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A Comparative Study on the Measurement of Urinary Kallikrein in the Rat

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The present study was carried out to assess the correlations among the results of four different assays of urinary kallikrein in the rat. The peptidase, esterase, vasodilator and kinin-forming activities were employed for the determination of kallikrein activity. In the urine from normal rats, the assay of vasodilator activity correlated well with those of peptidase activity ($r=0.86$) and esterase activity ($r=0.89$). Similarly, a good correlation ($r=0.99$) was obtained between the peptidase and kinin-forming activities. However, in the case of rats under furosemide treatment, the esterase activity was poorly correlated with the peptidase and vasodilator activities, although a good correlation ($r=0.93$) was obtained between the latter two activities. These results indicate that the peptidase and vasodilator activities should be employed for the estimation of urinary kallikrein excretion in the urine from rats under furosemide treatment.

Keywords——rat urinary kallikrein; prolyl-phenylalanyl-arginine-4-methylcoumaryl-7-amide; peptidase activity; *N*-α-tosyl-L-arginine methyl ester; esterase activity; vasodilator activity; kininogen; kinin-forming activity; rat uterus; furosemide

Introduction

Urinary kallikrein has characteristics similar to those of renal kallikrein,[1,2] and is of special interest because of the postulated role of this enzyme system in diuresis and in certain forms of cardiovascular diseases.[3-6]

The level of kallikrein activity in the urine has been determined by assaying either proteolytic or esterolytic activity of the enzyme, and various techniques for the measurement of its activity have been reported in experimental animals and humans. Recently, a radioimmunoassay[7-10] has been developed as the most sensitive method, but it is laborious and difficult to prepare the antibody against bradykinin or kallikrein. Generally, arterial blood-flow measurement seems to be a reasonable and sensitive assay for urinary kallikrein on the basis of the physiological action of the enzyme.[11,12] On the other hand, a colorimetric method using synthetic aromatic esters of arginine is easy to perform and commonly used. However, the method of esterolytic assay lacks specificity and quantitative accuracy. In fact, it has been shown that *N*-α-tosyl L-arginine methyl ester (TAME) esterase activity is largely due to non-kallikrein esterase in human urine.[13] More recently, a fluorometric method using prolyl-phenylalanyl-arginine-4-methylcoumaryl-7-amide was shown to be specific and sensitive for the measurement of urinary kallikrein[14,15] and was applied to urine samples from various patients.[15] However, there is little available data on the determination of rat urinary kallikrein using this method. It has been reported that rat urine contains both kallikrein and non-kallikrein esterases.[16] Thus, it seems important to exactly estimate kallikrein excretion under various conditions.

The aim of the present study is to assess the correlations among the results of four different assays of urinary kallikrein, *i.e.*, peptidase, vasodilator, kinin-forming and esterolytic determinations under normal or diuretic conditions in the rat.

Materials and Methods

Materials——The materials obtained from commercial sources were: prolyl-phenylalanyl-arginine-4-methylcoumaryl-7-amide (Pro-Phe-Arg-MCA), 7-amino-4-methylcoumarine (AMC) and bradykinin from the

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Protein Research Foundation (Minoh, Japan); N-α-tosyl-L-arginine methyl ester (TAME) from Sigma Chemical Co. (Saint Louis, U.S.A.); chromotropic acid from Merck Co. (Darmstadt, Germany). Other chemicals used were of reagent grade. A purified hog pancreatic kallikrein (49 KU/mg protein), which was kindly provided by Dr. H. Ohde, Fujimoto Pharmaceutical Co. (Osaka, Japan), was used as a standard preparation of kallikrein.

Collection of Rat Urine——Male Wistar rats weighing 210—240 g were used. For 1 week before the study the rats were fed a standard laboratory chow, Oriental MF (Oriental Yeast Co., Tokyo, Japan), and provided with tap water *ad libitum.*

After being fasted for 16 h, the animals were given physiological saline equal to 2.5% of body weight *via* a stomach tube. In the experiments using the diuretic, each rat was intraperitoneally given furosemide at a dose of 1, 5, or 25 mg/kg immediately after loading of physiological saline. All rats were housed individually in metabolic cages to collect urine samples for 4 h. Rats were given water but not food during the collection. The urine was centrifuged to remove solid debris, and analyzed for sodium and potassium with a Hitachi model 204-P flame photometer. Urine samples for the measurement of kallikrein were dialyzed against distilled water at 4°C for 24 h, and stored at −20°C.

Assay of Pro-Phe-Arg-MCA Peptidase Activity[15]——One milliliter of 0.1 mM Pro-Phe-Arg-MCA in 0.05 M Tris-HCl buffer (pH 8.0), containing 100 mM NaCl and 10 mM $CaCl_2$, was preincubated at 37°C for 2.5 min, then 20 μl of enzyme sample was added. After incubation at 37°C for 10 min, the enzyme reaction was stopped by the addition of 1.5 ml of 17% acetic acid. The amount of AMC liberated from the substrate was measured with excitation at 380 nm and emission at 460 nm using a Hitachi model 650-40 fluorescence spectrophotometer connected to a Hitachi 056 recorder. The instrument was standardized so that a 10 μM solution of AMC in Tris-HCl buffer (pH 8.0) and acetic acid gave 1.0 relative fluorescence. The activity was expressed in terms of peptidase units (U) equivalent to nmoles of Pro-Phe-Arg-MCA hydrolyzed per min during incubation at 37°C.

Assay of TAME Esterase Activity[11]——A mixture of 0.1 ml each of 0.1 M phosphate buffer (pH 8.0), 0.1 M TAME and test samples was incubated at 30°C for 30 min. The enzyme reaction was stopped by the addition of 0.2 ml of 15% trichloroacetic acid, then 0.1 ml of 2% $KMnO_4$ was added to oxidize the methanol generated enzymatically into formaldehyde. After exactly 1 min, 0.1 ml of 10% $NaHSO_3$ and 4.0 ml of 0.4% chromotropic acid, dissolved in 67% H_2SO_4, were added to stop the oxidation and to decolorize the solution. The reaction tubes were placed in a boiling water bath for 15 min then cooled to room temperature. The absorbance at 580 nm was measured with a Hitachi model 100-10 spectrophotometer. The amount of methanol liberated from the substrate was estimated from the calibration line for methanol (0—10 μmol/ml) by the same procedure. The activity was expressed in terms of esterase units (EU) equivalent to μmoles of TAME hydrolyzed per min during incubation at 30°C.

Assay of Vasodilator Activity——Mongrel dogs of either sex weighing 10 to 15 kg were anesthetized with sodium pentobarbital (30 mg/kg, *i.v.*). The left femoral artery was exposed through an incision, and the femoral blood-flow was measured with an electromagnetic flowmeter (MF-25, Nihon Koden) connected to a recorder (U-626, Nihon Denki Kagaku). An arterial cannula was inserted into the branch of femoral artery proximal to the flow probe for the injection of samples.

Urine samples were dialyzed against distilled water at 4°C for 20 h then diluted with sterilized saline. The femoral blood-flow was recorded for 2 min following the injection of 0.5 ml of diluted sample. The quantity of kallikrein was estimated from the calibration line obtained with 0.5 ml aliquots of standard kallikrein solutions in the range of 0.02 to 0.08 KU/ml. The activity was expressed in terms of kallikrein units (KU) equivalent to the standard kallikrein used.

Preparation of Dog Kininogen——Mongrel dogs of either sex were anesthetized with sodium pentobarbital (30 mg/kg, *i.v.*) and blood samples were collected without hemolysis from the femoral artery cannula into syringes with 15% disodium ethylenediaminetetraacetic acid (EDTA) or heparin solution. The blood samples were immediately cooled in ice and centrifuged at 10000 rpm for 20 min at 4°C. The plasma was heated at 60°C for 1 h, and centrifuged at 10000 rpm for 10 min at 0°C. The supernatant fraction was mixed with the same volume of saturated ammonium sulfate solution, and kept at 4°C for 24 h. After centrifugation at 10000 rpm for 10 min, the precipitate was dissolved in a cold physiological saline (half the volume of the original plasma) and the solution was dialyzed against distilled water for 48 h. The dialyzates were centrifuged, then the supernatant fraction was lyophilized and stored at −20°C until used. One milligram of the kininogen yielded 510 ng of bradykinin, when incubated with excess of trypsin (bovine Type III, Sigma).

Assay of Kinin-Forming Activity——The uterus was isolated from rats pretreated with estradiol (100 μg/animals, *s.c.*) 36 h before study, and suspended in a 20 ml bath of air-saturated Ringer–Locke solution. One-fifth milliliter of diluted dog kininogen solution was added to the bath, followed after 2 min by 0.2 ml of dialyzed urine sample. Uterine contractions for 2 min were recorded on a Nihon Denki Kagaku model U-626 recorder using an isotonic transducer (TD-112s, Nihon Koden). Synthetic bradykinin was used as the standard in the bioassay. The dog kininogen had no kininase activity and no contractile effect upon the uterus in the absence of diluted urine sample. Diluted urine sample did not cause the direct contraction of the rat uterus in the absence of dog kininogen when used at dose levels appropriate to the assay. The activity was expressed as ng of bradykinin equivalent.

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Results and Discussion

In the present study, we examined the correlations among the results of four different techniques for the estimation of urinary kallikrein excretion in the rat. As shown in Fig. 1, the assay of vasodilator activity correlated well with the assays of peptidase activity (r=0.86, p<0.001) and esterase activity (r=0.89, p<0.001) in the urine from normal rats. The measurement of arterial blood-flow increases in peripheral vasculature, which reflects the biological activity of kallikrein, has been used as a sensitive assay method. It was found that the peptidase assay using Pro-Phe-Arg-MCA could be applied to the determination of urinary kallikrein in rats as well as in humans.[15] This fluorometric method was easy to perform without using experimental animals and kininogen, and was much more sensitive than the assays of esterase and vasodilator activities. In addition, a good correlation (r= 0.99, p<0.001) was obtained between peptidase and kinin-forming activities (Fig. 2).

It has been reported that kallikrein excretion correlates directly with urine volume or urinary sodium excretion in humans and animals.[17,18] On the other hand, the presence of

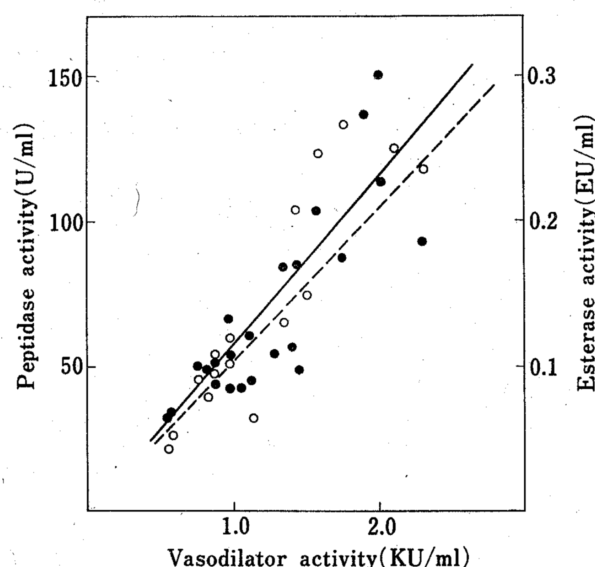

Fig. 1. Correlation of Vasodilator Activity with Peptidase and Esterase Activities

The three activities were measured in the dialyzed urine of normal rats. ●—●: correlation between vasodilator and peptidase activities. ○--○: correlation between vasodilator and esterase activities. For exprimental details, see the text.

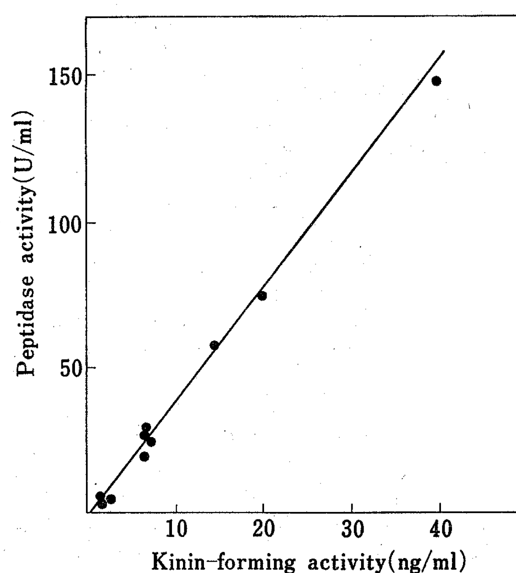

Fig. 2. Correlation between Kinin-Forming and Peptidase Activities in the Urine of Normal Rats

The two activities were measured in the dialyzed urine of normal rats.

TABLE I. Effect of Furosemide on the Urine Volume and Urinary Excretions of Sodium and Potassium

	Urine flow (ml/100 g/4 h)	$U_{Na}V$ (μeq/100g/4 h)	U_KV (μeq/100 g/4 h)
Control	1.91±0.19	239±31.6	69.5± 5.48
Furosemide			
1 mg/kg	2.71±0.29[a]	388±55.7[a]	97.7±10.0[a]
5 mg/kg	5.61±0.26[b]	683±21.4[b]	134.4± 6.36[b]
25 mg/kg	6.71±0.25[b]	856±31.8[b]	161.4±10.0[b]

All values are means±S.E. of 6 experiments. *a*) Values are significantly different from the control (p<0.05). *b*) Values are significantly different from the control (p<0.001). Abbreviations: $U_{Na}V$=urinary excretion of sodium, U_KV=urinary excretion of potassium.

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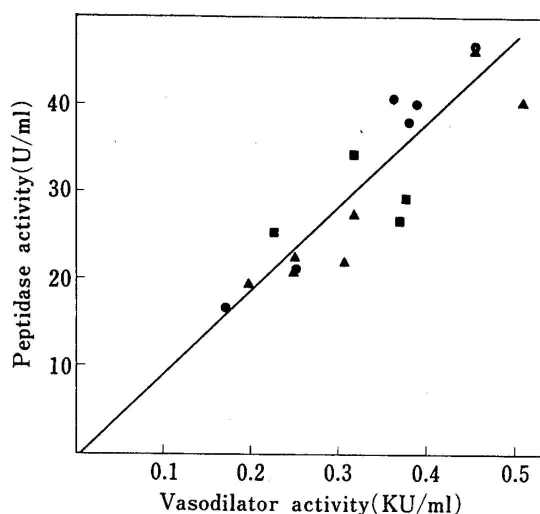

Fig. 3. Correlation between Vasodilator and Peptidase Activities in the Urine of Rats treated with Furosemide

The two activities were measured in the dialyzed urine of rats treated with furosemide at doses of 1 mg/kg (●), 5 mg/kg (▲) and 25 mg/kg (■).

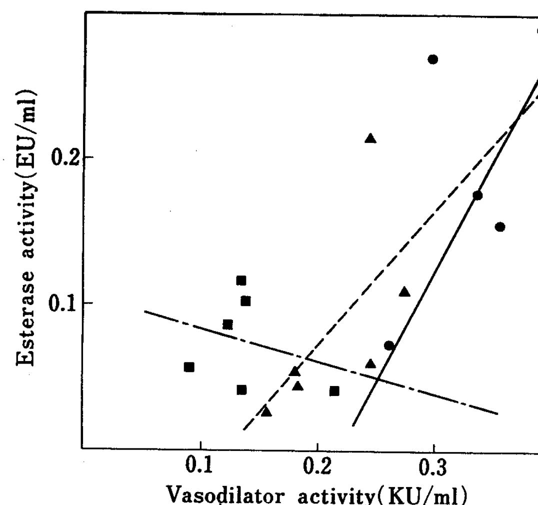

Fig. 4. Correlation between Vasodilator Activity and Esterase Activity in the Urine of Rats treated with Furosemide

The two activities were measured in the dialyzed urine of rats treated with furosemide at doses of 1 mg/kg (●—●, $r=0.92$, $p>0.1$), 5 mg/kg (▲---▲, $r=0.63$, $p>0.5$) and 25 mg/kg (■—■, $r=0.28$, $p>0.5$).

non-kallikrein esterase was observed in the rat urine. Therefore, there is a possibility that the esterolytic assay is not suitable for the quantitative determination of urinary kallikrein activity in the rat. We examined the validity of TAME esterase assay in comparison with the assays of vasodilator and peptidase activities under diuretic conditions.

Table I shows the diuretic effect of furosemide, a high-ceiling diuretic. This agent caused significant increases in urine volume and in urinary excretions of sodium and potassium in the rat. Although the vasodilator activity was well correlated with the peptidase activity ($r=0.93$, $p<0.001$) in the urine under diuretic conditions (Fig. 3), it showed a poor correlation with the TAME esterase activity (Fig. 4). The peptidase activity was also poorly correlated with the TAME esterase activity (Fig. 5). The correlations in the latter two cases became extremely poor and appeared to change in nature when furosemide was administered at a dose of 25 mg/kg (see Figs. 4 and 5). These findings suggest that some changes in urinary excretions of kallikrein and non-kallikrein esterase occur in rats after administration of diuretics. Further experiments are required to clarify the mechanism involved.

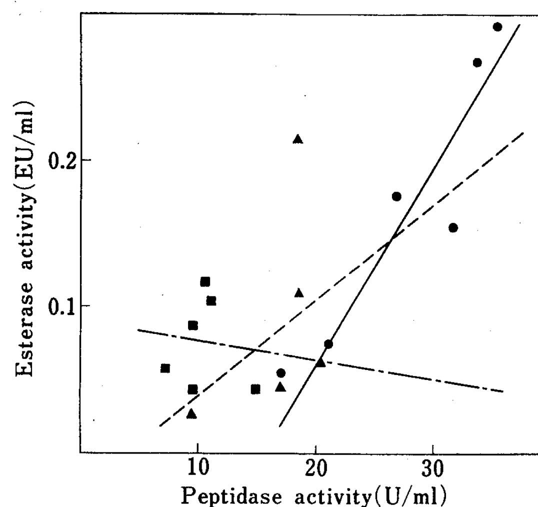

Fig. 5. Correlation between Esterase Activity and Peptidase Activity in the Urine of Rats treated with Furosemide

The two activities were measured in the dialyzed urine of rats treated with furosemide at doses of 1 mg/kg (●—●, $r=0.92$, $p>0.1$), 5 mg/kg (▲---▲, $r=0.39$, $p>0.8$) and 25 mg/kg (■—■, $r=0.10$, $p>0.8$).

In conclusion, under diuretic conditions in the rat, it is necessary to assess the urinary kallikrein excretion by means of the peptidase or vasodilator assay rather than by the esterase assay.

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