Urinary Kallikrein Excretion in Normotensive Aging Female Rats (43016)

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Abstract. **The effect of aging on the intrarenal kallikrein-kinin system activity was investigated in normotensive 3-, 10-, 20-, and 30-month-old female Wistar rats. Urinary kallikrein excretion was measured by three independent assays (immunoreactive concentration, kininogenase, and amidolytic activities) and was found to decrease progressively from 10 to 30 months. In the 30-month-old rats the urinary immunoreactive kallikrein excretion represented 40–44% of the level detected in 3-month-old rats. Active and total kallikrein exhibited the same magnitude of reduction. Furthermore, the active to inactive kallikrein ratio remained unchanged throughout the life period studied. The level of urinary kallikrein inhibitor was studied by measuring the recovery of purified rat urinary kallikrein added in the samples; no change was observed with aging. None of the factors known at present to influence kallikrein excretion could be evoked to explain this age-related decrease. It is therefore suggested that this decrease may reflect a progressive impairment of the intrarenal endocrine function or an alteration in the secretion of the enzyme.** [P.S.E.B.M. 1990, Vol 193]

Our understanding of the age-related changes in kidney function may be improved by the knowledge of the regulatory systems which control renal blood flow, glomerular filtration rate, and solute excretion. In addition to the well-characterized renin-angiotensin-aldosterone system, the vasoactive prostaglandin and kallikrein-kinin systems are involved in the regulation of renal functions, water and electrolyte balance, and blood pressure control (1, 2). In the course of aging, the various complex interactions between these regulations can be disturbed. For instance, with age renal blood flow and the renin-angiotensin system activity are reduced in the rat (3), as in man (4, 5). This corresponds to a lower plasma concentration of renin and angiotensinogen and to a decrease in the renin content of the kidney. It suggests that either the endocrine role of the kidney is impaired with age or that the vasoconstrictive role of the renin angiotensin system is reduced as the blood supply to the kidney diminishes with age. Comparable information on prostaglandins and the kallikrein-kinin system are not yet available. To investigate further the relationship between kidney function and intrarenal hormone production in aging rats, observations were extended in the present study to the parent kallikrein-kinin system.

Renal kallikrein (EC 3.4.21.35) is a serine protease located mainly in the connecting cells of the distal tubule of the nephron where the enzyme is essentially bound to the apical membrane (6–8). One of its main roles is the generation of bradykinin, a very potent vasodilator and natriuretic nonapeptide. This production of bradykinin occurs in the cortical collecting duct, where kallikrein reacts with the low molecular kininogen present in the tubular fluid. Since the plasma kinin filtered at the glomerulus is reabsorbed and inactivated along the proximal tubule, the physiologic effect of kinins on tubular function probably occurs distally to the site of their production. It has therefore been suggested that kinins are involved in electrolyte and water transport by the distal nephron. In a micropuncture study, kinin administered in the late proximal tubule, distal to the main site of kininase, increased sodium and water excretions (9). It has even been suggested that bradykinin may antagonize ADH by inhibiting the hydroosmotic response of the cortical collecting tubule to ADH, probably via a prostaglandin-mediated mechanism (10). Therefore, a greater secretion of kinins in old animals could be responsible for the age-related increase in diuresis associated with decrease in urine osmolality. Moreover, it is believed that the kallikrein-kinin system may also be involved in renal hemodynamic regulation. A positive correlation between intrarenal kinin activity and renal blood flow suggests that the kallikrein-kinin system acts as a vasodilator by reducing intrarenal vascular resistance (11). The recent development and use of kinin antagonist confirms this hypothesis (12), and finally a glomerular bradykinin binding site has been identified (13). It is suggested that the density of this bradykinin binding site is down-

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regulated by the activity level of the kallikrein-kinin system (14).

This investigation was therefore undertaken to determine whether the kallikrein-kinin system is stimulated with age to maintain renal blood flow and glomerular filtration or is reduced as a consequence of an age-related impairment of intrarenal hormone production. Urinary kallikrein excretion (UKE) was measured in follow-up experiments on 3-, 10-, 20-, and 30-month-old rats kept in metabolic cages, using various independent methods including direct measurements of immunoreactive kallikrein concentrations, amidolytic and kininogenase activities, and the proportion of active and inactive kallikrein.

Materials and Methods

Animals and Physiologic Procedure. Female Wistar rats (WAG/Rij) 3, 10, 20, and 30 months old were purchased from the Institute for Experimental Gerontology of the Organization for Health Research, TNO, Rijswick, The Netherlands and studied over the following month. Mean survival age of the colony was 30 months. Animals were fed a commercial diet (D.O4, U.A.R., Villemoisson, France) containing 17% protein, 0.62% potassium, and 0.27% sodium. Water was provided *ad libitum*. Daily input and output were measured in metabolic cages. Animals were first acclimatized for 2 days in individual metabolic cages with free access to food and water. On Day 3 urine collection was started at 10 AM. Urine was collected under oil and 0.01% NaN_3 to prevent bacterial degradation of the proteins. After volume determination, it was filtered and aliquots were frozen (−30°C) while awaiting protein and kallikrein determinations.

Proteinuria. Total proteinuria was calculated from urinary volume and protein concentration, which was assayed by Bradford's technique using serum albumin as standard (Sigma). Age-related changes in the molecular size distribution of the excreted proteins were determined by polyacrylamide gel electrophoresis as previously described in detail (15).

Urinary Kallikrein Measurements. Kallikrein concentration was assessed with a direct radioimmunoassay using purified rat urinary kallikrein (16) as standard and iodinated rat urinary kallikrein as tracer. The antibodies used were raised in rabbit against our purified rat urinary kallikrein; they recognized both the active and inactive kallikrein after high-performance liquid chromatography separation of these two forms (16). Trypsinization of the samples did not induce changes in the determination of immunoreactive kallikrein concentration. However kallikrein bound to a high molecular weight protein is not detectable, but no interference was observed with albumin. The results are expressed in micrograms of kallikrein in 24-hr urinary volume/g of kidney weight (μg kallikrein/24 hr/g kidney wt).

Amidolytic activity was determined as previously described (17) using the synthetic substrate Val-Leu-Arg-pNA (S2266 KABI VITRUM). Kallikrein is able to hydrolyze the ester or amide bond with arginine residue in C terminus position. The activity is estimated by the liberation rate of *p*-nitroaniline measured spectrophotometrically at 405 nm. The results are expressed in micromoles of substrate split per minute in 24-hr urinary volume and per g of kidney weight (μmol/24 hr/g kidney wt).

Kallikrein activity of the urine was measured by the kininogenase activity using a kinin radioimmunoassay (18). An aliquot of urine was incubated with inactivated citrated dog plasma as source of kininogen. This preparation was devoid of spontaneous kininogenase and kininase activities, but nevertheless the incubation buffer contained 0.3 m*M* orthophenanthroline and 0.3 m*M* EDTA as kininase inhibitors. The generated kinins were quantified by kinin radioimmunoassay, using the nonapeptide bradykinin as standard and a kinin antibody which recognizes bradykinin and longer analogs but not the bradykinin fragments. The results are expressed in micrograms of bradykinin generated per minute and per 24-hr urinary volume and per mg of kidney weight (μg bradykinin/24 hr/g kidney wt).

Total activatable kallikrein was measured after trypsin activation. Samples were incubated for 30 min with 5 μg of trypsin in 0.2 *M* Tris (pH 7.8) at 37°C. Trypsin activates prekallikrein by liberating a heptapeptide-inactive profragment. The action of trypsin was stopped by adding 20 μg of soybean trypsin inhibitor. Previous controls had demonstrated that in these conditions trypsin is blocked without any inhibitory effect of soybean trypsin inhibitor on kallikrein. The substrate was then added, the kinins determined, and the results expressed as described above.

Kallikrein Inhibitors. Kallikrein inhibitors were assayed by measuring the recovery of purified kallikrein added in the urine sample and then the total kallikrein of the respective samples was determined. For this, 1 ng of purified kallikrein (19) was added in an appropriate amount of each urine sample (from 0.5 to 5 μl) and kallikrein was measured as described above by its immunoreactive concentration and kininogenase and amidolytic activities.

Age-related change was statistically analyzed by one-way analysis of variance and the Neuman-Keul test. Significance was set at $P < 0.05$. Means are expressed ± SE.

Results

Kidney weight increased in proportion to body weight from 3 to 20 months. Between 20 and 30 months the kidneys still grew in size, despite a slight reduction in body weight (Table I). In this strain of rats (WAG/Rij), there is no evidence of major kidney disease or marked development of conjunctive tissue before 30 months; the renal hypertrophy corresponds to increases

Table 1. Age-Related Variations in Body Weight, Weight of Both Kidney and Daily Urinary Excretion

Age (months)	n	Body weight (g)	Two kidneys (g)	Urine volume (ml/24 hr)	Proteinuria[a] (mg/100 g body wt)
3	5	170 ± 5	1.14 ± 0.03	4.34 ± 0.54	1.30 ± 0.09
10	8	193 ± 4[b]	1.21 ± 0.03[b]	4.13 ± 0.27	1.31 ± 0.07
20	8	225 ± 9[b, c]	1.34 ± 0.03[b, c]	5.3 ± 0.66	1.45 ± 0.11
30	9	198 ± 7[b, d]	1.44 ± 0.03[b-d]	10.9 ± 2.04[b-d]	2.40 ± 0.27[b-d]

[a] Mean values from $n = 11$ (see ref. 15).
[b] Different from 3 months ($P < 0.05$).
[c] Different from 10 months ($P < 0.05$).
[d] Different from 20 months ($P < 0.05$).

in glomerular diameter, length of proximal tubule and medulla without changes in the number of glomeruli which remains constant throughout life (20). Since the excreted kallikrein is produced by cells of kidney tubules, it was decided in this article to refer the results to renal tissue mass rather than to body weight.

The urinary volumes collected over a 24-hr period were constant between 3 and 20 months but greater in 30-month-old rats (Table I). This greater volume in the oldest rats is associated with a lower urine osmolality, the osmotically active solute excretion thus remains constant (21). Total protein excretion is also affected by age but only in the 30-month-old group (15). The electrophoresis analysis of urinary protein patterns confirmed that age-related proteinuria was almost exclusively due to an increase in albumin excretion whereas the excretion of low molecular mass proteins was not modified (15).

As shown in Figure 1 the displacement curves obtained with dilution of urine of all groups were parallel to the standard curve. The urinary excretion of immunoreactive kallikrein exhibited a significant and age-dependent progressive reduction from 3 to 30 months (Fig. 2) (3 months: 30 ± 9, $n = 5$; 10 months: 21.4 ± 1.4, $n = 8$, $P < 0.05$; 20 months: 17.2 ± 0.9, $n = 8$, $P < 0.05$; 30 months: 12.8 ± 0.7, $n = 9$, $P < 0.05$,

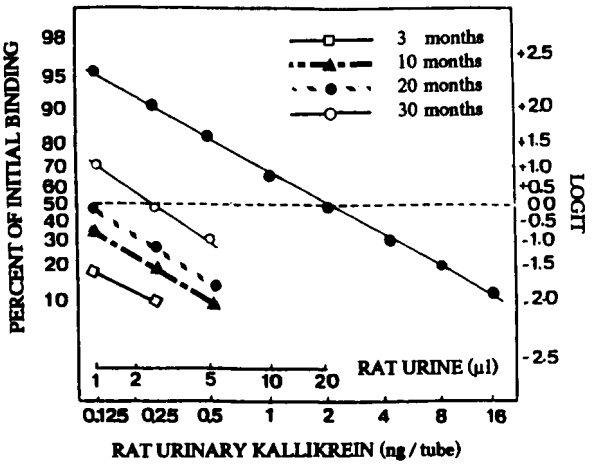

Figure 1. Logit-log transformation of the standard curve and of dilution of urine from 3 ($n = 5$)-, 10 ($n = 8$)-, 20 ($n = 8$)-, and 30 ($n = 9$)-month-old female rats. Each point represents the mean ± SE.

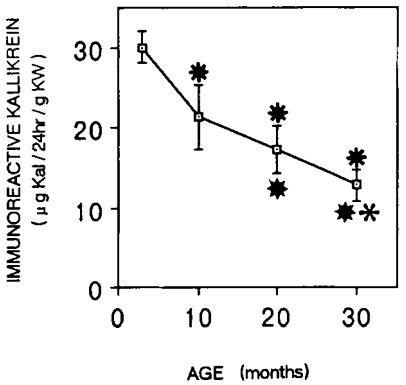

Figure 2. Immunoreactive kallikrein excreted in the urine of 3 ($n = 5$)-, 10 ($n = 8$)-, 20 ($n = 8$)-, and 30 ($n = 9$)-month-old female rats. Each point represents the mean ± SE. * Different from 3 months ($P < 0.05$); * Different from 10 months ($P < 0.05$); * Different from 20 months ($P < 0.05$).

µg kallikrein/24hr/g kidney wt). The level of immunoreactive kallikrein in 30-month-old rats represented 42.6 ± 2.3% of that of 3-month-old rats.

The changes in amidolytic activity are shown in Figure 3. The activity decreased significantly ($P < 0.05$) in 20- and 30-month-old rats (Fig. 3A), being only 59.1 ± 2.3% of that of 3-month-old rats. Total amidolytic activity exhibited a significant decrease in 10-month-old rats (Fig. 3B): in 30-month-old rats it was 52.8 ± 3.8% of that of 3-month-old animals. The ratio of active to total kallikrein, however, did not change over the life period studied (Fig. 3C).

The evolution with age of kininogenase activities is shown in Figure 4. Urinary excretions of active (Fig. 4A) and trypsin activatable (Fig. 4B) kininogenase activities demonstrated the same pattern of decrease in 10- to 30-month-old rats. In the 20- and 30-month-old rats, kininogenase activities represented 45.9 ± 5.5 and 47.2 ± 1.6% of their respective controls. The percentage of active form remained unchanged from 3 to 30 months (Fig. 4C).

The decreases in total amidolytic and total kininogenase activities exhibited highly significant and positive correlations ($r = 0.991$ and $r = 0.998$ [$P < 0.01$], respectively) with decreases in immunoreactive concentration. No correlation was found between the increase in daily urinary output and the decrease in UKE.

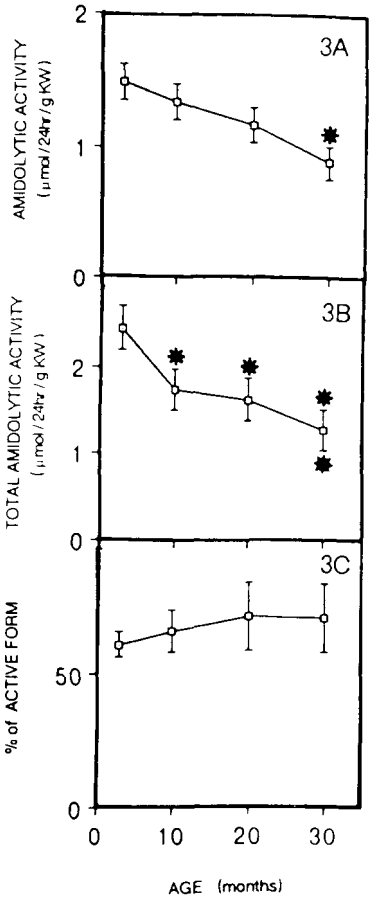

Figure 3. Amidolytic (A), total amidolytic (B), and percentage of active form (C) excreted in the urine of 3 (n = 5)-, 10 (n = 8)-, 20 (n = 8)-, and 30 (n = 9)-month-old female rats. Each point represents the mean ± SE. *Different from 3 months ($P < 0.05$); *Different from 10 months ($P < 0.05$); * Different from 20 months ($P < 0.05$).

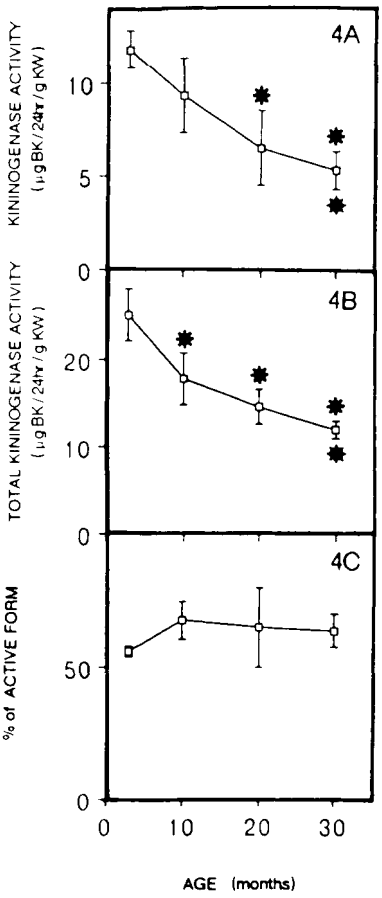

Figure 4. Kininogenase (A), total kininogenase (B), and percentage of active form (C) excreted in the urine of 3 (n = 5)-, 10 (n = 8)-, 20 (n = 8)-, and 30 (n = 9)-month-old female rats. Each point represents the mean ± SE. *Different from 3 months ($P < 0.05$); *Different from 10 months ($P < 0.05$); * Different from 20 months ($P < 0.05$).

The data of the recovery of purified kallikrein are given in Table II: no significant age-related decrease was detected, there is thus no age-dependent formation of kallikrein inhibitors.

Discussion

This study of kallikrein excretion in aging rats should be considered in the context of the broad investigation of kidney aging that we started 5 years ago. We first produced evidence that the female Wistar rats (WAG/Rij) fed with a 17% protein diet may be considered to be a unique model for studying kidney aging free of the interfering effects of severe chronic nephrosis. Even in the 30-month-old rats there was no loss of nephrons, no evidence of glomerulosclerosis, no gross degenerative pathology, and no great increase in protein excretion (15, 20). Furthermore, from various control experiments carried out during these last years, we are convinced that comparisons of the data from different cohorts of the inbred WAG/Rij colonies are valid. Thus, the present data on the kallikrein-kinin system may safely be compared with data concerning renal blood flow, glomerular filtration rate (21), proteinuria (15) and mean arterial pressure (3) already published from studies conducted in our laboratory.

Table 2. Age-Related Recovery of Purified Kallikrein Added to Urine

Age	n	Kallikrein concentration (% of recovery)	Kininogenase activity (% of recovery)	Amidolytic activity (% of recovery)
3	5	88 ± 8	94 ± 5	89 ± 10
10	8	92 ± 5	91 ± 7	92 ± 6
20	8	93 ± 9	96 ± 5	94 ± 5
30	9	92 ± 6	94 ± 7	93 ± 7

The reason why we have now examined the kallikrein system is that we feel a complete understanding of the age-related changes in kidney function must include the different regulatory systems acting on renal tissue. After an investigation on the renin-angiotensin system, we have turned our attention to the parent kallikrein-kinin system. Experiments with vasopressin are in progress.

Kallikrein is excreted into the urine in both active and inactive forms, which may be modified by urinary inhibitors of the enzyme. In relation to this, the use of

three different approaches for kallikrein measurements provides information on the age-related alteration in kallikrein-kinin system activity. The detection of immunoreactive kallikrein is highly specific and measures both active and inactive kallikrein (prekallikrein and/or kallikrein bound to an inhibitor). The kininogenase activity, very close to the physiologic activity of the protein, is very sensitive to inhibitors (pH, tubular proteins, and ionic composition) which are present in variable amounts in the course of aging. The amidolytic activity, which is based on the hydrolysis of a synthetic tripeptide, may exhibit differences in sensitivity to inhibitor compared with the kininogenase activity, especially under pathologic conditions. Trypsinization of the samples is used to activate prekallikrein and to assess the proportion of active to inactive kallikrein; the percentage of the active form gives complementary information about the secretion and activation processes of kallikrein. Thus, a general consideration of the results of these different assays eliminates possible inexactitude of any one method. In any case, all assays indicated a progressive age-related decrease in kallikrein excretion in rats without changes in the ratio of the active to inactive form of the enzyme. However, it may be noted that urinary amidolytic activity excretion only decreased at 20 months whereas immunoreactive kallikrein and kininogenase activity both decreased from 3 months onward. Urine, however, contains amidolytic enzymes other than kallikrein (22). Moreover, we recently demonstrated that kininogenase and amidolytic activities may exhibit a different sensitivity to inhibitors (23). These observations may account for the delayed age-related decrease in amidolytic activity as compared with immunoreactive kallikrein and kininogenase activity measurements. The relevance of age to the decrease of UKE has also been documented in the normotensive human and even more so in hypertensive elderly subjects (24, 25). Various studies suggest that this age-related reduction in urinary kallikrein results from a change in its production by the cells of the distal segments of the nephron rather than from a modification of filtered kallikrein. Clearance experiments have established that the filtered glandular kallikrein from other organs such as pancreas and salivary glands are reabsorbed along the proximal tubule and do not account for a significant urinary presence of the enzyme (26). This is confirmed by a less than 8% urinary recovery of labeled glandular kallikrein injected intravenously (27); also, stop flow technique and immunocytochemistry observations show that kallikrein is synthesized in renal tissue and enters the urine at the distal part of the nephron (28).

Why does kallikrein diminish with aging? Under normal conditions, tissue kallikrein contents exhibit dietary sodium, androgen, and sex-linked dependencies. A low sodium diet increases both urinary and renal kallikrein (29) by stimulating the relative rate of synthesis (30). The level of urinary and renal kallikrein is also increased by mineralocorticoids and reduced by glucocorticoids (31) in male, but not in female rats, although no sex differences could be detected in the kallikrein level of untreated control animals (30). In male rats, testosterone exercises a permanent inhibitory control of the renal biosynthesis of kallikrein by altering mRNA accumulation (32). Another factor inducing increase in UKE is a rise in renal perfusion pressure (33). UKE is also found to be reduced in hypertensive states (24, 25) in both man and experimental animals with the exception of the mineralocorticoid-dependent hypertension in which it is increased (1, 2).

None of these factors can explain the age-related decrease in UKE observed in our study. No change in food intake, plasma concentration, or urinary excretion of sodium occurred in a previously studied group of female Wistar rats from 3 to 30 months old (21). As female rats were used, it is unlikely that the observed age-related decrease in UKE was an aldosterone-mediated mechanism or that it was linked to changes in testosterone level. In a previous report (15), we showed that the glomerular filtration rate and renal blood flow as expressed per kidney weight did not change from 10 to 20 months and decreased between 20 and 30 months. This reduction in blood supply per gram of tissue without a change of the mean arterial pressure makes it unlikely that the age-related urinary kallikrein excretion rate is linked to a rise in renal perfusion pressure. It is well known that UKE is reduced in hypertension and in renal parenchymatous disease (1, 2), but no hypertension, marked proteinuria, glomerulosclerosis, or kidney disease was detected in 30-month-old WAG/Rij rats (15, 20). Finally, it seems that the decrease in UKE is independent of changes in kidney hemodynamics since these age-related changes only appear at the very end of the rat's life (21) whereas the decrease in UKE is progressive with age. This suggests that the kallikrein-kinin system does not play a major physiologic role in hemodynamics or electrolyte and blood pressure regulation in aging rats and certainly not in the age-related decrease of urine osmolality.

Although it is admitted that urinary parameters of the kallikrein-kinin system are a reliable index of the intrarenal system, dissociations between these parameters have been reported (29). If urine is the main outlet for renal excretion, venous and lymph excretions have also been reported (1, 2). Thus, a decrease in urinary kallikrein excretion not only suggests a reduction in the intrarenal system but may also indicate an age-related alteration in the excretory pathway mechanism. It is interesting that the age-related decrease in urinary kallikrein excretion is associated with a diminished activity of the renin-angiotensin system (3). However, this concomitant fall in urinary kallikrein excretion and renin-angiotensin system activities in old rats rather suggests a progressive impairment of the intrarenal endocrine function with age than an alteration in the excretory process of the enzyme.

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