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Interaction between Renin-Angiotensin and Endothelium-Derived Nitric Oxide Systems in Two-Kidney, One Clip Hypertensive Rats

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=ABSTRACT=

The present study was aimed to investigate the role of endothelium-derived nitric oxide (NO) in the control of renin release and to examine if NO is implicated in the development of two-kidney, one clip (2K1C) hypertension. Male Sprague-Dawley rats (150~200 g) were constricted at the left renal artery. They were then supplemented with N^G-nitro-L-arginine methyl ester (L-NAME, 5 mg/100 mL) or with L-arginine hydrochloride (400 mg/100 mL) in the drinking water. The control group was supplied with normal tap water. The sham-clipped rats were operated as in 2K1C rats except for that no clip was made. The kidneys were taken to examine *in vitro* release of renin at days 7 and 14 following clipping the renal artery. Northern blot analysis was also done to assess the expression of renin gene in the kidney. In sham-clipped rats, L-NAME caused a sustained increase of the blood pressure, whereas L-arginine was without effect. Neither L-NAME nor L-arginine-supplementation significantly affected the development of hypertension in 2K1C rats. Plasma renin concentration (PRC) measured on day 28 did not significantly differ among the L-NAME, L-arginine and control groups either in 2K1C or in sham-clipped rats. Renin contents (RRC) in the clipped kidney were increased, while those in the contralateral kidney were decreased. The release of renin *in vitro* from cortical slices was also enhanced in the clipped kidney, whereas it was attenuated in the contralateral. Comparing the RRC and *in vitro* release, the latter was more rapidly decreased than the former in the contralateral kidney. The renin mRNA levels in the contralateral kidney were almost at their nadir at days 7 and 14 in 2K1C rats. It is suggested that NO does not affect the development of 2K1C hypertension in which the renin-angiotensin system has been activated. The data also confirm that RRC and renin gene expression are increased in the clipped kidney and suppressed in the contralateral kidney in 2K1C rats.

Key Words: Nitric oxide, Renin, 2-Kidney, 1 clip hypertension

INTRODUCTION

Over the last decade, the vascular endothelium has been found to produce factors which modulate the vascular tone through constricting or dilating the underlying smooth muscle layer. With increasing numbers of the endothelial factors involved in vascular regulatory mechanisms, interactions between these and other hormonal systems have been subjects of much interest.

There is compelling evidence that endothelium-

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derived nitric oxide (NO) and renin-angiotensin systems are interactive at various levels, impinging on blood pressure regulation. Among others, NO has been found to play a role in the control of renin secretion (Johnson & Freeman, 1992; Munter & Hackenthal, 1991; Persson et al, 1993; Vidal et al, 1988). In addition, angiotensins are known to stimulate the synthesis of NO in endothelial cells and coronary vessels (Seyedi et al, 1995; Wiemer et al, 1993). It has been also reported that treatment with inhibitors of NO synthesis augments the pressor and vasoconstrictor responsiveness to angiotensin II (Conrad & Whittemore, 1992; Ito et al, 1991), and that a mechanism of vasodilation mediated by NO is preferentially manifested in angiotensin-dependent hypertension (Pucci et al, 1995). These findings support the concept that NO and angiotensin II function as antagonistic regulators of blood pressure, with NO serving as a counter-regulatory influence on the vascular actions of angiotensin II.

Two-kidney, one clip (2K1C) model of renal hypertension has been established to be renin-dependent. Taking account of an interaction between NO and renin, it may be hypothesized that 2K1C hypertension is affected by the activity of NO system. The pathophysiological role of NO has not been established in 2K1C hypertension.

It has been extensively confirmed in humans with renovascular hypertension as well as in experimental animals with hypertension (Mann & Pickering, 1992) that the renin secretion is enhanced in the clipped kidney, and suppressed in the contralateral kidney. However, the mechanism underlying the suppression in the contralateral kidney still remains unsettled. One may hypothesize that the inhibited renin secretion in the contralateral kidney is related with an altered activity of NO system.

The present study was aimed to further investigate the role of NO in the control of renin release and to examine if NO is implicated in 2K1C hypertension. In the first series of experiments, the effects of inhibition or stimulation of NO system on the developmental phase of hypertension were examined in 2K1C rats. For comparison, effects of inhibited or stimulated NO system on the development of hypertension were also examined in DOCA-salt rats. In another series of experiments, the expression of genes for renin was determined in the clipped and contralateral kidneys of 2K1C rats. The renin response to a blockade of NO synthesis was also examined using *in vitro* preparations from the isolated renal cortex.

METHODS

Development of 2K1C and DOCA-salt hypertension

Male Sprague-Dawley rats (150~200 g) were constricted at the left renal artery with a silver clip having an internal gap of 0.25 mm under ketamine anesthesia. The contralateral kidney was left untouched. They were then divided into three groups. The first group was supplemented with N^G-nitro-L-arginine methyl ester (L-NAME, Sigma; 5 mg/100 mL), and the second with L-arginine hydrochloride (Choongwae, 400 mg/100 mL) in the drinking water. The third group was supplied with normal tap water to serve as control. The sham-clipped rats were operated as in 2K1C rats except for that no clip was made. They were also divided into L-NAME, L-arginine, and control groups. Three to five rats of the same group were housed together in a cage.

Daily ingested amounts of L-NAME and L-arginine were calculated as milligrams per 100 g body weight of the animals. Systolic blood pressure (SBP) was measured at days 7, 14, 21, and 28 after clipping the renal artery. Basal blood pressure was taken as an average of three-consecutive-day values before clipping the artery.

Additional three groups of rats were subcutaneously implanted with silastic DOCA (Sigma, 200 mg/kg) strip, one week after unilateral neph-

rectomy. They were then supplied with 0.9 % NaCl drinking solution for 4 weeks, while supplemented with L-NAME, L-arginine, or none as in 2K1C rats.

Isolated renal cortical slices

The kidney was taken following decapitation and divided into two hemispheres. The 0.4-mm thick cortical slices were made, parallel to the capsular surface with a Staddie-Riggs microtome. The initial slice was discarded, and the next two were taken from one hemisphere. The flask containing the two slices was placed in an oscillating incubation bath while the medium was maintained at 37°C with 95% O_2-5% CO_2. After 30 min equilibration, the medium was replaced with fresh one. The slices were then incubated for 1/2 to 1 hour, after which a 200-μL aliquot was taken. Some specimens were treated with L-NAME (10^{-4} mol/L) or sodium nitroprusside (10^{-5} mol/L) during the incubation. Samples taken were centrifuged and frozen until analyzed for renin concentration, and the slices were weighed to determine the results by milligrams of wet weight. One pair of slices from the other hemisphere was homogenized in 0.1% bovine serum albumin in distilled water. The homogenate was centrifuged at 4°C, and the supernatant was stored at −20°C until analyzed for renin. Renin concentration was determined by radioimmunoassay as described previously (Cho et al, 1987).

The composition of the physiological salt solution was as follows (in mmol/L): NaCl 125, $NaHCO_3$ 19, KCl 4, $CaCl_2$ 2.6, NaH_2PO_4 1.2, $MgSO_4$ 0.8, and glucose 0.2 g/100 mL. The drugs used were all purchased from Sigma Chemical Company (St. Louis, MO), except sodium nitroprusside (Roche; Basel, Switzerland).

RNA isolation from kidneys

The kidneys were rapidly removed following decapitation. They were homogenized in 9 mL of 4-mol/L guanidine hydrothiocyanate and 1% β-mercaptoethanol using a Kinametic Polytron blender (Kriens) and layered onto a 4-mL 5.7-mol/L CsCl cushion. Total RNAs were pelleted by ultracentrifugation at 33,000 rpm for 20 hours in a 50-Ti rotor.

Northern blot analysis

Total RNAs (10 μg) were denatured with glyoxal, separated by size on 1.2% agarose gels, and transferred to GeneScreen (DuPont, Wilmington, DE) as previously described (Kim et al, 1994). For the renin probe, cDNA was radiolabeled by nick-translation. Hybridizations were for 10 h at 65°C in 4×SSC, 2×Denhardt's, 0.1% SDS, and 1 mg/mL salmon sperm DNA (20×SSC is 3 mol/L NaCl and 0.3 mol/L sodium citrate, pH 7.0; 20× Denhardt's is 0.4% (w/v) polyvinyl-pyrolidone, 0.4% (w/v) serum albumin, and 0.4% (w/v) Ficoll). Blots were wahsed at 65°C in 2×SSC, 0.1% SDS, and the signal was visualized by autoradiography at −70°C with an intensifying screen. In all experiments, the integrity of the RNA samples was established by UV shadowing of the blot prior to hybridization. Quality of the RNA samples was further confirmed by Northern analysis with a murine 18S rRNA oligonucleotide probe (5'-TCCATTATTCCTAGTGCGGTATCCAGGAG GATC-GGGCCTGCTTT-3').

Statistics

Results are expressed as means±SEM. The statistical significance of differences between groups was assessed by one-way ANOVA followed by Bonferroni's test for multiple comparisons.

RESULTS

Effects of L-NAME and L-arginine on 2K1C hypertension

Fig. 1 shows the development of hypertension in 2K1C and sham-clipped rats. No rats died during the period of observation. SBP progressively increased

in 2K1C rats, which was not significantly affected by either L-NAME or L-arginine. In sham-clipped control rats, L-NAME significantly increased SBP, whereas L-arginine was without effect. Daily amounts of L-NAME and L-arginine ingestion did not differ between the 2K1C and sham-clipped control rats (Table 1). PRC measured on day 28 following clipping the renal artery did not significantly differ among the groups either in 2K1C or in sham-clipped control rats (Fig. 2).

DOCA-salt rats showed a progressive increase of the blood pressure (Data not shown). No rats died in the control group supplied with saline only. L-Arginine-supplementation did not affect the development of hypertension, in which one of 8 rats died during the third week of observation. In the L-NAME-supplemented group, one of the 7 rats died at 2 weeks, three died at 2 weeks, another died at 4 weeks into the study, and only 2 rats survived at the end of the study. Before the deaths, they showed much higher blood pressures than the control.

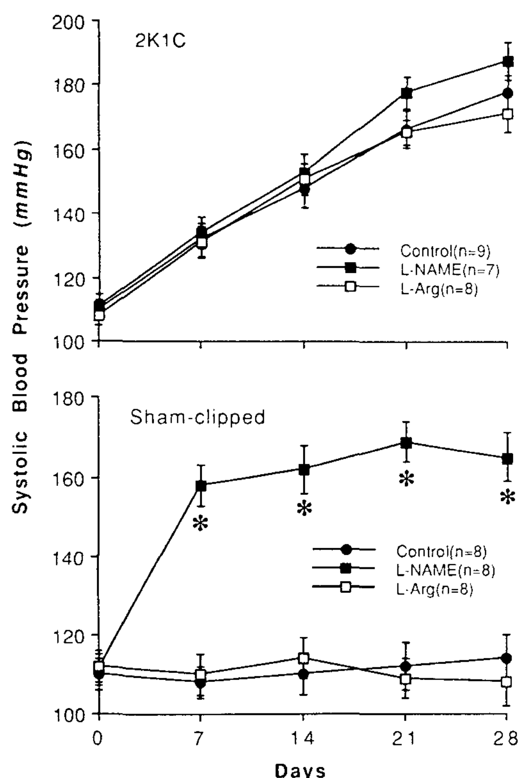

*Fig. 1. Effects of L-NAME and L-arginine ingestion on systolic blood pressure in 2K1C and sham-clipped rats. n=number of rats. * p<0.01, compared with control.*

Renal contents and release of renin *in vitro*

The renin contents in cortical slices (RRC) are presented in Fig. 3. RRC in the clipped kidney were increased, while those in the contralateral kidney were decreased. In the contralateral kidney, RRC were 66±18% of the clipped kidney at day 7, and 11±4% at day 14 after clipping the unilateral renal artery.

The release of renin *in vitro* from cortical slices was also enhanced in the clipped kidney, and attenuated in the contralateral kidney (Fig. 3). Comparing the RRC and the release, the latter was more rapidly decreased than the former. The renin release from the contralateral kidney was 28±5% of the clipped kidney at day 7, being more marked than the degree of decrease in RRC. At day 14 after clipping the renal artery, however, the release from the contralateral kidney was 10±4% of the clipped kidney, being comparable to RRC.

Effects of L-NAME on renin release *in vitro*

Fig. 4 shows the effects of acute treatment with L-NAME or sodium nitroprusside on renin release in the incubation media. L-NAME (10^{-4} mol/L) significantly increased basal release of renin, although sodium nitroprusside (10^{-5} mol/L) did not significantly affect the rate of the basal release.

Renal renin gene expression

Fig. 5 illustrates typical Northern blots of kidney RNA obtained from 2K1C and sham-clipped rats with a renin cDNA probe. The level of renin mRNA in the contralateral kidney was virtually abolished in 2K1C rats.

Table 1. Daily amounts of L-NAME and L-arginine ingestion in two-kidney, one clip (2K1C) and sham-clipped control rats

Days	L-NAME		L-Arginine	
	Control	2KIC	Control	2KIC
0~7	0.72±0.03	0.73±0.02	72.5±2.1	64.4±3.6
8~14	0.79±0.03	0.77±0.03	73.4±2.4	76.2±2.2
15~21	0.69±0.02	0.70±0.02	67.7±4.2	74.4±3.4
22~28	0.68±0.03	0.68±0.05	66.8±4.1	69.3±4.8

Numbers of rats are as in Fig. 1. Values represent means±SEM (mg per day per 100 g body weight).

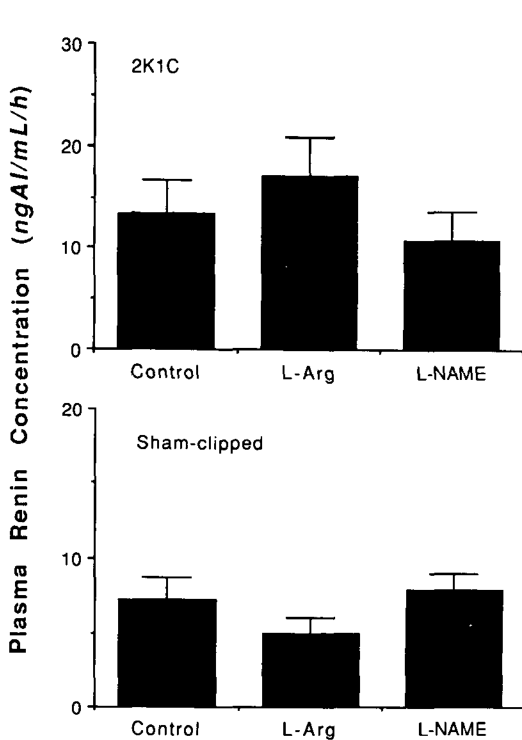

Fig. 2. Plasma renin concentrations in 2K1C and sham-clipped control rats. [L-Arg] and [L-NAME] denote the groups supplemented with L-arginine and L-NAME in their drinking water, respectively.

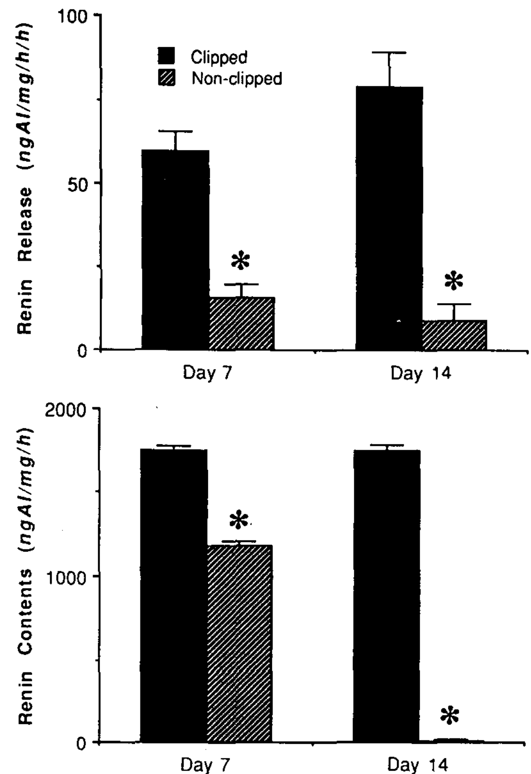

*Fig. 3. Cortical renal renin contents and renin release at days 7 and 14 after clipping the left renal artery. *p<0.01, compared with the clipped.*

The mRNA level of endothelial type of NO synthases was not affected by clipping the artery (data not shown).

DISCUSSION

The enzyme responsible for the synthesis of NO

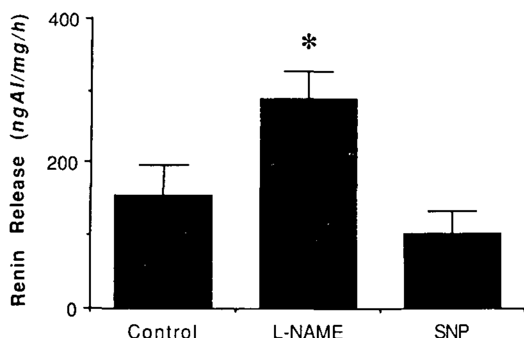

*Fig. 4. Effects of L-NAME and sodium nitroprusside (SNP) on renin release from the isolated renal cortical slice. [L-NAME] and [SNP] denote the results in the presence of L-NAME (10^{-4} mol/L) and sodium nitroprusside (10^{-5} mol/L), respectively. Values are means±SEM, from six experiments each, expressed in nanograms angiotensin I per hour per milligram per 30 min incubation. *p<0.05, compared with control.*

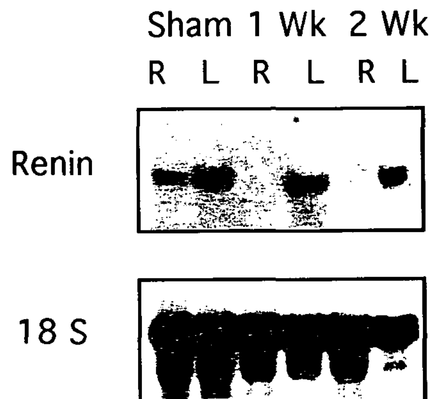

Fig. 5. Renin gene expression. Northern blots of kidney RNA of 2K1C and sham-clipped rats hybridized with renin cDNA probes. Sham-clipped rats were on 1 week after sham-clipping the left renal artery.

in the endothelial cell is the constitutive type of NO synthase, utilizing L-arginine as the substrate. It is competitively inhibited by L-arginine analogues such as N^G-monomethyl-L-arginine and L-NAME (Gardiner et al, 1990; Palmer et al, 1989). L-NAME has been observed to cause vasoconstriction in the renal, mesenteric, and hindquarter vascular beds (Gardiner et al, 1990), and increases in total peripheral resistance (Elsner et al, 1992).

We observed a sustained increase of blood pressure during ingestion of L-NAME in the present study, supporting a major role of NO in the long-term regulation of arterial pressure (Baylis et al, 1992; Dananberg et al, 1993; Gardiner et al, 1990). Since L-NAME has no direct constrictor or dilator effects on vascular smooth muscle, the increased blood pressure is attributed to the removal of endothelium-derived vasodilation through inhibition of NO production.

On the contrary, in 2K1C rats, L-NAME did not augment the hypertension. The final product of renin-angiotensin system, angiotensin II, has a pressor effect mainly through its potent vasoconstrictor action. The failure of L-NAME in modifying 2K1C hypertension suggests that the NO blockade and the early phase of 2K1C hypertension have the common pressor mechanism, possibly on the vasculature. The vasoconstrictor effect of angiotensin II could then mask an effect of L-NAME, or vice versa, in 2K1C rats. The dissociation of the L-NAME effect in the sham-clipped and 2K1C rats cannot be attributed to a difference in its dose, since the ingested amounts of L-NAME were virtually the same in the two sets of rats. By comparison, L-NAME may have augmented DOCA-salt hypertension, since five of seven rats in the L-NAME-supplemented group, which died during the experiment, showed much higher blood pressures than the control before their deaths. The discrepancy in the L-NAME effect points to different pressor mechanisms between 2K1C and DOCA-salt rats. The hypertension in the L-NAME-supplemented DOCA-salt rats may be mediated through vasoconstriction as well as through renal retention of the body fluid.

In the *in vitro* study, L-NAME significantly increased the basal release of renin, being consistent with the hypothesis that endogenous NO inhibits the

release. This finding has been extensively observed by previous investigators (Vidal et al, 1988), and supported by the enhanced renin release following inhibition of NO synthase (Beierwaltes & Carretero, 1992). The lack of a significant effect of sodium nitroprusside on the basal release of renin may be attributed to different experimental conditions. Sodium nitroprusside has been in fact found to have dual effects on renin release from isolated juxtaglomerular cells: both an inhibitory and a stimulatory effect (Schricker & Kurtz, 1993).

Nor was PRC significantly affected by L-NAME in 2K1C rats, as was not the blood pressure. Since L-NAME may increase the blood pressure, its effect on renin release has to be considered in association with the changes in systemic blood pressure. A combination of increased renal perfusion pressure activating the renal baroreceptor mechanism and a reflex reduction in renal sympathetic activity may decrease the secretion of renin. Therefore, L-NAME may not be effective in further augmenting PRC in 2K1C rats of which renin-angiotensin system has been activated. The effect of one factor may be obscured by that of the other. The levels of renin would then depend on the equilibrium between the opposing effects. The contradictory results among the studies may be the consequence of different opposing mechanisms participating in renin secretion.

On the other hand, L-arginine has been suggested to produce a vasodilator effect via stimulating synthesis and release of NO (Hashikawa et al, 1992). In the healthy as well as in patients with essential hypertension, L-arginine treatment caused a rapid reduction of systolic and diastolic pressures (Nakaki et al, 1990). Although supplementation with L-arginine was without significant effects in modifying the development of hypertension either in 2K1C or in DOCA-salt rats, this finding may not disprove that L-arginine is efffective in ameliorating the hypertension. Either L-arginine availability or the activity of NO synthase may be abnormal only in salt-sensitive rats, and L-arginine may therefore ameliorate a defect in NO production that manifests and results in hypertension while genetically susceptible rats are on a high-salt diet. Indeed, parenteral or oral administration of L-arginine prevents hypertension in Dahl salt-sensitive rats given a high salt-diet, while it does not affect development of hypertension in spontaneously hypertensive rats (Chen & Sanders, 1991).

On the contrary, it has been recently suggested that in models of angiotensin-dependent hypertension does NO participate in the implementation of vasodepressor responses to interruption of the renin-angiotensin system (Guan et al, 1996). Although this implies that NO improves a renin-dependent hypertension, it is uncertain whether it also applies to the developmental phase of 2K1C hypertension. The combination of increased activity of the renin-angiotensin system and prolonged hypertension may create conditions that favor involvement of NO in the mediation of vasodepressor responsiveness to interruption of the renin-angiotensin system (Guan et al, 1996).

It has been well known that the activation of renin-angiotensin system is due to triggering of renin release from the clipped kidney (Leenen et al, 1973). In the present study, renin contents and release from the renal cortical slice *in vitro* were, as expected, markedly enhanced in the clipped kidney of 2K1C rats. The hypertension was also associated with increased PRC, and enhanced mRNA and protein levels of renin in the clipped kidney. These findings are in accord with those reported in rats studied during the developmental phase of 2K1C hypertension (Von Thun et al, 1994). Conversely, the renin release from renal slices has been found low in the non-clipped kidney of 2K1C rats (De Jong, 1969). Haefliger et al (1995) further observed that the reduced release from the contralateral kidney was associated with decreased mRNA levels of renin. We also found that the reduced renin release from the contralateral kidney

was associated with decreased or virtually abolished renin mRNA levels. Furthermore, the *in vitro* renin release from the contralateral kidney was more markedly reduced than the degree of the reduced tissue renin contents at day 7 after clipping the unilateral renal artery, and they were comparable at day 14. This finding suggests that the contralateral kidney rapidly loses its contribution to the plasma renin activity in 2K1C rats, so that the clipped kidney only contributes to the plasma renin thereafter.

The suppression of renin gene expression manifest in the contralateral non-clipped kidney of 2K1C rats has been suggested to be primarily due to high circulating angiotensin II levels (Haefliger et al, 1995). However, the detailed mechanism remains to be established. Although it was hypothesized that the inhibited renin secretion in the contralateral kidney is associated with an altered activity of NO, we failed to find a relationship between the genes for renin and NO synthase. The expression of genes for endothelial type of NO synthases in the kidney was not affected in our study. This finding may not necessarily exclude a possible role of NO in modulating the expression of renin, however. Further studies using other techniques such as polymerase chain reaction may be required to delineate whether NO is implicated in the altered expression of renin in the clipped and contralateral kidneys.

In summary, although NO may have an inhibitory effect on renin release, NO system does not affect the development of 2K1C hypertension of which renin-angiotensin system is already activated. The renal renin contents and gene expression are suppressed in the contralateral kidney in 2K1C rats, of which mechanism has to be further examined.

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