Laboratory Studies

Vascular Renin-Angiotensin System in Two-Kidney, One Clip Hypertensive Rats

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SUMMARY The possible role of the renin-angiotensin system in the maintenance of hypertension in two-kidney, one clip hypertensive rats was studied. Plasma renin activity rose rapidly and markedly in association with the elevation of blood pressure and then decreased gradually, although blood pressure remained high. Renin activity in the lung, aorta, and mesenteric artery also increased with the development of hypertension and then decreased in a way similar to that of plasma renin activity at the chronic stage of hypertension. Plasma angiotensin converting enzyme activity did not change significantly until 16 weeks after unilateral renal artery clipping, whereas vascular angiotensin converting enzyme activity significantly increased at the chronic, but not the acute, stage of hypertension. In chronically renal hypertensive rats, 1-sarcosine, 8-isoleucine angiotensin II or enalapril, an angiotensin converting enzyme inhibitor, lowered the blood pressure and enalapril also lowered the angiotensin converting enzyme activity of vascular tissues. The constrictor effect of angiotensin I was greater in isolated arteries from chronically hypertensive rats than in those from age-matched normotensive rats. These results suggest that the vascular renin-angiotensin system plays an important role in the maintenance of two-kidney, one clip hypertension. Elevated vascular angiotensin converting enzyme activity appears to increase local production of angiotensin II, which results in vasoconstriction by acting directly and indirectly through adrenergic nerves on vascular smooth muscle.

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KEY WORDS • angiotensin converting enzyme • renal hypertension • angiotensin

THE renin-angiotensin (RA) system plays an important role in the regulation of blood pressure and salt-water balance. Although overacceleration of this system was once considered a major cause of hypertension, this view is no longer prevalent because of the existence of hypertension with low levels of plasma renin activity (PRA), as observed in spontaneous hypertension in rats and low renin essential hypertension in humans. Furthermore, the intriguing findings that angiotensin converting enzyme (ACE) inhibitors can effectively relieve low renin hypertension has directed attention to the kallikrein-kinin system and the prostaglandin system, because ACE inhibitors prevent the destruction of bradykinin and bradykinin lowers blood pressure directly or by stimulating the production of vasodilator prostaglandins.

The plasma RA system may not be the only RA system; components of the RA system are also present in extrarenal tissues, including blood vessels. It is possible that locally generated angiotensin II modulates the tissue function. To assess the role of the tissue RA system in hypertension, the present study compared the angiotensin II-generating system in plasma and tissue during the development and maintenance of hypertension in two-kidney, one clip (2K1C) hypertensive rats and examined alterations of the vascular RA system in prolonged hypertensive animals.

Materials and Methods

Six-week-old male Wistar rats (weight 200-225 g) were divided into two groups: an intact control group and a 2K1C renal hypertensive group. The rats were fed regular rat chow (Clea Japan, Tokyo, Japan), given free access to tap water, and housed under identical conditions until the start of the experiments.
Experimental Protocol

To induce 2K1C hypertension, the rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.), and the left renal artery was partially occluded by a silver clip (inside diameter, 0.2 mm) while the right renal artery was left intact. Only animals with a mean blood pressure (MBP) greater than 150 mm Hg were used. Rats were killed by exsanguination 2, 4, 8, and 16 weeks after clipping, under ether anesthesia. The lung, whole aorta, and mesenteric plus jejunal arteries were immediately removed to prepare samples for the assay of tissue renin and ACE activities. Before exsanguination, the MBP was measured and 1-ml blood samples were obtained for the determination of PRA and ACE activity.

For the measurement of MBP, rats were anesthetized with ether, and a cannula was inserted in the left femoral artery and connected to a pressure transducer (Nihon Kohden Kogyo, Tokyo, Japan). The MBP was obtained 3 to 5 hours after the recovery of anesthesia.

Fourteen weeks after clipping, five 2K1C rats were used to assess the effect of [Sar',Ile']angiotensin II (Protein Research Foundation, Osaka, Japan). Four age-matched control rats also were used for comparison. The MBP was monitored continuously while [Sar',Ile']angiotensin II was infused through a cannula inserted into the femoral vein at rates of 250, 500, and 1000 ng/min. Decreases in MBP from the preinfusion level and the time needed to attain the maximal effect at each dose were obtained.

To assess the effect of enalapril (MK 421; Merck-Japan, Tokyo, Japan) on chronic renal hypertension, enalapril (0.5 mg/kg bolus) was injected into the femoral vein of five 2K1C rats 16 weeks after clipping and the decreases in MBP and vascular ACE activity were determined.

To assess the effect of contractions induced by angiotensin I and angiotensin II, the aorta and mesenteric artery were isolated from 2K1C rats 16 weeks after clipping or age-matched control rats killed by exsanguination. The vessel was then cut into helical strips, which were fixed vertically between hooks in a muscle bath containing modified Ringer-Locke solution (composition: 118.3 Na+, 5.4 K+, 2.2 Ca²⁺, 1.0 Mg²⁺, 131.6 Cl⁻, 25.0 HCO₃⁻, 5.6 dextrose, pH 7.3–7.4) maintained at 37 ± 0.3°C and aerated with a mixture of 95% O₂, 5% CO₂. Resting tensions were adjusted to 2 and 1 g for the aorta and mesenteric artery, respectively. Isometric contractions and relaxations were displayed on an ink-writing oscillograph (Nihon Kohden Kogyo). Acetylcholine chloride (Daichi Pharmaceutical, Tokyo, Japan) was added to the arterial strips precontracted with prostaglandin E₂ (Ono Pharmaceutical, Osaka, Japan) to determine the existence of endothelium functionally. Only the arteries, in which marked relaxations were induced by acetylcholine, were used for further experiments. At first, the responses to angiotensin II (Protein Research Foundation), in a concentration known to induce maximal contraction (10⁻⁷ M), were taken three times to eliminate the influence of tachyphylaxis. Responses to angiotensin I (5 × 10⁻⁸ and 10⁻⁷ M; Protein Research Foundation) and angiotensin II (10⁻⁷, 2 × 10⁻⁸, 5 × 10⁻⁸, and 10⁻⁷ M) then were obtained. We have previously reported that angiotensin I is converted to angiotensin II by vascular ACE and that the octapeptide, but not the decapetide, produces contractions of isolated dog arteries.

Assay of Renin Activity

The PRA was measured by radioimmunoassay of angiotensin I using a CEA-IRE-SORIN kit (Paris, France). For assessment of tissue renin activity, fresh tissues were weighed, cut into small pieces with scissors, and homogenized in 10 mM tris (hydroxymethyl) aminomethane chloride (Tris-HCl) buffer (pH 7.4) containing a mixture of protease inhibitors: 5 mM phenylmethylsulfonyl fluoride (PMSF; Sigma Chemical Corp., St. Louis, MO, USA), 5 μg/ml leupeptin (Protein Research Foundation), and 1 mM ethylenediaminetetraacetic acid (EDTA; Sigma). After centrifugation at 800 g for 10 minutes, the supernatant was incubated at 37°C for 6 hours with bilaterally nephrectomized rat plasma in 0.2 M 2-(N-morpholino)ethanesulfonic acid buffer, pH 6.2, containing 5 mM PMSF, 10 mM EDTA, 35 μM captopril (Sankyo Pharmaceuticals, Tokyo, Japan), 15 μM bestatin (Protein Research Foundation), and 0.1% neomycin sulfate (Sigma).

Angiotensin I generated during incubation was measured by radioimmunoassay as described above. To exclude the contribution of nonspecific angiotensin I-generating enzymes other than renin, renin activity was defined as the difference in the sample's angiotensin I-generating activity with and without preincubation with specific anti-rat renin antibody. The tissue renin activity value was divided by protein concentration measured by the modified method of Bradford and expressed as a specific activity (ng/hr/mg protein). In preliminary experiments, we measured tissue renin activities in the lung, aorta, and mesenteric artery of rats that had been nephrectomized for 12, 24, and 48 hours and confirmed the existence of tissue renin 48 hours after nephrectomy: 0.05 ± 0.02 in the lung, 0.11 ± 0.03 in the aorta, 0.11 ± 0.02 in the mesenteric artery, and 0.002 ± 0.0007 ng/hr/mg protein in plasma.

Assay of Angiotensin Converting Enzyme Activity

The ACE activity was determined by using the modified method of Cushman and Cheung; the detailed procedure to increase the sensitivity has been described previously. Plasma ACE activity was determined as follows. Plasma, 50 μl, was incubated at 37°C for 30 minutes with hippuryl-L-histidyl-L-leucine (final concentration, 5 mM; Protein Research Foundation) in 200 μl of 10 mM potassium phosphate buffer, pH 8.3, containing 300 mM sodium chloride. The enzyme reaction was stopped by the addition of 750 μl of 3% metaphosphate. (The blank sample was prepared by adding metaphosphate before incubation.) The reaction mix-
ture then was centrifuged at 12,000 g for 10 minutes, and 20 μl of the supernatant was applied to a reversed-phase 4.6 × 250-mm column (TSK gel 120A; Toyo Soda, Tokyo, Japan) and eluted at 38°C with 10 mM KH₂PO₄/methanol (1:1; pH 3.0) at a rate of 0.7 ml/min. Hippurate was detected by ultraviolet absorbance at 228 nm. The activity of ACE was expressed in milliunits per milliliter; 1 unit of ACE activity was defined as the amount of enzyme that generated 1 μmol of hippurate in 1 minute at 37°C.

Tissue ACE activity was determined as follows. Fresh tissues (wet weight, 30–500 mg) were minced finely and immediately placed in an ice-cold glass homogenizer containing 10 volumes of homogenization buffer. This solution consisted of 20 mM Tris-HCl buffer (pH 8.3), 5 mM magnesium acetate, 30 mM KCl, 0.25 M sucrose, and 0.5% Nonidet P-40. The suspended solution was well homogenized on ice (approximately 0°C) and stored overnight at 4°C. The next morning, the homogenized sample was centrifuged for 20 minutes at 20,000 g and 4°C. The supernatant, 50 μl, was then incubated with substrate as already described. Specific activity (μU/mg protein) was then obtained using tissue protein concentration values determined as just described.

Statistical Analysis

Results are expressed as mean values ± SEM. Statistical analyses for more than two groups were made using Tukey’s method after one-way analysis of variance, and those for two groups were made by Student’s unpaired t test.

Results

Two weeks after clipping, MBP was significantly higher in 2K1C rats (158.3 ± 6.0 mm Hg; n = 9) than in control rats and remained significantly elevated 4, 8, and 16 weeks after clipping (Figure 1). Conversely, MBP in six 22-week-old control rats (age-matched to 2K1C rats clipped for 16 weeks) was not significantly different from that in 6-week-old control rats (n = 18; 122.3 ± 3.0 vs 112.7 ± 2.0 mm Hg).

As shown in Figure 1, mean PRA values were drastically increased in the early stage of hypertension, reaching a peak 4 weeks after clipping (119.5 ± 11.2 ng/ml/hr; n = 10). This value was significantly higher than that seen in 6-week-old control rats (9.1 ± 1.4 ng/ml/hr). The PRA gradually decreased to 56.5 ± 8.2 ng/ml/hr at Week 16, which was significantly lower than the peak value but did not differ significantly from the value in 6-week-old control rats.

Renin activity in the lung, aorta, and mesenteric artery tended to undergo changes similar to those seen with PRA. The tissue renin activity in 2K1C rats 4 weeks after clipping (lung, 0.66 ± 0.17; aorta, 0.94 ± 0.20; mesenteric artery, 1.03 ± 0.40 ng/hr/mg protein; n = 4) was significantly higher than that seen in 6-week-old control rats (lung, 0.07 ± 0.01; aorta, 0.31 ± 0.14; mesenteric artery, 0.37 ± 0.07 ng/hr/mg protein; n = 5). The tissue renin activity in

2K1C rats 8 and 16 weeks after clipping was significantly less than that seen 4 weeks after clipping, but it was not significantly different from that recorded in 6-week-old control rats (Figure 2).

Plasma ACE activity was not significantly different before or during the course of hypertension (Figure 3). On the other hand, lung ACE activity in 2K1C rats 2, 4, 8, and 16 weeks after clipping was 149.4 ± 13.6 (n = 9), 145.2 ± 17.7 (n = 10), 150.2 ± 16.8 (n = 10), and 124.0 ± 7.7 μU/mg protein (n = 11), respectively, which was significantly higher than that.
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seen in 6-week-old (81.6 ± 4.3; n = 7; p < 0.05) and age-matched control rats (p < 0.05; Figure 4). Aortic ACE activity in 2K1C rats up to 8 weeks after clipping was not significantly different from that seen in 6-week-old (9.4 ± 1.0 mU/mg protein; n = 7) or in age-matched control rats. However, the activity was significantly higher in 2K1C rats 16 weeks after clipping (19.7 ± 2.7 mU/mg protein; n = 5, p < 0.05) than in 6-week-old or age-matched control rats (10.5 ± 1.2 mU/mg protein; n = 7). Similar results were obtained for ACE activity in mesenteric arteries. Only the ACE activity in 2K1C rats 16 weeks after clipping (12.9 ± 2.7 mU/mg protein; n = 5, p < 0.05) was significantly higher than that found in 6-week-old (8.0 ± 0.4 mU/mg protein; n = 7) or age-matched control rats (6.2 ± 1.0 mU/mg protein, n = 7; see Figure 4).

The MBP in 2K1C rats 14 weeks after clipping (191 ± 14 mm Hg, n = 5) was lowered by infusions of [Sar¹, Ile⁸]angiotensin II in a dose-dependent manner; however, the level attained still was higher than the MBP in control rats (Figure 5). The MBP returned to the preinfusion level after the drug infusion was stopped. The time required to attain the maximal effect of the angiotensin II antagonist did not differ appreciably among these responses. In contrast, [Sar¹, Ile⁸]angiotensin II (250–1000 ng/min) did not alter MBP in three control rats (127 ± 4 mm Hg) and only slightly elevated MBP in a fourth control rat (preinfusion, 132; infusion at 1000 ng/min, 156; postinfusion, 140 mm Hg).

Intravenous bolus injection of enalapril (0.5 mg/kg), an ACE inhibitor, significantly lowered the MBP (from 224 ± 10 to 199 ± 5 mm Hg; n = 5, p < 0.05) after 4 hours in 2K1C rats 16 weeks after clipping. Aortic ACE activity 4 hours after the enalapril injection was reduced to 11.50 ± 1.50 mU/mg protein (n = 5), which was significantly lower than the activity in 2K1C rats that did not receive enalapril (19.70 ± 2.50 mU/mg protein; n = 7).

Equipotent concentrations of angiotensin II, to produce the same magnitude of contractions induced by angiotensin I (5 x 10⁻⁴ and 10⁻³ M), were calculated from the dose-response curve of angiotensin II in each preparation. Mean values of the equipotent concentrations for aortas and mesenteric arteries isolated from 2K1C rats 16 weeks after clipping and age-matched control rats are tabulated in Table 1. The equipotent concentrations of angiotensin II in both aorta and mesenteric artery were significantly higher in 2K1C rats than in age-matched control rats.
Tissue Hypertensive Rats 16 Weeks After Clipping and Age-Matched Non-
Aorta and Mesenteric Arteries from Two-Kidney, One Clip

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Control rats</th>
<th>2K1C rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aorta</td>
<td>2.2 ± 0.5 (8)</td>
<td>3.9 ± 0.3* (8)</td>
</tr>
<tr>
<td>2K1C rats</td>
<td>3.1 ± 0.4 (9)</td>
<td>7.9 ± 0.8† (8)</td>
</tr>
<tr>
<td>Mesenteric artery</td>
<td>1.3 ± 0.2 (8)</td>
<td>4.0 ± 0.9 (10)</td>
</tr>
<tr>
<td>Control rats</td>
<td>2.1 ± 0.2* (7)</td>
<td>8.0 ± 0.5† (9)</td>
</tr>
</tbody>
</table>

Values are means ± SEM. Number of rats is shown in parentheses.

ANGL = angiotensin; 2K1C = two-kidney, one clip.
*p < 0.05, †p < 0.01, compared with values in control rats.

Discussion

The RA system plays an important role in the control of blood pressure and fluid homeostasis. Recently, attention has been directed to the RA system localized in vascular tissues rather than to the circulating RA system in an attempt to explain certain types of hypertension. Thurston et al.22, 23 showed that ACE inhibitors lowered the blood pressure in 2K1C hypertensive rats even after bilateral nephrectomy and that the aortic reninlike activity 6 weeks after clipping was significantly elevated, as compared with that in normotensive rats. Garst et al.24 using a bioassay method for angiotensin I, suggested that the activity was increased in the aorta during both the acute and chronic phases of renal hypertension. The aortic tissue contains angiotensin I-generating enzymes other than renin25; therefore, changes in renin activity in vascular tissue have not been examined specifically in the course of hypertension.

In the present study, we determined the vascular renin activity by using anti-rat renin antibody.10 Renin activity was elevated during the acute development of hypertension and returned to a normal level at the chronically hypertensive stage. These findings suggest that the vascular RA system is involved in the maintenance of renal hypertension, although increased production of angiotensin I by renin in the vascular wall is not responsible for the maintenance of hypertension.

Activation of the RA system in systemic circulation is responsible for the development of hypertension in the early stage, since clipping of the unilateral renal artery increases the release of renin from the kidney and plasma levels of renin and angiotensin II are elevated.26 The elevated blood pressure at this stage is thought to depend on angiotensin II levels in circulating plasma, since renin inhibitors,27 ACE inhibitors,28 and angiotensin II analogues29 are quite effective in lowering the blood pressure. The present study also found hyperreninemia in the early stage (2–4 weeks after renal artery clipping) of 2K1C hypertension. In the chronic stage of hypertension, however, PRA did not differ significantly from that seen in normotensive rats. On the other hand, ACE activity in the lung, aorta, and mesenteric artery of 2K1C rats 16 weeks after clipping was significantly higher than that found in age-matched normotensive rats. Similar results have also been obtained with vascular ACE activity in experiments with 2K1C hypertensive dogs (M. Miyazaki, unpublished observation).

Thickening of the vascular wall in hypertensive animals may be related to the increased ACE activity. The conversion rate from angiotensin I to angiotensin II in the vascular tissue of chronic 2K1C hypertensive rats was accelerated, as compared with that in normotensive rats, since angiotensin II concentrations sufficient to produce the same magnitude of contractions caused by angiotensin I in the aorta and mesenteric artery were significantly higher in 2K1C rats 16 weeks after clipping than in control rats in vitro. Similar results were obtained in preliminary studies in vivo, in which the ratios of 50% effective doses of angiotensin I and II needed to elevate the blood pressure in 2K1C and control rats were compared. Plasma ACE activity did not change significantly until 16 weeks after clipping. These observations led us to speculate that the increase in vascular ACE activity plays an important role in the maintenance of high blood pressure in the chronic stage of hypertension in 2K1C rats. However, this enzyme cannot be regarded as rate-limiting in angiotensin II production unless the correlation between ACE activity and systemic blood pressure is demonstrated more completely.

This hypothesis is supported by the findings that converting enzyme inhibitors significantly lowered the blood pressure with a significant inhibition of vascular ACE activity and that injections of an angiotensin II analogue, [Sar1,Ile8]angiotensin II, also lowered MBP significantly in chronically hypertensive rats. Thurston and Swales30 have also demonstrated a hypotensive action of angiotensin II analogue in 2K1C hypertensive rats more than 4 months after clipping. Angiotensin II–dependent hypertension can be expected to develop even though the PRA and plasma ACE activity are not increased, if local production of angiotensin II in the vascular wall is increased or if angiotensin II receptor sensitivity is increased. These increments augment vascular contractility due to the octapeptide’s actions on smooth muscle and on the adrenergic nerve terminals innervating the muscle.31 Our recent data suggest that the sensitivity of prejunctional angiotensin II receptors is significantly higher in chronically (8 months) hypertensive 2K1C dogs than in acutely (1 month) hypertensive or normotensive dogs.32 The importance of local generation of angiotensin II in the blood vessels of spontaneously hypertensive rats has also been suggested by Antonacchio and Kerwin,33 who postulated that the inability of the angiotensin II analogue to lower MBP in spontaneously hypertensive rats is associated with its low accessibility to nerve terminals.

Further study is underway to clarify whether our
hypothesis on the role of locally produced angiotensin II can be extended to other types of chronic hypertension with low PRA, such as low renin human essential hypertension and spontaneous hypertension in rats, in which ACE inhibitors are effective.

Acknowledgment
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References
Vascular renin-angiotensin system in two-kidney, one clip hypertensive rats. 
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