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Differentially-Altered Vascular Guanylate Cyclase Isoforms in Experimental Hypertensive Rats

Pathophysiological implications of the vascular nitric oxide (NO)/cGMP pathway were investigated in various rat models of hypertension. The expression of brain and endothelial constitutive NO synthases (bNOS, ecNOS) was determined by Western blot analysis, and the biochemical activity of soluble and particulate guanylate cyclases (GC) was assessed by the amount of cGMP generated in the thoracic aortae of rats with deoxycorticosterone acetate (DOCA)-salt, two-kidney, one clip (2K1C), and spontaneous hypertension (SHR). Plasma nitrite/nitrate levels were decreased in DOCA-salt and 2K1C hypertension, and increased in SHR. The vascular expression of bNOS as well as that of ecNOS was decreased along with tissue nitrite/nitrate contents in DOCA-salt and 2K1C hypertension. The expression of both bNOS and ecNOS was increased in SHR with concomitant changes of tissue nitrite/nitrate contents. The activity of soluble GC was decreased, and that of particulate GC was increased in DOCA-salt hypertension. The soluble GC activity was increased, while the particulate GC activity was not affected in 2K1C hypertension. The soluble GC activity was not significantly changed, but the particulate GC activity was decreased in SHR. These results indicate that the high blood pressure is associated with differentially-altered vascular NO/cGMP pathway in different models of hypertension.

Key Words: *Nitric-oxide synthase; Guanylate cyclase; Desoxycorticosterone hypertension*

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INTRODUCTION

Endothelium-dependent vasorelaxation has been largely accounted for by the release of nitric oxide (NO) (1). The synthesis of NO is catalyzed by a family of NO synthases (NOS) (2), and its biological effect is in turn mediated by generating the second messenger, guanosine 3', 5'-monophosphate (cGMP) (3). In various models of hypertension, the vascular activity of NO/cGMP pathway has been found altered. It may be depressed as in mineralocorticoid or renal hypertension, or overactive as in some genetic models of hypertension (4, 5).

On the other hand, cGMP formation is not only mediated by activating the soluble guanylate cyclase (GC), but by activating the particulate isoform of GC (6). However, whether these two isoforms are affected differentially or concurrently has not been investigated in hypertension.

The present study was aimed at further investigating the pathophysiological implications of NO/cGMP pathway in hypertension. Specifically, the vascular expression of NOS isozymes and activities of GC isoforms were determined in different rat models of hypertension.

MATERIALS AND METHODS

Hypertensive rat models

Male Sprague-Dawley rats, weighing 150-200 g, were used to develop deoxycorticosterone acetate (DOCA)-salt and two-kidney, one clip (2K1C) hypertension. They were kept in accordance with "Institutional Guidelines for Experimental Animal Care and Use", which is a slight modification of those recommended by the American Physiological Society. DOCA-salt hypertension was induced by subcutaneous implantation of silicone rubber containing DOCA (200 mg/kg), one week after the unilateral nephrectomy. The rats were then supplied with 0.9% saline to drink. The control group was also unilaterally nephrectomized and supplied with saline to drink, but was not implanted with DOCA. Two-kidney, one

clip (2K1C) hypertension was induced by constriction of the left renal artery with a silver clip with internal gap of 0.25 mm. The control group was without clipping. DOCA-salt and 2K1C rats were used six weeks after inducing the hypertension. Spontaneously hypertensive rats (SHR) were also male and aged 12-15 weeks. Age-matched male Wistar-Kyoto (WKY) rats served as the control.

Systolic blood pressure was measured indirectly at the tail artery on the day of experiment. It was significantly higher in the experimental groups than in their controls (190±6 vs 122±9 mmHg in DOCA-salt rats, $p<0.01$; 182±9 vs 116±13 mmHg in 2K1C rats, $p<0.05$; 205 ±12 vs 126±7 mmHg in SHR and WKY, $p<0.01$; n=8 each).

Measurement of nitrite/nitrate and atrial natriuretic peptide

Trunk blood was taken into prechilled tubes containing aprotinin (500 KIU/mL) and ethylenediaminetetraacetic acid (EDTA, 1 mg/mL) by decapitation under a conscious state. Nitrite/nitrate were measured with a colorimetric assay kit (Oxford; Oxford, MI, U.S.A.). Eighty μL 3-(N-morpholino)propanesulfonic acid (50 mmol/L)/EDTA (1 mmol/L) buffer and 5 μL samples were put to wells in a microplate. Nitrate reductase (0.01 U) and 10 μL NADH (2 mmol/L) were also 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. The protein concentration of the tissue aliquot was determined by Bradford method (7), with bovine serum albumin as a standard.

The concentration of atrial natriuretic peptide (ANP) was measured by radioimmunoassay using a commercially available kit (Peninsula Laboratories; Belmont, CA, U.S.A.), following extraction of the plasma with Sep-Pak C18 cartridges (Waters Associates; Milford, MA, U.S.A.). The values were corrected with the extraction ratio (68.5 ±1.4%).

Protein preparation and Western blot analysis

The thoracic aorta was rapidly taken following the decapitation. It was 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). The supernatant was used for blotting brain (b) NOS, and the pellet was resuspended for blotting endothelial constitutive (ec) NOS.

An equivalent amount of total tissue protein (100 μg) was loaded on each lane. The protein sample was 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, U.S.A.) were used as size standard. After the separation, the protein was 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 hr. It was then incubated with 1:2,000 dilution of monoclonal mouse anti-bNOS and anti-ecNOS antibodies (Transduction Laboratories; Lexington, KY, U.S.A.) in 2% non-fat milk/TBST for one hr 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 two hr. 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℃. Rat pituitary and human endothelial cells were used as positive controls for bNOS and ecNOS, respectively.

Guanylate cyclase activity

The thoracic aorta was homogenized in ice-cold buffer (50 mmol/L Tris-HCl, pH 8.0, containing 1 mmol/L EDTA, 0.2 mmol/L PMSF, and 250 mmol/L sucrose), and centrifuged at 1,000 *g*. The supernatant was further fractionated by centrifuging at 100,000 *g* for one hr at 4℃. The resulting supernatant was used as the cytosolic fraction. The pellet was resuspended by ultrasonication in buffer containing 50 mmol/L Tris-HCl (pH 7.6) and 1 mmol/L EDTA, being employed as the membrane fraction. The activity of soluble GC was determined in the cytosolic fraction by the amount of cGMP accumulated in response to sodium nitroprusside (SNP), and that of particulate GC was assessed in the membrane fraction by the amount of cGMP accumulated in response to ANP. Ninety μL of working solution (50 mmol/L Tris-HCl, pH 7.6, containing 15 mmol/L phosphocreatine, 20 μg/mL creatine phosphokinase, 2 mmol/L 3-isobutyl-1-methylxanthine, and 1 mmol/L ATP) was warmed to 37℃. The test agent and the membrane fraction containing 20 μg of protein were added in succession. The reaction was started by adding $MgCl_2$ (4 mmol/L) and GTP (1 mmol/L), and stopped after 2.5

min by adding ice-cold 50 mmol/L sodium acetate (pH 4.0). After centrifugation at 1,000 *g* and 4℃ for 10 min, the supernatant was assayed for cGMP. ANP stimulated the membrane GC in a dose-dependent fashion, Km being 3.1×10^{-8} mol/L. The K_m value against SNP was 9.6×10^{-5} mol/L. ANP did not increase the cytosolic activity of GC. Contamination of cytosolic fraction was ruled out by the absence of response to SNP in the membrane fraction. All chemicals used were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.).

Statistics

Data were expressed as means±SEM. Comparisons between the hypertensive and control groups were made by unpaired t-test.

RESULTS

Plasma nitrite/nitrate and ANP

Table 1 shows plasma nitrite/nitrate and ANP levels. The nitrite/nitrate levels were decreased, and ANP increased in DOCA-salt and 2K1C hypertension. On the contrary, nitrite/nitrate as well as ANP was increased in SHR.

Vascular NOS expression and nitrite/nitrate contents

Representative Western blots of NOS isozymes and their densitometric analysis data are shown in Fig. 1 and 2. Anti-bNOS and anti-ecNOS monoclonal antibodies hybridized with proteins of approximately 155 kDa and 140 kDa, respectively. The expression of bNOS as well as that of ecNOS was decreased in DOCA-salt and 2K1C hypertension (Fig. 1). On the contrary, the expression of both bNOS and ecNOS was increased in SHR (Fig. 2).

Table 1. Plasma concentrations of nitrite/nitrate and ANP

	Nitrite/nitrate (μmol/L)	ANP (pg/mL)
DOCA-salt		
Control	55.1±5.7	34.3±6.9
Hypertensive	25.2±3.5†	59.0±7.4*
2K1C		
Control	47.2±3.1	16.5±5.5
Hypertensive	35.4±2.9*	42.8±2.0†
SHR		
WKY	33.2±2.5	23.8±5.7
SHR	52.6±3.8*	54.9±6.2*

Each datum represents the mean±SEM of 6 rats.
*$p<0.05$, †$p<0.01$; compared with control.

Accordingly, the vascular nitrite/nitrate contents were significantly decreased in rats with DOCA-salt and 2K1C hypertension, whereas they were increased in SHR (Fig. 3).

Soluble and particulate guanylate cyclase activities

The soluble and particulate GC activities were determined by the amounts of cGMP accumulated in responses to SNP and ANP, respectively. The soluble GC activity was decreased, and the particulate GC activity increased in DOCA-salt hypertension (Fig. 4). In 2K1C hypertension, the soluble GC activity was increased, whereas the particulate GC activity was not affected (Fig. 5). The soluble GC activity remained unchanged, while the particulate GC activity was decreased in SHR (Fig. 6).

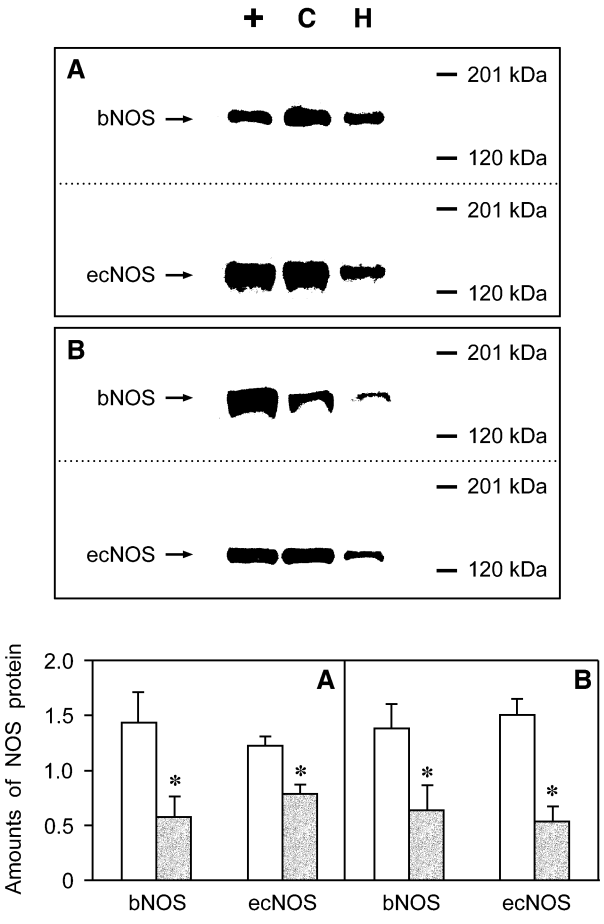

Fig. 1. Representative Western blots and densitometric analyses showing bNOS and ecNOS in the thoracic aorta of DOCA-salt (A) and 2K1C (B) rats. +, Positive control. C, Control group. H, Hypertensive group. In the bottom, the open column represents the control, and the hatched column depicts the hypertensive group. Amounts of NOS proteins are in arbitrary units. *$p<0.01$, compared with control (n=6 each).

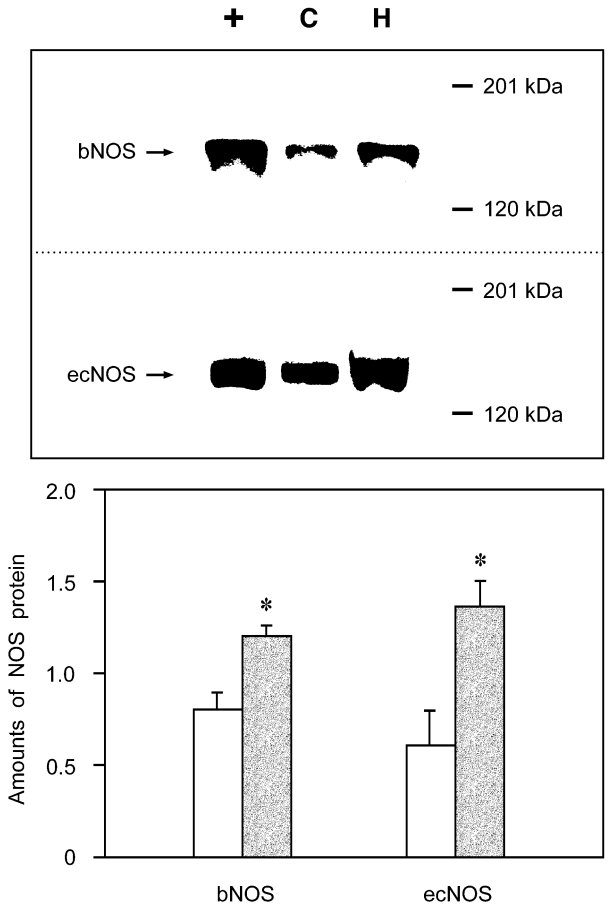

Fig. 2. Representative Western blots and densitometric analyses showing bNOS and ecNOS in the thoracic aorta of SHR. WKY rats were used as control. Legends as in Fig. 1. *p<0.01, compared with control (n=6 each).

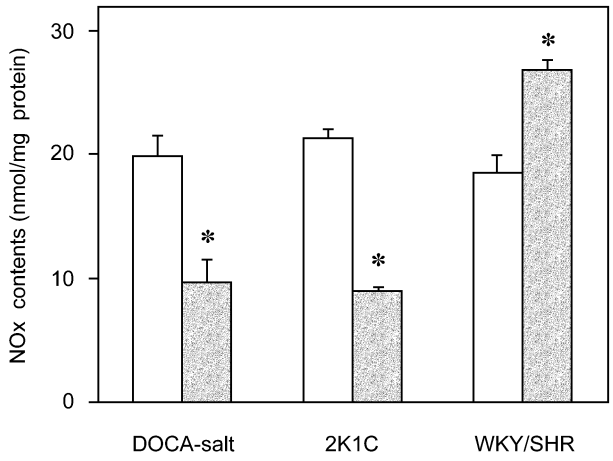

Fig. 3. Nitrite/nitrate levels in the thoracic aorta of DOCA-salt and 2K1C rats, and SHR. Legends as in previous figures. *p< 0.01, compared with control (n=6 each).

DISCUSSION

DOCA-salt hypertension has been ascribed to the volume-expansion induced by renal retention of body fluids, and 2K1C hypertension to the vasoconstriction induced by enhanced activity of the renin-angiotensin system. Despite the discrepancy in their pressor mechanisms, both models of hypertension were associated in common with decreases of NOS expression and tissue nitrite/nitrate levels in the present study. The reduced NO activity may in part be attributed to an enhanced elimination (8). However, the decreased nitrite/nitrate levels associated with diminished NOS expression may

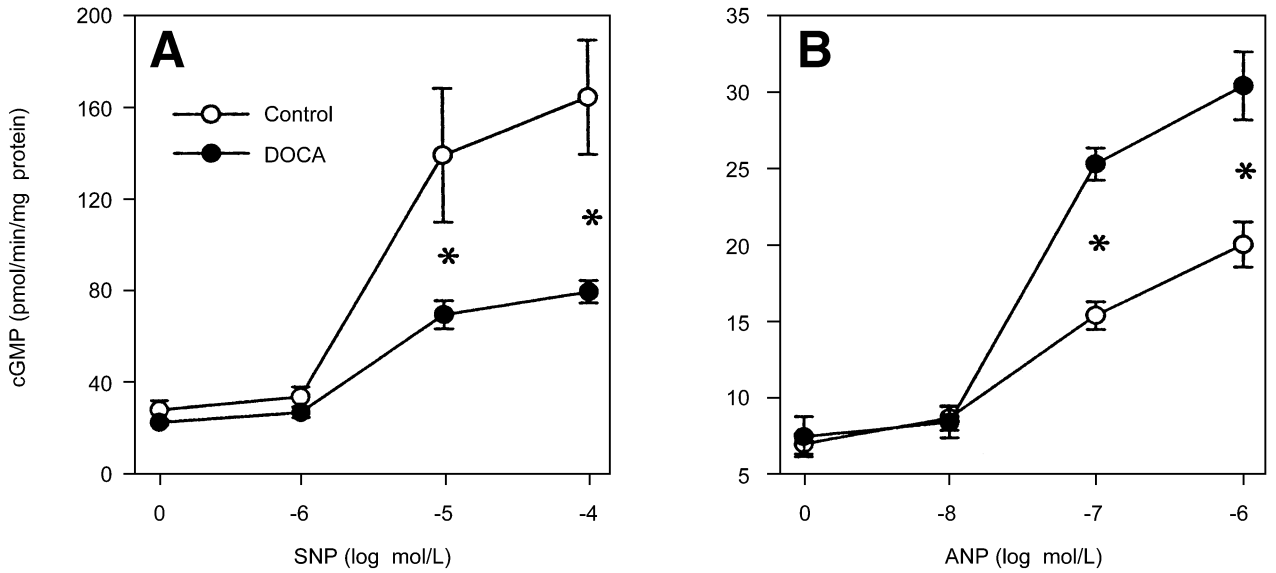

Fig. 4. cGMP accumulation in the vascular soluble (A) and particulate (B) fractions in DOCA-salt hypertension. *p<0.01, compared with control (n=6 each).

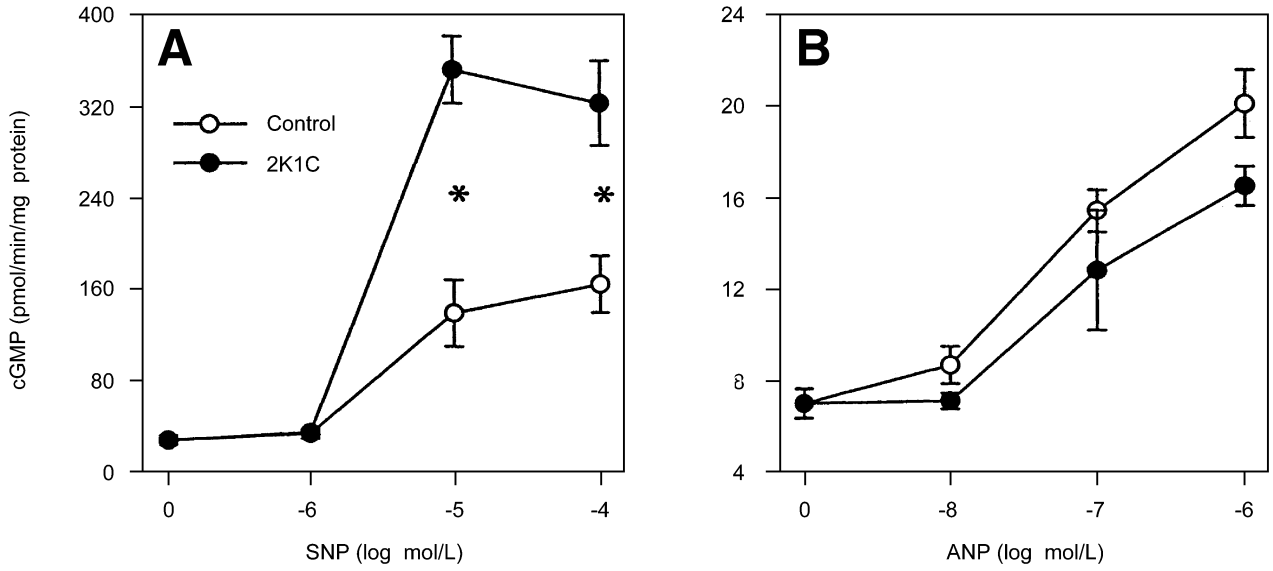

Fig. 5. cGMP accumulation in the vascular soluble (A) and particulate (B) fractions in 2K1C hypertension. *$p<0.01$, compared with control (n=6 each).

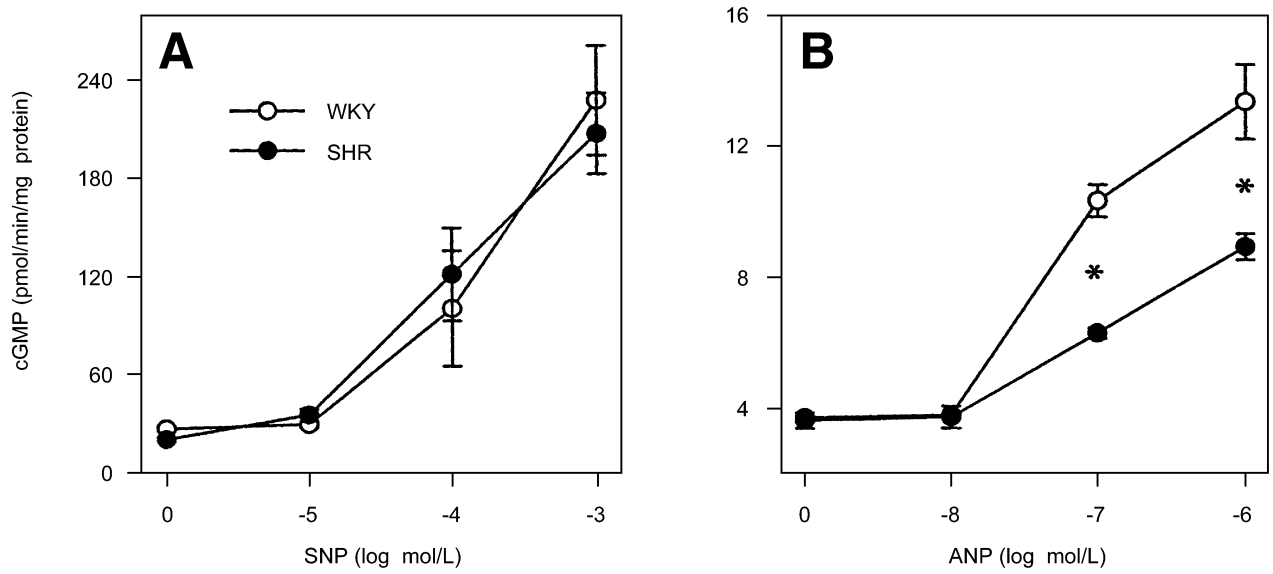

Fig. 6. cGMP accumulation in the vascular soluble (A) and particulate (B) fractions in SHR. *$p<0.01$, compared with WKY (n=6 each).

indicate a decreased synthesis of NO.

Our results are not in accord with the previous finding which showed increases of circulating nitrite/nitrate levels in 2K1C hypertension (9). However, the duration of hypertension differed between the studies, i.e., six weeks in the present study and four weeks in the other study. It is speculated that NO system becomes suppressed over longer periods of hypertension. The reduced activity of NO system may then contribute to the maintenance of elevated blood pressure (10, 11).

The results in SHR were contradictory to those in DOCA-salt and 2K1C rats. SHR, of which high blood pressure is genetically determined, showed increases in tissue nitrite/nitrate levels, along with concomitant increases of vascular NOS expression. This is in line with a recent study in which the activity of constitutive NOS and concentrations of nitrates in mesenteric resistance arteries are higher in SHR than in age-matched control rats (5). Since the hypertension is developed and maintained mainly by the increased peripheral vascular resistance in SHR, the increased vascular NO activity may represent a mechanism counteracting the high blood

pressure in this model of hypertension.

The vasorelaxation may also be affected by an alteration at the step beyond NO formation, such as GC activity. In DOCA-salt hypertension, the soluble GC activity was decreased, and the particulate GC activity was increased. The decreased responsiveness to SNP would be expected, when NO generated from the applied SNP is inactivated before it is able to activate GC (12). However, SNP may be directly accessible to GC, and the released NO is unlikely to be inactivated so rapidly in our experimental condition. The decreased cGMP formation may thus be attributed to an impaired activity of soluble GC. On the contrary, the particulate GC activity may be referred to a counter-regulatory mechanism stimulated by primary decreases in soluble GC activity.

2K1C hypertension was also associated with differential regulation of GC isoforms. The soluble GC activity was increased, and the particulate GC activity remained unchanged. The decreased endogenous NO production may result in a supersensitivity of the soluble GC to exogenously-derived NO. It has been recently shown that angiotensin II stimulates cGMP production in incubated aortic segments, which could be blocked by inhibition of soluble GC (13). Alternatively, therefore, the increased circulating angiotensin II in 2K1C hypertension may be directly responsible for the enhanced soluble GC activity. Nevertheless, the particulate GC activity may not be affected without a primary decrease of soluble GC activity in 2K1C hypertension.

On the other hand, SHR showed no significant changes of soluble GC activity, but significant decreases of the particulate GC activity. Some salt-resistant forms of essential hypertension have been shown to be associated with mutations in the GC-A gene (14). The diminished particulate GC activity may thus be a primary phenomenon genetically determined in SHR. It is unlikely that the circulating ANP is responsible for the altered particulate GC activity, since the plasma ANP levels were similarly increased in the three models of hypertension examined.

It has been suggested that the basal aortic cGMP is mainly dependent on NO and soluble GC activity, and only to a minor extent on particulate GC activity (15). We observed in the present study that the highest cGMP levels stimulated by ANP would be barely comparable to the basal cGMP level in the soluble fraction. The role of particulate GC may be minor in contributing to the overall vascular accumulation of cGMP. A previous study also showed a similar accumulation of cGMP in mesenteric resistant arteries between SHR and WKY rats (5). Although a higher expression of soluble GC mRNA has been observed in cultured smooth muscle of SHR (16), the expression of genetic message may not be directly indicative of its biological activity in vivo. Taken together, the pathophysiological implication of the decreased particulate GC activity may be negligible in SHR, especially when the soluble GC activity remained unaltered. On the other hand, the biological activity of soluble GC may not be affected by a primary change of particulate GC activity.

A dissociation was shown among the activities of NOS and GC, and subsequent formation of cGMP. The endogenous NO may be unable to proportionally raise cGMP levels in the vascular smooth muscle. Moreover, a parallelism between stimulation of GC and excretion of cGMP is lacking in certain pathophysiological situation (17), and no direct correlation has been found between the degree of vasorelaxation and the amount of cGMP accumulated (18). The increased cGMP formation may not be linearly transduced into an enhanced vasorelaxation as in 2K1C hypertension (19), or the vasorelaxation may be maintained in a certain extent despite the reduced formation of cGMP as in DOCA-salt hypertension. Additional mechanisms may also take place to prevent NO from accomplishing its normal hemodynamic actions. The unaltered cGMP response to SNP in SHR may support the notion that the attenuated endothelium-dependent vasorelaxation is not due to a decreased activity of NO/cGMP pathway, but to an enhanced constrictor mechanism (20).

In summary, our results indicate that high blood pressure is associated with differentially-altered vascular NOS and GC isozymes in different models of hypertension. Their pathophysiological implications need to be further defined.

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