Lysosomal Enzyme Activities in Rat Kidney treated with DOCA, especially with Reference to Renin, Cathepsin and β -Glucuronidase

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IT IS established that desoxycorticosterone acetate (DOCA) plus salt can produce hypertension, cardiovascular lesions and nephrosclerosis in rat. *Brown et al.* reported high plasma-DOC in hypertensive patients with normal plasma-aldosterone and low plasma-renin.¹ *Brunner et al.* demonstrated high incidence of heart attacks or strokes among essential hypertensive patients with normal or high plasma renin activity as compared to those with low plasma renin and suggested the vasculotoxic effects of renin.² Electron microscopic histochemical investigations have showed the presence of both acid phosphatase and β -glucuronidase in the juxtaglomerular cell granules.³⁻⁵ Renin activity was also shown in the lysosomal fraction of rat kidney.⁶ *Kira et al.* demonstrated the presence of hemoconcentrating substance and vascular permeability factor in the lysosomal fraction of rat kidney.⁷ The present investigation was undertaken to study the relation between lysosomal acid hydrolases and hypertension or hypertensive vascular lesions.

MATERIALS AND METHODS

Female Wistar rats weighing 80 to 110g were uninephrectomized in one group (Experiment 1) and not nephrectomized in another group (Experiment 2). One or two 30 mg of DOCA were

implanted subcutaneously. In Experiment 1, nine rats implanted with DOCA were given Oriental laboratory chow containing 0.6% NaCl with 0.9% saline as drinking fluid. Seven control sham-operated rats received Oriental Laboratory chow with tap water. In Experiment 2, twenty five rats implanted with DOCA were given high salt diet containing 7.6% NaCl with 0.9% saline. Twenty four control rats received standard normal diet containing 0.6% NaCl with tap water. Systolic blood pressure of rat was determined by the plethysmographic method.

Tissue fractionations: Rat kidney were removed under ether anesthesia after rats were fasted for 20 to 24 hours. Rat kidney isolated was placed in ice-cold 0.45 M sucrose and homogenized in a Potter-Elvehjem homogenizer with 0.45 M sucrose (1 : 8, w/v) by two up-and-down strokes within 30 seconds. The cytoplasmic fraction (C. F.) of rat kidney was obtained by centrifuging the homogenate for 2.5 min at 650g using Kubota KR-6P centrifuge. The supernatant after centrifuging C. F. for 5 min at 6600g was centrifuged again for 60 min at 100000g using Hitachi 65P Ultracentrifuge, and the soluble fraction (S₁) was obtained. In another procedure, the supernatant after centrifuging C. F. for 60 min at 18000g was used as supernatant fraction (S₂) and the precipitate was resuspended in 0.45 M sucrose as sedimentable fraction. Cytoplasmic and sedimentable fractions were used for the assay of lysosomal enzymes after ten times freezing and thawing.

Enzyme assays: In the assay of renal renin activity, incubation was carried out for 45 min at

Key Words:

Lysosomal enzyme activities
DOCA hypertension
Renin

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* This paper was presented on the II Conference of Pathogenesis of Hypertension, November 20, 1972, Fukuoka

† This investigation was supported by a grant-in-aid for scientific research from the Japanese Ministry of Education.

TABLE I-a DATA IN EXPERIMENT 1. ENZYME ACTIVITY IN THE CYTOPLASMIC FRACTION (C. F.) OF RAT KIDNEY

Systolic Blood pressure	(E)	184 ± 39	(9) mmHg****
	(C)	82 ± 9	(8) mmHg
Body Weight	(E)	88.9 ± 13.8	(9)
	(C)	100 ± 2.1	(8)
Heart Weight per 100g of body weight	(E)	163 ± 43	(9)****
	(C)	100 ± 7	(8)
Kidney Weight per 100g of body weight	(E)	175 ± 44.3	(9)****
	(C)	100 ± 4.7	(8)
Protein	(E)	88.9 ± 20.8	(9)
	(C)	100 ± 7	(8)
Renin	(E)	23.2 ± 21.9	(9)
	(C)	100 ± 21	(8)
Cathepsin	(E)	173.6 ± 40.4	(9)****
	(C)	100 ± 14.2	(7)
β-Glucuronidase	(E)	150.5 ± 33.4	(9)****
	(C)	100 ± 9.7	(8)
β-Glycerophosphatase	(E)	96.1 ± 7.9	(9)
	(C)	100 ± 3.2	(8)
Deoxyribonuclease	(E)	118.6 ± 30.8	(9)
	(C)	100 ± 14.7	(8)
Ribonuclease	(E)	95.5 ± 23.8	(9)
	(C)	100 ± 8.5	(8)

(E): the case of Experiment, (C): the case of Control.

Means ± SD are given. Control value is expressed as 100 except systolic blood pressure. The number of cases is shown in parenthesis.

* $P < 0.05$, ** $P < 0.025$, *** $P < 0.01$, **** $P < 0.005$

37°C, in 0.04 M acetate buffer, pH 5.5, containing dialyzed rat plasma as substrate and one drop of DFP (1 : 20 in isopropyl alcohol). The reaction was stopped by boiling for 10 min in a water bath and the filtrate was used for bioassay. Rat plasma as renin substrate was dialyzed against pure water and then against 3 mM EDTA solution, respectively for 24 hours. Cathepsin activity was determined by the modified method of Anson.⁸ Incubation was carried out for 30 min at 37°C, in 0.2 M acetate buffer, pH 3.6, containing denatured hemoglobin (Nutritional Biochemicals Corp., U.S.A., 2%, w/v) as substrate. The reaction was stopped by trichloroacetic acid (3.9 %, w/v), and the products of the reaction were assayed on the filtrate by the method of Lowry *et al.*⁹ using Shimadzu QV-50 spectrophotometer. β-Glucuronidase activity was assayed by the modified method of Fishman.¹⁰ Incubation was carried out for 2 hours at 37°C, in 0.08 M acetate buffer, pH 4.5, containing phenolphthalein monoglucuronide (Chugai Pharmaceutical Co., Japan) as substrate. The reaction was stopped by trichloroacetic acid (2.5%, w/v), and the phenolphthalein content was determined on the filtrate by

colorimetry. β-Glycerophosphatase activity was measured by the modified method of Gianetto *et al.*¹¹ Incubation was carried out for 30 min at 37 °C, in 0.13 M acetate buffer, pH 5, containing β-glycerophosphate (Merck, Germany, 0.053 M) as substrate. The reaction was stopped by trichloroacetic acid (8%, w/v), and inorganic phosphate content of the filtrate was assayed according to the method of Allen.¹² Deoxyribonuclease and ribonuclease activities were determined by the modified method of Romeo *et al.*¹³ Incubation was carried out for either one hour or 30 min at 37°C, in 0.09 M acetate buffer, pH 5, containing either deoxyribonucleic acid (type V from calf thymus, Sigma Chemical Co., U.S.A., 0.18%, w/v) or ribonucleic acid (type XI from yeast, Sigma, 0.18%) as substrate, respectively. The reaction was stopped by trichloroacetic acid (4.4 %, w/v) in deoxyribonuclease or by perchloric acid (15.6%, w/v) containing uranyl acetate in ribonuclease. The absorption of the filtrate was read at 260 mμ in a spectrophotometer. Protein concentration was assayed by the method of Lowry *et al.*⁹ Enzyme activities were expressed in units or ng per mg of protein.

TABLE 1-b DATA IN EXPERIMENT 2. ENZYME ACTIVITY IN THE CYTOPLASMIC FRACTION (C. F.) OF RAT KIDNEY

		2---3 days	5---8 days	8 days (1---2 days)	15---48 days after DOCA administra- tion (more than 4 days after the development of hypertension)
Systolic Blood Pressure	(E) (C)	107 ± 23 (6) 107 ± 14 (6)	104 ± 15 (6) 104 ± 11 (6)	165 ± 17 (4)**** 89 ± 10 (3)	181 ± 23 (9) mmHg**** 105 ± 19 (9) mmHg
Body Weight	(E) (C)	99 ± 6.5 (6) 100 ± 8.1 (6)	87.8 ± 5.9 (6)**** 100 ± 5.8 (6)	93 ± 9.6 (4) 100 ± 3.7 (3)	80.4 ± 8.8 (9)**** 100 ± 4.7 (9)
Heart Weight per 100g of body weight	(E) (C)	98.7 ± 5.5 (6) 100 ± 7.6 (6)	92.2 ± 13.6 (6) 100 ± 14.7 (6)	111.7 ± 12.3 (4) 100 ± 14.1 (3)	151.8 ± 20.6 (9)**** 100 ± 5.4 (9)
Kidney Weight per 100g of body weight	(E) (C)	106 ± 9.8 (6) 100 ± 3.5 (6)	117.6 ± 4.9 (6)**** 100 ± 6.3 (6)	124.5 ± 7.3 (4)**** 100 ± 4.2 (3)	159.5 ± 27.7 (9)**** 100 ± 5.1 (9)
Protein	(E) (C)	119.9 ± 18.2 (6) 100 ± 7.7 (6)	103.7 ± 8.9 (6) 100 ± 5 (6)	103.4 ± 18.3 (4) 100 ± 12.8 (3)	94.6 ± 10.6 (9) 100 ± 4.1 (9)
Renin	(E) (C)	83.6 ± 14.8 (6) 100 ± 15.8 (6)	44.5 ± 18.2 (6)**** 100 ± 17.1 (6)	9 ± 2.8 (4) 100 ± 41 (3)	3.1 ± 6.5 (8)**** 100 ± 15.2 (8)
Cathepsin	(E) (C)	118.6 ± 19.2 (6) 100 ± 5.3 (6)	124.3 ± 11.6 (6)**** 100 ± 3.9 (6)	138.3 ± 11.5 (4)* 100 ± 14.9 (3)	178 ± 60.5 (9)**** 100 ± 7.2 (9)
β-Glucuronidase	(E) (C)	96.6 ± 11.8 (6) 100 ± 7.9 (6)	100.7 ± 11.6 (6) 100 ± 6.3 (6)	102.9 ± 12 (4) 100 ± 15.9 (3)	128.3 ± 25.7 (8)** 100 ± 6.2 (9)
β-Glycerophosphatase	(E) (C)	— —	89.8 ± 14 (6) 100 ± 2.9 (6)	96 ± 8.2 (4) 100 ± 6.4 (3)	90.8 ± 6.5 (9)*** 100 ± 4.2 (9)
Deoxyribonuclease	(E) (C)	— —	— —	106.6 ± 13.7 (4) 100 ± 2.5 (3)	111.3 ± 25.1 (9) 100 ± 5.3 (9)
Ribonuclease	(E) (C)	— —	— —	94.1 ± 15.8 (4) 100 ± 11.9 (3)	84.6 ± 10.2 (5)** 100 ± 7 (6)

(E): the case of Experiment, (C): the case of Control. Means ± SD are given. Control value is expressed as 100 except systolic blood pressure. The number of cases is shown in parenthesis. * $P < 0.05$, ** $P < 0.025$, *** $P < 0.01$, **** $P < 0.005$

Fig. 1. The changes in rats without nephrectomy after the administration of both DOCA and high salt diet.

RESULTS

1. DOCA plus 0.9% saline in uninephrectomized rats (Experiment 1). Nine rats implanted with DOCA showed blood pressure levels of 184 ± 39 mmHg until 2.5 months after DOCA administration, while eight control rats 82 ± 9 mmHg (Table I-a). Both heart and kidney weights per 100g of body weight were increased in rats treated with DOCA. The results of enzyme activities in C. F. are shown in Table I-a. Renal renin activity was suppressed, but cathepsin and β -glucuronidase activities were increased in rats treated with

DOCA. Deoxyribonuclease, ribonuclease and β -Glycerophosphatase activities in rats treated with DOCA did not differ significantly from those in control rats.

2. DOCA, 0.9% saline plus high salt diet containing 7.6% NaCl in rats without nephrectomy (Experiment 2).

The results in experiment 2 are shown in Table I-b and graphically in Figure 1.

(a) During normotensive period until 8 days after DOCA administration, renal renin activity was decreasing. Both cathepsin activity in C. F. and kidney weight per body weight were slightly

TABLE II-a PROTEIN CONCENTRATION AND ENZYME ACTIVITY IN THE SOLUBLE FRACTION (S₁) OR IN THE SUPERNATANT FRACTION (S₂) OF RAT KIDNEY

	Experiment 1 (S ₁)	Experiment 2 (1---2 days) (S ₂)	Experiment 2 (more than 4 days after the development of hyper- tension) (S ₂)
Protein	(E) 82.4 ± 10.1 (4) (C) 100 ± 16.5 (4)	109 ± 19.8 (4) 100 ± 4.4 (3)	98.1 ± 3.2 (7) 100 ± 4.7 (8)
Cathepsin	(E) 112.9 ± 30.7 (4) (C) 100 ± 27.5 (3)	107.6 ± 12 (4) 100 ± 19.9 (3)	113.7 ± 14.7 (5) 100 ± 7.6 (5)
β-Glucuronidase	(E) 80.5 ± 5.6 (4)* (C) 100 ± 10.1 (4)	94.8 ± 14.7 (4) 100 ± 9.8 (3)	98.1 ± 16.6 (7) 100 ± 12 (8)
β-Glycerophosphatase	(E) 74.5 ± 19.5 (4) (C) 100 ± 21.5 (4)	100.6 ± 20 (4) 100 ± 6.6 (3)	84.2 ± 10.6 (5)* 100 ± 5.5 (5)

(E): the case of Experiment, (C): the case of Control. Means ± SD are given.
Control value is expressed as 100. The number of cases is shown in parenthesis. * $P < 0.05$

TABLE II-b THE PERCENTAGE OF THE SOLUBLE FRACTION (S₁) OR OF THE SUPERNATANT FRACTION (S₂) TO THE CYTOPLASMIC FRACTION (C. F.) IN PROTEIN CONCENTRATION AND ENZYME ACTIVITY

	Experiment 1 (S ₁)	Experiment 2 (1---2 days) (S ₂)	Experiment 2 (more than 4 days after the development of hyper- tension) (S ₂)
Protein	(E) 63.7 ± 11.3 (4) (C) 55.6 ± 7.2 (4)	57.9 ± 1.4 (4) 55 ± 0.02 (3)	57.5 ± 2.8 (8) 56.5 ± 2.3 (9)
Cathepsin	(E) 45.3 ± 10.4 (4) (C) 56.5 ± 11.1 (3)	29.9 ± 4.2 (4)** 38.9 ± 1.4 (3)	24.6 ± 3 (6)* 29.6 ± 4.4 (6)
β-Glucuronidase	(E) 33.9 ± 2.1 (4) (C) 51.2 ± 5.9 (4)	55.1 ± 4.1 (4) 61.4 ± 6.9 (3)	54.7 ± 7.3 (8)**** 67.1 ± 6.9 (9)
β-Glycerophosphatase	(E) 24.1 ± 5.2 (4) (C) 21.5 ± 3 (4)	37.5 ± 2.7 (4) 36.9 ± 1.6 (3)	33.2 ± 2.1 (6) 35.1 ± 3.1 (6)

Means ± SD are given. * $P < 0.05$, ** $P < 0.025$, *** $P < 0.01$, **** $P < 0.005$

increased at 5 to 8 days after DOCA administration.

(b) At 8 days after DOCA administration (one to 2 days after the development of hypertension), when systolic blood pressure became 165 ± 17 mmHg, kidney weight per body weight was increased in rats treated with DOCA, but heart weight per body weight was not increased as yet. Suppressed renin and elevated cathepsin activi-

ties were found in rats with DOCA as shown in Table I-b and in Figure 1.

(c) At 15 to 48 days after DOCA administration (more than 4 days after the development of hypertension), when systolic blood pressure averaged 181 ± 23 mmHg, both heart and kidney weights per body weight were more increased, though body weight of hypertensive rats was decreased. Suppressed renin, elevated cathepsin and increas-

ed β -glucuronidase activities were found in rats with DOCA, but β -glycerophosphatase and ribonuclease activities were rather decreased. Furthermore, cathepsin and β -glucuronidase revealed a tendency for increased activities when rat kidney was more enlarged.

(d) In C. F. from rat renal cortex, cathepsin activity was 174.2 ± 42.5 in eight hypertensive rats implanted with DOCA, when 100 ± 16.3 in eight control rats ($P < 0.005$). In the sedimentable fraction after centrifuging C. F. at 18000g for 60 min., cathepsin activity was 195 ± 78.9 in six hypertensive rats implanted with DOCA, when 100 ± 3.2 in six control rats ($P < 0.05$).

3. Soluble fraction (S_1) and supernatant fraction (S_2)

Cathepsin activity in both S_1 and S_2 did not show any significant difference between rats with DOCA hypertension and control rats (Table II-a), though the former rats showed elevated cathepsin activity in C. F. in comparison to the latter. β -Glucuronidase and β -glycerophosphatase in both S_1 and S_2 revealed a tendency for decreased activities in rats treated with DOCA, though not always (Table II-a). Therefore the percentage of S_1 or S_2 to C. F. was diminished in both cathepsin and β -glucuronidase activities in rats with DOCA hypertension (Table II-b). The percentage of S_1 or S_2 to C. F. in β -glycerophosphatase activity was nearly identical between rats treated with DOCA and control rats, because the former rats showed a tendency for decreased activity of β -glycerophosphatase in C. F. as compared with the latters. These findings indicate that the release of lysosomal enzymes into the soluble or the supernatant fraction was not increased in rats treated with DOCA plus salt.

DISCUSSION

It has been well known that uninephrectomized rats treated with DOCA plus salt develop hypertension with cardiovascular lesions. We could induce hypertensive rats without uninephrectomy by administration of DOCA with high salt diet (7.6% NaCl) plus 0.9% saline, and hypertensive rats only with high salt diet plus 0.9% saline!¹⁴ In the rats treated with DOCA, renal renin activities were already decreased before the development of hypertension and were more suppressed after that of hypertension. This finding indicates that renal renin activity may change in parallel with the alteration of salt-water metabolism. We have observed that renal renin activities were slightly increased in rats with DOCA plus high salt or

with high salt, though less than those in control normotensive rats, when renal vascular lesions were found intensely by microscopy!¹⁴ It has been also shown that vascular receptor affinity to angiotensin II is increased in high sodium intake and in DOCA hypertension!¹⁵ and that injection of renin or angiotensin in rats pretreated with DOCA plus salt induces intense renal vascular lesions!^{16,17} The slight increase of renin activity in rats treated with DOCA plus salt, observed by us, may result in a vicious cycle among renin, hypertension and vascular lesions. Renal cathepsin activity was slightly increased before the development of hypertension, when renal enlargement was found slightly. As hypertension developed, cathepsin activity was more increased. It has been reported that both collagenase and protease, such as cathepsin D, were necessary for collagen fibril degradation!¹⁸ In the inflammatory response, neutrophil cell granules will be able to release their lysosomal contents into the outside of the cell by exocytosis!¹⁹ Olsen reported the inflammatory cellular reaction in hypertensive vascular disease, in which mononuclear cells would become macrophages after penetration into vascular wall!²⁰ It is probable that elevated cathepsin activity results form cellular inflammatory reaction in rat kidney of DOCA hypertension.

β -Glucuronidase activity was increased in the rats with DOCA hypertension, especially when rat kidney weight was more enlarged. β -Glucuronidase relates to the metabolism of polysaccharides and mucopolysaccharides. It is also demonstrated that hyperplastic vascular changes of malignant hypertension involve both collagen fibers and acid mucopolysaccharides. Miller *et al.* reported increased activities of both β -glucuronidase and cathepsin in human atherosclerotic aortas!²¹ Therefore increased activities of both cathepsin and β -glucuronidase in rats with DOCA hypertension may relate to hypertensive vascular lesions. However it remains to be clarified whether increased activities of these two lysosomal enzymes are originated from renal vascular lesions or from the other renal tissue alterations. Morrison *et al.* reported that renal papillary cell granules, characterized as lysosomes electron microhistochemically, appeared in rats fed on potassium-deficient diet!²² and that acid phosphatase activity of papillae from the potassium-deficient rats was greater than that of the controls!²² Although hypokalemia occurs by DOCA administration, renal papillary dense granules due

to hypokalemia can not explain all about our results because we also observed elevated cathepsin activity in renal cortex of hypertensive rats. Further investigation is necessary to clarify the role of lysosomal enzymes, especially renin, β -glucuronidase and cathepsin, in hypertensive vascular lesions.

SUMMARY

In the cytoplasmic fraction (C. F.) of rats treated with DOCA plus salt, renal renin activity was already decreased before the development of hypertension and was more suppressed after that of hypertension. Renal cathepsin activity was slightly increased shortly before the development of hypertension and more elevated after that of hypertension. Increased renal β -glucuronidase activity was found after the longer duration of hypertension. Deoxyribonuclease activity was slightly increased in average after the development of hypertension, but not significantly. Ribonuclease and β -glycerophosphatase activities were not increased, rather slightly decreased after the development of hypertension.

The percentage of soluble fraction (S_1) or supernatant fraction (S_2) to cytoplasmic fraction (C. F.) was diminished in both cathepsin and β -glucuronidase activities in rats treated with DOCA plus high salt as compared with that in the controls, but was nearly identical with that in the controls in β -glycerophosphatase activity.

The probable role of lysosomal acid hydrolases in hypertensive vascular lesions was discussed.

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