Biochemical Evidence of a Kallikrein-like Activity in Rat Reproductive Tissues

Roberto Miatello, Maria Lama, Susana González, Teresa Damiani, Héctor Nolly

Abstract We performed this study to examine the presence of a kallikrein-kinin system in rat fetal and maternal tissues. Uteri and placentas from Wistar pregnant and nonpregnant rats were perfused to eliminate blood, and fetal membranes were washed several times with saline. Amniotic fluids were obtained without blood contamination by amniocentesis from eight rats. The different samples were homogenized and centrifuged (2000g during 20 minutes), and the supernatant was incubated with dog kininogen and 0.1 mol/L Tris-HCl buffer (pH 8.5) in the presence of peptidase inhibitors. Kinins released were measured by radioimmunoassay. Kininogenase activity was found in rat uteri, placental vessels, amniotic fluids, and fetal membranes. The enzymes were present in active but mostly in inactive forms. The kallikrein-like enzymes found in the different preparations and rat urinary kallikrein used as control had similar molecular weights, immunologic characteristics, and inhibition profiles with protease inhibitors.

We conclude that kallikrein-like enzymes are present in rat organs of reproduction. These data suggest that kinins released locally may act as paracrine hormones in the regulation of blood pressure during pregnancy. (Hypertension. 1994;23 [suppl I]:I-193-I-197.)

Key Words • kallikrein-kinin system • kallikrein • kinins • blood pressure • uterus • placenta • amniotic fluid • amnion

Methods

Adult female Wistar rats between 14 and 15 days' gestation (term rat pregnancy, 22 days) weighing 200 to 250 g were anesthetized with sodium pentobarbital (50 mg/kg IP). The animals were treated in accordance with the guiding principles approved by our Institutional Review Committee and the experimental procedures set forth in the Declaration of Helsinki. A catheter was introduced into the aorta, and the uteri were perfused by hydrostatic pressure with saline until the fluid flowing out of the inferior cava appeared free of blood (usually 5 minutes). Uteri from pregnant and nonpregnant rats were dissected free of placenta and fetal materials and were washed thoroughly to remove all blood. Amniotic fluid was obtained without blood contamination by amniocentesis. Placentas and fetal membranes were also removed and placed in a Petri dish containing saline, washed by renewing the buffer several times, and then minced, rinsed further, and frozen at −20°C until use.

Preparation of Rat Tissue Extracts

The homogenate (200 mg wet tissue/mL) was centrifuged at 1000g for 10 minutes to eliminate debris, and the supernatant was dialyzed overnight at 4°C against 0.01 mol/L Tris-HCl buffer (pH 7.4).

Incubation Procedure

The homogenate supernatant (400 µL) from different tissue extracts (80 mg wet tissue weight) was incubated with partially purified dog kininogen (2000 ng kinin-releasing capability) as previously described. The kinins generated during the 5-hour incubation period were measured by radioimmunoassay. Bradykinin recovery was 80±5% (n=6). Results are expressed as picograms bradykinin per milligram protein per minute of incubation. For determination of the optimum pH, aliquots of the homogenates were incubated with kininogen and peptidase inhibitors at pH levels ranging 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

For determination of whether inactive kallikrein was present, the homogenates were trypsinized as described previously.

Pregnancy is associated with striking physiological changes that enable the gravida to accommodate the growing products of conception. These changes—quite profound in the cardiovascular system, kidney, and uterus—have been characterized extensively but never understood completely. Despite increases in plasma volume and cardiac output, blood pressure actually declines. This reduction occurs because peripheral vascular resistance in pregnancy decreases significantly from normal nonpregnant levels. The renin-angiotensin-aldosterone system is activated markedly during pregnancy. Decreased blood pressure in the face of elevated plasma levels of angiotensin suggests that pregnant women are resistant to the pressor effect of angiotensin. Precisely why pregnancy reduces peripheral vascular resistance is unknown. Recently, it has been proposed that the reduced peripheral vascular resistance is due to an increased production of vasodilator substances. Kinins are potent vasodilator peptides released from kininogen by kininogenases. There is now clear evidence that part of the hemodynamic effect of kinins are mediated by prostaglandins and endothelium-derived relaxing factors. Kallikrein has been implicated in the regulation of uteroplacental blood flow; however, whether or not kallikrein can be found within these tissues is not known. Although kinin-forming activity has been reported in human organs of reproduction, further characterization is needed because proteases other than kallikrein can also release kinins. In this study we examined the presence and biochemical characteristics of kallikrein-like enzymes in the uteroplacental-fetal complex.

From the Argentine Council of Research (CONICET), School of Medicine, UNC Mendoza, Argentina.

Correspondence to Héctor Nolly, MD, López y Planes 743, V. Hipódromo–5547, Mendoza, Argentina.
A ratio equal to 0.2 μg trypsin per milligram tissue was found to be optimal. The reaction was stopped by addition of soybean trypsin inhibitor (SBTI) (1 μg/mg tissue), after which total (active plus inactive) kallikrein was measured.

**Immunologic Characterization**

Inhibition of kininogenase activity was assessed by incubation of the tissue homogenates with globulin purified from rabbit antiserum against rat urinary kallikrein or from nonimmunized rabbits. Before incubation with kininogen, 400 μL of the homogenate supernatant from the tissues (equivalent to 80 mg tissue) was incubated with 200 μg of either globulin, after which kininogenase activity was measured.

**Affinity Chromatography on Immobilized Kallikrein Antibodies**

Immunofinity chromatography of the different preparations was performed according to a technique previously described.

**Gel Filtration and Molecular Weight Determination**

Two milliliters of the supernatant homogenate (=35 mg protein) was applied to an Ultrogel AcA₄₅ column (100x1 cm) equilibrated and eluted with 0.1 mol/L phosphate buffer (pH 7.4). The column was eluted at a rate of 18 mL/h; 3-mL fractions were collected and kininogenase activity monitored. To determine molecular weight (MW), the elution volume of the present enzyme was compared with standards of known MW, namely, γ-globulin (MW 158 kD), ovalbumin (MW 43 kD), chymotrypsinogen (MW 25 kD), and myoglobin (MW 17 kD). Rat submandibular gland kallikrein, used as control, was purified using a modification of a previous method.

**Inhibition Studies**

Homogenates (equivalent to 80 mg wet tissue in 400 μL Tris) were preincubated at 37°C for 30 minutes together with SBTI (100 μg/mL), phenylmethylsulfonyl fluoride (PMSF, 2 mmol/L), aprotinin (1000 KIU), and D-Phe-Phe-Arg-chloromethyl ketone (10⁻⁵ mmol/L) (final concentrations). The inhibitors were dissolved in 0.1 mol/L Tris-HCl (pH 7.4), except for PMSF, which was dissolved in methanol. After the preincubation period, samples were incubated with kininogen for 5 hours at 37°C for assay of kininogenase activity. As a control, we used a dilution of purified rat submandibular kallikrein, which gave kininogenase activity similar to that observed in this study.

Proteins were determined by Bradford's method as previously described.

**Reagents**

MW markers (Bio-Rad, NY), Ultrogel (LKB, Piscataway, NJ), and trypsin and SBTI (Worthington Biochemical Corp, Worthington, NJ) were all analytical grade. The kininogen used was a gift from Dr G. Scicli.

**Statistics**

Two-sided, two-sample t tests were used for examination of kininogenase activity. Unless otherwise noted, all results are expressed as mean±SEM.

**Results**

Fig 1 shows active and total kininogenase activity in rat uterus. Most of the enzyme was present in an inactive form. After trypsin activation, kininogenase was increased almost fivefold. Incubation with kallikrein antibodies almost completely (88±3%) inhibited kininogenase activity, whereas it was resistant to SBTI. Gravid rat uterus (14 days' gestation, n=8) contained larger amounts of total kininogenase (334±16 pg bradykinin/mg per hour) than nongravid uterus (279±20 pg bradykinin/mg per hour; n=7) (P<.01). When assayed on AcA₄₅ Ultrogel, the uterus kininogenase eluted between ovalbumin (43 kD) and chymotrypsinogen (25 kD). Its apparent MW as calculated from the elution volume on the gel filtration column was 38±1 kD; for placenta, amnion and amniotic fluid also ranged between 36 and 38 kD. The kinin-generating activities of different tissues showed an optimum pH of 8.5. On immunoaffinity chromatography, kininogenase activity

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**Fig 1.** Bar graph shows kininogenase activity of rat pregnant uterus: active kallikrein (left bar), total kallikrein (middle bar), and trypsin-treated samples plus kallikrein antibodies (KK-Ab) (right bar). Bk indicates bradykinin; SBTI, soybean trypsin inhibitor.
eluted in a single peak with 0.1 mol/L sodium acetate buffer (pH 3.5) and 1 mol/L NaCl (data not shown).

Fig 2 shows the kininogenase activity present in amnion. The kininogenase activity remained unmodified after trypsin treatment (data not shown). Active kininogenase was blocked by kallikrein antibodies and was resistant to SBTI.

The kininogenase activity in rat placenta was partially blocked by incubation with SBTI. After trypsin activation, kininogenase increased threefold. Kallikrein antibodies completely inhibited the kininogenase activity (Fig 3).

Fig 4 shows low but detectable amounts of kininogenase activity in amniotic fluid. The activity increased threefold by incubation with trypsin, and it was completely inhibited by incubation with kallikrein antibodies.

The Table compares the sensitivity of kininogenases from maternal tissues and fetal membranes to several proteinase inhibitors.

**Discussion**

This study demonstrates the presence of kinin-forming enzymes in rat maternal tissues and fetal membranes. The insensitivity of these kininogenases to SBTI
readily distinguishes them from trypsin and plasma kallikrein. The kininogenases described here appear to be quite similar to rat glandular kallikrein. They have similar MWs and optimum pH values and inhibition profiles. We also examined the ability of kallikrein antibodies to neutralize the kallikrein-like activity. In every case, at least 80% of the kallikrein-like activity is inhibited by the anti-kallikrein antiserum, indicating that most of the activity is due to immunoreactive kallikrein. After passage through immunoaffinity columns followed by gel filtration chromatography, we obtained partially purified enzymes that allowed us to further study its physicochemical properties.

An inactive form of kallikrein was detected in rat uterus, placenta, and amniotic fluids but surprisingly not in rat amnion. The enzyme was activated by trypsin treatment. Inactive kallikrein is present in high concentrations, constituting approximately 90% of the total kininogenase activity in the different preparations. The lack of inactive kallikrein in amnion was an unexpected finding.

The presence of kinin-forming enzymes in rat organs of reproduction suggests but does not prove that the enzymes are locally synthesized in the uteroplacental complex. The mRNA coding for glandular kallikrein has been found in rat uterus (G. Valdés, J. Corthorn, O.A. Carretero, A.G. Sicil, personal communication). Evidence in support for mRNA for kallikrein in placenta, amniotic fluid, and fetal membranes is lacking at the present time. The kallikrein-like enzyme in the amniotic fluid can have several origins. The maternal kidney could be a reasonable source, because the maternal fluid is in constant equilibrium with amniotic and fetal fluid. Another possibility is that the fetal kidney can make a contribution through the urine that is eliminated in the amniotic fluid; or this enzyme may originate partly from the cells of the placenta, fetal membranes, or uterus and then be transferred to the amniotic fluid. This is a reasonable possibility because we have found kallikrein-like enzymes in these tissues.

These enzymes may participate directly in the processing of polypeptide hormones and indirectly through the release of kinins in the regulation of blood pressure during pregnancy and labor.

In summary, proteases that resemble tissue kallikrein are present in rat reproductive tissues. These enzymes are in both active and inactive forms, and the MWs, optimum pH values, and immunologic characteristics are similar to those of tissue kallikrein. This finding is consistent with an autocrine or paracrine role for these enzymes in the regulation of the utero-placental-fetal blood flow and the processing of polypeptide hormones.

<table>
<thead>
<tr>
<th>Effects of Proteinase Inhibitors on Kininogenase Activity of Fetal Membranes and Maternal Tissues</th>
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<tbody>
<tr>
<td><strong>Percent Inhibition</strong></td>
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<tr>
<td>------------------------</td>
</tr>
<tr>
<td>PMSF (2 mmol/L) (n=3)</td>
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<tr>
<td>D-PPACK (10⁻⁸ mmol/L) (n=3)</td>
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<tr>
<td>SBTI (100 μg/mL) (n=6)</td>
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<tr>
<td>Aprotinin (100 KIU) (n=3)</td>
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PMSF indicates phenylmethylsulfonyl fluoride; D-PPACK, d-Phe-Phe-Arg-chloromethyl ketone; and SBTI, soybean trypsin inhibitor.
References

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