ours have demonstrated that flavonoids decreased vascular tone and agonist-induced contraction in isolated rat arteries through EDNO-sGC-cGMP relaxant pathway coupled to stimulation of endogenous nitric oxide production from the endothelium [3–5]. Chronic administration of the antioxidant flavonoids such as quercetin was shown to reduce blood pressure as well as to enhance endothelium-dependent relaxation in various animal models of hypertension [6,7]. We recently demonstrated that chronic administration of flavone, a parent flavonoid molecule, exerts blood pressure lowering effects as well as improves endothelium-dependent and-independent aortic relaxation in spontaneously hypertensive...
rats (SHR) [8]. The vascular beneficial effects of quercetin and related bioflavonoids were suggested to derive from their protective antioxidant effects which lead to improved bioavailability of EDNO [6,7]. We and others have demonstrated that flavone, without any hydroxyl substitutions on its basic ring structure, show little or no free radical scavenging and antioxidant activity [9,10]. This raises the question as to whether flavone preserves EDNO bioavailability and thus confers vascular benefits in hypertensive vessels. The aim of this study, therefore, was to further investigate the mechanisms underlying the vascular benefits of flavone. For this purpose, thoracic aortic rings isolated from normal Wistar–Kyoto (WKY) rats were mounted in organ chambers and exposed to various drug treatments in the presence of flavone (10 μM). In addition, whether vascular responses of aortae isolated from SHR and streptozotocin-induced diabetic-WKY rats could be affected directly by flavone was also examined.

2. Materials and methods

2.1. Drugs and chemicals

The following drugs were used: flavone (TCI Chemicals, Japan), acetylcholine chloride, indomethacin, methylene blue, Nω-nitro-L-arginine methyl ester (L-NAME), phenylephrine-HCl, pyrogallol, reduced form of β-nicotinamide adenine di-nucleotide (β-NADH), streptozotocin (Sigma Chemicals Co., St. Louis, Mo., USA), sodium nitroprusside (BDH Limited, Poole, England). All the drugs were dissolved in distilled water with the exception of flavone, which was dissolved in dimethyl sulfoxide (DMSO), and indomethacin, which was dissolved in 0.5% (w/v) sodium carbonate. The final concentration of DMSO was adjusted to <0.05% (v/v), which was shown to be devoid of any effects on vascular tone [3–5].

2.2. Experimental animals

All experiments were reviewed and approved by the University of Malaya Animal Care and Ethics Committee. The rats were maintained five per cage at a constant temperature (24 ± 1 °C), with a 12 h dark/light cycle and supplied with a standard pelletted laboratory chow (Gold Coin Sdn. Bhd., Malaysia) and tap water ad libitum. Experiments were performed on thoracic aortae isolated from male (20–21 weeks old) normal WKY rats (systolic blood pressure (SBP) ≤130 mm Hg and blood glucose ≤7 mmol l−1), SHR with established hypertension (SBP ≥175 mm Hg), and WKY with established diabetes (blood glucose ≥17 mmol l−1). Diabetes was induced in WKY rats (12–13 weeks old) by a single dose of streptozotocin (STZ, 75 mg kg−1 of body weight, IP) dissolved in cold normal saline. The SBP was measured using the non-invasive tail-cuff method (ITTC Inc., USA) and blood glucose levels were assessed using the Advantage-II blood-glucose test strips (Roche Diagnostics, Germany).

2.3. Aortic ring preparation

The aortic rings were prepared as previously described [3,15,16]. Briefly, the rats were anaesthetized with pentobarbital (60 mg kg−1 of body weight, i.p.), descending thoracic aorta was dissected, cut into small rings (3–5 mm in width) and suspended in a 5 ml organ bath containing normal Krebs physiological salt solution (KPSS) of the following composition (mM): NaCl 118.2, KCl 4.7, CaCl2·2H2O 2.5, KH2PO4 1.2, MgCl2 1.2, glucose 11.7, NaHCO3 25.0, and EDTA 0.026. The bathing solution was gassed continuously with 95% oxygen and 5% carbon dioxide at 37 °C (pH 7.4). Isometric tension (g) was measured using a force displacement transducer connected to a Mac Lab recording system (ADI Instruments, Australia). Aortic rings were then progressively stretched to an optimal basal tension of 1 g and allowed to equilibrate for 45 min. During this period, the bathing solution was replaced every 15 min and, if needed, the basal tone was readjusted to 1 g. Aortic rings were then repeatedly stimulated with KCl solution (high K+ 80 mM) for 5 min at 10 min intervals until two consecutive equal contractions were attained—evidence of tissue stability.

2.4. Pharmacological studies

Following washout of high K+ responses, the aortic rings were incubated for 20 min with flavone (10 μM) or its vehicle (control), and cumulative concentration–response curves to the endothelium-dependent and -independent relaxant agonists acetylcholine (ACH, 10−10 to 10−5 M) and sodium nitroprusside (SNP, 10−11 to 10−6 M), respectively, or to the α1-receptor agonist phenylephrine (PE, 10−10 to 10−5 M) were then performed. To test the relaxation responses to ACh and SNP the aortic rings were pre-contracted with PE (1 μM). The flavone concentration of 10 μM was chosen based on previous studies, including ours, which showed that this concentration exerted both endothelium-dependent and -independent relaxation in pre-contracted rat arteries [3–5]. In addition, the physiologically achievable plasma concentrations of flavonoids are reported to be usually in the range of 0.1–10 μM [11]. Preliminary studies showed that 10 μM flavone or its vehicle did not modify basal tension of aortic rings during the time course of incubation, i.e., before addition of the agonists.

In experiments to characterize the mechanisms involved in the effects of flavone, the aortic rings were exposed to various pharmacological agents for 5 min before the incubation with flavone or its vehicle. Where indicated, endothelium was removed by gently rubbing the intimal surface of the vessel with the blunted forceps. The endothelium was considered effectively removed if ACh (1 μM) caused less than 10% relaxation of aortic rings pre-contracted with PE. To examine the possible role of nitric oxide, prostacyclin, and the cyclic GMP relaxant pathway in the effects of flavone, the concentration–response curves to ACh were performed in aortic rings incubated with and in continued presence of Nω-nitro-L-arginine methyl ester (L-NAME, 10 μM)—an eNOS inhibitor, indomethacin (10 μM)—a cyclooxygenase inhibitor, and methylene blue (10 μM)—a cyclic GMP inhibitor, respec-
To examine the contribution of endothelium-derived hyperpolarizing factor (EDHF) or potassium (K+) channels in the effects of flavone, the aortic rings were partially depolarized by increasing concentration of KCl in the KPSS (4.8–20 mM), and the concentration–response curves to ACh was then performed [12]. To confirm the dependence of flavone effects on endothelium, concentration response curves to ACh and SNP were performed in endothelium-denuded aortic rings.

Cumulative responses to ACh, SNP, and PE were also recorded in aortic rings from SHR and diabetic-WKY rats as described above. To examine the possible role of oxygen free radical scavenging in the effects of flavone, relaxant responses to ACh in WKY rat aortic rings incubated with flavone or with its vehicle (control) in the presence of pyrogallol (300 μM) or β-NADH (300 μM) were determined. Pyrogallol auto-oxidizes in tissue bath medium to release extracellular superoxide anions, while β-NADH stimulates release of intracellular superoxide anions from NADH/NADPH-oxidase activity [13,14]. Pyrogallol or β-NADH was added to tissue bath 5 min before constricting the aortic rings with phenylephrine (1 μM). In this set of experiments, following washout of responses to high K+, the KPSS was switched to indomethacin (10 μM)-containing KPSS for rest of the experiment. Indomethacin was added to the tissue bath to avoid possible influence of prostaglandins [15,16].

2.5. Calculations and statistical analysis

The concentrations indicated in the text or in the figures represent the final tissue-bath concentrations of respective agonists. The responses were recorded as mean ± standard error of the mean (S.E.M.) for the number of rats. The contractile responses of aortic rings to graded concentrations of PE are expressed as percentages of the maximum contractile effect of high K+ in respective tissues. The vasodilator effect of increasing concentrations of ACh or SNP were expressed as percent decrease of the peak PE (10−6 M) contraction. The concentration–response curve for each experimental condition was plotted and from it were deduced the values of maximal contraction (Cmax) or maximal relaxation (Rmax) and the concentration of the agonist (expressed as negative log molar) producing 50% of maximum contraction or relaxation (pEC50) recorded (Prism Version 2.0, GraphPad Software, USA). Statistical evaluation of the data was performed by ANOVA and Student’s t-test. The differences in pEC50 values were tested for statistical significance only if the maximal contraction or maximal relaxation of the respective concentration–response curves is not significantly different. A value of p < 0.05 was considered statistically significant.

3. Results

ACh and SNP caused concentration-dependent relaxation of PE (10−6 M)-induced contraction in control- or flavone-pretreated WKY rat aortic rings (Fig. 1). Flavone enhanced the relaxation observed at the lower concentrations of ACh (pEC50 = 7.83 ± 0.13) and SNP (pEC50 = 8.65 ± 0.1) in a similar manner and shifted concentration–response curves to the left compared with equivalent responses observed in control aortic rings (pEC50: ACh = 7.28 ± 0.19; SNP = 7.57 ± 0.1). Flavone enhanced the relaxation observed at the highest concentration of ACh (100.9 ± 1.15% versus 88.4 ± 5.57%), but did not modify the relaxation observed at the highest concentration of SNP (106.57 ± 1.10% versus 104.91 ± 1.06%) when
compared to control aortic rings. PE evoked concentration-dependent increases in smooth muscle tone in both control- and flavone-pretreated WKY aortic rings (Fig. 2). PE-induced maximal contraction was significantly reduced and concentration–response curve shifted slightly rightwards in flavone-pretreated WKY aortic rings ($C_{\text{max}} = 105.5 \pm 6.6\%$, $pEC_{50} = 6.93 \pm 0.11$) compared with respective control aortic rings ($C_{\text{max}} = 127.6 \pm 6.8\%$, $pEC_{50} = 7.18 \pm 0.12$).

In experiments to characterize the mechanism of action, inhibition of cyclooxygenase activity with indomethacin did not affect ACh-induced relaxation in control aortic rings nor did it in the flavone-pretreated aortic rings (Fig. 3A). In contrast, inhibition of nitric oxide synthesis with $l$-NAME (Fig. 3B) or inhibition of cGMP activity with methylene blue (Fig. 3C) totally abolished relaxation responses to ACh in control- as well as flavone-pretreated WKY rings. Presence of KCl (20 mM) significantly attenuated the relaxation observed at all the concentrations except the lowest concentration of ACh tested (0.1 nM) in control aortic rings (Fig. 3D). This attenuation was only observed at concentrations <1 $\mu$M ACh (i.e., below the sub-maximal concentration) in flavone-pretreated aortic rings. In addition, in the presence of KCl (20 mM) flavone was able to significantly reverse the impaired relaxation observed at >1 nM concentrations of ACh compared to control aortic rings (Fig. 3D). Denudation of endothelium completely abolished ACh-induced relaxation in control- as well as flavone-pretreated WKY aortic rings (Fig. 4A). Flavone enhanced SNP-induced relaxation in endothelium-denuded WKY aortic rings (Fig. 4B) similar to that observed in endothelium-intact WKY aortic rings (Fig. 1B).

Under control (vehicle-incubation) conditions, both ACh (SHR: $R_{\text{max}} = 72.24 \pm 2.04\%$, $pEC_{50} = 7.22 \pm 0.09$; SDR: $R_{\text{max}} = 64.09 \pm 5.14\%$, $pEC_{50} = 6.91 \pm 0.16$) and SNP (SHR: $R_{\text{max}} = 96.19 \pm 2.12\%$, $pEC_{50} = 7.70 \pm 0.07$; SDR: $R_{\text{max}} = 95.84 \pm 1.41\%$, $pEC_{50} = 7.29 \pm 0.41$) caused essentially similar patterns of relaxation of PE ($10^{-6}$ M)-induced contraction in SHR and diabetic-WKY rings. Flavone enhanced the relaxation observed at all the concentrations of ACh and SNP, shifting the concentration–response curves to the left in aortic rings from both SHR (ACh: $R_{\text{max}} = 85.9 \pm 1.7\%$, $pEC_{50} = 7.53 \pm 0.07$; SNP: $R_{\text{max}} = 104.59 \pm 3.03\%$, $pEC_{50} = 8.49 \pm 0.11$) and diabetic-WKY (ACh: $R_{\text{max}} = 98.0 \pm 2.1\%$, $pEC_{50} = 7.65 \pm 0.07$; SNP: $R_{\text{max}} = 106.33 \pm 1.37\%$, $pEC_{50} = 8.50 \pm 0.14$) rats (Fig. 5). PE evoked concentration-dependent increases in smooth muscle tone in aortic rings from both SHR and diabetic-WKY rats (Fig. 6) (SHR: $C_{\text{max}} = 116.2 \pm 4.5\%$, $pEC_{50} = 7.23 \pm 0.09$; SDR: $C_{\text{max}} = 148.9 \pm 9.0\%$, $pEC_{50} = 7.17 \pm 0.12$). Flavone significantly decreased maximal PE-induced contraction by about 36% and 54%, respectively, and shifted concentration–response curves rightwards in aortic rings from both SHR ($C_{\text{max}} = 80.9 \pm 12.6\%$, $pEC_{50} = 7.05 \pm 0.23$) and diabetic-WKY ($C_{\text{max}} = 94.5 \pm 10.6\%$, $pEC_{50} = 6.92 \pm 0.17$) rats (Fig. 6).

The presence of pyrogallol completely abolished ACh-induced relaxation in control aortic rings and flavone was without any significant effect on the ACh-induced relaxation (Fig. 7). Addition of $\beta$-NADH induced a transient contraction in control aortic rings (236.0 ± 0.05 mg) as well as in flavone-pretreated aortic rings (221 ± 0.25 mg). In the presence of $\beta$-NADH, relaxation to ACh were significantly reduced.

Fig. 3. relaxation responses to acetylcholine (ACh) in vehicle- or flavone (10 $\mu$M)-pretreated endothelium-intact aortic rings from normal Wistar–Kyoto (WKY) rats in the presence of (A) indomethacin (indo, 10 $\mu$M), (B) $l$-NAME (10 $\mu$M), (C) methylene blue (MB, 10 $\mu$M) and (D) 20 mM KCl. Symbols represent mean ± S.E.M. ($n = 4$ or 5).
in both control ($R_{\text{max}} = 27.8 \pm 4.6\%$; $\text{pEC}_{50} = 7.42 \pm 0.25$) and flavone ($R_{\text{max}} = 48.9 \pm 4.16\%$; $\text{pEC}_{50} = 6.71 \pm 0.21$)-pretreated aortic rings compared with respective β-NADH untreated aortic rings (Fig. 7). However, this reduction was significantly lesser in magnitude in flavone-pretreated aortic rings compared with control aortic rings.

4. Discussion

This study has identified the effects of a non-antioxidant flavonoid flavone on vascular reactivity in normal, hypertensive and diabetic rat isolated aortae. The present results show that pre-incubation with flavone (10 μM) enhanced endothelium-denuded aortic rings isolated from normal Wistar–Kyoto (WKY) rats. Symbols represent mean ± S.E.M. ($n=4$ or 5). *$p<0.05$ vs. vehicle-pretreated aortic rings.

Fig. 5. Relaxation responses to: (A) acetylcholine (ACh) and (B) sodium nitroprusside (SNP) in vehicle- or flavone (10 μM)-pretreated endothelium-intact aortic rings from spontaneously hypertensive rats (SHR) or streptozotocin-induced diabetic-WKY rats (SDR). Symbols represent mean ± S.E.M. ($n=6$ or 7). *$p<0.05$ vs. equivalent response in corresponding vehicle-pretreated aortic rings.

Fig. 6. Contractile responses to phenylephrine (PE) in vehicle- or flavone (10 μM)-pretreated endothelium-intact aortic rings from spontaneously hypertensive rats (SHR) or streptozotocin-induced diabetic-WKY rats (SDR). Symbols represent mean ± S.E.M. ($n=6$ or 7). *$p<0.05$ vs. equivalent response in vehicle-pretreated SHR aortic rings, $\#p<0.05$ vs. equivalent response in corresponding vehicle-pretreated aortic rings.

Fig. 7. Relaxation responses to acetylcholine (ACh) in vehicle- or flavone (10 μM)-pretreated endothelium-intact aortic rings isolated from Wistar–Kyoto (WKY) rats in the presence of superoxide anions generated by reduced form of β-nicotinamide adenine di-nucleotide (β-NADH, 300 μM) or pyrogallol (pyro, 300 μM). Symbols represent mean ± S.E.M. ($n=4$ or 5). *$p<0.05$ vs. equivalent response in control aortic rings, $\#p<0.05$ vs. equivalent response in corresponding vehicle-pretreated aortic rings.
dependent vascular relaxation induced by ACh in WKY rat isolated aortae, a finding which has not been reported previously. We also demonstrated that flavone did not alter the failure of ACh to induce relaxation in endothelium-denuded WKY aortic rings. Granted that vascular relaxation to ACh is mediated by endothelium-derived relaxant factors [17], it could be argued that the enhancement of ACh relaxation is an indication that flavone probably promotes such relaxation by some endothelium-dependent effects. The failure of flavone to enhance ACh-induced relaxation in WKY aortic rings treated with the eNOS inhibitor (l-NAME) is consistent with this speculation. We also found that flavone-induced enhancement of ACh-induced relaxation in WKY aortic rings was not altered by indomethacin, suggesting that release of prostacyclin and/or nitric oxide–prostacyclin interaction did not contribute to the flavone effect. In addition, flavone enhancement of ACh-induced relaxations, although abolished at concentrations less than 1 μM, was also observed in WKY aortic rings incubated with KCl (i.e., partial depolarization). Moreover, relaxations to ACh were significantly higher except at very low concentrations of ACh in flavone pretreated WKY aortic rings in comparison to control WKY aortic rings in the presence of KCl. These findings suggest that although participation of EDHF or K+ channels is involved in the flavone enhancement of ACh-induced relaxations, the effect of flavone does not appear to be merely mediated by EDHF or K+ channels. In summary, the findings from experimental conditions using l-NAME, indomethacin, and KCl suggest that nitric oxide synthesized from endothelium mediates predominantly the effects of flavone on endothelium-dependent relaxation in isolated WKY aortae.

Nitric oxide released from endothelium diffuses into adjacent smooth muscle cells and leads to guanylate cyclase activation, cGMP elevation and ultimately to vascular smooth muscle relaxation [17]. Flavone did not alter the inhibition of ACh relaxation by the cGMP inhibitor, methylene blue, but it enhanced endothelium-independent relaxation to SNP in both endothelium-intact and endothelium-denuded WKY aortic rings. SNP breaks down spontaneously to yield nitric oxide, thereby causing endothelium-independent vasodilatation by the same effector mechanism as nitric oxide released from endothelium [18]. The present data, therefore, suggest that the effects of flavone resides downstream the nitric oxide–sGC–cGMP cascade (i.e., enhancement in the bioactivity of nitric oxide) and not just an enhancement in synthesis of nitric oxide from endothelium. This view is consistent with earlier reports which showed that flavonoids accumulate in the interspatial gap between endothelial and vascular smooth muscle cells, hence interact with intracellular processes in endothelial cells or even in the vascular smooth muscle cells [19,20]. Direct measurement of sGC activity and/or cGMP concentrations in control and flavone pretreated aortic rings which we were unable to perform in this study will be useful in further clarifying these assumptions.

The present results demonstrated that flavone enhanced ACh- and SNP-induced relaxation and attenuated PE-induced contraction in isolated aortic rings from SHR and diabetic rats, a finding similar to that observed in normal WKY aortae. This indicates that the observed effects of flavone on vascular responses in isolated SHR or diabetic rat aortic rings could be due to mechanisms similar to that observed in normal WKY rings. The vascular beneficial effects of flavonoids in hypertension were thought to be due to inhibition of superoxide anions and/or its reactive oxygen metabolites [6,7]. We have recently demonstrated that pre-incubation with the flavonoid quercetin at a concentration similar to that of flavone used in this study (i.e., 10 μM) enhanced endothelium-dependent relaxations in SHR and diabetic rat aortae via scavenging of superoxide anions [15,16]. The possibility that flavone acted by the same mechanism was explored. Pretreatment with flavone significantly prevented β-NADH-induced attenuation of ACh relaxation in WKY aorta, suggesting that flavone inhibited the basal activity of NADH/NADPH-oxidase and/or scavenged superoxide anions following their release from NADH/NADPH-oxidase, the major enzymatic source for endogenous superoxide anions production in hypertensive and diabetic blood vessels [1,2]. If this speculation was correct, it should be expected, contrary to our finding, that flavone would inhibit the transient β-NADH contraction which is thought to be provoked by removal of the relaxant activity of basal nitric oxide through NAD(P)H oxidase-induced superoxide anion release [14,21]. In addition, we also found that flavone did not reverse failure of ACh to induce relaxations in the presence of exogenous superoxide anions generated by pyrogallol [13]. Moreover, quercetin, an antioxidant flavonoid, at 10 μM completely prevented the detrimental effects of β-NADH or pyrogallol on relaxations to acetylcholine in WKY aortae (data not shown). Putting these observations together, we suggest that flavone enhancement of ACh relaxation of WKY aortae in the presence of β-NADH as well as in SHR and diabetic aortae does not appear to be essentially due to preservation of EDNO from incapacitation by superoxide anions, but rather by the improvement in the bioactivity of available nitric oxide (i.e., increased sGC/cGMP activity), as described above.

In conclusion, the present results demonstrate that flavone modulates vascular responses in isolated aortic rings from normal, hypertensive, and diabetic rats. These effects probably result from enhanced bioactivity of nitric oxide rather than just the protection of nitric oxide from superoxide anions. Considering our earlier report that chronic administration of flavone (in vivo) enhanced endothelium-dependent and -independent vascular relaxations in SHR aortae [8], we suggest that the effects of flavone on vascular relaxations in SHR and possibly in human hypertension results from mechanisms similar to those observed in this in vitro study.

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