Reduced Ratio of Active-to-Total Urinary Kallikrein in Essential Hypertension

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SUMMARY Urinary kallikrein excretion was studied in 34 patients with mild, normal-renin, essential hypertension without evidence of target organ damage and in 23 normotensive controls, using assays that measure both active (kininogenase activity) and total (active plus inactive) kallikrein. There was no significant difference in either active or total kallikrein excretion between the two groups. However, the ratio of active-to-total enzyme was decreased in the hypertensives (0.83 ± 0.03 units/µg) compared to the normotensives (1.00 ± 0.05 units/µg) (p < 0.002). The active-to-total ratio was inversely related to sodium excretion in both groups, indicating that the proportion of active to inactive enzyme increased in response to reduced sodium intake. We conclude that, although absolute excretion of active and total kallikrein is not decreased, enzyme activity per microgram of total kallikrein excreted is reduced in mild, normal-renin essential hypertension. This abnormality may be due to a defective enzyme, or to a reduced excretion of active relative to inactive kallikrein. The latter could result from the presence of a urinary kallikrein inhibitor or to reduced activation of a proenzyme. (Hypertension 5: 603-609, 1983)

KEY WORDS • normal renin hypertension • prokallikrein • kallikrein inhibitor

Renal kallikrein is a proteolytic enzyme produced in the kidney and excreted in the urine which liberates physiologically active peptides, the kinins, from a plasma globulin, kininogen. Investigators have reported that urinary kallikrein excretion is decreased in essential hypertension. These findings have led to the hypothesis that abnormalities in the kallikrein-kinin system may lead to hypertension in some patients. In contrast, other studies have reported comparable excretion rates of urinary kallikrein in hypertensive and control subjects. It has been suggested that failure to control for variables, such as renal function and race, may account for the decrease in kallikrein excretion in hypertension found in some studies. However, several investigators have reported that kallikrein excretion is reduced in hypertensives with normal renal function, even if compared to normotensives matched for age, sex, and race. The reason for the difference among these studies remains unknown.

In previous reports only enzymatically active kallikrein was measured, in most cases by means of esterase assays. Recent work has demonstrated that approximately 60% of the kallikrein in randomly selected samples of human urine is present in an inactive form, which can be activated with trypsin. It is not known whether the inactive fraction represents a proenzyme or a kallikrein inhibitor complex.

We have developed a direct radioimmunoassay that measures total urinary kallikrein, i.e., both active and inactive forms. Using this method as well as a kininogenase assay for active kallikrein, we have measured total and active kallikrein excretion in subjects with mild, normal-renin essential hypertension. We find no difference in the absolute excretion rates of active or total enzyme between our hypertensive and control groups. However, the activity per microgram of total enzyme (A:T ratio) is reduced in the hypertensive group. This suggests either that the active enzyme in hypertension is abnormal or that the proportion of active to inactive enzyme is reduced. The latter could result either from reduced activation of a proenzyme or from the presence of an inhibitor in the urine of hypertensives.
Methods

We studied 34 patients with mild essential hypertension. Twenty-four were men, 10 were women, and all were white. Fifteen had never been treated; the remaining 19 had received no antihypertensive medication for at least 1 week prior to the study. Since there were no significant differences in results between untreated and previously treated subjects, data are not presented separately. The normotensive control group comprised 17 men and six women, all of whom were white with a normal medical history and physical examination.

Protocol

All subjects were studied as outpatients. They were instructed to drink approximately 2 liters of fluid a day. A 24-hour urine collection was obtained on an ad libitum diet for measurement of kallikrein, sodium, and potassium excretion. The subjects were then instructed by a diettian to ingest a diet containing 40 mEq of sodium. After 7 days on this diet, another 24-hour urine collection was obtained for kallikrein assay and electrolytes. In most subjects urine collections were obtained on both diets. In 12 hypertensives and in nine controls, however, urine was collected while the subject was upright and ambulatory for 4 hours, blood was drawn for measurement of plasma renin activity and aldosterone levels. Additional studies in the hypertensives included chest x-ray, electrocardiogram, serum electrolytes, serum creatinine, serum catecholamine levels, urinalysis, and urinary protein excretion.

Urine was stored at 4°C during the 24 hours of collection and then frozen at −70°C.

Assays

Materials

Materials used included a lactoperoxidase iodination kit and 125I-bradykinin (New England Nuclear Corporation, Boston, Massachusetts); goat antirabbit IgG and bovine gamma globulin (Miles Laboratories, Inc., Elkhart, Indiana), and polyethylene glycol (J.T. Baker Chemical Company, Philipsburg, New Jersey).

Kallikreinase Activity

Kallikrein activity in the urine was determined by a modification of a previously described method.14 Urine samples were incubated with partially purified bovine kallikrein in the presence of kallikrein inhibitors and the kinins generated were measured by kinin RIA. Low molecular weight (LMW) kallikrein was partially purified from bovine plasma by ammonium sulfate and zinc acetate precipitations, the first two steps of a procedure of Yano et al.15 The reaction was carried out in 0.1 M sodium phosphate buffer pH 8.5 containing 3.0 mM 1, 10-phenanthrol ine, and 30 mM Na2 ethylenediaminetetra-acetic acid (assay buffer). Various amounts of urine (2–20 μl) were brought to a volume of 0.4 ml with assay buffer and equilibrated at 37°C for 5 minutes. Then 1 mg of kininogen (approximately 350 ng kinin equivalents) dissolved in 0.1 ml assay buffer was prewarmed and added to each sample to initiate the reaction. Samples were incubated at 37°C for 20 minutes and the reaction terminated by heating in a boiling water bath for 15 minutes. In control reactions, substrate was incubated without urine (kallikrein blank) and an internal standard consisting of pooled urine from normal subjects was analyzed in each assay.

The kinins were measured by radioimmunoassay (RIA) using rabbit antibradykinin serum generously supplied by Dr. Colin Johnston (Melbourne, Australia). The RIA was performed in 0.1 M Tris-HCl buffer, pH 7.4, containing 10.0 mM Na2 ethylenediaminetetra-acetic acid, 1.0 mM 1, 10-phenanthrol ine, 0.2% gelatin, and 0.1% neomycin. The reaction mixture contained 0.025 ml of 125I-Tyr4-bradykinin (approximately 4000 cpm); 0.1 ml of normal (for nonspecific binding) or antibradykinin serum, both diluted 1:4000; 25 to 1,000 pg of unlabelled bradykinin, or 0.01 to 0.05 ml of sample; and the RIA buffer to yield a total volume of 0.5 ml. Samples were incubated for 18 hours at 4°C and counted for radioactivity. Antibody-bound 125I bradykinin was precipitated with polyethylene glycol as follows; 0.1 ml bovine gamma globulin (1 g/100 ml dissolved in RIA buffer) followed by 0.6 ml polyethylene glycol (25 g/100 ml in 0.1 M Tris-HCl pH 7.4 at 4°C) was added to each tube, which was then vortexed and centrifuged at 3000 rpm for 30 minutes. The supernatant was decanted and the precipitate containing the antibody-bound 125I bradykinin was counted for radioactivity. A standard curve was obtained by plotting the percent of initial binding (B/Bo) vs the bradykinin standards. Nonspecific binding determined in each assay by substituting nonimmune serum for the antibradykinin serum, ranged 2.8%–3.2%, and was subtracted from all experimental data. All samples were assayed in duplicate. The concentration of kinin in the unknown sample was determined by interpolation on the standard curve. In 10 standard curves, the B/Bo produced by 250 and 1000 pg of unlabelled bradykinin was 91% ± 1.2% and 32% ± 0.60%, respectively. Dose response curves for lysyl-bradykinin and methionyl-lysyl bradykinin were not different from those for bradykinin. The metabolites of bradykinin produced by incubating bradykinin with chymotrypsin did not cross react with the antibradykinin antibody.

When incubated without any enzyme, 1 mg of kininogen read as 1 ng kinin equivalent. When incubated with an excess of trypsin, the same amount of the substrate yielded 350 ng kinin equivalents. Thus, cross reactivity between the bradykinin antiserum and kininogen was 0.3%. The kininogen blank was subtracted from each sample value. If the blank exceeded 20% of the kinins generated by the sample, the incubation was repeated using a larger aliquot of urine. Also, if a sample consumed more than 10% (35 ng kinin equivalents) of substrate, the incubation was repeated with a smaller aliquot of urine.

Twenty-three randomly selected samples from this study (8 normotensive and 15 hypertensive urines) were assayed directly by kinin RIA. The level of en-
The plasma renin status of hypertensive subjects was determined by the Core Laboratory of the Specialized Hypertension Service. The variability of these data is shown to measure total (active and inactive) kallikrein. Thus, endogenous kinins in the urine samples were not subtracted from the experimental data because they represented a negligible fraction of the generated kinins. The interassay coefficient of variation of the kinin RIA was 5% (n = 13). The interassay variation of the kininogenase assay (kinin generation and RIA) was 14% (n = 12). The recovery of bradykinin added to seven urine samples prior to the incubation step of the kininogenase assay was 99% ± 5% (n = 7).

Kinin generation was linear with increasing amounts of urine (from 0.5 to 10 μl) added to the incubation mixture. Kinin generation was also proportional to the time of incubation (from 5 to 60 minutes).

Kininogenase activity is expressed as units/ml. One unit is defined as the amount of enzyme that generates 1 μg of bradykinin equivalents per minute of incubation under the conditions of assay.

Urinary Kallikrein RIA

The radioimmunoassay for human urinary kallikrein has been described previously in detail,13 and has been shown to measure total (active and inactive) kallikrein.

Other Analyses

Tame-esterase activity was determined by the modification of the radiochemical method of Beavan et al.,14 described by Levy et al.5 Serum creatinine,17 plasma renin activity,18 and plasma aldosterone levels19 were determined by previously described techniques. The plasma renin status of hypertensive subjects was determined by the Core Laboratory of the Specialized Center of Research in Hypertension by plotting plasma renin activity on both high and low salt diets on a nomogram relating plasma renin activity to 24-hour urinary sodium excretion.18 Serum and urinary sodium and potassium were measured by flame photometry using lithium as an internal standard. The age of the two groups was comparable; hypertensives were 42 ± 2 years and normotensives, 40 ± 2 years. Blood pressure in the hypertensive group was 150 ± 2/100 ± 1 mm Hg and in the normotensives was 121 ± 4/75 ± 3 mm Hg. Sodium excretion ranged between 9 and 300 mEq/day in both groups (fig. 1). Sodium excretion in the two groups on the 40 mEq sodium diet (normotensives = 48 ± 13 mEq/day; hypertensives = 42 ± 7 mEq/day) and on the ad libitum diet (normotensives = 167 ± 11 mEq/day; hypertensives = 131 ± 12 mEq/day) was not different. Potassium excretion in the two groups on the low sodium intake (normotensives = 71 ± 7 mEq/day; hypertensives = 82 ± 6 mEq/day) and ad libitum diet (normotensives = 77 ± 6 mEq/day; hypertensives = 82 ± 9 mEq/day) was also comparable. In addition, there was no difference within either group in potassium excretion on the ad libum diet or low sodium intake.

In all the hypertensive subjects, plasma renin activity in relation to 24-hour urinary sodium excretion was normal (ad libitum diet = 3.2 ± 0.02 ng/ml/hr; low salt diet = 7.0 ± 0.7 ng/ml/hr). Plasma aldosterone levels in the hypertensives were also normal (11.1 ± 1.3 ng/dl on the ad libitum diet; 29.5 ± 4 ng/dl on the low salt diet). None of the hypertensive subjects had evidence of left ventricular hypertrophy by electrocardiogram or chest roentgenograms. Fundal examination was normal in 25% of the subjects, grade 1 retinopathy was found in 56%, grade 2 in 19%; no subjects had exudates, hemorrhages, or papilledema. Catecholamine levels, serum potassium, serum creatinine, and urine protein excretion were normal in all subjects.

Twenty-four-hour creatinine excretion was the same in the hypertensives (1421 ± 86 mg/day) as in the normotensives (1434 ± 86 mg/day). Within each group, however, creatinine excretion was lower in the females (hypertensives = 883 ± 72 mg/day; normotensives = 1150 ± 64 mg/day) as compared to the males (hypertensives = 1645 ± 80 mg/day; normotensives = 1534 ± 104 mg/day), p < 0.05.
Kininogenase (Active Kallikrein) and Total Kallikrein Excretion

Neither active nor total kallikrein excretion in the hypertensives was different from that in the control group (table 1). The means for these data adjusted by covariance analysis do not differ substantially from the unadjusted means. (Active kallikrein = 115 units/day in hypertensives, 129 units/day in normotensives; total kallikrein = 148 μg/day in hypertensives, 131 μg/day in normotensives.)

Active-to-Total (A:T) Kallikrein Ratio

We calculated the active to total kallikrein ratio (units/μg) for each urine sample to be able to compare the activity per total weight of enzyme in each subject of the hypertensive and control groups. The A:T ratio in the hypertensives was significantly lower than the AT ratio in the normotensives (p < 0.002). The means adjusted by covariance analysis (0.82 and 1.01 for hypertensives and normotensives respectively) were essentially the same as the arithmetic means.

Correlation of Kallikrein Excretion with Sodium Excretion

Kininogenase excretion in the normotensive group correlated inversely with sodium excretion (log y = 2.61 - 0.26 log x, r = -0.47, p < 0.003). In the hypertensive group, there was no significant correlation between kininogenase excretion and sodium excretion (r = -0.13, p > 0.3). Total kallikrein excretion did not correlate with sodium excretion in either the normotensives (r = -0.07, p > 0.6) or the hypertensives (r = +0.09, p > 0.4) groups. The A:T ratio correlated negatively with sodium excretion in both the normotensives (y = 2.02 - 0.53 log x, r = -0.69, p < 0.001) and the hypertensive populations (y = 1.38 - 0.29 log x, r = -0.42, p < 0.001) (fig. 1). The slope of the regression line for the A:T ratio vs sodium excretion in the hypertensives tended to be lower than that in the normotensive group (p < 0.1).

Correlation of Kallikrein Excretion with Age and Sex

Hypertensives

Active and total kallikrein within the hypertensive group were higher in the males (135 (45, 323) units/day; 170 (62, 468) μg/day) than females (85 (33, 209) units/day; 107 (22, 512) μg/day) (p < 0.01). The A:T ratio did not correlate with sex. There was a negative correlation between active kallikrein excretion and age (log y = 2.39 - 0.008 x; r = -0.39, p < 0.01) and between total kallikrein excretion and age (log y = 2.48 - 0.007 x; r = -0.35, p < 0.01). The A:T ratio was not related to age (r = -0.04, p > 0.8).

Normotensives

There was no correlation between active or total kallikrein excretion or the A:T ratio and age or sex in the normotensive group.

TAME-esterase Excretion

We also measured TAME-esterase excretion to compare our results with other studies of hypertensive subjects since a majority of the studies utilized esterase activity as a measure of kallikrein activity. Esterase activity in the normotensive group correlated positively with sodium excretion (log y = 2.61 - 0.26 log x, r = 0.47, p < 0.003). In the hypertensive group, there was no significant correlation between TAME-esterase excretion and sodium excretion (r = 0.13, p > 0.3).

Table 1. Active and Total Kallikrein Excretion and the Ratio of Active to Total Kallikrein in Hypertensive and Normal Subjects

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Kininogenase excretion (units/day)</th>
<th>Total kallikrein excretion (μg/day)</th>
<th>Active Total (units/μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normotensives (n = 23)</td>
<td>129(40,417)</td>
<td>136(47,389)</td>
<td>1.00 ± 0.05</td>
</tr>
<tr>
<td>Hypertensives (n = 34)</td>
<td>119(38,380)</td>
<td>151(51,446)</td>
<td>0.83 ± 0.03*</td>
</tr>
</tbody>
</table>

Active and total kallikrein excretion are expressed as the geometric mean, with the 95% confidence limits for the data in parenthesis. The active-to-total ratio is expressed as the arithmetic mean ± SEM (see Statistics in Methods section).

*p < 0.002 compared to normotensives.
excretion was comparable in the normotensives (9.9 (2.48) EU/day) and the hypertensives (10.3 (2.44) EU/day). Esterase activity per microgram of total kallikrein was also not different between the two groups (normotensives = 0.09 ± 0.01 EU/μg; hypertensives = 0.08 ± 0.01 EU/μg, p > 0.4).

Discussion

We have examined kallikrein excretion in a group of white subjects with mild essential hypertension and normal renal function. We measured kallikrein activity by a kinogenase assay and in addition determined total kallikrein by a direct radioimmunoassay. This is the first study reported of total kallikrein excretion in hypertensive patients. We find no difference in either active or total kallikrein excretion between the normotensive and hypertensive groups (table 1). Esterase excretion was also the same in the two groups. Thus, our findings are similar to those of Lawton and Fitz and Holland et al., who found no reduction in esterase excretion in a group of subjects with normal renal function and with mild essential hypertension. Since creatinine excretion (which is related to total muscle mass) was lower in both normotensive and hypertensive females than in males of the same group (see Results section).

While absolute excretion rates of active and total kallikrein were not reduced in hypertensive subjects, the A:T ratio (an index of the activity of the enzyme per microgram of total kallikrein) was lower in the hypertensive population than in the normotensives. The absolute difference between the two groups, though small (17%), was highly significant (p < 0.002).

The reduced A:T ratio in hypertensives could be due to a number of factors. It is possible that the enzyme in hypertensives is intrinsically abnormal so that it has a lower catalytic rate than the enzyme in normal subjects. Another possibility is that the proportion of active-to-inactive enzyme in hypertensives is reduced either because of defective activation of a pro-enzyme, or because of the presence of an inhibitor. We have determined that our purified standard human urinary kallikrein, which is entirely in an active form, has an A:T ratio of 1.5 ± 0.1 units/μg. Thus, if the active enzyme in hypertensives is not intrinsically abnormal, we can estimate that in the hypertensives 55% (0.83/1.5 × 100) of the kallikrein in urine was in the active form as compared to 67% (1.0/1.5 × 100) in the normotensive controls. Although the mean difference between the groups is not large, it is possible that the abnormality in the kallikrein kinin system is present only in a subgroup of the hypertensive population. In that case, the relatively small reduction in the A:T ratio of an unselected group of hypertensives might conceal a much greater abnormality in a subset. As shown in figure 2, the A:T ratio in seven urines from six hypertensive patients falls below the 95% confidence limits for the relationship between the A:T ratio and sodium excretion in the normotensives. Thus, 18% of the hypertensives fall below the 95% confidence limits, rather than 2.5% (one subject) predicted by a normal distribution. This group of patients did not differ from the rest of the hypertensives with regard to severity of hypertension, age or sex distribution.

Interestingly, while the A:T ratio is decreased in hypertensives, the esterase to total kallikrein ratio is not reduced. We propose two possible explanations for this discrepancy. Recent evidence indicates that a significant fraction of human urinary esterase activity may be due to enzymes other than kallikrein. The activity of such enzymes might offset a reduction in the esterase activity of kallikrein itself. Alternatively, it is possible that an inhibitor is present that interferes with the kinogenase but not the esterase activity of urinary kallikrein. An inhibitor with this property has been isolated from rat kidney.

A qualitatively comparable discrepancy between changes in esterase and kinogenase activities has been found in the Dahl salt-sensitive hypertension model in rats, in which urinary kinogenase activity is reduced to a greater extent than esterase activity.

Our data also suggest that, in hypertensives, kinogenase excretion responds abnormally to dietary sodium restrictions. A number of studies have demonstrated that active kallikrein excretion is stimulated by a low salt diet in normals. Our finding of an inverse

![Figure 2](image_url). Relationship between the log of sodium excretion and the active-to-total kallikrein ratio in hypertensives. The solid lines represent the 95% confidence limits for this relationship in the normotensives.
correlation between kininogenase excretion and sodium excretion in the normotensive group is in agreement. In contrast, in our hypertensive population, there was no correlation between active kallikrein excretion and sodium excretion, suggesting that the hypertensives respond abnormally to a potent stimulus to kallikrein excretion. Plasma aldosterone levels increased normally in the hypertensive group (see Results, Group Characteristics). Therefore, the failure of salt restriction to stimulate active kallikrein does not appear due to failure to stimulate aldosterone secretion. Our findings are similar to those of Margolius et al., who found that esterase excretion did not increase as much in hypertensives as in normotensives in response to a low salt diet. Lawton et al. reported a normal response to low salt diet in a group of white hypertensives, but noted that in a subgroup of patients kallikrein (esterase) excretion actually decreased in response to dietary restriction despite a normal rise in serum aldosterone levels. Both Levy et al. and Holland et al. reported that urinary kallikrein (esterase) excretion increased less on a low salt diet in black hypertensives than in black controls but found no such abnormality in white hypertensives.

There are no previous observations on the effect of variations in salt intake on the A:T ratio in hypertensives. We have found that the proportion of active kallikrein in urine increases in normotensive subjects during salt restriction. In our present study, the A:T ratio in both the hypertensive and normotensive groups correlated inversely with sodium excretion, indicating that the proportion of active to inactive kallikrein rose in response to dietary sodium restriction in both groups (fig. 1). This response to dietary sodium restriction tends to be greater in the normotensive than the hypertensives, but the difference in slopes of the relation between A:T ratio and sodium excretion was not quite significant (p < 0.1).

The significance of our findings in terms of the possible role of the kallikrein-kinin system in the pathogenesis of hypertension remains to be determined. The absolute excretion rates of active total enzyme are not reduced in the hypertensive subjects. However, the presence of an apparently normal level of enzyme excretion in the presence of established hypertension does not necessarily exclude a role for the renal kallikrein kinin system in the pathogenesis of hypertension. It is possible, for example, that activity of the system is reduced in certain prehypertensive persons. This abnormality might lead to the development of hypertension either because salt excretion is reduced or because decreased kinin activity results in vasoconstriction. Since changes in renal arterial pressure appear to alter kallikrein excretion, once hypertension becomes established active and total kallikrein excretion might increase toward normal. However, the low ratio of active to total kallikrein would signal the presence of the underlying enzyme defect. Obviously, such a sequence, or indeed any pathogenetic role for the kallikrein kinin system in a subgroup of hypertensives, is entirely hypothetical. While our hypertensive subjects had no gross evidence of target organ damage, renal insufficiency or proteinuria, it is still possible that the abnormalities of the kallikrein kinin system we have reported here are merely the consequence of a subtle effect of hypertension on the kidney. Further studies are needed to determine whether abnormalities of the renal kallikrein kinin system are important in the pathogenesis of hypertension.

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