Oxytocic Effect of Trypsin on the Isolated Rat Uterus

Gabriel Orce, Graciela Castillo, Sergio Esteban

Abstract To study the oxytocic effect of trypsin, we measured the force of isometric contraction in uteri isolated from estrogenized rats exposed to trypsin (8.8×10^-10 to 1.7×10^-8 mol/L) either alone or in the presence of receptor antagonists to angiotensin II (saralasin ([Sar^1]Angiotensin II) or DuP 753 [losartan]) or to kinins (d-[Arg^8]Hyp^3,Thi^5,D-Phe^7]-bradykinin). We found that saralasin or DuP 753, but not the kinin antagonist, displaced the dose-response curve to the right without altering the effects of oxytocin or bradykinin. Enalaprilat (an angiotensin I converting enzyme inhibitor) or pepstatin A (a renin inhibitor) also displaced the dose-response curve to trypsin to the right, without altering the effects of oxytocin or angiotensin II. Our results indicate that the response to trypsin is mediated by an agent produced from a substrate present in the uterus and acting on the angiotensin II type 1 receptor and are consistent with both renin and angiotensin I converting enzyme being involved in its mechanism of action, thus supporting the notions that the renin-angiotensin system may be important in the late stages of pregnancy and that serine proteases existing in the uterus may contribute to its activation. (Hypertension. 1994;23[suppl 1]: I-250-I-255.)

Key Words • angiotensin • bradykinin • enalaprilat • oxytocin • renin-angiotensin system • pepstatins

The isolated rat uterus contracts when exposed to the serine proteases kallikrein and trypsin even if no exogenous substrate is added to the incubation solution. Recently, we reported that the oxytocic effect of kallikrein, believed to be due to a direct action of the enzyme, was mediated at least in part by kinins generated by the enzyme acting on a substrate present in the uterus. The oxytocic effect of trypsin exhibits similarities to that of kallikrein: (1) Trypsin also generates kinins from kininogens of various origins, (2) after repeated exposure to either enzyme, the uterus loses its responsiveness to it, and (3) uteri desensitized to trypsin also become nonresponsive to kallikrein (although desensitization to kallikrein does not imply loss of responsiveness to trypsin). This gave rise to the notion that the oxytocic action of trypsin was mediated via kinin generation. The oxytocic effect of trypsin on uteri rendered nonresponsive to kallikrein was assumed to be due to the lower specificity of the former, which allowed it to generate kinins from kallikrein-resistant substrates even after the kallikrein-sensitive substrate was exhausted.

We performed dose-response curves (DRCs) to trypsin in the absence or presence of receptor antagonists for other oxytocic agents (bradykinin and angiotensin II) and report here that the effect of trypsin involves the enzymatic release from a substrate present in the uterus of a mediator acting on the Ang II type 1 (AT_1) receptor.

Methods

[Val^3]Ang II amide (Hypertension, Ang II) and human angiotensin I (Ang I) (Bachem, Bubendorf, Switzerland)

were gifts from CIBA-GEIGY, Basel, Switzerland. N-(1-S-carboxy-3-phenylpropyl)l-ala-l-pro (enalaprilat; Merck Sharp & Dohme, West Point, Pa), oxytocin (Sytocinon, Sandoz Argentina), [Sar^1]Ang II (saralasin, Norwich-Eaton, Norwich, NY), and 2-n-butyl-4-chloro-5-(hydroxy-methyl)-1-[[2'-(1H-tetrazol-5-yl)biphenyl-4-yl]methyl]imidazole, potassium salt (DuP 753, Du Pont Merck, Wilmington, Del) were generous gifts from the producers. Sodium amobarbital, dexam (average molecular weight, 18400), bradykinin, [d-Arg^8]Hyp^3,Thi^5,D-Phe^7]-bradykinin (bradykinin antagonist), and isovaleryl-Val-Val-Ala-Ala-Sta (pepstatin A) were from Sigma Chemical Co, St Louis, Mo; bovine pancreatic trypsin (3x crystallized, dialyzed salt-free, lyophilized; molecular weight, 23 200) was from Millipore Corp, Freehold, NJ; and estradiol valerate in oil (Progynon Depot) was from Schering Argentina. Water-soluble drugs were dissolved in de Jalon's solution (see below) containing 0.1% bovine serum albumin and were diluted whenever necessary with the same solution. Pepstatin A dissolved in ethanol was added directly to the bath (ethanol volume was <0.3% of total bath volume, far below that causing alteration of rat myometrial contractility). Trypsin, pepstatin A, and enalaprilat were prepared shortly before use and discarded at the end of the experiment; stock solutions (1 mmol/L or higher) of the rest of the drugs were kept for several days at -20°C. Female rats (150 to 200 g) were injected subcutaneously with estradiol (200 µg/kg body wt) 24 to 48 hours before the experiment. The animals were killed by a sharp blow to the head; the uterine horns were dissected free of mesenteric attachments in situ, and a 2-cm length was tied to a wire holder and placed in an isolated organ bath containing 3 mL of de Jalon's solution (composition in g/L: NaCl, 9.00; KCl, 0.42; glucose, 0.50; NaHCO_3, 0.50; and CaCl_2, 0.03, pH 7.4) kept at 27°C and bubbled with 95% O_2-5% CO_2. Force of isometric contraction was measured with a model 1030 force transducer (UFI, Morro Bay, Calif) connected to a paper strip recorder (Gilion Medical Electronics Inc, Middleton, Wis). The preparation was allowed to rest under a tension of 1 g for 1 hour, during which the solution was changed several times. Given the rhythmic nature of the response, the preparation was rinsed three times with fresh solution as soon as the first contraction reached its peak and was allowed to rest until the recorder tracing returned to baseline value before the next dose.

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3.0

2.0

1.0

\[ \text{F (g)} \]

\[ \text{LOG [TR]} \]

**FIG 1.** Line graphs show displacement of dose-response curve to trypsin (TR) by 2.5 \( \times 10^{-10} \) mol/L saralasin (SAR) (left) and 5.0 \( \times 10^{-8} \) mol/L SAR (right) added to the bath 1 minute before trypsin. Curves were significantly different from each other by ANOVA (overall, \( P < .002 \) left, \( P < .01 \) right). *\( P < .05 \) vs control preparation.

was applied. Tissue reactivity was assessed by recording responses to oxytocin and Ang II or bradykinin at regular intervals. The responses were measured (in grams) as force developed at the peak of contraction.

In some experiments, one uterine horn was perfused to reduce its plasma content. The rats were anesthetized with sodium amobarbital (100 mg/kg body wt), and a PE-10 catheter was placed in the jugular vein. String ties were placed loosely around the tail and hind limbs, the abdomen was opened, and ligatures were placed loosely around the aorta and vena cava. The renal pedicles, as well as both ends and all mesenteric attachments of one uterine horn, were tightly ligated, taking care to include all vessels supplying them. Heparin (500 IU) was injected via the jugular vein, and 10 minutes later the rat was killed by bilateral pneumothorax. The ligatures around the limbs, tail, aorta, and vena cava were tightened, and a PE-50 catheter was inserted in each vessel below the ligature. Kreb's solution (composition in g/L: NaCl, 6.9; KCl, 0.35; MgSO\(_4\), 0.14; KH\(_2\)PO\(_4\), 0.16; glucose, 2.0; NaHCO\(_3\), 2.0; and CaCl\(_2\), 0.2, pH 7.4, gassed with 5% CO\(_2\)-95% O\(_2\)) containing dextran (18.4 g/L) was perfused through the aortic catheter at 5 mL/min with a perfusion syringe (Harvard Apparatus, South Natick, Mass); perfusion was continued for 10 minutes after the perfused horn became uniformly pale (15 to 20 minutes total perfusion time). The horns were dissected free without interrupting perfusion (to prevent backflow of blood from surrounding territories) and mounted in standard fashion. The procedure removes more than 85% of the plasma contaminating the isolated horn without causing visible edema.\(^6\) DRCs were routinely obtained by recording responses to increasing concentrations of each agent, achieved by adding aliquots of its stock solution to the bath in the absence and presence of a concentration of the antagonist devoid of effect per se and known by preliminary experiments to inhibit responses to its respective agonist.
Data are reported as mean±SEM. Statistical analysis was performed by means of three-way ANOVA, using a model incorporating type of drug, concentration, and preparation variability as factors; responses to individual doses were compared using Student's t test for paired data. Animals were at all times treated according to the directives set forth by the National University of Tucumán regarding the humane treatment of research animals.

**Results**

**Effect of Saralasin**

The DRC to trypsin was displaced to the right by the peptide Ang II analogue saralasin (2.5×10⁻¹⁰ mol/L, Fig 1, left); at 5.0×10⁻⁹ mol/L, saralasin also caused a depression of the maximal response to trypsin (Fig 1, right), reminiscent of a similar behavior compared with Ang II (data not shown). Full responsiveness to trypsin was restored by rinsing the preparation.

**Effect of DuP 753**

The DRC to trypsin was also displaced to the right in the presence of the nonpeptide AT₁ receptor antagonist DuP 753 at 4.8×10⁻⁸ mol/L (Fig 2). Rinsing the preparation restored full responsiveness to trypsin.

**Effect of Angiotensin I**

Exposure of the preparation to Ang I greatly reduced further responses to the peptide, as well as to Ang II and trypsin, even after the preparation was rinsed.
repeatedly; no change was observed in the responses to either bradykinin or oxytocin (Fig 3).

Influence of Changes in the Dose-Response Curve Protocol on the Response to Trypsin

On the assumption that changes in the protocol should alter the pattern of substrate consumption, thus presumably modifying substrate availability and the response to trypsin, the usual protocol (nine increasing doses of the enzyme) was changed either to exposure to the same nine doses in random order or to four increasing doses, including the ones yielding the lowest and highest responses. No difference was found by ANOVA in the magnitude of the maximal responses (values were 2.31±0.55 g, \( P<.01 \), \( n=6 \); 2.74±0.32 g, \( P<.01 \), \( n=6 \); and 2.61±0.37 g, \( P<.01 \), \( n=5 \), respectively).

Lack of Effect of the Kinin Antagonist

The response to trypsin was not altered in the pres- ence of the kinin antagonist at 5.0×10⁻⁷ mol/L, a concentration known to inhibit responses to kallikrein and bradykinin⁶ (Fig 4).

Effect of Trypsin in Perfused Uteri

The DRC to trypsin in uterine horns previously perfused with Krebs' solution exhibited no significant

<p>| Effect of 2.0×10⁻⁴ mol/L Enalaprilat or 9.7×10⁻⁷ mol/L Pepstatin A on the Response of Isolated Uterus to Individual Doses of Oxytocin or Bradykinin |
|---------------------------------|-----------------|-----------------|--------------|</p>
<table>
<thead>
<tr>
<th><strong>Agent and Dose</strong></th>
<th><strong>Alone</strong></th>
<th><strong>Plus Inhibitor</strong></th>
<th><strong>P</strong></th>
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</thead>
<tbody>
<tr>
<td>Oxytocin</td>
<td></td>
<td></td>
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<tr>
<td>3 mU/mL</td>
<td>1.20±0.14*</td>
<td>1.28±0.10*</td>
<td>NS</td>
</tr>
<tr>
<td>20 mU/mL</td>
<td>1.86±0.18*</td>
<td>2.10±0.18*</td>
<td>NS</td>
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<tr>
<td>Oxytocin</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>3 mU/mL</td>
<td>1.11±0.13*</td>
<td>1.04±0.24*</td>
<td>NS</td>
</tr>
<tr>
<td>20 mU/mL</td>
<td>2.09±0.23*</td>
<td>1.91±0.20*</td>
<td>NS</td>
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<tr>
<td>Bradykinin</td>
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<tr>
<td>1.0×10⁻⁷ mol/L</td>
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<td>2.0×10⁻⁸ mol/L</td>
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<td>2.45±0.35*</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are oxytocic responses to agents listed, first alone and then after a 30-minute exposure to either enalaprilat or pepstatin A; inhibitor remained in the bath throughout the response. \( P \) indicates statistical significance of the difference between the two values.

*\( P<.01 \), †\( P<.05 \), different from zero (\( n=4 \)).
differences compared with nonperfused paired controls (Fig 5).

**Effect of Enalaprilat**

Enalaprilat (2.0 x 10^-4 mol/L) displaced the DRC to trypsin to the right without change in the maximal effect of the enzyme (Fig 6, left). In contrast, the DRC to Ang II was not affected by enalaprilat (Fig 6, right), nor were the responses to individual doses of oxytocin affected (Table).

**Effect of Pepstatin A**

Pepstatin A (9.7 x 10^-7 mol/L) strongly inhibited the contractile response to trypsin (Fig 7, left); the DRC to Ang II was not modified (Fig 7, right). The responses to individual doses of bradykinin or oxytocin were not altered (Table).

**Discussion**

Our findings strongly suggest the oxytocic activity of trypsin on the rat uterus to be mediated by an agent different from kinins, because the response was not affected by a kinin antagonist and was decreased by enalaprilat. Furthermore, the observation that both tachyphylaxis to Ang I and exposure to Ang II antagonists inhibit the response to trypsin indicates that the latter is mediated by an agent acting on AT_1 receptors. The substrate appears neither to be removed by perfusion nor to be a limiting factor in the response, even after repeated exposure to trypsin.

The details of the mediator generation process are thus far uncertain, particularly because trypsin does not generate Ang II from any of the usual substrates. The mechanism might involve activation by trypsin of an enzyme capable of so doing, such as teomine. Conversely, activation of renin—known to be relatively abundant in the uterine wall—and to increase considerably during pregnancy—could be the first step of another such mechanism. This would require the Ang I generated to be converted to Ang II, a process heretofore considered unlikely, given the low Ang I converting enzyme activity ascribed to the rat uterus. Nevertheless, the inhibiting influences of pepstatin A and enalaprilat suggest that the latter mechanism may be involved in the action of trypsin.

Our results support the notion that the renin-angiotensin system may be involved in the late stages of pregnancy and that serine proteases existing in the uterus may participate in its activation.

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**References**

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