

고혈압에서 혈관 산화질소 합성 동위 효소 발현 변화

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=Abstract=

Altered Vascular Expression of Nitric Oxide Synthase Isozymes in Hypertension

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Background: The endothelium-dependent vasorelaxation has been largely accounted for by the release of nitric oxide (NO). Three distinct isoforms of NO synthases (NOS) have been characterized, i.e., brain(bNOS), inducible (iNOS), and endothelial constitutive (ecNOS). Although hypertension has been associated with a vascular endothelial dysfunction, changes in the vascular expression of NOS isoforms have not been established. The present study was aimed at exploring the vascular expression of NOS isozymes in hypertension. **Material and Method:** Two-kidney, one clip (2K1C) and deoxycorticosterone acetate (DOCA)-salt hypertension were induced in rats. The expression of different NOS isozymes in the thoracic aorta was determined by Western blot analysis. The vascular tissue contents of nitrites were measured by colorimetric assay. **Result:** Arterial blood pressure was significantly higher in experimental groups of 2K1C and DOCA-salt rats compared with their corresponding control rats. The vascular expression of bNOS as well as that of ecNOS was decreased in both models of hypertension. iNOS was not changed in DOCA-salt hypertension, but was also decreased in 2K1C hypertension. The vascular contents of nitrites were significantly decreased in DOCA-salt as well as in 2K1C hypertension. **Conclusion:** These results suggest that 2K1C and DOCA-salt hypertension are associated with decreases in the vascular expression of NOS isozymes and nitrite contents.

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Key word : 1. Nitric oxide
2. Hypertension

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본 논문의 저작권 및 전자매체의 지적소유권은 대한흉부외과학회에 있다.

INTRODUCTION

The endothelium-dependent vasorelaxation has been largely accounted for by the release of nitric oxide (NO) synthesized from a semiessential amino acid, L-arginine¹. Three distinct isoforms of NO synthases (NOS) have been so far characterized, i.e., brain (bNOS), inducible (iNOS), and endothelial constitutive (ecNOS)². ecNOS has been documented in the vascular endothelium³. Although bNOS has been found to be localized primarily in organs containing neural elements, it is also found in aortic extracts⁴. iNOS expression may be induced by specific stimuli such as bacterial lipopolysaccharides⁵.

Konishi and Su⁶ first reported a reduced endothelium-dependent vasorelaxation in spontaneously hypertensive rats (SHR). Although a vascular endothelial dysfunction associated with hypertension has been widely accepted in humans as well as in experimental animals, the responses to endothelium-independent dilators may not be affected in hypertension⁷⁻¹². These findings suggest that the mechanisms inducing the endothelial synthesis and release of NO may be impaired in hypertension.

An altered NO synthesis and release may be attributed to an altered activity of NOS. Aortic calcium-dependent NOS activity is reduced in salt-sensitive Dahl rats compared with their normotensive controls¹³. However, *in vitro* studies have demonstrated that hemodynamic forces such as shear stress and cyclic strain increase vascular NO production by increasing endothelial NOS expression and NOS activity^{14,15}. The vascular calcium-dependent NOS activity is increased¹⁶, and the expression of ecNOS has been found to be increased along with NO synthesis in SHR¹⁷. However, the vascular expression of isoforms of NOS has not been established in various models of hypertension.

The present study was aimed at exploring the vascular expression of NOS isozymes in hypertension. Two-kidney, one clip (2K1C) and deoxycorticosterone acetate (DOCA)-salt hypertension were made in rats, and the expression of NOS isozymes in the thoracic aorta was determined by Western blot analysis.

MATERIAL AND METHOD

Development of hypertension

Male Sprague-Dawley rats (150~200 g) were used throughout the study. They were maintained according to the Institutional Guidelines for Experimental Animal Care and Use. 2K1C hypertension was made under ketamine (50 mg/kg, IP) anesthesia by constricting the left renal artery with a silver clip having an internal gap of 0.2 mm. The sham-clipped rats were operated as were 2K1C rats, except

for that no clipping was made.

To develop DOCA-salt hypertension, rats were subcutaneously implanted with silicone rubber containing DOCA (200 mg/kg), one week after they had been unilaterally nephrectomized, under ketamine anesthesia. They were then given with 0.9% saline to drink. Control rats were also unilaterally nephrectomized and subjected to saline drinking, but were not implanted with DOCA.

Protein preparation and Western blot analysis

The thoracic aorta was rapidly taken and stored at -70°C until used. The aorta was homogenized with Polytron homogenizer at 3,000 rpm in a solution containing 250 mmol/L sucrose, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.1 mmol/L phenylmethylsulfonyl fluoride and 5 mmol/L potassium phosphate buffer, at pH 7.6. Large tissue debris and nuclear fragments were removed by two low speed spins in succession ($3,000\times\text{g}$, 5 min; $10,000\times\text{g}$, 10 min). Protein concentration of the homogenate was determined by the method of Bradford¹⁸, with bovine serum albumin as a standard. The pellet was resuspended for protein blotting of ecNOS, and the supernatant was used for blotting of bNOS and iNOS.

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

Measurements of nitrites

Plasma and vascular tissue nitrite(+nitrate) levels were measured with a colorimetric assay kit (Oxford; Oxford, MI, USA). A microplate was used to perform enzyme reactions

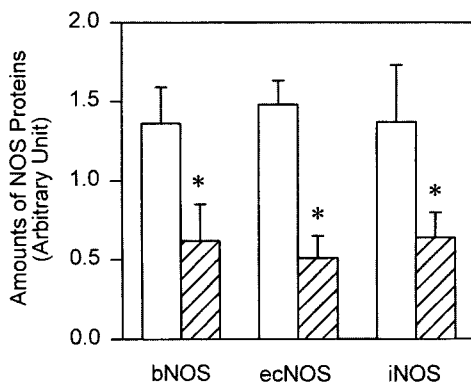
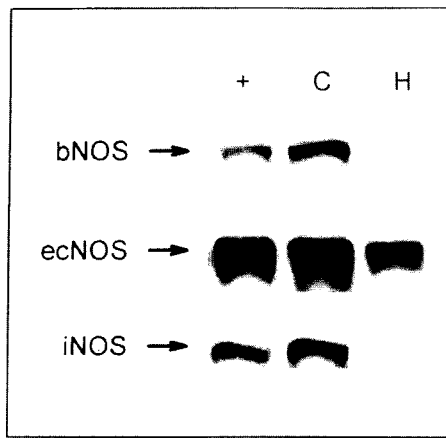


Fig. 1. Representative Western blot of the thoracic aorta and its densitometric analysis in 2K1C rats. One hundred μ g of proteins were loaded into each lane. Rat pituitary (bNOS), human endothelial cell (ecNOS), and inducible macrophage NOS (iNOS) were used as positive control (+). [H] denotes hypertension, and [C] corresponding control. Open column indicates the control and hatched column denotes the hypertensive groups. Each column represents mean \pm SEM of 4 experiments. * $p < 0.05$, compared with control.

in vitro. Eighty mL MOPS (50 mmol/L)/EDTA (1 mmol/L) buffer and 5 μ L samples were added to wells. Nitrate reductase (0.01 U) and 10 μ L 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 in a microtiter plate reader. The concentration of nitrite was determined from a standard curve which was constructed by standard reagents included in the kit. The protein concentration of the tissue aliquot was determined by the method of Bradford¹⁸.

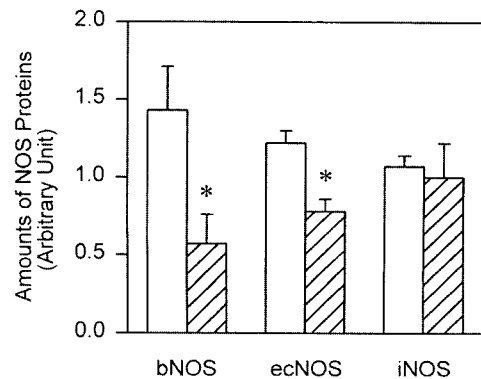
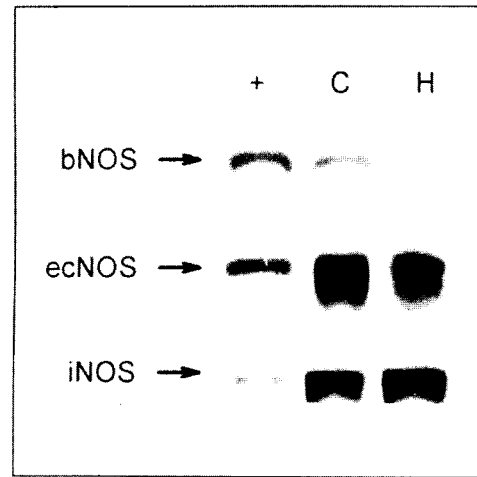


Fig. 2. Representative Western blot of the thoracic aorta and its densitometric analysis in DOCA-salt rats. Each column represents mean \pm SEM of 4 experiments. Legends as in Fig. 1. * $p < 0.05$, compared with control.

Statistics

Data were expressed as mean \pm SEM. Comparisons between hypertensive and control rats were made by non-paired t-test.

RESULT

Four weeks after developing the hypertension, mean arterial pressure measured from the femoral artery under thiopental (50 mg/kg, IP) anesthesia was significantly higher in hypertensive groups than in their corresponding controls (167 ± 8 vs 105 ± 6 mmHg in 2K1C rats, $n=6$ each, $p < 0.01$; 163 ± 6 vs 109 ± 5 mmHg in DOCA-salt rats, $n=6$ each, $p < 0.01$).

Anti-bNOS, anti-ecNOS, and anti-iNOS monoclonal antibodies hybridized with proteins of approximately 155 kDa, 140 kDa, and 130 kDa, respectively. bNOS as well as ecNOS and iNOS were decreased in 2K1C hypertension (Fig. 1). In DOCA-salt hypertension, iNOS was not changed, but ecNOS and bNOS were decreased (Fig. 2).

The vascular contents of nitrites were significantly decreased in the hypertensive groups than in controls in DOCA-salt (9.7 ± 2.7 vs 20.8 ± 4.2 nmol/mg protein, $n=5$ each, $p<0.05$) as well as in 2K1C rats (9.2 ± 1.5 vs 23.5 ± 2.4 nmol/mg protein, $n=5$ each, $p<0.01$).

DISCUSSION

An altered NO synthesis and release may be attributed to an altered activity of NOS. Indeed, the impaired endothelium-dependent relaxation in response to acetylcholine in hypertensive Dahl salt-sensitive rats¹⁹⁾ has been found to be associated with a diminished NOS activity¹³⁾. On the contrary, in hypertensive SHR, endothelium-dependent relaxation mediated by NO is normal, and aortic calcium-dependent NOS activity is rather increased¹⁶⁾. Whereas inducible NO production is impaired in association with a mutation of iNOS gene in salt-sensitive Dahl rats²⁰⁾, the vascular expression of iNOS is also increased in SHR²¹⁾. The expression of NOS isozymes has not been established in various models of hypertension.

The present study showed that the vascular expression of bNOS as well as that of ecNOS was decreased in 2K1C and DOCA-salt hypertension. The expression of iNOS was decreased in 2K1C hypertension, and it remained unaltered in DOCA-salt hypertension. The vascular contents of nitrites were decreased in both models of hypertension. Taken together, it is suggested that the attenuated activity of NO system in hypertension is, at least in part, attributed to a diminished expression of NOS and subsequent decrease of NO synthesis.

It has been found that a short-term infusion of angiotensin II leads to graded increases in vascular resistance and NO generation, as evidenced by an increased urinary excretion of nitrites²²⁾. In addition, angiotensin II does not directly influence glomerular endothelial cell calcium concentration or NO generation²³⁾. In this context, the pressor effect associated with an increased circulating angiotensin II in 2K1C hypertension may not be attributed to a primary decrease of NOS expression and NO synthesis in the vasculature.

The endothelial abnormalities observed in hypertensive blood vessels can be reversed by appropriate antihypertensive therapy^{13,24)}. This finding suggests that the reduced endothelium-dependent relaxation responsiveness does not play a primary role in initiating the hypertensive process, but

it rather results from a chronic exposure of the vasculature to the high blood pressure. The endothelial dysfunction may not be a cause but a consequence of the hypertension. At later stages of the disease, however, the reduced relaxation could contribute to the maintenance of an elevated peripheral vascular resistance and favor the occurrence of complications such as atherosclerosis²⁵⁾. On the contrary, increased NOS activity in SHR^{16,17,26)} would represent physiological adaptation to the increased hemodynamic forces in hypertensive states. It is likely that the vascular NOS activity which differs among various models of hypertension partly explain the different rates of occurrence of end-organ disease in hypertension. Indeed, some previous studies demonstrated that NOS activity was negatively correlated with end-organ disease in hypertension^{16,26)}.

In summary, 2K1C and DOCA-salt hypertension are associated with decreases in the vascular expression of NOS and NO production. The attenuated activity of NO system could contribute to an elevated peripheral vascular resistance, maintaining the hypertension.

REFERENCES

1. Palmer RM, Ferrige AG, Moncada S. *Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor*. Nature 1987;327:524-6.
2. Förstermann U, Schmidt HH, Pollock JS, Sheng H, Mitchell JA, Warner TD. *Isoforms of nitric oxide synthase: characterization and purification from different cell types*. Biochem Pharmacol 1991;42:1849-57.
3. Sessa WC, Harrison JK, Barber CM, et al. *Molecular cloning and expression of a cDNA encoding endothelial cell nitric oxide synthase*. J Biol Chem 1992;267:15274-6.
4. Sessa WC, Harrison JK, Luthin DR, Pollock JS, Lynch KR. *Genomic analysis and expression patterns reveal distinct genes for endothelial and brain nitric oxide synthase*. Hypertension 1993;21:934-8.
5. MacNaul KL, Hutchinson NI. *Differential expression of iNOS and cNOS mRNA in human vascular smooth muscle cells and endothelial cells under normal and inflammatory conditions*. Biochem Biophys Res Commun 1993;196:1330-4.
6. Konishi M, Su C. *Role of endothelium in dilator responses of spontaneously hypertensive rat arteries*. Hypertension 1983;5:881-6.
7. Panza JA, Quyyumi AA, Brush JE, Epstein SE. *Abnormal endothelium-dependent vascular relaxation in patients with essential hypertension*. N Engl J Med 1990;323:22-7.
8. Dohi Y, Criscione L, Lüscher TF. *Renovascular hypertension impairs formation of endothelium-derived relaxing factors and sensitivity to endothelin-1 in resistance arteries*. Br J Pharmacol 1991;104:349-54.
9. Tuncer M, Vanhoutte PM. *Response to the endothelium-*

- dependent vasodilator acetylcholine in perfused kidneys of normotensive and spontaneously hypertensive rats.* Blood Press 1993;2:217-20.
10. Taddei S, Virdis A, Mattei P, Salvetti A. *Vasodilatation to acetylcholine in primary and secondary forms of human hypertension.* Hypertension 1993;21:929-33.
 11. Lee J, Choi KC, Yeum C-H, Kim W, Yoo K, Park JW, Yoon PJ. *Impairment of endothelium-dependent vasorelaxation in chronic two-kidney, one clip hypertensive rats.* Nephrol Dial Transplant 1995;10:619-23.
 12. Kelm M, Preik M, Hafner DJ, Strauer BE. *Evidence for a multifactorial process involved in the impaired flow response to nitric oxide in hypertensive patients with endothelial dysfunction.* Hypertension 1996;27:346-53.
 13. Hayakawa H, Coffee K, Raij L. *Endothelial function and cardiorenal injury in experimental salt-sensitive hypertension: effects of antihypertensive therapy.* Circulation 1997;96:2407-13.
 14. Buga GM, Gold ME, Fukuro JM, Ignarro LJ. *Shear stress-induced release of nitric oxide from endothelial cells grown on beads.* Hypertension 1991;17:187-93.
 15. Awolei MA, Widmann MD, Sessa WC, Sumpio BE. *Cyclic strain increases endothelial nitric oxide synthase activity.* Surgery 1994;116:439-44.
 16. Hayakawa H, Raij L. *The link among nitric oxide synthase activity, endothelial function, and aortic and ventricular hypertrophy in hypertension.* Hypertension 1997;29:235-41.
 17. Vaziri ND, Ni Z, Oveisi F. *Upregulation of renal and vascular nitric oxide synthase in young spontaneously hypertensive rats.* Hypertension 1998;31:1248-54.
 18. Bradford MM. *A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding.* Anal Biochem 1976;72:248-54.
 19. Lüscher TF, Raij L, Vanhoutte PM. *Endothelium-dependent vascular responses in normotensive and hypertensive Dahl rats.* Hypertension 1987;9:157-63.
 20. Chen PY, Gladish RD, Sanders PW. *Vascular smooth muscle nitric oxide synthase anomalies in Dahl-Rapp salt-sensitive rats.* Hypertension 1998;31:918-24.
 21. Wu CC, Hong HJ, Chou YA, Yen MH. *Evidence for inducible nitric oxide synthase in spontaneously hypertensive rats.* Biochem Biophys Res Commun 1996;228:459-66.
 22. Deng X, Welch WJ, Wilcox CS. *Renal vasoconstriction during inhibition of nitric oxide synthase: effects of dietary salt.* Kidney Int 1994;46:639-46.
 23. Marsden PA, Brock TA, Ballermann BJ. *Glomerular endothelial cells respond to calcium-mobilizing agonists with release of EDRF.* Am J Physiol 1990;258:F1295- F303.
 24. Lüscher TF, Vanhoutte PM, Raij L. *Antihypertensive treatment normalizes decreased endothelium-dependent relaxations in salt-induced hypertension of the rat.* Hypertension 1987;9(suppl III):III193-7.
 25. Shimokawa H, Vanhoutte PM. *Impaired endothelium-dependent relaxation to aggregating platelets and related vasoactive substances in porcine coronary arteries in hypercholesterolemia and atherosclerosis.* Circ Res 1989; 64:900-4.
 26. Hayakawa H, Raij L. *Nitric oxide synthase activity and renal injury in genetic hypertension.* Hypertension 1998; 31:266-70.

=국문초록=

배경: 혈관 내피층에서 분비되어 평활근층 이완을 일으키는 물질의 본체는 산화질소(nitric oxide, NO)이며 NO synthase(NOS)에는 뇌형(brain NOS, bNOS), 내피세포형(endothelial constitutive NOS, ecNOS) 및 유도형(inducible NOS, iNOS) 등 세 가지 동위효소가 있음이 알려져 있다. 고혈압은 혈관 내피층 기능 이상을 보임이 알려져 있으나 NOS 동위 효소의 변화를 포함한 세포내 기전은 아직 확실치 않다. 저자들은 고혈압 기전을 구명하기 위한 일환으로 고혈압 혈관에서 NOS 동위효소가 어떻게 변화되는가 조사하고자 하였다. **대상 및 방법:** 흰쥐에서 two-kidney, one clip (2K1C) 고혈압과 deoxycorticosterone acetate(DOCA)-salt 고혈압을 일으켰다. 4주 뒤 고혈압이 일어난 것을 확인하고 적출 흉부 대동맥 표본에서 Western blot 분석에 의한 NOS 동위효소 발현 조사 및 비색법에 의한 조직내 산화질소 정량을 하였다. **결과:** 2K1C 및 DOCA-salt 흰쥐에서 실험군은 각각의 대조군에 비해 유의하게 높은 혈압을 보였다. 두 고혈압군에서 모두 적출 대동맥 표본의 bNOS 및 ecNOS 단백질 발현이 감소되었다. iNOS 단백질은 DOCA-salt 고혈압에서 변화를 보이지 않으나 2K1C 고혈압에서는 역시 감소를 보였다. 혈관조직내 산화질소 함량은 두 고혈압에서 모두 유의하게 감소되었다. **결론:** 2K1C 및 DOCA-salt 고혈압에서 혈관의 NOS 발현과 산화질소 함량이 감소되어 있으며 이는 고혈압의 유지 기전에 공헌하리라 추측되었다.

중심단어: 1. 산화질소
2. 고혈압