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In vivo transfer of antisense oligonucleotide against urinary kininase blunts deoxycorticosterone acetate-salt hypertension in rats

*,[1]Izumi Hayashi, [1]Masataka Majima, [1]Tomoe Fujita, [1]Toshiaki Okumura, [1]Yuji Kumagai, [2]Naruya Tomita, [2]Ryuichi Morishita, [2]Jitsuo Higaki & [2]Toshio Ogiwara

[1]Department of Pharmacology, Kitasato University School of Medicine, 1-15-1 Kitasato, Sagamihara, Kanagawa 228-8555, Japan and [2]Department of Geriatric Medicine, Osaka University School of Medicine, Suita, Osaka 565-0871, Japan

1 We have previously reported that the renal kallikrein-kinin system suppressed the development of deoxycorticosterone acetate (DOCA)-salt hypertension. Kinins were degraded in the kidney mainly by carboxypeptidase Y (CPY)-like kininase. Blockade of renal kinin degradation may reduce hypertension in the developmental stage.

2 We constructed an antisense oligonucleotide against rat CPY homologue (5′-CAT-CTC-TGC-TTC-CTT-GTG-TC-3′, AS) and its randomized control oligonucleotide (5′-TCC-TTC-CTG-CTT-GAG-TTC-CT-3′, RC), and prepared an HVJ-liposome complex that prolongs and increases the effectiveness of the antisense oligonucleotide. Antisense oligonucleotide was transfected (25 nmole rat^{-1}, in terms of nucleotide) into the kidney from the renal artery.

3 Blood pressure was measured through a catheter inserted into the abdominal aorta. Mean blood pressure (MBP) in DOCA-salt treated (for 2 weeks) Sprague Dawley strain rats was 130 ± 3 mmHg ($n=11$), and was reduced significantly ($P<0.05$) more by AS transfection (122 ± 4 mmHg, $n=6$) than by RC treatment (137 ± 6 mmHg, $n=5$) 4 days after the transfection.

4 This reduction in MBP was accompanied by increased urinary sodium excretion (AS, 8.4 ± 1.5 mmole day^{-1}; RC, 4.6 ± 0.5 mmole day^{-1}, $P<0.05$) and a reduction in urinary CPY-like kininase activity.

5 Ebelactone B (5 mg kg^{-1}, twice a day, p.o.), an inhibitor for urinary CPY-like kininase, also reduced MBP and induced natriuresis to the same degree as AS.

6 Lisinopril, an inhibitor for angiotensin converting enzyme (ACE) failed to reduce the elevated MBP.

7 These results suggest that CPY-like kininase may have more contribution than ACE to degrade kinin in the kidney, and that knockdown of CPY-like kininase in the kidney may partly prevent rat DOCA-salt hypertension.

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Abbreviations: ACE, angiotensin converting enzyme; AS, antisense oligonucleotide; BK, bradykinin, BN-Ka, Brown Norway Katholiek; CMC, carboxymethyl cellulose; CPY, carboxypeptidase Y; DOCA, deoxycorticosterone acetate; HVJ, haemagglutinating virus of Japan; MBP, mean blood pressure; ODN, oligodeoxyribonucleotide; RC, randomized control oligonucleotide

Introduction

We have previously reported that the kallikrein-kinin system has a suppressive role in the development of hypertension (Katori & Majima, 1998). When sodium was retained or loaded, the suppressive effect of this system was observed (Majima *et al*., 1993; 1994b). When the blood pressure in conscious, unrestrained rats receiving continuous intravenous infusion of sodium solution was measured, simple volume expansion with physiological saline did not increase mean blood pressure in Brown Norway Katholiek (BN-Ka) rats which lack the capacity for kinin generation (Majima *et al*., 1995a). However, continuous infusion of 0.3 M sodium chloride solution increased blood pressure in BN-Ka rats with sodium retention. Normal rats did not show any hypertensive response or sodium retention even with 0.3 M sodium chloride solution. These results indicate that sodium retention, but not volume expansion, is relevant to the development of hypertension, when kinin generation is lacking (Majima *et al*., 1995a). On the basis of this result, we have proposed that a primary function of the renal kallikrein-kinin system is to excrete excess sodium by cancellation of sodium reabsorption in the renal tubules, when sodium has been loaded or been accumulated by mineralcorticoids (Katori & Majima, 1998). Excess sodium in normal rats can be excreted through the inhibition of sodium reabsorption by kinin. However, once kinin generation is lacking, excess sodium cannot be excreted and tends to be accumulated in the body. Recent results from another group using bradykinin B2 receptor knockout mice support our previous results (Alfie *et al*., 1997).

Thus facilitation of the renal kallikrein kinin system may prevent hypertension by inhibiting sodium retention. If the degradation of kinin is inhibited in the kidney, a possible suppression of salt-related hypertension would be supposed. We reported carboxypeptidase Y (CPY)-like kininase, a novel

*Author for correspondence: E-mail: hayashii@med.kitasato-u.ac.jp

urinary kininase in rat urine that in some characteristics, resembled a carboxypeptidase from yeast, (Kuribayashi *et al*., 1993). This serine protease was a major kininase in terms of kinin-degrading activity (Kuribayashi *et al*., 1993), and was also secreted in human urine (Saito *et al*., 1995). Subsequently, ebelactone B, a microbial product that was isolated from *Actinomycetes*, was found to be a potent and specific inhibitor of CPY-like kininase (Majima *et al*., 1994a). Ebelactone B exhibited kinin-dependent diuretic and natriuretic actions in anaesthetized rats, and reduced the high blood pressure in a DOCA-salt model (Majima *et al*., 1994a; 1995b; Ito *et al*., 1999).

In the present experiment, we further tested the effect of blockade of CPY-like kininase to provide further evidence for the preventive roles of renal kallikrein-kinin system in hypertension. For this purpose, we used an antisense strategy to inhibit renal CPY-like kininase activity selectively. Antisense oligodeoxyribonucleotides (ODNs) are widely used as inhibitors of specific gene expression because they offer the exciting possibility of blocking the expression of a particular gene without changing the functions of other genes (Helene & Toulme, 1990). Antisense ODNs are useful tools in the study of gene function and may be potential therapeutic agents, although antisense ODNs have several weak points such as their short-life, low efficiency of uptake, and degradation by endocytosis and nucleases (Akhtar & Juliano, 1992; Marcus-Sekura, 1988; Agrawal *et al*., 1991). We recently developed an efficient gene transfer method mediated by a viral liposome complex (Kaneda *et al*., 1989a,b; Tomita *et al*., 1992; 1993; Morishita *et al*., 1993b). This delivery system enhances the efficacy and prolongs the half-life of antisense ODNs *in vitro* and *in vivo* (Morishita *et al*., 1993a; 1994).

The present report demonstrates that selective renal *in vivo* transfection of antisense ODNs against rat CPY homologue partly prevents the development of hypertension in DOCA-salt treated rats. This antihypertensive strategy was also compared to the effects of CPY-like kininase inhibitor, ebelactone B, and ACE inhibitor, lisinopril, to clarify the roles of these kininases in salt-related hypertension. Inhibition of kininase in urine by antisense ODNs will provide further evidence on the pivotal suppressive roles of the renal kallikrein kinin system.

Methods

Animals

Male Sprague-Dawley (SD) rats (specific pathogen-free, SLC, Hamamatsu, Japan) 6-weeks-old were used. All animals were housed at constant humidity ($60 \pm 5\%$) and temperature ($25 \pm 1^{\circ}C$), and kept on a 12 h light/dark cycle throughout the experiments. The number (n) 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.

Construction of ODNs

Phosphorothioated ODNs were synthesized on a 391 DNA synthesizer (Applied Biosystems) with standard DNA synthesis techniques, purified with high-performance liquid chromatography, washed with 70% ethanol, and resuspended in TE buffer (10 mmol l^{-1}, Tris, pH 7.5, and 1 mmol l^{-1} EDTA, pH 8.0). For thiation of the phosphate linkages, 0.2 M solution of 3*H*-1.2-benzodithiole-3-one, 1,1-dioxide was used as the oxidizing agent (Iyer *et al*., 1990). Concentration was determined with a spectrophotometer. The following sequences were used: antisense rat CPY homologue (Sato-Prior *et al*., 1997), 5′-CAT-CTC-TGC-TTC-CTT-GTG-TC-3′ (AS), and its randomized control oligonucleotide, 5′-TCC-TTC-CTG-CTT-GAG-TTC-CT-3′ (RC).

Preparation of haemagglutinating virus of Japan–liposome solution

Haemagglutinating virus of Japan (HVJ; Sendai virus, Z strain) was propagated in chorioallantoic fluid of embryonated eggs as described previously (Kaneda *et al*., 1989a,b). HVJ was collected by centrifugation at $27{,}000 \times g$ for 40 min and suspended in balanced salt solution overnight. This procedure was repeated at least twice. The suspended HVJ was stored at 4°C and used within 1 week after purification. The haemagglutinating activity of HVJ was determined as described previously (Kaneda *et al*., 1989a,b). One absorbance at 540 nm of HVJ suspension contained 1 mg ml^{-1} protein and was equivalent to 15,000 haemagglutinating units (HAU) ml^{-1} as an index of fusogenic property. The liposomes were prepared from the lipid mixture (phosphatidylcholine, phosphatidylserine, and cholesterol), as reported previously (Tomita *et al*., 1995a). Antisense ODN and randomized control ODN were incorporated into the liposome by shaking and sonication. The liposomes and HVJ, inactivated by UV irradiation (110 erg mm^{-2} s^{-1}) for 3 min just before use, were incubated at 4°C for 10 min and then at 37°C for 30 min with gentle shaking (two strokes per second). This solution was centrifuged by sucrose gradient. The top layer was collected for use.

Induction of hypertension in rats

At 6-weeks-of-age, unilateral nephrectomy was done under light ether anaesthesia. The right renal artery and vein of rat were partially exposed and ligated. The right kidney was removed after a flank incision. The drinking water was replaced with 1% NaCl solution after the nephrectomy, and deoxycorticosterone acetate solution (10 mg kg^{-1} $week^{-1}$, 10 mg ml^{-1} in physiological saline, containing 50 mg/ml of gum arabic) was subcutaneously administered twice a week as reported previously (Majima *et al*., 1995b; Ito *et al*., 1999). Two weeks after the start of DOCA-salt treatment, the rats were anaesthetized with sodium pentobarbital (50 mg kg^{-1}, i.p.) and the left renal artery was exposed surgically. A catheter was placed in the abdominal aorta *via* the left femoral artery to measure mean arterial blood pressure (MBP). Rats with a mean blood pressure above 125 mmHg were used for the experiments.

Administration of antisense ODN or kininase inhibitors

At 2 days after the measurements of blood pressure as described above, on the day of transfection, the catheter was removed and a new catheter for transfection was inserted, which was introduced to the left renal artery *via* the femoral artery under anaesthesia with sodium pentobarbital (50 mg kg^{-1}, i.p.). For transfection, clipping the abdominal aorta occluded the blood flow to the kidney. Physiological saline was perfused from the left renal artery to the left kidney through a catheter placed to wash out blood before transfection. Removal of blood was visible. HVJ-liposome complex containing antisense ODN or randomized control ODN (15 nmole rat^{-1}) was introduced selectively into the left kidney of the DOCA-salt treated rats, and incubated for 10 min. After completion of transfection, adequate reperfu-

sion was also visible, since the colour of the left kidney was changed by blood flow. The catheter for the transfection was removed and another catheter to measure mean blood pressure was inserted from the left femoral artery and exteriorized in the interscapular region. The dose of antisense ODN and randomized control ODN used was decided by the data reported previously (Tomita *et al*., 1995a). Control rats were not treated with unilateral nephrectomy and DOCA-salt.

In two other sets of DOCA-salt treated rats, either ebelactone B (5 mg kg^{-1}, suspended with 1% of carboxymethyl cellulose (CMC) in distilled water at a concentration of 15 mg ml^{-1}), a specific inhibitor for CPY-like kininase (Majima *et al*., 1994b; 1995b; Ito *et al*., 1999) or the angiotensin converting enzyme (ACE) inhibitor lisinopril (5 mg kg^{-1}, suspended with 5% of CMC in distilled water at a concentration of 15 mg ml^{-1}) was administered orally twice a day for 4 days. Control animals received only vehicle solution (5% of CMC in distilled water). The dose of ebelactone B used in the present experiment was selected according to the results of the previous experiment (Ito *et al*., 1999). We confirmed that the dose of 5 mg kg^{-1} was sufficient to inhibit a rise in blood pressure in salt-related hypertension (Ito *et al*., 1999) and to induce significant diuresis and natriuresis in saline-infused anaesthetized rats, since three times the dose of ebelactone B had shown no more than the same degree of prevention of blood pressure rise in rats (Majima *et al*., 1994b).

MBP was determined in conscious, unrestrained rats, as reported previously (Majima *et al*., 1993; 1994a). Briefly, a catheter, which was inserted into the abdominal aorta through the femoral artery as described above, was attached to a blood pressure transducer (TP-400T, Nihon Koden). MBP was monitored on a polygraph (Thermal Array Recorder RTA-1100M, Nihon Koden). Starting 30 min after the connection of the transducer, recording was carried out for more than 30 min on the rats, which were housed in separate cages. The values of MBP are shown as means over the 30 min-period.

Collection of urine and measurement of urinary levels of sodium

Twenty-four-hour urine samples from individual rats after administration of ODNs or kininase inhibitors were collected using metabolic cages. The volume of urine was recorded at the end of the 24 h period. Urinary sodium levels were determined electrometrically using ion-selective electrodes selective for sodium (Majima *et al*., 1995b; Ito *et al*., 1999).

Measurement of urinary kininase activity

The activity of CPY-like kininase in urine samples collected in metabolic cages over 24 h was measured by generation rates of a bradykinin (BK) metabolite, des-Arg^9-BK, from BK.

Rat urine (5 μl), 395 μl of 0.05 M Tris-HCl buffer containing 0.1 M NaCl and 0.01 M $CaCl_2$ (pH 8.0), and a solution of appropriate inhibitors or saline (50 μl) were added into the plastic tubes, and after preincubation for 10 min at 37°C, the mixtures were incubated for 1 h at 37°C with 1 nmol/50 μl of BK. The reaction was terminated by addition of 20% trichloroacetic acid (100 μl), and the mixture was centrifuged at $1500 \times g$ at 4°C for 15 min. The supernatant (300 μl) was transferred to other plastic tubes, containing 300 μl of buffer for enzyme-linked immunosorbent assay (ELISA) (Majima *et al*., 1996), and the mixture was subjected to ELISA for des-Arg^9-BK. The des-Arg^9-BK-generating activity secreted in 24-h urine samples was expressed as pmol h^{-1} 24 h^{-1}.

Development of an ELISA for des-Arg^9-bradykinin

A polyclonal antibody against des-Arg^9-BK was raised in rabbits by subcutaneous injection of an immunogen prepared by conjugating its amino terminus to bovine serum albumin, as reported previously (Majima *et al*., 1996).

Des-Arg^9-BK was conjugated with horseradish peroxidase (type VI, Sigma, Chemical Corp., St. Louis, MO, U.S.A.) using glutaraldehyde, as reported previously (Majima *et al*., 1996).

One hundred μl of antiserum dilute 1 : 1000 was incubated at 25°C with immunoplate coated with anti-rabbit IgG for 1 h. Fifty μl of solutions of standard peptide (9.8 pg–10 ng ml^{-1}) or of sample solution were added to the wells of the plate. After incubation at 25°C for 1 h, 50 μl of the solution of enzyme-labelled des-Arg^9-BK was added, and incubation was carried out at 4°C for 18 h. After removal of the contents of the wells, 100 μl of substrate solution (0.087% (w v^{-1}) o-phenylenediamine dihydrochloride and 0.02% (w v^{-1}) H_2O_2) was added to each well, and the absorbance at 492 nm was measured after 1 h of incubation at 37°C. The concentrations of des-Arg^9-bradykinin in the samples were determined by reference to the standard calibration curves for authentic peptide (Peptide Institute, Osaka, Japan). ELISA (Markit M, Dainippon Pharmaceut. Corp. Ltd., Osaka, Japan) for BK was used.

Statistical analysis

Statistical significance of differences between groups was determined by a one-way ANOVA or ANOVA followed by *post-hoc* Bonferroni test. A probability (P) value of 0.05 or less was taken to indicate statistical significance. Values were expressed as means with s.e.mean with the number of observations indicated in parentheses.

Results

Effects of renal transfection of antisense oligodeoxyribonucleotide against rat CPY homologue on mean blood pressure in DOCA-salt treated rats

MBP of non-treated SD strain rats (6-weeks-old) was 102 ± 2 mmHg ($n = 12$), rats with uninephrectomized was 103 ± 3 mmHg ($n = 12$), and DOCA-salt treatment for 2 weeks gradually increased MBP to 132 ± 3 mmHg ($n = 12$). When antisense ODN against rat CPY homologue was transfected to the kidney, the mean blood pressure was gradually decreased (Figure 1A). By contrast, the transfection of randomized control ODN did not reduce the mean blood pressure, and the slight increase in the blood pressure still continued. The difference of the mean blood pressure between antisense and randomized control ODNs-treated rats was statistically significant at day 4. However the blood pressure of rats transfected with antisense ODN did not fall to the level in control rats that were not treated with unilateral nephrectomy and DOCA-salt. Although the blood pressure was increased in the antisense treated group at 6 days transfection, a significant difference between the antisense and the randomized ODNs was still observed (Figure 1A).

MBP in the antisense-treated group of normal SD rats without uninephrectomized and DOCA-salt treatment was 103.3 ± 1.5 mmHg ($n = 4$) and that in the randomized group was 104.7 ± 1.5 ($n = 4$).

Daily oral administration of lisinopril to the rats under DOCA-salt treatment did not reduce MBP at all. However, the

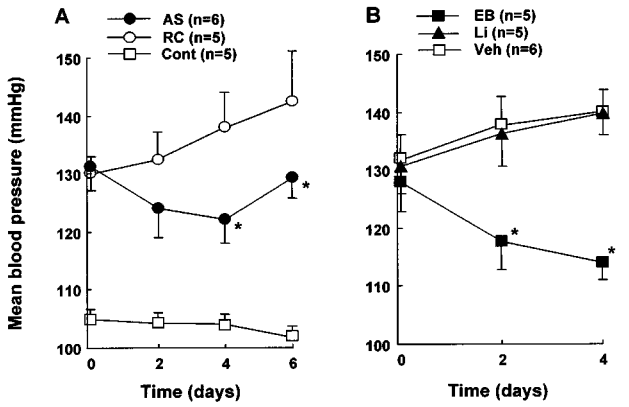

Figure 1 The effects of transfection of antisense oligonucleotide against rat carboxypeptidase Y homologue on the development of deoxy-corticosterone acetate-salt hypertension. After right unilateral nephrectomy at 6-weeks-of-age, deoxycorticosterone acetate (5 mg/kg, s.c.) was administered twice a week for 2 weeks. (A) Antisense oligonucleotide against rat carboxypeptidase Y homologue (AS) or randomized oligonucleotide (RC) was transfected on day 0. Control rats (Cont) were not treated with unilateral nephrectomy and DOCA-salt. Values are means ± s.e.mean of the mean blood pressures measured in various numbers (n) of rats. $^*P<0.05$, a one-way ANOVA, *c.f.* randomized control oligonucleotide-transfected animals. (B) For other groups of rats, ebelactone B (EB, 5 mg kg^{-1}), lisinopril (Li, 5 mg kg^{-1}), or 5% CMC (Veh, 3 ml kg^{-1}) was administered orally twice a day from day 0. Values are means ± s.e.mean of the mean blood pressures measured in various numbers (n) of rats. $^*P<0.05$, ANOVA followed by *post hoc* Bonferroni test, *c.f.* vehicle solution-administered animals (Veh).

administration of ebelactone B significantly reduced MBP, compared with that observed in vehicle-treated rats (Figure 1B).

Effects of renal transfection of antisense oligodeoxyribonucleotide against rat CPY homologue on urine volume and urinary sodium excretion in DOCA-salt treated rats

Figure 2 depicts the 24-h urine volumes of the rats 2 days after the ODN transfection. Although these volumes were not significantly increased in the rats treated with antisense ODN against rat CPY homologue, compared with those in rats receiving randomized control ODN, the urinary sodium excretion was significantly increased with the transfection of the antisense (Figure 3).

Daily oral administration of lisinopril or ebelactone B did not significantly increase the urine volume (Figure 2). However, the administration of ebelactone B significantly increased the excretion of sodium, compared with that of vehicle. Lisinopril did not affect the sodium excretion in the present experiment (Figure 3).

Effects of renal transfection of antisense oligodeoxyribonucleotide against rat CPY homologue on excretion of urinary CPY-like kininase

CPY-kininase degrades BK to des-Arg^9-BK in rat urine, and this degradation was completely inhibited by ebelactone B (Majima *et al.*, 1994b). So, to confirm the efficacy of transfection of antisense ODN against rat CPY homologue, the rates of des-Arg^9-BK generation from BK as markers of CPY-like kininase activity were determined in the urine collected from the rats kept in metabolic cages. In the present experiment, 10–30% of BK was degraded. The generation

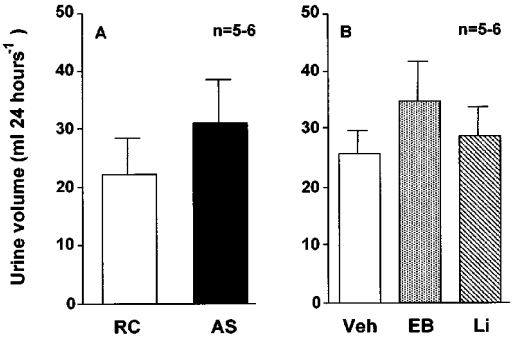

Figure 2 The effects of transfection of antisense oligonucleotide against rat carboxypeptidase Y homologue on urine volume in deoxycorticosterone acetate-salt treated rats. After right unilateral nephrectomy at 6-weeks-of-age, deoxycorticosterone acetate (5 mg kg^{-1}, s.c.) was administered twice a week for 2 weeks. (A) Antisense oligonucleotide against rat carboxypeptidase Y homologue (AS) or randomized oligonucleotide (RC) was transfected on day 0. Two days after the start, the urine was collected in the metabolic cages on day 2 to 3. Values are means ± s.e.mean of the urine volume collected for 24 h in various numbers (n) of rats. A one-way ANOVA, *c.f.* randomized control oligonucleotide-transfected animals. (B) For other groups of rats, ebelactone B (EB, 5 mg kg^{-1}), lisinopril (Li, 5 mg kg^{-1}), or 5% CMC (Veh, 3 ml kg^{-1}) was administered orally twice a day from day 0. Two days after the start, the urine was collected in the metabolic cages on day 2 to 3. Values are means ± s.e.mean of the urine volume collected for 24 h in various numbers (n) of rats. ANOVA followed by Bonferroni test, *c.f.* vehicle solution-administered animals (Veh).

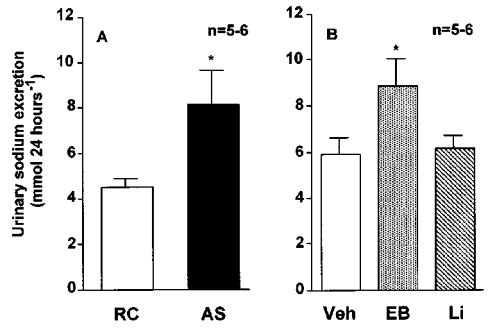

Figure 3 The effects of transfection of antisense oligonucleotide against rat carboxypeptidase Y homologue on urinary sodium excretions in deoxycorticosterone acetate-salt treated rats. After right unilateral nephrectomy at 6-weeks-of-age, deoxycorticosterone acetate (5 mg kg^{-1}, s.c.) was administered twice a week for 2 weeks. (A) Antisense oligonucleotide against rat carboxypeptidase Y homologue (AS) or randomized oligonucleotide (RC) was transfected on day 0. Two days after the start, the urine was collected in the metabolic cages on day 2 to 3. Values are means ± s.e.mean of the urinary sodium excretion secreted for 24 h in various numbers (n) of rats. $^*P<0.05$, a 1-way ANOVA, *c.f.* randomized control oligonucleotide-transfected animals. (B) For other groups of rats, ebelactone B (EB, 5 mg kg^{-1}), lisinopril (Li, 5 mg kg^{-1}), or 5% CMC (Veh, 3 ml kg^{-1}) was administered orally twice a day from day 0. Two days after the start, the urine was collected in the metabolic cages on day 2 to 3. Values are means ± s.e.mean of the urinary sodium excretion secreted for 24 h in various numbers (n) of rats. $^*P<0.05$, ANOVA followed by Bonferroni test, *c.f.* vehicle solution-administered animals (Veh).

rates of des-Arg^9-BK in the urine samples, which were obtained from 2 to 3 days after the transfection of antisense ODN against rat CPY homologue, were markedly suppressed, compared with those of rats receiving the randomized control ODN (Figure 4). The addition of ebelactone B to the incubation mixtures inhibited the generation of des-Arg^9-BK in the urine from the randomized control ODN-treated rats (Figure 4). However, this inhibitory effect was not seen in the

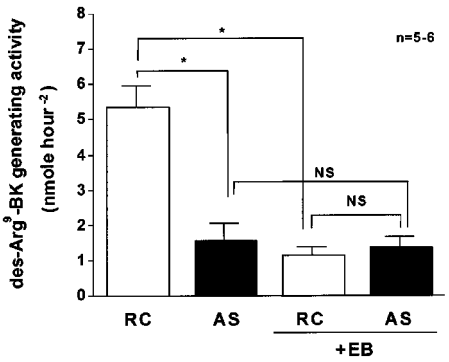

Figure 4 The effects of transfection of antisense oligonucleotide against rat carboxypeptidase Y homologue on des-Arg^9-bradykinin generating activity in urine in deoxycorticosterone acetate-salt treated rats. After right unilateral nephrectomy at 6-weeks-of-age, deoxycorticosterone acetate (5 mg kg^{-1}, s.c.) was administered twice a week for 2 weeks. Antisense oligonucleotide against rat carboxy peptidase Y homologue (AS) or randomized oligonucleotide (RC) was transfected. Two days after the start of antisense oligonucleotide, the urine was collected in the metabolic cages. Des-Arg^9-bradykinin generating activity in urine was determined in the absence (RC, AS) or the presence (RC + EB, AS + EB) of ebelactone B (10^{-5} M). Values are means ± s.e.mean of des-Arg^9-bradykinin generating activity in urine secreted for 24 h in various numbers (n) of rats. $^*P<0.05$, ANOVA followed by Bonferroni test for multiple comparisons.

urine from the rats receiving the antisense ODN, suggesting that the blockade of the expression of CPY-like kininase by the present antisense ODN treatment.

Discussion

BK is a potent biologically active peptide, which can induce hypotension, increases in renal blood flow and sodium excretion from the kidney (Katori & Majima, 1998). We have previously reported that BK 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 BK-(1-6), whereas that in rat plasma was BK-(1-5) (Katori & Majima, 1998). Our previous gel filtration study revealed that the predominant kinin-degrading enzymes in rat urine was CPY-like exopeptidase, which has a molecular weight of approximately 93 kDa, cleaves C-terminal arginine to generate des-Arg^9-BK as the first step of degradation (Kuribayashi *et al*., 1993). It has been believed that all the kininases belonged to the metal enzymes (Ura *et al*., 1987). However, we found that a large amount of kininase, which was resistant to the chelating agents, was secreted into the rat urine and human urine (Kuribayashi *et al*., 1993; Saito *et al*., 1995). We named it as CPY-like kininase from the inhibitory profiles by known inhibitors (Kuribayashi *et al*., 1993). That study was the first to report the presence of CPY-like exopeptidase in rat urine, although members of the CPY family were reported to be protective proteins 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). The presence of abundant kininase on the renal tubules was reported (Katori & Majima, 1998). Even when a large amount of BK was infused into the renal artery, the urinary secretion of BK was not increased. Kinin secretion into the urine depended on the kinin generating system present in the connecting tubules and/or collecting ducts, and the kinin degrading systems.

Thus, blocking the kinin degradation in the renal tubules and enhancing the action of BK in the kidney is quite a useful strategy for prevention of salt-related hypertension (Majima *et al*., 1994b; 1995b; Ito *et al*., 1999). In terms of kinin degradation rate, CPY-like kininase has the most potent activity in kidney. To block the CPY-like kininase, we tried the following two manoeuvres in the present experiment: (1) administration of a low molecular weight inhibitor of the enzyme, ebelactone B, which was isolated from *Actinomycetes* and was originally reported to inhibit a methylesterase (Tan & Rando, 1992) and an acylpeptide hydrolase (Scaloni *et al*., 1992) by modification of the active site serine; and (2) *in vivo* antisense ODN transfection against rat CPY homologue (Sato-Prior *et al*., 1997). Ebelactone B did not inhibit kininases in rat plasma (Majima *et al*., 1994a), since the major kininases in human plasma or rat plasma are ACE (kininase II) and CPN (kininase I). However, the specificity of this inhibitor was not guaranteed completely. To perform more specific inhibition, we constructed an antisense ODN against rat CPY homologue. CPY was characterized as a protective protein in humans from the primary structure and the enzymatic activity (Galjart *et al*., 1988). Although the cDNAs from mouse and human were also cloned, the full-length of cDNA encoding rat CPY has not been characterized and is not available. Recently preferentially expressed cochlear genes from a rat cochlea cDNA library were identified, and one of them, which was partially cloned and sequenced, was matched to mouse protective protein, i.e. CPY (Sato-Prior *et al*., 1997). Therefore we used the information of a partial nucleotide sequence as 'rat' CPY, to design the antisense ODN in the present study. In our previous paper, we reported obtaining the antibody against mouse CPY, which was raised by subcutaneous injection of the synthetic peptide with the 17 amino acid sequence corresponding to the amino terminal of mouse CPY conjugated with Keyhole Limpets haemocyanin. This antibody could inhibit the kinin degrading activity of CPY-like kininase isolated from rat urine (Kuribayashi *et al*., 1993). In the present experiment, the antisense ODN against rat CPY homologue certainly inhibited CPY-like kininase activity in rat urine, judging from the reduced generation of des-Arg^9-BK from BK in the urine after its transfection. Thus, the reduction in the mean blood pressure may have been caused by CPY-like kininase inhibition by the antisense ODN. Natriuresis was also induced by treatment with the antisense. The cancellation of sodium retention, which is relevant to the prevention of DOCA-salt hypertension, may be introduced by treatment with the antisense ODN against rat CPY homologue.

ACE inhibitor, lisinopril failed to reduce blood pressure and to promote natriuresis in DOCA-salt hypertensive rats in the present study. We have previously reported that inhibitory effect of ACE inhibitor on degradation of bradykinin in rat urine is less than that of CPY inhibitor or neutral endopeptidase inhibitor (Majima *et al*., 1995b). These results strongly suggest that CPY-like kininase would be more important than ACE as a local degradation enzyme of kinin in kidney. However, in the present study, blood pressure of rats transfected with antisense ODN against CPY-like kininase did not fall to the level in control rats that were not treated with DOCA-salt. This suggests a possible involvement of other kinin-degrading enzymes than CPY-like kininase at the developmental hypertensive stage of DOCA-salt rats used here, since neutral endopeptidase has also been shown to have some contribution to a degradation of kinin in rat kidney (Majima *et al*., 1995b). Otherwise, other factors, e.g. a central mechanism such as increased arginine vasopressin, may sustain the hypertension. The present study was completed in the

relatively early phase of DOCA-salt hypertension. Further assessment of the antisense ODN in the chronic phase of the model would be also important.

There have been many studies in which antisense ODNs were shown to suppress the expression of various mammalian genes *in vivo* (Stein & Cheng, 1993). It is of note that successful inhibition of the progression of tumour growth by antisense ODNs was reported in an animal cancer model (Kitajima *et al*., 1992). A major problem with antisense ODN was their inability to cross cellular membranes and accumulate effectively in the nuclei. The half-life of phosphorothioated oligonucleotides alone is generally known to be around 1 day. An efficiency of the incorporation into cultured cells and tissues is less. In this experiment, we took the *in vivo* introduction of CPY-antisense ODN by HVJ–liposome method, because the method has been reported to be highly efficient and little toxicity in many tissues such as kidney (Tomita *et al*., 1992). The reason for the high efficiency involves the entrapment of ODN or DNA within liposomes and an enhancement of liposomes to fuse to cell membrane. HVJ-liposome-mediated gene transfer has been shown to increase efficiency in delivering antisense ODN into the target cells and to sustain the stability of the transfected ODNs as compared with conventional methods using cationic liposomes (Morishita *et al*., 1994). We previously reported that effects of antisense ODNs introduced by the method is longer than ODN alone and can be continued until 4–6 days (Tomita *et al*., 1992; 1995a,b). We measured the blood pressure (BP) until 6 days transfection in the CPY-antisense treated group in the present study. Although the blood pressure was increased in the antisense treated group at the sixth day, a significant difference between the antisense ODN and the randomized ODN was still observed, suggesting a higher efficiency in the transfection.

In conclusion, the selective *in vivo* transfection of antisense ODNs against rat CPY homologue partly prevents the development of hypertension in DOCA-salt treated rats with marked inhibition of urinary CPY-like kininase. Inhibition of kininase in urine by antisense ODNs would provide further evidence of the pivotal suppressive roles of the renal kallikrein kinin system during the developmental stage of hypertension.

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