Diuretic and Natriuretic Effect of Ebelactone B in Anesthetized Rats by Inhibition of a Urinary Carboxypeptidase Y-Like Kininase

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ABSTRACT—Ebelactone B (EB) ($10^{-7}-10^{-5}$ M) inhibited dose-dependently carboxypeptidase (CP) Y-like exopeptidase, one of the major kininases separated from rat urine, whereas it inhibited neither CPA, CPB or neutral endopeptidase (NEP). Degradation of bradykinin (BK) to BK-(1-8) in rat urine was completely inhibited by EB (10^{-5} M) with the increased generation of BK-(1-7). Intraduodenal administration of EB (3 mg/kg) to anesthetized rats caused marked diuresis (by 110%) and natriuresis (130%), in parallel with the increase in urinary kinin levels (110%). Intravenous infusion of a BK antagonist, Hoe140 (3 mg/kg/hr), strongly blocked both EB-induced diuresis and natriuresis. EB may be a novel type of diuretic and natriuretic agent that acts by increasing urinary kinin levels.

Keywords: Ebelactone B, Carboxypeptidase Y, Diuresis

Bradykinin (BK) is very active in renal function because it participates in increasing the renal blood flow and in diuresis and natriuresis (1). We have reported that the degradation pathway of BK in rat urine is quite different from that in plasma because of the difference in kininases present (2). The major kininases in rat urine were neutral endopeptidase (NEP) and carboxypeptidase (CP) Y-like exopeptidase (3), whereas angiotensin converting enzyme (ACE) and CPN mainly degrade BK in rat plasma (2).

Ebelactones, isolated from actinomycetes, were reported to inhibit some enzymes such as esterase, lipase and *N*-formylmethionine aminopeptidase (4). In the present paper, we report that Ebelactone B (EB) selectively inhibited not only CPY from yeast but also CPY-like exopeptidase in rat urine without inhibition of other kininases in plasma and urine. Furthermore, administration of EB to anesthetized rats caused diuresis and natriuresis via increased urinary kinin excretion. This suggests that EB is a candidate for a new type of diuretic and natriuretic agent.

NEP and CPY-like exopeptidase in rat urine were separated by using a Superdex 200 column as described in the previous paper (3). Plasma samples were prepared from blood collected from the carotid artery under ether anesthesia (2). Enzyme activities of isolated CPA, CPB and CPY were determined with peptide substrates: Z-Gly-Phe for CPA (5), Bz-Gly-Arg for CPB (6) and Z-Phe-Leu for CPY (7). Assays of kininase activity and detection of BK fragments were carried out by HPLC (2, 3). The in vivo effects of EB were studied in male Sprague-Dawley strain rats (SPF, 280-350 g, 8- to 10-week-old, anesthetized with sodium pentobarbital, 40 mg/kg, i.p.). Urine was collected through a polyethylene cannula inserted into the bladder to estimate the volume of urine and urinary sodium and potassium levels. Physiological saline was infused (6 ml/kg/hr) via the jugular vein throughout the experimental period. Urine volume was estimated by its weight, and urinary sodium and potassium levels were assayed by flame photometry (8). For the assay of kinin in the urine, urine was collected directly into plastic tubes containing absolute ethanol through a polyethylene cannula inserted into both ureters under pentobarbital anesthesia, since kinin is rapidly degraded during its stay in the bladder. The amounts of kinin in the extracts were determined by enzyme immunoassay kits (Markit M bradykinin; Dainippon Pharmaceutical Corp., Osaka) (9). EB, supplied by Institute of Microbial Chemistry (Tokyo) was administered (1 or 3 mg/kg, dissolved in DMSO at 3.3 or 10 mg/ml) intraduodenally. Control rats were given DMSO (0.3 ml/kg) alone. Urine was collected for 15 min before and 1 hr after the drug treatment.

The following enzymes and inhibitors were used: CPA from bovine pancreas and CPB from porcine pancreas (Sigma Chemical Co., St. Louis, MO, USA), CPY from yeast (Oriental Yeast Co., Ltd., Tokyo), MGTA (D,L-2-mercaptomethyl-3-guanidinoethyl-thiopropanoic acid), an inhibitor of CPN (Carbiochem Corp, La Jolla, CA, USA), Z-Phe-Leu (Bachem Corp., Rubendorf, Switzerland), phosphoramidon, an inhibitor of NEP, Z-Gly-Phe and Bz-Gly-Arg (Peptide Institute, Osaka).

The paired *t*-test was used to evaluate the significance of difference, and a P value less than 0.05 was considered to be significant.

Table 1. Effect of ebelactone B on CPA, B, Y and kininases from rat urine

Enzymes	Inhibition (%)	Concentration of ebelactone B
CPA	0.0±0.0	10^{-5} M
CPB	0.0±0.0	10^{-5} M
CPY	100.0±0.0	10^{-5} M
Neutral endopeptidase (from rat urine)	0.0±0.0	10^{-5} M
CPY-like exopeptidase (from rat urine)	10.4±1.8	10^{-7} M
	83.9±3.8	10^{-6} M
	95.4±1.8	3×10^{-6} M
	99.2±1.3	10^{-5} M

Each value represents the mean±S.E.M. from three experiments.

As shown in Table 1, EB completely inhibited the activity of CPY at 10^{-5} M, whereas it had no effect on those of CPA and CPB at the same dose. EB also inhibited the activity of CPY-like exopeptidase (IC_{50}: 3.3×10^{-7} M), separated from rat urine, in a concentration-dependent manner (10^{-7}–10^{-5} M) without affecting the activity of NEP. Rat urine degraded BK at the first step into BK-(1–8) (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe) through the action of a CPY-like exopeptidase and into BK-(1–7) (Arg-Pro-Pro-Gly-Phe-Ser-Pro) by NEP (3). As shown in Table 2a, the generation of BK-(1–8) from BK in rat urine was inhibited by EB (10^{-5} M) with an increased amount of BK-(1–7). This increase in BK-(1–7) generation may be attributable to the slow degradation of the substrate BK by CPY inhibition. The generation of BK-(1–7) was inhibited by phosphoramidon (10^{-4} M). The combination of both inhibitors completely inhibited the generation of both BK fragments. Although we previously reported that BK-(1–6) was a stable metabolite of BK in rat urine (2), the amount of BK-(1–6) generated in the present experiment was scanty, suggesting less total kininase activity in the present urine samples. Captopril (Sankyo Pharmaceutical Co., Ltd., Tokyo) had no effect on the generation of both degradation products of BK, indicating that the kininase activity in rat urine was attribut-

Table 2. Effect of ebelactone B on the degradation of bradykinin in rat urine or plasma

		Bradykinin fragment formed (nmol)		
		Bradykinin-(1–8)	Bradykinin-(1–7)	Bradykinin-(1–5)
a) Rat urine				
No inhibitor		2.5±0.2	1.7±0.1	
Ebelactone B	(10^{-5} M)	— (0)	2.8±0.2 (164)	
Phosphoramidon	(10^{-4} M)	2.0±0.2 (79)	— (0)	
Ebelactone B + Phosphoramidon		— (0)	— (0)	
Captopril	(3×10^{-5} M)	2.6±0.5 (103)	1.6±0.4 (96)	
b) Rat plasma				
No inhibitor		6.1±0.6	4.1±1.4	4.6±0.7
Ebelactone B	(10^{-5} M)	6.0±0.4 (99)	3.9±0.2 (96)	5.0±0.4 (108)
MGTA	(10^{-5} M)	— (0)	4.3±0.1 (104)	5.2±0.4 (114)
Captopril	(3×10^{-5} M)	6.3±0.9 (109)	0.2±0.02 (5)	0.2±0.05 (4)
MGTA + Captopril		— (0)	0.1±0.03 (2)	0.1±0.02 (2)

Bradykinin (40 nmol) was incubated with 450 ml of saline-diluted rat urine (60-fold, final concentration of protein: 0.04±0.01 mg/ml) or plasma (15-fold, 4.1±0.1 mg/ml) for 1 hr (urine) or 30 min (plasma). Twenty-four percent of the bradykinin was degraded in urine and 47% of the bradykinin was degraded in plasma in the absence of inhibitors (No inhibitor). — indicates that the amount of bradykinin fragment formed was below the detection limit (less than 0.01 nmol). The values in parentheses are rates (%) of generated amounts of bradykinin fragments compared with those in the absence of inhibitors (No inhibitor). Each value represents the mean±S.E.M. from three experiments.

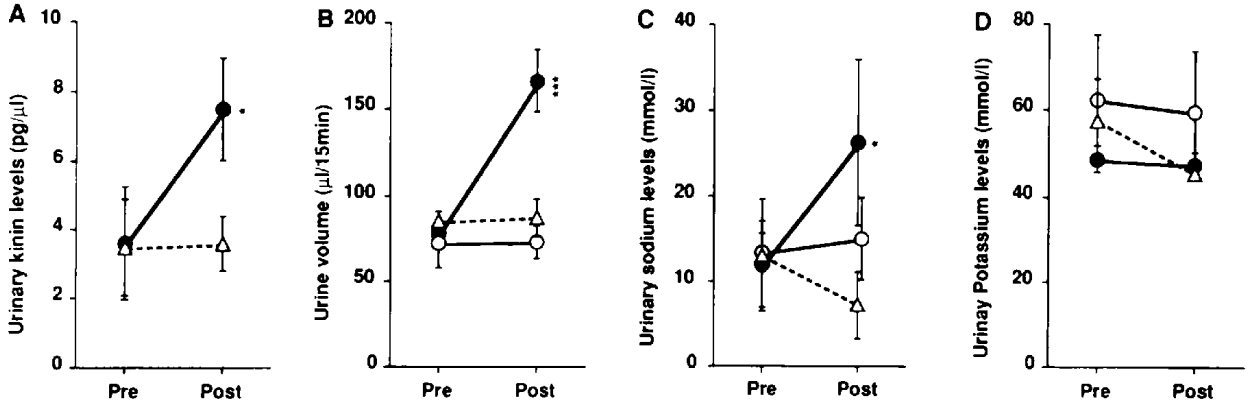

Fig. 1. Effects of Ebelactone B (EB) on urinary kinin levels (A), urine volume (B), urinary sodium levels (C) and urinary potassium levels (D) in anesthetized rats. EB (3 mg/kg) was administered intraduodenally. Urine samples for 15 min were collected 15 min before EB (Pre) and 1 hr after EB (Post). Hoe140, a bradykinin antagonist, was infused (3 mg/kg/hr) intravenously from 15 min before EB for 75 min. Each value represents the mean ± S.E.M. from four animals. The post-value was compared with the pre-value in each group of animals (paired *t*-test, $^{*}P<0.05$, $^{***}P<0.001$). -●- Ebelactone B, - _ - Ebelactone B + Hoe140, - -△- - Vehicle.

able to both NEP and the CPY-like exopeptidase, but not ACE. In rat plasma (Table 2b), BK was degraded to BK-(1-8) by CPN and to BK-(1-7) by ACE, and both fragments were further degraded to BK-(1-5) (Arg-Pro-Pro-Gly-Phe) by ACE (2). EB had no effect on the generation of these BK fragments. Captopril inhibited the generation of BK-(1-7) and BK-(1-5), whereas MGTA inhibited the generation of BK-(1-8) alone.

Treatment of anesthetized rats with EB (3 mg/kg, intraduodenally) markedly increased the kinin levels in urine, collected from ureters 1 hr after the administration of EB, from 3.6 ± 1.6 to 7.5 ± 1.4 pg/μl (by 110%, $P<0.05$), whereas the control rats receiving only the vehicle did not show any changes in urinary kinin excretion (Fig. 1A). This increase in kinin excretion was accompanied with the marked increase in urine volume from 78.0 ± 7.9 to 166.4 ± 18.0 μl/15 min (by 110%, $P<0.001$) (Fig. 1B). When a low dose of EB (1 mg/kg) was administered, the increase in the urine volume was from 84.0 ± 11.5 to 124.6 ± 14.6 μl/15 min (by 48%, $n=3$, $P<0.05$). The urine volume of the vehicle control rats was not changed, and the values were 85.2 ± 5.8 and 87.2 ± 10.8 μl/15 min before and 1 hr after the treatment, respectively (Fig. 1B). Urinary sodium levels were markedly increased by EB (3 mg/kg) treatment by 110% (Fig. 1C) ($P<0.05$), whereas urinary potassium levels were kept fairly constant (Fig. 1D). Even a low dose of EB (1 mg/kg) caused a significant ($P<0.05$) increase (by 56%) in urinary sodium levels. The intraduodenal administration of EB (3 mg/kg) treatment reduced the total kininase activity by 25%. EB administration (3 mg/kg) did not change the systemic blood pressure, and the mean blood pressure before and 1 hr after the EB-treatment were 112 ± 15 mmHg and 109 ± 12 mmHg ($n=3$), respectively.

Intravenous infusion of a sufficient amount (8) of the bradykinin antagonist Hoe140 (3 mg/kg/hr, Hoechest, Frankfult, Germany) from 15 min before EB administration (3 mg/kg) for 75 min cancelled the EB-induced increase in urine volume and urinary sodium levels (Fig. 1, B and C). Urinary potassium levels were not affected by an antagonist treatment (Fig. 1D).

We reported in a previous paper (3) the presence of a CPY-like exopeptidase in rat urine, although members of the CPY family were also reported to act as a protective protein in association with lysosomal beta galactosidase and neuraminidase in mouse kidney (10) and a deaminase from human platelets (11). However, the physiological roles of urinary CPY-like exopeptidase were not clear. In the present paper, we found that EB, which was originally reported to inhibit a methylesterase (12) and an acylpeptide hydrolase (13) by modification of an active site serine, inhibited urinary CPY-like exopeptidase in rats. EB selectively inhibited CPY, not CPA and CPB, so that it inhibited urinary the CPY-like exopeptidase (Table 1). EB did not inhibit kininases in plasma (Table 2). As EB did not inhibit rat urinary kallikrein (data not shown), which is also a serine esterase, the increased excretion of urinary kinin by EB was due to the kininase inhibition. The increased excretion of urinary kinin by EB was accompanied with an increase in urine volume and urinary sodium levels, but not with urinary potassium levels, and these increases were completely suppressed by Hoe140. These results strongly suggest that urinary kinin induces diuresis and natriuresis and inhibition of degradation of BK by compounds such as EB may accelerate the excretion of urine volume and urinary sodium, since the BK receptor

(B_2) is present mainly on the collecting tubules (14), which is different from the site of action of loop diuretics or thiazide derivatives. As we reported that the lack or reduced activity of urinary kallikrein-kinin system accelerated the development of hypertension through reduced excretion of sodium and water in several models (8, 9, 15), EB or other kininase inhibitor in the urine may be a candidate for a novel type of anti-hypertensive drug.

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