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Mechanisms of Phytoestrogen Biochanin A-Induced Vasorelaxation in Renovascular Hypertensive Rats

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혈관이완 기전

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CONTENTS

CONTENTS OF FIGURES

국문초록

신혈관성 고혈압쥐에서 Biochanin A에 의한 혈관이완 기전

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식물성 estrogen으로 알려진 biochanin A는 혈관 이완효과가 있음이 보고되었으나 고혈압 상태에서의 작용기전에 대해서는 명확히 알려져 있지 않다. Biochanin A가 two-kidney, one clip (2K1C) 신혈관성 고혈압 모델에서 혈관 반응에 미치는 영향을 검토하고 그 기전이 정상혈압동물과 차이가 있는지 구명하고자 본 연구를 시행하였다.

흰쥐 일측 신동맥에 clip을 장치한 고혈압군과 대조군의 적출 흉부 대동맥 표본에서 biochanin A의 이완반응을 확인하고 약물의 전처치가 biochanin A의 이완반응에 미치는 영향을 양군에서 비교 검토하였다.

Biochanin A에 의한 이완반응은 2K1C 고혈압군에서 대조군에 비해 항진되었다. 혈관내피층 제거시 biochanin A의 이완반응은 고혈압군에서는 약화되었으나 대조군은 차이가 없었다. Nw-nitro-L-arginine methyl ester 및 indomethacin 전처치는 biochanin A의 이완반응에 영향을 미치지 않았다. Biochanin A의 이완반응은 glibenclamide 및 tetraethylammonium에 의해 고혈압군과 대조군에서 양군 모두 억제되었다. 4-aminopyridine의 전처치

는 biochanin A의 이완반응을 고혈압군에서 억제시켰으나 대조군은 영향받지 않았다.

이상의 실험결과는 biochanin A의 이완반응이 혈관 평활근 세포의 K^+ 통로 활성화에 기인하며 신혈관성 고혈압 상태에서는 혈관 내피층 유래인자에 의한 막전압 의존성 K^+ 통로가 이완작용과 관련됨을 시사한다.

Ⅰ. 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 strategy is greatly limited owing to carcinogenic effects of estrogens on the endometrium in women and feminizing effects in men. Hence, there is a strong interest in finding alternative estrogen like agents that are noncarcinogenic and nonfeminizing, yet induce cardioprotective effects. In this regard, phytoestrogens are naturally occurring plant-derived nonsteroidal estrogens which are present in the human diet. Their chemical structure is similar to that of estrogen, what enables them to bind the estrogen receptor thus acting as estrogen agonists or antagonists[3,4].

It has been shown that dietary soy-derived estrogens are vasoactive and soya intake is thought to have beneficial cardiovascular effects[5]. Both dietary soy-derived estrogens[6] and estradiol-17 β replacement therapy[7] enhance the dilator response to acetylcholine of atherosclerotic arteries from female monkeys, while isoflavones from red clover improve systemic arterial compliance in menopausal women[8]. Biochanin A is an estrogen-like compounds that occurs naturally in soybean and clovers[9]. In vitro experiments have shown that biochanin A relaxes rabbit basilar artery[10] and induces significant,

gender-independent relaxation in rabbit coronary arteries[11]. Its potency is greater than other isoflavonoid[10,11]. Furthermore, Wang et al. subsequently confirmed that biochanin A induces an endothelium-independent relaxation in rat aortic rings and the mechanism is involve the blockage of Ca^{2+} entry through the cell membrane and the activation of K^{+} channels[1]. In addition, biochanin A-induced vasorelaxation was augmented in spontaneously hypertensive rats than in normotensive rats and the greater relaxation in hypertensive rats is mediated by the release of endothelium-derived substances that may open both voltage-dependent and Ca^{2+}-activated K^{+} channels in vascular smooth muscle[12]. Under these backgrounds, previous study reported 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 contributes to the 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 actions induced by biochanin A on the vasculature in the thoracic aorta isolated from 2K1C renovascular hypertensive rats and to contribute to the vascular pathophysiology of hypertension.

Ⅱ. METHODS

1. Induction of 2K1C renovascular hypertension

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

2. Tissue preparation

The thoracic aorta between the aortic arch and diaphragm was carefully removed and placed in cold, standard physiological salt solution (PSS) of the following composition (in mM): NaCl 118.3, KCl 4.7, $NaHCO_3$ 25, $MgCl_2$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5 and glucose 11.1. Vessels were cleaned of adherent fat and connective tissue, 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 of PSS maintained at 37±0.05 ℃, aerated with a mixture of 95 % O_2 and 5 % CO_2 to maintain a pH 7.4±0.01. One of the holders was fixed at the bottom of the chambers and the other was connected to a force displacement transducer (Grass FTO3) for measurement of isometric tension development (Fig. 1). Before initiating specific experimental protocols, the aortic rings were stretched to the point of their optimal length-tension relationship 2 g, determined in similar preliminary experiments using repeated exposure to 60 mM KCl solution (obtained by equimolar replacement of NaCl by KCl in the physiological solution), and allowed to equilibrate during the period of at least 90 min.

ISOLATED TISSUE BATH

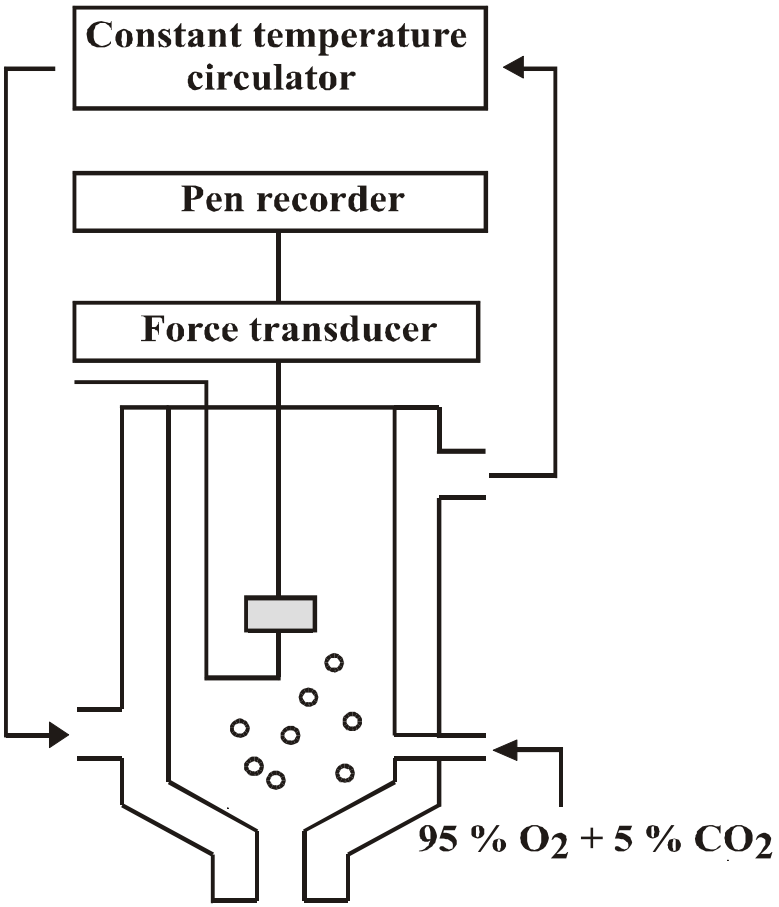

Fig. 1. A schematic representation of the recording system for isometric contraction with 15 mL tissue bath.

3. Protocols

At the beginning of the experiments, aortic rings were stimulated with 60 mM 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 (3×10^{-7} M in sham and 4×10^{-8} 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 (10^{-7} to 10^{-4} M), acetylcholine (10^{-9} to 10^{-5} M) or sodium nitroprusside (SNP; 10^{-10} to $10^{-6.5}$ M) were determined. In an alternate set of experiments, the aorta was pretreated for 10 min with biochanin A (3×10^{-5} M) before the addition of phenylephrine in the case of acetylcholine- and sodium nitroprusside-induced relaxation. To verify the role of functional endothelium in the vascular relaxant effects of biochanin A, the endothelium of some thoracic aortae was removed by gently rubbing the intimal surface with a moistened cotton swab. Successful removal of endothelial cells from aortic rings was confirmed by the inability of acetylcholine to induce relaxation.

In another experiments, the nitric oxide synthase (NOS) inhibitor N_w-nitro-L-arginine methyl ester (L-NAME, 10^{-4} M) or the cyclo-oxygenase inhibitor indomethacin (10^{-5} M) , and K^+ channel blockers glibenclamide (3×10^{-6} M), tetraethylammonium (TEA, 10^{-3} M) or 4-aminopyridine (10^{-3} M), were added to the

bath 10 min before the addition of phenylephrine.

4. Drugs and chemicals

Drugs used were acetylcholine, 4-aminopyridine, biochanin A, glibenclamide, indomethacin, L-NAME, SNP and TEA. They were purchased from Sigma Chemical Co. (St. Louis, Mo). All other chemicals were of analytical grade. Biochanin A, glibenclamide and indomethacin were dissolved in dimethylsulfoxide (DMSO) and the others were prepared in distilled water. Final bath concentrations of DMSO were less than 0.05 %, which did not alter contraction or relaxation responses.

5. Statistical analysis

Values presented in the figures are expressed as the means and standard error of the means. Relaxant responses are given as the percent change in phenylephrine-induced contractile tension. The concentration of causing half-maximal relaxation (IC_{50}) was determined by a plot of the percentage of responses and expressed as the mean of negative log molar (pD_2) for individual tissue response, using Origin software. Statistical comparisons were performed by Student's t-test or analysis of

variance (ANOVA) followed by Duncan's test for multiple comparisons. Probability values of <0.05 were considered statistically significant.

Ⅲ. RESULTS

Ten weeks after the operative intervention, the systolic blood pressure were 190±5 mmHg (n=44,) and 137±4 mmHg (n=40) in 2K1C hypertensive and sham-clipped control rats, respectively ($P<0.05$, Fig. 2).

1. Vasorelaxant responses to biochanin A

The tension induced by phenylephrine was enhanced in aortic rings from 2K1C rats (1.45±0.07 g, $P<0.05$) than in those from sham rats (1.12±0.06 g). Biochanin A completely relaxed aortic rings from 2K1C and sham rats when they were precontracted with phenylephrine. However, the relaxation was augmented in rings from 2K1C rats compared with sham rats (pD_2: 5.05±0.08 vs 4.67±0.07, $P<0.05$). This effect was more pronounced at the high concentration of biochanin A (Fig. 3). Biochanin A-induced relaxation was significantly attenuated by removal of endothelium in aortic rings from 2K1C rats (pD_2: 4.69±0.06, $P<0.05$), while no significant differences were shown in rings from sham rats (Fig. 4, Fig. 5).

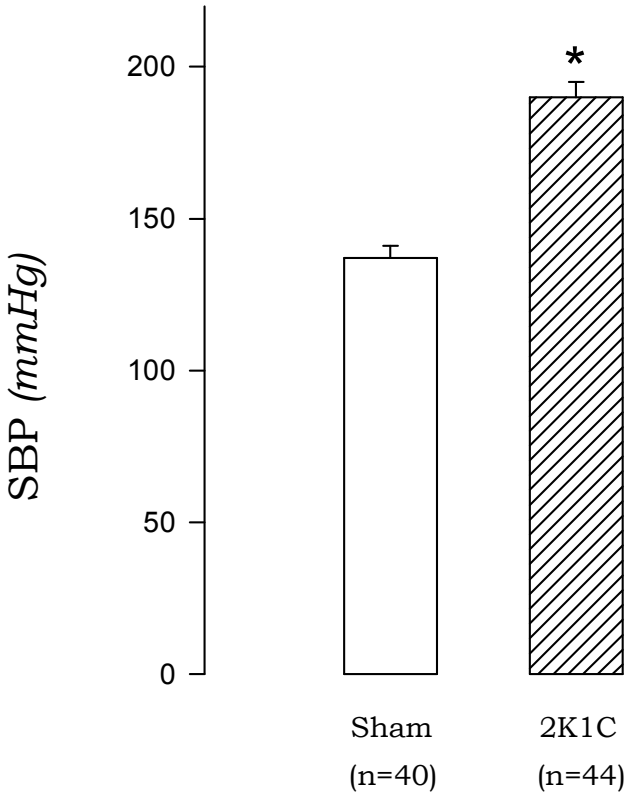

Fig. 2. Systolic blood pressure in 2K1C hypertensive and sham-clipped control rats.

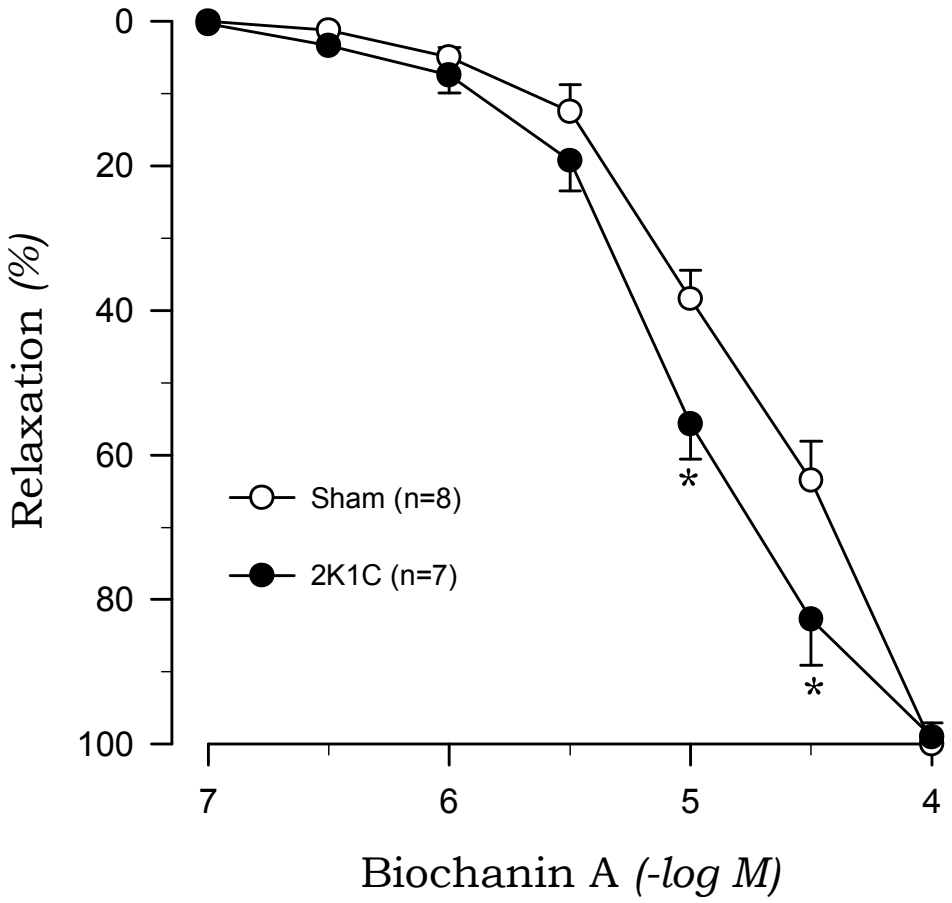

Fig. 3. Biochanin A-induced vasorelaxation in phenylephrine-precontracted aortic rings from 2K1C hypertensive and sham-clipped control rats. Points represent means±SE for number(n) of experiments in parentheses. * P <0.05, compared with sham values.

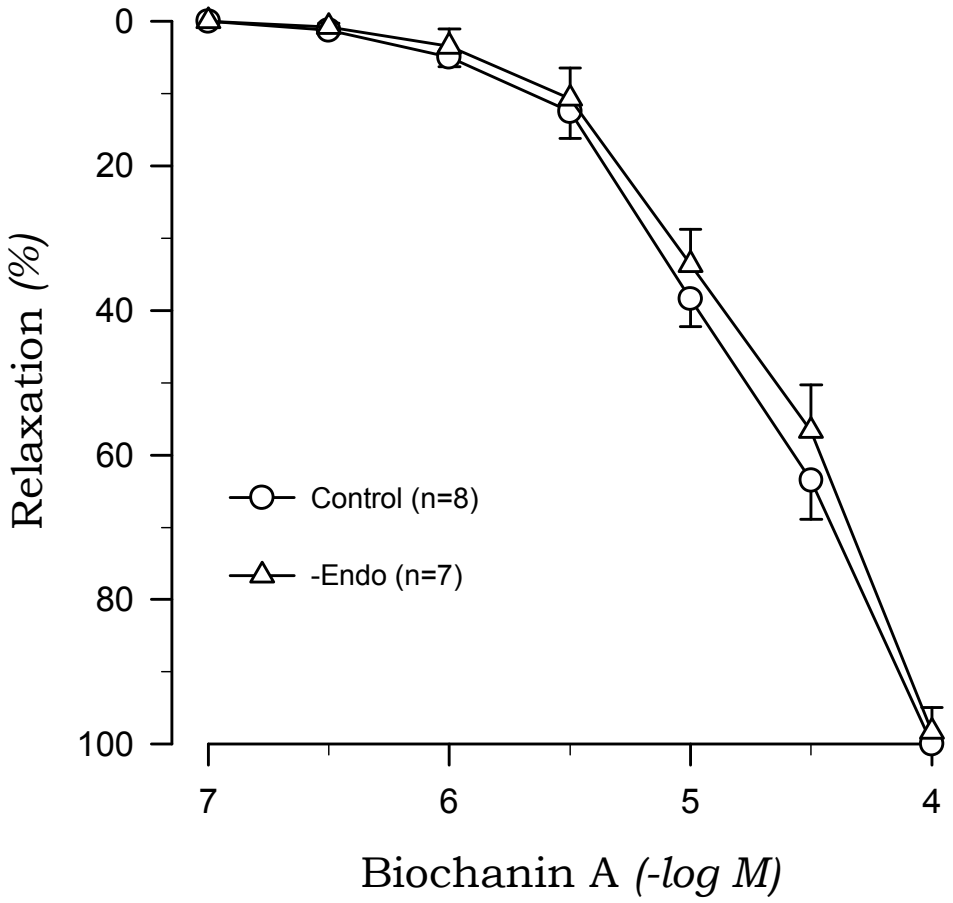

Fig. 4. Biochanin A-induced vasorelaxation in phenylephrine-precontracted aortic rings without endothelium (-Endo) from sham rats.

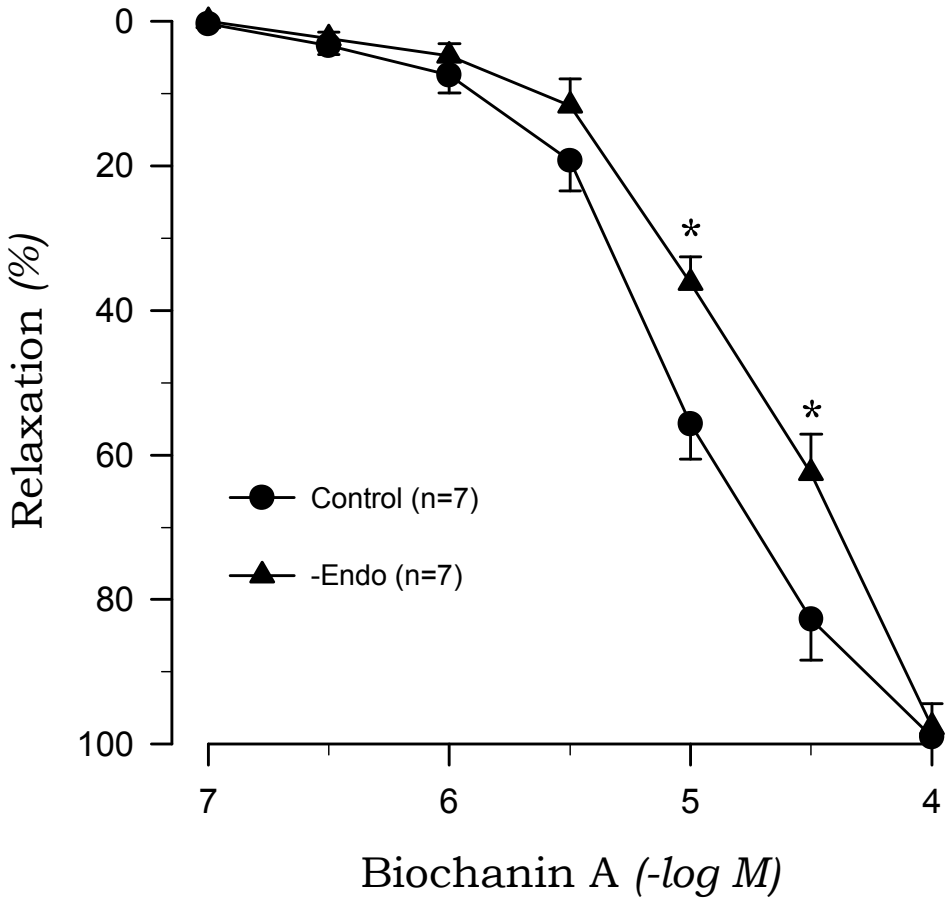

Fig. 5. Biochanin A-induced vasorelaxation in phenylephrine-precontracted aortic rings without endothelium (-Endo) from 2K1C hypertensive rats. * P <0.05, compared with control values.

2. Vasorelaxant responses to acetylcholine and SNP

Acetylcholine-induced endothelium-dependent vasodilation was significantly attenuated in aortic rings from 2K1C rats compared to those from sham rats. Treatment with L-NAME (10^{-4} M) completely inhibited acetylcholine-induced vasodilatory effect in both 2K1C and sham groups (data not shown). Biochanin A (3×10^{-5} M) had no effect on acetylcholine-induced vasodilation either in sham or in 2K1C rats (Fig. 6). The endothelium-independent vasorelaxation to SNP was not altered in 2K1C rats. Biochanin A did not affect SNP-induced vasodilation (Fig. 7).

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

Pre-incubation with L-NAME or indomethacin had effect on biochanin A (3×10^{-5} M)-induced vasodilation neither in sham nor in 2K1C rats (Fig. 8, Fig. 9).

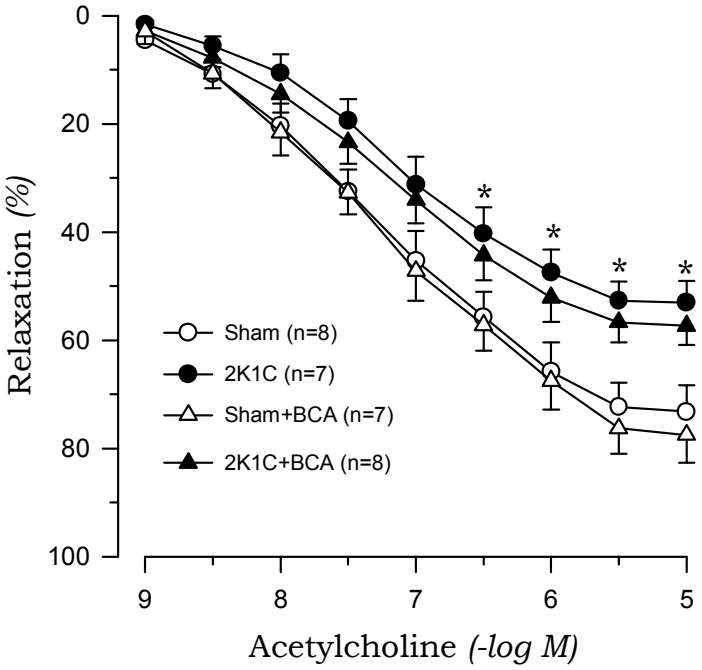

Fig. 6. Effects of biochanin A (BCA) on vasodilatory responses induced by acetylcholine in aortic rings from 2K1C hypertensive and sham-clipped control rats. * P <0.05, compared with corresponding sham values.

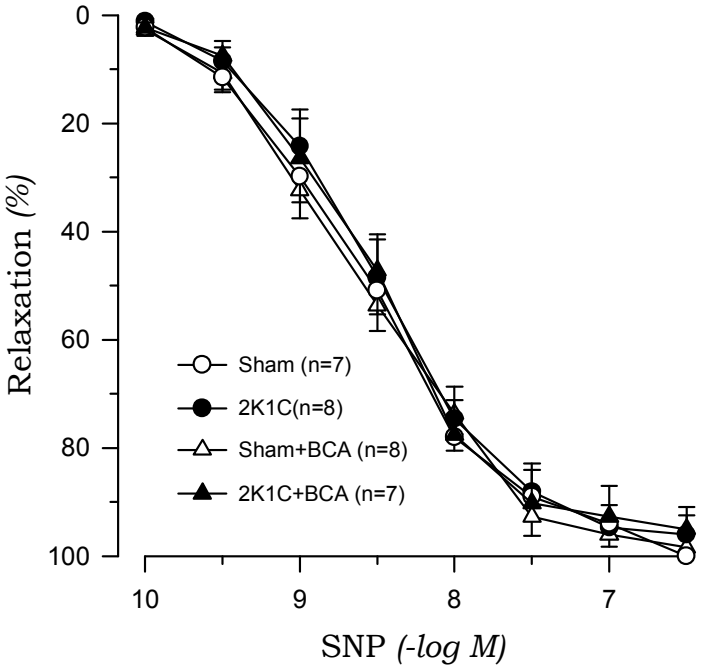

Fig. 7. Effects of biochanin A (BCA) on vasodilatory responses induced by sodium nitroprusside (SNP) in aortic rings from 2K1C hypertensive and sham-clipped control rats.

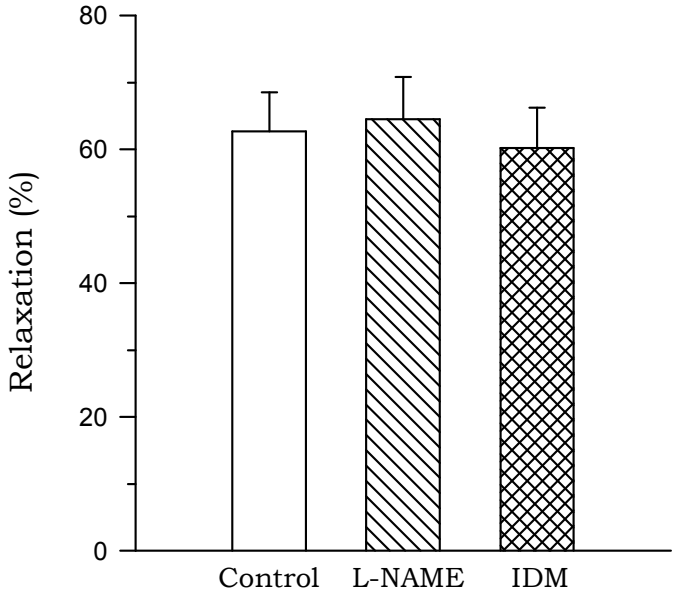

Fig. 8. Effects of N_w-nitro-L-arginine methyl ester (L-NAME) and indomethacin (IDM) on the relaxation induced by biochanin A in aortic rings from sham-clipped control rats. Data are attained from six to nine experiments.

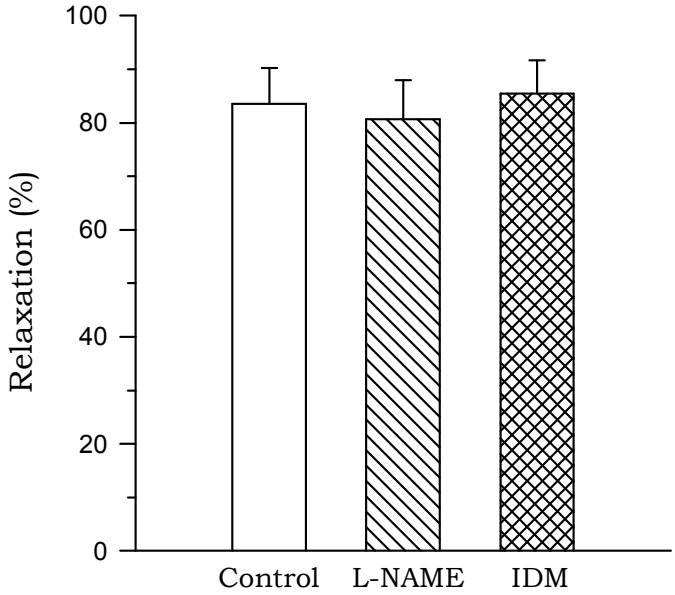

Fig. 9. Effects of N_w-nitro-L-arginine methyl ester (L-NAME) and indomethacin (IDM) on the relaxation induced by biochanin A in aortic rings from 2K1C hypertensive rats. Other legends as in Fig. 8.

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

Pretreatment with glibenclamide, an inhibitor of 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 aortic rings from both sham and 2K1C rats (Fig. 10, Fig. 11). In contrast, 4-aminopyridine, an inhibitor of voltage-dependent K^+ channels, inhibited biochanin A-induced relaxation only in aortic rings from 2K1C rats and had no effect on the relaxation of aortic rings from sham rats (Fig. 12).

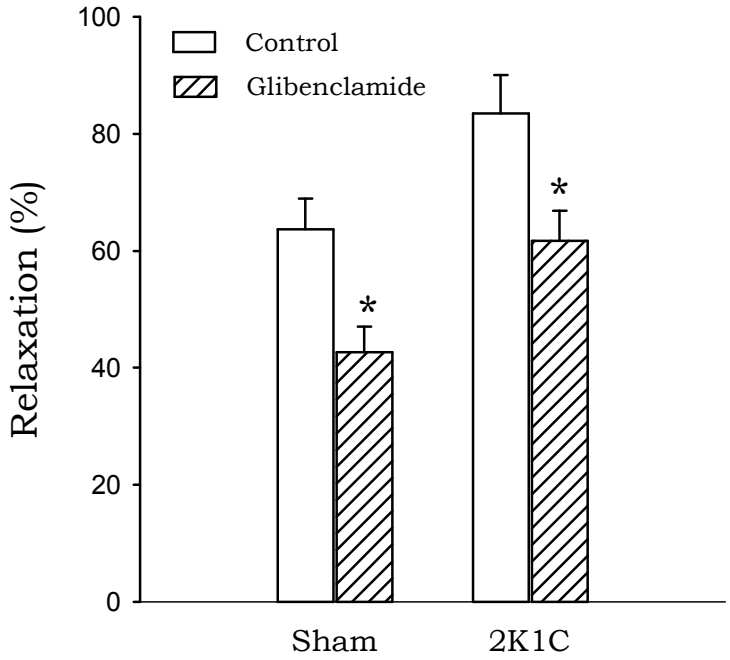

Fig. 10. Effects of glibenclamide on the relaxation induced by biochanin A in aortic rings from 2K1C hypertensive and sham-clipped control rats. Data are attained from six to nine experiments. * P <0.05, compared with corresponding the control value.

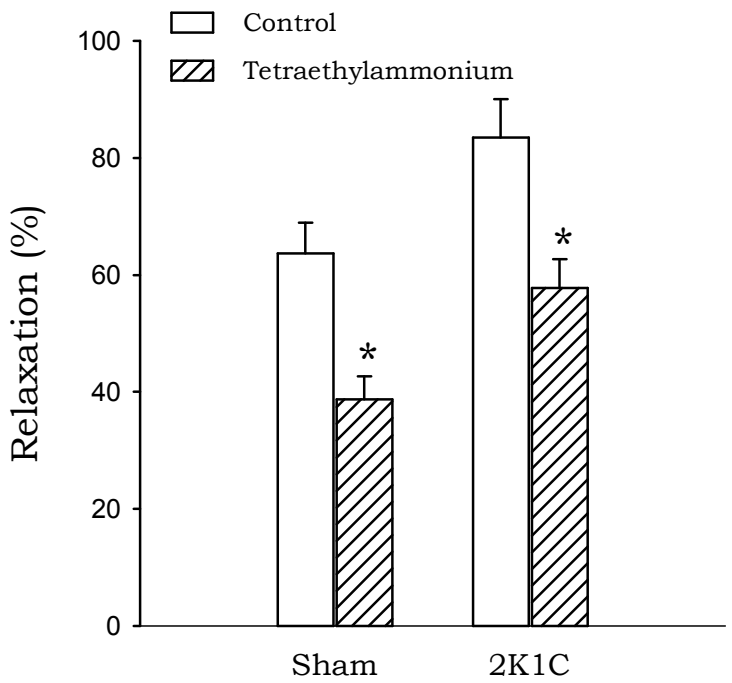

Fig. 11. Effects of tetraethylammonium on the relaxation induced by biochanin A in aortic rings from 2K1C hypertensive and sham-clipped control rats. Data are attained from six to nine experiments. Other legends as in Fig. 10.

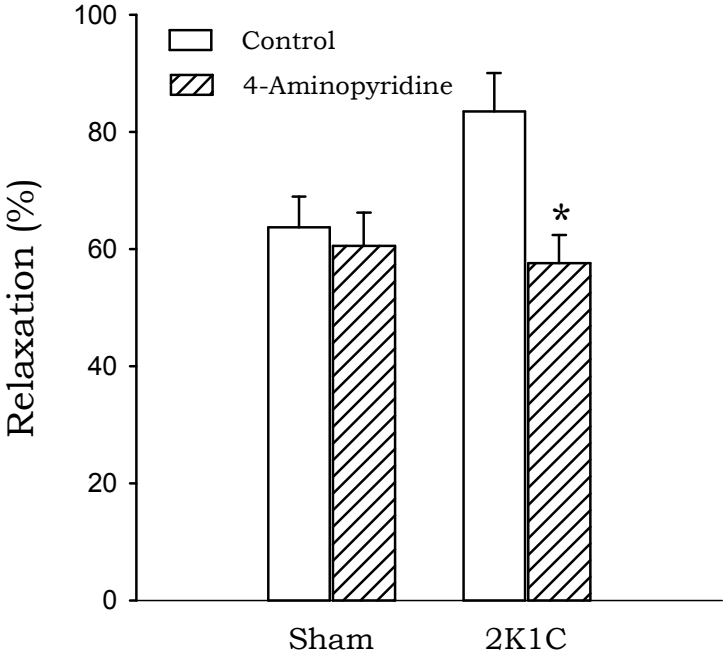

Fig. 12. Effects of 4-aminopyridine on the relaxation induced by biochanin A in aortic rings from 2K1C hypertensive and sham-clipped control rats. Data are attained from six to nine experiments. Other legends as in Fig. 10.

Ⅳ. DISCUSSION

Previous study have demonstrated that vascular reactivity to contractile agonist is enhanced in disease states such as hypertension[16]. As has been shown previously, the contractile response to phenylephrine was augmented in 2K1C hypertensive rats as compared to sham-clipped normotensive rats. In the present study, biochanin A induced a dose-dependent relaxation in phenylephrine-precontracted aortic ring preparations with an intact endothelium isolated from 2K1C and sham rats and the relaxation was augmented in hypertensive rats. In addition, the relaxant effect of biochanin A was comparable between in aortic rings with or without endothelium from sham rats. The results indicate that biochanin A-induced relaxation is endothelium-independent and biochanin A may directly affect vascular smooth muscle in normotensive rats. Similar results have been attained in rabbit coronary[11] and 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 endothelium removal in aortic rings from 2K1C hypertensive rats. These observations imply that the vasorelaxing effect of biochanin A on phenylephrine-induced contractions is partially endothelium dependent in hypertensive aortae differs from its endothelium-independent in normotensive rats.

Acetylcholine causes NO release through activation of specific endothelial receptors, resulting in activation of endothelial NOS[17]. The present study confirmed earlier observations[15,18], that endothelium-dependent relaxations to acetylcholine are markedly depressed in 2K1C hypertensive rats as compared with sham-clipped control rats. Treatment with L-NAME completely inhibited the acetylcholine-induced vasodilatory effect in both groups, suggesting that the acetylcholine-induced vasodilation is largely due to NOS-derived NO. Although the relaxation induced by acetylcholine was attenuated in hypertensive rats, biochanin A did not affect the acetylcholine-induced endothelium-dependent vasorelaxations in both 2K1C and sham rats. In addition, in the experiment using an NO donor in aortic rings from 2K1C and sham rats, biochanin A had no effect on SNP-induced endothelium-independent vasodilation. These results indicate that the functions of NO synthesis in the vascular endothelium and NO-mediated relaxation in the smooth muscle in aortic ring preparations are not altered by biochanin A in both normotensive and hypertensive rats.

Although biochanin A did not affect the acetylcholine-induced vasorelaxations in both 2K1C hypertensive and sham rats in this experiments, in order to clarify whether the vasodilator NO is involved in biochanin A-induced relaxation, effects of L-NAME on the relaxation induced by biochanin A were examined. L-NAME failed to affect the relaxation induced by biochanin A in aortic rings from 2K1C and sham rats. The results again

imply that the role of NO is not involved in biochanin A-induced relaxation in both groups. The results of the present study are in agreement with previous data from the coronary artery[11] and aorta[12], which suggest that vasodilator NO is not candidate contributors to biochanin A-induced relaxation. Conversely, Torregrosa et al[10]. reported that L-NAME may partly reduce the inhibitory effect of biochanin A on phenylephrine-induced contraction, which indicate that the relaxant effect of biochanin A in aortae with endothelium from rabbits can be due to the release of NO. One possible explanation may be attributed to differences in experimental conditions, species or strains[12]. In addition, endothelial cells play a role in the control of vascular homeostasis by releasing prostacyclin and endothelium-derived hyperpolarizing factor (EDHF), as well as NO[19]. In the present study, as has been shown previously[11,12], indomethacin, which inhibits the synthesis of prostaglandins[20], had little effect on the biochanin A-induced relaxation in aortic rings from 2K1C and sham rats. The results indicate that the release of prostanoids is not involved in biochanin A-induced relaxation in both groups. Taken together, denudation of the endothelium reduced the relaxant effect of biochanin A in aortic rings from 2K1C hypertensive rats in this experiment, whereas L-NAME and indomethacin did not affect the relaxation, suggesting the involvement of an EDHF, which is acting through K^+ channels and associated with hyperpolarization of vascular smooth muscle cells[21]. Furthermore,

previous studies have reported that EDHF-mediated vasorelaxation preserved in animal model of renovascular hypertension[22,23].

It has been known that endothelium-derived substances, such as EDHF, are able to pass through internal elastic lamina, reach underlying vascular smooth muscle at a concentration sufficient to activate ion channels, and initiate smooth muscle hyperpolarization and relaxation[24]. In endothelium and smooth muscle, K^+ channels are considered crucial effector proteins in the control of vascular tone and arterial blood pressure[25]. Direct activation of K^+ channels in arterial smooth muscle cells hyperpolarizes the membrane and thus inhibits Ca^{2+} influx through voltage-gated Ca^{2+} channels. Therefore, K^+ channels provide an important negative feedback on smooth muscle Ca^{2+} signaling and arterial tone by promoting relaxation[26]. Vascular smooth muscle contains several types of K^+ channel which can be modulated by various factors[27]. In the present study, to test the hypothesis that K^+ channels contribute to biochanin A-induced vasodilation in vascular smooth muscle, endothelium-intact rings were pretreated with K^+ channel blockers glibenclamide, TEA and 4-aminopyridine. Biochanin A-induced relaxation was significantly reduced by treatment with glibenclamide, a blocker of ATP-sensitive K^+ channels, in aortic rings from both 2K1C hypertensive and sham rats. In addition, TEA, which blocks large-conductance Ca^{2+}-activated K^+ channels when used at appropriate concentrations[12], also

inhibited the relaxant effect of biochanin A in both groups. These results imply that an 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, while no significant differences were shown in sham rats. Therefore, an augmented relaxation induced by biochanin A in rings from 2K1C rats than sham rats in this study, may be due, in part, to the release of endothelium-derived substances that may open voltage-dependent K^+ channels and evoke an hyperpolarization of the smooth muscle cells. Similar results were also reported in genetically hypertensive rats, which suggest that an endothelium-derived activation of smooth muscle cell K^+ channels contributes to the vasorelaxation in hypertension[12].

In summary, biochanin A causes both endothelium-dependent and -independent relaxation in aortae from 2K1C hypertensive rats, while only endothelium-independent relaxation in sham-clipped normotensive rats. The relaxant effect of biochanin A is augmented in 2K1C rats compared with sham 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, 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 cells.

V. SUMMARY

The plant-derived estrogen biochanin A is known to cause vasodilation, but its mechanisms of action in hypertension remain unclear. This study was undertaken to investigate the effects and mechanisms of biochanin A on thoracic aorta in two-kidney, one clip (2K1C) renovascular hypertensive rats. 2K1C hypertension was made by clipping the left renal artery and age-matched rats received a sham treatment served as control. Thoracic aortae were mounted in tissue baths for measurement of isometric tension. Biochanin A caused a concentration-dependent relaxation in aortic rings from 2K1C hypertensive and sham rats and the relaxation was augmented in 2K1C rats compared with sham rats. Biochanin A-induced relaxation was significantly attenuated by removal of endothelium in aortic rings from 2K1C rats, but not sham rats. N_w-nitro-L-arginine methyl ester, a nitric oxide synthase inhibitor, or indomethacin, a cyclooxygenase inhibitor, did not affect the relaxation induced by biochanin A in aortic rings from 2K1C and sham rat. Treatment with glibenclamide, a selective inhibitor of ATP-sensitive K^+ channels, or tetraethylammonium, an inhibitor of Ca^{2+}-activated K^+ channels, significantly reduced the 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, while no significant differences were shown in sham rats. 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.

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