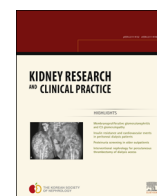




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Original Article

Mechanisms of phytoestrogen biochanin A-induced vasorelaxation in renovascular hypertensive rats

Seok Choi¹, Won Suk Jung², Nam Soo Cho², Kwon Ho Ryu¹, Jae Yeoul Jun¹,
Byung Chul Shin³, Jong Hoon Chung³, Cheol Ho Yeum^{1,*}¹ Department of Physiology, College of Medicine, Chosun University, Gwangju, Korea² Department of Emergency Medicine, College of Medicine, Chosun University, Gwangju, Korea³ Department of Internal Medicine, College of Medicine, Chosun University, Gwangju, Korea

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Background: The plant-derived estrogen biochanin A is known to cause vasodilation, but its mechanism of action in hypertension remains unclear. This study was undertaken to investigate the effects and mechanisms of biochanin A on the thoracic aorta in two-kidney, one clip (2K1C) renovascular hypertensive rats.**Methods:** Hypertension was induced by clipping the left renal artery, and control age-matched rats were sham treated. Thoracic aortae were mounted in tissue baths to measure isometric tension.**Results:** Biochanin A caused concentration-dependent relaxation in aortic rings from 2K1C hypertensive and sham-treated rats, which was greater in 2K1C rats than in sham rats. Biochanin A-induced relaxation was significantly attenuated by removing the endothelium in aortic rings from 2K1C rats, but not in sham rats. N^o-Nitro-L-arginine methyl ester, a nitric oxide synthase inhibitor, or indomethacin, a cyclooxygenase inhibitor, did not affect the biochanin A-induced relaxation in aortic rings from 2K1C and sham rats. By contrast, treatment with glibenclamide, a selective inhibitor of adenosine triphosphate-sensitive K⁺ channels, or tetraethylammonium, an inhibitor of Ca²⁺-activated K⁺ channels, significantly reduced biochanin A-induced relaxation in aortic rings from both groups. However, 4-aminopyridine, a selective inhibitor of voltage-dependent K⁺ channels, inhibited the relaxation induced by biochanin A in 2K1C rats, whereas no significant differences were observed in sham rats.**Conclusion:** These results suggest that the enhanced relaxation caused by biochanin A in aortic rings from hypertensive rats is endothelium dependent. Vascular smooth muscle K⁺ channels may be involved in biochanin A-induced relaxation in aortae from hypertensive and normotensive rats. In addition, an endothelium-derived activation of voltage-dependent K⁺ channels contributes, at least in part, to the relaxant effect of biochanin A in renovascular hypertension.© 2014. The Korean Society of Nephrology. Published by Elsevier. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Introduction

Estrogen replacement therapy markedly reduces the risk of cardiovascular disease in postmenopausal women [1,2]. However, the use of hormone replacement therapy as a cardioprotective

* Corresponding author. Department of Physiology, College of Medicine, Chosun University, 309, Pilmundaero, Dong-Gu, Gwangju 501-759, Korea.

E-mail address: chyum@chosun.ac.kr (CH Yeum).

strategy is greatly limited owing to carcinogenic effects in women and feminizing effects in men. Phytoestrogens are naturally occurring plant-derived nonsteroidal estrogens, which also bind to the estrogen receptor, acting as estrogen agonists or antagonists [3,4].

It has been shown that dietary soy-derived estrogens are vasoactive and have beneficial cardiovascular effects [5]. Both dietary estrogens [6] and estradiol replacement therapy [7] enhance the vasorelaxant response to acetylcholine in female monkeys, whereas systemic arterial compliance was improved by a red clover diet in menopausal women [8]. Biochanin A that naturally occurs in soybeans and clover is estrogen-like [9]. It has been shown to relax the rabbit basilar artery [10] and to induce significant sex-independent relaxation of rabbit coronary arteries [11]. Furthermore, Wang et al [1] subsequently confirmed that biochanin A induces endothelium-independent relaxation in rat aortic rings, of which mechanism involves blockage of Ca^{2+} entry through the cell membrane and activation of K^{+} channels. In addition, biochanin A-induced vasorelaxation was increased in spontaneously hypertensive rats than in normotensive rats, which was mediated by the release of endothelium-derived substances that may open voltage-dependent and Ca^{2+} -activated K^{+} channels in the vascular smooth muscle [12]. Under these backgrounds, we have previously shown that an activation of Ca^{2+} -activated K^{+} channels in vasorelaxation is altered in two-kidney, one clip (2K1C) renal hypertension [13]. Although an endothelium-derived activation of smooth muscle cell K^{+} channels, which contributes to biochanin A-induced vasorelaxation, was observed in spontaneously hypertensive rats, the effect of biochanin A on vascular function in 2K1C renovascular hypertension remains unclear.

The present study was designed to examine the mechanisms of the relaxing effects induced by biochanin A in the thoracic aorta isolated from 2K1C renovascular hypertensive rats.

Methods

Induction of 2K1C renovascular hypertension

Renovascular hypertension was induced in rats following the 2K1C Goldblatt model [14]. Briefly, male Sprague–Dawley rats (Samtako Inc., Osan, Korea), weighing 160–180 g, were anesthetized with an intraperitoneal injection of sodium thiopental (40 mg/kg). Under antiseptic conditions, an incision was made on the left flank to access the left renal artery, which was separated from the renal vein and cleaned of connective tissue. A U-shaped solid silver clip with an internal diameter of 0.2 mm was applied to the exposed renal artery, resulting in the partial occlusion of renal perfusion. The contralateral kidney remained untouched, and the wound was closed. A group of age-matched rats were sham treated and served as the control group. The control animals underwent an operation similar to the 2K1C rats except that no clip was used. All animals were fed normal chow and were given tap water. They were used for the experiments at 10 weeks after the clipping, because endothelial dysfunction is associated with the duration of hypertension [15]. Hypertensive rats were selected based on their systolic blood pressure measured in a conscious state by the tail cuff method.

Tissue preparation

The thoracic aorta between the aortic arch and diaphragm was carefully removed and placed in cold, standard physiological salt

solution with the following composition: NaCl 118.3mM, KCl 4.7mM, NaHCO_3 25mM, MgCl_2 1.2mM, KH_2PO_4 1.2mM, CaCl_2 2.5mM, and glucose 11.1mM. Vessels were cleaned of adherent fat and connective tissue, and then cut into 2–3 mm long cylindrical rings under a dissecting microscope. The rings were suspended between two triangle-shaped stainless steel holders in the vessel lumen in organ baths containing 15 mL physiological salt solution maintained at $37 \pm 0.05^\circ\text{C}$, aerated with a mixture of 95% O_2 and 5% CO_2 , and maintained at a pH of 7.4 ± 0.01 . One of the holders was fixed at the bottom of the chamber, and the other was connected to a Grass force displacement transducer (FTO3, Quincy, MA, USA) to measure isometric tension development. Prior to initiating specific experimental protocols, the aortic rings were stretched to the point of their optimal length–tension relationship 2 g, which was determined in similar preliminary experiments using repeated exposure to 60mM KCl (obtained by equimolar replacement of NaCl by KCl in the physiological solution), and equilibration for a period of at least 90 minutes.

Protocols

At the beginning of the experiment, aortic rings were stimulated with 60mM KCl to test their functional integrity. In all experiments, aortic rings from 2K1C and sham-operated rats were precontracted to 50% effective concentration (EC_{50}) with phenylephrine ($3 \times 10^{-7}\text{M}$ in sham and $4 \times 10^{-8}\text{M}$ in 2K1C), which were obtained in preliminary experiments. When the contractile response achieved a steady state, relaxation–response curves to the cumulative addition of biochanin A (from 10^{-7}M to 10^{-4}M), acetylcholine (from 10^{-9}M to 10^{-5}M), or sodium nitroprusside (SNP; from 10^{-10}M to $10^{-6.5}\text{M}$) were determined. In an alternate set of experiments, the aorta was pretreated for 10 minutes with biochanin A ($3 \times 10^{-5}\text{M}$) prior to the addition of phenylephrine in the case of acetylcholine- and SNP-induced relaxation. To verify the role of functional endothelium in the vascular relaxant effects of biochanin A, the endothelium was removed from some thoracic aortae by gently rubbing the intimal surface with a moistened cotton swab. The successful removal of the endothelial cells from aortic rings was confirmed via the inability of acetylcholine to induce relaxation.

In another experiments, the nitric oxide synthase (NOS) inhibitor *N*^o-nitro-L-arginine methyl ester (L-NAME, 10^{-4}M) or the cyclo-oxygenase inhibitor indomethacin (10^{-5}M), and K^{+} channel blockers glibenclamide ($3 \times 10^{-6}\text{M}$), tetraethylammonium (TEA, 10^{-3}M), or 4-aminopyridine (10^{-3}M), were added to the bath 10 minutes prior to the addition of phenylephrine.

Drugs

Acetylcholine, 4-aminopyridine, biochanin A, glibenclamide, indomethacin, L-NAME, SNP, and TEA were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of analytical grade. Biochanin A, glibenclamide, and indomethacin were dissolved in dimethylsulfoxide, and the others were prepared in distilled water. The final concentrations of dimethylsulfoxide were $< 0.05\%$, which did not alter the contraction or relaxation responses.

Statistical analysis

The values presented in the figures are the means and standard errors of the means. Relaxant responses are presented

as the percent change in phenylephrine-induced contractile tension. The concentration causing half-maximal relaxation (IC_{50}) was determined by plotting the percentage of responses and expressed as the mean of negative log molar (pD_2) for individual tissue response, using the Origin software (Northampton, MA, USA). Statistical comparisons were performed using Student *t* test or analysis of variance followed by Duncan's test for multiple comparisons. A *P* value < 0.05 was considered statistically significant.

Results

The systolic blood pressure was 190 ± 5 mmHg ($n=44$, $P < 0.05$) and 137 ± 4 mmHg ($n=40$) in 2K1C hypertensive and sham-clipped rats, respectively.

Vasorelaxant responses to biochanin A

The tension induced by phenylephrine was enhanced in the aortic rings from 2K1C rats (1.45 ± 0.07 g, $P < 0.05$) compared with those from sham rats (1.12 ± 0.06 g). Biochanin A completely relaxed the aortic rings from 2K1C and sham rats when they were precontracted with phenylephrine. However, the relaxation was augmented in the aortic rings from 2K1C rats than in the rings from sham rats (pD_2 : 5.05 ± 0.08 vs. 4.67 ± 0.07 , $P < 0.05$). This effect was more pronounced at the higher concentration of biochanin A (Fig. 1). Biochanin A-induced relaxation was significantly attenuated by the removal of the endothelium in the aortic rings from 2K1C rats (pD_2 : 4.69 ± 0.06 , $P < 0.05$), whereas no significant differences were observed in rings from sham rats (Fig. 2).

Vasorelaxant responses to acetylcholine and SNP

Acetylcholine-induced vasodilation was significantly attenuated in the aortic rings from 2K1C rats than in those from sham rats. Treatment with L -NAME (10^{-4} M) completely blocked the acetylcholine-induced vasodilatory effect in both 2K1C

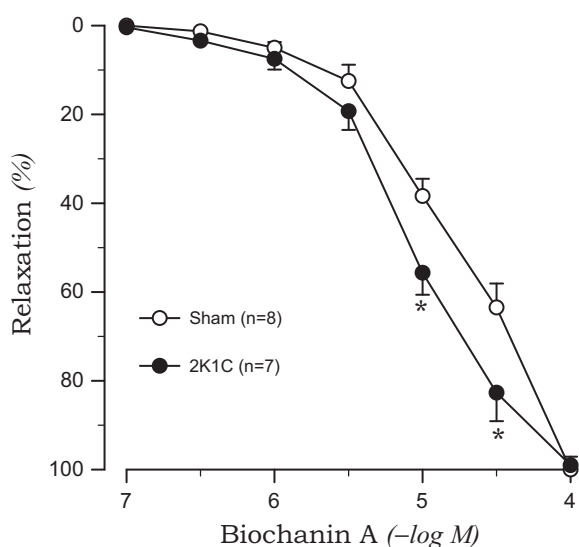


Figure 1. Biochanin A-induced vasorelaxation in phenylephrine-precontracted aortic rings from sham-clipped control and 2K1C hypertensive rats. Points represent means \pm SE for the number (*n*) of experiments in parentheses. * $P < 0.05$, compared with sham values. 2K1C, two-kidney, one clip.

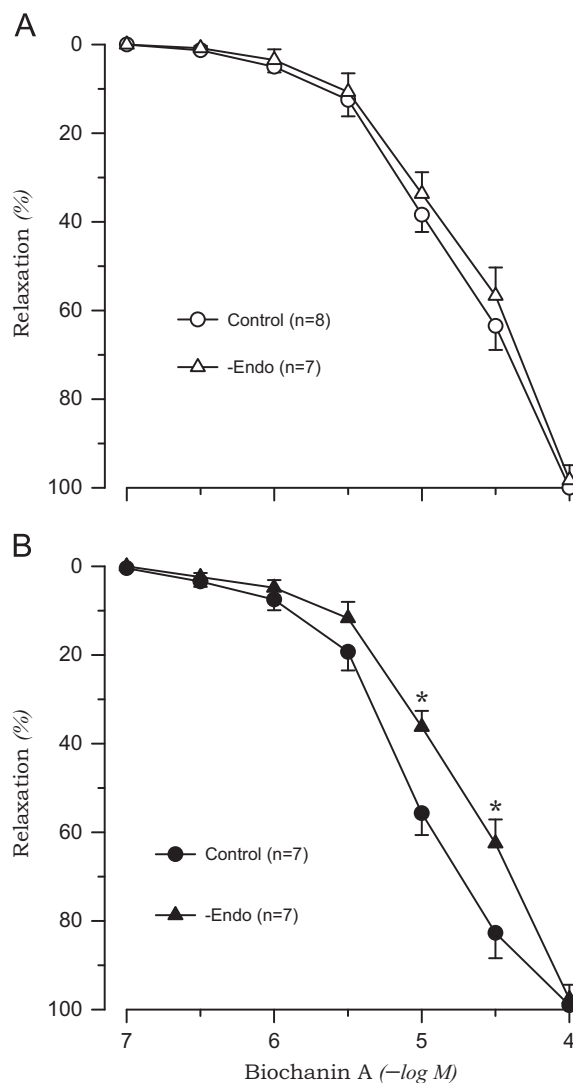


Figure 2. Biochanin A-induced vasorelaxation in phenylephrine-precontracted aortic rings without endothelium from sham-clipped control (A) and 2K1C hypertensive rats (B). * $P < 0.05$, compared with corresponding control values. 2K1C, two-kidney, one clip; -Endo, without endothelium.

and sham groups (data not shown). Biochanin A (3×10^{-5} M) had no effect on acetylcholine-induced vasodilation in either sham or 2K1C rats (Fig. 3). The vasorelaxation response to SNP was not altered in 2K1C rats. Biochanin A did not affect SNP-induced vasodilation (Fig. 4).

Effects of *L*-NAME and indomethacin on biochanin A-induced vasorelaxation

Preincubation with *L*-NAME or indomethacin had no effect on biochanin A (3×10^{-5} M)-induced vasodilation in either sham or 2K1C rats (Fig. 5).

Effects of K^+ channel blockers on biochanin A-induced vasorelaxation

Pretreatment with glibenclamide, an inhibitor of adenosine triphosphate (ATP)-sensitive K^+ channels, and TEA, an inhibitor of Ca^{2+} -activated K^+ channels, significantly reduced biochanin A (3×10^{-5} M)-induced relaxation in 2K1C and sham-clipped rats.

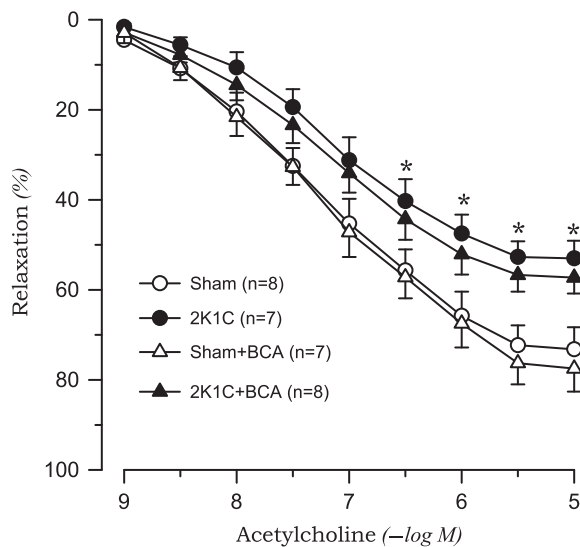


Figure 3. Effects of biochanin A on vasodilatory responses induced by acetylcholine in aortic rings from sham-clipped control and 2K1C hypertensive rats. * $P < 0.05$, compared with corresponding sham values. 2K1C, two-kidney, one clip; BCA, biochanin A.

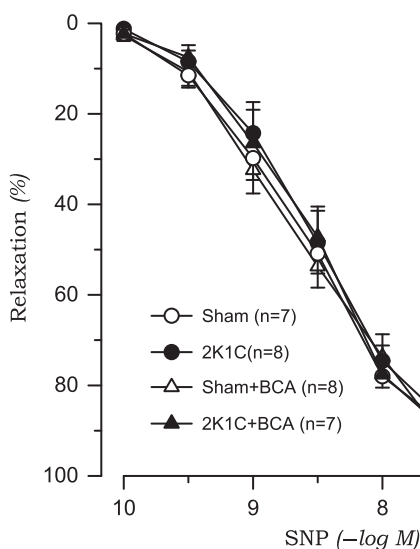


Figure 4. Effects of biochanin A on vasodilatory responses induced by sodium nitroprusside in aortic rings from sham-clipped control and 2K1C hypertensive rats. 2K1C, two-kidney, one clip; BCA, biochanin A; SNP, sodium nitroprusside.

However, 4-aminopyridine, an inhibitor of voltage-dependent K^+ channels, inhibited biochanin A-induced relaxation only in 2K1C rats with no effects in sham rats (Fig. 6).

Discussion

We previously confirmed that vascular reactivity to contractile agonist is enhanced in disease states such as hypertension [16]. As shown in our previous findings, the contractile response to phenylephrine was augmented in 2K1C hypertensive rats than in sham-clipped normotensive rats. In the present study, biochanin A induced dose-dependent relaxation in phenylephrine-precontracted aortic rings with intact endothelium isolated from 2K1C and sham rats, of which the

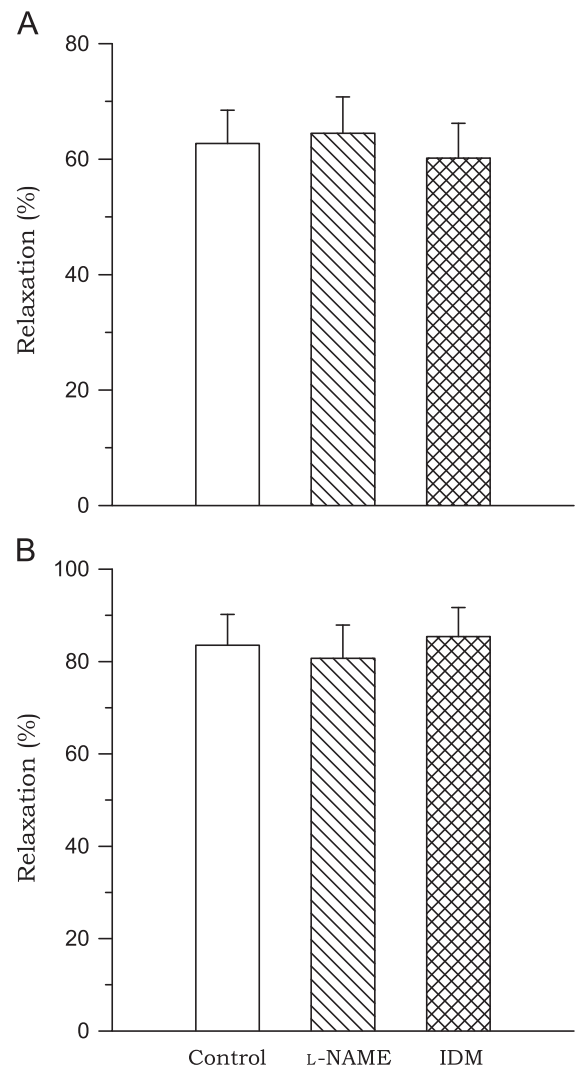


Figure 5. Effects of N^{ω} -nitro-L-arginine methyl ester and indomethacin on the relaxation induced by biochanin A in aortic rings from sham-clipped control (A) and 2K1C hypertensive (B) rats. Data are obtained from six to eight experiments. 2K1C, two-kidney, one clip; IDM, indomethacin; L-NAME, N^{ω} -nitro-L-arginine methyl ester.

magnitude was augmented in hypertensive rats. The relaxant effect of biochanin A in aortic rings from sham rats with or without endothelium was comparable, suggesting that biochanin A-induced relaxation is endothelium-independent and may directly affect the vascular smooth muscle in normotensive rats. Similar results have been demonstrated in rabbit coronary [11] or basilar arteries [10] and in rat aortae [12]. However, as has been shown previously in genetically hypertensive rats [12], the relaxant effect of biochanin A was significantly attenuated by the removal of the endothelium in aortic rings from 2K1C hypertensive rats. These observations imply that the vasorelaxing effect of biochanin A is partially endothelium dependent in hypertensive aortae, whereas it is endothelium independent in sham rats.

Acetylcholine causes NO release via the activation of specific endothelial receptors, resulting in the activation of endothelial NOS [17]. The present study confirmed earlier observations [15,18] that endothelium-dependent relaxation to acetylcholine is markedly depressed in 2K1C hypertensive rats compared with that in sham-clipped control rats. Treatment with L-NAME

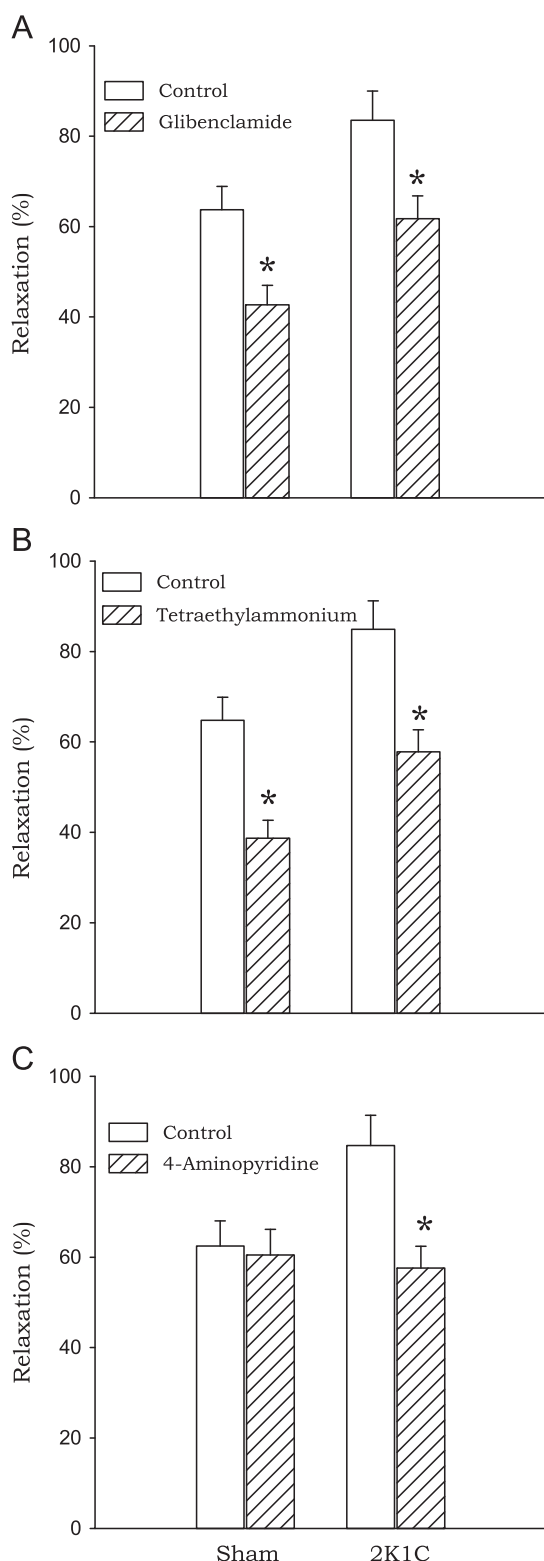


Figure 6. Effects of glibenclamide (A), tetraethylammonium (B), and 4-aminopyridine (C) on the relaxation induced by biochanin A in aortic rings from sham-clipped control and 2K1C hypertensive rats. Data are obtained from six to eight experiments. * $P < 0.05$, compared with corresponding control values. 2K1C, two-kidney, one clip.

completely inhibited the acetylcholine-induced vasodilatory effect in both groups, suggesting that acetylcholine-induced vasodilation is largely attributable to NOS-derived NO. Although the relaxation induced by acetylcholine was attenuated in

hypertensive rats, biochanin A did not affect acetylcholine-induced endothelium-dependent vasorelaxation in either 2K1C or sham rats. In addition, biochanin A had no effect on SNP (which is an NO donor)-induced endothelium-independent vasodilation in aortic rings from 2K1C and sham rats. These results indicate that the functions of NO synthesis or NO-mediated relaxation in aortic rings from hypertensive and normotensive rats are not altered by biochanin A.

Although biochanin A did not affect acetylcholine-induced vasorelaxation in 2K1C hypertensive and sham rats in our experiments, in order to clarify whether the vasodilator NO is involved in biochanin A-induced relaxation, the effects of L-NAME on the relaxation induced by biochanin A were examined. L-NAME did not affect biochanin A-induced relaxation in 2K1C and sham rats. The results again imply that the role of NO is not involved in biochanin A-induced relaxation in either group. The results of the present study are in agreement with previous data from the coronary artery¹¹ and aorta,¹² which suggest that vasodilator NO is not a candidate contributor to biochanin A-induced relaxation. Conversely, Torregrosa et al [10] reported that biochanin A-induced relaxation is partially reduced by L-NAME treatment, which indicates that the relaxant effect of biochanin A in aortae with endothelium from rabbits is due to the release of NO. One possible explanation may be attributed to the differences in the experimental conditions, species, or strains [12]. Alternatively, the endothelial release of prostacyclins or endothelium-derived hyperpolarizing factor (EDHF) other than NO may be responsible [19]. In the present study, however, indomethacin, an inhibitor of the synthesis of prostaglandins [20], had little effect on biochanin A-induced relaxation in aortic rings from 2K1C and sham rats. Taken together, an involvement of an EDHF, acting through K^+ channels to cause hyperpolarization of vascular smooth muscle cells [21], may be raised. It has been indeed known that the EDHF-mediated vasorelaxation was preserved in an animal model of renovascular hypertension [22,23].

The EDHF passes through the internal elastic lamina and vascular smooth muscle, and causes an activation of ion channels, thus initiating smooth muscle hyperpolarization and inducing relaxation [24]. In the endothelium and smooth muscle, K^+ channels control the vascular tone and arterial blood pressure [25]. The activation of K^+ channels causes a hyperpolarization of cell membranes in vascular smooth muscle and thus inhibits Ca^{2+} influx via voltage-gated Ca^{2+} channels. Therefore, an activation of K^+ channels may play a role in Ca^{2+} signaling and modulate the arterial tone [26]. There are several types of K^+ channels preserved in vascular smooth muscle [27]. In the present study, to examine whether the K^+ channels contribute to biochanin A-induced vasodilation in vascular smooth muscle, we pretreated with the K^+ channel blockers glibenclamide, TEA, and 4-aminopyridine in endothelium-intact aortic rings from 2K1C hypertensive and sham rats. Biochanin A-induced relaxation was significantly reduced by glibenclamide, a blocker of ATP-sensitive K^+ channels, in 2K1C and sham rats. In addition, TEA, which blocks large-conductance Ca^{2+} -activated K^+ channels when used in appropriate concentrations [12], also inhibited the relaxant effect of biochanin A in both groups. These results imply that activation of both ATP-sensitive and Ca^{2+} -activated K^+ channels may be involved in biochanin A-induced relaxation in hypertensive and normotensive rats. Interestingly, 4-aminopyridine, an inhibitor of voltage-dependent K^+ channels, significantly

attenuated biochanin A-induced relaxation in aortic rings from 2K1C rats, but no significant differences were observed in sham rats. Therefore, the greater relaxation induced by biochanin A in rings from 2K1C rats compared with those from sham rats in our experiment, may in part be responsible for the voltage-dependent K^+ channels and evoke hyperpolarization of the smooth muscle cells. Similar results were also observed in genetically hypertensive rats, in which the activation of K^+ channels contributes to the vasorelaxation in hypertension [12]. In addition, a previous study reported that TEA inhibits the vasorelaxant effect of biochanin A in normotensive rats [12], whereas the effects of TEA on biochanin A-induced relaxation were comparable between 2K1C and sham rats in this study. A possible discrepancy may be attributed to the different animal species used in this study.

In summary, the relaxant effect of biochanin A is augmented in aortae of 2K1C hypertensive rats compared with those of sham-clipped control rats, and the relaxation is endothelium-independent in sham rats, whereas it is both endothelium-dependent and -independent in 2K1C rats. An activation of vascular smooth muscle K^+ channels may be involved in biochanin A-induced relaxation in both 2K1C and sham rats. In addition, the enhanced relaxation by biochanin A in renovascular hypertension is mediated by endothelium-derived substances that may evoke an activation of voltage-dependent K^+ channels in vascular smooth muscle.

Conflict of interest

The authors have no conflict of interest to declare.

Acknowledgments

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