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Increased Expression of Nitric Oxide Synthase Coincides with Reversal of Renovascular Hypertension

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The present study was aimed at investigating whether there are changes in the expression of nitric oxide synthase (NOS) in relation with the unclipping-induced fall of blood pressure in two-kidney, one clip (2K1C) hypertension. Male Sprague-Dawley rats were made 2K1C hypertensive by clipping the left renal artery for four weeks. Sham-clipped rats served as control. The expression of endothelial constitutive (ec) NOS proteins and tissue levels of NO metabolites were determined in the kidney. Systolic blood pressure was significantly increased in clipped rats compared with that in the control. The development of hypertension was associated with decreases in the expression of ecNOS proteins and tissue levels of NO metabolites in the clipped kidney. The blood pressure at twenty-four hours after removal of the renal arterial clip fell to the control level. Accordingly, in the unclipped kidney, the expression of ecNOS proteins and tissue contents of NO metabolites were increased to the control level. The contralateral kidney was not affected by the development or reversal of hypertension. It is suggested that an enhanced expression of ecNOS in the unclipped kidney is an important component in the reversal of renovascular hypertension.

Key Words: Two-kidney, One clip hypertension, Unclipping, Nitric oxide synthase

INTRODUCTION

It has long been known that two-kidney, one clip (2K1C) hypertension is rapidly and completely reversed by removal of the renal arterial clip. Although the hypertension has been primarily attributed to an enhanced activity of the renin-angiotensin system, its blockade does not fully restore the normal blood pressure (Russell et al, 1982; Huang & Navar, 1983). In addition, the replacement of the urinary volume lost following the removal of the arterial clip does not prevent the depressor response (Neubig & Hoobler, 1975). Changes in cardiac output or vascular structure cannot also account for the unclipping-induced fall of blood pressure (Hallbäck-Nordlander et al, 1979; Lundgren & Weiss, 1979). The mechanisms underlying the unclipping-induced fall of blood pressure remain to be further determined.

With the recent emergence of its diverse roles in the body fluid and blood pressure homeostasis, nitric oxide (NO) has been of particular attraction in relation with the unclipping-induced reversal of 2K1C hypertension. An acute reversal of 2K1C hypertension is completely eliminated by inhibiting the synthesis of NO with an L-arginine analogue, N^{ω}-nitro-L-arginine methyl ester (Beierwaltes et al, 1995). The hypotensive response to the unclipping is blunted by the blockade of NO synthesis in one-kidney, one clip hypertensive rats (Fenoy et al, 1997; Huang & Tasi, 1998). These findings suggest a role of NO in mediating the unclipping-induced reversal of hypertension.

The synthesis of NO is catalyzed by a family of NO synthases (NOS). Therefore, an altered NO activity may be a reflection of an altered expression of NOS. We have indeed shown that tissue NO contents are decreased along with the expression of endothelial constitutive (ec) NOS in the clipped kidney in 2K1C hypertension (Yang et al, 1998). However, the expression of NOS has not been determined in relation with

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the unclipping-induced reversal of 2K1C hypertension. The present study was aimed to investigate whether the unclipping in experimental 2K1C hypertension alters the expression of NOS. Rats were made 2K1C hypertensive and their expression of NOS proteins was determined.

METHODS

Animals

Male Sprague-Dawley rats weighing 160～190 g were used. 2K1C hypertension was induced by clipping the left renal artery with a silver clip having an inner gap of 0.25 mm through an abdominal incision under anesthesia with ketamine (50 mg/kg, IP). Sham-clipped rats served as control. To avoid early acute phase of renin-dependent period, the rats were used four weeks after inducing the hypertension.

Systolic blood pressure was measured under a conscious state using a tail-cuff method. After measurement of the blood pressure, some rats were removed of their renal arterial clips under ketamine anesthesia. Twenty-four hours after removal of the clip, the rats were again measured of their systolic blood pressure. The rats were then killed by decapitation under a conscious state to collect the trunk blood. The plasma was kept at $-20^{\circ}C$. Kidneys, thoracic aorta and brain were rapidly taken, and stored at $-70^{\circ}C$ until assayed.

Protein preparation and Western blot analysis

The kidneys, thoracic aorta and brain were thawed and homogenized with Polytron homogenizer at 3,000 rpm in a solution containing 250 mmol/L sucrose, 1 mmol/L ethylenediamine tetraacetic acid (EDTA), 0.1 mmol/L phenylmethylsulfonyl fluoride and 20 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 g, 5 min; 10,000 g, 10 min). The total protein solution was centrifuged at 100,000 g for 1 h. The supernatant was used for blotting of brain (b) NOS, and the pellet was resuspended for blotting of ecNOS. The protein concentration was determined by the method of Bradford (1976) with bovine serum albumin as a standard.

Western blotting was done according to the method of Mattson & Higgins (1996), with a slight modification. Protein samples (70 μg) were electrophoretically size-separated with a discontinuous system consisting of 6% polyacrylamide resolving gel and 5% polyacrylamide stacking gel, and transferred to a nitrocellulose membrane at 20 V and 100 mA overnight. The membrane was washed, blocked, incubated with a 1 : 2,500 dilution of monoclonal mouse anti-NOS antibodies (Transduction Laboratories; Lexington, KY, USA), and then incubated with a horseradish peroxidase-labeled goat anti-mouse IgG (1 : 1,000). The bound secondary antibody was detected by enhanced chemiluminescence (Amersham; Buckinghamshire, England). The protein level was determined by quantifying the signals of autoradiograms.

Colorimetric assay of nitrite/nitrate

Nitrite/nitrate levels were measured with a colorimetric NO assay kit (Oxford; Oxford, MI, USA). A microplate was used to perform enzyme reactions in vitro. For spectrophotometric assay of nitrites with Griess reagent, 65 μL 3-[N-morpholino]profanesulfonic acid (50 mmol/L)/EDTA (1 mmol/L) buffer and 20 μL tissue samples were added to wells in duplicates. 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 absorbance values at 540 nm were read in a microtiter plate reader (Bio-Rad; Hercules, CA, USA).

Statistical analysis

Results were expressed as means$\pm$SEM. The statistical significance of differences between the groups was determined using Student t-test.

RESULTS

Systolic blood pressure was significantly increased in clipped rats compared with that in the control (168 $\pm$3 mmHg, n=7; vs 127$\pm$3 mmHg, n=7, $p<0.01$). The blood pressure at twenty-four hours after removal of the renal arterial clip fell to 129$\pm$4 mmHg (n=7), being comparable to that in the control.

The expression of ecNOS proteins in the clipped kidney is shown in Fig. 1, and that in the contralateral kidney is depicted in Fig. 2. Along with the high blood pressure, the abundance of ecNOS proteins was

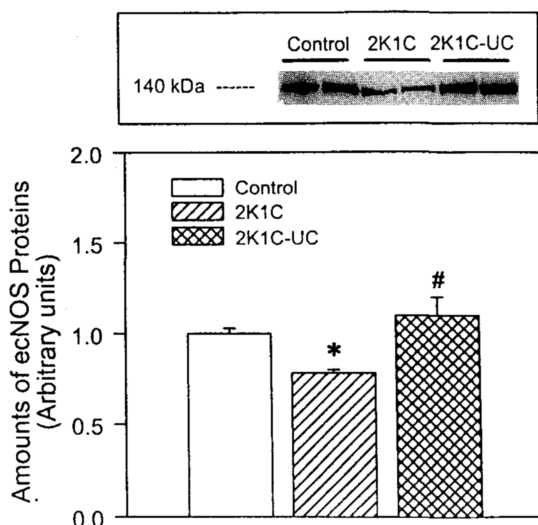

Fig. 1. Representative autoradiograms and densitometric data of ecNOS proteins in the clipped kidney. The renal arterial clip was intact in 2K1C, and was removed 24 hours before the experiment in 2K1C-UC. Each column shows mean±SEM of 7 rats. *$p<0.01$, compared with control. #$p<0.01$, compared with 2K1C.

Table 1. Nitrite/nitrate levels in the clipped and contralateral kidneys, thoracic aorta, and plasma

	Control	2K1C	2K1C-UC
Clipped kidney (nmol/mg protein)	1.14±0.12	0.69±0.09*	1.07±0.08#
Contralateral kidney (nmol/mg protein)	0.94±0.09	0.98±0.22	0.95±0.09
Thoracic aorta (nmol/mg protein)	1.22±0.14	1.11±0.14	1.08±0.13
Plasma (mol/L)	38.9±7.0	42.1±12.3	40.4±8.4

Numbers of rats were 7 in each group. The arterial clip was intact in 2K1C, and was removed 24 hours before the experiment in 2K1C-UC. *$p<0.01$, compared with control. #$p<0.01$, compared with 2K1C.

decreased in the clipped kidney, whereas it remained unaltered in the contralateral non-clipped kidney. Following removal of the arterial clip, the expression of ecNOS proteins was increased to the control level in the unclipped kidney, while not significantly altered in the contralateral kidney. Neither the expression of ecNOS in the thoracic aorta nor that of bNOS in the brain stem was significantly affected by the development or reversal of hypertension (data not shown).

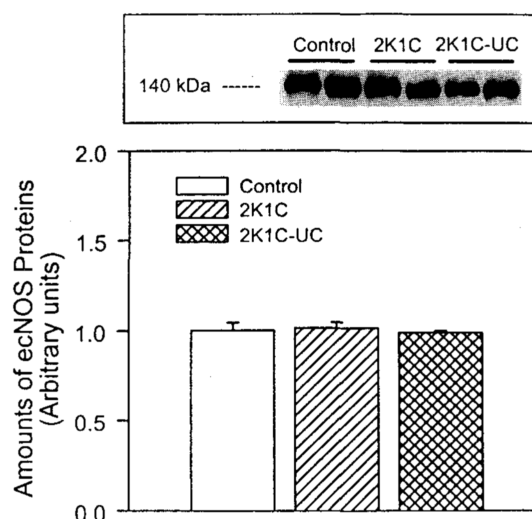

Fig. 2. Representative autoradiograms and densitometric data of ecNOS proteins in the contralateral kidney. Each column shows mean±SEM of 7 rats. Legends as in Fig. 1.

Tissue levels of NO metabolites are presented in Table 1. They were significantly decreased in the clipped kidney, which was normalized by unclipping. NO metabolites in the contralateral non-clipped kidney remained unaltered by the development or reversal of hypertension. Neither in the thoracic aorta nor in the plasma, NO metabolite levels were significantly altered.

DISCUSSION

The blood pressure was significantly increased in clipped rats. Along with the hypertension, tissue contents of NO metabolites and ecNOS expression were decreased in the clipped kidney. Following removal of the arterial clip, the blood pressure fell to the control level. Accordingly, the tissue contents of NO metabolites and the abundance of ecNOS proteins increased to the control level in the unclipped kidney. In the contralateral kidney, tissue contents of NO metabolites and ecNOS expression were not affected by the development or reversal of hypertension.

It has been shown that an inhibition of NOS produces a greater reduction of vasorelaxation to acetylcholine and an exaggerated hypertension in spontaneously hypertensive rats (SHR) (Lacolley et al, 1991; Lee & Webb, 1992). Furthermore, NO synthesis has been found increased in SHR (Nava et al, 1996; Kim et al, 1999). The enhanced NO activity has been

regarded as a mechanism counteracting the high blood pressure in this model of hypertension. It is speculated that an augmentation of ecNOS expression and NO production in the unclipped kidney contributes to reversal of the hypertension. An acute increase of perfusion pressure due to unclipping may result in an augmented endothelial shear stress and NOS expression to facilitate the local synthesis of NO in the unclipped kidney. The speculation may be supported by the previous observation that the unclipping reduces the vasoconstrictor response in *ex vivo* blood-perfused tail arteries only when the endothelium is intact (Gerkens, 1989).

However, the linkage between the altered NO activity and the depressor effect is not clear. Medullipin, a polar lipid released from the renal medulla, may be one of candidates for the fall of blood pressure after unclipping. Bergström et al (1992) observed that N^w-nitro-L-arginine, an inhibitor of NO synthesis, inhibits the renomedullary vasodepressor response. Göthberg (1994) also showed that the medullary depressor mechanism would not be activated when NO activity was blocked. A blockade of NO activity decreases Po_2 in the renal medulla, which in turn reduces the local medullipin release (Brezis et al, 1991). Taken together, an increased renomedullary blood flow or flow-related increase in local oxygen tension may trigger the medullipin release via the action of NO.

In the central nervous system, neurons containing bNOS immunoreactivity and producing NO exist in the nucleus of the tractus solitarius (NTS) and the rostral ventrolateral medulla, known as pressor area (Ciriello et al, 1986; Ohta et al, 1993). However, the expression of bNOS may not be related with the development of hypertension. For instance, SHR showed no differences in bNOS levels in the brain stem from control rats (Clavier et al, 1994). There also have been no significant changes in the expression of bNOS in the dorsal medulla, including NTS, after clipping the unilateral renal artery (Krukoff et al, 1995). The present study also showed that the expression of bNOS in the brain stem was not altered by either development or reversal of 2K1C hypertension.

Furthermore, the expression of ecNOS in the thoracic aorta was not affected by the development or reversal of hypertension. Accordingly, the contents of NO metabolites in the thoracic aorta or in the plasma were not significantly altered. An altered vascular NO activity is unlikely to be involved in the reversal of 2K1C hypertension. The kidney is also known to express bNOS and iNOS immunoreactivities, and their physiological functions in relation with renin release and tubuloglomerular feedback have been suggested (Mundel et al, 1992; Tojo et al, 1994). Future studies will be needed to explore whether an altered role of different isoforms of NOS other than ecNOS is related with the reversal of 2K1C hypertension.

In summary, it is suggested that an enhanced ecNOS expression and NO synthesis in the unclipped kidney is an important component in the unclipping-induced reversal of renovascular hypertension.

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