A Local Kallikrein-Kinin System Is Present in Rat Hearts

Héctor Nolly, Luis A. Carbini, Gloria Scicli, Oscar A. Carretero, A. Guillermo Scicli

Abstract  It has been reported that kinins mediate part of the beneficial cardiac effects induced by treatment with angiotensin-converting enzyme inhibitors in situations such as ischemia-reperfusion injury, myocardial infarction, and cardiac hypertrophy. However, it is not known whether the heart contains an independent kallikrein-kinin system. We measured kallikrein in tissue and in the incubation medium of heart slices. Heart slices released active and total (tryptic-activatable) kallikrein into the medium (46±5 and 380±18 pg bradykinin/mg, respectively, after 1 hour and 78±6 and 654±14 pg bradykinin/mg after 2 hours, n=7). Release was not due to tissue damage because lactate dehydrogenase, a cytosolic marker, decreased from 8.9±2.9 to 2.9±1.0 U/mg per hour. Although kallikrein was released, total tissue kallikrein in the slices did not change (423±25 pg bradykinin/mg in nonincubated slices and 370±42 pg bradykinin/mg after 2 hours, P=NS), suggesting pool replenishment. Cardiac kallikrein activity was inhibited by incubation with anti-glandular kallikrein antibodies. Pretreatment with the protein synthesis inhibitor puromycin (10 mg IP) lowered release of active kallikrein from 78±6 to 22±4 pg bradykinin/mg and total kallikrein from 654±14 to 113±9 pg bradykinin/mg (P<.001). By using reverse transcription polymerase chain reaction with kallikrein family oligonucleotide primers and a specific kallikrein probe, we found that mRNA for tissue kallikrein is present in both atrial and ventricular RNA. Kallikrein activity was also detected in primary cultures of neonatal rat atrial and ventricular cardiocytes and their incubation medium. Kallikrein mRNA was present in both cell types. Heart slices also released kininogen (1375±78 pg bradykinin/mg). Release was inhibited by puromycin (P<.001). These data demonstrate that the heart contains an independent kallikrein-kinin system. Locally generated kinins may help regulate cardiac function. (Hypertension. 1994;23(pt 2):919-923.)

Key Words  • heart • kallikrein-kinin system • kininogens • vasodilation

Methods  Preparation of Rat Cardiac Extracts

Male Wistar rats (250 to 300 g) were anesthetized with sodium pentobarbital (50 mg/kg IP). Catheters were placed in the left ventricle and vena cava, and phosphate-buffered saline (pH 7.4) containing heparin (12.5 U/mL) was infused through the ventricle at a rate of 10 mL/min with blood collected via the vena cava. The infusion was continued until the effluent appeared to be blood-free. The heart was removed, placed in a Petri dish containing Krebs-Henseleit buffer, and washed by renewing the buffer several times. It was minced, rinsed further, and frozen at −20°C until used, at which time it was twice homogenized in the cold room for 10 seconds with a Polytron homogenizer. The homogenate was centrifuged at 1000×g for 10 minutes and the supernatant dialyzed overnight at 4°C against 0.01 mol/L Tris-HCl buffer (pH 7.4).

Determination of Cardiac Kininogenase

The homogenate supernatant (400 μL) from the tissue extract (80 mg wet tissue weight) was incubated for 5 hours at 37°C with 200 μL partially purified dog kininogen (2000 ng kinin-releasing capacity) in the presence of 1000 μL fresh 0.1 mol/L Tris-HCl buffer (pH 8.5) containing a cocktail of peptidase inhibitors. Soybean trypsin inhibitor (SBTI) was used to inhibit plasma kallikrein or trypsin-like enzymes that might contaminate the preparations. Generated kinins were measured by radioimmunoassay. Bradykinin recovery was 81±4% (n=6). To determine the optimum pH of the cardiac kininogenase, we incubated aliquots of the cardiac homogenate with kininogen and peptidase inhibitors at pH levels ranging from 5 to 9 using either 0.1 mol/L acetate, 0.1 mol/L phosphate, or 0.1 mol/L Tris-HCl. Results are expressed as picograms bradykinin per milligram protein per minute incubation.
Determination of Inactive Kallikrein

To determine whether inactive kallikrein was present, we incubated the homogenates with 0.2 μg trypsin/mg wet tissue for 30 minutes at 37°C and pH 8.5, after which the reaction was stopped with SBTI (100 μg).

Immunologic Characterization

Inhibition of kininogengase activity was assessed by incubating the tissue homogenates with globulin purified from rabbit antiserum against rat urinary kallikrein or obtained from nonimmunized rabbits.8

Affinity Chromatography on Immobilized Kallikrein Antibodies

Anti-kallikrein-CH-Sepharose 4B gel (Pharmacia) was equilibrated with 0.1 mol/L sodium phosphate buffer (pH 7.4). The cardiac homogenate supernatant was mixed with the gel for 2 hours at room temperature and then for 24 hours at 4°C. Unbound proteins were eliminated 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). The kininogengase was eluted with 0.1 mol/L sodium acetate buffer (pH 3.5) containing 1 mol/L NaCl as described previously.5

Gel Filtration

Two milliliters of the homogenate supernatant (35 mg protein) was applied to an Ultrogel AcA40 column (100 x 1 cm) equilibrated and eluted with 0.1 mol/L phosphate buffer (pH 7.4) at a rate of 18 mL/h; 3-ml fractions were collected and kallikreinase activity monitored. To determine molecular weight, we compared the elution volume of the cardiac enzyme with standards as described previously.5

Discontinuous Polyacrylamide Gel Electrophoresis

Disc electrophoresis was performed on 10% polyacrylamide gels as described previously.2 We chromatographed 200 μL of the eluate from immunoaffinity chromatography (150 μg protein). Purified submandibular gland kallikrein (20 μg) was run in parallel for comparison of electrophoretic mobility. Each rod was cut into 2-mm-thick slices; each slice was homogenized in 1 mL of 0.1 mol/L Tris-HCl buffer (pH 7.4) and centrifuged at 4000g for 20 minutes, and the supernatant was used for kallikreinase assay.

Inhibition Profile

Cardiac homogenates (80 mg wet tissue per 400 μL buffer) were preincubated at 37°C for 30 minutes together with phenylmethylsulfonyl fluoride (PMSF, 2 mmol/L), aprotinin (1000 KIU), D-Phe-Phe-Arg-chloromethyl ketone (D-PPACK, 10−6 mol/L), and SBTI (100 μg/mL final concentration). Then samples were incubated with kininogen for 5 hours at 37°C to assay kallikreinase activity.

Release of Kallikrein From Heart Slices

Slices 2 mm thick were obtained from the surface of ventricles taken from controls and rats treated with puromycin (10 mg IP, 3 hours before the experiment). After several rinses with Krebs-Henseleit buffer, the slices were incubated in oxygenated Krebs-Henseleit solution at 37°C. The buffer was renewed four times every 30 minutes. Kallikreinase activity was measured in the supernatants and tissue. Cell damage was monitored by the release pattern of lactate dehydrogenase (LDH) as measured by standard protocols.

Measurement of Kininogen

Kininogen was assayed by a modification of the technique of Diniz and Carvalho.9 The samples (1000 μL) were boiled for 60 minutes at 37°C (pH 8.5) in the presence of peptidase inhibitors. The incubation was ended by adding SBTI (100 μg).

Extract of RNA, Reverse Transcription, Polymerase Chain Reaction Amplification, and Southern Blot

DNA was extracted as described previously.7 The RNA concentration was determined by measuring the optical density at 260 nm. The upstream (KALPCRU2) and downstream (KALPCRU4) primers used for amplification and the probe (PSPCRex2) oligonucleotides have been described previously.6; the amplified product has a predicted size of 430 bp. Reverse transcription polymerase chain reaction (RT-PCR) was carried out with the rTth polymerase (Perkin-Elmer) following the manufacturer's recommended protocol. Briefly, 250 ng total RNA was reverse-transcribed at 60°C for 20 minutes and PCR-amplified according to the following protocol: 1 minute at 94°C, 30 seconds at 55°C, and 1 minute at 72°C (with 1-second extension per cycle) for 35 cycles. Fifteen picomoles of each primer was used. The RT controls were performed with all reagents except MnCl2. Fifty micromers of the PCR products was run in a 1.5% agarose gel at 5 V/cm. At the end of the run, the gel was denatured (1.0 mol/L NaCl/0.5 mol/L NaOH, 30 minutes) and neutralized (0.5 mol/L Tris, pH 7.4/1.5 mol/L NaCl, 30 minutes), and the DNA was transferred to a Nytran membrane by capillary blotting. UV cross-linking was carried out for 2 minutes. The membrane was prehybridized for 2 hours and then hybridized overnight at 50°C. The oligonucleotide probe was end-labeled with 32P by the terminal transferase method. At the end of the hybridization, the blots were washed four to five times for 15 minutes each in 0.1 x SSC/0.1% sodium dodecyl sulfate at 40°C and exposed to x-ray film.

Statistics

Two-sided, two-sample t tests were used to assay kininogengase activity in rat heart slices. Bonferroni's adjustment for multiple comparisons was used. Unless otherwise noted, all results are expressed as mean±SEM.

Results

Homogenates from rat hearts contained SBTI-resistant kininogengase activity (Fig 1). Approximately 10% was in an active form; the rest required activation by

![Fig 1. Bar graph shows kininogengase activity resistant to inhibition by soybean trypsin inhibitor in cardiac tissue. Total kininogengase activity was determined after activation of inactive kallikrein with trypsin. KK Ab indicates kallikrein antibodies; Bk, bradykinin.](http://hyper.ahajournals.org/doi/abs/10.1161/01.HYP.23.6.920)
trypsin. Cardiac kininogenase activity was inhibited by preincubation with anti-glandular kallikrein antibodies. On immunoaffinity chromatography, kininogenase activity was eluted in a single peak with 0.1 mol/L sodium acetate buffer (pH 3.5) and 1 mol/L NaCl (Fig 2). On disc polyacrylamide gel electrophoresis, mobility of both cardiac kininogenase and urinary kallikrein was similar (Fig 3). The molecular weight of the cardiac kininogenase was calculated from its elution volume on gel filtration and found to be approximately 35 000 D (n=2).

Analysis of the effect of proteinase inhibitors on cardiac kininogenase activity showed that the enzyme was inhibited 92±5% by PMSF, 90±2% by aprotinin, 88±4% by D-PPACK, and only 10±3% by SBTI (n=5). The cardiac enzyme had an optimum pH of 8.5±0.5 (not shown).

Kininogenase activity was also found in homogenates of cultured neonatal rat cardiomyocytes obtained from both atria and ventricles as well as in the incubation medium (Table). The presence of mRNA for kallikrein in adult rat cardiac tissue was determined by RT-PCR using primers that amplify all members of the kallikrein family and kallikrein-specific probes. A hybridization band of the predicted size was obtained from atrial and ventricular RNA. Kallikrein mRNA was also present in atrial and ventricular cardiomyocytes (Fig 4).

Both active and trypsin-activatable kininogenses were released into the medium by heart slices. Release was continuous during the 2-hour incubation period. Release was not due to tissue damage, because LDH, a cytosolic marker, decreased from 8.9±2.9 to 2.9±1.0 U/mg per hour. Although kallikrein was released, total tissue kallikrein in the slices did not change (423±25 in nonincubated slices and 370±42 pg bradykinin/mg after 2 hours, P=NS), suggesting pool replenishment. Pretreatment of the donor rat with the protein synthesis inhibitor puromycin significantly lessened the release of cardiac kininogenase (Fig 5).

Discussion

The present data demonstrate that an enzyme having the characteristics of glandular kallikrein is present in the rat heart. The cardiac enzyme was isolated from rat heart homogenates by affinity chromatography on immobilized kallikrein antibodies and shown to be an active serine protease with kininogenase activity. The cardiac kininogenase and rat urinary kallikrein had a similar elution volume when chromatographed on an Ultrogel AcA34 column and exhibited similar mobility on 10% polyacrylamide gel electrophoresis. Moreover, the isoelectric point, molecular mass, optimum pH, immunologic properties, and susceptibility to inhibitors were indistinguishable from those of glandular kallikrein. The mRNA coding for glandular kallikrein was present in the atria and ventricles and in cultured primary neonatal rat atrial and ventricular cardiomyocytes. Thus, the presence of both the enzyme and its mRNA substantiates the existence of a kallikrein-kinin pathway in the heart. This extends our previous observations and confirms the report of Xiong et al.

When epicardial slices were incubated in vitro, kallikrein was released into the medium in concentrations too high to be the result of nonspecific release due to damage, as indicated also by the lack of association between kallikrein and LDH activity. Kallikrein release was significantly decreased by pretreatment with the protein synthesis inhibitor puromycin. This suggests that the released kallikrein originates from a pool that requires de novo synthesis.

The hypothesis that the heart contains an independent kallikrein-kinin system is also strengthened by the fact that kallikrein was found in epicardial slices and in the medium bathing the slices (Fig 6). Kallikrein release into the medium after 2 hours of incubation was three times higher than with nonincubated slices (1375±78 versus 423±28 pg bradykinin/mg, n=7). Release of kallikrein from cardiac slices was significantly inhibited by pretreatment with puromycin (P<.001), again suggesting de novo synthesis. These data suggest that cardiac tissue synthesizes and releases both kallikrein and kallikrein.

Kininogenase Activity (SBTI-Resistant) in Cultured Rat Neonatal Cardiocytes

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<tr>
<th>Tissue</th>
<th>Medium</th>
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<tr>
<td>Atria</td>
<td>Ventricles</td>
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<tr>
<td>Active</td>
<td>600±32</td>
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<td>Total</td>
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Activity is shown in picograms bradykinin per milligram protein per hour. Data are mean±SD of cardiocytes from two pools of cardiomyocytes obtained from 30 hearts each. SBTI indicates soybean trypsin inhibitor.
Rats have three types of kininogen: low-molecular-weight and high-molecular-weight kininogen and T-kininogen. Only low-molecular-weight and high-molecular-weight kininogen are substrates for glandular kallikrein. The present data do not allow us to distinguish which type of kininogen is present in the heart; nevertheless, this is, to our knowledge, the first time that evidence of a cardiac kininogen has been reported. Plasma contains substantial amounts of kininogens. Thus, cardiac kallikrein may generate kinins from plasma kininogen and possibly from locally synthesized kininogen as well.

It has been reported that the mRNA for high-molecular-weight kininogen is present in endothelial cells and that vascular smooth muscle cells in culture release both kallikrein and kininogen. In addition, we have previously demonstrated that kallikrein and its mRNA are present in vascular tissue. Indeed, the presence of kinin-generating enzymes in the heart and vascular tissue strengthens the hypothesis that locally generated kinins can act as a paracrine hormone, regulating blood flow within the heart. A recent study suggests that when the system is stimulated by blocking degradation of endogenous kinins with ACEi, kinins mediate some of the functional changes induced by these drugs. For example, it has been reported that in isolated perfused rat hearts with myocardial ischemia, bradykinin increases coronary flow and reduces the incidence and duration of ventricular fibrillation after reperfusion. The incidence of arrhythmias was suppressed by treatment with an ACEi, and this protective effect was negated if a kinin receptor antagonist was also present. Recently, it was reported that a kinin antagonist obliterated the reduction in infarct size provided by ACEi in an ischemic reperfusion injury model. These data suggest that ACEi act by a kinin-mediated mechanism in these models. These kinins may be generated locally by the cardiac kallikrein-kinin system described here.

In summary, glandular (tissue) kallikrein, a potent kinin-generating enzyme, is present in the rat heart and in cultured primary neonatal ventricular cardiocytes. Epicardial slices released both glandular kallikrein and kininogen. This was inhibited by puromycin. RT-PCR demonstrated the mRNA coding for glandular kallikrein in both atria and ventricles, as well as in cardiocytes in culture. The presence of a local kallikrein-kinin system suggests that this pathway may act as a paracrine system contributing to the regulation of cardiac function.

References


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Hypertension. 1994;23:919-923
doi: 10.1161/01.HYP.23.6.919

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1994 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

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