DISTRIBUTION OF RENIN IN SUBCELLULAR FRACTIONS FROM THE RABBIT KIDNEY

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Abstract—Subcellular localization of renin in the rabbit kidney cortex was investigated using two centrifugation techniques. Renin was indirectly assayed on the basis of pressor activity and the reference enzymes such as succinic dehydrogenase, acid phosphatase and D-glucose-6-phosphatase for the other subcellular particulates were biochemically determined. Renin activity was found mainly in the mitochondrial fraction with very little in the microsomal fraction by a differential centrifugation. By a discontinuous sucrose density gradient centrifugation, renin granules were mainly recovered in the fraction corresponding to 1.5 M sucrose, while most of mitochondria, lysosomes and microsomes equilibrated in the upper fractions. This renin granular fraction contained approximately 50% of total granular renin activity having a specific activity about six times that seen in the homogenate. After recentrifugation of the renin granular fraction, most of renin activity was recovered in the sediment. Repeated freezing and thawing of this fraction resulted in an increase of renin activity. On the basis of these experimental data it is assumed that renin located in the different subcellular particulates from mitochondria, lysosomes and microsomes in the rabbit kidney cortex.

It is widely acknowledged that renin is stored as granules in the juxtaglomerular cells of the kidney and that renin release from the kidney is associated with many factors which are mediated through blood vessels, the sympathetic nervous system and macula densa. However, there is no unified concept on the mechanism underlying renin release from the juxtaglomerular cells. In order to approach the mechanism of renin release at a subcellular level, we have previously shown that a large amount of dog renin granules almost free from other subcellular granules could be isolated (1). Furthermore, we have reported that release of renin from the dog renin granules is stimulated by addition of dibutyryl cyclic AMP (2) and calcium ion (3) to the incubation medium.

On the other hand, there is little data available on the subcellular localization of renin in the rabbit kidney cortex except for the report by Cook and Pickering (4), who demonstrated that renin and succinic dehydrogenase were found in the different subcellular particulate fraction.

The present investigation was an attempt to procure detailed information on the subcellular localization of renin in the rabbit kidney cortex, as revealed by differential and gradient centrifugation techniques.

MATERIALS AND METHODS

Animals and preparation of homogenate

Male albino rabbits weighing from 2.5 to 3.0 kg were maintained in a room with a
constant temperature of 25°C and humidity of 60%, and fed ordinary rabbit pellets (Ori-
etal RC-4) and tap water ad libitum. Under urethane anesthesia (1 g/kg), the kidneys
were removed and immediately perfused with cold isotonic saline solution through the
renal artery to flush out any remaining blood. After decapsulation the kidney was longi-
tudinally cut through the hilus and the medulla was removed. The cortex was finely cut
with a blade, under low temperature, and then portions of 2 g were homogenized in 16 ml
of freshly prepared 0.45 M sucrose solution. In order to minimize the rupture of renin
granules, homogenization was gently carried out in a chilled Potter-Elvehjem homogenizer
with a loose-fitting pestle at 800 rpm for 40 sec. After separation of the unbroken cells,
cell debris, and nuclei by centrifugation for 10 min at 500×g, the homogenate was sub-
jected to subcellular fractionation.

Fractionation of the homogenates by differential centrifugation

Various subcellular fractions were prepared from the homogenates by differential
centrifugation in a Hitachi model 20 PR centrifuge with a RPR 20 rotor and a Hitachi
model 65 P ultracentrifuge with a PR 65T rotor. Three particulate fractions namely heavy
mitochondrial, light mitochondrial, and microsomal, were successively isolated by sedi-
mentation at 6,000×g for 5 min, 20,000×g for 20 min and 100,000×g for 60 min, re-
spectively. The final supernatant obtained after separation of the microsomes was termed
the ‘cell sap fraction’. All procedures were carried out at 0°C. The particulate frac-
tions were carefully resuspended in 0.45 M sucrose solution by means of the Potter-Elveh-
jem homogenizer. The suspensions and the cell sap fraction were analysed for protein,
renin and reference enzymes of principal subcellular particulate, respectively.

Fractionation of the homogenate by a discontinuous sucrose density gradient centrifugation

Discontinuous sucrose gradients were prepared in a centrifuge tube by layering 7 ml
each of sucrose solutions from 1.2 M to 1.7 M. Ten ml of the homogenate were care-
fully layered on top of the gradient and centrifuged at 60,000×g for 90 min in a Hitachi
model 65 P ultracentrifuge with a RPS 25-2A rotor. Following centrifugation, the tubes
were carefully cut corresponding to the interfacial bands. Six particulate fractions and
a supernatant were obtained. These particulate fractions were designated as fractions
1 to 6 from the bottom layer.

Assays of enzymatic activities and protein

The reference enzymes used to assess the purity of the fractions were: renin (EC
3.4.4.15) for renin granules, succinic dehydrogenase (EC 1.3.99.1) for mitochondria, acid
phosphatase (EC 3.1.3.2) for lysosomes and D-glucose-6-phosphatase (EC 3.1.3.9) for
microsomes.

Renin activity was measured by incubating the sample with renin substrate and as-
saying the amount of angiotensin formed. As masking of enzyme activity may occur
in vesicular structures, the homogenate and fractions were frozen and thawed before ass-
say, and then diluted 10- and 50 fold, respectively, in a cold isotonic saline solution. One-
fifth ml of 0.05 M disodium ethylenediaminetetraacetic acid (EDTA), 0.02 ml of 5 % di-
isopropyl fluorophosphate (DFP), which was dissolved in isopropanol, and 0.7 ml of 0.15 M
RABBIT KIDNEY RENIN GRANULES

phosphate buffer, pH 7.4, containing 0.9% sodium chloride, were added to 0.1 ml of diluted sample. The mixture was preincubated at 37°C for 20 min in order to completely inhibit the angiotensinase activity. The reaction was started by addition of 0.2 ml of rabbit renin substrate. After 4 hr of incubation at 37°C, the reaction mixture was placed in a boiling water bath for 10 min, cooled, and centrifuged. Angiotensin formation system contained 0.02% of neomycin, by which angiotensin produced in the incubation mixture was protected from bacterial destruction. The angiotensin formed during the incubation was biossayed in pentobarbital-anesthetized rats, pretreated with i.p. injections of pentolinium and atropine. As reference standard for angiotensin, Val8-angiotensin II-amide (Hypertensin, CIBA) was used. Renin activity was expressed as the equivalent to micrograms of angiotensin II per ml of the fraction formed during the 4 hr incubation.

Renin substrate was prepared from the plasma of a nephrectomized rabbit according the previously reported method (5). Neither renin activity nor angiotensinase activity was detected in the substrate solution. More than 1,380 ng of angiotensin was liberated from 1 ml of the substrate when incubated with excess rabbit renin.

Succinic dehydrogenase was assayed at 25°C in a final incubation mixture of 3.0 ml by measuring the rate of reduction of potassium ferricyanide in the presence of potassium cyanide following the method of Slater and Bonner (6). The activity was expressed in arbitrary units as a decrease of absorbancy at 400 nm per ml of fraction per hr.

Acid phosphatase was assayed at 37°C in a final incubation mixture of 1.0 ml containing 10 mM β-glycerophosphate and 0.2 M tris-acetate, pH 5.0. The reaction was terminated by the addition of ice-cold 60% perchloric acid. After the precipitated protein was discarded by centrifugation, the amount of released inorganic phosphate in the supernatant was determined following the method of Fiske and Subbarow (7) with modifications that have been described in detail elsewhere (5).

D-glucose-6-phosphatase was assayed in a manner similar to that of acid phosphatase assay, except that 2 mM D-glucose-6-phosphate was substituted for β-glycerophosphate and the reaction was run in 0.2 M tris-acetate buffer, pH 6.5.

Acid phosphatase and D-glucose-6-phosphatase activities were expressed as micromoles of inorganic phosphate released from each substrate per ml of the fraction per hr.

The protein content of the fraction was determined colorimetrically by the method of Lowry et al. (8), using crystalline bovine albumin as the standard.

RESULTS

Subcellular fractionation by differential centrifugation

Since our previous study has indicated that dog renin granules are prone to rupture under mechanical agitation, the rabbit kidney cortex homogenate was prepared by a gentle procedure (800 rpm, 40 sec) to obtain intact renin granules.

Table 1 shows the percent distribution of renin, succinic dehydrogenase, acid phosphatase, D-glucose-6-phosphatase and protein in each subcellular fraction obtained by differential centrifugation of kidney cortex homogenate. The heavy mitochondrial frac-
TABLE 1. Distribution of renin and reference enzymes in subcellular fractions by
differential centrifugation

|                | Absolute Values of Homogenatea | Percent Distribution |
|----------------|--------------------------------|----------------------|
|                | M     | L     | P       | S       |
| Protein        | 8.84±0.22 | 18.29±1.81 | 17.48±0.93 | 9.67±0.38 | 54.55±1.92 |
| Renin          | 91.31±13.3 | 25.91±0.83 | 14.13±2.72 | 4.54±1.28 | 55.16±3.93 |
| Succinate DH   | 14.53±1.22 | 58.12±2.99 | 34.74±2.21 | 6.35±0.53 | 0         |
| Acid P-ase     | 102.8±4.90 | 34.56±2.98 | 29.11±0.77 | 17.19±1.15 | 16.98±1.81 |
| G6P-ase        | 151.3±7.02 | 24.13±1.88 | 26.83±3.29 | 43.31±4.08 | 5.73±0.94 |

Values are means±SE of 7 experiments.
M—Heavy mitochondrial fraction; L—Light mitochondrial fraction;
P—Microsomal fraction; S—Final supernatant;
* Absolute values are given in mg/ml of the kidney cortex homogenate for protein
and in units per ml of the kidney cortex homogenate for enzymes.

Succinate dehydrogenase showed the highest activity of succinic dehydrogenase accounting for about 58% of
total activity and a fourfold increase in specific activity. The remaining 35% of succinic
dehydrogenase activity was found in the light mitochondrial fraction. Approximately
64% of acid phosphatase was recovered in the heavy and light mitochondrial fraction with a
twofold increase in specific activity. About 43% of total D-glucose-6-phosphatase
activity corresponding to a fourfold increase in specific activity was found in the micro-
somal fraction. These data were qualitatively similar to those obtained in the rat (9, 10,
11) and dog (1) kidney homogenate.

About 40% of total renin activity was recovered with a twofold increase in specific
activity in the heavy and light mitochondrial fraction. However, the supernatant also
showed approximately half of the total activity.

Subcellular fractionation by discontinuous sucrose density centrifugation

Table 2 shows activities of renin and reference enzymes and concentration of pro-
tein in each fraction obtained by discontinuous sucrose density centrifugation. D-glu-

TABLE 2. Concentration of protein and activities of renin and reference enzymes in
homogenate and fractions after a sucrose density gradient centrifugation

|                | Protein mg/ml | Renin ng*/ml/4 hr | Succinate DH A4°| Acid P-ase Pt μg/ml/hr | G6P-ase Pt μg/ml/hr |
|----------------|---------------|-------------------|-----------------|------------------------|-------------------|
| Homogenate     | 11.78±0.24    | 132.8±15.76       | 25.21±2.56      | 108.2±4.49             | 252.3±11.79       |
| Supernatant    | 5.93±0.21     | 50.59±9.79        | 2.21±0.19       | 34.7±5.64              | 52.0±5.21         |
| Fraction 6     | 3.63±0.12     | 10.49±2.42        | 6.43±1.07       | 37.4±1.33              | 185.3±7.77        |
| Fraction 5     | 1.93±0.14     | 4.72±0.63         | 7.77±0.48       | 17.7±1.31              | 83.8±6.33         |
| Fraction 4     | 2.40±0.11     | 18.68±4.43        | 12.93±1.71      | 15.3±0.83              | 61.6±5.09         |
| Fraction 3     | 0.65±0.028    | 50.62±4.23        | 4.40±0.59       | 13.1±1.07              | 12.6±2.46         |
| Fraction 2     | 0.32±0.019    | 15.58±2.42        | 1.91±0.18       | 12.9±1.43              | 3.1±0.91          |
| Fraction 1     | 0.25±0.020    | 6.79±1.12         | 0               | 13.8±0.57              | 1.3±0.61          |

Values are means±SE of 7 experiments. *Angiotensin equivalents. **Decrease of absorbancy at 400 mg/ml. † Released inorganic phosphate from substrate.
cose-6-phosphatase showed a single peak activity in fraction 6 (1.2 M sucrose fraction), with an increase in specific activity of about three times that of the original homogenate. A higher activity of acid phosphatase was found in fraction 6 as compared with the other fractions, which also exhibited somewhat fewer activities. These results indicate a heterogeneity of the lysosomes. Succinic dehydrogenase was mainly located in fraction 4 with an increased specific activity of three times, showing that most of the mitochondria equilibrated in this fraction. On the other hand, renin activity was mostly recovered in fraction 3, i.e., 50.62 ± 4.23 μg of angiotensin equivalents were liberated per 4 hr with 1 ml of this fraction. The specific activity of renin in this fraction was 64.27 ± 12.48 μg angio-

Fig. 1. Responses of arterial blood pressure to intravenous injection of synthetic angiotensin II and fraction 3.

Fig. 2. Distribution patterns of renin and reference enzymes of subcellular particulates following a discontinuous sucrose density gradient centrifugation of renal cortex homogenates. Acid P-ase: acid phosphatase; Succinate DH: succinate dehydrogenase; G6P-ase: D-glucose-6-phosphatase. Abscissa: fraction (F) number. Ordinate: percent of total enzyme activity obtained from fraction 1 to 6.
tensin equivalents per mg of protein per 4 hr, being six times the homogenate. When this fraction was directly injected into the femoral vein, typical pressor action by renin was observed as shown in Fig. 1. The concentration of protein was high in fraction 4 and 6, corresponding to the mitochondria, lysosome and microsome, but low in fraction 1 to 3.

Fig. 2 presents the distribution patterns of renin and the other reference enzymes, which were calculated from the recovered volumes of fractions 1–6 and their respective enzyme activities. Approximately 50% of D-glucose-6-phosphatase and 30% of acid phosphatase were recovered in fraction 6 and 42% of succinic dehydrogenase in fraction 4. On the other hand, fraction 3 contained approximately 50% of total granular renin activity. These results indicate that renin granules can isolate themselves from the other subcellular granules and have a higher density.

When fraction 3 was recentrifuged at 100,000 g for 60 min, most of the renin activity was recovered in the sediment. Part of fraction 3 was frozen at −20°C and thawed below 5°C. This procedure was repeated six times. The renin activity of the treated fraction was markedly augmented, being eight times that of the nontreated fraction. These findings suggest that renin is isolated in the form of granule in this fraction.

DISCUSSION

The present study was made on the subcellular localization of renin in the rabbit kidney cortex. The separation of renal renin granules from other subcellular particulates was evaluated by biochemically determining the activities of the respective reference enzymes. Renin was indirectly assayed on the basis of its pressor activity. Since the various forms of angiotensinase present in kidney tissue (12, 13, 14) may interfere with renin assay, their influence was investigated in preliminary experiments; the angiotensinase activity of each fraction was completely inactivated by preincubation of the fractions with EDTA and DFP at 37°C for 20 min.

Differential centrifugation experiments showed that renin granules sedimented mainly in the heavy and light mitochondrial fraction and very little in the microsomal fraction. Early studies (15, 16) using differential centrifugation also demonstrated renin activity in the mitochondrial fraction of dog, pig, and rat kidney homogenate. On the other hand, Fisher (17) and Gomba and Soltesz (18) have shown in combined histochemical and electronmicroscopic techniques that juxtaglomerular granules are indeed lysosomes. This is supported by Ogino et al. (19) on the basis of a marked pressor effect of the kidney fraction having the highest acid phosphatase observed in the same fraction. Recently Onoyama et al. (11) also reported that lysosomal fraction of rat kidney contained the largest amount of renin in all of the subcellular fractions. In fact, in the present study, a major portion of acid phosphatase activity was associated with the mitochondrial fraction. However, it is difficult to conclude that renin is localized in the mitochondria or lysosomes because of the methodological incompleteness of separation of renin granules in differential centrifugation.
The cell sap fraction obtained by a differential centrifugation showed a high percentage of total renin activity, which was higher by comparison with that obtained from the dog kidney cortex by the same experimental conditions. Apparently rabbit renin is stored intracellularly in more mechanically fragile granules than the dog renin granules, as the former ruptured readily during homogenization and centrifugation of the kidney tissue.

The present studies on discontinuous sucrose density gradient centrifugation demonstrated that renin is distributed differently than other principal reference enzymes. Renin granules were mainly recovered in the fraction corresponding to 1.5 M sucrose. This fraction contained approximately 50% of total renin activity which appeared in the particulate fractions. The specific activity of renin in this fraction was about six times that found in the homogenate. The possibility that renin in this fraction is separated in the form of granules was suggested by the following findings: 1) most of renin activity was recovered in sediment following recentrifugation of the separated fraction and 2) the renin activity of this fraction increased to eight times with freezing and thawing.

In addition, it is noteworthy that the rabbit renin granules have a lower density than the dog renin granules, which were recovered mostly in the fraction corresponding to 1.6 M sucrose (1). This suggests that renin granules of renal cortex vary species by species.

On the other hand, most of succinic dehydrogenase, D-glucose-6-phosphatase and acid phosphatase equilibrated in the fractions over the renin granular fraction. Cook and Pickering (4) also reported the similar results with respect to the separation of succinic dehydrogenase and renin. Accordingly, it is assumed that renin in the rabbit kidney is stored in granules which have a higher density than other subcellular particulates such as mitochondria, lysosomes and microsomes.

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