Analysis of Relationship Between Renin-Angiotensin System and Blood Pressure in Experimental Hypertension Induced by Kidney Extract

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JIN YAMAMOTO, AND KOICHI OGINO

Kidney extract (I), (II) and (III) were obtained from rats which were adrenalectomized and given tap water, adrenalectomized and given 10 g/l NaCl, and intact, respectively. When each extract was subcutaneously injected every 12 hours into uninephrectomized rats, the blood pressure was gradually elevated. This hypertension-inducing action was observed more or less in every extracts. The potency was (I) > (II) > (III) while no apparent disparity was found in renin content of the three extracts. During and after the development of hypertension, plasma renin level was not increased 12 hours after the injection except in the recipients of extract (I), even in the presence of a high blood pressure. However, from an aspect of changes following each individual injection, the effect of the kidney extract on the plasma renin level appeared to be parallel with its hypertension-inducing effect. The latter effect, therefore, could be attributed to a cumulative effect of an excessive fluctuation of the renin-angiotensin level or the blood pressure, although implication of other renal substances than renin would not be excluded. Continuous high level of plasma renin or angiotensin may accelerate the development of hypertension.

In spite of extensive studies by a number of investigators, the role of renin-angiotensin system in the pathogenesis of hypertension remained unestablished. Results from recent approaches to renal hypertension are rather conflicting. Interfering with renin-angiotensin system by angiotensin-antagonists, converting enzyme inhibitors, renin inhibitors and antirenin, or by passive immunization against angiotensin was reported successful in lowering the blood pressure – particularly in two-kidney type of renal clip hypertension. On the other hand, active immunization against angiotensin II did not either prevent the development of renovascular hypertension or ameliorate it.

Some investigators intended to produce sustained hypertension by administering angiotensin or renin into experimental animals and substantiate an excess of these substances as a cause of hypertensive disorders. Masson and associates were successful in producing a hypertensive disease with vascular changes similar to those of renovascular hypertension, by subcutaneous administration of renin-rich materials into the rat. However, Hirano and Masson found

Key Words:
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Renal renin
Plasma renin
Angiotensin II
Hypertension
Anesthesia

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low levels of plasma and renal renin in those hypertensive rats after 35 days of treatment. Kira et al., in a similar experiment, observed a discrepancy between renin content and hypertension-inducing potency of various kinds of kidney extracts. These findings throw doubt on the involvement of renin-angiotensin system in renal as well as "renin-induced" hypertension, and prompted us to study the latter kind of experimental hypertension in more detail with the hope of finding a clue to the nature of the hypertension-inducing action of renal substance. Kidney extract from adrenalectomized rats was chosen as a subject of this study, since it had a potent hypertension-inducing action for its renin content.

**Methods**

Closely inbred Wistar rats (Amimal Center of Kyoto University) were used as experimental animals.

**Preparation of Kidney Extract:** Rats were bled to death from the abdominal aorta under light ether anesthesia. Kidneys were removed immediately and stored frozen at $-20^\circ$C. They were thawed and homogenized with 1 ml of deionized water per gram of tissue in a Waring blender. The homogenate was frozen and thawed five times and adjusted to pH3.0 with 5 mol/l HCl. After 30 minutes, it was re-adjusted to pH7.0 with 5 mol/l NaOH and centrifuged at 10,000 g for 20 minutes. The supernatant was referred to as kidney extract and stored at $-20^\circ$C until it was used. All the procedures were performed below 4°C.

**Sources of Kidney Extracts:** Male or female rats weighing 170 to 200 g were divided into three groups. The first was adrenalectomized and given tap water. The second was also adrenalectomized but given 10 g/l NaCl. The third group was intact and given tap water. Six days after the operation all the three groups were killed and kidneys were taken for the preparation of extract. Kidney extracts obtained from the first, the second and the third groups were referred to as extract (I), (II) and (III), respectively.

**Plasma Angiotensin II Concentration:** It was determined by radioimmunoassay method of Gocke et al., using kits from CEA-CEN-Sorin.

**Plasma Renin Activity:** Angiotensin I released during incubation at 37°C for 30 minutes was measured using radioimmunoassay kits (Schwarz-Mann or CEA-CEN-Sorin) according to the method of Haber et al.

The activity was expressed in nanograms of angiotensin I released by 1 ml of plasma in 1 hour.

**Plasma Renin Concentration:** Partially purified rat renin substrate was prepared by the method of Boucher et al. with minor modifications and lyophilized. On the day of the assay, this was dissolved in phosphate buffer (0.1 mol/l, pH6.5) which contained 0.01 mol/l EDTA, 0.15 mol/l NaCl and 5 μmol/l phenylmercuric acetate. This buffer was used in common for assays of renal renin and renal substrate. The incubation mixture consisted of 0.1 ml of plasma, 5 μl of DFP (50 g/l solution in isopropyl alcohol), 10 μl of dimercaprol (100 g/l solution in peanut oil) and 0.5 ml of the buffer containing substrate equivalent to approximately 1,500 ng of angiotensin. The mixture was incubated at 37°C for 18 hours, and then adjusted to pH5.5 with hydrochloric acid. Tubes were placed in boiling water for 7 minutes and precipitated proteins were centrifuged off. The supernatant was bioassayed in pentolinium-treated rats anesthetized with amobarbital, against angiotensin II amide (Hypertensin-Ciba) as the standard. Unit was expressed in nanogram of angiotensin II equivalent produced by 1 ml of plasma in 1 hour.

**Renin Content of the Kidney and Kidney Extract:** The kidney extract was diluted to 1/100 with 9 g/l NaCl. Kidney was homogenized with 1 ml of deionized water per 100 mg of tissue in a Potter-Elvehjem homogenizer and diluted to 1/10 with saline. A 0.1 ml of sample was incubated with 15 μl of DFP (50 g/l solution) and 0.5 ml of the buffer containing substrate equivalent to approximately 3,000 ng of angiotensin, at 37°C for 15 minutes. Released angiotensin was bioassayed in the rat. Unit was nanogram of angiotensin II equivalent produced by 1 mg of kidney tissue or by 1 μl of the extract in 15 minutes.

**Determination of Renin Substrate:** It was expressed by the amount of angiotensin bioassayed after complete conversion of substrate by incubating 0.1 ml of a sample with excess rat renin in the buffer containing angiotensinase inhibitors as described above.

**Experiment 1**

Female rats weighing 90 to 100 g were uninephrectomized. Six days after the operation, they were divided into four groups and injected subcutaneously with 0.5 ml of one of the three kidney extracts or saline every 12 hours. The blood pressure was measured before the injection.
TABLE 1  RENIN CONTENT OF KIDNEY EXTRACTS

A: first batch

<table>
<thead>
<tr>
<th>Extract</th>
<th>I</th>
<th>II</th>
<th>III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Renin</td>
<td>146</td>
<td>148</td>
<td>117</td>
</tr>
<tr>
<td>(ng/15 min/μl)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B: second batch

<table>
<thead>
<tr>
<th>Extract</th>
<th>I</th>
<th>II</th>
<th>III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Renin</td>
<td>151</td>
<td>167</td>
<td>135</td>
</tr>
<tr>
<td>(ng/15 min/μl)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

every morning by tail plethysmography. On the third, fifth or sixth, and tenth day, after the measurement of blood pressure, six to eight rats of each group were anesthetized with amobarbital. Renal vessels were cramped immediately after laparotomy and blood was taken from the aorta into a tube which was pre-cooled in melting ice and contained EDTA-Na₂ to make the final concentration 5 mmol/l. Plasma was separated for the assay of angiotensin II, renin activity, renin concentration and renin substrate. The kidney and the heart were removed and weighed, and the former was kept frozen until the determination of renal renin.

Experiment 2

In order to determine plasma renin level without any influence of anesthesia, a polyethylene catheter was implanted to the abdominal aorta through the femoral artery at least one day before blood sampling. The rat was let move freely in a cage. Three or four rats of each group in the experiment 1 were employed in this experiment. When an elevation of blood pressure became manifest on the sixth to tenth day, each 0.5 ml of blood was taken through the catheter into a syringe moistened with ammonium EDTA (150 g/l), before (in other words 12 hours after) and 1, 4 and 8 hours after the injection. The blood was replaced with the same volume of saline. Mean blood pressure was recorded by connecting the catheter to a pressure transducer and an electromanometer (Nihon-Koden). Effect of single injection of the kidney extract was observed by injecting 0.7 ml of each extract into four female rats weighing approximately 170 g which were unilaterally nephrectomized and catheterized the day before the injection. Blood sampling was done before and 1, 4, 8 and 12 hours after the injection.

Fig.1. Course of blood pressure in three groups of rats which received kidney extracts.
The rats were injected subcutaneously with 0.5 ml of extract (I), (II), or (III) every 12 hours. Cross bars indicate standard errors of the mean.

Significance of the difference between two means was determined by Student’s t-test, or by Fisher-Behrens’ test when the variances were not homogenous.

RESULTS

1. Hypertension-inducing Effect and Renin Content of Kidney Extracts

The blood pressure was gradually elevated more or less in the three groups of rats which received kidney extracts. No evident disparity was found in the renin content of the three extracts, although that of extract (III) seemed slightly less than the other two (Table I). Nevertheless, a definite difference was observed in the hypertension-inducing potency of the extracts. After the 6th day blood pressure of the three groups of recipients was significantly different from each other (p < 0.05). Both of the rapidity of rising and the final value of blood pressure were \( (I) > (II) > (III) \) as is seen in Figure 1. In a separate experiment, each 0.35 ml of extract (I) and 0.5 ml of extract (III) were injected to the corresponding recipients. In this case the renin content of the injected extract (I) was certainly slightly less than that of extract (III). Here again extract (I) showed definitely greater hypertension-inducing effect than extract (III) (Figure 2). Therefore the difference in the hypertension-inducing potency cannot be attributed directly to the difference in the renin content of kidney extracts.

2. Plasma Renin and Angiotensin of the Recipient Rats

Plasma renin and angiotensin II were determined approximately 12 hours after the preceding injection at three stages — the 3rd day when the blood pressure was still on the control level, the 5th or 6th day when it began to rise, and the 10th day when hypertension was stabilized at least in the recipients of extract (I). The results were summarized in Table II. Also shown is concentration of plasma renin substrate on the 10th day, which was not affected by the administration of kidney extracts. Both of renin and angiotensin II of the recipients of extract (I) were significantly higher than those of the other two groups (p < 0.01 or < 0.05), with the exception of the third day and renin activity of the 5th or 6th day. There was no significant difference between the two groups received extract (II) and (III). Although control group had higher renin and angiotensin levels, this is attributed to an influence of anesthesia. Renin

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### TABLE II
PLASMA RENIN AND ANGIOTENSIN IN THE RECIPIENTS OF KIDNEY EXTRACTS AND UNTREATED CONTROLS

#### A: (3RD DAY)

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>I</th>
<th>II</th>
<th>III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiotensin II (pg/ml)</td>
<td>74 ± 38</td>
<td>69 ± 39</td>
<td>68 ± 43</td>
<td>69 ± 36</td>
</tr>
<tr>
<td>Renin Activity (ng/ml/hr)</td>
<td>9 ± 2</td>
<td>8 ± 2</td>
<td>5 ± 4</td>
<td>5 ± 4</td>
</tr>
<tr>
<td>Renin Concentration (ng/ml/hr)</td>
<td>19 ± 6</td>
<td>16 ± 3</td>
<td>8 ± 5</td>
<td>11 ± 7</td>
</tr>
<tr>
<td>Blood Pressure (mmHg)</td>
<td>134 ± 6</td>
<td>135 ± 15</td>
<td>130 ± 6</td>
<td>125 ± 5</td>
</tr>
</tbody>
</table>

#### B: (5–6TH DAY)

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>I</th>
<th>II</th>
<th>III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiotensin II (pg/ml)</td>
<td>45 ± 24</td>
<td>34 ± 10</td>
<td>12 ± 8</td>
<td>15 ± 7</td>
</tr>
<tr>
<td>Renin Activity (ng/ml/hr)</td>
<td>15 ± 7</td>
<td>11 ± 4</td>
<td>6 ± 4</td>
<td>5 ± 4</td>
</tr>
<tr>
<td>Renin Concentration (ng/ml/hr)</td>
<td>32 ± 15</td>
<td>21 ± 4</td>
<td>7 ± 3</td>
<td>8 ± 4</td>
</tr>
<tr>
<td>Blood Pressure (mmHg)</td>
<td>127 ± 6</td>
<td>182 ± 19</td>
<td>157 ± 18</td>
<td>142 ± 15</td>
</tr>
</tbody>
</table>

#### C: (10TH DAY)

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>I</th>
<th>II</th>
<th>III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiotensin II (pg/ml)</td>
<td>60 ± 24</td>
<td>63 ± 37</td>
<td>10 ± 8</td>
<td>17 ± 14</td>
</tr>
<tr>
<td>Renin Activity (ng/ml/hr)</td>
<td>12 ± 5</td>
<td>12 ± 6</td>
<td>1 ± 1</td>
<td>5 ± 3</td>
</tr>
<tr>
<td>Renin Concentration (ng/ml/hr)</td>
<td>26 ± 11</td>
<td>30 ± 5</td>
<td>7 ± 2</td>
<td>12 ± 10</td>
</tr>
<tr>
<td>Renin Substrate (ng/ml)</td>
<td>669 ± 80</td>
<td>724 ± 70</td>
<td>693 ± 128</td>
<td>660 ± 90</td>
</tr>
<tr>
<td>Blood Pressure (mmHg)</td>
<td>127 ± 6</td>
<td>199 ± 12</td>
<td>168 ± 17</td>
<td>147 ± 10</td>
</tr>
</tbody>
</table>

Means and standard deviations are shown.

Concentration of the recipients of extract (II) and (III) are on the same level as of conscious controls which was determined in experiment 2. Renin activity and angiotensin II concentration were parallel with renin concentration, so that only the latter was measured in experiment 2. Changes in Plasma Renin Level Following Injection of Kidney Extracts

hours. The higher peak was obtained with extract (II). In the recipients of extract (I) the peak of plasma renin was attained in either 1 hour or 4 hours and the highest of the three. It remained on a higher level even 12 hours after the injection. Any apparent influence of the blood sampling alone was not detected in the control animals. The pre-injection value of each group, except the control group, coincides with that observed in experiment 1.

When the same experiment was done following a single injection of kidney extract, results were somewhat different. The peak of plasma renin was attained in 4 hours with either extract, but the effectiveness of the three extracts was in the same order as the above (Figure 4a). In order to see whether the difference in the effect on plasma renin is due to intrinsic renin release, rats of the same size were bilaterally nephrectomized and 0.4 ml of each extract was injected on the next day. Plasma renin rose much higher and continuously, indicating an important role of the kidney in removal of plasma renin. However, the effectiveness of the three extracts was again (I) > (II) > (III) (Figure 4b).

4. Renal Renin Content of the Recipients

It was determined on the 3rd day and the 5th or 6th day (Table III). Renal renin was significantly decreased after 5 or 6 days of injection of either extract (P < 0.01). Difference between the three recipient groups was not significant. A tendency of decrease was already seen on the 3rd day, but not statistically significant.

**DISCUSSION**

Increase in plasma renin is not always observed either clinically or experimentally even in renovascular hypertension in which implication of renin-angiotensin system is expected. Failure in abolishing renal hypertension by active immunization against angiotensin gives another ground for speaking against the involvement of renin-angiotensin system. In the present study also observed are discrepancies between blood pres-

TABLE III RENAL RENIN CONTENT IN THE RECIPIENTS OF KIDNEY EXTRACTS AND UNTREATED CONTROLS

<table>
<thead>
<tr>
<th>Group</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>3rd Day</td>
<td>285 ± 99</td>
<td>248 ± 61</td>
<td>285 ± 57</td>
<td>351 ± 47</td>
</tr>
<tr>
<td>5–6th Day</td>
<td>109 ± 57</td>
<td>151 ± 37</td>
<td>181 ± 61</td>
<td>274 ± 45</td>
</tr>
</tbody>
</table>

Means and standard deviations are shown.

Fig. 4. Course of plasma renin concentration following single injection of kidney extracts.
a: unilaterally nephrectomized rats
b: bilaterally nephrectomized rats
Control group received saline. Mean values are shown.

pressure and renin-angiotensin level. First, between hypertension-inducing potency and renin content, determined in vitro, of the kidney extracts. An extract with higher renin content was not always more potent in inducing hypertension. This is consistent with the observation by Kira et al.\textsuperscript{19} Secondly, the blood pressure of the recipients was elevated without any increase in plasma

*Japanese Circulation Journal Vol. 38, December 1974*
renin level, except the recipients of extract (I), when the both were measured 12 hours after the preceding injection. Hirano and Masson\textsuperscript{18} observed less plasma renin than the control and depleted renal renin in the hypertensive rats treated with crude renin for 35 days. Similar findings were obtained in the present study even in much shorter period of the treatment. Higher plasma renin and angiotensin levels of the control rats in their experiment and in our experiment I are attributed to an effect of anesthesia. Ether or amobarbital anesthesia markedly increases plasma renin of untreated rats\textsuperscript{23,24} However, the plasma renin concentrations of the rats which received kidney extracts were on the same level as of the conscious rats in experiment 2. In those animals, release of intrinsic renin due to anesthesia would have been suppressed by the administration of excessive renin. Accordingly the results of experiment I can be interpreted as follows: plasma renin of the recipients of extract (II) and (III) falls within normal range while that of recipients of extract (I) is higher than the normal.

For an explanation of these discrepancies one could assume the existence of a hypertension-inducing substance in the kidney independently of renin-angiotensin system. Broader effectiveness of antirenin (its antigen is not pure renin) than angiotensin inhibitors in remitting hypertension\textsuperscript{4} suggests a possibility of involvement of some other renal substances than renin in the pathogenesis of hypertension. Renal pressor substances different from renin or angiotensin were reported\textsuperscript{25,26} although evidence for their hypertension-eliciting action is lacking.

Alternative explanations are also possible. A renal substance, such as sensitizing factor\textsuperscript{27} might have increased vascular sensitivity to pressor substances and the normal renin-angiotensin level would have been enough to maintain the high blood pressure. A gradual potentiation of reactivity to angiotensin or renin by repeated administration of kidney extract or crude renin has been found in the rat\textsuperscript{28,29}

Another way of explanation seems more attractive. As was seen in experiment 2, changes in plasma renin level during 12 hours following the injection did not necessarily reflect the renin content of the injected extract. Influence of intrinsic renin release on the plasma renin, if any, would not be so much, since similar difference in the effect of the three extracts was also observed in nephrectomized recipients. Adrenalectomy probably produced some changes in the property of renin or related substance like pro-renin or in the content of the latter in the donor’s kidney. Substitution with salt would have ameliorated these changes at least to some extent. From Figure 3 and 4, the extent of change in plasma renin level, when integrated, appears to be parallel to hypertension-inducing effect of each kidney extract. Hence it is likely that an excessive fluctuation of plasma renin or angiotensin level elicited the hypertension. The most effective hypertension-inducer was extract (I) which produced relatively continuous elevation of plasma renin. Therefore, although continuous high level of plasma renin or angiotensin is not indispensable for the development of hypertension, it would at least accelerate or aggravate hypertension. Cumulative effect of intermittent rise of plasma renin could gradually elevate blood pressure by means of some other actions than direct vasoconstrictor effect of angiotensin. Nervous, adrenal or intrarenal effect of angiotensin may contribute to this, directly or by increasing vascular reactivity. This assumption is supported by experiments of Kolersky et al\textsuperscript{30} and of Saleh and Heintsz\textsuperscript{31} in which intermittent infusion of a pressor amount of angiotensin produced sustained hypertension with or without renal vascular changes. It is also possible that intermittent elevation of blood pressure, not of renin or angiotensin per se, elicited hypertension by inducing vascular structural changes and increasing responsiveness to pressor substances\textsuperscript{32}

In conclusion, subcutaneous administration of kidney extracts induced a hypertension in the rat which was not always accompanied by an elevation of plasma renin level. This is likely to be due to a cumulative effect of an excessive fluctuation of plasma renin-angiotensin level or/and blood pressure. Continuous increase in plasma renin or angiotensin appeared to potentiate this effect. Implication of other renal substances than renin, however, cannot be excluded.

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Japanese Circulation Journal Vol. 38, December 1974

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Discussion:
Chairman: Dr. T. OMAE

Dr. FUKUCHI: You used very crude renal extract containing many substances other than
renin. Have you ever used the extract of other organs for the same kind of experiment?
Dr. MATSUNAGA: We have assumed that some factor(s) other than renin may exist in the kidney to produce hypertension. Therefore, the very crude extract containing many substances was used for our experiment. We do not think that the effect of depressor factor plays a significant role. We have an experience of using the extract of the liver, but hypertension could not be produced by injecting it. When the kidney extract was more purified, a difference between hypertension-producing effect and renin content became smaller. Therefore, there is less possibility that renal pressor factor exists independently of renin, but the possibility cannot be denied that some renal factor exists to alter the effectiveness of renin-angiotensin system. So far, however, it will not be impossible to explain our data by the effect of renin-angiotensin system.
Dr. KANEKO: Was the difference of PRA or blood pressure significant between group II and III? Did you examine renal function in your animals? It is advisable to consider renal function because in the chronic experiment change in renal function may affect blood pressure level.
Dr. MATSUNAGA: Yes, difference of blood pressure between group II and III was significant. Difference of PRA was not significant 12 hours following the injection, but the peak value of PRA was higher in group II than in group III. Renal function was not examined. I agree with your opinion.
Dr. ONOYAMA: How did you draw blood samples? When you took blood samples five times, hypovolemia may have affected the renal pressor activity. How did you handle with a dead space when you drew blood?
Dr. MATSUNAGA: In control rats, we only took blood samples, but PRA was not significantly changed. We had a dead space of approximately 0.1 ml. Blood samples were taken, of course, after discarding the fluid in the dead space. Then, an enough volume of saline was injected through the catheter to replace the lost blood.
Dr. OMAE: How do you explain the fact that PRA was higher in the controls? If PRA was lower in experimental groups than in the controls, it is not understandable that renin plays a role in the development of hypertension.
Dr. MATSUNAGA: In controls, the effect of anesthesia may have increased PRA. In the experimental rats injected with the renal extract, as indicated by the depressed renal renin content, intrinsic renin release may have been suppressed. And the effect of anesthesia may not have been manifested. This is understandable when compared with the data in the conscious state.
Dr. FUKUCHI: Even when renal renin release is suppressed by exogenous renin, PRA must be high because the procedure of determining PRA detects exogenous renin.
Dr. MATSUNAGA: It is not certain.
Dr. SOKABE: In the rat, drawing of blood itself much affects PRA. Therefore, we must be careful in performing this kinds of experiment in the rat.