ALTERED PHOSPHOLIPASE C ACTIVITY IN RENAL MEDULLA OF DOCA-SALT HYPERTENSIVE RATS

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We investigated the alterations of phospholipase C activity in the kidney and arterial wall of deoxycorticosterone acetate (DOCA)-salt hypertensive rats. Neither DOCA nor salt administration alone affected phospholipase C activity in the kidney and arterial wall. In contrast, when the rats were given both DOCA and salt and developed high blood pressure, the phospholipase C activity became greater in the outer and inner medulla (papilla) while the enzyme activity was reduced significantly in the renal cortex and arterial wall. Such an increase in phospholipase C activity was not observed in the kidney of a genetic rat for spontaneous hypertension. Moreover, the phospholipase A₂ activity was also stimulated in the renal medulla of DOCA-salt hypertensive rats. Thus, these data indicate that DOCA-salt hypertension is associated with an increase in phospholipase C activity in the renal medulla, which seems primary to the development of DOCA-salt hypertension.

THERE is a considerable amount of evidence that phospholipid metabolism is related to intracellular signal transmission!¹⁻³ In addition to adenosine 3':5' -cyclic monophosphate (cyclic AMP) and protein kinase A, phosphoinositides turnover has recently been emphasized as an intracellular calcium messenger system which modulates cytosolic free calcium concentration, and mediates subsequent transmission of the signals.³ Among the family of phosphoinositides metabolism-related enzymes, phospholipase C is assumed to play a key

role in initiating the signal transduction.

There is also much evidence that facilitated adrenergic nerve discharge or vasopressin secretion has an important role in the development of DOCA-salt hypertension;⁴⁻⁶ α_1 adrenoceptor reportedly modulates sodium and water handling in the distal tubules.^{7,8} Vasopressin exerts potent vasoconstrictor and antidiuretic effects as well. In addition, these hormone actions are mediated by phosphoinositides metabolism!¹⁻³

In order to understand the high sensitivity of DOCA-salt rats to these vasoactive substances, it seems appropriate to examine the alterations of phospholipase C activity in the kidney because renal factors are integral in volume-dependent hypertension. Thus, in

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TABLE 1 AVERAGED SYSTOLIC BLOOD PRESSURES AND BODY WEIGHTS
DOCA-salt rats:

Variables	Control	Salt	DOCA	DOCA-salt
Body Weight (gram)	266±4 (13)	271±2(9)	270±3 (11)	258±2 (11)
Blood Pressure (mmHg)	135±1 (13)	136±5(9)	136±3 (11)	167±3 (11)*

Control, Salt, DOCA and DOCA-salt represent control rats, salt rats, DOCA rats and DOCA-salt hypertensive rats, respectively. Number in parentheses represents the number of rats. * $p < 0.001$ vs the value of control rats.

SHR and WKY:

Variables	SHR	WKY
Body Weight (gram)	277±6(14)	267±10(11)
Blood Pressure (mmHg)	173±3(14)	136±2(11)*

* $p < 0.01$ vs the respective values of SHR. Number in parentheses represent the number of rats.

Fig.1. Phospholipase C activity in the kidney of DOCA-salt hypertensive rats.

C=uninephrectomized rats given water, S=uninephrectomized rats given saline, D=uninephrectomized rats given deoxycorticosterone acetate emulsion, DS=uninephrectomized rats given both deoxycorticosterone acetate and saline. N represents the number of rats. The group difference was assessed by one way analysis of variance. F values were 1.14 for cortex ($p > 0.1$), 5.06 for outer medulla ($p < 0.005$) and 5.96 for inner medulla ($p < 0.002$). NS represents statistically not significant when compared with control value.

this study, we investigated alterations in phospholipase C activity in the renal medulla and in the renal cortex, compared to the vascular wall of DOCA-salt hypertensive rats.

MATERIALS AND METHODS

Development of DOCA-salt hypertensive rats
Forty four 6-week-old Wistar rats, fed a

0.6% NaCl chow and given tap water after weaning, were uninephrectomized under pentobarbital sodium (25 mg/kg body weight) anesthesia. After two weeks of recovery, the rats were randomly assigned to (1) control (C) rats which received tap water ad lib for 4 weeks, (2) salt (S) rats which were given 1% saline solution for 4 weeks, (3) DOCA (D) rats which were given tap water, and subcutaneously injected deox-

TABLE II ACTIVITY OF PHOSPHOLIPASE C IN THE VASCULAR WALL

Control	Salt	DOCA	DOCA-salt
$0.234 \pm 0.018(13)$	$0.183 \pm 0.018(9)$	$0.223 \pm 0.011(11)$	$0.187 \pm 0.007(11)^*$

Activity is expressed as nmoles substrate/min/mg dried weight of aorta. Control, Salt, DOCA and DOCA-salt represent uninephrectomized control rats, rats given salt alone, rats given DOCA alone and rats given both DOCA and salt, respectively. Number in parentheses represents the number of rats. The group difference was assessed by one way analysis of variance. F value was 3.12 ($p < 0.05$). * $p < 0.05$ vs the value of control rats.

Fig.2. Phospholipase A₂ activity in the kidney of DOCA-salt hypertensive rats.

C=uninephrectomized rats given water, S=uninephrectomized rats given saline, D=uninephrectomized rats given deoxycorticosterone acetate emulsion, DS=uninephrectomized rats given both deoxycorticosterone acetate and saline. N represents the number of rats. The group difference was assessed by one way analysis of variance. F values were 0.23 for cortex ($p > 0.1$), 3.62 for outer medulla ($p < 0.025$) and 2.97 for inner medulla ($p < 0.05$). NS represents statistically not significant when compared with control value.

ycorticosterone acetate (DOCA; 10 mg/kg body weight) emulsified with arabic gum once a week for 4 weeks, or (4) DOCA-salt (DS) rats which were given 1% saline solution, and injected DOCA (10 mg/kg body weight) once a week for 4 weeks. Blood pressure was measured by the tail-cuff method at the end of feeding period. The kidneys and descending thoracic aorta were obtained from the rats under anesthesia with pentobarbital sodium (25 mg/kg body weight). The aorta was rinsed with fresh, ice-chilled saline solution. The kidney was immediately perfused with 20 ml of ice-chilled fresh saline and plunged in liquid nitrogen. The organs were stored at -70°C until further preparation.

Spontaneously hypertensive rats

Fourteen spontaneously hypertensive rats and eleven Wistar-Kyoto rats were used in this study. They were originally from Okamoto-Aoki strain and bred at Kantou-Teishin Hospital, Tokyo, Japan. After weaning, they were fed a 0.6% salt diet for 10 weeks. At the end of the feeding period, blood pressure was measured by the tail-cuff method. The kidneys were obtained and processed in the same way as described above.

Preparation for enzymatic solution of phospholipases

The frozen kidneys were warmed up to -4°C , and then sliced to about 1.0 mm. The renal outer cortex, outer medulla, and

Fig.3. Phospholipase C activity in the kidney of spontaneously hypertensive rats.

The figure in parentheses represents the number of rats. NS represents statistically not significant when compared with WKY.

inner medulla (papilla) were punched out and the excised tissues homogenized with a Polytron blender. The homogenate was then spun at 10^4 g at 4°C for 30 min and the supernatant used as the enzymatic solution for measuring phospholipase. The precipitate was dried and weighed. The aorta was minced and homogenized using the same procedures and the supernatant used as the enzymatic solution.

Assay of phospholipase C activity

Based on the modified method of Rittenhouse⁹⁻¹¹ phospholipase C activity was measured. Briefly, $50\ \mu\text{l}$ of 10^4 g supernatant was preincubated at 4°C for 15 min in $200\ \mu\text{l}$ of 50 mM N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (hepes) buffer at pH 7.0 including 0.1 M sodium chloride (NaCl), 5 mM calcium chloride (CaCl_2) and 1.25 mM deoxycholic acid sodium. The reaction was started by adding the substrate, $50\ \mu\text{l}$ of 3×10^4 dpm of L-3-phosphatidyl ($U\text{-}^{14}\text{C}$) inositol (5–6 mCi/mmol), and terminated with Folch's solvent (chloroform : methanol = 2 : 1, v/v) 5 min after the incubation. After adding 0.1

ml of 1 N hydrochloride (HCl) solution, the reaction mixture was vigorously vortexed. The mixture was spun at low temperature and 700 g for 30 min. The aqueous phase (upper phase) was placed in a 10 ml scintillation vial, and a trace of chloroform evaporated under dry nitrogen. Radioactivity of (^{14}C) inositol 1-phosphate in the aqueous phase was counted by an automatic scintillation counter. The enzymatic activity was related to the dried tissue weight.

Assay of phospholipase A_2 activity

Phospholipase A_2 activity was measured according to the modified method of Billah et al.¹⁰⁻¹² Briefly, $50\ \mu\text{l}$ of 10^4 g supernatant was preincubated at 37°C for 30 min in $200\ \mu\text{l}$ of 0.1 M glycine buffer at pH 8.0 containing 0.2 M NaCl, 2 mM CaCl_2 and 1.25 mM deoxycholic acid sodium. The reaction was initiated by adding $50\ \mu\text{l}$ of 3×10^4 dpm of 1-stearoyl-2-(^{14}C) arachidonyl L-3-phosphatidylcholine (5–6 mCi/mmol) and terminated using Folch's solvent after 60 min of incubation. (^{14}C) arachidonate which was produced from the radioactive phosphatidylcholine was extracted twice using Folch's solvent, and separated with silicic acid thin layer chromatography. The separation pattern was analyzed by an Aloca radioactive TLC scanner system (Aloca Co., Tokyo, Japan). The (^{14}C) arachidonate zone was scraped off, and its radioactivity determined with an automatic liquid scintillation counter. The enzymatic activity was related to the dried tissue weight.

Reagents

Reagents were all of analytical grade. The radioactive materials were purchased from Amersham International plc, Buckinghamshire, England.

Statistical analysis

Values were expressed as mean $\pm$ SE. The difference was analyzed by the Student's t test and one way analysis of variance.

RESULTS

Average body weight and systolic blood pressure are shown in Table I. Although DOCA-salt hypertensive rats tended to have

a lower body weight than control rats, the difference was statistically not significant. Average blood pressure of the control rats was equal to that of both salt rats and DOCA rats. The blood pressure increased significantly up to 167 mmHg when the rats were challenged with both DOCA and salt. 14-week-old SHR exhibited sustained hypertension, and the blood pressure level was equal to that of DOCA-salt hypertensive rats.

Renal phospholipase C activity was as shown in Fig. 1. The phospholipase C activity was greater in the renal medulla than in the renal cortex. In the medulla, the phospholipase C activity was found to be highest in the papilla, tenfold that in the renal cortex. As shown in Fig. 1, DOCA-salt hypertensive rats exhibited a significantly lowered phospholipase C activity in the renal cortex by 17%, as compared to the control rats (0.158 ± 0.012 vs 0.191 ± 0.011 nmoles/min/mg dry tissue weight). In contrast, phospholipase C activity was increased significantly by 26% in the outer medulla and by 44% in the inner medulla, compared to the control rats (0.738 ± 0.030 vs 0.586 ± 0.027 nmoles/min/mg dry tissue weight in the outer medulla and 1.386 ± 0.090 vs 0.959 ± 0.111 nmoles/min/mg dry tissue weight in the inner medulla, respectively).

Phospholipase C activity in the aortic wall is shown in Table II. DOCA-salt rats exhibited a decrease in the enzymatic activity compared with the control rats.

Renal phospholipase A_2 activity is presented in Fig. 2. Phospholipase A_2 was found mainly in the renal papilla and outer medulla. In the renal cortex, there was no difference in phospholipase A_2 activity between DOCA-salt hypertensive rats and the controls. Phospholipase A_2 activity in DOCA-salt hypertensive rats was enhanced significantly by 24% in the outer medulla and by 66% in the inner medulla, compared to control rats (3.73 ± 0.21 vs 2.99 ± 0.16 pmoles/min/mg dry tissue weight in the outer medulla and 12.75 ± 1.80 vs 7.66 ± 1.33 pmoles/min/mg dry tissue weight in the inner medulla, respectively).

In contrast, phospholipase C activity declined significantly in the inner medulla of SHR although the blood pressure level was

equal to that of DOCA-salt hypertensive rats (2.55 ± 0.40 vs 5.37 ± 0.96 nmoles/min/mg dry tissue weight, Fig. 3).

DISCUSSION

In this study, we measured phospholipase C activity in 10^4 g supernatant mainly because phospholipase activity is widespread in the various intracellular organelles.¹³ The substrate specificity and assay condition made it feasible to use this approach.⁹⁻¹² We employed a Vmax condition for the assay of phospholipase C. In such experimental circumstances, enzymatic activity reflects enzymatic content in the tissue.

We clearly demonstrated that phospholipase C activity in the renal medulla was increased in DOCA-salt hypertensive rats. Since neither rats given DOCA nor rats given salt alone exhibited an increase in phospholipase activity, the DOCA-salt hypertensive state may be connected to enhanced activity. DOCA-salt hypertensive rats exhibit high sensitivity to adrenergic nerve stimulation or vasopressin action. Since these hormone actions are mediated by phosphoinositides metabolism, the increased phospholipase C activity, seen in DOCA-salt hypertensive rats, possibly participates, to some extent, in enhanced responsiveness to these vasoactive hormones.

Indeed, there is evidence that protein kinase C, activated by diacyl glycerol, a product of phosphoinositides hydrolyzed by phospholipase C, stimulates Na^+ H^+ antiporter system,¹⁴ thereby facilitating sodium reabsorption in the kidney.^{15,16} Thus, it seemed probable that increased phospholipase C activity in the renal medulla enhances sodium and water reabsorption, thereby contributing to the development of volume-dependent hypertension in the DOCA-salt rat model. Moreover, SHR exhibited decreased phospholipase C activity in the renal medulla although the averaged systolic blood pressure was almost equal to that of DOCA-salt hypertensive rats. These data indicate that enhanced phospholipase C activity in the renal medulla is unique to DOCA-salt hypertensive rats and this property is not secondary to the hypertensive state. However, there is some variation in this enzyme activity between rat strains. The exact im-

plications of enhanced phospholipase C activity in hypertensive mechanisms remain to be elucidated.

Troyer et al² have reported that phospholipase C is observed in the brush border membrane of the proximal tubules of the renal cortex. Much phospholipase C activity also occurs in the arterial walls^{8,9}. Since the renal cortex is rich in glomeruli or vascular vessels, phospholipase C activity here may reflect this activity in the vascular wall. Indeed, we found that phospholipase C activity in the aortic wall was decreased in DOCA-salt hypertensive rats. The mechanism for the decrease in phospholipase C activity in the vascular wall remains to be elucidated. However, since there is evidence that volume expansion produces a factor (s) in rats which inhibits phospholipases in the platelets, this inhibitory factor may be related to reduced phospholipase C activity.¹⁰

In this study we also demonstrated that phospholipase A₂ activity was increased in the renal medulla of DOCA-salt hypertensive rats. Since phospholipase A₂ was measured in a Vmax condition^{10,11} the increased activity was expected to reflect an increase in the enzyme content, but not an activation of this enzyme. Thus, the increase in phospholipase A₂ activity could not be explained by calcium mobilization caused by rapid turnover of phosphoinositides, mediated by enhanced phospholipase C activity. Enhanced phospholipase A₂ activity would increase free arachidonate and subsequently stimulate the synthesis of vasodepressor prostaglandin. Since vasodepressor prostaglandins reduce the sodium reabsorption in the collecting tubules^{17,18} increased prostaglandin synthesis would lower the facilitated sodium reabsorption in DOCA-salt hypertensive rats, a sort of feedback mechanism for the enhanced phospholipase C activity.

There are few studies on the relationship between phospholipases in the kidney and the hemodynamics or tubular functions of the kidney. At this time we are unable fully to relate the alterations observed in this study to the functional changes in the kidney of DOCA-salt hypertensive rats. We are now beginning to expand on our experiments to investigate a genetic rat model for salt-induced hypertensionlike Dahl strain.

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