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2013年 2月  
博士學位申請論文

Effects of Oxidative Stress on Endothelial  
Modulation of Contractions in Aorta  
from Renal Hypertensive Rats

朝鮮大學校 大學院  
醫學科  
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Modulation of Contractions in Aorta  
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신성 고혈압쥐에서 내피의존 혈관수축반응에  
미치는 산화적 스트레스 영향

2013 年 2 月 日

朝鮮大學校 大學院

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Endothelium-Dependent Vasodilation by  
Ferulic Acid in Aorta from Chronic Renal  
Hypertensive Rats

指導教授 金 相 勳

이 論文을 醫學博士學位 申請論文으로 提出함

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신 혜 랑

신 혜 랑의 博士學位論文을 認准함

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## 국문초록

### 신성 고혈압쥐에서 내피의존 혈관수축반응에 미치는 산화적 스트레스 영향

#### 신혜량

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혈관내피세포는 평활근층 긴장을 조절함으로써 혈압조절에 관여하며 고혈압상태에서는 혈관내피세포 기능 변화가 일어남이 알려져 있다. 저자는 신혈관성 고혈압상태에서 산화적 스트레스가 내피의존 혈관수축반응에 미치는 영향이 차이가 있는지 밝히고자 본 연구를 시행하였다.

체중 160~180 g 흰쥐 (Sprague-Dawley, 수컷)의 일측 신동맥에 직경 0.2 mm 의 clip을 장치하고 10 주 동안 사육하여 2-kidney, 1 clip (2K1C) 고혈압을 유발시켰다. 대조군은 개복한 후 바로 봉합하였다. 실험당일 흉부 대동맥을 채취하여 수조에 현수하고 그 등장성 장력변화를 생리기록기에 기록하였다.

적출 흉부 대동맥 표본은 norepinephrine에 의해 용량의존 수축반응을 보였으며 그 반응은 고혈압군에서 항진되었다. Norepinephrine에 의한 수축반응은 혈관 내피층을 제거하거나 N<sup>ω</sup>-nitro-L-arginine methyl ester 처치시 증가되었으며 그 크기는 고혈압군에서 대조군에 비해 약화되었다. Vitamin C 는 norepinephrine의 수축반응을 고혈압군에서 억제시켰으나 대조군

은 영향받지 않았으며 diphenyleiodonium 이나 apocynin 전처치 시에도 유사한 양상을 보였다. Hypoxanthine과 Xanthine oxidase 병용처치시 대조군은 norepinephrine의 수축반응이 항진되었으나 고혈압군은 영향이 없었으며 수축반응은 Vitamin C에 의해 양군 모두 억제되었다.

이상의 실험결과로 보아 혈관내피세포는 nitric oxide를 유리시킴으로써 수축반응을 조절하며 2K1C 신성고혈압상태에서 혈관내피층 기능의 변이는 부분적으로 NADH/NADPH oxidase에 의한 superoxide anion 생성의 증가와 관련될 것으로 사료된다.

## I . INTRODUCTION

Initially regarded as an inert between blood and blood vessels, the vascular endothelium plays a pivotal role in maintaining the vascular tone by generating various vasoactive substances<sup>1)</sup>. Under basal conditions, the endothelium releases relaxing and contracting factors, of which balance contributing to the local regulation of vascular tone<sup>2)</sup>. Hypertension is associated with altered endothelial function in large conduit and small resistance arteries<sup>3-5)</sup>, which is characterized by impaired endothelium-dependent relaxation in response to vasodilators such as acetylcholine. The endothelium-dependent vasodilation is indeed impaired in a number of experimental models, including two-kidney, one clip (2K1C) renal hypertension<sup>6-8)</sup>. A decreased production of endothelial relaxing factors or an increased production of endothelial contractile substances may be responsible for the depressed endothelium-dependent relaxations in hypertension<sup>9)</sup>.

Besides the direct relaxing or contracting effects of the endothelium, it also can modulate the effects of vasoconstrictor substances. It is widely recognized that the vascular endothelium plays an important role in the response of isolated vascular segments to several vasoconstrictors, including norepinephrine<sup>7, 10)</sup>. The constrictors apparently interact with the endothelium to cause the release of relaxing factors, which then have an inhibitory effect on vascular smooth muscle tone<sup>7, 11)</sup>. We have reported previously

that the endothelium negatively modulates contractions of rat aorta by releasing nitric oxide (NO)<sup>12)</sup>. The inhibitory effect of the endothelium against contractions induced by norepinephrine was markedly deteriorated in 2K1C hypertensive rats compared to sham-clipped control rats<sup>12)</sup>. However, the mechanisms that underlie the impaired endothelial modulation have not been extensively elucidated.

It is well known that the development of endothelial dysfunction is linked to an exaggerated production of superoxide anion. The evidence has accumulated that increased oxidative inactivation of NO by an excess of superoxide anions may account for the decrease in available NO and endothelial dysfunction observed in hypertension<sup>13, 14)</sup>. Miyagawa et al<sup>15)</sup> have shown that vascular oxidative stress may contribute to the altered circulation by impairing endothelial modulation of vascular contractions in spontaneously hypertensive rats. In addition, an increase in oxidative stress systemically plays a major role in maintaining high arterial blood pressure and sympathetic drive in renal hypertension<sup>16)</sup>, and we also observed that hydrogen peroxide may partly contribute to an altered vascular relaxation in 2K1C hypertensive rats<sup>17)</sup>.

Therefore the purpose of present study was designed to examine the possible role of oxidative stress in the impaired endothelium dependent modulation of vascular contractions in 2K1C renal hypertensive and sham-clipped normotensive rats isolated aortic ring preparations.

## II. METHODS

### 1. Induction of 2K1C renal hypertension

Male Sprague-Dawley rats (160~180 g) were anesthetized with sodium thiopental (40 mg/kg, IP). The left posterior side of the animal was shaved and sterilized with 70 % ethanol. An incision about 2 cm long was made through the skin and muscles just below the ribs. The left kidney was then exposed and retracted to expose the renal artery. A silver clip with an internal diameter of 0.2 mm was applied on the exposed renal artery. The clip was then turned so that the slit opening faces the abdomen. The contralateral kidney was left intact. The muscles and skin were sutured and the rats were allowed to recover from anaesthesia. Control rats received a sham treatment: they were operated as in 2K1C rats except for that no clip was made. All rats were maintained on standard chow with free access to drinking water. They were used at 10 weeks after clipping, since the endothelial dysfunction is associated with a duration of hypertension<sup>18)</sup>. Hypertensive rats were selected on the basis of the systolic blood pressure measured obtained in conscious rats by use of the tail-cuff method with a piezoelectric pneumotaxic pulse transducer.

## 2. Tissue preparation

The thoracic aorta between the aortic arch and diaphragm was rapidly removed and placed into a physiological salt solution (PSS) of the following composition (mM): NaCl 118.3, KCl 4.7, NaHCO<sub>3</sub> 25, MgCl<sub>2</sub> 1.2, KH<sub>2</sub>PO<sub>4</sub> 1.2, CaCl<sub>2</sub> 2.5 and glucose 11.1. Vessels were cleaned of adhering tissue and cut into rings (2 mm in width) under a dissection microscope. Care was taken not to stretch the artery or damage the luminal surface. In some preparations, the endothelium was removed by gentle rubbing of 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.

The rings were suspended by means of two triangle-shaped stainless steel holders in the vessel lumen in organ chambers containing 15 ml of PSS maintained at 37 °C, aerated with a mixture of 95 % O<sub>2</sub> and 5 % CO<sub>2</sub>. 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, aortae were equilibrated under a resting tension of 2 g for at least 90 min. This tension was found to be optimal for contraction of the preparation as assessed by repeated exposure to a 60 mM KCl solution (obtained by equimolar replacement of NaCl by KCl in the

## ISOLATED TISSUE BATH

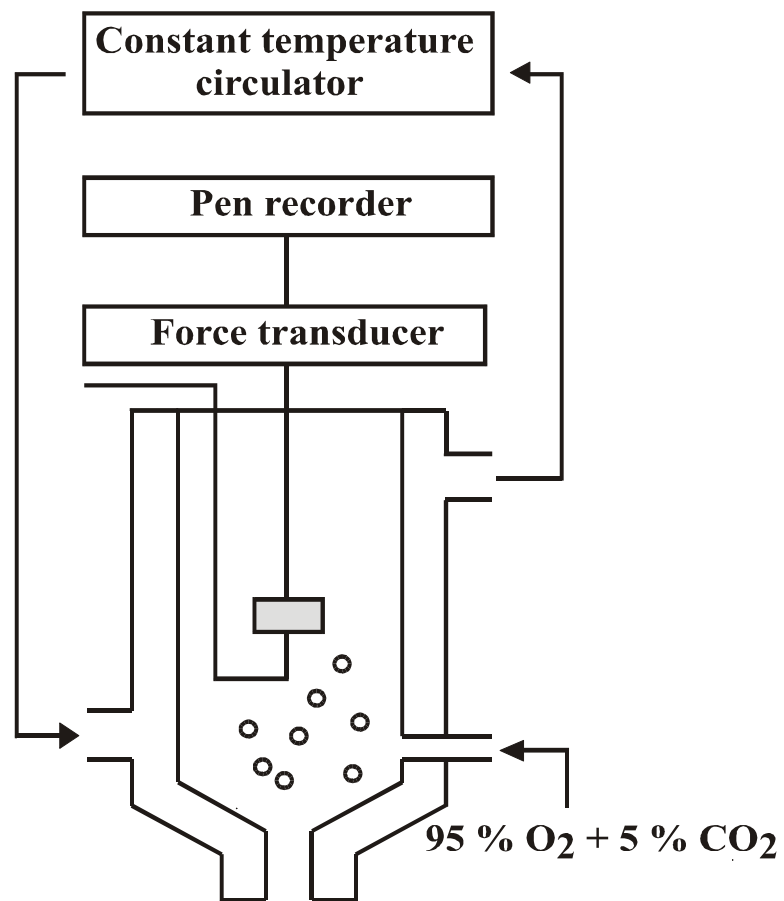


Fig. 1. A schematic representation of the recording system for isometric contraction with 15 mL tissue bath.



physiological solution) under various resting tensions. After the equilibration period, the rings were maximally contracted by the 60 mM KCl solution two times at 45 min intervals.

### 3. Protocol

Concentration-response curves to norepinephrine ( $10^{-10}$  to  $10^{-5}$  M) were obtained in rings with or without endothelium. To obtain  $\alpha$ -adrenoceptor mediated responses to norepinephrine, aortic rings were pretreated with  $\beta$ -adrenoceptor antagonist timolol ( $3 \times 10^{-7}$  M). The contribution of NO to the endothelium-dependent modulation of vascular contractions was assessed by analyzing the effect of N $\omega$ -nitro-L-arginine methyl ester (L-NAME  $10^{-4}$  M), which inhibits the endogenous production of NO from L-arginine, on the contractile response to norepinephrine. To examine a possible role of oxidative stress and the involvement of nicotinamide adenine dinucleotide/nicotinamide adenine dinucleotide phosphate (NADH/NADPH) oxidase in the impaired modulation of contractions by the endothelium in 2K1C rats, we obtained concentration-response curves to norepinephrine of aortic rings with endothelium in the absence and presence of vitamin C ( $10^{-4}$  M) or inhibitors of NADH/NADPH oxidase, diphenyleneiodonium (DPI,  $10^{-5}$  M) or apocynin ( $3 \times 10^{-5}$  M)<sup>15</sup>. The potential role of other enzymatic sources of superoxide was assessed by

examining the effects of allopurinol ( $3 \times 10^{-4}$  M), a xanthine oxidase inhibitor, on concentration - response curves to norepinephrine. The effects of superoxide on the contractile response to norepinephrine were examined using the hypoxanthine - xanthine oxidase system, which generates superoxide anion and hydrogen peroxide<sup>19</sup>. Concentration - response curves for norepinephrine were constructed in the absence and presence of hypoxanthine ( $2 \times 10^{-4}$  M)/xanthine oxidase (20 U/L) plus catalase (1000 U/mL).

#### **4. Drugs**

Drugs used were acetylcholine chloride, apocynin (Calbiochem, California, USA), catalase, diphenyleiodonium, hypoxanthine, L-norepinephrine bitartrate, L-NAME, timolol maleate, vitamin C (L-ascorbic acid) and xanthine oxidase. They were purchased from Sigma Chemical Co (St. Louis, MO, USA) unless otherwise stated. Drug concentrations are expressed as the final molar concentration within the organ chamber and all drugs were administered in volumes not exceeding 0.5 % of the bath volume.

#### **5. Statistics**

Contractions were expressed as a percentage of the contraction developed by the 60 mM KCl. Data are presented as means and standard error of the mean for the number of aortic rings indicated in parentheses. Statistical comparisons were performed by means of unpaired t test or analysis of variance with repeated measurements and Fischer post-hoc test. Differences were considered to be statistically significant when P value was less than 0.05.

### III. RESULTS

Ten weeks after the operative intervention, the systolic blood pressure were  $136 \pm 3$  mmHg (n=38) and  $190 \pm 4$  mmHg (n=40) in sham-clipped control and 2K1C hypertensive rats, respectively ( $P < 0.05$ , Fig. 2). The magnitude of KCl (60 mM)-induced isometric tension development was comparable between the two groups ( $1.41 \pm 0.10$  g in control;  $1.49 \pm 0.13$  g in 2K1C).

Norepinephrine evoked a concentration-dependent contraction of aortic rings from sham-clipped and 2K1C rats. The contractile response to norepinephrine of aortic rings with endothelium was greater in 2K1C than in those from sham rats. Removal of the endothelium augmented the norepinephrine-induced contraction, and this effect was more pronounced for aortic rings from sham rats compared to 2K1C rats. Treatment with L-NAME mimicked the effects of endothelium removal on norepinephrine-induced contraction (Fig. 3, Fig. 4).

To investigate whether oxidative stress is involved in the impaired endothelial inhibition of norepinephrine-induced contraction in 2K1C hypertensive rats, we examined the effects of vitamin C on norepinephrine-induced contraction in aortic rings with endothelium. Vitamin C reduced the contractile response to norepinephrine in 2K1C rats but not sham rats (Fig. 5, Fig. 6).

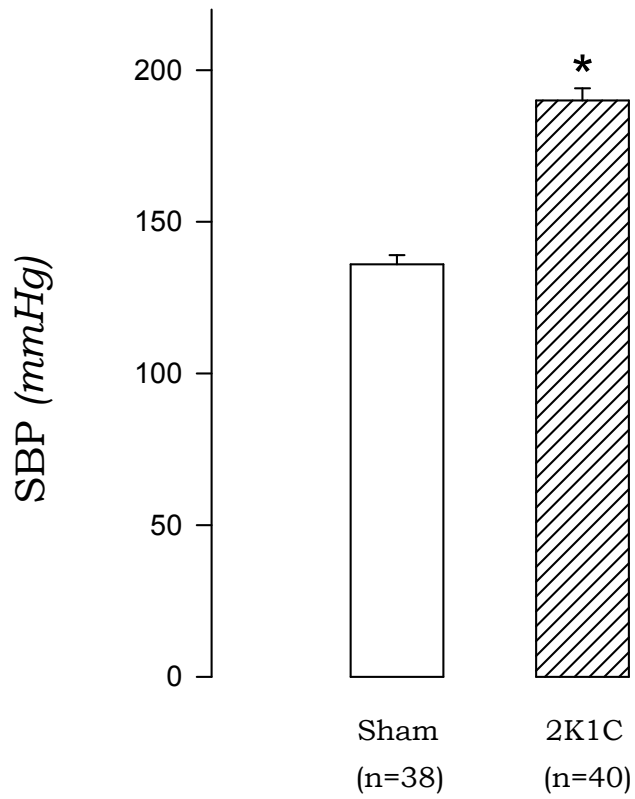


Fig. 2. Systolic blood pressure (SBP) in 2K1C hypertensive and sham-operated control rats. The values were obtained at 10 weeks after inducing the hypertension. \*  $P < 0.05$ , compared with the sham value.

### Sham

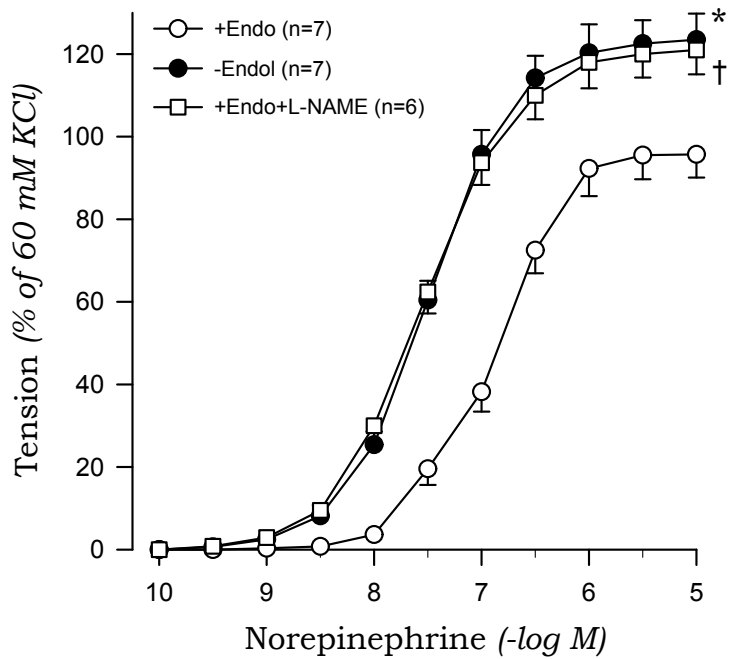


Fig. 3. Concentration-response curves to norepinephrine in aortic rings with (+) and without (-) endothelium (Endo) from sham rats. The results obtained in rings with endothelium in the presence of L-NAME ( $10^{-4}$  M) are also shown. Points represent means $\pm$ SE for number(n) of experiments in parentheses. \*, † P < 0.05, compared with the +Endo value, respectively.

## 2K1C

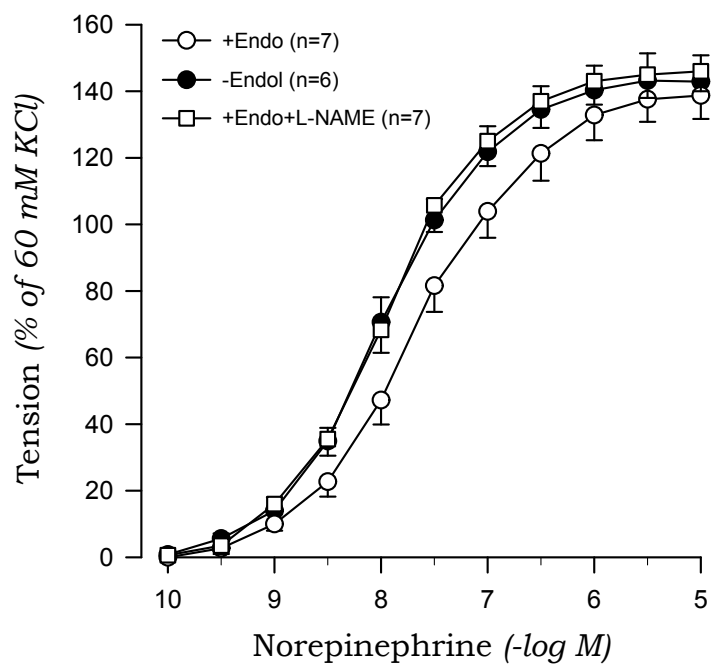


Fig. 4. Concentration-response curves to norepinephrine in aortic rings with (+) and without (-) endothelium (Endo) from 2K1C rats. The results obtained in rings with endothelium in the presence of L-NAME ( $10^{-4}$  M) are also shown.

Sham

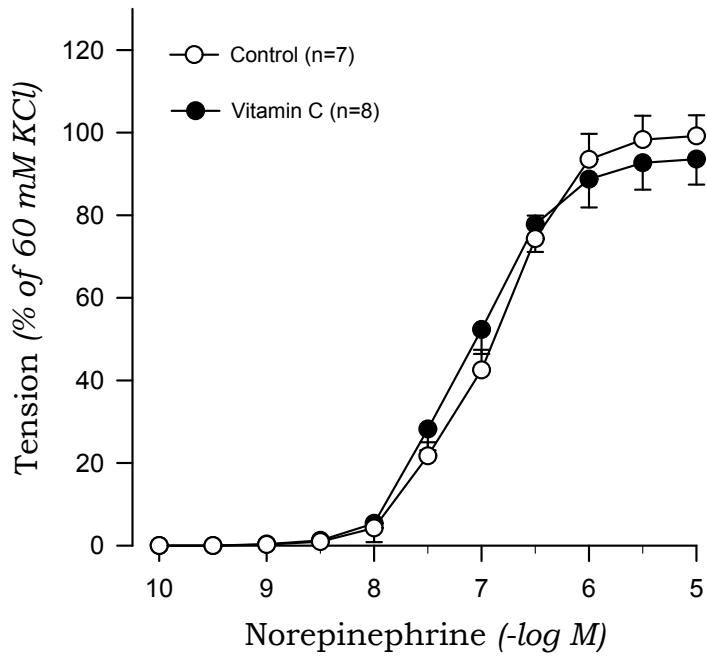


Fig. 5. Effects of Vitamin C on concentration-response curves to norepinephrine in aortic rings with endothelium from sham rats.



2K1C

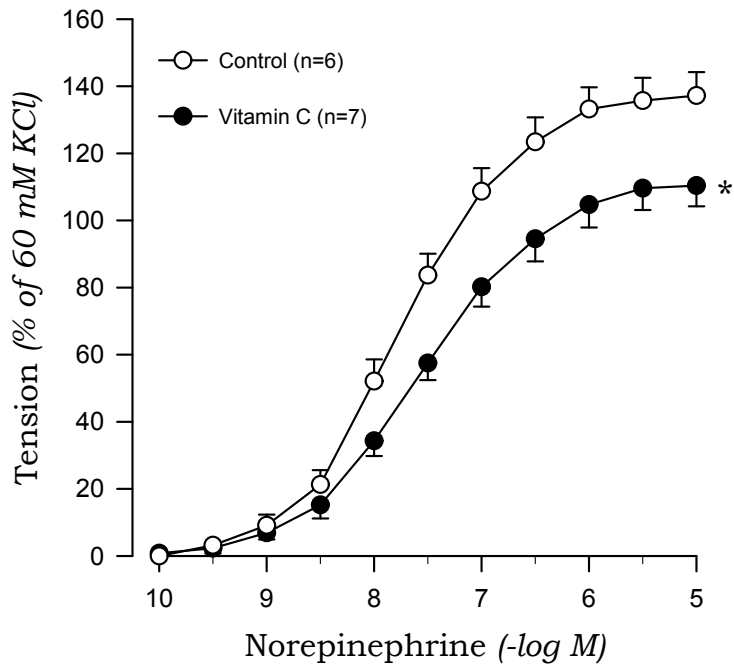


Fig. 6. Effects of Vitamin C on concentration-response curves to norepinephrine in aortic rings with endothelium from 2K1C rats. \* P<0.05, compared with the control value.

To investigate the origin of oxidative stress, we examined the effects of DPI and apocynin on norepinephrine-induced contraction in aortic rings from 2K1C and sham rats. DPI markedly suppressed the contractile response to norepinephrine of aortic rings with endothelium from 2K1C rats but not sham rats (Fig. 7, Fig. 8). Similarly, apocynin inhibited the norepinephrine-induced contraction in aortic rings with endothelium obtained from 2K1C rats but not sham rats (Fig. 9, Fig. 10).

A xanthine oxidase inhibitor, allopurinol affected the contraction to norepinephrine neither in 2K1C nor in sham rats (Fig. 11, Fig. 12).

In aortic rings with endothelium from sham rats, xanthine oxidase in the presence of hypoxanthine enhanced norepinephrine-induced contraction. This enhancement disappeared in the presence of vitamin C (Fig. 13). In contrast, the hypoxanthine - xanthine oxidase system did not augment the contractile response to norepinephrine in 2K1C rats (Fig. 14).

### Sham

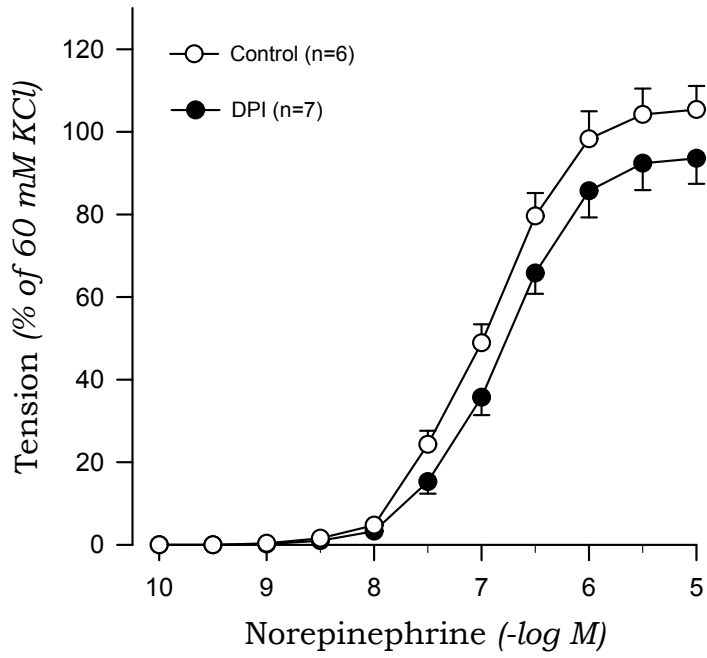


Fig. 7. Effects of diphenyleioidonium (DPI) on concentration-response curves to norepinephrine in aortic rings with endothelium from sham rats.

2K1C

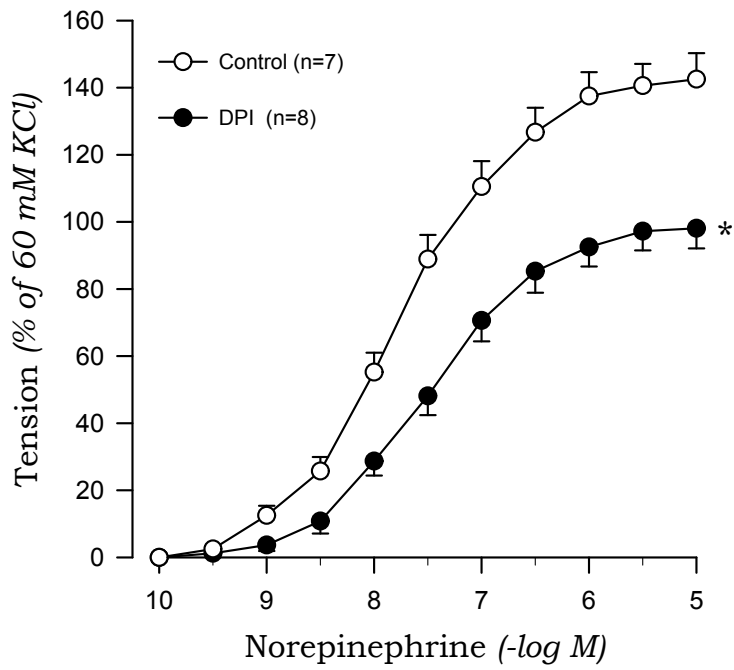


Fig. 8. Effects of diphenyleiiodonium (DPI) on concentration-response curves to norepinephrine in aortic rings with endothelium from 2K1C rats. \*  $P < 0.05$ , compared with the control value.

### Sham

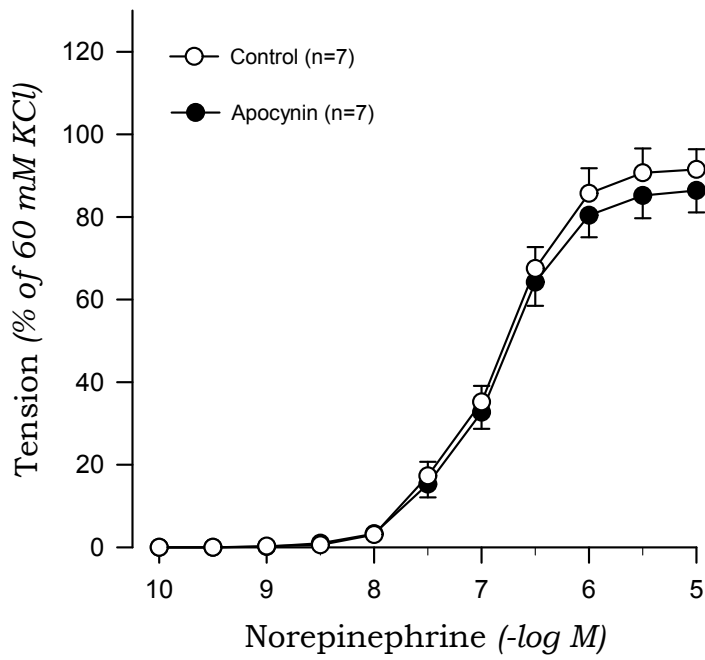


Fig. 9. Effects of apocynin on concentration-response curves to norepinephrine in aortic rings with endothelium from sham rats.

2K1C

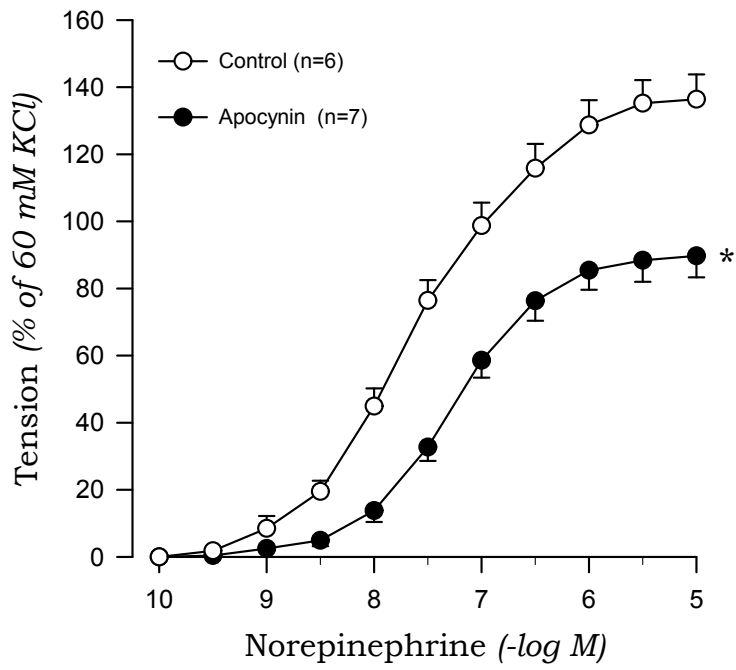


Fig. 10. Effects of apocynin on concentration-response curves to norepinephrine in aortic rings with endothelium from 2K1C rats. \*  $P < 0.05$ , compared with the control value.

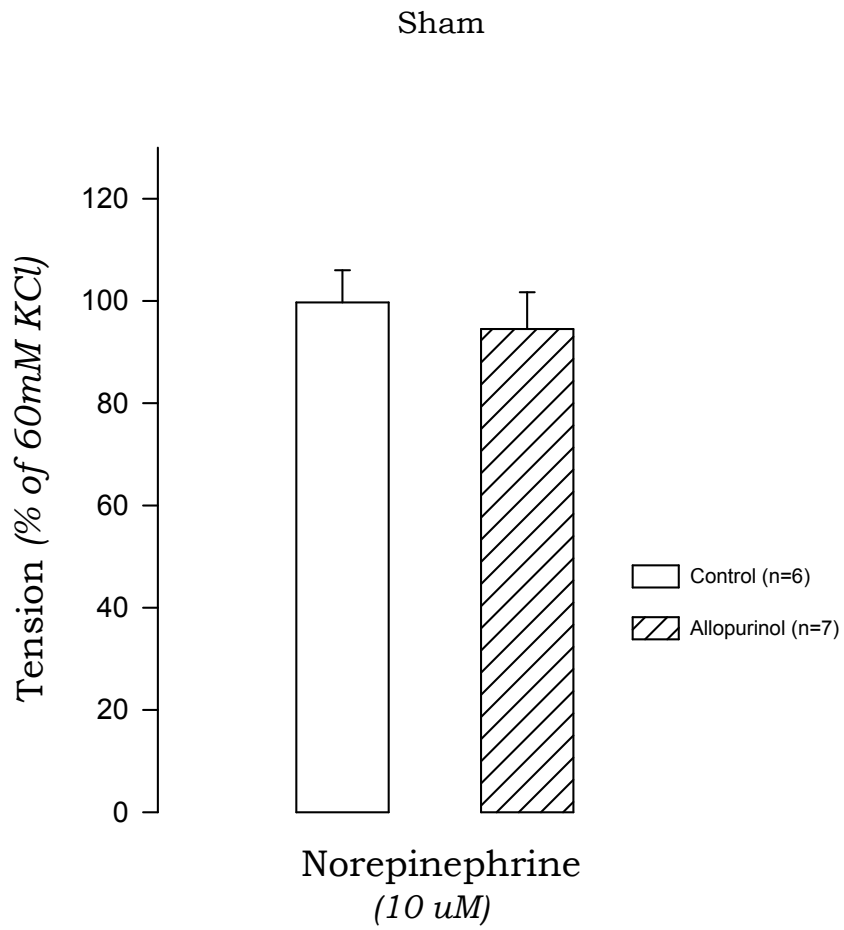


Fig. 11. Effects of allopurinol on the contractile response to norepinephrine in aortic rings with endothelium from sham rats.

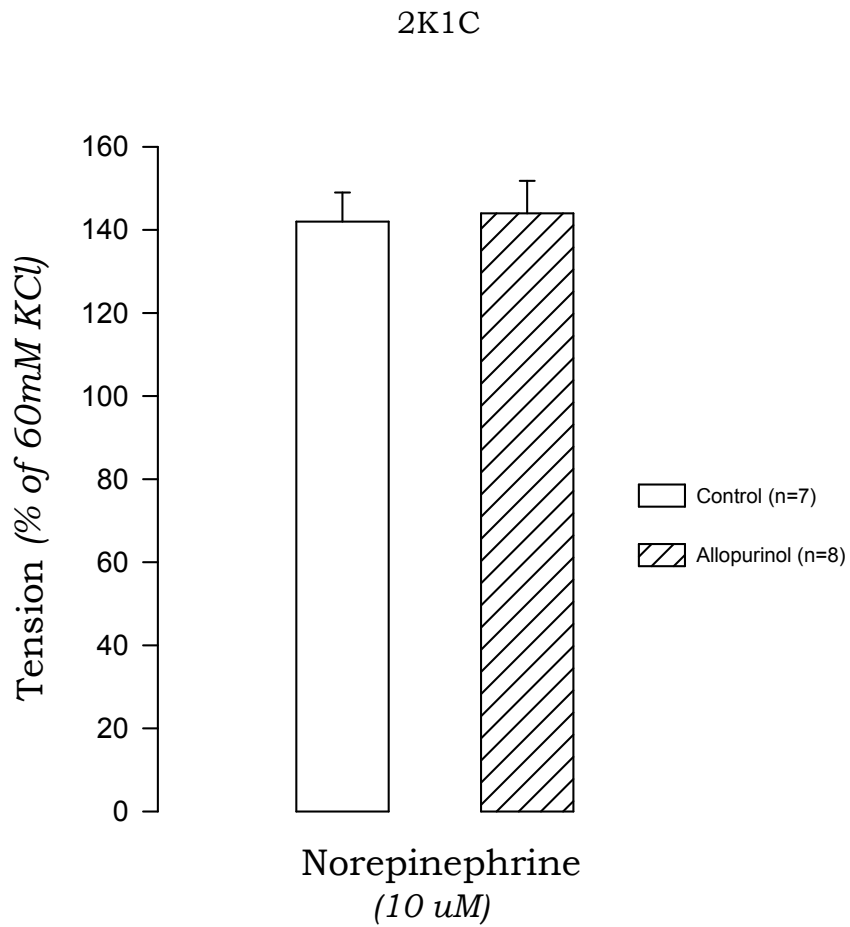


Fig. 12. Effects of allopurinol on the contractile response to norepinephrine in aortic rings with endothelium from 2K1C rats.



### Sham

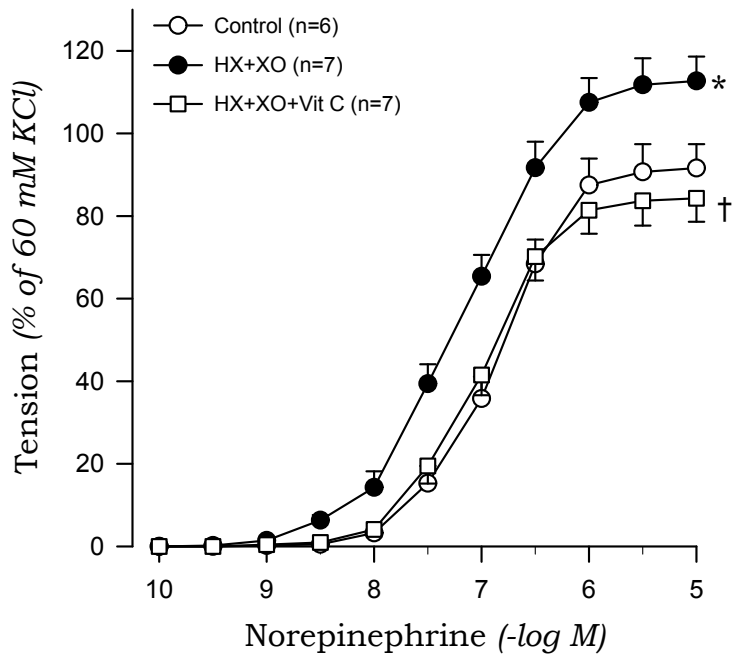


Fig. 13. Effects of hypoxanthine plus xanthine oxidase (HX+XO) and hypoxanthine plus xanthine oxidase plus vitamin C (HX+XO+Vit. C) in the presence of catalase on concentration-response curves to norepinephrine in aortic rings with endothelium from sham rats. \*  $P < 0.05$ , compared with the control value. †  $P < 0.05$ , compared with HX+XO values.

2K1C

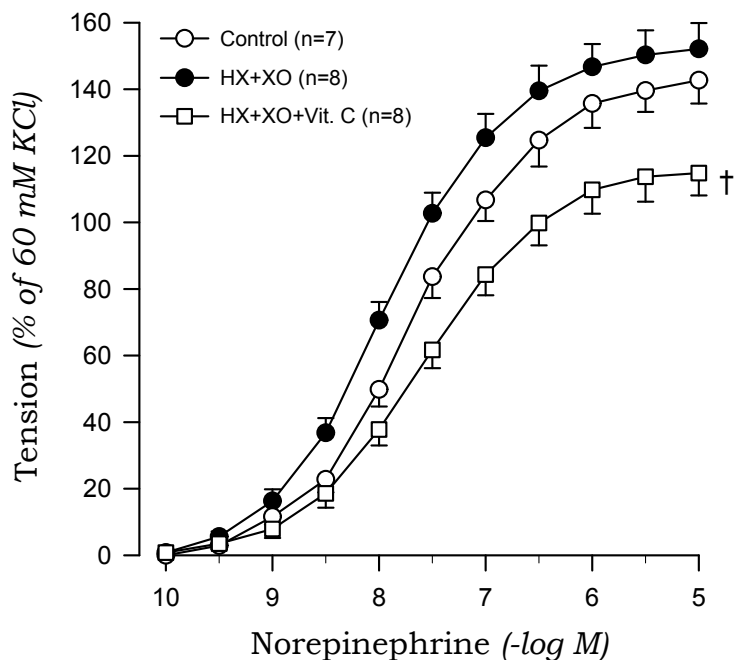


Fig. 14. Effects of hypoxanthine plus xanthine oxidase (HX+XO) and hypoxanthine plus xanthine oxidase plus vitamin C (HX+XO+Vit. C) in the presence of catalase on concentration-response curves to norepinephrine in aortic rings with endothelium from 2K1C rats. † P < 0.05, compared with HX+XO values.

#### IV. DISCUSSION

The vascular responsiveness induced by norepinephrine was enhanced in 2K1C hypertensive rats compared to sham-clipped control rats, as has been shown in our previous findings<sup>11,12</sup>). Augmented contractile responses in hypertension may be attributed to an augmented phosphoinositide hydrolysis, a greater release of intracellular  $Ca^{2+}$  from a cellular pool, an increased activation of protein kinase C, or alterations in number of affinity of inositol trisphosphate receptors on the endoplasmic reticulum of vascular smooth muscle<sup>20</sup>). Many of these processes may also be responsible for the increased sensitivity of vascular response to norepinephrine in 2K1C hypertension. Our observation that L-NAME mimicked the effects of endothelium removal on norepinephrine-induced contraction indicates that NO plays a pivotal role in the endothelial modulation of norepinephrine-induced contraction. The inhibitory role of the endothelium was markedly impaired in 2K1C rats, confirming our previous data<sup>12</sup>). This clearly demonstrates that 'endothelial dysfunction' indicates not only impaired relaxations but also exaggerated contractile responses in hypertension. The impaired endothelial modulation of vascular contractions induced by norepinephrine in 2K1C hypertensive rats could be due to a decrease in the production of NO, an increase in the inactivation of NO, and/or altered NO-cGMP signaling in the hypertensive

vascular tissue. However, an altered NO-cGMP signaling is not likely to be involved in the impaired endothelial modulation in 2K1C rats, because in our previous study<sup>12)</sup>, relaxations induced by sodium nitroprusside were not different for aortic rings obtained from 2K1C and sham rats. On the other hand, Miyagawa et al<sup>15)</sup> have demonstrated that NO levels are not altered after contractions induced by norepinephrine, although the inhibitory role of the endothelium is impaired in hypertensive rats compared to normotensive rats. In addition, we have also found that plasma concentrations of NO is comparable between 2K1C and sham rats<sup>21)</sup>. These observations imply that the production of NO is preserved in hypertensive endothelium, and it may be speculated that the inactivation of NO is more pronounced in aortic rings from 2K1C hypertensive rats. In line with this view, contractile responses to norepinephrine in aortic rings with endothelium obtained from 2K1C rats were significantly depressed after the treatment with vitamin C in this study. Vitamin C is an antioxidant that improves impaired endothelium-dependent relaxation induced by acetylcholine in hypertension; this effect is attributed to the scavenging of free radicals by vitamin C<sup>22)</sup>. Therefore, production of reactive oxygen species is likely involved in the impaired endothelial modulation of arterial contractions in 2K1C hypertensive rats, although vitamin C may have increased endothelial NO synthase (eNOS) activity or reduced the production of superoxide by inhibiting NADH/NADPH oxidase<sup>23)</sup>. An enhanced production of

super oxide anion may result in inactivation of NO and generation of peroxynitrite<sup>16)</sup>. The resulting decrease in NO availability might be involved in the impaired endothelial modulation of arterial contractions.

Exaggerated production of superoxide anions by the vascular wall has been observed in different animal models of hypertension, including 2K1C rats<sup>15,16,24)</sup>. There are several possible sources of superoxide anion, including NADH/NADPH oxidase, eNOS, and xanthine oxidase. However, NADH/NADPH oxidase accounts for most of the superoxide generated within the vessel wall<sup>15)</sup> and was reported to be involved in superoxide production in the arteries obtained from 2K1C hypertensive rats<sup>25)</sup>. In the present study, the NADH/NADPH oxidase inhibitor DPI reduced norepinephrine-induced contraction in aortic rings with endothelium obtained from 2K1C rats, but had no such effect on rings from sham rats. The results suggest that hypertensive vascular tissue produces superoxide anions via a NADH/NADPH oxidase-dependent mechanism during the contractions induced by norepinephrine. Although DPI may also inhibit eNOS, NADH/NADPH oxidase is the most likely candidate for the source of excessive superoxide production in the present study because the other specific inhibitor of NADH/NADPH oxidase, apocynin, also reversed the impaired endothelial modulation in 2K1C rats. On the other hand, allopurinol, a xanthine oxidase inhibitor, did not affect the norepinephrine-induced contraction either in sham or in 2K1C

rats, as has been shown previously in genetically hypertensive rats<sup>15</sup>). Therefore, it is unlikely that xanthine oxidase is involved for the source of superoxide production under the experimental conditions in this study.

Renovascular hypertension caused by renal artery stenosis leads to the stimulation of the renin-angiotensin system, thereby elevating circulating angiotensin II (Ang II) levels. However, despite the fact that the 2K1C model has been extensively investigated, the exact mechanisms responsible for the maintenance of this type of hypertension remain unclear. One hypothesis is that an increase in Ang II can activate the NADH/NADPH oxidase that will produce superoxides, modifying the bioavailability of NO and increasing vasoconstriction, in addition to generating an increase in the sympathetic activity<sup>16</sup>). In association of this notion, Ang II has been shown to stimulate the generation of superoxide anion radical in cultured vascular smooth muscle cells<sup>26</sup>) and in intact aortae of rats made hypertensive by chronic Ang II infusion<sup>27</sup>). Taken together, it can not be ruled out the possibility that Ang II may affect on the production of superoxide anion in 2K1C hypertensive rats, although we did not confirm the concentration of Ang II in this study.

To further verify that superoxide is responsible for the impaired endothelial modulation of arterial contractions in 2K1C rats, we conducted experiments using a superoxide generation system. Oxidant stress conditions can be created in isolated

aortic rings by the generation of superoxide anions via the hypoxanthine-xanthine oxidase system<sup>28)</sup>. However, these conditions are complicated by the fact that superoxide anion generating systems also given rise to other reactive oxygen species such as hydrogen peroxide, hydroxyl radical and peroxynitrite which can damage the endothelium and thereby impair endothelial modulation<sup>29)</sup>. We wished to examine specifically the interaction between NO and superoxide anion and, consequently, catalase was included<sup>15)</sup> in our experiment. Hypoxanthine-xanthine oxidase in the presence of catalase enhanced the norepinephrine-induced contraction of aortic rings with endothelium from sham-clipped control rats; this effect was reversed by the application of vitamin C, which imply that increased production of superoxide anions can impair the endothelial modulation of arterial contractions. Effects of hypoxanthine-xanthine oxidase was negligible in 2K1C rats, possibly because most of the NO released is inactivated by endogenous superoxide derived from NADH/NADPH oxidase and, thus, exogenous superoxide generated by hypoxanthine-xanthine oxidase system does not have additional inhibitory effects against NO.

In summary, modulation of vascular contractions is one of the most important regulatory roles of the endothelium in the circulation, and this feature is impaired in the aorta of 2K1C hypertensive rats. The impairment is related to increased production of superoxide anions by NADH/NADPH oxidase in

response to norepinephrine. Vascular oxidative stress may contribute to the altered circulation in renal hypertension by impairing the regulation of contraction by the endothelium.



## V. SUMMARY

The endothelium modulates vascular contractions. This study was conducted to examine the effects of oxidative stress on endothelial modulation of contractions in chronic two-kidney, one clip (2K1C) renal 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. The contractile response to norepinephrine of aortic rings with endothelium was greater in 2K1C rats than in sham rats. Endothelium removal augmented the norepinephrine-induced contraction and the augmentation was more pronounced in sham rats than in 2K1C rats. N $\omega$ -nitro-L-arginine methyl ester, which inhibits the production of nitric oxide (NO), mimicked the effect of endothelium removal. Vitamin C suppressed the contraction of aortae with endothelium from 2K1C rats but not from sham rats. Diphenyleneiodonium and apocynin, inhibitors of nicotinamide adenine dinucleotide/nicotinamide adenine dinucleotide phosphate (NADH/NADPH) oxidase, attenuated the contraction of aortae with endothelium from 2K1C rats but not sham rats. Superoxide generated by xanthine oxidase/hypoxanthine enhanced the norepinephrine-induced contraction of aortae with endothelium from sham rats, while it did not affect on 2K1C rats. Enhanced contractile responses to

norepinephrine by xanthine oxidase/hypoxanthine in sham rats were reversed by vitamin C. These results suggest that the endothelium modulates vascular contraction by releasing NO and the endothelial modulation is impaired in 2K1C renal hypertension. The impairment is, at least in part, related to increased production of superoxide anions by NADH/NADPH oxidase.

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