Urinary Kallikrein in Rats Bred for Their Susceptibility and Resistance to the Hypertensive Effect of Salt

A New Radioimmunoassay for Its Direct Determination

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SUMMARY Urinary kallikrein was measured in rats bred to be susceptible (S) or resistant (R) to the hypertensive effect of salt. To determine kallikrein, three different methods were used: (1) a new direct radioimmunoassay (RIA) for the enzymic protein; (2) a method based on the capability of kallikrein, when incubated with kininogen, to generate kinins which were then measured by RIA (kininogenase activity); and (3) a method based on the capability of kallikrein to break the ester bond of p-tosyl-L-arginine methyl ester HCl (TAME). A significant correlation ($r = 0.90$) was found between the direct RIA and the kininogenase method. It was found that urinary kallikrein was significantly decreased in the S as compared to the R rats by the use of these three methods. Urinary kallikrein in the S rats was much lower when measured by the kininogenase method than by direct RIA or esterolytic assay. This difference could be due to excretion of pre-kallikrein and/or kallikrein bound to an inhibitor (inactive kallikrein). It is suggested that the decrease of urinary kallikrein excretion (active and inactive) in the S rats could be a consequence of a genetic defect that may affect the development of hypertension perhaps through the alteration of sodium and water excretion by the kidney.

DAHL et al. developed two strains of rats, one which is highly susceptible (S) and the other which is highly resistant (R) to the hypertensive effect of salt. Although the inherited susceptibility or resistance to the effect of salt is polygenic, the kidney appears to play a primary role in the determination of blood pressure levels. Because the S rats develop hypertension only when fed a high-sodium diet, it is reasonable to assume that an abnormal renal factor or factors in the kidney may be involved in regulating sodium and water excretion.

It was of interest to investigate urinary kallikrein excretion in these two strains of rats, since we had previously postulated that renal kallikrein could be involved in the regulation of sodium and water excretion by the kidney. Therefore, urinary kallikrein excretion was measured in S and R rats by three different methods based on three different principles:

1. Direct Radioimmunoassay (RIA) for the Enzymic Protein

A RIA for the direct measurement of urinary kallikrein was developed using antibodies against purified enzyme and $^{125}$I-kallikrein. This method provides a direct and highly sensitive measurement of rat urinary kallikrein, and thus could be used in further studies of the kallikrein-kinin system in physiological and pathological situations. A description of this method is included here.

2. Kininogenase Activity

In this method, Kallikrein is measured by its capacity to generate kinins when incubated with kininogen (kallikrein substrate), and the kinins generated are measured by RIA.

3. Esterolytic Activity

In this procedure, the enzyme is determined by its capability to break an ester bond of a synthetic substrate.

Methods

Six S and six R male rats, 16 weeks of age, were obtained from the Brookhaven National Laboratory. They were placed in individual metabolic cages and urine was collected for 6 days. Urinary volume was recorded and samples were stored at $-20^\circ$C for kallikrein, protein, sodium, and potassium determinations. At the end of the collection period, body weight was recorded and blood pressure was measured by the tail-cuff method. All of the rats were given tap water ad libitum and fed rat chow containing 0.45% sodium (normal sodium diet) and 0.89% potassium.

Urinary kallikrein was measured by the following methods:

1. Direct RIA for the enzymic protein: Purified urinary
kallikrein and its antiserum were prepared as previously described. Kallikrein was iodinated by a modification of the method of Hunter and Greenwood. For this, 87 μg of chloramine T (25 μl), 35 μg of the purified kallikrein (100 μl), and 1 mCi of 125I NaI (50 μl) were incubated at room temperature for 10 seconds. The reaction was stopped by the addition of 480 μg of sodium metabisulfite (0.05 M phosphate buffer, pH 7.5). The mixture was then diluted with 200 μl of carrier potassium iodide containing 1 mg of dextran blue 2000 and 10% bovine serum albumin (BSA). The separation of labeled kallikrein from the reaction mixture was carried out by gel filtration using Sephadex G15 (10-ml bed volume). Equilibration and elution from the gel were carried out in 0.05 M phosphate buffer, pH 7.5, and the void fraction with the dextran blue was collected. Two hundred microliters of this fraction were purified further on a column of Sephadex G100 (50 x 1 cm). Equilibration and elution from the gel were carried in 0.05 M Tris-HCl buffer, pH 7.5, containing 0.1% BSA (Tris buffer). All subsequent dilutions for the RIA procedure were made with this buffer except the kallikrein antiserum, which was diluted with Tris buffer containing 1:40 normal rabbit serum instead of BSA.

For the radioimmunoassay procedure, 1-40 ng of purified urinary kallikrein (standard curve) or 1-10 μl of rat urine (unknown) were transferred by pipette into disposable polyethylene tubes. To each tube, 100 μl of the 125I-kallikrein having approximately 3000 counts/min and 100 μl of 1:40,000 dilution of kallikrein antiserum were added, and the final volume was adjusted to 500 μl with Tris buffer. After 4 hours of incubation at room temperature, 100 μl of a 3:100 dilution of a previously titered sheep antirabbit gamma globulin antiserum were added to each tube. The tubes were subsequently incubated for 18 hours at 4°C. After the incubation, they were centrifuged at 3000 g for 40 minutes and the precipitate counted in an automatic, well-type gamma counter. Both the supernatant extract and precipitate of the first two tubes, in which antibody against kallikrein was omitted, were counted to determine total count and nonspecific binding. Each point on the standard curve, as well as for the unknown samples, was assayed in duplicate. In addition, an internal standard of crude rat urinary kallikrein was run in every assay.

Recovery studies were done by adding 2 ng of purified kallikrein to 2, 3, 4, and 5 μl of rat urine. The percent recovery was calculated by using the following equation: \( \frac{U_2 - U_1}{PUK} \times 100 = \text{percent recovery} \). \( U_1 \) is the enzyme quantity in the urine sample, \( U_2 \) is the enzyme quantity in the urine sample containing the purified kallikrein, and \( PUK \) is the enzyme quantity of the purified kallikrein. Results were calculated by computer using "logit-log" linearization of the standard plot as described by Rodbard et al. Results were expressed as micrograms of enzymic protein per 24-hour urinary volume.

2. Kininogenase activity: The kininogenase activity of the urine was determined as previously described. Results were expressed as micrograms of kinins generated per minute of incubation per 24-hour urinary volume.

3. Esterolytic activity: In this procedure, kallikrein was determined by the capacity of the enzyme to break an ester bond of \( p \)-tosyl-\( L \)-arginine methyl ester HCl (TAME). The unhydrolyzed ester was measured by a modification of Robert's colorimetric method. Results were expressed in micromoles of substrate consumed per minute per 24-hour urinary volume.

Sodium and potassium were measured by flame photometry using lithium as the internal standard. Urinary protein concentration was determined by the Lowry procedure.

Esterolytic activity, protein, sodium, and potassium in the urine were determined only on days 1, 3, and 5 of the urine collection. Coefficient of correlation or percent calculation was calculated with the data collected from these 3 days. Results in the text and figures are expressed as mean ± SE. Differences between mean values were evaluated by Student's t-test.

Results

Direct RIA for the Enzymic Protein

A typical standard curve showing significant displacement of \(^{125}\text{I}-\text{kallikrein with only 2 ng of purified kallikrein}

FIGURE 1 Standard curve for the direct RIA of kallikrein. Upper right panel shows linearization of this plot by using "logit" scale in the ordinate of B/Bo and log scale of standards in the abscissa.
of correlation between the three different methods used to measure the urinary kallikrein and between kallikrein and urinary protein. The \( r \) was calculated for the S and R group combined and separately. In addition, 11 different dilutions of pure kallikrein ranging from 100 ng to 100 \( \mu \)g/ml were measured by the three different methods. The correlation among the results obtained by these three methods were: enzymic protein (direct RIA) vs. kininogenase, \( r = 0.98 \); enzymic protein vs. esterolytic activity, \( r = 0.99 \); and kininogenase vs. esterolytic activity, \( r = 0.99 \).

**Urinary Kallikrein, Protein, Sodium, and Potassium Excretion by the S and R Rats**

Figure 5 shows: urinary kallikrein excretion as measured by the three methods; 24-hour urinary protein excretion; and blood pressure measured at the end of the experiment. Figure 6 shows kallikrein excretion by the S rats expressed as a percent of the kallikrein excretion in the R rats. Urinary sodium excretion per day was 3.28 ± 0.1 mEq in the S rats and 3.09 ± 0.2 mEq in the R rats. Urinary potassium was 3.75 ± 0.1 mEq and 3.41 ± 0.2 mEq, and urinary volume was 22 ± 0.6 ml and 36 ± 8 ml in the S and R rats, respectively. Body weight at the end of the experiment was 383 ± 5 g in the S rats and 353 ± 9 g in the R rats.

**Discussion**

There has been no report, up to the present, of a direct RIA for urinary kallikrein. The purification of rat urinary kallikrein and the production of antibodies against this enzyme were instrumental in developing the direct RIA. The antiserum, having a very high titer, was used in a final dilution of 1:200,000. This RIA is able to detect 1 ng of

**TABLE 1**  
Correlation between Kallikrein Values Obtained by Three Different Methods and between These and Total Urinary Protein

<table>
<thead>
<tr>
<th></th>
<th>S&amp;R*</th>
<th>S</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzymic protein vs. kininogenase</td>
<td>0.90</td>
<td>0.53</td>
<td>0.71</td>
</tr>
<tr>
<td>Enzymic protein vs. esterolytic activity</td>
<td>0.85</td>
<td>0.08</td>
<td>0.57</td>
</tr>
<tr>
<td>Kininogenase vs. esterolytic activity</td>
<td>0.84</td>
<td>0.21</td>
<td>0.52</td>
</tr>
<tr>
<td>Enzymic protein vs. urinary protein</td>
<td>-0.78</td>
<td>0.05</td>
<td>-0.32</td>
</tr>
<tr>
<td>Kininogenase vs. urinary protein</td>
<td>-0.74</td>
<td>-0.66</td>
<td>-0.16</td>
</tr>
<tr>
<td>Esterolytic activity vs. urinary protein</td>
<td>-0.66</td>
<td>0.23</td>
<td>0.16</td>
</tr>
</tbody>
</table>

* S&R indicates that \( r \) was calculated for the S and R groups combined.
Kallikrein was measured by three different methods (abscissa) and the values obtained for the R rats were compared with each of the methods were arbitrarily assigned 100%. Therefore, at the present time, the use of this RIA is limited only to the determination of rat urinary kallikrein.

The validity of this RIA method was verified as follows.

1. The displacement produced by rat urine was similar to that produced by purified rat urinary kallikrein (Fig. 2);
2. When the same urine samples were tested repeatedly, results were consistent (Fig. 3);
3. Recovery of purified kallikrein added to untreated urine was 100%; and
4. A highly significant correlation was found between urinary kallikrein activity measured by direct RIA and by kininogenase activity (kinin RIA) (Fig. 4).

When correlations were made between the results obtained by the direct RIA and by the esterolytic assay, the correlation was strong only when the results of the S and R rats were combined (Table 1). The correlation between the direct RIA or the kininogenase assay and the esterolytic assay for the S rats was not significant (P > 0.1). It may be that when the levels of urinary kallikrein are low, as in the case of the S rats, most of the esterolytic activity of the urine may not be due to urinary kallikrein but to other enzymes.

Urinary kallikrein excretion, measured by the three different methods, was significantly lower in the S rats when compared to the R rats (P < 0.01) (Fig. 5). Urinary volume in the S rats was also lower than in the R rats, but this difference was not statistically significant (P > 0.05). The fact that urinary kallikrein excretion was lower in the S rats could not be explained by lower urinary volume, since the concentration of urinary kallikrein per milliliter of urine in the S rats also was lower. This was most clear when the kallikrein was measured by the kininogenase method. The blood pressure in the S and R rats was similar. Hypertension did not develop in the S rats because they were fed a diet containing only 0.45% sodium and they had been selectively bred to develop hypertension only when fed a high-sodium diet (8% sodium content). Thus, the conspicuously lower urinary kallikrein excretion in the S rats was not secondary to an increase in blood pressure.

It is noteworthy that, when kallikrein excretion in the S rats was measured by the kininogenase method, it was much lower than when measured with the direct RIA or the esterolytic method (Fig. 5). This difference remained even after the normalization of the data (Fig. 6). The presence of an inhibitor in the urine of the S rats could explain this phenomenon. This hypothetical inhibitor may either be attached to a site in the kallikrein necessary for the binding of the kininogen (natural substrate) or it may induce an allosteric change in the enzyme that impairs the binding of the kininogen. However, this inhibitor could leave the antigenic site of the enzyme unaffected, allowing the direct RIA to detect this inactive kallikrein. In the case of the esterolytic method, it could be, as mentioned previously, that most of the esterolytic activity in the urine of the S rat is not due to kallikrein but to other enzymes. It is also possible that the inhibitor-kallikrein complex dissociates in the presence of a high concentration of TAMe, or that the inhibitor does not occupy a site close to the active region of the enzyme. This would allow the binding of a low molecular weight substrate such as TAMe (mol wt 379) but would still impair the binding of the high molecular weight substrate such as the kininogen (mol wt 40,000–60,000). This assertion is, in part, supported by a recent study which showed that a specific kallikrein inhib-
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itor was isolated from the rat kidney tubules. This inhibitor was able to inhibit the kininogenase activity of the urinary kallikrein but not the esterolytic activity. However, it was not reported whether this inhibitor is secreted in the urine.

In the present study, we found an increase in the urinary protein in the S rats (Fig. 6) and a significant ($P < 0.01$) negative correlation between the proteinuria and the kininogenase activity. It could be that these urinary proteins are responsible for the inhibition of the kininogenase activity, since they are probably plasmatic proteins, and plasma contains a considerable amount of kallikrein inhibitors.

Another possible explanation of the disproportionately lower kininogenase activity in the S rats is that part of the kallikrein excreted in the urine is inactive pre-kallikrein that cross-reacts with the antibody against kallikrein. Recently, Corthorn et al. reported that a significant amount of pre-kallikrein is excreted in the urine.

It appears that, in certain situations, the direct RIA measures total kallikrein (active and inactive) and that the kininogenase method measures only active kallikrein. From the physiological point of view, one could postulate that measurement of kallikrein by the kininogenase method is preferable, since the possible effects of this enzyme in the kidney are not direct but mediated through the release of kinins. However, it has not yet been determined whether the amount of kallikrein is the limiting factor in the intrarenal formation of kinins. It could be that in the S rats with higher urinary protein, more kininogen is present in the distal nephron where kallikrein is incorporated into the urine and kinins are formed.

Then the S rats with low kallikrein excretion could be forming the same amount of kinins as the R rats if the reaction is of first order in relation to the substrate. It seems unlikely, however, that the decrease of 10 to 20 times in kininogenase activity found in the S rat, when compared to R rats, could be explained by an increase in the availability of kininogen. The answers to many of these questions will have to wait until accurate procedures to measure intrarenal kinin formation are developed.

Dahl et al., using interstrain renal transplants, concluded that genetically controlled factors operating through the kidney are responsible for part of the hypertension in the S rats. Since the S rats develop hypertension only when fed a high-sodium diet, it is reasonable to assume that at least one of these abnormal renal factors in the kidney is involved in regulating sodium and water excretion. It may be that one of these loci controls the production of urinary kallikrein. The alteration of this locus could be at the level of the distal tubule, where it was found that urinary kallikrein is produced. Furthermore, it is possible that this is the primary alteration in the kidney and, perhaps, is also the cause of the hypertension. On the basis of our results, however, we cannot exclude the possibility that the decrease in urinary kallikrein excretion in the S rats was secondary to an unidentified alteration in the kidney.

In conclusion, urinary kallikrein excretion was measured by three different methods and found to be significantly decreased in the S rats when compared to the R rats. A significant correlation was observed between kallikrein measurement by a new direct RIA for the enzymic protein and by the kininogenase method. However, when urinary kallikrein in the S rats was measured by its kininogenase activity, the decrease in urinary kallikrein excretion was more pronounced than when measured by the direct RIA. It is postulated that this phenomenon was due to the excretion of inactive kallikrein by the S rat. This inactive kallikrein could be detected by the direct RIA but not by the kininogenase method. The decrease of urinary kallikrein excretion in the S rats could be a consequence of a genetic defect that may play a role in the development of hypertension, perhaps through the alteration of sodium and water excretion by the kidney.

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