Failure of the Oxytocin-Induced Increase in Secretion of Urinary Kallikrein in Young Spontaneously Hypertensive Rats

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ABSTRACT—Urinary kallikrein excretion during oxytocin (OT) infusion were studied in anesthetized (sodium pentobarbital, 50 mg/kg, i.p.) young (4-weeks-old) spontaneously hypertensive rats (SHR) and Wistar Kyoto rats (WKY). OT-infusion (30 nmol/kg/30 min) to WKY significantly increased urinary excretion of the active kallikrein from the basal levels (25.4±5.6 10^{-2} x AU/15 min, n=5) to 37.3±5.0 10^{-2} x AU/15 min (P<0.05, n=5) and 50.7±17.1 10^{-2} x AU/15 min (P<0.05, n=5) 15 and 30 min after the start of OT-infusion, respectively. In SHR, OT-infusion did not increase the urinary excretion of active kallikrein, but did decrease the urine volume and sodium excretion. The concentration of the active kallikrein in the kidney of WKY was not changed by OT-infusion, but that of SHR was slightly increased. The OT-infusion resulted in significantly higher concentrations of the active kallikrein in SHR kidney than in WKY kidney. These results suggest that less excretion of urinary kallikrein in SHR during OT-infusion may be attributable to a lower response in the secretion of kallikrein from the kidney.

Keywords: Renal kallikrein, Spontaneously hypertensive rat, Oxytocin, Diuresis, Natriuresis
OT-induced natriuresis was mediated by this increased urinary kallikrein (15).

The present experiments were performed to test whether kallikrein levels are lower in SHR than in WKY, when the excretion is stimulated by infusion of OT and report that the increased secretion of urinary kallikrein stimulated by OT in SHR was lower than that in WKY, whereas the concentrations of active kallikrein contained in the kidney tissue of SHR was more than those in WKY after OT stimulation, suggesting that the lower excretion of urinary kallikrein may be attributable to lower responses in secretion of renal kallikrein in the kidney.

MATERIALS AND METHODS

Animals

Male SHR and male normotensive WKY (specific pathogen-free, 4-week-old; Hoshino-Sankyo Lab., Service, Saitama) were used. All rats were given normal rat chow (NMF; Oriental Yeast Corp., Tokyo) and tap water ad libitum immediately after weaning and were housed at constant humidity (60±5%) and temperature (25±1°C) and kept on a continuous 12-hr light/12-hr dark cycle (16). The number of animals used for each experiment are as described in the corresponding sections.

This study was performed in accordance with the guidelines for animal experiments of Kitasato University School of Medicine.

Preparation of infusion experiments

Four-week-old rats were anesthetized initially with sodium pentobarbital (Nembutal; Abbott Lab., North Chicago, IL, USA) (50-70 mg/kg, i.p.). Tracheostomy was performed, and a polyethylene cannula (PE-100; Clay Adams, Persippany, NJ, USA) was inserted into the trachea. The right femoral vein was cannulated with a polyethylene cannula (PE-l0, Clay Adams) through a small abdominal skin incision. The exposed area and the cannula exteriorized for collection of urine was then covered with plastic wrap (Saran Wrap; Asahi Chemical Industries, Tokyo) for prevention of insensible perspiration from the exteriorized for collection of urine was then covered with plastic wrap (Saran Wrap; Asahi Chemical Industries, Tokyo) for prevention of insensible perspiration from the

Urine volume was measured by weight, which was converted to volume by taking its specific gravity as 1. After centrifugation at 1,500 x g for 15 min at 4°C to remove solid debris, the supernatants were repeated at 15-min intervals sequentially (time 0 min and time 45 min). Physiological saline (0.9% (W/V) NaCl) solution was switched to 0.9% NaCl solution containing oxytocin (OT; Peptide Institute, Minoh, Osaka), which was infused (30 nmol/kg/30 min) for 30 min, from time 15 min to time 45 min (15).

To test the effects of a bradykinin B2-receptor antagonist, Hoe 140 (D-Arg[Hyp3,Thi5,D-Tic',Oic8]BK) (a gift from Hoechst AG, Frankfurt, Germany) was administered to another group of animals by infusion (6 ml/kg/hr) of 0.9% NaCl solution and OT solution (in 0.9% NaCl), both of which contained Hoe 140 at the final concentration of 0.5 mg/ml. The first solution (Hoe 140 solution) was infused for a 15-min period, from time 0 min to time 15 min, and the second solution (Hoe 140+OT solution) was infused for a 30-min period, from time 15 to time 45 min without the interruption of the infusion when the solutions were changed (Fig. 3). In this group, urine samples were also collected at 15-min intervals for 45 min to determine the urine volume and the urinary excretions of sodium and potassium. For the saline control group of animals, 0.9% NaCl solution and OT solution (in 0.9% NaCl) were infused (6 ml/kg/hr) as described above.

Measurement of urine volume, urinary sodium, potassium and creatinine

Urine volume was measured by weight, which was converted to volume by taking its specific gravity as 1. After centrifugation at 1,500 x g for 15 min at 4°C to remove solid debris, the supernatants were taken, and their urinary sodium and potassium levels were measured by the ion-selective electrode (Fuji Dri-Chem Slide Na-K-Cl; Fuji Dri-Chem 800V; Fuji Film Co., Ltd., Tokyo) after dilution (fivefold) with distilled water (15). Excreted electrolytes were expressed in nmol per 15 min.

After tenfold dilution of the supernatants with distilled water, the urinary creatinine levels were determined by the Jaffe's reaction (Creatinine-Test Wako; Wako Pure Chemical Industries, Ltd., Osaka), and they were also
expressed in micrograms per 15 min (15).

**Measurement of serum creatinine levels**
Immediately after the urine collection, blood (0.5 ml) was collected through a cannula inserted into the femoral artery into the glass tube, without anticoagulant, left at room temperature for 2 hr, and then centrifuged at 1,500 x g for 15 min at 25°C. From another group of rats (4-weeks-old) without OT-infusion, blood samples were also collected under anesthesia to prepare the sera. The creatinine levels of the serum samples were determined by the same method as used for urinary creatinine.

**Measurement of urinary kallikrein**
The activity of the active kallikrein in urine collected over 24 hr was measured using a peptidyl fluorogenic substrate selective for glandular kallikrein, Pro-Phe-Arg-methyl-coumarinylamide (Peptide Institute) (17, 18). After a tenfold dilution of the original urine with 0.2 M Tris-HCl buffer (pH 7.8), 10 μl of the diluted urine was incubated with 1 ml of 5 x 10^{-5} M substrate solution in 0.05 M Tris-HCl buffer containing 0.1 M NaCl and 0.01 M CaCl₂ (pH 8.0). One arbitrary unit (AU) was defined as the amount of urinary kallikrein that released 1 x 10^{-10} M 7-amino-4-methyl-coumarin (Peptide Institute) for 10 min/μl urine at 37°C. Kallikrein activity excreted over 24 hr was determined in the present experiment. Active kallikrein activity was calculated as the difference between amidase activity in the presence of soy bean trypsin inhibitor (SBTI, 0.5 μg/μl urine; Worthington Biochem., Corp., Halls Mill Road, NJ, USA) and that in the presence of aprotinin (0.5 μg/μl urine, Wako Pure Chemical Industries, Ltd.). This determination method may exclude the contamination of proteases other than kallikrein in the urine (15).

**Measurement of the concentration of kallikrein in the kidney tissues**
Immediately after terminating OT-infusion (time 45 min), the kidney was taken out and then homogenized on ice with Tris-HCl buffers (0.2 M, pH 8.5, containing 10 mM of benzamidine), which was 4 vol. (ml) per gram of wet kidney tissue. The supernatant after the centrifugation (1,500 × g, 4°C, 30 min) was used as a sample solution of a specific urinary kallikrein enzyme-linked immunosorbent assay (ELISA), which was selective to the active kallikrein (19). The kidney before OT-infusion (time 15 min) was also removed to determine the concentrations of kallikrein before OT administration.

The rat urinary kallikrein was purified 25,500-fold from 7.6 liters of urine from Wistar rats by a modification of the prokallikrein purification method reported by Takaoka et al. (20). Antibody for rat urinary kallikrein was raised in rabbits by immunization following the described procedures for human urinary kallikrein (21). With this antibody, Western blot analysis (22) of the kidney homogenate of SD strain rats (8-weeks-old, male) revealed a single positive band with an apparent molecular weight of approximately 41 kDa (Fig. 1). In the cases of SHR and WKY, Western blot analysis of kidney homogenate (4-weeks-old, male) also showed a single positive band with the same apparent molecular weight. This antibody specific for rat renal kallikrein was used in the following ELISA and immunohistochemical study.

A sandwich ELISA was developed following the previous report (19) with some modifications. The modifications were as follows: 1) the labeling enzyme was changed to β-galactosidase from horseradish peroxidase, 2) the substrate and the enzyme reaction terminator were changed to β-galactoside nitropurpure and NaOH from o-phenylenediamine+hydrogen peroxide and HCl solution, respectively.

The sensitivity of this ELISA system was approximately 0.05 ng/well, and a good linear relationship was obtained up to 3 ng/well. The above-mentioned supernatant of the kidney homogenate was diluted from 100- to 400-

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Fig. 1. Western blot analysis of kallikrein in kidney homogenates. Marker proteins: a, phosphorylase B (106 kDa); b, bovine serum albumin (80 kDa); c, ovalbumin (49.5 kDa); d, carbonic anhydrase (32.5 kDa); e, soy bean trypsin inhibitor (27 kDa); f, lysozyme (17 kDa).
fold, and then an aliquot of the diluted sample was analyzed by ELISA. In comparison with the standard curve, which was obtained by using known mounts of purified urinary kallikrein, the concentrations of the active kallikrein in the kidney were determined. The recovery of a known amount of purified rat urinary kallikrein added to the diluted (400-fold) kidney homogenate was 78±7% (n=3).

For determination of prokallikrein, 1 ml of the above supernatant was incubated with 1 ml of trypsin (Worthington Biochem., Corp.) solution (1 mg/ml, in 0.1 M Tris-HCl buffer containing 2 mM CaCl₂, pH 8.0) at 30°C for 20 hr. Then 1 ml of SBTI solution (3 mg/ml, in physiological saline) was added, and the amount of the active kallikrein that was converted from prokallikrein by trypsin in the incubation mixture was determined (19).

**Immunohistochemical studies**

Immunohistochemical studies were performed according to the previous report (23) on the kidneys isolated from rats before the infusion of oxytocin (at 15 min). Briefly, the kidneys were immediately fixed with 4% paraformaldehyde in 0.1 M phosphate buffer solution (pH 7.4). After fixation, the kidneys were dehydrated with a graded series of ethanol solutions and then embedded in paraffin. The sections (3 μm) from the paraffin-embedded kidney were mounted on the glass slides and then deparaffinized with xylene and placed in cold (4°C) acetone for immunostaining.

Steps in the staining of dehydrated sections using a Vectastain ABC Kit (Vector Lab., Burlingame, CA, USA) were as follows: 1) incubation with diluted normal horse serum, 2) incubation with anti-rat renal kallikrein serum (10 μg/ml), 3) incubation with biotinylated anti-rabbit IgG (7 μg/ml), 4) incubation with avidin-biotin peroxidase complex, 5) placement in 0.02% 3,3'-diaminobenzidine (DAB) and 0.3% nickel ammonium sulfate in 50 mM Tris-HCl buffer (pH 7.4), 6) color development by immersion in the DAB solution containing 0.005% H₂O₂, 7) examination and photography with a light microscope.

**Statistical analyses**

Values are expressed as means±S.E.M.

For the results from multiple groups, factorial ANOVA, followed by Sheffe’s test, or repeated ANOVA was used.

A P value less than 0.05 was considered to be significant.

**RESULTS**

Changes in urine volume and urinary excretions of kallikrein, sodium and potassium in oxytocin-infused rats

Urine volume 15 min after the start of the experiment (time 15 min, the basal value) in WKY was 94.8±27.4 μl/15 min (n=5). As shown in Fig. 2A, OT-infusion (30 nmol/kg/30 min) significantly increased urine volume 15 and 30 min after the start of OT-infusion (time 30 and 45 min) to 213.5±52.2 μl/15 min (P<0.05, n=5) and 292.4±121.1 μl/15 min (P<0.01, n=5), respectively. The increase in the urine volume at time 45 min was 308% of the basal value. In SHR, the increase in the urine volume by OT-infusion was small, and the difference between the basal value (45.6±14.4 μl/15 min, n=5) and the OT-infused value (time 30 min, 72.8±27.6 μl/15 min, n=5; time 45 min, 131.9±43.1 μl/15 min, n=5) was not statistically significant.

Urinary excretion of sodium 15 min after the start of the experiments (time 15 min, the basal value) in WKY was 5.1±1.5 pmol/15 min (n=5), and OT-infusion (30 nmol/kg/30 min) significantly increased sodium excretion at 15 and 30 min after the start of OT-infusion (time 30 and 45 min) to 20.1±5.9 pmol/15 min (P<0.05, n=5) and 44.8±6.2 pmol/15 min (P<0.01, n=5), respectively (Fig. 2B). The basal sodium excretion in SHR was 1.9±0.5 pmol/15 min (n=5). Although the increased level (16.4±4.5 pmol/15 min, n=5) became significant (P<0.05) at time 45 min, the increase by OT-infusion in SHR was small, compared with those in WKY. The difference between SHR and WKY was statistically significant (P<0.05) at time 45 min (Fig. 2B).

Urinary excretion of potassium tended to be increased slightly by OT-infusion in both SHR and WKY, but the values in SHR was kept lower throughout the experiment, compared with those in WKY. The difference between SHR and WKY was statistically significant at time 30 (P<0.01) and 45 min (P<0.05) (Fig. 2C).

The basal urinary excretions of active-kallikrein in WKY rats was 25.4±5.6 10⁻²×AU/15 min (n=5). As shown in Fig. 2D, OT-infusion significantly increased the active-kallikrein 15 and 30 min after the start of OT-infusion (at time 30 and time 45 min) to 37.3±5.0 10⁻²×AU/15 min (P<0.05, n=5) and 50.7±17.1 10⁻²×AU/15 min (P<0.05, n=5), respectively. However, SHR did not respond to this OT-infusion with any urinary excretion of active-kallikrein at all (at time 15 min, 21.2±10.9 10⁻²×AU/15 min (n=5); at time 45 min, 22.9±5.1 10⁻²×AU/15 min (n=5), although a slight, but not significant, increase was observed at time 30 min (27.5±11.7 10⁻²×AU/15 min, n=5) (Fig. 2D).

The MBP of WKY before OT-infusion (time 0 min) was 101±2 mmHg (n=4), which was not changed during
OT-infusion (102 ± 1 mmHg, n=4, at time 30 min). In the case of SHR, OT-infusion did not cause any significant changes in MBP (103 ± 3 mmHg, n=4, at time 0 min; 104±2 mmHg, n=4, at time 30 min).

Urinary creatinine excretions in WKY were kept constant throughout OT-infusion (75±17 μg/15 min, 70±10 μg/15 min and 68±5 μg/15 min, at time 15, 30 and 45 min, respectively; n=4). This was also true in SHR (50±18 μg/15 min, 49±12 μg/15 min and 44±6 μg/15 min, at time 15, 30 and 45 min, respectively; n=4). Infusion of OT did not induce any significant changes in serum creatinine levels in WKY (3.8±0.3 μg/ml, n=5, without treatment; 4.1±0.3 μg/ml, n=3, at time 45 min) and SHR (4.6±0.4 μg/ml, n=5, without treatment; 3.8±0.3 μg/ml, n=3, at time 45 min).

**Effects of Hoe 140 infusion on oxytocin-induced increases in urine volume and urinary excretions of sodium and potassium**

Continuous infusion of Hoe 140, a bradykinin B2 antagonist, caused significant reduction of the OT-induced increase in urine volume (at time 30 and time 45 min) in both SHR (by 93%) and WKY (70%), but the inhibitory effect was small in SHR (Fig. 3A). Almost the same results were obtained in the OT-induced natriuresis (Fig. 3B). However, Hoe 140 did not cause any changes in the potassium excretion under OT-infusion (Fig. 3C). The administration of Hoe 140 did not significantly change the MBP during OT-infusion (data not shown).
Kallikrein concentrations in the kidney before and after the oxytocin infusion

Concentrations of the immunoreactive active kallikrein in the kidney in WKY at time 15 min was 550±85 ng/g wet tissue (n=8), which was not changed after OT-infusion (at time 45 min, 565±51 ng/g wet tissue, n=8) (Fig. 4). In contrast, the concentration of kallikrein in the kidney in SHR tended to be increased by OT-infusion from 660±65 ng/g wet tissue (at time 15 min, n=8) to 768±33 ng/g wet tissue (at time 45 min, n=8). At time 45 min after OT-infusion, the concentration of kallikrein in SHR kidney was significantly (P<0.05) higher than that in WKY kidney.

Concentration of the prokallikrein in the kidney in WKY and SHR at time 15 were 831±104 ng/g wet tissue (n=4) and 1,360±224 ng/g wet tissue (n=4), respectively.

Immunohistochemical localization of kallikrein in kidneys

Rat urinary kallikrein immunostaining was observed over some cells of the connecting tubules (as indicated by arrows) and contrasted with the clear unstained background (Fig. 5). No immunostaining was observed in other segments of the nephron. The immunoreactive cells were intermingled with immunonegative cells corresponding to the intercalated cells. In SHR, before OT-infusion (Fig. 5, panel 2), the immunoreactive kallikrein was located throughout most of the cytoplasma. Even after OT-infusion, no apparent reduction or increase in the
positive staining was observed in SHR. In WKY, before OT-infusion (Fig. 5, panel 1), reduced positive staining in the connecting tubular cells was observed, but the OT-infusion did not cause any marked changes in positive staining as in SHR.

DISCUSSION

It is widely accepted that the source of urinary kallikrein is the connecting tubules of the kidney (24, 25). In the present immunohistochemical studies, we confirmed that the kallikrein is located solely in the connecting tubules (Fig. 5). Our previous report revealed less urinary excretion of kallikrein in young SHR (4–6 weeks of age) only before the blood pressure reached a plateau. This result on the urinary kallikrein activities was consistent with the previous reports by other researchers that SHR excrete less urinary kallikrein (4, 8–13). In the present experiment, sensitivity for secretion of kallikrein was tested using OT as a releaser of the kallikrein.

A low sodium diet or salt deprivation always accelerates kallikrein synthesis and excretion (3, 26–28), and the increased excretion of kallikrein was reported after aldosterone administration (29). Patients with hyperaldosteronism excrete higher amounts of kallikrein in urine (2, 27). The same phenomenon was seen in patients with Bartter’s syndrome (30). High potassium intake increased the excretion of kallikrein with hypertrophy and hyper-
plasia of the kallikrein-containing cells (31). Intravenous infusion of vasopressin was reported to stimulate both release of urinary kallikrein and intrarenal formation of kinin (32).

Among stimuli to increase the secretion of urinary kallikrein, we recently found that a pituitary hormone, OT, increased the excretion of kallikrein (15). This OT-increased kallikrein secretion was accompanied with increases in urinary excretion of sodium, potassium and chloride, among which the increases in urinary excretions of sodium and chloride were kinin-dependent (15). In the present experiment, the increase in the urinary excretion of kallikrein was negligible in SHR, compared with WKY (Fig. 2D). This absence of response of kallikrein excretion was accompanied by lower excretion of sodium, potassium and water (Fig. 2: A–C). Hoe 140 lessened water and sodium excretions (Fig. 3: A and B), but not potassium excretion (Fig. 3C), in both WKY and SHR, and the effect was more potent in WKY than in SHR (Fig. 3: A and B). These indicate the increased kallikrein has a role in the excretion of water and sodium, but not excretion of potassium. Thus, the lower excretion of kallikrein may be relevant to the lower excretions of water and sodium in SHR.

The SHR were reported to have a lower kallikrein secretion response during the reduction of renal perfusion pressure (8). In the present study, the systemic blood pressure was not increased throughout the infusion of OT at the dose used. Furthermore, the urinary creatinine excretion rates remained fairly constant during OT infusion in both SHR and WKY. Since after OT infusion, there was no marked changes in the serum creatinine concentration, it was plausible that filtration by the glomerulus may not be changed with OT infusion.

Kallikrein was determined in the kidney (10), and SHR contained more active kallikrein in the kidney from 0 to 12 weeks of age (10). Our antibody recognized the active form of rat urinary kallikrein, but not the pro-form, as reported previously (19). To determin prokallikrein using this antibody, trypsin treatment to convert the pro-form to the active form was necessary (19). The tendency for SHR to contain more immunoreactive kallikrein in the kidney than WKY was observed before OT-infusion, although the difference of the tissue concentration between SHR and WKY was not statistically significant (Fig. 4). However, after OT-infusion, the kallikrein concentrations in SHR was increased, and the difference became significant as a consequence of their failure to release kallikrein (Fig. 3D). Although the part of the kallikrein synthesized in the connecting tubules was secreted through the basolateral membrane, the main portion of the synthesized kallikrein was secreted into the lumen of the connecting tubules (24). In the present immuno-

h histochemical studies, the intense staining of kallikrein in the connective tubules in SHR may reflect this failure of secretion of kallikrein (Fig. 4). Since prokallikrein concentrations in the kidney of SHR tended to be a little higher than those of WKY, the possibility of the reduced conversion of prokallikrein to active kallikrein can not be excluded at present.

It is possible that the lack of response in the kallikrein excretion may be explained by the reduced number of OT receptors in the renal tissues of SHR. This possibility can not be ruled out in the present experiment, although the hypertensive responses of anesthetized rats to a high dose of OT (single i.v. injection, 0.03–10 μmol/kg) were not different between SHR and WKY (ED₅₀: 282±29 nmol/kg (n=4) and 250±28 nmol/kg (n=4), respectively) in separate experiment (M. Majima et al., data will be published separately). The sites of action of OT in the kidney tissues were reported to be the renal tubules (33–35), although some reported the specific [³H]OT binding to the glomerulus (36) and the macula densa (37).

The basal plasma OT concentrations in normal males do not differ from those in nonpregnant females and pregnant females before labor (15–42 weeks of gestation) (14). However, these levels were very low, compared with those used in the present experiment. Thus, the involvement of endogenous OT in the excretions of kallikrein, water and electrolytes may not be physiological.

In conclusion, the increased secretion of urinary kallikrein in SHR by OT was less than that in WKY, whereas the concentration of kallikrein contained in the kidney tissue of SHR was more than that in WKY after OT stimulation, suggesting that lower excretion of urinary kallikrein induced by OT may be attributable to a failure of the kallikrein secretion response in the kidney.

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