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Renin Release and Lipid Peroxidation in Renin Granules of the Rat

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The present study was carried out to determine the effect of lipid peroxidation on renin release from renin granules of the rat. Renin granules were isolated from the kidney cortex by discontinuous sucrose density gradient centrifugation. Renin activity was measured by radioimmunoassay and lipid peroxidation was estimated by means of the thiobarbituric acid test. Renin release from isolated renin granules was markedly stimulated by incubation at 37°C in the presence of ascorbic acid or ferrous ions, accompanied by increased formation of lipid peroxide in renin granules. These simultaneous increases in lipid peroxidation and renin release were abolished by the addition of *N,N'*-diphenyl-*p*-phenylenediamine. On the other hand, dehydroascorbic acid or ferric ions caused no change in lipid peroxidation and renin release. In addition, the molecular weight of renin released from the granules was 40000 and its activity was not changed after acidification. From these findings, it is assumed that ascorbic acid and ferrous ions stimulate the peroxidation of endogenous lipids that form part of the membrane of renin granules, and this process results in a breakdown of renin granules.

Keywords——rat renin granules; renin release; lipid peroxidation; ascorbic acid; dehydroascorbic acid; ferrous ions; ferric ions; *N,N'*-diphenyl-*p*-phenylenediamine; molecular weight of renin; acidification

Introduction

There have been many studies on renin release from the kidney to circulating blood, and several factors are known to stimulate renin release *in vivo*. Previously, Yamamoto *et al.* have shown that the release of renin from isolated renin granules is stimulated by the addition of dibutyryl cyclic-AMP[1] and calcium ions[2] to the incubation medium. Furthermore, Funakawa *et al.*[3] have reported that mechanical or osmotic shock, and freezing–thawing provoke renin release from renin granules. However, the molecular mechanism underlying renin release is unknown.

Lipid peroxidation is believed to be important as a factor causing membrane leakage or lysis. Several investigations have shown the close association between lipid peroxidation and increased permeability both in red blood cells[4–6] and in subcellular particulates such as mitochondria[7–9] and lysosomes.[10–12] However, there is little information on lipid peroxidation in renin granules.

The present study was undertaken, therefore, to evaluate the effect of lipid peroxidation on renin release from renin granules.

Materials and Methods

Preparation of Renin Granules——Male Wistar rats weighing 180—200 g were used. Renin granules were prepared from the kidney cortex by discontinuous sucrose density gradient centrifugation.[13,14] The kidney cortex was gently homogenized with ice-cold 0.45 M sucrose. After the separation of unbroken cells, cell debris and nuclei by centrifugation at $500 \times g$ for 10 min, the homogenate was subjected to fractionation by discontinuous sucrose density gradient centrifugation. The gradient was prepared 1 h before use by layering 7 ml each of sucrose solutions from 1.2 M to 1.7 M in a centrifuge tube. Ten ml of the homogenate was layered on top of the sucrose density gradient and then the tubes were centrifuged at $60000 \times g$ for 90 min with a RPS 25-2A rotor in a Hitachi model 65P ultracentrifuge. Renin granules were recovered in the fraction corresponding to 1.5 M sucrose.

Incubation System for Renin Granules——One ml of the renin granules fraction was suspended in 1.0 ml of 0.1 M Tris–HCl buffer (pH 7.0) containing 0.15 M KCl. Ascorbic acid, ferrous sulfate, dehydroascorbic acid and ferric chloride were dissolved in the same buffer. *N,N'*-Diphenyl-*p*-phenylenediamine (DPPD) was dissolved in ethanol and diluted with the same buffer. These agents were added to the suspension of renin granules. The suspension was incubated at 37°C for 0—90 min, and separated into the supernatant and sediment by centrifugation at 105000 × *g* for 60 min.

Renin Assay——Renin-containing samples were incubated with partially purified rat renin substrate. Disodium ethylenediaminetetraacetic acid (20 mM), 8-hydroxyquinoline (1.6 mM), dimercaprol (4.0 mM) and diisopropylfluorophosphate (1.0 mM) were added to the incubation medium to inhibit converting-enzyme and angiotensinase. After 15 min of incubation at 37°C, the mixture was placed in a boiling bath for 5 min to terminate the renin reaction. Angiotensin I (AI) generated in the incubation mixture was determined by radioimmunoassay[15] using the CEA-IRE-SOLIN kit. Neither ascorbic acid nor ferrous ions influenced the generation rate of AI and the trapping of AI by antibody. Renin activity was expressed as μg of AI/ml/h.

Preparation of Renin Substrate——The renin substrate was prepared from the plasma of nephrectomized rats according to the procedure described previously.[16] Contamination of renin and angiotensinase in the renin substrate was estimated according to the method described previously,[17] but none was observed. The substrate yielded 12.5 μg of AI per ml when incubated with an excess of rat renin.

Measurements of Lipid Peroxides and Protein Contents——Lipid peroxides were measured in terms of the formation of 2-thiobarbituric acid (TBA)-reacting substances, presumed to be malondialdehyde (MDA), by means of a fluorometric assay described by Ohkawa *et al.*[18] The protein contents were determined by the method of Lowry *et al.*[19] with modifications as described by Bensadoun and Weinstein[20] to eliminate the interference by Tris buffer.

Determination of Molecular Weight of Renin released from Renin Granules——The molecular weight of renin was estimated by gel filtration. One ml of sample was applied to a Sephadex G-100 column (1.6 × 90 cm, Pharmacia) equilibrated with 40 mM sodium phosphate buffer (pH 7.4) containing 0.1 M NaCl and 3 mM NaN_3 as an antimicrobial agent at 4°C. The flow rate was 8.8 ml/h and 1 ml fractions were collected. The void volume of the column was estimated by the use of blue dextran, and bovine serum albumin (molecular weight, 67000), ovalbumin (molecular weight, 45000), α-chymotrypsinogen A (molecular weight, 25000) and cytochrome c (molecular weight, 12900) were used as standards for molecular weight.

Acidification——Aliquots of the supernatant after incubation of renin granules were dialyzed against 0.05 M glycine–HCl buffer (pH 3.0) containing 0.1 M NaCl for 20 h at 4°C and subsequently against 0.04 M sodium phosphate buffer (pH 7.4) containing 0.1 M NaCl for 20 h at 4°C. As a control, a sample was dialyzed separately against sodium phosphate buffer (pH 7.4) for 40 h at 4°C.

Results

Renin granules prepared from the kidney cortex of normal rats contained a small amount (1.08±0.15 nmol MDA/mg protein) of endogenous lipid peroxides. Incubation of these granules at 37°C for 90 min caused a slight increase in the lipid peroxide content (Table I). When the suspension of renin granules was incubated in the presence of 5 μM ascorbic acid or 5 μM ferrous ions, the lipid peroxidation was greatly enhanced, the lipid peroxides level being

Table I. Effects of Ascorbic Acid and Ferrous Ions on Lipid Peroxide Formation and Renin Release from Renin Granules

Conc. of drugs	Lipid peroxide formation (nmol of MDA/mg of protein)		Renin release (%)	
(μM)	Ascorbic acid	Ferrous ions	Ascorbic acid	Ferrous ions
0	1.95±0.12	1.95±0.12	24.7±1.91	24.7±1.91
5	4.55±0.12[a]	4.94±0.42[a]	56.0±3.45[a]	43.7±1.45[a]
10	8.23±0.29[a]	6.25±0.37[a]	76.3±4.21[a]	43.4±1.77[a]
50	11.33±0.69[a]	10.00±0.36[a]	96.9±0.32[a]	49.4±3.08[a]
100	12.54±0.51[a]	10.84±0.60[a]	95.6±1.06[a]	62.7±2.14[a]

A suspension of renin granules (0.98±0.07 mg of protein) was incubated with ascorbic acid or ferrous ions at 37°C for 90 min and then separated into the supernatant and sediment by centrifugation at 105000 × *g* for 60 min. The sediment was analyzed for lipid peroxides, the level of which is expressed as nmol of MDA per mg of protein. The supernatant and sediment were analyzed for renin activity. Total renin is taken to be sum of renin content in the supernatant and the sediment, and the amount of renin release during incubation is expressed as a percentage of total renin. All values are means ± S.E. of five separate experiments.

a) Values are significantly different from the control value ($p < 0.001$).

about 2.5 times the control. Increasing the concentrations of these agents resulted in a dose-related increase in lipid peroxide formation in renin granules. At a concentration of 50 μM, the amount of lipid peroxide reached an extremely high value, which was 5—6 times the control. On the other hand, renin release from the granules was strongly stimulated by the addition of ascorbic acid or ferrous ions to the incubation medium. The stimulatory effect of ascorbic acid was more potent than that of ferrous ions at various concentrations, 5—100 μM. In particular, most of the renin in the granules could be released in the presence of 50 μM ascorbic acid.

Figure 1 shows the time course of lipid peroxide formation and renin release during incubation of renin granules with 50 μM ascorbic acid or 50 μM ferrous ions at 37°C. In the presence of ascorbic acid, the rate of lipid peroxide formation in renin granules had increased greatly by 30 min after the start of incubation, showing a 230% increase in the lipid peroxide content. Simultaneously, renin release from the granules occurred rapidly and reached a maximum level of more than 95% of the total renin with in 30 min after the start of incubation. Ferrous ions also provoked time-dependent increases in lipid peroxide formation and renin release, though at a considerably slower rate than ascorbic acid.

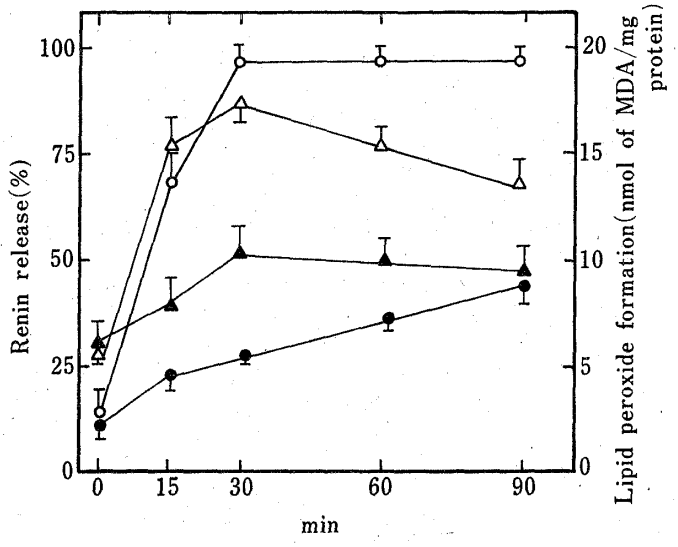

Fig. 1. Time Course of Lipid Peroxide Formation and Renin Release during Incubation of Renin Granules with Ascorbic Acid or Ferrous Ions

The concentration of ascorbic acid (open symbols) or ferrous ions (closed symbols) was 50 μM in all experiments. Other experimental details and the expression of renin release (circles) and lipid peroxide formation (triangles) were the same as in Table I. Points and bars represent mean values ± S.E. of four separate experiments.

Furthermore, we examined the correlation between lipid peroxide formation and renin release. When 100 nM DPPD, an antioxidant, was added to a suspension of renin granules and incubated at 37°C for 30 min in the presence of ascorbic acid or ferrous ions, the stimulation of lipid peroxidation by ascorbic acid or ferrous ions was completely inhibited. Simultaneously, renin release from the granules was also decreased to the control level by 100 nM DPPD. In addition, at a concentration of 50 μM, dehydroascorbic acid or ferric ions caused no significant change in lipid peroxide formation or renin release compared to the control (Table II).

Figure 2 shows the gel filtration profile of renin released from the granules in the presence of 50 μM ascorbic acid. The peak of renin activity corresponded to a molecular weight of 40000. A similar result was obtained for renin release during incubation of renin granules with 50 μM ferrous ions. Furthermore, when renin granules were ruptured by freezing and thawing five times or by suspending them in Triton X-100 according to the method of Funakawa *et al.*,[3] the

TABLE II. Effects of Various Agents on Lipid Peroxide Formation and Renin Release from Renin Granules

Addition and its concentration	Lipid peroxide formation (nmol of MDA/mg of protein)	Renin release (%)
No addition	2.14±0.32	17.5±1.41
Dehydroascorbic acid (50 μM)	2.29±0.21	18.8±1.71
Ascorbic acid (50 μM)	13.30±0.10[a)]	96.9±2.35[a)]
Ascorbic acid (50 μM) +*N*,*N*′-diphenyl-*p*-phenylenediamine (100 nM)	2.44±0.38	22.2±2.12
Ferric ions (50 μM)	3.09±0.31	19.6±3.20
Ferrous ions (50 μM)	9.11±0.23[a)]	30.9±1.00[b)]
Ferrous ions (50 μM) +*N*,*N*′-diphenyl-*p*-phenylenediamine (100 nM)	3.12±0.40	20.1±1.85

A suspension of renin granules (0.98 ± 0.07 mg of protein) was incubated at 37 °C for 30 min. Other experimental details and the expression of renin release and lipid peroxide formation were the same as in Table I. All values are means ± S.E. of four separate experiments.
a), *b*) Values are significantly different from the control value ([a)] $p<0.001$, [b)] $p<0.01$).

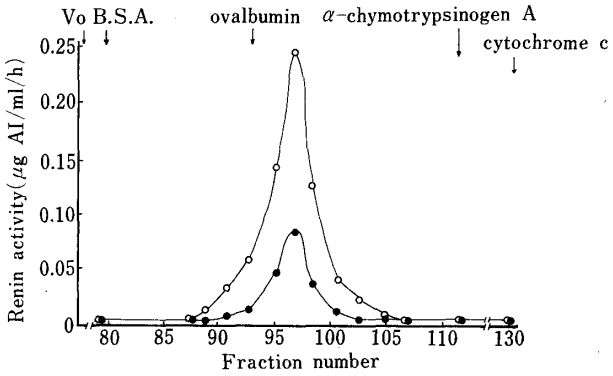

Fig. 2. Elution Profile of Renin released from the Granules

A suspension of renin granules (0.98 ± 0.07 mg of protein) was incubated with 50 μM ascorbic acid ○ or 50 μM ferrous ions ● at 37 °C for 30 min and then separated into the supernatant and sediment by centrifugation at 105000× *g* for 60 min. One ml of the supernatant was applied to a Sephadex G-100 column. Elution positions of molecular weight standards are indicated by arrows; void) volume (Vo), bovine serum albumin (B.S.A.; molecular weight, 67000), ovalbumin (molecular weight, 45000), α-chymotrypsinogen A (molecular weight, 25000), cytochrome c (molecular weight, 12900).

TABLE III. Effects of Acidification on the Activity of Renin released from Renin Granules

Treatment	Renin activity (μg/ml/h)	
	Ascorbic acid	Ferrous ions
Dialysis at pH 7.4 (control)	6.67±0.88	2.53±0.49
Dialysis at pH 3.0 (experimental)	6.82±1.15	2.58±0.65

A suspension of renin granules (0.98 ± 0.07 mg of protein) was incubated with 50 μM ascorbic acid or 50 μM ferrous ions at 37 °C for 30 min and then separated into the supernatant and sediment by centrifugation at 105000 × *g* for 60 min. A part of the supernatant was dialyzed against the buffer (pH 3.0 or pH 7.4). Values are means ± S.E. of three separate experiments.

elution profiles of renin from the granules still showed a single peak with a molecular weight of 40000.

To examine whether masked enzyme activity appears on acidification, the supernatant of the medium after incubation of renin granules with 50 μM ascorbic acid was acidified. As shown in Table III, no significant change in the activity of renin was observed. The same result was obtained with the supernatant when renin granules were incubated in the presence of 50 μM ferrous ions.

Discussion

Previous studies[4–12,21,22] have shown that biomembrane and subcellular organelles are major sites of lipid peroxidation and that the lysis of cells or subcellular structures is frequently attributable to peroxidation of the lipid components of their membrane. It is of interest, therefore, to determine the relation of lipid peroxidation in renin granules to renin release, since this may provide information on the molecular mechanism underlying renin release.

It has been shown [3] that dog renin granules are stable during incubation in 10 mM potassium phosphate buffer, pH 7.0, *i.e.*, approximately 20% of total renin appears in the medium during incubation of renin granules at 37°C for 180 min. In the present study, similar amounts of renin release were observed when a suspension of rat renin granules was incubated in 0.1 M Tris–HCl buffer, pH 7.0, at 37°C for 90 min. However, the addition of ascorbic acid resulted in a dramatic increase of renin release into the suspending medium and an increased formation of lipid peroxide in renin granules during incubation at 37°C. These effects of ascorbic acid on renin granules were dose-dependent and occurred rapidly during incubation at 37°C. On the other hand, ferrous ions also stimulated the lipid peroxide formation and renin release from the granules, but were found to be less effective than ascorbic acid. At concentrations of 5—50 μM ferrous ions the increases in lipid peroxidation were not parallel with those in renin release, but the reason for this is unclear.

It is well known that DPPD shows an antioxidative effect on lipid peroxide formation in subcellular organelles. In the present study, DPPD inhibited the lipid peroxide formation due to ascorbic acid or ferrous ions. Further, the stimulatory effects of ascorbic acid or ferrous ions on renin release were not observed in the presence of DPPD. Dehydroascorbic acid and ferric ions caused no significant changes in lipid peroxidation and renin release. From these findings, it is assumed that ascorbic acid and ferrous ions stimulate the peroxidation of endogenous lipids that form part of the membrane of renin granules, and this process results in breakdown of the renin granules.

Recently, several studies have been carried out to investigate the storage form of renin in the granules of rats and dogs. Morris and Johnston[23] reported that isolated rat renin granules contained both active renin with a molecular weight of 37000 and acid-activatable renin with a molecular weight of 44000. On the other hand, Sagnella *et al.*[24] demonstrated that rat renin granules contained only the low molecular weight (molecular weight, 42000) renin and that its activity was not activated by acidification. Similar results were reported in dog renin granules.[25,26] These discrepancies in the storage form and molecular weight of renin might be due to the differences of experimental conditions and species. In the present study, the molecular weight of renin released from the granules by ascorbic acid- or ferrous ions-induced lipid peroxidation was 40000 and its activity was not changed after acidification. Furthermore, the same results were obtained with renin released from the granules by freezing and thawing or treatment with Triton X-100. These results indicate that renin in the granules of rats exists as active renin with a molecular weight of 40000.

In this work, we demonstrated that the release of renin was markedly stimulated by lipid peroxidation at a subcellular level. Further studies are required on the relationship between renin release and lipid peroxidation *in vivo*.

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