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Ebelactone B, an inhibitor of urinary carboxypeptidase Y-like kininase, prevents the development of deoxycorticosterone acetate-salt hypertension in rats

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Abstract

Kininogen-deficient Brown Norway Katholiek rats (BN-Ka) excrete little urinary kinin, compared with normal rats of the same strain (BN Kitasato rats (BN-Ki)). Deoxycorticosterone acetate-salt treatment increased systolic blood pressure in both rats, but much faster in BN-Ka than in BN-Ki. Daily subcutaneous administration of ebelactone B (15 and 5 mg/kg/day), a rat urinary carboxypeptidase Y-like kininase inhibitor, significantly reduced systolic blood pressure in BN-Ki, but not in BN-Ka. This treatment significantly increased urinary Na^+ excretion and reduced Na^+ concentration in the erythrocytes in BN-Ki, but not in BN-Ka. An angiotensin-converting enzyme inhibitor, lisinopril (5 mg/kg/day s.c.), did not reduce the systolic blood pressure in either BN-Ki or BN-Ka. These results suggested that ebelactone B has promise as a preventive agent for the development of hypertension acting through the inhibition of urinary kinin degradation.

Keywords: Ebelactone B; Carboxypeptidase Y, urinary; Kininogen-deficient rat; Deoxycorticosterone acetate-salt hypertension; Na^+ excretion

1. Introduction

Bradykinin, a biologically active peptide, is well known to induce increases in renal blood flow and in water and Na^+ excretion. Kinin is generated by the action of kallikrein secreted in the distal tubules, and its receptors are distributed on the tubular cells of the distal tubules (Scicli and Carretero, 1986; Tomita and Pisano, 1984). It has been claimed that less urinary kallikrein is secreted in patients with essential hypertension, and the renal kallikrein-kinin system may play a suppressive role in hypertension in human (Margolius et al., 1971, 1974; Schneider et al., 1973; Levy et al., 1977) and animal hypertensive models (Margolius et al., 1972; Carretero et al., 1978; O'Connor et al., 1982; Handa et al., 1983; Moore et al., 1984; Marchetti et al., 1984; Arbeit and Serra, 1985; Ader et al., 1985, 1987; Praddaude et al., 1989; Mohsin et al., 1992).

We previously reported that, using kininogen-deficient Brown Norway-Katholiek (BN-Ka) rats and normal rats of the same strain (BN-Kitasato, BN-Ki), the urinary kallikrein-kinin system may contribute to lowering of the systemic blood pressure in the initial phase of the development of deoxycorticosterone acetate-salt hypertension in uninephrectomized rats by accelerating the excretion of Na^+ and water (Majima et al., 1991).

As already reported (Majima et al., 1988, 1993a; Shima et al., 1992; Kuribayashi et al., 1993), the degradation pathway of bradykinin in urine collected from the rat ureter was quite different from that in rat or human plasma. The kininases, enzymes that degrade bradykinin in rat urine, were neutral endopeptidase and carboxypeptidase Y-like kininase (Kuribayashi et al., 1993; Majima et al., 1994b), whereas those in the plasma of rats and man were kininase I (carboxypeptidase N) and kininase II (angiotensin I-converting en-

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zyme) (Shima et al., 1992; Majima et al., 1993a). Ebelactone B, isolated from *Actinomycetes*, was reported to inhibit some enzymes such as esterase, lipase and *N*-formylmethionine aminopeptidase (Umezawa et al., 1980). Our earlier study, however, revealed that ebelactone B selectively inhibited not only carboxypeptidase Y from yeast, but also carboxypeptidase Y-like kininase in rat urine without inhibiting other kininases in plasma and urine (Kuribayashi et al., 1993). In our previous report (Majima et al., 1994b), ebelactone B showed kinin-dependent diuretic and natriuretic effects in saline-infused anesthetized rats, suggesting that ebelactone B is a novel type of diuretic and natriuretic agent. These results prompted us to test the antihypertensive effect of ebelactone B in some hypertensive models.

The present paper reports that ebelactone B, a urinary carboxypeptidase Y-like kininase inhibitor, reduced the systemic blood pressure of normal BN-Ki rats during development of deoxycorticosterone acetate-salt hypertension by inhibiting kinin degradation in urine.

2. Materials and methods

2.1. Animals

BN-Ka rats (*Rattus norvegicus*, BN/fMai) were initially obtained from the Katholiek Universiteit of Leuven, Belgium and were fed in our animal facilities (Majima et al., 1991, 1993b, 1994a). Normal rats of the same strain have been kept in Kitasato University (BN-Ki). Male rats 6–10 weeks of age were used. In a separate experiment, male Sprague-Dawley strain rats (specific pathogen-free, SLC, Hamamatsu, Japan) 6 weeks of age were used. All animals were housed at a constant humidity ($60 \pm 5\%$) and temperature (25 ± 1°C), and kept on a 12-h light/12-h dark cycle throughout. The number of animals used for each experiment is stated in the corresponding section. This study was performed in accordance with the guidelines for animal experiments of Kitasato University School of Medicine.

2.2. Induction of hypertension and administration of ebelactone B

From a few days after weaning, BN-Ka and BN-Ki rats were fed ad libitum with a low NaCl diet (NMF, Oriental Yeast Corp., Tokyo, Japan) containing 0.3% NaCl. The rats were given free access to distilled water for drinking. At 7 weeks of age, the drinking water was replaced with 1% NaCl solution after left-side uninephrectomy and weekly subcutaneous administration of deoxycorticosterone acetate solution (5 mg/kg/week, 5 mg/ml in physiological saline, containing 50 mg/ml of gum arabic) was started, as reported previously (Majima et al., 1991). Seven-week-old male Sprague-Dawley strain rats were also treated with the deoxycorticosterone acetate-salt treatment.

One week after the start of deoxycorticosterone acetate-salt treatment (when the rats were 8 weeks old), ebelactone B (a gift from the Institute of Microbial Chemistry, Tokyo, Japan) or lisinopril (a gift from Shionogi Pharmaceutical Corp., Osaka, Japan) was administered (15 or 5 mg/kg/day, dissolved with dimethyl sulfoxide (DMSO) at a concentration of 45 or 15 mg/ml) once a day for 7 days by subcutaneous injection. Control animals received only vehicle solution (0.33 ml/kg/day).

The dose of ebelactone B used in the present experiment was selected according to the previous experiment, in which these doses induced sufficient diuresis and natriuresis in saline-infused anesthetized rats (Majima et al., 1994b).

2.3. Measurement of systemic blood pressure

The systolic blood pressure of unanesthetized rats was determined with a tail-cuff plethysmograph (Ueda model UR-1000, Ueda Seisakusho, Tokyo, Japan), as reported previously (Majima et al., 1991, 1993b, 1994a).

Mean arterial blood pressure was also determined in conscious and unrestrained rats as described in our previous report (Majima et al., 1993b, 1994a). Briefly, a polyethylene cannula (PE-10, Clay-Adams, Parsippany, NJ, USA) was inserted into the abdominal aorta through the femoral artery under light ether anesthesia and the cannula was connected to a PE-50 cannula (Clay-Adams, Parsippany, NJ, USA) and exteriorized in the interscapular region. A blood pressure transducer (TP-200T, Nihon Kohden, Tokyo, Japan) was attached to the other end of the intra-arterial cannula, and the mean arterial blood pressure was monitored on a polygraph (WS-641-G, Nihon Kohden, Tokyo, Japan). Starting 30 min after the connection of the transducer, recordings were made for over 1 h in the rats, which were kept in separate cages.

2.4. Blood collection

Under light ether anesthesia, blood was collected from the carotid artery of each strain of rats through the cannula (PE-50) into glass tubes without anticoagulant 5 days after the start of ebelactone B treatment. Collected blood was left at room temperature for 2 h, and then centrifuged at $1500 \times g$ for 15 min at 25°C so as to obtain serum. Blood was also collected directly into the tubes containing ice-chilled iso-osmotic

lithium chloride solution to determine the Na^+ concentration of the erythrocytes. In order to prepare citrated plasma, blood was collected into the plastic tubes containing 1/10 volume of 3.8% sodium citrate, and was centrifuged at $1500 \times g$ for 15 min at 25°C. The surpernatant was used for citrated plasma. For disodium ethylenediaminetetraacetate (EDTA)-treated plasma, 1 ml of blood was also collected directly into plastic tubes containing 0.1 mg of EDTA. After centrifugation at $1500 \times g$ for 15 min at 25°C, supernatant was obtained as EDTA-treated plasma.

2.5. Collection of urine and measurement of urinary levels of creatinine, Na^+ and K^+

Twenty-four-hour urine samples from individual rats after administration of ebelactone B were collected using metabolic cages. The volume of urine was recorded at the end of the 24-h period. Urinary creatinine levels were measured by a kinetic method using Jaffe's reaction (Majima et al., 1991, 1993b, 1994a). Urinary Na^+ and K^+ levels were determined electrometrically using coated wire electrodes selective for Na^+ and K^+, respectively (Majima et al., 1991, 1993b, 1994a).

2.6. Measurement of urinary kinin

Free kinin was measured in the urine collected via catheters (PE-10, Clay Adams, Parsippany, NJ, USA) inserted into both ureters of rats of BN-Ka and BN-Ki strains under Na^+ pentobarbital anesthesia (60 mg/kg s.c.). The kinin levels were determined with a bradykinin enzyme immunoassay kit (Markit M, Dainippon Pharmaceutical Corp., Osaka, Japan) after extraction with ethanol (Majima et al., 1991, 1993b, 1994a). The amounts of kinin secreted were expressed in ng/24 h.

2.7. Measurement of kininogen levels in plasma

Plasma kininogen levels in citrated plasma were determined by the amount of kinin released from the plasma, as described in previous reports (Majima et al., 1991, 1993b, 1994a), and the levels were expressed in nanograms of released bradykinin per milligram of plasma protein.

2.8. Measurement of levels of creatinine, Na^+ and K^+ in serum and Na^+ concentrations in erythrocytes

The levels of creatinine, Na^+ and K^+ in the sera were determined by the same methods as were used for those in urine, as described above. The Na^+ concentration in the erythrocytes ($RBC[Na]_i$) was determined using atomic absorption spectrophotometry (McCormic et al., 1989), as reported previously (Majima et al., 1993b, 1994a). The Na^+ concentrations in the erythrocytes were expressed as mmol/l RBC.

2.9. Measurement of levels of Na^+ in cerebrospinal fluid

Cerebrospinal fluid from rats under light ether anesthesia was obtained by aspiration from the cisterna magna with a 26-gauge needle. The levels of Na^+ in the cerebrospinal fluid were determined with an atomic absorption spectrophotometer (Majima et al., 1994a).

2.10. Measurement of plasma renin activity

EDTA-treated plasma was incubated at 37°C for 90 min and the amounts of angiotensin I generated were measured by radioimmunoassay kit (Gamma Coat ^{125}I Plasma Renin Activity Kits, Baxter Healthcare Corp., Cambridge, MA, USA) (Majima et al., 1991, 1993b, 1994a). The plasma renin activity was expressed in terms of nanograms of angiotensin I generated per milliliter plasma per hour.

2.11. Statistical analysis

Values were expressed as means ± S.E.M., and Student's t-test was used to evaluate the significance of differences. When variances were heterogeneous, statistical analyses were performed by the Aspin-Welch method or by Wilcoxon's rank sum test. For the paired results, the paired t-test was used. A P value less than 0.05 was considered to be significant.

3. Results

3.1. Plasma kininogen levels and urinary kinin levels

The plasma levels of high molecular weight (HMW) kininogen in deficient BN-Ka rats at 7 weeks of age were very low (0.14 ± 0.14 ng bradykinin equivalent/mg plasma protein, $n = 5$) and those of low molecular weight (LMW) kininogen were also very low (0.06 ± 0.01 ng bradykinin equivalent/mg plasma protein, $n = 5$) in deficient BN-Ka rats, whereas the plasma concentrations of HMW and LMW kininogens in normal BN-Ki rats were 15.7 ± 0.4 ($n = 7$) and 9.0 ± 0.4 ($n = 7$) ng bradykinin equivalent/mg plasma protein, respectively. The amount of immuno-reactive free kinin excreted in the uretral urine in normal BN-Ki rats 7 weeks of age was 112.7 ± 39.8 ng bradykinin/24 h ($n = 5$), but was very low in deficient BN-Ka rats (less than 4.8 ng bradykinin/24 h, $n = 4$). The results from BN-Ka and BN-Ki rats in the present experiments were essentially the same as those in our previous reports (Majima et al., 1991, 1993b, 1994a).

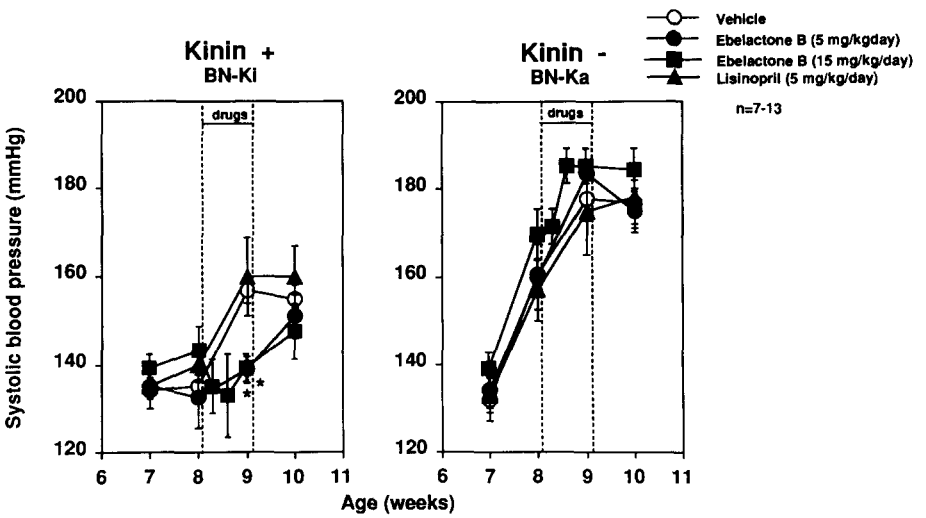

Fig. 1. Effects of ebelactone B and lisinopril on the developmental stage of deoxycorticosterone acetate-salt hypertension. Values (systolic blood pressure) are means ± S.E.M. of the numbers (n) of rats. After uninephrectomy at 7 weeks of age, deoxycorticosterone acetate (5 mg/kg s.c.) was administered once a week. From 8 weeks of age, ebelactone B (5, 15 mg/kg/day) or lisinopril (5 mg/kg/day) was administered (s.c.) for a week to deoxycorticosterone acetate-salt-treated normal BN-Ki rats and kininogen-deficient BN-Ka rats. Values from rats receiving ebelactone B or lisinopril were compared with those of rats receiving vehicle at the same age. * $P < 0.05$.

3.2. *Effects of ebelactone B on systemic blood pressure of deoxycorticosterone acetate-salt treated BN-Ka and BN-Ki rats*

The systolic blood pressure of 7-week-old deficient BN-Ka rats was 132 ± 2 mm Hg ($n = 7$), which was not significantly different from that of normal BN-Ki rats (129 ± 3 mm Hg, $n = 13$) (Fig. 1). From the first week after the start of deoxycorticosterone acetate-salt treatment, the systolic blood pressure of deficient BN-Ka rats increased significantly to 161 ± 9 mm Hg ($n = 7$), and continued to rise quickly thereafter until the second week of treatment, when it reached a plateau (178 ± 7 mm Hg, $n = 7$), whereas the systolic blood pressure of normal BN-Ki rats given the same treatment showed a gradual increase that was still in progress 3 weeks after the start of the treatment (Fig. 1). The systolic blood pressure of normal BN-Ki rats peaked 11 weeks after the start of the treatment.

When the rats were treated daily with the angiotensin converting enzyme inhibitor, lisinopril (5 mg/kg/day s.c.), neither deficient BN-Ka nor normal

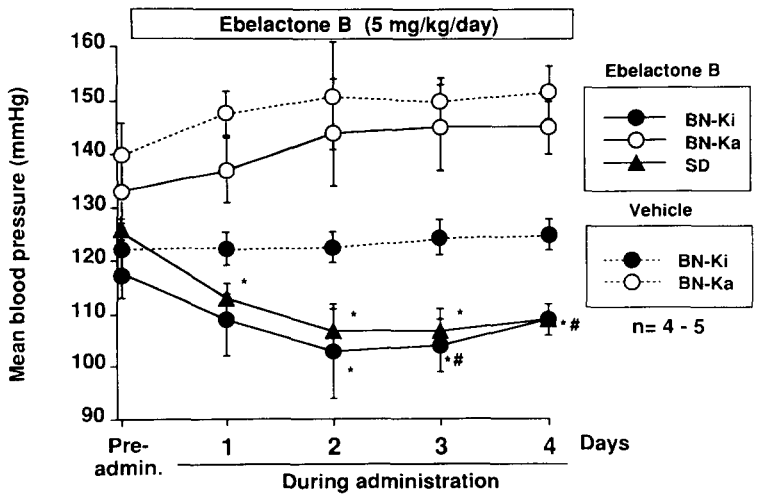

Fig. 2. Effects of ebelactone B on the mean blood pressure of deoxycorticosterone acetate-salt-treated rats. Values (MBP) are means ± S.E.M. of the numbers (n) of rats. From 8 weeks of age, ebelactone B (5 mg/kg/day s.c.) or vehicle was administered for 4 days to normal BN-Ki rats, kininogen-deficient BN-Ka rats and SD rats under deoxycorticosterone acetate-salt treatment. Values from rats before administration of ebelactone B or vehicle were compared with those after administration. * $P < 0.05$ (paired t-test). Values from rats receiving ebelactone B were compared with those of rats receiving vehicle on the same day. # $P < 0.05$.

BN-Ki rats showed any significant antihypertensive response, compared with those receiving vehicle solution (Fig. 1). In contrast, the daily subcutaneous administration of ebelactone B (5 mg/kg/day) for one week resulted in a significant reduction of systolic blood pressure in normal BN-Ki rats (Fig. 1). A higher dose of ebelactone B (15 mg/kg/day s.c.) also induced a fall in systolic blood pressure, although the efficacy of this dose was almost the same as that of 5 mg/kg/day. However, the deficient BN-Ka rats did not show any antihypertensive response to the administration of ebelactone B (Fig. 1). The 5 mg/kg/day dose was therefore selected for further analysis of the effect of ebelactone B.

The mean blood pressure, measured in conscious and unrestrained rats through the indwelling cannula, was gradually and significantly reduced from a high level (118 ± 4 mm Hg, $n = 4$) by daily administration of ebelactone B (5 mg/kg/day s.c.) in normal BN-Ki rats during the 4-day administration period. However, there was no reduction in mean blood pressure in deficient BN-Ka rats (Fig. 2).

This was also true when another strain of rat was used. The mean blood pressure of deoxycorticosterone acetate-salt-treated Sprague-Dawley strain rats at 9 weeks of age was also reduced by administration of ebelactone B (5 mg/kg/day s.c.) for 4 days (Fig. 2).

The mean blood pressure of normal BN-Ki rats receiving only vehicle solution was not changed throughout the experimental period (Fig. 2). The mean blood pressure of deficient BN-Ka rats was kept at higher levels and was not changed by administration of vehicle solution during the experimental period (Fig. 2).

3.3. Effect of ebelactone B on urine volume and urinary excretions of Na^+, K^+ and creatinine

Deoxycorticosterone acetate-salt treatment resulted in significant increases in urine volume and in urinary excretion of Na^+ in both BN-Ki and BN-Ka rats, compared with those observed in rats without deoxycorticosterone acetate-salt treatment (Fig. 3). When ebelactone B (5 mg/kg/day) was given subcutaneously to deoxycorticosterone acetate-salt-treated rats, the urine volume of normal BN-Ki rats for 24 h, which was determined on the second day of ebelactone B treatment, tended to be increased, but the difference was

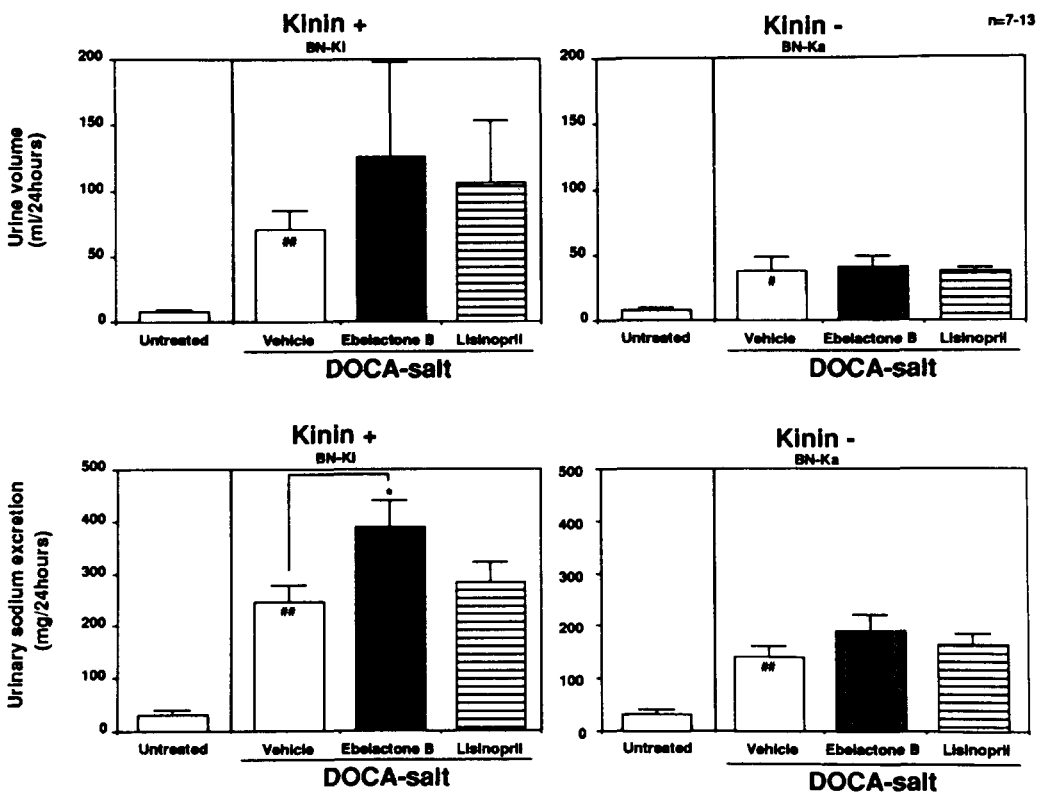

Fig. 3. Effect of ebelactone B and lisinopril on urinary Na^+ excretion and urine volume in deoxycorticosterone acetate-salt-treated rats. Values are means ± S.E.M. of the numbers (n) of rats. From 8 weeks of age, ebelactone B (5 mg/kg/day) or lisinopril (5 mg/kg/day) was administered (s.c.) for one week to deoxycorticosterone acetate-salt-treated normal BN-Ki rats and kininogen-deficient BN-Ka rats. Values from rats receiving ebelactone B or lisinopril were compared with those from rats receiving vehicle solution. * $P < 0.05$. The open columns on the left (untreated) indicate the values from rats without deoxycorticosterone acetate-salt treatment (7 weeks of age). Values from deoxycorticosterone acetate-salt-treated rats receiving vehicle solution were compared with those without deoxycorticosterone acetate-salt treatment. $^{\#}$ $P < 0.05$; $^{\#\#}$ $P < 0.01$.

Table 1
Changes in urinary excretions of K^+ and creatinine in deoxycorticosterone acetate-salt-treated rats during the subcutaneous administration of ebelactone B or lisinopril

Measurements	Rats	Untreated (7 weeks old)	DOCA-salt-treated rats (9 weeks old)		
			Vehicle	Ebelactone B	Lisinopril
Urinary K^+	BN-Ki	38.0 ± 0.4	50.9 ± 5.1 [a]	59.8 ± 7.6	56.2 ± 4.4
(mg/24 h)	BN-Ka	39.7 ± 1.3	56.0 ± 7.3 [a]	55.1 ± 4.4	64.0 ± 3.3
Urinary creatinine	BN-Ki	9.4 ± 0.5	11.5 ± 0.7	13.1 ± 1.8	11.4 ± 0.4
(mg/24 h)	BN-Ka	8.8 ± 0.8	12.4 ± 0.3	13.9 ± 1.5	14.6 ± 1.3

BN-Ka, Brown Norway Katholiek rats; BN-Ki, Brown Norway Kitasato rats. Each value represents the mean ± S.E.M. from 7–13 animals. Ebelactone B (5 mg/kg/day s.c.) or lisinopril (5 mg/kg/day s.c.) was administered daily to deoxycorticosterone acetate-salt-treated rats for one week. Values from deoxycorticosterone-acetate-salt treatment (untreated). [a] $P < 0.05$.

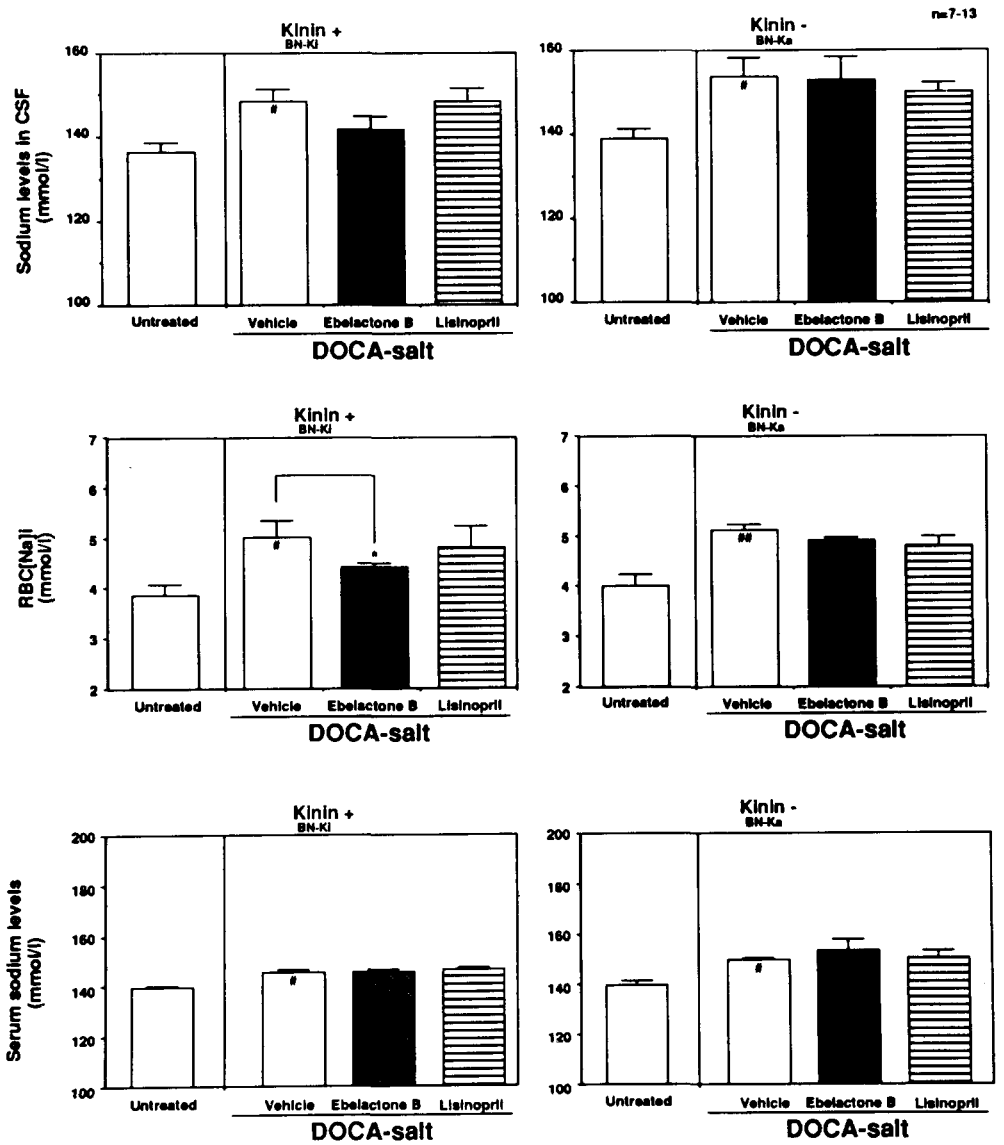

Fig. 4. Effect of ebelactone B and lisinopril on the Na^+ levels in cerebrospinal fluid, erythrocytes and serum in deoxycorticosterone acetate-salt-treated rats. Values are means ± S.E.M. of the numbers (n) of rats. From 8 weeks of age, ebelactone B (5 mg/kg/day) or lisinopril (5 mg/kg/day) was administered (s.c.) for one week to deoxycorticosterone acetate-salt-treated normal BN-Ki rats and kininogen-deficient BN-Ka rats. Values from deoxycorticosterone acetate-salt-treated rats receiving ebelactone B or lisinopril were compared with those receiving vehicle solution. * $P < 0.05$. The left-hand open columns (untreated) indicate the values from rats without deoxycorticosterone acetate-salt treatment (7 weeks of age). Values from deoxycorticosterone acetate-salt-treated rats receiving vehicle solution were compared with those in rats without deoxycorticosterone acetate-salt treatment. # $P < 0.05$; ## $P < 0.01$.

Table 2
Changes in serum K^+ and creatinine levels in deoxycorticosterone acetate-salt-treated rats during the subcutaneous administration of ebelactone B or lisinopril

Measurements	Rats	Untreated (7 weeks old)	DOCA-salt-treated rats (9 weeks old)		
			Vehicle	Ebelactone B	Lisinopril
Serum K^+	BN-Ki	4.5 ± 0.2	4.5 ± 0.2	4.5 ± 0.2	4.5 ± 0.2
(mmol/l)	BN-Ka	4.2 ± 0.2	4.7 ± 0.5	4.3 ± 0.1	4.3 ± 0.1
Serum creatinine	BN-Ki	4.7 ± 0.5	4.0 ± 0.1	3.8 ± 0.1	3.9 ± 0.1
(mg/l)	BN-Ka	4.5 ± 0.3	3.8 ± 0.2	4.3 ± 0.3	4.2 ± 0.2

BN-Ka, Brown Norway Katholiek rats; BN-Ki, Brown Norway Kitasato rats. Each value represents the mean ± S.E.M. from 7–13 animals. Ebelactone B (5 mg/kg/day s.c.) or lisinopril (5 mg/kg/day s.c.) was administered daily to deoxycorticosterone acetate-salt-treated rats for one week.

not significant due to the marked variance of the values under ebelactone B treatment (Fig. 3). However, the amount of Na^+ excreted in the urine for 24 h was significantly increased by ebelactone B in normal BN-Ki rats under deoxycorticosterone acetate-salt treatment. Lisinopril (5 mg/kg/day s.c.) did not alter the urine volume or the urinary excretion of Na^+ in normal BN-Ki rats. Neither of those parameters in deficient BN-Ka rats was changed significantly by ebelactone B or lisinopril (Fig. 3). There was no difference in excretion of urinary K^+ or creatinine between the vehicle-treated and the ebelactone B-or lisinopril-treated rats (Table 1).

3.4. Effect of ebelactone B on Na^+ concentration of cerebrospinal fluid and erythrocytes

Na^+ concentrations in the cerebrospinal fluid of deoxycorticosterone acetate-salt-treated normal BN-Ki rats, which were collected on the fifth day of vehicle administration, were significantly increased, when compared with those of the BN-Ki rats 7 weeks of age that did not receive deoxycorticosterone acetate-salt treatment (Fig. 4). A significant increase was also observed in the deoxycorticosterone acetate-salt-treated deficient BN-Ka rats. The increased Na^+ levels in the cerebrospinal fluid of normal BN-Ki rats tended to drop on ebelactone B treatment (5 mg/kg/day s.c.). No reduction in the Na^+ concentration of the cerebropspinal fluid was observed in deficient BN-Ka rats after administration of ebelactone B. Lisinopril treatment (5 mg/kg/day s.c.) did not induce any changes in the cerebrospinal fluid Na^+ levels.

The Na^+ concentration in the erythrocytes in untreated normal BN-Ki rats showed no significant difference from that in deficient BN-Ka rats, and the deoxycorticosterone acetate-salt treatment resulted in significant increases in both strains. Daily ebelactone B administration caused a significant reduction in the erythrocyte Na^+ concentration in the deoxycorticosterone acetate-salt-treated normal BN-Ki rats on the fifth day of treatment, but not in deficient BN-Ka rats at the same stage of treatment (Fig. 4). Lisinopril treatment did not induce any changes in the erythrocyte Na^+ levels in either BN-Ki or BN-Ka rats under deoxycorticosterone acetate-salt treatment (Fig. 4).

3.5. Effect of ebelactone B on serum concentrations of Na^+, K^+ and creatinine

The serum concentrations of Na^+ in the deoxycorticosterone acetate-salt-treated normal BN-Ki and deficient BN-Ka rats were slightly but significantly increased, compared with the untreated rats of each strain. The serum concentrations of Na^+ in the deoxycorticosterone acetate-salt-treated normal BN-Ki rats and deficient BN-Ka rats, which were determined in the blood samples collected on the fifth day of treatment, showed no significant reduction with ebelactone B (5 mg/kg/day s.c.), and there was no reduction in those treated with lisinopril (5 mg/kg/day s.c.), compared with the vehicle-treated BN-Ki or BN-Ka rats (Fig. 4). There were no significant differences in the serum concentration of K^+ or of creatinine in vehicle-treated rats and ebelactone B-or lisinopril-treated rats (Table 2).

3.6. Plasma renin activity in deoxycorticosterone acetate-salt treated rats

No difference in plasma renin activity was seen between untreated BN-Ki rats and BN-Ka rats at 10

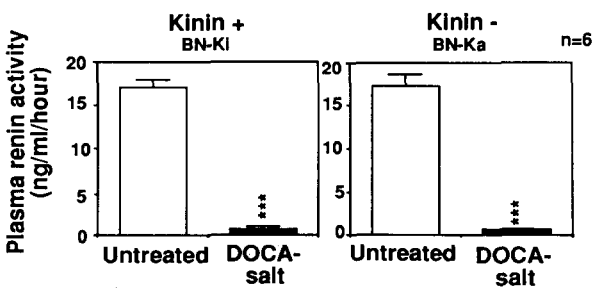

Fig. 5. Plasma renin activities in rats with and without deoxycorticosterone acetate-salt treatment. Values are means ± S.E.M. of the numbers (n) of rats at 10 weeks of age. Values from rats under deoxycorticosterone acetate-salt treatment were compared with those from untreated rats. *** $P < 0.001$.

weeks of age (Fig. 5). Deoxycorticosterone acetate-salt treatment for 3 weeks (from 7 weeks of age) reduced the plasma renin activity markedly in both BN-Ki rats and BN-Ka rats (Fig. 5).

4. Discussion

We have reported that bradykinin was degraded in rat urine collected from the ureter in a manner quite different from that in rat plasma, and that its main degradation product in rat urine was bradykinin-(1–6), whereas that in rat plasma was bradykinin-(1–5) (Majima et al., 1993a). Our previous gel filtration study revealed that the two major kinin-degrading enzymes in rat urine were neutral endopeptidase and carboxypeptidase Y-like exopeptidase, the latter of which has a molecular weight of approximately 93 kDa (Kuribayashi et al., 1993). It was the first report on the presence of carboxypeptidase Y-like exopeptidase in rat urine, although members of the carboxypeptidase Y family were reported to be a protective protein in association with lysosomal β-galactosidase and neuraminidase in mouse kidney (Galjart et al., 1990) and with a deaminase from human platelets (Jackman et al., 1990). To clarify the physiological roles of urinary carboxypeptidase Y-like exopeptidase, we found that a low molecular weight inhibitor of the enzyme, ebelactone B, which was isolated from *Actinomycetes* and was originally reported to inhibit a methylesterase (Tan and Rando, 1992) and an acylpeptide hydrolase (Scaloni et al., 1992) by modification of the active site serine. Ebelactone B selectively inhibited carboxypeptidase Y, but not carboxypeptidase A and carboxypeptidase B from the pancreas, so that it inhibited urinary carboxypeptidase Y-like exopeptidase and the degradation of bradykinin by rat urine (Majima et al., 1994b). Ebelactone B did not inhibit kininases in rat plasma (Majima et al., 1994b), as the major kininases in human plasma or rat plasma were angiotensin converting enzyme (kininase II) and carboxypeptidase N (kininase I). Interestingly, ebelactone B did not inhibit rat urinary kallikrein (data not shown), which is also a serine esterase.

Oral administration of ebelactone B to the saline-infused anesthetized rats (Sprague-Dawley strain) resulted in a significant increase in urine volume accompanied with increased levels of urinary kinin and Na^+, but not with increased urinary K^+ levels (Majima et al., 1994b). The increases in these parameters were completely suppressed by the bradykinin B_2 receptor antagonist, Hoe140 (Majima et al., 1994b). These results suggest that urinary kinin induces diuresis and natriuresis and the inhibition of bradykinin degradation by ebelactone B may accelerate the excretion of urine volume and urinary Na^+, since the bradykinin receptor (B_2) is present mainly on the collecting tubules (Tomita and Pisano, 1984), a location different from the site of action of loop diuretics or thiazide derivatives. We have reported repeatedly that the lack or reduced activity of the urinary kallikrein-kinin system accelerates the development of hypertension through the reduced excretion of Na^+ and water in several models (Majima et al., 1991, 1993b, 1994a; Mohsin et al., 1992), and so chose to study the effect of ebelactone B.

It is well known that deoxycorticosterone acetate-salt treatment causes gradual, but marked increases in the systolic blood pressure in rats. In a previous report (Majima et al., 1991), we demonstrated this in normal BN-Ki rats. It took more than 10 weeks for systolic blood pressure to peak in normal BN-Ki rats. However, the gradual increase in the systolic blood pressure was replaced by a rapid increase to near the maximum level within 2 weeks of the initiation of the same treatment in deficient BN-Ka rats, in which the urinary kinin level was negligible (Majima et al., 1991). This provides evidence that the kallikrein-kinin system plays a definite role in suppression of the early developmental stage of deoxycorticosterone acetate-salt hypertension. This hypothesis is further strengthened by the significant increase in the systolic pressure of normal BN-Ki rats seen during continuous infusion of a kallikrein inhibitor, aprotinin, subcutaneously for 7 days from the seventh day after the onset of the deoxycorticosterone acetate-salt treatment (Majima et al., 1991). During the developmental stage of deoxycorticosterone acetate-salt hypertension, urinary kallikrein excretion was increased markedly to levels 5–6 times higher than those in rats receiving no deoxycorticosterone acetate-salt treatment, and peaked 3 weeks after the start of treatment in normal BN-Ki and deficient BN-Ka rats (Katori et al., 1992). Normal BN-Ki rats increased Na^+ excretion in urine concomitantly with increases in the urinary kallikrein excretion, whereas deficient BN-Ka rats showed no such increase in Na^+ excretion despite the increase in urinary kallikrein excretion.

Administration of ebelactone B to deoxycorticosterone acetate-salt treated rats resulted in significant reductions in systolic blood pressure and mean blood pressure during the development of hypertension in normal BN-Ki and SD strain rats, which can generate kinin in urine (Fig. 1 and Fig. 2). This reduction in the blood pressure was kinin-dependent, since kininogen-deficient BN-Ka rats, which secreted negligible amounts of kinin in the urine, showed no reduction in blood pressure. The dose of ebelactone B used in the previous experiment (1 and 3 mg/kg) caused kinin-dependent diuresis in saline-infused anesthetized rats in a dose-dependent manner. This effect was not due to the changes in hemodynamic state, since the systemic blood pressure was not changed during the experiment

(Majima et al., 1994b). In the present experiment, 5 and 15 mg/kg/day of ebelactone B were selected as sufficient doses to induce kinin-dependent diuresis and natriuresis. Fifteen mg/kg/day of ebelactone B was equipotent to 5 mg/kg/day of the inhibitor in normal BN-Ki rats (Fig. 1), suggesting that 5 mg/kg/day may provide the maximal effect. The fact that deficient BN-Ka rats did not show any hypotensive response even at a higher dose of ebelactone B suggested lack of direct action of this inhibitor on the resistance vessels. However, the possibility that another effect, such as a blood pressure lowering effect, may attenuate the ebelactone B-induced renal effect in normal BN-Ki rats cannot be ruled out in the present experiment.

Ebelactone B significantly increased Na^+ excretion in normal BN-Ki rats, but its effect on urine volume was not statistically significant in normal BN-Ki rats, as there was a great variance in urine volume in the deoxycorticosterone acetate-salt-treated normal BN-Ki rats. By contrast, deficient BN-Ka rats showed no increase in urinary excretion of Na^+ or in urine volume during the administration of ebelactone B (Fig. 3).

Hypertension was readily induced in deficient BN-Ka rats by the loading of a mild salt diet (Majima et al., 1993b) and by subcutaneous infusion of a non-pressor dose of angiotensin II (Majima et al., 1994a), although the hypertension was not induced in normal BN-Ki rats even by the same treatments. The hypertension was accompanied with an increase in the Na^+ levels in the erythrocytes and cerebrospinal fluid. We are therefore proposing the hypothesis that the Na^+ accumulation in the cerebrospinal fluid and in cells such as smooth muscle may be caused by a reduction of Na^+ excretion in the renal tubules due to absence or reduction of activities of the renal kallikrein-kinin system, and may lead to hypertension. In the present deoxycorticosterone acetate-salt hypertension, the Na^+ concentrations in the erythrocytes and in the cerebrospinal fluid were increased in both normal BN-Ki rats and deficient BN-Ka rats. It should be emphasized that the ebelactone B treatment reduced the Na^+ concentration in the erythrocytes and tended to reduce that in the cerebrospinal fluid in normal BN-Ki rats. The lack of this reduction in deficient BN-Ka rats during the treatment indicated that the reduction was related to kinin generation.

The lack of any antihypertensive effect of angiotensin converting enzyme inhibitors in deoxycorticosterone acetate-salt hypertension in rats is widely accepted (Pham et al., 1993). This is not surprising, since plasma renin activity was suppressed markedly by deoxycorticosterone acetate-salt treatment in both normal BN-Ki rats and deficient BN-Ka rats. Although angiotensin converting enzyme (kininase II) is a predominant kininase in rat plasma (Majima et al., 1993a), and administration of the angiotensin converting enzyme inhibitor captopril causes a significant increase in blood kinin levels (Majima and Katori, 1994), lisinopril induced no hypotensive response either in deficient BN-Ka rats or in normal BN-Ki rats (Fig. 1), suggesting that the increased blood kinin level was not linked to the hypotensive response. These may be reasons for the lack of an antihypertensive effect on administration of angiotensin converting enzyme inhibitors in deoxycorticosterone acetate-salt hypertension.

It has been reported previously (Ura et al., 1987; Kuribayashi et al., 1993; Majima et al., 1994b) that another major kininase in rat urine is neutral endopeptidase. The lack of inhibition of urinary kininase activity by metal chelating agents has led to our discovery of a novel type of rat urinary kininase, carboxypeptidase Y-like kininase, a non-metal enzyme kininase which was a serine protease (Kuribayashi et al., 1993). We were unable to detect in rat urine any kininases sensitive to the metal chelating agents, other than neutral endopeptidase (Majima et al., 1993a, 1994b; Kuribayashi et al., 1993)

Poststatin (Majima et al., 1993a), isolated from *Streptomycetes viridochromogenes*, which was originally reported to inhibit prolyl endopeptidase, was first reported by us as an inhibitor of the activities of both neutral endopeptidase and carboxypeptidase Y, resulting in the complete inhibition of total kininase in rat urine (Majima et al., 1993a). This agent also reduced the blood pressure of deoxycorticosterone acetate-salt-treated normal BN-Ki rats, but not that of deficient BN-Ka rats so treated (Majima and Katori, 1994). Inhibition of both carboxypeptidase Y and neutral endopeptidase may lead to more potent hypotensive responses in this model. The combination of a neutral endopeptidase inhibitor (Smits et al., 1990; Sybert et al., 1990; Bralet et al., 1991) with ebelactone B is under investigation and the results will be reported separately.

In conclusion, the present results suggested that ebelactone B, a urinary carboxypeptidase Y-like kininase inhibitor, may be a promising agent for the development of rat deoxycorticosterone acetate-salt hypertension acting through the abolishment of Na^+ retention due to the inhibition of kinin degradation.

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