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Diminished Vascular Guanylyl Cyclase Activity in Deoxycorticosterone Acetate-Salt Hypertension

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Pathophysiological implications of the vascular nitric oxide (NO)/cGMP pathway in hypertension were investigated. Sprague-Dawley rats were made deoxycorticosterone acetate (DOCA)-salt hypertensive for six weeks. The protein expression of endothelial constitutive NO synthase (ecNOS) and the tissue content of NO were determined in the thoracic aorta. The protein expression and catalytic activity of soluble guanylyl cyclase (GC) were also determined. Systolic blood pressure measured on the day of experiment was significantly higher in the experimental group than in the control. The hypertension was associated with decreases in the vascular tissue content of NO metabolites, concomitantly with the expression of ecNOS proteins. The protein expression of GC was not affected, while its catalytic activity was significantly decreased in hypertension. These results indicate that the high blood pressure is associated with a decreased activity of vascular NO/cGMP pathway in DOCA-salt hypertension.

Key Words: Nitric oxide synthase, Guanylyl cyclase, Deoxycorticosterone acetate-salt hypertension

INTRODUCTION

The endothelium-dependent vasorelaxation has been largely accounted for by the release of nitric oxide (NO) of which synthesis is catalyzed by a family of NO synthases (NOS). However, NO is not biologically active until it causes a secondary formation of guanosine 3', 5'-monophosphate (cGMP) catalyzed by soluble isoform of guanylyl cyclase (GC) (Förstermann et al, 1986). Therefore, the biological activity of NO system may be represented by the tissue activity of GC.

The activity of NO/cGMP pathway may be altered in different models of hypertension (Lüscher et al, 1987; Dominiczak & Bohr, 1995). Furthermore, along concomitant changes of NO concentrations, the vascular expression of NOS has been found either decreased or increased (Nava & Lüscher, 1995; Lee et al, 1999). However, whether there is an altered regulation of GC has not been established in hypertension. The present study was aimed at investigating the pathophysiological implications of NO/cGMP pathway in hypertension. The expression of endothelial constitutive (ec) isoform of NOS and the tissue content of NO were determined in the thoracic aorta in a rat model of experimental hypertension. The protein expression and the catalytic activity of GC were also determined.

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METHODS

Development of hypertension

Male Sprague-Dawley rats, weighing 150−200 g, were used. 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. The experimental group was, one week after unilateral nephrectomy, subcutaneously implanted with silicone rubber containing deoxycorticosterone acetate (DOCA, 200 mg/kg). 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 without DOCA. They were kept for 6 weeks until the day of experiment. Systolic blood pressure was measured indirectly at the tail artery.

Measurement of nitrite/nitrate

The thoracic aorta was rapidly taken following the decapitation in a conscious state. 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 determination of the tissue content NO using a colorimetric assay kit (Oxford; Oxford, MI, USA). 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 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 (Bradford et al, 1976), with bovine serum albumin as a standard.

Western blot analysis

The tissue protein preparation was used for blotting ecNOS and soluble isoform of GC. An equivalent amount of total protein (100 μg) was loaded on each lane. It 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, USA) 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 1 hr. It was then incubated with 1 : 2,000 dilutions of monoclonal mouse anti-ecNOS antibodies (Transduction Laboratories; Lexington, KY, USA) or with 1 : 1,000 dilutions of polyclonal mouse anti-GC antibodies (Calbiochem-Novabiochem; San Diego, CA, USA) in 2% non-fat milk/TBST for 1 hr at room temperature. It was further incubated with horseradish peroxidase-labeled goat anti-mouse IgG (1 : 1,000) in 2% non-fat milk in TBST for 2 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°C.

Guanylyl 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 and 4°C. The supernatant was further fractionated by centrifugation at 100,000×g for 1 hr. The resulting supernatant was used as the cytosolic fraction. The activity of soluble GC was determined in the cytosolic fraction by the amount of cGMP accumulated in response to sodium nitroprusside (SNP). 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°C. The test agent and the cytosolic fraction containing 20 μg of protein were added in succession in the presence of SNP. 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°C for 10 min, the supernatant was assayed for cGMP. The Km value against SNP was 9.6×10^{-5} mol/L. ANP did not increase the activity of GC in this cytosolic fraction. All chemicals used were purchased from Sigma Chemical Company (St. Louis, MO, USA).

Statistics

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

RESULTS

Vascular NOS expression and NO contents

Systolic blood pressure measured on the day of

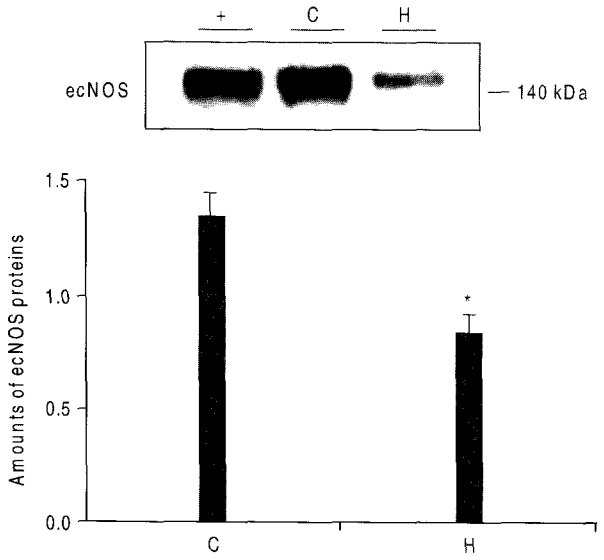

Fig. 1. Representative Western blots and densitometric analysis showing endothelial constitutive isoform of nitric oxide synthase (ecNOS) in the thoracic aorta. (+): Positive control. (C): Control. (H): Hypertensive. Amounts of proteins are in arbitrary units. *P<0.01, compared with the control (n=6 each).

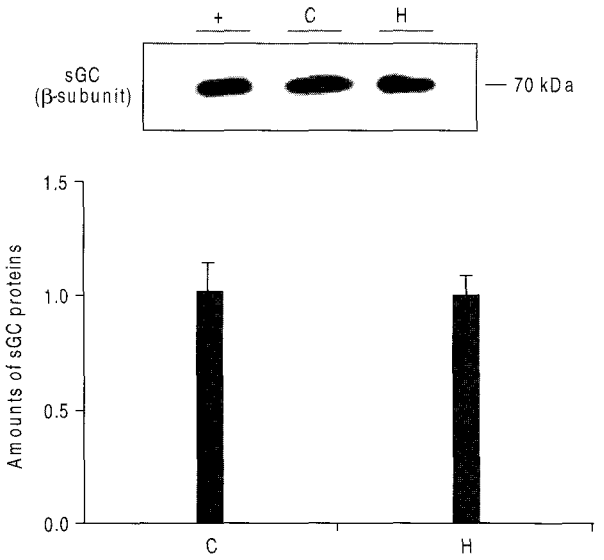

Fig. 2. Representative Western blots and densitometric analysis showing soluble isoform of guanylyl cyclase (sGC) in the thoracic aorta. Legends as in Fig. 1. n=3 each.

experiment was significantly higher in the experimental group than in the control (190±6 vs 122±9 mmHg, n=9 each, P<0.01). Fig. 1 shows representative Western blots of ecNOS and their densitometric data. Anti-ecNOS monoclonal antibodies hybridized with proteins of approximately 140 kDa. The expression of ecNOS was significantly decreased in hypertension. Accordingly, the vascular NO content was significantly lower in hypertension than in control (9.2±2.1 vs 19.8±1.9 nmol/mg protein, n=6 each, p <0.01).

Vascular expression and activity of GC

Representative immunoblots of GC and their densitometric data are shown in Fig. 2. Anti-GC polyclonal antibodies mainly hybridized with β-subunit proteins of GC at approximately 70 kDa. The expression of GC was not significantly altered in hypertension. However, its catalytic activity determined by the amount of cGMP accumulated in response to SNP was significantly attenuated in hypertension (Fig. 3).

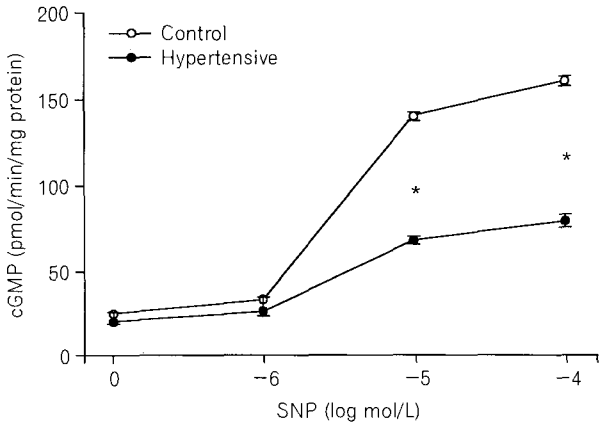

Fig. 3. cGMP accumulation stimulated by a series concentrations of sodium nitroprusside (SNP) in the soluble fraction of the thoracic aorta. *P<0.01, compared with control (n=6 each).

DISCUSSION

The vascular content of NO was significantly decreased in six-week-hypertensive DOCA-salt rats. This finding confirms our previous study that showed decreases of circulating NO levels and vascular NO contents in two-kidney, one clip (2K1C) and DOCA-salt hypertensive rats (Lee et al, 1999). The reduced NO activity may be attributed either to a diminished

synthesis or to an enhanced degradation of NO. However, the decrease associated with a concomitant decrease in the expression of ecNOS proteins suggests a decreased synthesis.

It has been previously shown that circulating NO levels are increased in 2K1C rats (Dubey et al, 1996). The discrepancy may be accounted for by the different duration of hypertension between the studies, i.e., 6 weeks in the present study and 4 weeks in the previous study. It is speculated that NO becomes suppressed over a longer period of hypertension, contributing to the maintenance of high blood pressure in chronic hypertension. On the other hand, vascular NOS activities as well as NO concentrations are increased in spontaneously hypertensive rats (Nava & Lüscher, 1995; Lee et al, 1999). The elevation of blood pressure is genetically determined in this model of hypertension, and the increased vascular NO activity may represent a mechanism counteracting the high blood pressure.

The biological activity of NO may not become apparent until it activates soluble GC and hence results in a secondary generation of cGMP (Arnal et al, 1992). In the present study, the vascular accumulation stimulated by SNP was decreased in DOCA-salt hypertension. The decreased responsiveness to SNP would be expected, when NO generated from the applied SNP is inactivated before it is able to activate GC (Bouloumié et al, 1997). However, SNP may be directly accessible to GC, and the NO released is unlikely to be inactivated so rapidly in our experimental condition. In addition, the protein expression of soluble GC was not significantly altered. It has been observed that the expression of soluble GC mRNA in the cultured smooth muscle is higher in spontaneously hypertensive rats than in control (Papapetropoulos et al, 1994). On the contrary, the accumulation of cGMP is similar in mesenteric resistant arteries between spontaneously hypertensive and control rats (Nava & Lüscher, 1995). Taken together, the expression of genetic message may not be a direct indicator of its biological activity. A decrease of cGMP formation despite the unaltered expression of GC suggests an impairment of the biochemical activity of GC *per se* with its genetic message maintained.

Finally, it may be worthwhile to point out a lack of parallelism between stimulation of GC and formation of cGMP in certain pathophysiological situations (Rapoport & Murad, 1988). In addition, no direct correlation has been found between the degree of vasorelaxation and the amount of cGMP accumulated (Hu et al, 1992). Therefore, a reduced activity of NO/cGMP pathway may not completely explain the increased peripheral resistance in hypertension. Certain mechanisms intrinsic to the vasculature may exist to keep the vascular tone relatively constant when the formation of cGMP is reduced. Alternatively, other mechanisms may also take place to prevent the normal vasodilator action of NO/cGMP pathway from accomplishing its hemodynamic actions in hypertension.

In summary, it is suggested that DOCA-salt hypertension is associated with decreases in the vascular expression of NOS and NO generation. Although the vascular expression of GC remains unaltered, its biochemical activity may be decreased in this model of hypertension.

ACKNOWLEDGEMENTS

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