Altered Urinary Kallikrein Excretion in Rats with Hypertension

By Harry S. Margolius, Ronald Geller, Wybren de Jong, John J. Pisano, and Albert Sjoerdsm

ABSTRACT

The urinary excretion of kallikrein, an enzyme which cleaves a vasodilator kinin from kininogen substrate, was examined in three types of hypertensive rats. Enzyme activity was measured by both esterase activity (isotopic assay) and bioassay with purified rat urinary kallikrein as a standard. Excretion of kallikrein was found to increase with age in spontaneously hypertensive rats, but levels were lower and stable in normotensive Wistar control animals. Rats with desoxycorticosterone-salt hypertension excreted markedly more, whereas rats with renal hypertension excreted significantly less kallikrein than did control animals. The alterations in kallikrein excretion were unrelated to changes in urine volume and protein excretion. The findings indicate that the kallikrein-kinin system may be involved in different forms of hypertension in the rat.

KEY WORDS spontaneous hypertension renal hypertension desoxycorticosterone-salt hypertension kinins urinary protein kallikrein bioassay kallikrein esterase activity (isotopic assay)
KALLIKREIN EXCRETION IN HYPERTENSION

### TABLE 1

<table>
<thead>
<tr>
<th>Animals</th>
<th>Age (wk)</th>
<th>Kallikrein esterase assay (EU/24 hr)*</th>
<th>Kallikrein bioassay (EU/24 hr)*</th>
<th>Systolic blood pressure (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wi</td>
<td>4</td>
<td>1.7 ± 0.7</td>
<td>2.1 ± 0.5</td>
<td>124 ± 1</td>
</tr>
<tr>
<td>SH</td>
<td>4</td>
<td>2.6 ± 0.6</td>
<td>2.8 ± 0.4</td>
<td>150 ± 5</td>
</tr>
<tr>
<td>Wi</td>
<td>8</td>
<td>2.2 ± 0.2</td>
<td>2.8 ± 0.4</td>
<td>130 ± 2</td>
</tr>
<tr>
<td>SH</td>
<td>8</td>
<td>3.6 ± 0.3†</td>
<td>3.9 ± 0.5†</td>
<td>201 ± 4†</td>
</tr>
<tr>
<td>Wi</td>
<td>6</td>
<td>2.9 ± 0.6</td>
<td>3.9 ± 0.5</td>
<td>123 ± 3</td>
</tr>
<tr>
<td>SH</td>
<td>6</td>
<td>7.9 ± 0.8†</td>
<td>7.3 ± 0.3†</td>
<td>213 ± 1†</td>
</tr>
<tr>
<td>Wi</td>
<td>7</td>
<td>2.4 ± 0.5</td>
<td>2.9 ± 0.6</td>
<td>124 ± 2</td>
</tr>
<tr>
<td>SH</td>
<td>7</td>
<td>5.6 ± 1.2†</td>
<td>7.1 ± 2.2†</td>
<td>218 ± 4†</td>
</tr>
</tbody>
</table>

Means ± se are shown. *EU = esterase units as defined in Methods. †Differs from control value with P < 0.01. ‡Differs from control value with P < 0.05.

Twenty-four-hour urine collections were obtained from individual rats by using glass metabolism cages which allowed urine to be collected separately from feces. Urine was collected at room temperature under toluene and stored at 4°C until it was assayed, within 2 weeks of collection. The enzyme appears to be stable at 4°C for at least 1 month. Animals were fasting during the collection period but allowed access to distilled water. Prior to the urine collection period, rats were weighed and the awake blood pressure determined by a tail plethysmographic technique (14). Urine volume and urine protein excretion (15) were also determined.

### MEASUREMENT OF URINARY KALLIKREIN

In addition to producing a kinin from kininogen, kallikrein can also hydrolyze synthetic ester substrates (7). The ratio of these two activities during purification has been shown to be constant (16). To determine if only kinin-generating esterase is excreted in rat urine, both kallikrein esterase and kinin-generating activities were measured in this study. Esterolytic activity was determined according to the method of Beaven et al. (9), with minor modifications. A 2.0-ml aliquot of the 24-hour urine collection was desalted by passage through 12 ml of hydrated (Tris HCl, pH 8.0, 0.01M) Sephadex G-25 fine, using a centrifugal filter holder as described by Determann (17). The desalted filtrate was diluted with deionized water 100–1000 times before assay. The incubation mixture contained 0.014 µc of p-tosyl-l-arginine-[3H] methyl ester ([3H]-TAME), 280 mc/mmmole, 0.01–0.03 ml of diluted urine filtrate, and 0.2M Tris HCl buffer, pH 8.5, to a total volume of 0.1 ml. The tubes were incubated at 37°C for 30 minutes, and the reaction was stopped with 0.5 ml of ice-cold deionized water. The mixture was immediately passed over a column of Bio-rex 70, H⁺ form, 200–400 mesh, which retained the unhydrolyzed ([3H]-TAME) but not the released tritiated methanol, which was eluted with 0.4 ml of water. The entire effluent solution was counted in 10 ml of Bray’s solution in a Packard Tri-Carb scintillation spectrometer at 29% efficiency. A standard curve of esterase activity was constructed by incubating 2.5 × 10⁻⁵ to 1.0 × 10⁻⁴ esterase units of purified rat urinary kallikrein in the assay.† One esterase

### TABLE 2

<table>
<thead>
<tr>
<th>Animals</th>
<th>Age (wk)</th>
<th>Urine protein (mg/24 hr)</th>
<th>Urine volume (ml/24 hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wi</td>
<td>7</td>
<td>75 ± 5</td>
<td>28.8 ± 0.4</td>
</tr>
<tr>
<td>SH</td>
<td>6</td>
<td>60 ± 2</td>
<td>5.3 ± 0.5*</td>
</tr>
<tr>
<td>Wi</td>
<td>11</td>
<td>126 ± 11</td>
<td>22.3 ± 2.3</td>
</tr>
<tr>
<td>SH</td>
<td>11</td>
<td>77 ± 4*</td>
<td>14.8 ± 1.8†</td>
</tr>
<tr>
<td>Wi</td>
<td>16</td>
<td>56 ± 6</td>
<td>21.9 ± 2.2</td>
</tr>
<tr>
<td>SH</td>
<td>16</td>
<td>82 ± 3*</td>
<td>11.3 ± 1.8*</td>
</tr>
<tr>
<td>Wi</td>
<td>35</td>
<td>68 ± 26</td>
<td>14.3 ± 2.7</td>
</tr>
<tr>
<td>SH</td>
<td>35</td>
<td>100 ± 12</td>
<td>13.2 ± 1.9</td>
</tr>
</tbody>
</table>

Means ± se are shown. Data from animals shown in Table 1. *Differs from control value with P < 0.01. †Differs from control value with P < 0.05.
unit is defined as that amount of enzyme which hydrolyzes 1 μmole of TAME per minute at pH 8.0 and 30°C in a titrimetric assay (16). Each urine sample was assayed in duplicate, as were recoveries of the standard kallikrein preparation from each sample. Each experimental run (6–8 urine samples) had its own standard curve. A urine sample was assayed on at least two different days. All results were expressed in terms of esterase units of the standard rat urine kallikrein as excreted per 24-hour urine sample. Data were corrected for recovery of added kallikrein. Recoveries ranged from 70 to 100%.

Undiluted Sephadex filtrates of urine (10–100 μl) were bioassayed on a guinea pig ileum in the presence of excess kininogen substrate (100 μl) prepared from human plasma (10). Purified rat urine kallikrein was also used as the standard in the bioassay. Male Hartley guinea pigs, 150–200 g, were killed by a blow on the head and a segment of terminal ileum 2–4 cm long was immediately removed, rinsed, and mounted in a 10-ml bath of continually aerated Tyrode’s solution at 37 ± 0.1°C. Tyrode’s solution had the following composition: NaCl 8 g/liter, KCl 0.2 g/liter, CaCl₂·6H₂O 0.1 g/liter, NaH₂PO₄·H₂O 0.05 g/liter, NaHCO₃ 1.0 g/liter, glucose 1.0 g/liter, and atropine sulfate 500 μg/liter. Isometric contractions were measured with a Grass force displacement transducer (FT 03) and recorded on a Hewlett Packard oscillograph. An initial tension of 1 g was placed on the ileum, and the tissue was allowed to equilibrate from 30 minutes to 1 hour. A dose–response curve (grams of tension developed vs. esterase units of standard kallikrein added) was constructed at the onset and end of each individual experiment. Experimental data were accepted only when these two curves were not different from each other. Using this standard curve, the grams of tension developed per volume of unknown urine added were expressed as units of esterase activity. The lower limit of sensitivity of the bioassay was about 0.1 esterase units/ml rat urine. Validation of kinin generation by urinary kallikrein was confirmed in each experiment by inhibition of the tissue response with carboxypeptidase B, which inactivates kinin, and by pancreatic trypsin inhibitor, an inhibitor of urinary kallikrein. Soybean trypsin inhibitor, which blocks kinin generation by plasma kallikrein, did not block kinin generation in any rat urine specimen. All samples from comparable control and hypertensive groups were assayed as unknowns on at least two separate ileum preparations on different days. Data were expressed as means ± SE and analyzed for significance with Student’s t-test.

Results

As shown in Table 1, with both the esterolytic assay and bioassay, SH rats were found to excrete more kallikrein than normotensive Wistar control rats. Differences were statistically significant at 11 through 35 weeks of age, and there was a close correlation between urinary kallikrein levels as measured by both assays. Elevated blood pressures were measured in SH rats at 7 weeks and reached a plateau after 11 weeks of age. At all ages, body weight of SH rats did not differ from controls. There was a slight increase in urinary protein excretion with age in SH rats (Table 2). Urine volumes of SH rats were significantly less than those of Wistar rats except at 35 weeks of age.

In contrast to the elevated kallikrein excretion in SH rats, rats with renal hypertension excreted significantly less kallikrein than did sham-operated controls (Table 3). With the bioassay we were unable to detect any urinary kallikrein activity in six of the seven rats with renal hypertension. Urinary protein excretion

| TABLE 3 |

| Urinary Kallikrein, Protein, and Volume in Renal Hypertension |
|---|---|---|---|
| **Animals** | **Kallikrein esterase (EU/24 hr)** | **Kallikrein bioassay (EU/24 hr)** | **Systolic blood pressure (mm Hg)** | **Urine protein (mg/24 hr)** | **Urine volume (ml/24 hr)** |
| Sham-operated | 7 | 4.3 ± 0.6 | 3.3 ± 0.9 | 127 ± 2 | 33 ± 4 | 27.6 ± 3.2 |
| Renal hypertensive | 7 | 1.1 ± 0.3† | ND | 190 ± 7† | 125 ± 35‡ | 25.5 ± 4.6 |

Means ± SE are shown. Rats were 16-week-old Wistar females. ND = nondetectable, below limits of sensitivity of bioassay (≤ 0.1 Eu/ml).

*EU = esterase units as defined in Methods.
†Differs from control with P < 0.01.
‡Differs from control with P < 0.05.
was significantly elevated in the group with renal hypertension, but no changes in urine volume or body weight occurred.

A marked increase in urinary kallikrein excretion was observed in rats with DOCA-salt hypertension (Table 4). Rats drinking only saline had somewhat higher urinary kallikrein levels than did control animals. The group with DOCA-salt hypertension and the saline-treated group excreted about three times as much urine as rats drinking water. A similar increase in protein excretion was observed in the groups treated with saline and DOCA-salt.

**Discussion**

The present data show that the urinary excretion of kallikrein, as measured by esterolytic assay and bioassay, is altered in rats with chronic hypertension. Although measurement of kallikrein esterase activity has been used routinely in isolation and purification studies (16), an esterase assay sensitive enough to measure kallikrein without concentrating large volumes of urine has not been previously available. The present study shows that, using the method of Beaven et al. (9), an aliquot of gel filtrate equivalent to as little as $1 \times 10^{-5}$ ml of an individual rat urine sample can be assayed for esterase activity and the results will be in close agreement with results of a bioassay procedure. The use of a purified rat urinary kallikrein allowed the data to be expressed in terms of standard esterase units and corrected for recovery when necessary. Bioassay enabled us to confirm that urine esterase activity was analogous to kinin-generating activity. Furthermore, studies with kallikrein inhibitors showed that kinin generation was due to the action of urinary and not plasma kallikrein.

With these two independent assay procedures, we observed significantly increased kallikrein excretion in two forms of hypertension and decreased excretion in a third. SH rats excreted more kallikrein than did Wistar controls after 7 weeks of age despite generally smaller 24-hour urine volumes. Rats with DOCA-salt hypertension excreted markedly increased amounts of kallikrein which could not be attributed to increased urine volume or protein excretion. Rats with renal hypertension excreted significantly less kallikrein than sham-operated controls but had similar urine volumes and significantly higher protein excretion. Therefore, differences in 24-hour urine volume or protein do not account for altered kallikrein excretion in hypertensive rats.

Measurements of urinary kallikrein have not been reported previously in rats with spontaneous or DOCA-salt hypertension. A decrease in urinary kallikreinlike activity, as measured by bioassay, was recently reported in rats made hypertensive by extirpation of one kidney and with a figure-of-eight ligature in the other (18).

Some evidence had been gathered previously indicating that the kallikrein-kinin system may be altered in human hypertension. Elliot and Nuzum (19) demonstrated 37 years ago that patients with essential hypertension excreted less kallikrein than normotensive controls. This finding was confirmed subsequently by Frey et al. (20), but further investigations of the kallikrein-kinin system in

**Table 4**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Kallikrein esterase assay (EU/24 hr)*</th>
<th>Kallikrein bioassay (EU/24 hr)*</th>
<th>Systolic blood pressure (mm Hg)</th>
<th>Urine protein (mg/24 hr)</th>
<th>Urine volume (ml/24 hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>$5$</td>
<td>$2.6 = 0.4$</td>
<td>$3.5 = 1.1$</td>
<td>$122 = 3$</td>
<td>$62 = 14$</td>
</tr>
<tr>
<td>1% Saline</td>
<td>$10$</td>
<td>$5.8 = 1.1$</td>
<td>$4.3 = 0.7$</td>
<td>$132 = 9$</td>
<td>$165 = 27$</td>
</tr>
<tr>
<td>DOCA + 1% Saline</td>
<td>$10$</td>
<td>$14.9 = 2.8$</td>
<td>$11.4 = 1.9$</td>
<td>$185 = 5$</td>
<td>$185 = 21$</td>
</tr>
</tbody>
</table>

Means ± SE are shown. Rats were 16-week-old Sprague-Dawley males.

*EU = esterase units as defined in Methods.
†Differs from water-fed animals with $P < 0.01$.
hypertensive patients were not forthcoming.
However, we have completed a preliminary
survey of kallikrein excretion in patients with
essential and secondary hypertension. The
findings of Elliot and Nuzum and Frey et al.
have been confirmed and extended to show
that in patients with some forms of secondary
hypertension, there is elevated urinary excre-
tion of kallikrein (8). Viewed collectively,
these data in both rats and humans show that
urinary kallikrein excretion can be markedly
altered in different forms of hypertensive
disease.

The meaning of altered kallikrein excretion
in hypertension is not clear. The present data
and the provocative fact that both kallikrein
activity and renin activity are closely associat-
ed in the kidney (21) and submaxillary gland
(22) point to the necessity for further studies
into the relationships between the renin-
angiotensin and kallikrein-kinin systems. The
possibility that kallikrein and the kinins are
involved in regulation of systemic blood
pressure or intrarenal vascular control awaits
further investigation.

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