Effect of Glandular Kallikrein on Renin Release in Isolated Rat Glomeruli

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SUMMARY Numerous studies have suggested that a functional relationship may exist between the kallikrein-kinin and the renin-angiotensin systems within the kidney. We investigated the effects of glandular kallikrein on renin release by using an in vitro preparation of isolated rat glomeruli with their attendant arterioles. The effect of kallikrein was studied in the presence or absence of 0.1% bovine serum albumin (BSA) in Krebs superfusion fluid. We also studied the effect of inactivating kallikrein by treatment with phenylmethylsulfonyl fluoride or by inhibiting it with aprotinin. In the absence of BSA, kallikrein caused a 12-fold increase in renin release, from 5.1 ± 1.2 ng angiotensin I (ANG I)/min to 66.0 ± 2.27 ng ANG I/min (p < 0.025). In the presence of BSA, renin release increased twofold, from 13.0 ± 1.8 ng ANG I/min to 24.3 ± 4.8 ng ANG I/min (p < 0.025). The basal level of renin measured when the glomeruli were superfused with BSA-Krebs was two to three times greater than when they were superfused with Krebs alone (p < 0.001). This finding suggests that media protein inhibited renin loss during either the superfusion or storage of renin samples. Neither phenylmethylsulfonyl fluoride–inactivated nor aprotinin-inhibited kallikrein stimulated renin release. We propose that kallikrein can stimulate renin release in isolated glomeruli. Although media protein did modify measured basal and kallikrein-stimulated renin release, the action of kallikrein appears to be a function of its enzymatic activity as the inactive enzyme did not stimulate renin release. (Hypertension 7: 27–31, 1985)

Key Words • aprotinin • esterase • juxtaglomerular • kidney • protease • protein

SUZUKI and colleagues1,2 have reported that rat urinary kallikrein and esterase A2 stimulate renin release from superfused rat kidney slices. They also found that trypsin was not effective under these experimental conditions, that aprotinin (a serine protease inhibitor) blocked the effect of kallikrein, and that bradykinin had no effect on renin release. They suggested that kallikrein could act directly and specifically to stimulate renin release. Recently, however, that laboratory has retracted these findings.3 They now indicate that kallikrein’s effect on renin was due to a nonspecific protective action exerted by protein on renin, which was otherwise lost during sample storage. Renin loss was prevented by adding either kallikrein or bovine serum albumin (BSA) to the samples. Aprotinin abolished the protective effect of both kallikrein and BSA, although no clear explanation was given for this observation. The concentration of BSA needed to prevent renin from being lost was 100 µg/ml; at 10 µg/ml it exerted a negligible protective effect. Since only 2 to 3 µg/ml of kallikrein was present, the possibility of its exerting a nonspecific protective effect on renin is unlikely.

We have further investigated the effects of glandular kallikrein on renin release by using an in vitro preparation of rat-isolated glomeruli with part of their attendant arterioles.4 The effect of kallikrein was studied in the presence or absence of 0.1% BSA in the superfusion fluid. We also have studied the effect of inactivating kallikrein by treatment with phenylmethylsulfonyl fluoride (PMSF), a negatively charged inhibitor, or aprotinin, a positively charged inhibitor. This study presents evidence for a direct stimulatory action of kallikrein on renin release as a function of its enzymatic activity.

Materials and Methods

Male Sprague-Dawley rats weighing 200 to 250 g were fasted overnight. They were anesthetized with sodium pentobarbital (5 mg/100 g body weight, i.p.; Abbott Pharmaceuticals, North Chicago, IL). The kidneys were exposed through a midventral incision and were flushed of blood with a modified Krebs-Ringer buffer solution by retrograde perfusion through the aorta. The kidneys were then removed and decapsul-
ed and the cortex was cut away. From minced cortical tissue, isolated glomeruli were harvested by means of a passive sieving technique, as previously described.4

Glomeruli from a group of 6 to 12 rats were pooled on a given experimental day. Equal aliquots of glomeruli weighing approximately 40 to 50 mg (wet weight) were suspended in Krebs medium and placed into plastic superfusion chambers constructed from 1/4-inch polypropylene T-connectors (Bel-Art, Pequannock, NJ) and enclosed with nylon monofilament mesh (Tetko, Inc., Elmsford, NY). Glomeruli were superfused with aerated Krebs buffer in parallel within identical chambers by a Manostat Cassette Pump (Manostat, NY, NY) at a rate of 300 µl/minute. This procedure allowed control and experimental manipulations to be paired from aliquots of the same tissue pool. All chambers were superfused initially for 50 minutes to obtain a stable baseline of renin release, after which two consecutive 10-minute collections of the chamber effluent were made into siliconized glass tubes with a modified Gilson FC-100 fraction collector (Gilson Medical Electronics, Inc., Middleton, WI). After the initial control period, the chambers were superfused by Krebs medium modified according to one of the following seven experimental protocols.

1. Krebs time control (n = 17). Chambers containing glomeruli were superfused during both the control and experimental periods with Krebs buffer.

2. Kallikrein superfusion (n = 14). After the initial control period, glomeruli were superfused with Krebs medium containing purified hog pancreatic kallikrein at concentrations of 3.0 µg/ml with an esterase activity of 170 mEU/ml. In a separate experiment, the kallikrein concentration in the collected sample was 3.0 µg/ml.

3. PMSF-kallikrein superfusion (n = 5). This group was similar to group 2 except that the kallikrein had been inactivated with PMSF, as previously described.5 Esterase activity of the PMSF-kallikrein was undetectable.

4. BSA-Krebs time control (n = 15). The glomeruli were superfused during the control and experimental periods with Krebs medium containing 0.1% heat-inactivated BSA (Difco Laboratories, Detroit, MI).

5. BSA-Krebs kallikrein superfusion (n = 11). After an initial control period with BSA-Krebs, glomeruli were superfused with BSA-Krebs medium containing kallikrein, 3.0 µg/ml, with an esterase activity of 170 mEU/ml. In a separate experimental series (n = 6) glomeruli were superfused with BSA-Krebs containing partially purified rat submandibular gland kallikrein with an esterase activity of 160 mEU/ml.

6. BSA-Krebs aprotinin-kallikrein superfusion (n = 4). This group was similar to group 5 except that the superfusion medium used during the control and experimental periods was modified by adding 500 kallikrein-inhibitory units (KIU) of aprotinin.

7. Krebs superfusion collected into tubes containing kallikrein (n = 7). To test if the effect of kallikrein was due to the protective action of the enzyme protein in the buffer, chambers containing glomeruli were superfused with Krebs medium and collected over four consecutive periods. One group was collected into tubes without kallikrein (n = 7), while another group was collected into tubes containing kallikrein (n = 7) such that the final kallikrein concentration in the collected sample was 3.0 µg/ml.

Analytical Methods

Renin concentration was determined in the samples by a method previously reported.6 Superfusion samples were stored overnight at 4°C before analysis. Samples were incubated for 3 hours at pH 6.5 with 48-hour nephrectomized rat plasma (substrate) in a quantity sufficient to generate 1000 ng of angiotensin I (ANG I). Angiotensinase inhibitors included 1.45 x 10^-7 M PMSF (Sigma Chemical Co., St. Louis, MO), 3.4 x 10^-4 M 8-hydroxyquinoline (Becton, Dickinson & Co., Orangeburg, NY), and 5.1 x 10^-4 M ethylenediaminetetraacetic acid (J.T. Baker Chemical Co., Phillipsburg, NJ). The ANG I generated was measured by radioimmunoassay (RIA) after the methods of Haber and co-workers.7 Renin release was calculated as renin concentration times milliliters of superfusion fluid per minute per 50 mg of glomeruli (wet weight). Renin concentration was calculated as nanograms of ANG I generated per hour of incubation per milliliter of superfusion fluid. (Renin release hereafter is expressed as ng ANG I/min.)

The enzymatic activity of the kallikrein was determined by measuring its esterolytic activity.6 To determine whether endogenous kinin formation could occur during the superfusion of the glomeruli with kallikrein, we determined substrate concentration within glomerular tissue and kinins in the superfusion samples. Kininogen in the glomerular homogenate was determined by the method of Fasciolo and colleagues.9 In brief, glomeruli were boiled for 15 minutes to destroy kininases, cooled in ice, and then homogenized. This solution was mixed and centrifuged at 20°C for 10 minutes, and the supernatant was removed and incubated with trypsin. After incubation, the samples were placed into four volumes of 100% ethanol, centrifuged, and the supernatant was decanted into siliconized glass tubes. The samples were evaporated under nitrogen for later determination of kinins by RIA, as previously described.10 The same RIA was used to determine kinin concentration in superfusion samples. A 0.5-ml aliquot from each collection sample was placed into four volumes of 100% ethanol and processed as just described. The BSA was treated by incubating for 3 hours at 58°C. The heat-inactivated BSA contained neither kininogenase activity nor measurable kinins. When the heat-inactivated BSA was tested for kininogenase inhibitors with purified hog kallikrein, neither the kallikrein esterase nor kininogenase activity was found to be reduced.

All values are presented as the arithmetic mean ± 1 standard error. Changes from the first (control) to the
second (experimental) period were evaluated with Student's paired \( t \) test. Values with and without added BSA were compared with an unpaired \( t \) test \((p < 0.05\) was considered significant).

**Results**

For group 1, time control, renin release in the first control period was \(4.2 \pm 1.0\) ng ANG I/min and was unchanged at \(3.6 \pm 1.0\) ng ANG I/min (NS) in the experimental period with continued Krebs superfusion (Figure 1).

In group 2, when glomeruli were superfused with kallikrein, renin increased 12-fold from \(5.1 \pm 1.2\) ng ANG I/min in the control period to \(66.0 \pm 22.7\) ng ANG I/min in the experimental period \((p < 0.025;\) Figure 1).

Renin release in group 3 during the control period was \(5.3 \pm 0.8\) ng ANG I/min and, when inactivated PMSF-kallikrein was added in the experimental period, it was \(5.1 \pm 1.0\) ng ANG I/min (NS; Figure 1).

In group 4 (time controls superfused with Krebs containing \(0.1\%\) BSA) renin release in the control period was \(16.03 \pm 2.82\) ng ANG I/min and was \(15.21 \pm 3.04\) ng ANG I/min in the experimental period \((p < 0.001)\). During the control period when renin release in the presence of BSA was compared with equivalent samples that had been superfused with Krebs only, renin release in the BSA-treated control was significantly higher \((p < 0.001)\).

In group 5, when hog kallikrein was added to the superfusate of BSA-Krebs, renin release increased significantly some twofold from \(13.04 \pm 1.81\) ng ANG I/min to \(24.30 \pm 4.76\) ng ANG I/min \((p < 0.025;\) Figure 2). In a separate series with rat kallikrein added to BSA-Krebs, renin release increased significantly from \(5.30 \pm 0.69\) to \(9.90 \pm 1.37\) ng ANG I/min \((p < 0.025)\). Matched BSA-Krebs controls did not increase \((5.14 \pm 0.79\) to \(3.22 \pm 0.95;\) \(n = 5)\).

In group 6, aprotinin was added to the BSA-Krebs superfusion fluid during the control and experimental periods. Renin released during the control period was \(15.67 \pm 3.23\) ng ANG I/min and was \(12.37 \pm 2.20\) ng ANG I/min \((p > 0.05)\) in the experimental period when kallikrein was added. Aprotinin exerted no apparent effect on the basal level of renin released during the control period, but it totally inhibited stimulation of renin released by kallikrein.

In group 7, the addition of kallikrein into the collection tubes did not affect the measurable renin concentration when compared with renin concentration of matched collections made without kallikrein (Figure 3). Over the four periods there was a steady decrease in renin concentration in both control (65%) and kallikrein-containing (56%) samples. Superfusion with Krebs containing \(0.1\%\) BSA \((n = 15)\) resulted in significantly greater renin concentrations in all periods, which decreased similarly approximately 54% over the four consecutive collections.

No measurable kininogen was found in the glomeruli incubated with trypsin. Similarly, no kinins were detected in the superfusion samples.

**Discussion**

We found that kallikrein could directly stimulate renin release from isolated glomeruli, and this effect appears to be a function of its enzymatic activity. This finding is similar to the initial reports of Suzuki and co-workers, who found that rat kallikrein and urinary esterase A could stimulate renin release from kidney slices. As in those reports, we found that aprotinin, a positively charged protease inhibitor, blocked the ac-
tion of kallikrein. We found that inactivation of kalli-
krein by PMSF, a negatively charged inhibitor, also
blocked the effect of kallikrein on renin release. Fur-
thermore, we were unable to detect the presence of
kininogen within the glomeruli, or of kinins within the
superfusion fluid, so that stimulation of renin release
by kallikrein could not have been due to kinin genera-
tion. Instead, we think kallikrein’s action is more
direct.

Although Suzuki and associates2 initially sug-
gested a direct kallikrein-renin interaction, Doi and col-
leagues3 subsequently retracted those findings and sug-
gested that the proteins — BSA or kallikrein —
prevented the loss or destruction of renin rather than
stimulated renin release. In contrast, we found that
kallikrein could induce renin release in the absence or
presence of buffer protein, and that this occurred with
purified hog pancreatic kallikrein as well as with a
partially purified rat submandibular kallikrein. When
0.1% BSA was added to the medium, we found that
basal renin levels were in fact increased two- to three-
fold, compared with matched samples obtained with
out BSA in the medium, similar to the results of Doi
and associates.3 Furthermore, this increase was consis-
tent over consecutive collections, as shown in Figure
3; however, addition of only kallikrein to collected
samples did not demonstrate any significant protective
action as proposed by Doi and colleagues. Because it is
known that some proteins in very dilute solution are
destroyed, undergo conformational changes, or adhere
to glassware, the added BSA may have preserved buff-
er renin by one of these mechanisms. Although some
of the effect of kallikrein could be explained by such a
protective effect, concentrations of BSA lower than 10
μg/ml were shown by Doi and co-workers1 to exert
little or no protective action. The kallikrein concentra-
tion used by Doi and associates,3 as well as that used in
our own studies, was only in the 2 to 3 μg/ml range,
which also should not be expected to exert any protec-
tive effect.

In the absence of albumin, renin release increased
approximately 12-fold in response to kallikrein; when
albumin was present, only a twofold increase was ob-
served. We cannot explain why the effect of kallikrein
on renin release was reduced when albumin was pres-
ent. Possibly, the albumin preparation contains some
kallikrein inhibitor; however, the addition of BSA did
not diminish either the measured esterase or kinino-
genase activity of the kallikrein. Alternatively, some
protein-protein interaction between the albumin and
kallikrein may affect the enzyme’s action on the jux-
taglomerular cells. The higher absolute renin response
to kallikrein with Krebs medium alone cannot be ex-
plained as a protective effect only, because equivalent
or greater levels of renin would be expected in samples
with 0.1% BSA and kallikrein. Further, if the effect of
kallikrein was due only to renin protection during su-
perfusion or storage, this protective effect would not
have disappeared when kallikrein was inactivated. It
could be reasoned that aprotinin eliminated the protec-
tive effect of the enzymatic protein (kallikrein) because
of its highly positive charge; however, inactivation
with either the positively charged aprotinin or the neg-
atively charged PMSF blocked renin stimulation.

We used the isolated glomeruli preparation, while
Doi and colleagues3 used a kidney slice preparation.
This difference may be important as the isolated glo-
meruli are essentially free of tubular elements, which
contain a rich supply of proteases11 that could partici-
pate in the destruction of renin.

A functional relationship between the kallikrein-
kinin and renin-angiotensin systems has been suggest-
ed by the proximity of kallikrein in the distal tubule to
the renin-producing juxtaglomerular cells and by the
fact that kallikrein can activate inactive renin.12,14
More precise localization has shown that kallikrein is
present in the connecting tubule (late distal and early
collecting tubules) and is not found in the macula
densa.15–17 It has also been reported, however, that the
distal tubule (after the macula densa) returns to the
afferent arteriole that makes contact with this vessel.18
Thus, it is possible that there is an anatomical rela-
tionship between the segment of the distal tubule that pro-
duces kallikrein and the afferent arteriole. In vivo evi-
dence indicates that inhibition of serine proteases with
aprotinin alters renin release.19 Aprotinin may inhibit
circulating kallikrein or other serine proteases not pre-
rent in the isolated glomeruli preparation. The physio-
logical significance of the direct action of kallikrein on
renin release remains unclear. Also, the high concen-
trations of the enzyme that were used in these in vitro
experiments may exert some nonspecific action on the
juxtaglomerular cells, thereby eliciting renin re-
lease. It is also possible that kallikrein mimics the
effect of another, more effective endogenous serine
protease.

In conclusion, our results indicate that in the ab-
sence of kininogen, active kallikrein can stimulate
renin release in the isolated glomeruli.

### Figure 3: Basal renin release from isolated glomeruli over four consecutive periods superfused with Krebs buffer containing 0.1% BSA, Krebs buffer without BSA, or Krebs buffer collected into tubes containing 3 μg/ml kallikrein (Periods 2, 3, and 4)
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