Antipain and leupeptin restrict uterine DNA synthesis and function in mice
(protease inhibitors/plasma membrane/fertility)

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ABSTRACT As in rats, administration of estradiol to ovariectomized mice results in a trypsin-like proteolytic activity in the uterus. After fractionation of uteri from estradiol-treated ovariectomized mice the protease activity was found in the 12,000 × g pellet and the nucleus, appearing first in the former. Further fractionation of the pellet by discontinuous sucrose gradient centrifugation resulted in sedimentation of the protease with S'-nucleotidase, a marker enzyme for plasma membrane and separate from mitochondrial and lysosomal enzyme markers. Solubilization was best accomplished by lysis at 37°C. The soluble enzyme from mouse uterus had optimal activity at about 43°C and pH 8.3 and was inhibited by diisopropylfluorophosphate, tosylarginine methyl ester, antipain, and leupeptin, but not by soybean trypsin inhibitor. Inhibition in vitro by antipain and leupeptin, two low molecular weight peptides, prompted the study of their effect in vivo on the mouse uterus. After intact, cycling female mice received subcutaneous injections of antipain and leupeptin for 16 days, their uteri showed significant diminution in weight and total DNA when compared to untreated controls. Fertility rates were also diminished. Trypsin-like protease activity may be essential to normal uterine metabolism and function.

A recent publication from this laboratory presented evidence for the presence of hormone-stimulated trypsin-like proteolytic activity in the rat uterus (1). This enzyme is inhibited by antipain and leupeptin, two peptides of low molecular weight which have elicited interest because of their ability to inhibit DNA synthesis in regenerating mouse liver (2), to retard the onset of chemically induced tumors (3), and to block the degradation in vitro of uterine histones (4). Antipain is [(S)-L-carboxy-2-phenylethyl]carbamoyl L-arginyl-L-valyl-L-argininal; leupeptin is propionyl- and acetyl-L-leucyl-L-leucyl-L-argininal. The present study was designed to test the effect of antipain and leupeptin on uterine function. Because of limitations in material, the mouse rather than the rat was used as the test animal, necessitating an inquiry into the properties of the uterine protease in mice. The results in this paper show that trypsin-like proteolytic activity is stimulated in mice by estradiol, that the protease is inhibited by antipain and leupeptin, and that administration of these protease inhibitors interferes with uterine function.

MATERIALS AND METHODS

Animals. Young adult female Swiss mice were obtained from Blue Spruce, Alta Mont, N.Y.

Materials. Protamine chloride was obtained from Sigma and succinylated (5). 4-Phenylsopiro[fun-2(3H),1'-phthalan]-3,3'-dione (Fluram) was bought from Fisher, and diisopropylfluorophosphate from Aldrich. Human plasminogen and urokinase were gifts from Alan Johnson. Soybean trypsin inhibitor (type 1-s) was obtained from Sigma. Antipain and leupeptin were donated by The United States-Japan Cooperative Medical Science Program.

Preparation of Subcellular Fractions. Differential centrifugation separated mouse uterine homogenates into 600 × g, 12,000 × g, 100,000 × g, and cytosol fractions as described (1). As in rats, only the 600 × g and 12,000 × g particulate fractions of mouse uteri exhibited protease activity, and these were investigated further.

Protease-Fluram Assay for Protease Activity. The test substance was incubated with succinylated protease and the amino groups formed were determined by the fluorescence generated on reaction with Fluram (6). To test for plasminogen activator, we preincubated the test substance with plasminogen, after which the protease-Fluram assay was carried out. The increment in fluorescence units due to plasminogen is the measure of plasmigen generated.

Localization of Extranuclear Protease Activity. The 12,000 × g extranuclear pellet was fractionated further on a discontinuous sucrose gradient. Activity of the protease in fluorescence units/4 hr per 100 μg of protein in each fraction was determined and compared to the appropriate enzymes specific for subcellular organelles (7). The 12,000 × g pellet from 10 to 12 uteri was homogenized in 2.5 ml of 0.25 M sucrose in 10 mM Tris (pH 7.4), layered on a sucrose gradient consisting from top to bottom of 3.5 ml each of 20, 40, and 60% sucrose, and centrifuged for 2 hr at 38,000 rpm in a Beckman model L-2 ultracentrifuge with an SW 41 rotor. Particulate fractions were found on top of the 20% sucrose (A band), the 40% sucrose (B band), and 60% sucrose (C band) phases. The solid fractions were separated and each was tested for cytochrome oxidase (8, 9), β-N-acetylgalactosaminidase (10, 11), S'-nucleotidase (12), and protease (6). Relative concentrations of the enzyme activities in the A, B, and C bands were calculated.

Solubilization of Extranuclear Protease. Soluble protease was prepared by lysing the 12,000 × g pellet at 37°C for 1.5 hr in 0.1 M phosphate buffer (pH 8.5) (0.4 ml per uterus). The mixture was centrifuged at 34,000 rpm for 1.5 hr in an SW 39L rotor. The supernatant and particulate fractions were assayed for protease and plasminogen activator. In one study the pH and temperature optima of the protease were determined.

Enzyme Studies of Inhibition. Antipain, leupeptin, tosylarginine methyl ester, soybean trypsin inhibitor, and diisopropylfluorophosphate were examined for their capacity to inhibit the solubilized protease. An aliquot of protease was added to 0.3 ml of 0.1 M phosphate buffer (pH 8.5) in the

Abbreviations: Fluram, 4-phenylsopiro[furan-2(3H),1'-phthalan]-3,3'-dione; antipain, ([S]-L-carboxy-2-phenylethyl]carbamoyl L-arginyl-L-valyl-L-argininal; leupeptin, propionyl- and acetyl-L-leucyl-L-leucyl-L-argininal.

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presence or absence of inhibitor at molarities ranging from 10^{-2} to 10^{-8}. After 15 min at room temperature, 100 μg of succinylated protamine was added. The sample (0.4 ml) was incubated at 37° for a specified time up to 4 hr and submitted to the protamine-Fluram assay. In some experiments the inhibitor was tested against trypsin in parallel with the uterine protease. The K_i for antipain and leupeptin was determined by the method of Dixon (15). In these experiments, the protamine-Fluram assay was run with soluble protease and inhibitor in the usual way, except that at each molarity of inhibitor three different concentrations of succinylated protamine were used. Moreover, the incubations were extended to 6 or 12 hr.

**Time-Course of Onset of Protease Activity in Estradiol-Treated Ovariectomized Mice.** Ovariectomized mice received subcutaneous injections of 0.15 μg of estradiol in 0.1 ml of sesame oil at 0 and 12 hr. After 0, 6, 12, 18, or 24 hr, four animals were sacrificed and the uteri were excised, weighed, and combined in pairs. Nuclear and 12,000 × g fractions were analyzed for protease activities by the protamine-Fluram assay.

**Effect of In Vivo Administration of Antipain and Leupeptin on Uterine Weight and DNA Content.** Intact female mice under light control (14 hr light, 10 hr dark) were given subcutaneous injections twice daily of a mixture of 3 mg of antipain and 2.5 mg of leupeptin in 0.2 ml of water adjusted to pH 7.0 with phosphate buffer. Controls received water only. After 16 days the animals were sacrificed by cervical dislocation and the uteri were immediately removed and weighed. Individual uteri were then minced and homogenized in 1 ml of Tris buffer (pH 7.5) in a Dounce glass homogenizer (Kontes Glass Co.). The homogenate was centrifuged at 12,000 × g for 20 min, and the resulting pellet was rehomogenized in 1 ml of the buffer. Fifty-microliter aliquots were analyzed for DNA (14).

**Effect of Antipain and Leupeptin on Fertility.** Intact female breeder mice, three to a cage, under light control were given subcutaneous injections of antipain and leupeptin for 16 days. A male mouse was then placed in each cage and the injections of the females were continued for 14 days. The males were placed in different cages every other day. The females were then sacrificed and the uteri examined for fetuses and implantation sites.

### Table 1. Appearance of uterine protease activity in estradiol-treated ovariectomized mice

<table>
<thead>
<tr>
<th>hr after treatment</th>
<th>Uterine weight, mg</th>
<th>Protease activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>12,000 × g pellet</td>
</tr>
<tr>
<td>6</td>
<td>22, 27</td>
<td>0.0</td>
</tr>
<tr>
<td>12</td>
<td>47, 47</td>
<td>4.8</td>
</tr>
<tr>
<td>18</td>
<td>56, 50</td>
<td>2.2</td>
</tr>
<tr>
<td>24</td>
<td>89, 75</td>
<td>10.6</td>
</tr>
<tr>
<td></td>
<td>70, 102</td>
<td>10.0</td>
</tr>
</tbody>
</table>

* Mice received 0.15 μg of estradiol at 0 and 12 hr. At the times indicated four mice were sacrificed. Uteri were removed, weighed, combined in the pairs shown, homogenized, and fractionated by centrifugation. There were no changes in the weights or protease activities in the uteri between 0 and 6 hr.

† Values are based on less than 1 fluorescent unit and may not be significant.

Table 2. Localization of uterine protease activity and marker enzymes in subfractions of the 12,000 × g pellet after separation by discontinuous sucrose gradient centrifugation

<table>
<thead>
<tr>
<th>Band</th>
<th>Protease</th>
<th>5'-Nucleotidase</th>
<th>β-N-Acetylglucosaminidase</th>
<th>Cytochrome oxidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
<td>0.033</td>
<td>0.225</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>150</td>
<td>0.490</td>
<td>0.56</td>
<td>-</td>
</tr>
<tr>
<td>C</td>
<td>0</td>
<td>0.068</td>
<td>0.410</td>
<td>+</td>
</tr>
</tbody>
</table>

In each experiment 10 ovariectomized mice were treated with 0.15 μg of estradiol daily for 2 days. Inexplicably, in Exp. 1 enzyme activities were 6-10 times greater than in Exp. 2. Note that β-N-acetylglucosaminidase (lysosomal enzyme) was distributed differently in the two experiments, but the profile differs from that of the protease. Units of enzyme activities are as follows: protease, fluorescence units per 4 hr/100 μg of protein; 5'-nucleotidase, μmol of phosphate released per hr/100 μg of protein (12); β-N-acetylglucosaminidase, μmol of p-nitrophenol released per hr/100 μg of protein (10, 11); cytochrome oxidase, presence (+) or absence (-) of activity determined by decrease in A at 560 nm (8, 9).

### RESULTS AND DISCUSSION

Ovariectomy results in the disappearance from the mouse uterus of protease activity capable of hydrolyzing protamine. Subsequent administration of estradiol causes the expression of the activity, initially in the 12,000 × g pellet and then in the nucleus (Table 1). The increase in uterine weight paralleled the increase in protease activity in the extranuclear pellet. There are striking similarities between the enzyme activity shown in this paper and that reported previously for rats (1). In both rodents the activity is seen first in the extranuclear 12,000 × g pellet and subsequently in the nucleus, although these events occur earlier in mice than in rats.

Since the 12,000 × g pellet includes an array of subcellular organelles, this fraction was subdivided further in an attempt to define more precisely the localization of the protease. Table 2 shows the distribution of protease activity and of typical enzyme markers in three subfractions of the 12,000 × g pellet obtained by discontinuous sucrose density centrifugation. Protease activity was concentrated in the B band, as was the 5'-nucleotidase activity which is a marker enzyme for plasma membranes. β-N-Acetylglucosaminidase and cytochrome oxidase activities, markers, respectively, for lysosomes and mitochondria, were distributed differently from the protease. In one study band B was purified further on a shallow continuous sucrose gradient, and again the equilibrium distribution of the protease closely paralleled the distribution of 5'-nucleotidase. In addition to hydrolyzing protamine, the membrane-bound protease activity is capable of activating plasminogen.

To guard against the possibility that the protease redistributed during the homogenization process, a control was run in which soluble protease was added to the medium in which the uterus was homogenized. Then subcellular fractions were prepared. The added proteolytic activity was recovered quantitatively from the supernate, in which the activity does not appear normally; the addition did not influence the proteolytic activity in the 12,000 × g pellet or nuclei. These studies suggest, but do not prove, that the estrogen-stimulated protease is bound to uterine cell membranes. The enzyme presents the...
The 12,000 × g pellets from uteri of intact or estradiol-treated ovariectomized mice were lysed and soluble protease was separated by centrifugation at 100,000 × g. The protease-Fluoram assay was run in the presence or absence of the inhibitors at the concentrations indicated.

* Percent decrease in fluorescence units when inhibitor was present. Protease equivalent to about 1 ng of trypsin was used.

† Percent decrease in fluorescence units when inhibitor was present in an incubating containing 0.04 μg of trypsin (10 nM).

The mixture of antipain and leupeptin, whereas control mice received water. After 16 days (about four cycles), vaginal smears were examined to determine the phase of the estrus cycle. The mice were then sacrificed and the uterus were weighed and assayed for DNA content. Fig. 2 shows that the DNA content and weights of the treated mice averaged about one-half those of the controls. The distributions were such that the result for a treated mouse rarely reached the average of the untreated mouse. The differences were significant at P < 0.001. Three other studies were conducted with 15 to 20 animals in each group and the results were essentially the same. The fraction of animals in diestrus correlated with the uterine weight. In experiment 1, 5 of 18 controls were in diestrus, whereas 13 of 22 treated animals were in diestrus. In experiment 2 the corresponding values were 7 of 24 for the controls and 13 of 25 for the treated animals.
Table 4. Influence of antipain and leupeptin on fertility in female mice

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Regimen</th>
<th>Pregnant/total</th>
<th>Control</th>
<th>Treated</th>
<th>( P^* )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5 mg leupeptin(^1)</td>
<td>10/15</td>
<td>17/30</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>5 mg leupeptin(^1) plus 6 mg antipain</td>
<td>18/20</td>
<td>6/20</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>5 mg leupeptin(^1) plus 6 mg antipain</td>
<td>13/20</td>
<td>7/22</td>
<td>0.05</td>
<td></td>
</tr>
</tbody>
</table>

Inhibitors were given subcutaneously for 16 days without males and then 14 days with males. Animals were sacrificed and uteri were examined.

* According to fourfold contingency test (18).

\(^1\) Single injection daily.

Fertility rate was another index of uterine function investigated. After treatment with antipain and leupeptin for 16 days, males were introduced into the cages and the treatments were continued for another 14 days. The appropriate controls were run. The data in Table 4 demonstrate a significantly lower fertility rate in the treated animals. Mice that conceived did not have fewer fetuses or abortives than the untreated gravidas.

The in vitro studies described suggest that proteases are involved with the metabolism and possibly uterine function. Antipain and leupeptin are two short-chain peptides capable of penetrating cells and of inhibiting a variety of neutral and acid proteases. Lysosomal cathepsin B1 (19), papain, trypsin (3), and the uterine protease described in this paper are representative examples. The administration of antipain and leupeptin to normal cycling rats results in a dramatic diminution in uterine weight and uterine DNA content (Fig. 2), and in statistically significant decreased fertility rates (Table 4). The mechanism(s) of action of these inhibitors requires elucidation. Some alternatives deserve consideration. First, active protease such as that described in this paper may be directly involved in normal uterine function. A preliminary study indicated that antipain and leupeptin given to ovariecctomized mice did not prevent a response to injected estradiol in terms of increases in uterine weight and uterine DNA. These results argue against a direct effect. However, these experiments should be expanded and perhaps extended to an immature mouse model before ruling out this hypothesis. A second possibility is that the inhibitor interferes with the normal function of the pituitary-ovarian axis and that depressed uterine function is secondary. A significant increase in plasminogen activator in response to gonadotropins in rat ovarian granulosa cells, particularly those destined to ovulate, has been reported in vitro and in vivo (20). If plasminogen activator is associated with ovulation, inhibition of the enzyme could result in inadequate uterine stimulation and the biological consequences described in this paper.

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