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Subcellular Localization of Renin in the Mouse Kidney

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A centrifugation study was carried out to investigate the subcellular localization of renin in the mouse kidney cortex. Renin activity was radioimmunoassayed and found mainly in the heavy mitochondrial fraction, with very little in the microsomal fraction, by differential centrifugation. Upon discontinuous sucrose density gradient centrifugation, renin granules were recovered mainly in the fraction corresponding to 1.5 M sucrose, while most of the mitochondria, lysosomes and microsomes equilibrated in the upper fractions. This renin granular fraction contained 53% of the total granular renin activity and had a specific activity 3.2 times that found in the unfractionated homogenate. After recentrifugation of the renin granular fraction, most of the renin activity was recovered in the sediment. Repeated freezing and thawing of this fraction resulted in an increase of renin activity, showing structure-linked latency. On the basis of these findings, it is assumed that renin in the mouse kidney cortex is located in granules which have a higher density than other subcellular particulates such as mitochondria, lysosomes and microsomes.

Keywords—mouse kidney cortex; renin granules; differential centrifugation; discontinuous sucrose density gradient centrifugation; renin; succinate dehydrogenase; acid phosphatase; D-glucose-6-phosphatase

Introduction

A number of studies have demonstrated that the juxtaglomerular cells of the kidney are the main site of renin biosynthesis and storage in most mammals.²⁻⁶⁾ The renin system of mice differs in several ways from that of other animals. In mice, the submaxillary gland is one of the richest source of renin, as well as the kidneys.^{7,8)} The submaxillary renin content varies among different strains of mice, and some mice have as much as 98% of their total renin in the submaxillary glands.⁸⁻¹⁰⁾ In addition, it has been shown that while norepinephrine stimulates release of submaxillary as well as renal renin, isoproterenol only stimulates renal renin release in mice.^{11,12)}

On the other hand, morphological studies¹³⁻¹⁵⁾ showed that mouse kidney renin was stored in the granules of juxtaglomerular cells. Recently, Soltész *et al.*¹⁶⁾ demonstrated the lysosomal nature of these granules using a histochemical technique. However, there is little data available on the subcellular localization of renin in the mouse kidney cortex. The present study was an attempt to procure detailed information on the subcellular localization of renin, and began with the fractionation of renin granules from the mouse kidney cortex.

Materials and Methods

Experimental Animals and Preparation of Tissue Homogenate—Male albino mice of ddY strain weighing 25–30 g were used. For 1 week before the study the mice were fed a standard laboratory chow (Oriental Yeast Co., MF) and provided with tap water *ad libitum*.

The animals were decapitated without anesthesia. Immediately, both kidneys were removed and placed in cold physiological saline. Each kidney was weighed, then the medulla was removed. The cortex was sectioned into thin slices with a blade at low temperature and rinsed thoroughly with cold physiological saline to remove as much blood as possible. Cortical slices were transferred to ice-cold 0.45 M sucrose (1:8, wt/vol), and gentle homogenization was performed in a Potter-Elvehjem homogenizer with a loose-fitting pestle at 800 rpm for 40 sec. After the separation of unbroken cells, cell debris, and nuclei by centrifugation

for 10 min at $500 \times g$, the homogenate was subjected to fractionation by differential centrifugation or discontinuous sucrose density gradient centrifugation.

Subcellular Fractionation of the Homogenate—In the first series of experiments, the homogenate was differentially centrifuged in a Hitachi model 20 PR centrifuge with a PRR 20 rotor or in a Hitachi model 65 P ultracentrifuge with a PR 65 T rotor. Three particulate fractions, namely heavy mitochondrial, light mitochondrial, and microsomal, were successively isolated by sedimentation at $6000 \times g$ for 5 min, at $20000 \times g$ for 20 min and at $100000 \times g$ for 60 min, respectively. All procedures were carried out at 0° . The particulate fractions were carefully resuspended in 0.45 M sucrose solution by means of the Potter-Elvehjem homogenizer. The suspensions and final supernatant were analysed for protein, renin and reference enzymes of principal subcellular particulates.

In the second series of experiments, the homogenate was fractionated by 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 was carefully layered on top of the gradient and centrifuged at $60000 \times g$ for 90 min in a Hitachi model 65 P ultracentrifuge with a RPS 25-2A rotor. After 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.

Preparation of Renin Substrate—Rats, dogs and rabbits were bilaterally nephrectomized 24 hr before bleeding, and blood samples were collected without hemolysis from an arterial cannula into syringes moistened with heparin solution or 15% disodium ethylenediaminetetraacetic acid (EDTA). The blood samples were immediately cooled in ice and centrifuged at 10000 rpm for 20 min at 0° . Renin substrate was prepared from the plasma according to the procedure described previously.¹⁷⁾ Angiotensin 1 was not detectable on incubating each renin substrate for 1 hr at 37° . To 0.5 ml of renin substrate, 100 ng of angiotensin 1 was added and the mixture was incubated for 15 min at 37° . The total angiotensin 1 in the samples was determined. Mean recovery of added angiotensin 1 was $98.5 \pm 5.1\%$. Therefore, the substrate was free of renin and angiotensinases. The substrate prepared from rats, dog, rabbits yielded 10.3, 3.56 or 1.95 μ g of angiotensin 1 per ml, respectively, when incubated with homologous renin.

Assays of Enzymatic Activities and Protein Concentration—The reference enzymes used to assess the purity of the fractions were: renin (EC 3.4.99.19) for renin granules, succinate 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 contents of the fractions and homogenate were determined by the procedure described previously.¹⁸⁾ Briefly, samples were diluted with 0.1% Triton X-100 solution, pretreated with 3 mM CuSO_4 , and incubated with rat renin substrate in the absence of activities of converting enzyme and angiotensinases. Generated angiotensin 1 was determined by radioimmunoassay. Renin content was expressed as μ g of angiotensin 1 generated per ml of the fractions and homogenate per hr of incubation. Activities of succinate dehydrogenase, acid phosphatase, and D-glucose-6-phosphatase were assayed according to the method described previously.¹⁹⁾ Succinate dehydrogenase activity was expressed in arbitrary units as a decrease of absorbancy at 400 nm per ml of fractions and homogenate per hr of incubation. Acid phosphatase and D-glucose-6-phosphatase activities were expressed as μ moles of inorganic phosphate released from each substrate per ml of the fractions and homogenate per hr of incubation. The protein contents of fractions and homogenate were determined by the method of Lowry *et al.*²⁰⁾ with crystalline bovine serum albumin as the standard.

Results

Species Specificity of the Reaction between the Mouse Kidney Renin and Renin Substrates from Rats, Dogs and Rabbits

The reactions of mouse kidney renin with renin substrates of the three animal species were evaluated in the same assay system at pH 7.0 and the results are given in Table I. Rat renin substrate showed the highest susceptibility to cleavage by mouse kidney renin. Accordingly, in the present study, rat renin substrate was used for determining the renin contents of the fractions and homogenate.

Subcellular Fractionation by Differential Centrifugation

Since our previous study¹⁹⁾ indicated that dog renin granules are prone to rupture under mechanical agitation, the mouse kidney cortex homogenate was prepared by a gentle procedure (800 rpm, 40 sec) in order to obtain intact renin granules.

Table II shows the percent distribution and specific activities of renin, succinate dehydrogenase, acid phosphatase and D-glucose-6-phosphatase in each subcellular fraction obtained by differential centrifugation of kidney cortex homogenate. The heavy mitochondrial fraction

TABLE I. Species Specificity of the Reaction between Mouse Kidney Renin and Renin Substrates from Rats, Dogs and Rabbits

Renin substrate	Generated angiotensin 1 ng/ml/hr
Rat	29.1 ± 1.27
Dog	16.6 ± 0.48
Rabbit	9.84 ± 0.29

Values are means ± S.E. of 4 experiments. The renin substrate from rats, dogs or rabbits was diluted with 0.25 M phosphate buffer, pH 7.0, containing 25 mM EDTA. This diluted substrate solution yields 400 ng of angiotensin 1 per ml. The incubation system consisted of: 1) diluted substrate solution, 0.5 ml; 2) 6.6% 8-hydroxyquinoline sulfate, 10 μ l; 3) 10% 2,3-dimercapto-1-propanol, 3 μ l; 4) 5% diisopropylfluorophosphate dissolved in propanol, 20 μ l; 5) mouse renin solution, 0.05 ml. The mixture was incubated at 37° for 15 min, and reaction was stopped by the addition of 0.1 ml of 1N HCl, followed by heating for 5 min in a boiling water bath. Subsequently, 0.35 ml of 0.67 M Na₂HPO₄ was added to the mixture and the whole was centrifuged at 3000 rpm for 20 min. Generated angiotensin 1 was determined by radioimmunoassay.

TABLE II. Percent Distribution and Specific Activities of Renin and Reference Enzymes in Subcellular Fractions obtained by Differential Centrifugation of Mouse Kidney Cortex

Enzymes	Homogenate	M ^{a)}	L ^{b)}	P ^{c)}	S ^{d)}
Percent distribution ^{e)}					
Renin		41.26 ± 2.01	18.35 ± 0.98	6.41 ± 0.50	32.78 ± 1.30
Succinate DH ^{f)}		65.76 ± 2.12	28.23 ± 1.47	6.01 ± 0.74	0
Acid P-ase ^{g)}		39.16 ± 2.48	31.26 ± 1.85	11.06 ± 1.47	18.52 ± 2.97
G6P-ase ^{h)}		24.99 ± 2.22	29.45 ± 2.55	43.09 ± 1.35	2.58 ± 0.48
Specific activities ⁱ⁾					
Renin	9.98 ± 0.96	19.75 ± 0.99	8.03 ± 0.41	6.33 ± 0.48	9.54 ± 0.41
Succinate DH	1.38 ± 0.14	5.39 ± 0.51	1.58 ± 0.08	0.62 ± 0.09	0
Acid P-ase	0.98 ± 0.03	1.58 ± 0.10	1.45 ± 0.08	1.06 ± 0.14	0.48 ± 0.07
G6P-ase	1.24 ± 0.08	1.10 ± 0.09	1.65 ± 0.14	5.45 ± 0.17	0.09 ± 0.01

Values are means ± S.E. of 8 experiments. a) Heavy mitochondrial fraction. b) Light mitochondrial fraction. c) Microsomal fraction. d) Final supernatant. e) Percent distribution was calculated as the activity in each fraction divided by the total activity recovered in M, L, P, and S times 100. f) Succinate dehydrogenase. g) Acid phosphatase. h) D-Glucose-6-phosphatase. i) See the text for units.

showed the highest activity of succinate dehydrogenase, accounting for about 66% of the total activity, with a specific activity about four times that of the homogenate. The remaining 28% of succinate dehydrogenase activity was found in the light mitochondrial fraction. Approximately 70% of acid phosphatase was recovered in the heavy and light mitochondrial fractions with a slight 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 microsomal fraction. These data are qualitatively similar to those obtained in the rat,²¹⁻²³⁾ dog¹⁹⁾ and rabbit²⁴⁾ kidney homogenates.

About 40% of total renin activity was recovered with a two-fold increase in specific activity in the heavy mitochondrial fraction. However, the supernatant also contained approximately 40% of total activity.

Subcellular Fractionation by Discontinuous Sucrose Density Centrifugation

Table III shows the activities of renin and reference enzymes and the concentration of protein in each fraction obtained by discontinuous sucrose density centrifugation. D-Glucose-6-phosphatase showed a single peak activity in fraction 6 (1.2 M sucrose fraction), with a specific activity 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. These results indicate a heterogeneity of the lysosomes. Succinate dehydrogenase activity was mainly concentrated in fraction 4 (1.4 M sucrose fraction) with a fourfold increase of specific activity, showing that

TABLE III. Protein Concentration and Activities of Renin and Reference Enzymes in the Homogenate and Fractions after Sucrose Density Gradient Centrifugation of Mouse Kidney Cortex

	Protein mg/ml	Renin $\mu\text{g}^a/\text{ml/hr}$	Succinate DH $-\text{A}^b/\text{ml/hr}$	Acid P-ase $\mu\text{mol}^c/\text{ml/hr}$	G6P-ase $\mu\text{mol}^c/\text{ml/hr}$
Homogenate	9.75 ± 0.52	71.99 ± 8.68	10.51 ± 1.19	8.00 ± 0.33	10.48 ± 0.63
Supernatant	4.79 ± 0.14	38.01 ± 5.68	1.35 ± 0.06	3.41 ± 0.24	1.23 ± 0.22
Fraction 6	3.02 ± 0.20	7.40 ± 0.65	3.09 ± 0.57	3.67 ± 0.32	9.70 ± 0.93
Fraction 5	1.98 ± 0.23	1.57 ± 0.20	3.11 ± 0.22	1.26 ± 0.12	5.13 ± 0.21
Fraction 4	1.31 ± 0.09	1.67 ± 0.12	5.45 ± 0.31	0.89 ± 0.08	3.01 ± 0.22
Fraction 3	0.62 ± 0.05	17.61 ± 2.76	2.30 ± 0.48	0.63 ± 0.04	1.21 ± 0.28
Fraction 2	0.52 ± 0.06	3.36 ± 0.28	1.37 ± 0.39	0.44 ± 0.04	0.33 ± 0.12
Fraction 1	0.29 ± 0.03	2.35 ± 0.31	0	0.28 ± 0.01	0

Values are means \pm S.E. of 8 experiments. a) Angiotensin 1. b) Decrease of absorbancy at 400 nm. c) Inorganic phosphate released from substrate.

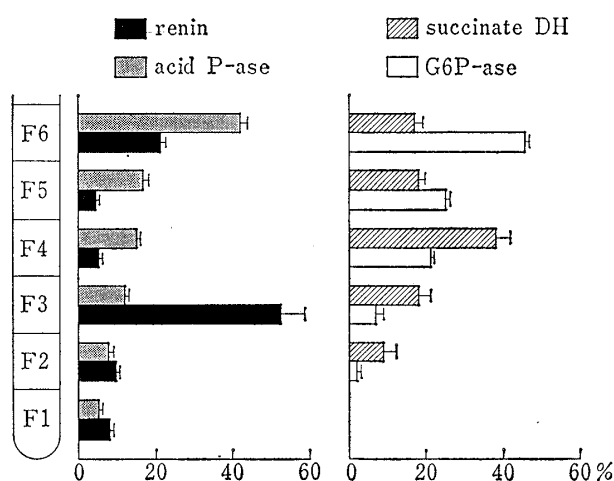


Fig. 1. Distribution Patterns of Renin and Reference Enzymes of Subcellular Particulates after Discontinuous Sucrose Density Gradient Centrifugation of Renal Cortex Homogenate

Acid P-ase, acid phosphatase; G6P-ase, D-glucose-6-phosphatase; Succinate DH, succinate dehydrogenase. Ordinate, fraction (F) number. Abscissa, percent of the total enzyme activity obtained from fractions 1–6.

6-phosphatase and 42% of acid phosphatase were recovered in fraction 6 and 38% of succinate dehydrogenase in fraction 4. On the other hand, fraction 3 contained approximately 53% of total granular renin activity. These results demonstrate that renin granules can be isolated from the other subcellular granules on the basis of higher density.

When fraction 3 was recentrifuged at $100000 \times g$ for 60 min, most of the renin activity was recovered in the sediment. Part of fraction 3 was frozen at -20° and thawed below 5° . This procedure was repeated six times. The renin activity of the treated fraction was markedly augmented, being seven times that of the nontreated fraction. These findings suggest that renin is isolated in the form of granules in this fraction.

Discussion

The present study was carried out to investigate the subcellular localization of renin in the mouse kidney cortex. The separation of renin granules from other subcellular particulates was evaluated by biochemically determining the activities of appropriate reference enzymes.

most of the mitochondria equilibrated in this fraction. On the other hand, renin activity was mostly recovered in fraction 3 (1.5 M sucrose fraction); $17.61 \pm 2.76 \mu\text{g}$ of angiotensin 1 was liberated per hr with 1 ml of this fraction. The specific activity of renin in this fraction was $28.40 \pm 4.45 \mu\text{g}$ of angiotensin 1 per mg of protein per hr, being 3.2 times that of the homogenate. When this fraction was directly injected into the femoral vein of the rat, typical pressor action by renin was observed. The concentration of protein was high in fractions 4–6, corresponding to the mitochondria, lysosomes and microsomes, but low in fractions 1 to 3.

Fig. 1 shows the distribution patterns of renin and the other reference enzymes, as calculated from the recovered volumes of fractions 1 to 6 and their enzyme activities. Approximately 46% of D-glucose-

Renin contents of the fractions were determined, based on radioimmunoassay of angiotensin 1 generated during incubation of the fractions and excess renin substrates. Since there are various forms of angiotensinases and angiotensin 1-converting enzyme in the kidney tissue, angiotensin 1 breakdown during incubation was prevented by the presence of inhibitors of these enzymes. In order to select the renin substrate best suited for determining the renin contents of mouse kidney cortex, we investigated the species specificity of the reaction between mouse kidney renin and various renin substrates. The results indicated that rat renin substrate showed the highest specificity at pH 7.0.

Differential centrifugation experiments showed that renin granules sedimented mainly in the heavy mitochondrial fraction and only slightly in the microsomal fraction. Early studies²⁵⁻²⁷) by differential centrifugation also demonstrated renin activity in the mitochondrial fraction of rat, pig and rabbit kidneys. On the other hand, Ogino *et al.*²⁸⁾ have suggested the lysosomal nature of rat renin granules on the basis that the highest activities of renin and acid phosphatase were noted in the same fraction. This is supported by Gomba and Soltész,^{16,29)} who demonstrated histochemically the presence of acid phosphatase, β -glucuronidase, aryl sulfatase and N-acetyl- β -glucosaminidase in the juxtaglomerular granules of mice. In fact, in the present study, a considerable amount of acid phosphatase was included in 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 by differential centrifugation.

The final supernatant obtained by differential centrifugation showed high renin activity. This would be derived mainly from the disruption of renin granules. In a preliminary experiment, when the force of homogenization was increased, renin activity shifted from the sedimentable fractions to the supernatant. It seems that mouse renin is stored in mechanically fragile granules.

The present discontinuous sucrose density gradient centrifugation studies demonstrated that renin is distributed differently from other principal reference enzymes. Renin granules were mainly recovered in the fraction corresponding to 1.5 M sucrose. This fraction contained 53% of the total renin activity that appeared in particulate fractions. The specific activity of renin in this fraction was 3.2 times that found in the unfractionated homogenate. The possibility that renin in this fraction is separated in the form of granules was suggested by the following results; 1) most of the renin activity was recovered in the sediment after recentrifugation of the separated fraction and 2) the renin activity of this fraction increased seven times upon repeated freezing and thawing. Furthermore, we (Nakamura *et al.* unpublished data) found that the molecular weight of renin in this renin granule fraction was 40000 ± 1000 by gel filtration and that no significant changes in its molecular weight and activity occurred after acidification. Therefore, it is assumed that only active renin of the usual molecular weight (37000—43000) is stored in renin granules of the mouse kidney. Previously we have demonstrated that renin granules of rabbit²⁴⁾ and rat¹⁸⁾ were also recovered in the fraction corresponding to 1.5 M sucrose, while dog¹⁹⁾ renin granules were recovered mostly in the fraction corresponding to 1.6 M sucrose. These findings indicate that the density of renin granules varies from species to species.

On the other hand, most of the succinate dehydrogenase, acid phosphatase and D-glucose-6-phosphatase equilibrated in the upper layers of the gradient above the renin granular fraction. Accordingly, it is assumed that renin in the mouse kidney is stored in granules which have a higher density than other subcellular particulates such as mitochondria, lysosomes and microsomes.

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