Release of Activatable Kallikrein by Isolated Rat Kidneys

JENNY CORTHORN, LIC. BIO, AND JUAN ROBLERO, PH.D.

SUMMARY Rat isolated kidneys were perfused for 60 minutes with a modified Krebs-Henseleit bicarbonate solution. Perfusate and urine samples showed klinogenase activity (active kallikrein) which could be enhanced by activation with trypsin (activatable kallikrein). Identification of the klinogenase activity generated by trypsin was made with rat renal kallikrein antiserum, aprotinin, lima bean trypsin inhibitor (LBTI), soybean trypsin inhibitor (SBTI), and oromucoid. A sample of perfusate was partially purified through DEAE-Sephael chromatography. Intraarterial injection of this fraction decreased blood pressure in the perfused hind limb of a rat. (Hypertension 3 (suppl II): II-39-II-41, 1981)

KEY WORDS rat kidney • trypsin activatable kallikrein • prokallikrein • renal activatable kallikrein

KALLIKREINS are serine proteases that release kinins from kinogen. Glandular kallikreins are found in the exocrine glands, and their secretions, in the kidney, and in urine. Renal kallikrein is involved in the regulation of blood flow, sodium and water excretion, and blood pressure, as well as in the pathogenesis of experimental and clinical hypertension. Experiments performed in our laboratory have shown that isolated rat kidneys, perfused with a saline solution free of kallikrein or its precursor, release kallikrein into the perfusate and urine, suggesting that renal kallikrein is released into the blood circulation where it may exert a systemic action. Recently, it has also been shown that human urine contains prokallikrein, which may be of renal origin. All these facts have led us to study the release of an activatable form of kallikrein by the perfused kidney.

Materials and Methods

Sprague-Dawley male rats (220-300 g b.w.) were anesthetized with sodium-pentobarbital (40 mg/kg i.p.). Renal circulation was isolated by exposing the inferior vena cava, abdominal aorta, and superior mesenteric artery. All the small blood vessels of the kidney area were tied off. The right renal artery was cannulated by introducing a PE-50 tubing into the superior mesentric artery advancing into the renal artery, and tying off the abdominal aorta. The inferior vena cava was cannulated with PE-240 tubing so that the tip of the cannula reached the right renal vein, to collect the renal venous outflow. The ureter was cannulated with PE-10.

The kidneys were perfused with pulsatile pressure at a constant flow rate which was set and maintained by means of a Roller Flex Pump. Perfusion pressure was measured by a strain-gauge manometer (Statham P23) and recorded on a Grass polygraph.

The perfusion system was a single-pass circuit to which a Millipore filter of 0.8 μm was inserted in line. A pH electrode was placed in the reservoir where the perfusion fluid equilibrated with the gas mixture, thus allowing a constant checking of pH (7.40-7.45). The perfusion fluid was a modified bicarbonate Krebs-Henseleit solution: 120 mM NaCl, 20 mM NaHCO₃, 5 mM KCl, 0.4 mM KH₂PO₄, 1 mM CaCl₂, 1 mM MgSO₄, 3 mM Na-pyruvate, 5 mM urea, 15 mM glucose, 0.7 mM creatinine, 4.7% Ficoll-70 (Pharmacia Fine Chemicals), and 0.3% bovine albumin fraction V (Calbiochem Company). This solution was passed through a Millipore filter of 0.45 μm and equilibrated with an O₂/CO₂ mixture (95%/5%) for 30 minutes, and through the experiment. All kidneys were perfused for 10 to 15 minutes (washing period) until the perfusate was completely free of blood. The glomerular filtration rate (GFR) was determined as creatinine clearance, and analyzed using a Wiener Lab Kit.

The percent of sodium reabsorbed by the isolated kidney was calculated from values of GFR, perfusate and urine sodium concentration. Sodium concentrations were measured by an Eppendorf flame photometer.

Determination of Active, Activatable, and Total Kallikrein

Total kallikrein equals active plus activatable kallikrein. Total kallikrein was determined after activation with trypsin in perfusate and urine samples. Active kallikrein was determined in the absence of trypsin. Kinin-generating activities in urine and perfusate samples were determined by bioassay in the rat uterus. A fraction of dog plasma obtained by precipitation with 25% to 45% saturated ammonium sulfate
was used as substrate. The lyophilized material was dissolved in buffer 0.05 M Na phosphate, 3 mM EDTA, 1 mM phenanthroline, pH 7.4 buffer PEP, its concentration was expressed as the amount of kinins formed when incubated with an excess of trypsin and rat urine.

Samples of perfusate (2 ml) of urine (1-5 μl) brought to a volume of 2 ml with PEP buffer were incubated with 5 μl of trypsin (1 mg/ml) for 15 minutes. Then, 10 μl of ovomucoid (4 mg/ml), a trypsin inhibitor, was added, and the samples were again incubated for 15 minutes. Finally, 1000 ng bradykinin (BK) equivalents of kininogen in 0.1 ml PEP buffer was added, and incubation was continued for 30 minutes. Incubation temperature was 37°C. The reaction was stopped by placing the tubes in a boiling water bath for 10 minutes. Control tubes were simultaneously run, consisting of buffer or fresh perfusion fluid instead of urine and perfusate. Kininogenase activity was expressed as μg BK equivalents generated in 30 minutes.

Identification of the Activatable Kininogenase Activity as Kallikrein

Before being mixed with the kininogen substrate, the following protease inhibitors were incubated with activated samples of perfusate and urine: aprotinin, in doses of 100 to 500 KIU/ml (Trasylol, Bayer);① soybean trypsin inhibitor, lima bean trypsin inhibitor, and ovomucoid, in doses of 20 to 100 μg/ml (Sigma Chemical Company).② Renal kallikrein antiserum was used for inhibiting kininogenase activity. Rabbit antiserum, developed against purified rat renal kallikrein,③ was incubated with activated samples of perfusate and urine during 4 hours at room temperature. Controls were run with normal rabbit serum.

Partial purification was carried out with 400 ml of perfusate from one kidney, which showed a large proportion of activatable kallikrein (11 μg BK eq of total kallikrein and 1 μg BK eq of active kallikrein). The perfusate was applied on a diethylaminoethyl (DEAE)-sephacel column (9 X 5 cm, Pharmacia Fine Chemical Company) equilibrated with 0.1 M Na phosphate pH 6.0. The trypsin-activatable kallikrein was eluted with 0.35 M Na phosphate pH 6.0. The active fraction was pooled and concentrated in dialysis tubing against N,a pressure. Kininogenase and esterase activity of active and total kallikrein were determined in the fraction thus obtained.

Esterase activity of active and total kallikrein was determined by the radiochemical esterolytic method, using 3H-TAME.④ Purified rat urinary kallikrein was used as standard. One TAME unit (TU) is defined as the amount of enzyme that hydrolyzes 1 μmole of TAME per minute at pH 8.0 at 30°C.

The partially purified fraction was injected intravenously into the perfused hindlimb of an anesthetized rat. Perfusion was made at a constant rate with autologous blood.⑤ The hypotensive effect of this fraction was compared to that produced by bradykinin and active urinary kallikrein purified through DEAE-cellulose.

Results

Ten kidneys were perfused for 60 minutes in a single pass system with a systolic perfusion pressure of 114 ± 12 mm Hg, (mean ± se). The perfusion flow rate was 15 ± 0.2 ml/min, and the resulting urine flow was 18 ± 9 μl/min. The mean value for a GFR of 0.77 ± 0.07 ml/min and for sodium reabsorption of 91% indicates an adequate functional state of the isolated perfused kidney. The kininogenase activity of perfusate and urine was increased by trypsin activation. The total amount of kininogenase activity released into the perfusate, after 60 minutes perfusion, was 4.6 ± 0.7 μg BK eq, (mean ± se) of active kallikrein and 10.3 ± 2.1 μg BK eq of total kallikrein, where 55% of the activity corresponds to activatable kallikrein evidenced by trypsin activation. The amount released into the urine was: 11.0 ± 1.2 μg BK eq of active kallikrein and 19.1 ± 5.4 μg BK eq of total kallikrein, where 42.5% of the activity corresponds to activatable kallikrein (fig. 1).

The kininogenase activity generated by trypsin in samples of perfusate and urine was inhibited by aprotinin and rat renal kallikrein antiserum and partially by soybean trypsin inhibitor (SBTI), whereas no effect was found for lima bean trypsin inhibitor (LBTI) and ovomucoid (table 1). Therefore, the enzymatic activity observed was identified as renal kallikrein. Figure 2 shows the DEAE-sephacel chromatography of a perfusate sample. Active kallikrein was not detected although it has been shown to elute in the same fractions as activatable kallikrein.

The active material was concentrated to 0.5 ml showing a kininogenase activity of 0.49 μg BK eq/ml active kallikrein and 1.15 μg BK eq/ml total kallikrein. The esterase activity of this fraction was 9.57 m TAME units (TU)/ml active kallikrein and 50.40 m TU/ml total kallikrein.

The intraarterial injection of this fraction into the perfused hindlimb of a rat caused a hypotensive effect like that produced by bradykinin and rat urinary active kallikrein (1.78 μg BK eq/ml; 25 m TU/ml) (fig. 3).

![Figure 1. Mean kininogenase activity of active and total kallikrein released into the perfusate, and urine after 60 minutes of kidney perfusion. Vertical lines indicate ± se; n = 10 AK = active kallikrein; TK = total kallikrein](file)
The present results show that isolated and perfused rat kidneys release an activatable form of kallikrein into the urine and perfusate. The kininogenase activity generated by trypsin was identified as kallikrein, because it is inhibited by rat renal kallikrein antiserum and aprotinin, and not inhibited by LBTI, or ovomucoid. The possibility that the kininogenase activity generated by trypsin could be due to plasma prokallikrein activation was ruled out since the perfusate collected was completely free of blood. Furthermore, antibodies to glandular kallikrein do not cross-react with plasma kallikrein and plasma kallikrein is inhibited by ovomucoid.

As shown in DEAE-sephacel chromatography, the activatable kallikrein obtained from the perfusate behaves very similarly to urinary kallikrein. The partial purified fraction of activatable kallikrein, although containing some active kallikrein, may have been activated in contact with the arterial blood, to cause a hypotensive effect of the same magnitude as fully active urinary kallikrein. This suggests the presence of an activator in the blood. Although our data are not conclusive, it seems likely that the trypsin-activatable enzyme is a true proenzyme rather than an enzyme-inhibitor complex, since a trypsin-activated prokallikrein has been found in human urine.

**Discussion**

The present results show that isolated and perfused rat kidneys release an activatable form of kallikrein into the urine and perfusate. The kininogenase activity generated by trypsin was identified as kallikrein, because it is inhibited by rat renal kallikrein antiserum and aprotinin, and not inhibited by LBTI, or ovomucoid. The possibility that the kininogenase activity generated by trypsin could be due to plasma prokallikrein activation was ruled out since the perfusate collected was completely free of blood. Furthermore, antibodies to glandular kallikrein do not cross-react with plasma kallikrein and plasma kallikrein is inhibited by ovomucoid.

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**References**


**Table 1. Identification of the Activatable Kininogenase Activity**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Conc. in incubation mixture</th>
<th>Inhibition of activatable kininogenase in</th>
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<tr>
<td></td>
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<td>Per fusate (%)</td>
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<td>Aprotinin</td>
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The activities of the activatable kininogenase of the control tubes of perfusate and urine were 22 ng BK eq/ml and 70 ng BK eq/ml, respectively.

**Figure 2. DEAE-sephacel chromatography**

Column = 9 × 5 cm. Sample = 400 ml of perfusate pH 6.0. Eluents = 600 ml of 0.1 M Na phosphate pH 6.0. 500 ml of 0.35 M Na phosphate pH 6.0. Fraction volumes = 13.5 ml; 5.5 ml. Flow rate = 37 ml/hr.

**Figure 3. Blood pressure effects in perfused hindlimb of an anesthetized rat.** 20 µl of perfusate correspond to 23 ng BK eq total kallikrein, 9.8 ng BK eq active kallikrein; 20 µl of urine correspond to 35 ng BK active kallikrein.
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