ENDOMETRIAL ARYLAMIDASE SECRETION:
A SENSITIVE PARAMETER OF STEROID HORMONE ACTION AND OF EMBRYO-MATERNAL INTERRELATIONSHIPS IN THE RABBIT

HANS-WERNER DENKER
Abteilung Anatomie der RWTH, Melatener Str. 211, D-5100 Aachen (West Germany)

ABSTRACT
Amino acid arylamidase I (in earlier work also referred to as leucine aminopeptidase) appears in the uterine epithelium and is extruded into the uterine lumen in considerable quantities after 3 d p.c. (days post coitum) in the rabbit. In the uterine secretion, the activity of this enzyme shows a very sharp and high peak at 5 d p.c., i.e. in the middle of the preimplantation phase, and it declines again rapidly until 8 d p.c. Preliminary experiments on the hormonal regulation indicate that arylamidase accumulates in the endometrium after progesterone treatment of non-ovariectomized animals, while the significance of the minor increase seen after estradiol remains uncertain. Extrusion into the uterine lumen, on the other hand, is clearly progesterone-dependent. In fact, the sharp peak of activity in the uterine fluid at 5 days (including the steep increase before and the decline thereafter) can be mimicked by progesterone treatment of non-ovariectomized animals. Comparative investigations of pseudopregnant and pregnant uteri indicate that the blastocyst seems to stimulate, by an unknown signal, the extrusion of arylamidase I from the uterine epithelium into the uterine lumen. Since quantitative biochemical tests of arylamidase activity are easy to perform, this enzyme may serve as a useful and particularly sensitive parameter of the action of steroid hormones, particularly of progesterone, on the endometrium.

INTRODUCTION
One enzyme of the aminopeptidase class, the so-called amino acid arylamidase I, has received interest because it becomes detectable in the uterine epithelium of the rabbit at the time when
the embryo is expected to enter the uterus, and the activity reaches very high levels here during the later preimplantation phase. In the middle of the preimplantation phase, considerable quantities of this enzyme are being extruded into the uterine lumen. As described earlier, the level of activity in the uterine epithelium and in the secretion responds also sensitively to the presence of a blastocyst. We are now reporting the results of a more detailed study of the time course of activity changes in the endometrium and the uterine secretion, and of a preliminary study of its hormonal regulation.

MATERIALS AND METHODS

Animals. Sexually mature virgin rabbits, cross-bred, were kept under standardized conditions and were mated to fertile bucks as described previously. Pseudopregnancy was induced by mating with vasectomized males.

Hormone treatments. Non-mated, non-ovariectomized females were injected subcutaneously once daily, for the indicated period, with 1.0 ml sesame oil containing either 50 µg of estradiol benzoate (Progynon B oleosum, Schering), or 4 mg of progesterone (E. Merck, Darmstadt, No. 24614).

Biochemical tests. Uterine flushings and endometrial tissue were obtained as described previously. Enzyme activity was determined in 3000 g supernatants, using L-leucine-β-naphthylamide (0.8 mM) as substrate, veronal-acetate Michaelis buffer pH 7.0, incubation at 37°C. Liberated β-naphthylamine was measured with the Goldbarg-Rutenburg type Bratton-Marshall reaction, and the protein content of the samples was determined by the biuret method of Westley and Lambeth.

Histochemical tests. Pieces of uteri were frozen with liquid nitrogen and sectioned on a cryostat. For accurate localization, the following methods proved satisfactory:

A. Section freeze-substitution, substrate L-leucine-β-naphthylamide or L-leucine-4-methoxy-β-naphthylamide, liquid incubation medium, simultaneous coupling with Fast Garnet GBC or with Fast Blue B.

B. Native sections, substrate L-leucine-4-methoxy-β-naphthylamide, simultaneous coupling with Fast Blue B, either in liquid
incubation medium or membrane method (the latter allows to retain the uterine secretion, which is easily diffusible, on the sections) 6.

RESULTS

Biochemical properties of amino acid arylamidase I

Amino acid arylamidase I is one of various (probably at least 3) related aminopeptidase type enzymes found in rabbit uterine tissues, uterine secretion and blastocyst tissues 6. Since this group of enzymes was found to split particularly well certain synthetic arylamide substrates (amino acid β-naphthylamides or p-nitroanilides) we refer to them as amino acid arylamidases as it has become customary in recent biochemical literature 2,3,6.

TABLE 1

BIOCHEMICAL PROPERTIES OF RABBIT UTERINE SECRETION ARYLAMIDASE (I)

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Arylamides (β-naphthylamides, p-nitroanilides) of various amino acids (e.g. leucine). Little hydrolysis of and little competitive inhibition by leucine amide and leucine hydrazide.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibitors</td>
<td>Puromycin, EDTA (biochemical and histochemical test system). Not inhibited by aprotinin (Trasylol) (10⁻³ M), soybean trypsin inhibitor (10⁻³ M) or iodoacetamide (10⁻² M) (histochemical test system).</td>
</tr>
<tr>
<td>Activating ions</td>
<td>Co⁺⁺ (barely influenced by Mg⁺⁺).</td>
</tr>
<tr>
<td>pH optimum</td>
<td>pH 7.0 - 8.0 (substrate: L-leucine-β-naphthylamide).</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>ca. 250,000 (gel filtration).</td>
</tr>
</tbody>
</table>

Arylamidase I hydrolyzes the arylamides of various different amino acids 6. Since the substrate leucine-β-naphthylamide had been used in the original investigations, this enzyme was initially referred to as "leucine aminopeptidase (LAP)" 1,7. More detailed biochemical investigations revealed, however, that there is only very little activity of classical leucine aminopep-
Fig. 1. Histochemical demonstration of amino acid arylamidase I activity in rabbit endometrium at 5 d p.c. Section freeze-substitution method, substrate L-leucine-β-naphthylamide, diazonium salt Fast Garnet GBC. The section freeze-substitution technique allows to localize the enzyme activity precisely in the apical parts of uterine epithelial cells. The uterine secretion, however, is only moderately well preserved with this technique (slightly arylamidase-positive granules). Its high activity would be very obvious with a membrane method (not shown), which on the other hand is not as good for intracellular localisation.

tidase (LAP, α-aminocylpeptide hydrolase, cytosol, EC 3.4.11.1) in rabbit uteri, and that the described arylamidase differs from LAP in several biochemical properties (see Tab. 1): showing only little activity towards leucine hydrazide, being activated by Co++, being inhibited by puromycin, showing a pH optimum between pH 7.0 and 8.0 (substrate: leucine-β-naphthylamide) (van Hoorn and Denker, unpublished). In addition to the histochemical studies mentioned above 6, these biochemical investigations also provided strong evidence that rabbit endometrial tissues contain more than one arylamidase capable of hydrolyzing the used substrates (leucine-β-naphthylamide,
leucine-p-nitroanilide etc.). However, arylamidase I is the predominant enzyme of this group in the uterine epithelium and in the uterine secretion, during the middle and late preimplantation phase.

Physiological changes in endometrial and uterine secretion arylamidase activities in early pregnancy

Most fascinating about rabbit uterine arylamidase I are the remarkable changes in activity which take place in the uterine epithelium and secretion during the preimplantation phase. In the uterine epithelium, the activity is nearly non-detectable, by histochemical tests, before and at 2 d p.c., but increases at 3 d p.c. \(^1\), i.e. at the time when embryos enter the uterus in regular pregnancy. This increase is, however, independent of the presence of embryos, because the same happens in pseudopregnancy \(^2,3,5\). In histochemical tests, the most intense reaction is found in the uterine epithelium (apical part of cytoplasm, Fig. 1) at 5-6 d p.c., and the reaction declines thereafter \(^1\). These histochemical findings are confirmed by biochemical investigations (Fig. 2), although the decline after 5-6 d p.c. is not as obvious, in the endometrial homogenates, as seen in the histochemical tests, at least in interblastocyst segments of the uterus (the more pronounced changes at blastocyst sites are discussed below). This may be due to the fact that endometrial stroma cell and blood plasma arylamidases may contribute to the activity measured in homogenates, while the histochemical test allows to study uterine epithelial cell activity alone. The differences in histochemical reactivity between non-pregnant and 6 d p.c. pregnant endometria have likewise been described by Petry et al. \(^8\).

Even more prominent and probably more significant for the physiology of preimplantation development are the changes in arylamidase I activity seen in the uterine secretion (Fig. 3). The activity is barely detectable in the flushings of non-pregnant uteri and until 2 d p.c., but begins to increase at 3 d p.c. \(^9\) (again the time when the embryos are expected to enter the uterus in normal pregnancy) and reaches a sharp and high peak at 5 d p.c. (Fig. 3). Interestingly, peak activity lasts for only approximately one day (see also \(^9\)). From 6 d p.c. on, the decline is considerably slowed down so that at 8 d p.c. the activity is still considerably higher than at or before 2 d p.c.
Fig. 2 - 5 (on opposite page). Amino acid arylamidase activity in rabbit endometrial tissue and uterine secretion during early pregnancy and after estradiol and progesterone treatments. Biochemical tests, substrate L-leucine-β-naphthylamide (see Text).

Fig. 2: Early pregnancy, endometrial homogenates.
Fig. 3: Early pregnancy, uterine flushings.
Fig. 4: Hormone treatments, endometrial homogenates.
Fig. 5: Hormone treatments, uterine flushings.

Values (mU/mg protein, mean ± SE, n = number of animals):

**Fig. 2:** Estrus: 10.5 (n = 1); 2 d p.c.: 14.1 ± 2.0 (n = 3); 5 d p.c.: 47.8 ± 5.3 (n = 3); 6 d p.c.: 45.9 (n = 2); 7 d p.c.: 44.5 (n = 1); 8 d p.c.: 40.3 ± 5.8 (n = 3); 8 1/2 d p.c.: 39.0 (n = 1).

**Fig. 3:** Estrus: 28.4 (n = 1); 2 d p.c.: 16.0 ± 2.6 (n = 3); 5 d p.c.: 986.4 ± 54.7 (n = 3); 6 d p.c.: 240.8 (n = 2); 7 d p.c.: 99.8 (n = 1); 8 d p.c.: 199.7 ± 58.4 (n = 3); 8 1/2 d p.c.: 56.4 (n = 1).

**Fig. 4:** Estrus as in Fig. 2. Estradiol treatment: day 2: 16.6 (n = 2); day 5: 22.6 (n = 2); day 8: 11.4 (n = 2). Progesterone treatment: day 2: 10.5 (n = 2); day 5: 37.5 (n = 2); day 8: 33.7 (n = 2).

**Fig. 5:** Estrus as in Fig. 3. Estradiol treatment: day 2: 8.1 (n = 2); day 5: 9.3 (n = 2); day 8: 10.5 (n = 2). Progesterone treatment: day 2: 19.2 (n = 2); day 5: 713.7 (n = 2); day 8: 158.3 (n = 2).

**Hormonal regulation**

The described increase in arylamidase activity in the endometrium and the specific pattern of changes in the uterine secretion (increase after the second day, peak around the fifth day, decrease until the eighth day) can be mimicked very well by treating non-ovariectomized, non-pregnant females with progesterone (see Materials and Methods) (Fig. 4 and 5).

Estradiol induces a slight increase in activity in endometrial homogenates but does not cause any changes in the uterine secretion (Fig. 4 and 5). The significance of the slight and transitory increase seen in the endometrial homogenates is not clear, and this observation needs to be confirmed by more extensive investigations and perhaps be studied more in detail, before drawing any conclusions from it. With the data at hand it appears possible that this phenomenon is attributable to stroma cell or blood plasma arylamidase(s), particularly because it is not seen likewise in histochemical tests, and because it is more obvious, in the biochemical tests, when L-leucine-p-nitroanilide is used as a substrate^9^ which
appears to be hydrolyzed slightly better by stromal arylamidase(s) than by uterine epithelial/secretion arylamidase I (van Hoorn and Denker, unpublished).

It should be noted that the animals used in these experiments had not been ovariectomized so that endogenous estrogen levels had not been eliminated. Therefore it cannot be excluded that estrogens may perhaps play a role in induction of the enzyme even in the experiments with progesterone application. However, it becomes very clear from the described experiments that the typical pattern of increase in arylamidase I activity in the endometrium (uterine epithelium) and particularly the extrusion into the uterine lumen are progesterone-dependent. This is confirmed by qualitative histochemical tests and by zymograms of uterine flushings (van Hoorn and Denker, unpublished; 7,10).

Actinomycin, injected intravenously (three injections, i.e. at 2, 3 and 4 d p.c., 0.1 mg/kg body weight each), partially inhibits the increase in endometrial and uterine secretion arylamidase I activity as seen in the controls until 5 d p.c. (van Hoorn and Denker, unpublished). Certainly, results of such experiments in which inhibitors are administered systemically should be evaluated with caution keeping in mind the possibility of side-effects and indirect actions via changes of e.g. hormone synthesis. These observations suggest, nevertheless, that the progesterone action may involve changes in arylamidase I messenger RNA synthesis. On the other hand, progesterone may also regulate the process of arylamidase extrusion into the uterine lumen.

The short duration of the arylamidase wave in the uterine secretion is most interesting. Uteroglobin, e.g., which is being extruded into the uterine lumen at approximately the same stage as arylamidase I, has been reported to keep a high concentration there for a few more days 7. However, exact comparison is hampered by the fact that this was based on semiquantitative tests of uteroglobin levels, while the photometric tests of arylamidase activity reported here may monitor changes more sensitively. If the difference in the profiles of both proteins should prove to be real, it will have to be investigated further whether this reflects a higher turnover rate of the enzyme. However, recent data on uteroglobin concentrations obtained with radioimmunoassays (14; Bullock, this
Symposium) indicate that its concentration profiles may be more similar to arylamidase I than thought before. This would mean that the hormonal regulation of synthesis and extrusion of both proteins in the rabbit uterus may in fact be very similar.

Influence of the blastocyst on endometrial arylamidase activity

The mechanism behind the rapid decline of arylamidase activity in the uterine secretion after 5 d p.c. is not clear. Interestingly there is evidence that the preimplantation blastocyst stimulates the discharge of the enzyme from the uterine epithelium. This is suggested by comparative investigations of the activity in the endometrium and in the uterine secretion of normally pregnant (blastocysts present) and pseudopregnant (blastocysts absent) animals.\(^2\) The blastocyst obviously signalizes its presence in the uterus via diffusible substances. It induces a decline in uterine epithelial arylamidase I activity in its vicinity already before the blastocyst coverings are dissolved. The fact that no cellular contact between trophoblast and uterine epithelium is needed for the induction of this reaction is impressively demonstrated in experiments in which the dissolution of the blastocyst coverings is prevented by administration of proteinase inhibitors (like aprotinin = Transylol, or antipain) in vivo.\(^3\) The blastocyst which remains non-attached in this case is still able to induce arylamidase depletion in the adjacent uterine epithelium (Denker, unpublished). The (hormonal?) nature of the signal provided by the blastocyst is still obscure since it has not been possible, so far, to mimic the effect in experiments with various hormone-containing IUDs.\(^1\)

The physiological role of arylamidase I is so far unknown. Being an exopeptidase, the enzyme might participate in the conversion of uterine proteins and peptides from inactive into active forms or v. v. as suggested earlier.\(^3,\)\(^1\) Since there is a physiological need for uptake of fixed nitrogen in the form of amino acids or protein by the blastocyst at this phase (for literature see), liberation of amino acids by endometrial and uterine secretion arylamidase may be physiologically significant. On the other hand there is a considerable change in the composition of the extracellular blastocyst coverings around 5 d p.c., i.e. at the time of the arylamidase I peak. At present it is impossible to say whether this coinci-
dence has any physiological meaning. Since the enzyme is also found in the trophoblast it should be interesting to see whether it is taken up by the blastocyst from the uterine secretion.

ACKNOWLEDGEMENTS

The biochemical arylamidase tests were performed by Gerhild van Hoorn during her work in our previous group (Arbeitsgruppe Prof. Dr. G.H.M. Gottschewski) at the Max-Planck-Institut für Immunbiologie, Freiburg. The author wishes to thank Mrs. Mathieu for typing the manuscript and Mr. W. Graulich for drawing the diagrams. These investigations were supported by Deutsche Forschungsgemeinschaft grants De 181/1-4, 7 and 8 (Schwerpunktprogramm "Physiologie und Pathologie der Fortpflanzung" and "Biologie und Klinik der Reproduktion").

REFERENCES


STEROID INDUCED UTERINE PROTEINS

Proceedings of the International Symposium on Steroid Induced Uterine Proteins held in Marburg, West Germany, 28-29 September, 1979

M. Beato Editor

1980

Elsevier/North-Holland Biomedical Press
Amsterdam · New York · Oxford
DEVELOPMENTS IN ENDOCRINOLOGY VOLUME 8

Other volumes in this series:
Volume 1
Multiple Molecular Forms of Steroid Hormone Receptors
M.K. Agarwal Editor (1977)
Volume 2
Progress in Prolactin Physiology and Pathology
C. Robyn and M. Harter Editors (1978)
Volume 3
Hormones and Brain Development
G. Dörner and M. Kawakami Editors (1978)
Volume 4
Lipoprotein Metabolism and Endocrine Regulation
L.W. Hessel and H.M.J. Krans Editors (1979)
Volume 5
Psychoneuroendocrinology in Reproduction
L. Zichella and P. Pancheri Editors (1979)
Volume 6
Proteases and Hormones
M.K. Agarwal Editor (1979)
Volume 7
Progress in Ecdysone Research
J.A. Hoffmann Editor (1980)

Forthcoming volumes in this series:

Neuroactive Drugs in Endocrinology
E.E. Müller Editor (1980)

Recent Advances in Invertebrate Reproduction