Adrenal Kallikrein

Hector Nolly, Ghassan Saed, Oscar A. Carretero, Gloria Scicli, and A. Guillermo Scicli

Kallikrein was identified in the adrenal glands of the rat. The enzyme was present in active and inactive forms (n=9), since preincubation with trypsin increased kallikreinogenase activity from 54.8±11.8 to 230±23 pg bradykinin per milligram protein per minute. Adrenal kallikreinogenase activity was inhibited by 91% by phenylmethylsulfonyl fluoride (2 mM), 81% by d-Phe-Phe-Arg-chloromethyl ketone (1 μM), 88% by aprotinin (1,000 KIU), and only 16% by soybean trypsin inhibitor (50 μM). Preincubation with antibodies against rat urinary kallikrein resulted in over 90% inhibition of kallikreinogenase activity. Immunoreactive glandular kallikrein was 30.7±4.8 ng/mg protein (n=11). The apparent molecular weight of the adrenal kallikreinogen was gel filtration chromatography was 33,000±500 D. Both the adrenal enzyme and the purified submandibular gland kallikrein used as a control had the same mobility on alkaline polyacrylamide gel electrophoresis. To determine whether messenger RNA (mRNA) for glandular kallikrein is present in adrenal gland RNA, we used the polymerase chain reaction employing oligonucleotide primers and glandular kallikrein 3P complementary DNA (cDNA) as a probe, which should give a cDNA fragment of 370 bp. Southern blots of the amplified products revealed a fragment of the predicted size. In conclusion, glandular kallikrein has been identified in the adrenal glands. The presence of mRNA for glandular kallikrein suggests that kallikrein is synthesized locally in this tissue. This provides an anatomic basis for possible participation of a local kallikrein-kinin pathway in the regulation of adrenal function. (Hypertension 1995;21:911–915)

KEY WORDS • kallikrein-kinin system • kininogenases • kallikrein • prekallikrein • kinins • adrenal cortex • adrenal medulla • blood pressure

Glandular kallikreins are serine proteases located in a number of organs, among them the brain and pituitary gland. Glandular kallikrein may be involved in bioregulatory processes, either directly by participation in enzymatic processing of polypeptide hormones1 or indirectly through the release of kinins.2,4-5 Because the adrenal gland is known to respond to kinins by releasing catecholamines4,6 and aldosterone, we questioned whether it might contain kallikrein. It has been postulated that glandular kallikrein acts as a paracrine enzyme, generating kinins locally.7 In the present study, we found that kallikrein and its messenger RNA (mRNA) are present in the adrenal gland, suggesting the existence of an independent adrenal kallikrein-kinin system. Tissue kallikrein in the adrenal gland may contribute to regulation of its function.

Methods

Preparation of Rat Adrenal Extracts

Male Wistar rats (Charles River, Wilmington, Mass.) weighing 250–300 g were anesthetized with sodium pentobarbital (5 mg/100 g i.p.). Catheters were placed in the left ventricle and vena cava, and phosphate-buffered saline (pH 7.4) containing heparin (12.5 units/mL) was infused through the ventricle at a rate of 200 ml/min with blood collected via the vena cava. The infusion was continued until the effluent appeared to be blood free. The adrenal glands were excised and perfused with phosphate-buffered saline saline containing 0.9% NaCl and washed by renewing the saline solution several times. The glands were then frozen at −20°C, thawed four times, and twice homogenized in the cold for 10 seconds with a Polytron homogenizer (position 7). The homogenate (200 mg wet tissue per milliliter) was centrifuged at 1,000 g for 10 minutes to eliminate debris, and the supernatant dialyzed overnight at 4°C against 10 mmol/L Tris(hydroxymethyl)aminomethane (Tris)-HCl buffer (pH 7.4). 

Incubation Procedure

Kinin-generating activity was determined by incubation of homogenate supernatant (300 μL) from tissue extract (60 mg wet tissue wt) for 4 hours at 37°C with 200 μL partially purified dog kininogen (2,000 ng kinin-releasing capability) in the presence of 1,000 μL of 0.1 mol/L Tris-HCl fresh buffer (pH 8.5) containing EDTA (15 mg/mL), 1–10 phenanthroline (1 mg/mL), 8-OH-quinoline (1 mg/mL), and soybean trypsin inhibitor (S BTI) (100 μg/mL). SBTI was used to inhibit plasma kallikrein or trypsinlike enzymes that might contaminate the preparations. The kinins generated during the 4-hour incubation period were measured by radioimmunoassay (RIA).8 Bradykinin recovery was 81±4% (n=6). Results are expressed as picograms bradykinin per milligram protein per minute of incubation. To determine optimum pH, aliquots of the adrenal homogenate were incubated with kininogen and peptidase
inhibitors at pH values varying from 5 to 9 using different buffers (0.1 mol/L acetate, 0.1 mol/L phosphate, and 0.1 mol/L Tris-HCl).

**Trypsin Activation**

To determine whether inactive kallikrein was present, the homogenates were incubated with trypsin. To establish the best ratio between trypsin and tissue homogenate for activation of inactive kininogenase, a fixed amount of tissue was preincubated for 30 minutes at 37°C with varying concentrations of trypsin ranging from 0.05 to 1.0 μg/mg tissue in 500 μL of 0.1 M Tris-HCl buffer (pH 8.5). A ratio equal to 0.2 μg trypsin per milligram tissue was found to be optimal for our use. In short, the equivalent of 100 mg tissue homogenate in 500 μL Tris was incubated with trypsin (20 μg) in 0.5 mL of 0.1 M Tris-HCl buffer (pH 8.5) for 30 minutes at 37°C, after which the reaction was stopped by adding SBTI (100 μg). In control experiments this ratio of SBTI to trypsin resulted in 100% inhibition of the esterolytic activity of trypsin against [3H]tosyl-arginine methyl ester, a trypsin substrate.

**Immunological Characterization**

Inhibition of kininogenase activity was assessed by incubating the tissue homogenates with globulin purified from rabbit antiserum against rat urinary kallikrein or from nonimmunized rabbits. 

Before incubation with kininogen, 300 μL of the homogenate supernatant from the adrenal gland (equivalent to 60 mg tissue) was incubated with 200 μg of either globulin, after which kininogenase activity was measured. Immunoreactive glandular kallikrein was measured by RIA as described previously.

**Affinity Chromatography on Immobilized Kallikrein Antibodies**

Immunoadfinity chromatography of the rat adrenal homogenate was performed as described previously. The anti-kallikrein–Sepharose gel was equilibrated with 0.1 mol/L sodium phosphate buffer (pH 7.4). The adrenal homogenate supernatant was mixed with the gel for 2 hours at room temperature, followed by 24 hours at 4°C. Unbound proteins were separated out by successive washings, first with 0.1 mol/L sodium phosphate buffer alone (pH 7.4) and then with 1 mol/L NaCl added (pH 6.0). Kininogenase activity was eluted with 0.1 mol/L sodium acetate buffer (pH 3.5) containing 1 mol/L NaCl. Samples were dialyzed against distilled water, then concentrated and frozen at -10°C.

**Gel Filtration on Ultrogel AcA64**

Two milliliters of the supernatant homogenate (35 mg protein) was applied to an Ultrogel AcA64 column (100×1 cm), equilibrated, and eluted with 0.1 mol/L phosphate buffer (pH 7.4). The column was eluted at a rate of 18 mL/hr; 3-mL fractions were collected and kininogenase activity monitored. To determine molecular weight (MW), the elution volume of the adrenal homogenate was compared with standards of known MW, namely gamma globulin (MW 158,000), ovalbumin (MW 43,000), chymotrypsinogen (MW 25,000), and myoglobin (MW 17,000). Rat submandibular gland kallikrein, used as a control, was purified using a modification of a previous method. 

**Discontinuous Polyacrylamide Gel Electrophoresis**

Disk electrophoresis was performed on 10% polyacrylamide gel as described previously. We chromatographed 200 μL of the eluate from immunoadfinity chromatography (150 μg protein) in each rod, placing purified submandibular gland kallikrein (20 μg) in a different rod to compare electrophoretic mobility. After electrophoresis, each rod was cut into 2-mm-thick slices; each slice was homogenized in 1 mL of 0.1 M HCl buffer (pH 7.4), centrifuged at 4,000g for 20 minutes, and the supernatant used for kininogenase assay.

**Inhibition Studies**

Adrenal homogenates (equivalent to 100 mg wet tissue in 500 μL Tris) were preincubated at 37°C for 30 minutes together with SBTI (100 μg/mL final concentration), phenylmethylsulfonyl fluoride (PMSF) (2 mmol/L), aprotinin (1,000 KIU), and D-Phe-Phe-Arg-chloromethyl ketone (D-PPACK) (10⁻⁸ M). The inhibitors were dissolved in 0.1 M Tris-HCl (pH 7.4), except for PMSF, which was dissolved in methanol. After the preincubation period, samples were incubated with kininogen for 4 hours at 37°C to assay kininogenase activity. Proteins were determined by Bradford’s method. As a control, we used a dilution of purified rat submandibular kallikrein that gave kininogenase activity similar to that observed with adrenal kininogenase.

**Extraction and Detection of Kallikrein Messenger RNA in Adrenal Glands**

To detect the presence of kallikrein mRNA in the adrenal gland, we amplified RNA using the reverse transcriptase–polymerase chain reaction (RT-PCR). The method used to extract RNA from the adrenal glands and the reagents and methods used for RT-PCR of adrenal RNA have been described in detail. For RT-PCR, the following oligonucleotide primers were used: 5' primer: Ex2PSKal, 3'-TCATCATTGCCT-GAAATACCTA-3') represents nucleotide positions 169-189 within the second exon of the mature glandular kallikrein mRNA sequence; 3' primer: Ex34PSKal, 5'-TCCAATTCGTTCAGGTGTAGTGATG-3') represents nucleotide positions 519-539 within the third and fourth exons of the mature glandular kallikrein mRNA sequence.

These regions are highly divergent among the various members of the kallikrein gene family and are specific for glandular kallikrein (named PS by MacDonald). Amplification of glandular kallikrein mRNA by PCR using the above primers would give a 370-bp fragment. All oligonucleotide primers were synthesized by phosphoramidite chemistry on an automated DNA synthesizer (model 380 B, Applied Biosystems, Inc., Foster City, Calif.). As a probe, we used a recombinant plasmid, pXP39, bearing a rat pancreatic kallikrein complementary DNA (cDNA) insert. The probe sequences contained the 3' 550-bp region of rat pancreatic kallikrein mRNA, which encodes 167 amino acids at the carboxyl terminal of
kallikrein plus the untranslated region. For the hybridization studies, the plasmid was digested with HindIII (Boehringer Mannheim, Indianapolis, Ind.), subjected to electrophoresis on a low-melting-point agarose gel, and the insert band extracted. The kallikrein insert was radiolabeled with 32P using the random primer method.

**Reagents**

Molecular weight markers (BioRad, N.Y.), Ultrogel (LKB, N.J.), trypsin and SBTI (Worthington Biochemical Corp., N.J.), captopril (Squibb), aprotinin (Bayer), and polyacrylamide (Eastman Kodak, N.Y.) were all analytical grade.

**Results**

Figure 1 shows active and total kininogenase activity in rat adrenal homogenates. After trypsin activation, kininogenase activity was increased almost fourfold. For comparison, parallel experiments were run with rat submandibular kallikrein. Incubation with kallikrein antibodies resulted in 90% and 92% inhibition of active and total adrenal kininogenase activity, respectively (Figure 1, left panel), and 96% inhibition of kallikrein standard (Figure 1, right panel). Kininogenase activity was also measured in pools of adrenal tissue from the cortex and medulla (n=5). Active kininogenase (picograms bradykinin per milligram per minute) was higher in the medulla (62±10 versus 53±8), as was total kininogenase activity (304±34 versus 236±42).

Adrenal kininogenase was inhibited 91±6% by PMSF, 81±5% by D-PPACK, 88±5% by aprotinin, and only 16±2% by SBTI (Table 1). The kinin-generating activity of the adrenal enzyme had an optimum pH between 8.0 and 9.0 (not shown). Quantitation of immunoreactive kallikrein by RIA in the original homogenates was not possible because of the lack of parallelism of the displacement of the adrenal samples with that of the purified rat glandular kallikrein standard; however, gel filtration removed the interference. Adrenal kallikrein was measured by RIA after gel filtration and found to be 30.7±4.8 ng/mg protein (n=11).

When assayed on AcA54, Ultrogel, the adrenal kininogenase eluted between ovalbumin (43,000 D) and chymotrypsinogen (25,000 D). Its apparent molecular weight, as calculated from the elution volume on the gel filtration column, was 33,000±500 D.

On immunoaffinity chromatography (Figure 2), kininogenase activity was eluted in a single peak with 0.1 M sodium acetate buffer (pH 3.5) and 1 M NaCl. PAGE of the adrenal gland preparation showed two peaks with kininogenase activity (Figure 3). Kallikrein antibodies inhibited only the second peak, which exhibited mobility similar to purified rat submandibular kallikrein. Both peaks resisted inhibition by SBTI.

PCR-amplified RNA from the whole adrenal gland showed a signal of the predicted size on Southern blots (Figure 4). A similar signal was observed with PCR-amplified kidney RNA.
TABLE 1. Effects of Proteinase Inhibitors on the Kininogenase Activity of Adrenal Homogenates and Submandibular Gland Kallikrein

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Rat adrenal kininogenase inhibition (%)</th>
<th>Rat glandular kallikrein inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aprotinin</td>
<td>64±7</td>
<td>91±4</td>
</tr>
<tr>
<td>100 KIU (n=3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1,000 KIU (n=5)</td>
<td>88±5</td>
<td>100±1</td>
</tr>
<tr>
<td>Soybean trypsin inhibitor</td>
<td>9±5</td>
<td>5±5</td>
</tr>
<tr>
<td>10 µg/ml (n=3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 µg/ml (n=10)</td>
<td>16±2</td>
<td>9±3</td>
</tr>
<tr>
<td>D-Phe-Phe-Arg-chloromethyl ketone</td>
<td>81±5</td>
<td>97±3</td>
</tr>
<tr>
<td>10^{-6} M (n=4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenylmethylsulfonyl fluoride</td>
<td>91±6</td>
<td>97±1</td>
</tr>
<tr>
<td>2 mM (n=4)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values in parentheses are the number of experiments.

Discussion

These findings demonstrate that a kininogenase is present in the adrenal glands. Several data indicate that this kininogenase resembles tissue kallikrein. Preincubation of rat adrenal kininogenase with aprotinin, PMSF, and D-PPACK resulted in inhibition of kininogenase, thus identifying the enzyme as a serine protease. Gel chromatography indicated that this adrenal kininogenase has an apparent molecular weight of 33,000±500 D. Its sensitivity to proteinase inhibitors as well as its molecular weight are those of glandular kallikrein: it is inhibited by aprotinin and D-PPACK but resistant to SBTI. Antibodies raised against rat urinary kallikrein recognized the adrenal kininogenase: the adrenal enzyme was strongly bound to immobilized antibodies against rat urinary kallikrein. After gel filtration, different volumes of the adrenal homogenate displaced labeled rat glandular kallikrein in the same manner as the standards in a radioimmunoassay for rat glandular kallikrein. These data indicate that the enzyme responsible for SBTI-resistant kininogenase in the adrenals closely resembles tissue kallikrein. However, we cannot exclude the possibility that we are dealing with a member of the kallikrein family of proteases with biochemical properties very similar to glandular kallikrein. In the rat there is a superfamily of kallikrein-related enzymes that may contain as many as 15 to 20 members, characterized by different substrate specificities. Amino acid sequence homology can be as much as 90%, so it is conceivable that antibodies raised against one member of the family cross-react with some other members. However, mobility of adrenal kininogenase on 10% PAGE was indistinguishable from purified rat glandular kallikrein. We also found an unidentified SBTI-resistant kininogenase that was not affected by preincubation with kallikrein antibodies, suggesting that it is not kallikrein. The lack of complete inhibition of kininogenase activity after PAGE is not consistent with the data shown in Figure 1, in which 90% of kinin-generating activity was blocked by kallikrein antibodies. The reasons for this discrepancy are not clear. PAGE was performed after immunoaffinity while the experiment shown in Figure 1 was performed with the crude adrenal extract. It is possible that the immunoaffinity column had adsorbed both kallikrein and another enzyme. The elution conditions used here would not have discriminated between true kallikrein and another enzyme still retained by the resin despite several washings. It is also possible that after immunoaffinity there was activation of a kininogenase recognized by the anti-kallikrein antibody but not blocked by it. We did not attempt to characterize this enzyme further because of the scarcity of materials.

To ascertain whether the adrenal kininogenase was indeed glandular kallikrein, we studied whether the mRNA coding for kallikrein was present in adrenal gland total RNA. For this we used the highly sensitive PCR technique. The enzymes of the kallikrein family are encoded by a multigene family; among them are glandular kallikrein, tonin, the enzyme we named submandibular enzymatic vasoconstrictor, and other related enzymes. A large number of kallikrein family member mRNAs have been sequenced in Ray MacDonal's laboratory; as far as we can determine, the...
oligonucleotides we used as primers for the RT-PCR reactions are specific, since they do not recognize other known kallikrein genes. A clear signal indicating the presence of mRNA for kallikrein was found when Southern blots were performed with RNA from the whole adrenal gland. The data indicating the presence of a kininogenase with the biochemical characteristics of glandular kallikrein, together with the RT-PCR results, clearly suggest that the glandular kallikrein-kinin pathway is present in the rat adrenal gland. We examined whether glandular kallikrein was present in the adrenal cortex, medulla, or both, measuring kininogenase activity in several pools of adrenal tissue. Active and total kininogenase were slightly higher in the medulla compared with the cortex; however, the difference was not statistically significant. Most of the enzyme appears to be in an inactive form, since incubation with trypsin did not enhance SBTI-resistant adrenal kininogenase activity nearly fourfold. Kallikrein and its mRNA are known to be present in the brain parenchyma and pituitary gland, and we have now found them in the adrenal glands as well. The presence of kallikrein in the adrenal gland is further evidence that it may be involved in bioregulatory mechanisms in the nervous system.

The function of such a kallikrein-like enzyme in the adrenal glands is not clear. Kinins induce the release of catecholamines from the rat adrenal medulla and sympathetic ganglia, so that locally generated kinins may participate in modulation of neurotransmitter release. Also, kinins, which are potent vasodilators, may help regulate medullary blood flow. The adrenal medulla contains vasoactive peptides synthesized in situ as inactive polypeptides and processed to the active form within the cell. It has been proposed that tissue kallikrein participates in processing of substances such as neural and epidermal growth factors and also high-molecular-weight atrial natriuretic factor, and we have demonstrated that it can process methylenkephalin-containing peptides as well. A serine protease able to cleave the Lys-Arg bonds of proenkephalins has been identified in the bovine adrenal medulla. It is possible that this enzyme is in fact glandular kallikrein. Other polypeptides have also been shown to be possible substrates for kallikrein-like enzymes. Thus, the kallikrein-like enzyme in the adrenal glands could also participate in local processing of polypeptide hormones in neural tissue. In addition, we found an SBTI-resistant kallikrein-like enzyme in the canine adrenal gland and ganglia stellata (Orce and Scici, unpublished data). Thus, kallikrein or kallikrein-related enzymes may be present in the adrenal glands and ganglia of species other than the rat. Furthermore, it has recently been reported that kinins can release aldosterone and cortisol from adrenocortical cells in vitro. The presence of an adrenal kallikrein-kinin system suggests that another possible function of this system is regulation of corticosteroid release.

In summary, an enzyme closely resembling glandular kallikrein in its enzymatic and immunological properties as well as its mobility on PAGE has been identified in the adrenal glands. In addition, mRNA coding for kallikrein was found in the whole adrenal gland. The presence of a local kallikrein-kinin system suggests that this pathway may contribute to regulation of adrenal function.

References
Adrenal kallikrein.
H Nolly, G Saed, O A Carretero, G Scicli and A G Scicli

Hypertension. 1993;21:911-915
doi: 10.1161/01.HYP.21.6.911

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1993 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/21/6_Pt_2/911

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org/subscriptions/