Partial Purification of a Transcellular-Fluid-Forming Factor from the Renal Cortex

SHUICHI NAMBA, YOSHIO HIRAMATSU, FUKUCHI KUSUNOKI, KATSUTOSHI OKUDA*, AND HIROFUMI SOKABE**

Existence of a transcellular-fluid-forming factor in the renal cortex has been reported. Purification of this factor by DEAE-cellulose column chromatography have yielded preparations 5.6 times greater in the ratio of this activity to that of renin than the crude tissue extract.

A factor in the renal cortex which forms the transcellular (pleural and peritoneal) fluid when it is injected into the bilaterally nephrectomized animals has been reported. A similar factor has been investigated with particular reference to hemoconcentrating or pulmonary edema inducing activity. The action has been attributed to renin, because of inseparability of this factor from renin preparation. But it may be different from renin, since angiotensin infusion into the nephrectomized animals does not produce an equivalent amount of transcellular fluid as renin, and since there is a discrepancy between the renin content and pleural effusion forming activity of the kidney extracts originated in some experimental conditions.

We have purified this factor, and increased the ratio of this activity to renin 5.6 times. It is called the transcellular fluid forming factor (TFFF), because the mechanism of action is not known.

MATERIALS AND METHODS

Pig kidney obtained at the abattoir and kept frozen was used for extraction. Frozen kidney tissue of the whales obtained from Taiyo Fishery Co. was also used.

Purification Procedures

All operations were carried out at 4 to 6°C.

Step 1. Crude preparation: Frozen kidneys were decapsulated, defatted, minced, and homogenized in 4 volumes of 0.9 per cent NaCl in a Waring blender. The homogenate was stirred for 3 hrs, and centrifuged. The supernatant fluid was adjusted to pH 4.6 by dropwise addition of 1 N HCl. After the precipitate was removed by centrifugation, the clear supernatant was brought to pH 6.5 with 1 N NaOH. To this solution was added solid (NH₄)₂SO₄ to give 0.6 saturation, and then the mixture was kept overnight. The precipitate was collected by centrifugation and dissolved in a minimum amount of water. This solution was dialyzed against running tap water for 2 days and then deionized water with several changes of outer solution. The precipitate resulted during dialysis was centrifuged off, and acetone was added to the supernatant to a concentration of 80 per cent (v/v). After standing for 30 mins., the precipitate was separated by decantation and centrifugation, washed with acetone and ether, and dried in vacuum.

Step 2. DEAE-cellulose column chromatography: This procedure is a modification of the method reported by Peart et al. Twenty-five g of crude preparation from step 1 was stirred for 2 hrs with 500 ml of 0.005 M phosphate buffer, pH 7.0. The suspension was centrifuged and the sediment extracted twice more by stirring for 2 hrs with 200 ml of the same buffer. The combined supernatants were applied to a DEAE-cellulose column (5 x 75 cm) equilibrated with the same buffer. The column was washed with the same buffer, and then eluted with the following solutions at a flow rate of 30 ml per hr: 0.05 M phosphate buffer of pH 6.5; 0.15 M phosphate-saline buffer of pH 6.0 (0.05 M phosphate buffer + 0.1 M NaCl), and 0.6 M phosphate-saline.

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buffer of pH 4.0 (0.1 M NaH₂PO₄ + 0.5 M NaCl). Effluents were taken every 20 ml with a fraction collector, and the optical density at 280 nm was measured with a Shimadzu QR-50 spectrophotometer. The peak fractions were pooled, and precipitated with acetone. The precipitate was dissolved in, and dialyzed against deionized water. A precipitate present in the dialyzed material was removed by centrifugation, and the supernatant was lyophilized to dryness.

Step 3. 2nd DEAE-cellulose column chromatography: The fraction CE₂₋₃ obtained by step 2 was subjected to a DEAE-cellulose column chromatography using NaCl and pH gradient system. The column (2 x 35 cm) was equilibrated with 0.1 M phosphate buffer, pH 6.0, and 300 mg of the material was added to the column. A gradient of NaCl concentration and pH was started, in a 500 ml mixing flask containing the 0.1 M buffer of pH 6.0, by gradual introduction of 0.6 M phosphate-saline buffer of pH 4.0 (0.1 M NaH₂PO₄ + 0.5 M NaCl). The flow rate was 7 ml per hr. Effluents were collected in 3 ml aliquots, and the peak fractions were pooled, dialyzed and lyophilized.

Miscellaneous Procedures

Sephadex gel filtration and CM-Sephadex chromatography were carried out according to the method of Peart et al.⁸

Calcium phosphate gel chromatography was also performed. Calcium phosphate gel on cellulose was prepared by modifying the method of Price and Greenfield⁹. To 15 g of cellulose powder (Whatman standard grade) suspended in 100 ml of water were added 50 ml of a solution containing 0.5 M of CaCl₂, 0.335 M of KH₂PO₄, and 0.165 M of HCl. After stirring for 2 mins, 50 ml of 4 M NH₄OH were added, and the mixture was left overnight. The supernatant fluid was decanted and the gel-cellulose precipitate was washed by decantation with about 2 L of water until it becomes negative to Nessler's reagent. The gel-cellulose was equilibrated with 0.005 M phosphate buffer of pH 6.8, and then packed into the column (2.3 x 30 cm). Three hundreds mg of CE₂₋₃ from step 2 was applied to the column, and the gradient elution was carried out by the use of a 500 ml mixing flask containing 0.005 M buffer and a 500 ml reservoir containing a solution of 0.05 M phosphate buffer, pH 6.8. Final elution was achieved with 0.06 M phosphate buffer, pH 6.8. Each peak was collected and dialyzed against deionized water, then was lyophilized to dryness.

Electrophoresis was carried out at a constant current of 30 mA for 3 hrs in a 25.5 x 7.5 x 0.5 cm slab of 10 per cent polyacrylamide gel, in the horizontal apparatus using the standard Tris-EDTA-borate buffer of pH 8.9.⁹ Samples were dissolved in the same buffer, and the amount representing about 0.2 mg of protein were applied to the gel.

Bioassays

Transcellular fluid (TF) forming activity (P-activity) was determined in a group of 5 female rats, Donryu strain, weighing 135 to 165 g and bilaterally nephrectomized 30 mins. before. Samples were dissolved in 5.0 ml of 0.9 per cent saline and injected intraperitoneally. The rats were caged in a group, given rat chow and tap water ad libitum, and kept in a room of constant temperature (23 to 25°C) and humidity (50 to 60 per cent). TF (pleural and peritoneal fluid) volume of each animal was measured 18 hrs after the injection, and averaged. One unit of P-activity was defined to form 5.0 ml of TF. Standard curve of P-activity (Fig. 1) was prepared by using a lot of crude preparation of hog kidney (HC-2).

Renin activity (R-activity) was determined in female Donryu rats, weighing 180 to 200 g, and bilate-
rally nephrectomized 18 hrs. before\textsuperscript{11} and anesthetized with sodium pentobarbital (40 mg/kg). Samples were injected in 5.0 ml of saline, 0.1 ml of which was injected intravenously into the rat. Determination was carried out in duplicate. Maximum elevation of blood pressure in each animal was measured, and averaged. One hundredth unit of R-activity was defined to increase 30 mmHg of blood pressure. Standard curve of R-activity (Fig. 2) was prepared by using the same lot of crude preparation (HC-2).

P:R ratio was calculated as the index of relative purification of TFFF to renin.

**RESULTS**

P- and R-activities of the starting materials used were shown in Table I. The whale kidney had relatively less renin. The crude preparations used for the further purification studies are also listed in the table.

Table II shows the changes in activity of TFFF and renin at various steps in pig kidneys. The values represent average results from 5 to 10 experiments. The crude preparation from step 1 had 37 to 101 m units of P-activity and 25 to 112 m units of R-activity per mg, and P:R ratio was 1.39.

By the DEAE-cellulose column chromatography, the crude preparation was divided into 4 fractions, named CE\textsubscript{-1}, -2, -3, and -4 (Fig. 3). P- and R-activities were concentrated in CE\textsubscript{-3}, but P:R ratio was not increased in this stage.

For further purification, CE\textsubscript{-1}-3 from step 2 was rechromatographed on DEAE-cellulose column using NaCl and pH gradient system (step 3). By this step, the activities were separated into two peaks (CE\textsubscript{2}-A and CE\textsubscript{2}-B in Fig. 4). Most of the R-activity was found in the front fraction, CE\textsubscript{2}-A, and P-activity was distributed into two fractions resulting 0.68 and

![Optical Density of 280nm](image)

**Fig. 3.** Chromatogram of crude preparation (25 g) on DEAE-cellulose with a 5 x 75 cm column. Elution was performed with the following solvents: A. 0.005 M phosphate buffer of pH 7.0, B. 0.05 M phosphate buffer of pH 6.5, C. 0.15 M phosphate-saline buffer of pH 6.0 (0.05 M phosphate buffer + 0.1 M NaCl), and D. 0.6 M phosphate-saline buffer of pH 4.0 (0.1 M Na H\textsubscript{2}PO\textsubscript{4} + 0.5 M NaCl).

<table>
<thead>
<tr>
<th>Table I</th>
<th>Activity of Starting Materials</th>
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<tbody>
<tr>
<td></td>
<td>Yield (%)</td>
</tr>
<tr>
<td>Rat kidney</td>
<td>4.8</td>
</tr>
<tr>
<td>Pig kidney</td>
<td>2.7</td>
</tr>
<tr>
<td>Crude preparation (HC-2)</td>
<td>1.35</td>
</tr>
<tr>
<td>Whale kidney</td>
<td>1.8</td>
</tr>
<tr>
<td>Crude preparation (WC-1, 2, 3)</td>
<td>1.5</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Table II</th>
<th>Purification of the Renal TFFF by DEAE-Cellulose Chromatography (from 1.0 kg of pig kidney)</th>
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<tbody>
<tr>
<td>Step</td>
<td>Yield (mg)</td>
</tr>
<tr>
<td>1. ((NH\textsubscript{4}))\textsubscript{2}SO\textsubscript{4} and acetone ppt (Crude preparation)</td>
<td>13,400</td>
</tr>
<tr>
<td>2. 1st DEAE-cellulose chromatography</td>
<td>CE\textsubscript{1}-1</td>
</tr>
<tr>
<td></td>
<td>CE\textsubscript{1}-2</td>
</tr>
<tr>
<td></td>
<td>CE\textsubscript{1}-3</td>
</tr>
<tr>
<td></td>
<td>CE\textsubscript{1}-4</td>
</tr>
<tr>
<td>3. 2nd DEAE-cellulose chromatography</td>
<td>CE\textsubscript{2}-A</td>
</tr>
<tr>
<td></td>
<td>CE\textsubscript{2}-B</td>
</tr>
</tbody>
</table>

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5.56 in P:R ratio, respectively. The results clearly indicate that P- and R-activities are separable. Recovery of renin activity from step 2 was nearly complete (96.2%) and of TFFF was about 77 per cent, but increase in specific activity was not seen.

Gel electrophoretic patterns of the fractions obtained by DEAE-cellulose chromatography are shown in Fig. 5. These fractions were heterogeneous, but the pattern of CE₂-B was distinguishable from CE₂-A.

Sephadex G-100 gel filtration, CM-Sephadex chromatography, and calcium phosphate gel chromatography (Table III, Fig. 6) were also tried. In these procedures, however, the separation of two activities was not observed, although specific activity fairly increased.

![Fig. 4. Chromatogram on DEAE-cellulose column of CE₂-3 obtained by step 2. The column (2 x 35 cm) was equilibrated with 0.1 M phosphate buffer of pH 6.0, and the material (300 mg) was applied to the column. A gradient of pH and salt concentration was started by introducing 0.6 M phosphate-saline buffer of pH 4.0 (0.1 M NaH₂PO₄ + 0.5 M NaCl) into a 500 ml mixing flask containing the 0.1 M buffer of pH 6.0. The dotted line shows the change of salt concentration.](image)

![Fig. 5. Electrophoretic patterns on polyacrylamide gel of CE₂-3 (A), CE₂-A (B), CE₂-B (C), and bovine serum albumin as a marker (D). The gel was stained with Amidoblack 10-B.](image)

![Fig. 6. Calcium phosphate gel column chromatogram of CE₂-3 from step 2. The material (300 mg) was applied to the column (2.3 x 30 cm) equilibrated with 0.005 M phosphate buffer, pH 6.8. A gradient of salt concentration was started by introducing 0.05 M phosphate buffer, pH 6.8, into a 500 ml mixing flask containing the 0.005 M buffer. Salt gradient is shown with the dotted line.](image)

**DISCUSSION**

We have purified the renal TFFF to obtain a maximum of 5.6 in the relative potency to renin activity (P:R ratio). Specific activity was increased to 724 m units per mg. Changes in P:R ratio with the source of materials and the procedures of purification are further evidence that this factor is not renin.

**TABLE III  Purification of the Renal TFFF by Calcium Phosphate Gel Chromatography (from CE₂-3 in step 2‡)**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Yield (%)</th>
<th>Specific activity (m units/mg)</th>
<th>Recovery of activity (%)</th>
<th>P:R</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA-1</td>
<td>7.1</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>HA-2</td>
<td>14.7</td>
<td>724</td>
<td>584</td>
<td>36.8</td>
</tr>
<tr>
<td>HA-3</td>
<td>11.8</td>
<td>348</td>
<td>489</td>
<td>14.2</td>
</tr>
<tr>
<td>HA-4</td>
<td>12.9</td>
<td>—</td>
<td>—</td>
<td>—</td>
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</table>

* Specific activity: P 289 and R 309; P: R 0.94

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The mechanism of TF formation by this factor is not known. TF is formed as a result of the relative increase in edudation from the peritoneum and pleura to the absorption. Increase in the vascular permeability may produce TF by augmenting edudation, but decrease in the absorption by this factor may also possibly produce TF. Therefore, we have used the term TFFF instead of the vascular permeability factor.

The presence of sodium excreting factor, which inhibits sodium reabsorption in the renal tubules, has been reported as in the kidney. TFFF might have some relationship with the renal sodium excreting factor, because both factor would inhibit sodium reabsorption mechanism. TFFF was decreased in the kidneys of some experimental hypertensive rats parallel with renin. This might suggest participation of TFFF in the pathogenesis of hypertensive cardiovascular disease. Further studies are necessary to elucidate these relationships.

Summary

1. A factor in the renal cortex which forms the transcellular (pleural and peritoneal) fluid when injected into the bilaterally nephrectomized (BN) animals has been known. We called it the transcellular fluid forming factor (TFFF), and tried its purification.

2. Transcellular forming activity (P-activity) was bioassayed in BN rats by the amount of transcellular fluid formed. Renin activity (R-activity) was determined by the blood pressure elevation in BN rats. P:R ratio in an extract was calculated as the index of relative purification of TFFF to renin.

3. DEAE-cellulose column chromatography of the crude preparation made from pig kidneys by ammonium sulfate and acetone precipitation resulted in four fractions, of which P- and R-activities were concentrated in the third. Rechromatography on DEAE-cellulose of this fraction gave two peaks. Most of the R-activity was found in the front fraction, and P-activity was distributed into two fractions resulting in P:R ratio of 0.68 and 5.6 respectively.

4. From the facts that P:R ratio changes with the source of materials and the procedures of purification, it is suggested that the renal TFFF may not be renin.

Acknowledgement

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References


