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Altered Renal Nitric Oxide System in Experimental Hypertensive Rats

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The present study was aimed at investigating whether the development of hypertension is related with an altered expression of nitric oxide synthases (NOS) in the kidney. By Western blot analysis, the expression of bNOS and ecNOS isoforms was determined in the kidney of deoxycorticosterone acetate (DOCA)-salt and two-kidney, one clip (2K1C) rats. In DOCA-salt hypertension, the expression of both bNOS and ecNOS was decreased, along with tissue contents of nitrites. In 2K1C hypertension, the nitrite content of the clipped kidney was decreased along with ecNOS levels, whereas neither the nitrite content nor the expression of NOS isoforms was significantly altered in the contralateral non-clipped kidney. These results suggest that the development of hypertension is associated with an altered renal expression of NOS and nitric oxide generation in DOCA-salt and 2K1C rats.

Key Words: Kidney; Nitric oxide synthases; Nitrite contents; Deoxycorticosterone acetate-salt hypertension; Two-kidney, One clip hypertension

INTRODUCTION

Endothelium-derived nitric oxide (NO) serves as a locally acting vasodilator and constitutes one of main factors in the short- and long-term regulation of blood pressure. It is synthesized from L-arginine by a group of enzymes known as NO synthases (NOS). There have been thusfar three distinct isoforms of NOS characterized, i.e., neuronal (nNOS, bNOS, or NOS I), inducible (iNOS or NOS II), and endothelial (ecNOS, eNOS, or NOS III) (Förstermann et al, 1991). ecNOS is expressed in the renal vasculature, and bNOS as well as iNOS have been identified in the tubular cells (Ahn et al, 1994; Bachman & Mundel, 1994; Mohaupt et al, 1994).

A pharmacologic inhibition of NO synthesis for 4–6 weeks produces renal vasoconstriction, renal dysfunction and progressive severe hypertension (Baylis et al, 1992; Ribeiro et al, 1994). The hypertension induced is maintained, at least in part, by activation of renin-angiotensin system (Qiu et al, 1994). However, the effect of NO on renin secretion has been contradictory: either inhibitory or stimulatory (He et al, 1995; Schricker & Kurtz, 1993; Sigmon et al, 1992; Stoos et al, 1992).

NO generation is also altered in other models of hypertension. Hirata et al (1995) have found that nitrite production in the kidney is suppressed and the immunoreactivities for both bNOS in macula densa and ecNOS in renal vessels are decreased in deoxycorticosterone acetate (DOCA)-salt hypertensive rats. In 2-kidney, 1 clip (2K1C) hypertensive rats, the endothelium-dependent vasodilation is impaired in the non-clipped kidney (Ortenberg et al, 1992), and more severe stenosis is associated with lesser degree of responses to inhibition of NOS in the clipped kidney (Sigmon & Beierwaltes, 1994).

Although an altered NO system may play a role in the development of certain forms of hypertension, the pathophysiological implications of different NOS isoforms in the development of hypertension have not been established. The present study was aimed at determining whether the expression of NOS is altered in the kidney during the development of hypertension.

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Two different models of hypertension, ie, DOCA-salt and 2K1C hypertension were made in rats, and the expression of the constitutive isoforms of NOS were determined by Western blot analysis.

METHODS

Development of hypertension

Male Sprague-Dawley rats (150~200 g) were kept in accordance with the Institutional Guidelines for Experimental Animal Care and Use, which was a slight modification from those recommended by the American Physiological Society. To develop DOCA-salt hypertension, rats were subcutaneously implanted with silicone rubber containing DOCA (200 mg/kg) one week after unilateral nephrectomy, and were then given with 0.9% saline to drink. Control rats were also unilaterally nephrectomized and given with saline drinking, but were not implanted with DOCA. 2K1C hypertension was made by constricting the left renal artery with a silver clip having an internal gap of 0.2 mm. Sham-clipped rats without clipping of the artery served as control. They were used four weeks after initiating the hypertension, when systolic blood pressure measured indirectly in a conscious state was significantly higher in the experimental groups than in the corresponding controls (190 ± 6 vs 122 ± 9 mmHg in DOCA-salt rats, $p<0.01$; 182 ± 9 vs 116 ± 13 mmHg in 2K1C rats, $p<0.05$, $n=12$ each).

Plasma concentrations of renin and nitrites

Trunk blood was taken into prechilled tubes containing aprotinin (500 kallikrein inhibiting unit/mL) and ethylenediaminetetraacetic acid (EDTA, 1 mg/mL) by decapitation under conscious state. Plasma renin concentration (PRC) was determined using an excess of homologous angiotensinogen and a radioimmunoassay kit for angiotensin I (New England Nuclear; Boston, MA, USA). Plasma and tissue nitrite(+nitrate) levels were measured with a colorimetric assay kit (Oxford; Oxford, MI, USA). For the assay with Griess reagent, 80 mL MOPS (50 mmol/L)/EDTA (1 mmol/L) buffer and 5 μL samples of the plasma or tissue aliquots were used. Nitrate reductase (0.01 U) and 10 μL NADH (2 mmol/L) were added to the reaction mixture, and the plate was shaken for 20 min at room temperature. Color reagents, sulfanilamide, and N-(1-naphthyl) ethylenediamine dihydrochloride were added, and the absorbance was read at 540 nm in a microtiter plate reader.

Protein preparation and Western blot analysis

The kidney was rapidly isolated following decapitation under a conscious state. No gross morphological changes were noted in the clipped kidney of 2K1C rats, except that it was reduced in size. The contralateral non-clipped kidney of 2K1C rats and the remnant kidney of DOCA-salt rats were hypertrophied. The separately dissected cortex, outer medulla, and inner medulla of the kidney, and aorta were homogenized at 3,000 rpm in a solution containing 250 mmol/L sucrose, 1 mmol/L EDTA, 0.1 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 5 mmol/L potassium phosphate buffer, at pH 7.6. Large tissue debris and nuclear fragments were removed by two consecutive low speed spins (3,000 g, 5 min; 10,000 g, 10 min). In the case of cortex, the supernatant was further centrifuged at 100,000 g for 60 min to concentrate membrane-bound protein. The pellet was resuspended for protein blotting of ecNOS and the supernatant was used for blotting of bNOS. The protein concentration was determined by the method of Bradford (1976), with bovine serum albumin as a standard.

Protein samples were electrophoretically size-separated with a discontinuous system consisting of 7.5% polyacrylamide resolving gel and 5% polyacrylamide stacking gel. High-range molecular weight markers (BioRad; Hercules, CA, USA) were used as size standard. An equivalent amount of total tissue protein (100 μg) was loaded on each lane. After separation, the protein was electrophoretically transferred to a nitrocellulose membrane at 20 V overnight. The membrane was washed in Tris-based saline buffer (pH 7.4) containing 1% Tween-20 (TBST) and blocked with 5% non-fat milk in TBST for one hour. It was then incubated with a 1 : 2,000 dilution of monoclonal mouse anti-bNOS and anti-ecNOS antibodies (Transduction Laboratories; Lexington, KY, USA) in 2% non-fat milk/TBST for one hour at room temperature. It was further incubated with a horseradish peroxidase-labelled goat anti-mouse IgG (1 : 1,000) in 2% non-fat milk in TBST for 2 hours. The bound antibody was detected by enhanced chemiluminescence on X-ray film (Amersham; Little Chalfont, Buckinghamshire, England). The membrane

was stripped between incubations with different antibodies in a Tris-buffered solution containing 2% sodium dodecyl sulfate and 100 mmol/L β-mercaptoethanol at 50°C.

Table 1. Plasma concentrations of renin (PRC) and nitrites

		PRC (ngAI/h/mL)	NO (μmol/L)
DOCA-salt			
	Control	2.4±1.5	45.2±5.5
	Hypertensive	0.7±0.4	25.2±3.5*
2K1C			
	Control	13.7±2.2	54.4±3.4
	Hypertensive	43.3±4.5**	40.8±3.2*

Number of rats in each group was 6~9. *p<0.05, **p<0.01; compared with control.

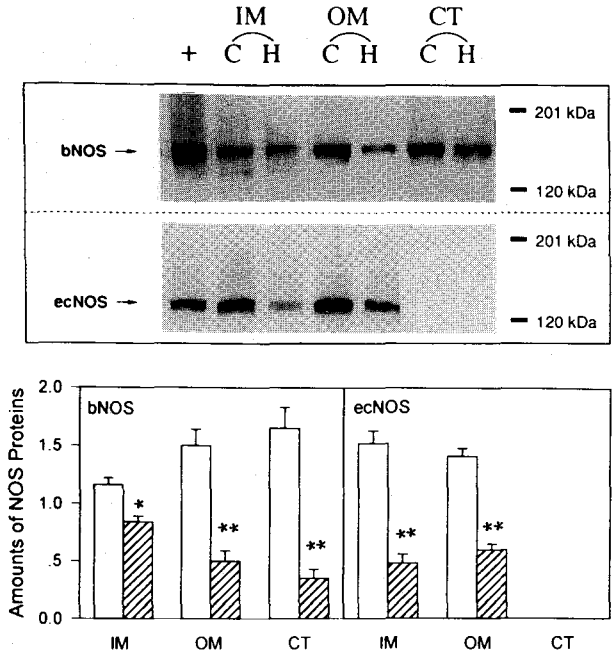

Fig. 1. Representative Western blot and densitometric analysis showing bNOS and ecNOS in the kidney of DOCA-salt rats. Amounts of NOS proteins in ordinates are in arbitrary units. Open and hatched columns depict control and hypertensive groups, respectively (mean± SEM of 6 experiments each). IM: inner medulla, OM: outer medulla, CT: cortex. C: control group. H: hypertensive group. *p<0.05, **p<0.01; compared with control.

Statistics

Data were expressed as mean±SEM. Comparisons between the hypertensive and control groups were made by Student's t-test.

RESULTS

Plasma concentrations of renin and nitrites

Table 1 shows plasma concentrations of renin and nitrites. PRC was significantly higher in the experimental group than in the control in 2K1C rats. On the contrary, it was decreased both in experimental and control groups of DOCA-salt rats, with no significant difference between the two. The plasma nitrites were decreased in both models of hypertension.

Renal expression of NOS isozymes and tissue contents of nitrites

ecNOS was not detected in the cortex, but was expressed in abundance in the medulla. bNOS was expressed both in the cortex and medulla. In DOCA-

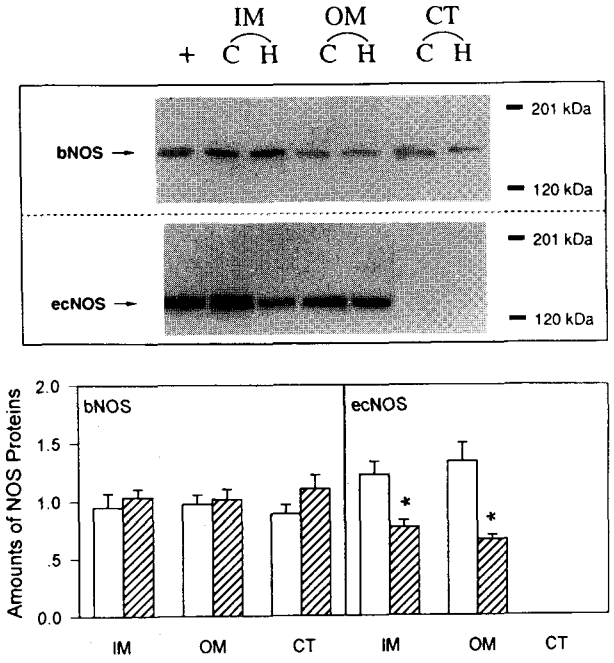

Fig. 2. Representative Western blot and densitometric analysis showing bNOS and ecNOS in the clipped kidney of 2K1C rats (n=6 each). Legends as in Fig. 1.

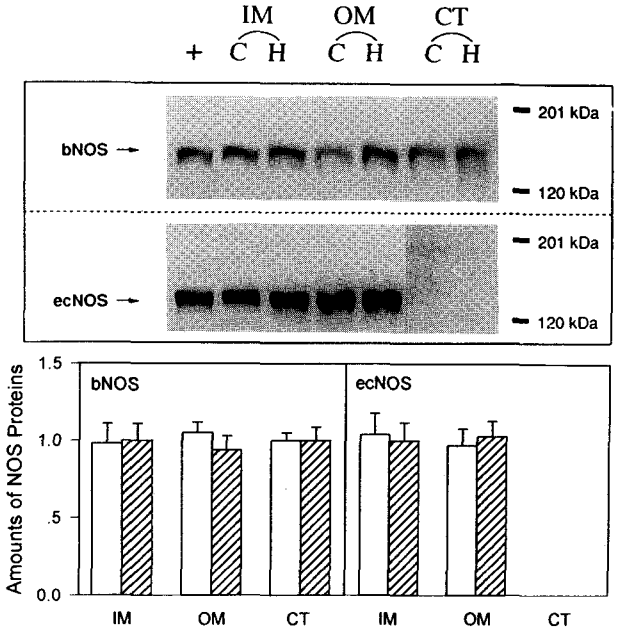

Fig. 3. Representative Western blot and densitometric analysis showing bNOS and ecNOS in the contralateral non-clipped kidney of 2K1C rats. Legends as in Figs. 1 and 2.

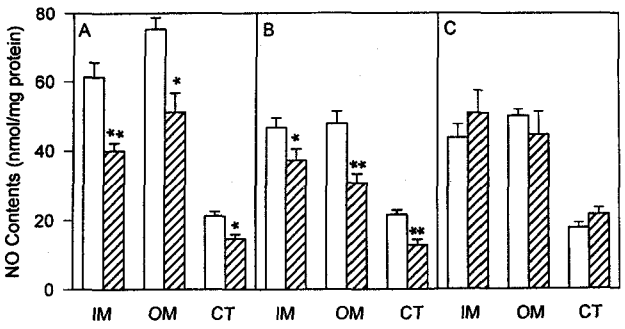

Fig. 4. Tissue contents of nitrites (NO) in the kidney. A: DOCA-salt hypertension. B, C: clipped and non-clipped kidneys of 2K1C hypertension, respectively n=6 each. Other legends as in Fig. 1.

salt hypertension, bNOS as well as ecNOS was decreased (Fig. 1). Renal tissue contents of nitrites were also significantly reduced in DOCA-salt hypertension (Fig. 4). In 2K1C hypertension, ecNOS was significantly decreased in the clipped kidney (Fig. 2), whereas it remained unaltered in the contralateral kidney (Fig. 3). Renal tissue contents of nitrites in the clipped kidney were decreased and those in the contralateral kidney were without significant changes (Fig. 4).

DISCUSSION

The kidney is one of major sites of NOS expression. bNOS is expressed in the macula densa and some adjacent thick ascending limb cells, neurons, efferent arterioles, Bowman's capsular cells and tubules (Terada et al, 1992; Tojo et al, 1995), and ecNOS is in the vascular endothelia of efferent and afferent arterioles, glomerular capillaries, vasa recta, and other arteries and veins (Wilcox et al, 1992). It has been also known that NOS activity is differentially distributed in the kidney. The activity in the medulla is nearly 10-fold higher than that in the cortex (Moridani & Kline, 1996), and the medulla has a greater capacity to produce NO than the cortex (Mattson et al, 1992). The present study also demonstrated an existence of NOS isoforms in the kidney, especially being prominent in the medulla.

Rats are normally resistant to the increased dietary salt intake, with a sustained and adaptive increase of NO production (Tolins & Schultz, 1994). The relative levels of NOS isozymes increased when rats are placed on a high salt diet (Ujiie et al, 1994). These findings indicate that NOS may play a major role in the regulation of sodium and water homeostasis during an increased dietary salt intake. In this process, the kidney protects against the development of salt-sensitive hypertension by rapid and precise adjustments of salt excretion in response to the intake. When NOS activity is chronically inhibited by treatment with NOS inhibitors, on the contrary, rats may become sensitive to the increased salt intake, developing hypertension and subsequent renal injury (Mattson et al, 1992; Mattson & Higgins, 1996; Schultz & Tolins, 1993). Taken together, an important regulatory role has been implicated for the activity of NOS and subsequent generation of NO in the regulation of renal function, control of urinary sodium and water excretion, and long-term regulation of arterial pressure. The primary decrease of NOS expression and subsequent reduction of NO release may result in renal vasoconstriction, and decreases of tubular sodium and water excretion, resulting in the development of hypertension.

DOCA-salt and 2K1C hypertension have been well known to differ in their pressor mechanisms, as was also shown in the present study by a decreased PRC in the former and an increased PRC in the latter. However, in both models of hypertension, plasma

levels of nitrites were uniformly decreased. Although the measurement of plasma and tissue nitrites on a free diet may be of limited value and significance, the diminished nitrites along with the expression of NOS suggests a decreased synthesis of NO. The decreased renal expression of NOS and hence NO release in DOCA-salt rats may allow them become sensitive to the pressor effect of the increased salt intake, resulting in hypertension. The decrease may not be attributed to the high-salt diet, since control rats were also on a high-salt diet. In addition, our results are in accord with previous findings which showed a markedly decreased NO release in DOCA-salt hypertensive rats compared with their normotensive controls (Hayakawa et al, 1993). It has been known that endothelin stimulates NO release via activation of endothelin subtype B (ET_B)-receptors (Hirata et al, 1993), and ET_B-receptor mRNA is significantly decreased in DOCA-salt rat kidneys (Hirata et al, 1995). In this context, a decreased ET_B-mediated NO release may in part account for the reduced tissue nitrites in DOCA-salt hypertension.

It has been shown that NOS inhibition can increase renin secretion in the whole animal, when the inhibitory effects of the increased renal perfusion pressure and the subsequent reflex reduction in renal sympathetic nerve function are prevented (Sigmon et al, 1992). The predominant effect of NO on renin release may thus be an inhibition, although a stimulatory effect is also apparent (Schricker & Kurtz, 1993). Sigmon and Beierwaltes (1994) have found in 2K1C rats that a more severe stenosis is associated with a lesser response to inhibition of NOS in the clipped kidney, suggesting that the stenosis decreases the degree of shear stress on the endothelium and NO generation. On the contrary, it has been also found that bNOS and renin are increased in parallel in the macula densa of the clipped kidney in 2K1C rats (Bosse et al, 1995). The discrepancy between the studies may indicate a differential change of different NOS isoforms in this model of hypertension.

In the present study, ecNOS levels were decreased with no significant changes in bNOS in the clipped kidney. A diminished arterial perfusion and hence decreased degree of shear stress on the vascular endothelium may have decreased NO generation in this kidney. The decreased NO activity may in turn result in an attenuation of its tonic inhibitory role on the renin release, causing increases of renin release and PRC. Taken together, the augmented renin-angiotensin system and decreased NOS expression may be causally related. The normally maintained bNOS in the presence of decreased ecNOS expression may augment the release of renin in 2K1C hypertension. On the other hand, the normal NOS activity and NO generation may allow the normal perfusion in the contralateral non-clipped kidney. This may also explain differential effects of blockade of NOS between the clipped and contralateral kidneys in 2K1C rats (Sigmon & Beierwaltes, 1994).

It has been found that a short-term infusion of angiotensin II leads to graded increases in vascular resistance and NO generation, as reflected by increased excretion of nitrate and nitrite (Deng et al, 1994). In addition, angiotensin II does not directly influence glomerular endothelial cell calcium concentration or NO generation (Marsden et al, 1990). In this context, the high circulating levels of angiotensin II may not be directly related with the altered NOS expression in the clipped kidney in 2K1C rats.

In summary, the present study suggests that the development of hypertension is causally related with an altered renal NO system in DOCA-salt and 2K1C rats. The failure of the Western blotting technique to detect ecNOS in the cortex may indicate a limited sensitivity of our technique, however. We could have missed important changes in ecNOS in the cortex where the vasculature is heavily loaded. Further detailed studies are needed to establish a direct relation between NOS and hypertension.

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