HORMONAL REGULATION OF PROTEINS IN THE UTERINE SECRETION OF OVARIECTOMIZED RATS AND THE IMPLICATIONS FOR IMPLANTATION AND EMBRYONIC DIAPAUSE

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Summary. Uterine secretions were obtained from long-term ovariectomized rats given progesterone for 10 days followed by oestrogen and progesterone. Analysis of the protein content was carried out using polyacrylamide disc gel electrophoresis at pH 8.9 and also of the protein-sodium dodecyl sulphate complexes. When progesterone was given alone, there was a lack of high molecular weight proteins. When oestradiol was also given, a protein (mol. wt 70,000) was detected within 1 hr. At 13 to 20 hr after oestrogen, there was a shift towards the secretion of high molecular weight proteins when the secretion profiles were similar to those of normal pregnant animals at Day 5, the day of implantation in the rat. The hormonal regulation of the secretion proteins is discussed. The physiological aspects of implantation in terms of zona lysis, embryonic diapause and the release from diapause after oestrogen treatment are discussed with reference to the changes in the intraluminal proteins.

INTRODUCTION

In some rodents, embryos enter a state of metabolic dormancy during lactation or when the pregnant females are ovariectomized and treated daily with progesterone (Cochrane & Meyer, 1957). In such embryos, mitotic activity ceases (Surani, 1975), and RNA synthesis, protein synthesis (Gulyas & Daniel, 1969; Weitlauf & Greenwald, 1965) and glucose metabolism are depressed (Menke & McLaren, 1970). If an injection of oestrogen is given, there is a resumption of RNA, protein and DNA synthesis (Prasad, Dass & Mohla, 1968; Dass, Mohla & Prasad, 1969) followed by implantation 24 hr after the hormone treatment (Psychoyos, 1969).

The embryos appear to be blocked in the G1 phase of the cell cycle during dormancy (Sanyal & Meyer, 1972; Sherman & Barlow, 1972; Surani, 1975). The reasons for embryonic diapause and the manner of oestrogen intervention on the release from diapause are unclear (see McLaren, 1973). Several workers have suggested that a uterine fluid component may inhibit embryo metabolism during diapause (Psychoyos, 1969; Psychoyos & Bitton-Casimir, 1969; Gulyas & Daniel, 1969) and that this component may be removed following oestrogen injection. For the resumption of RNA synthesis (Psychoyos & Bitton-
Casimiri, 1969) and trophoblastic outgrowth (Gwatkin, 1966) in vitro, however, the presence of serum in the medium is sufficient. Alternatively, oestrogen may directly stimulate embryo metabolism in utero (Prasad et al., 1968).

In this study, qualitative changes in the proteins of rat uterine secretions were observed in an attempt to determine if such changes could explain embryonic diapause and the release from diapause.

MATERIALS AND METHODS

Chemicals

Acrylamide was obtained from Eastman Kodak, Rochester, N.Y., N,N'-methylenebisacrylamide and NNN'N'-tetramethylethlenidiamine were obtained from B.D.H. Chemicals Ltd, Poole, Dorset, progesterone (4-pregnen-3,20-dione) and oestradiol-17β (1,3,5(10)-estratrien-3,17β-diol 3-benzoate) were obtained from Koch-Light Laboratories Ltd, Colnbrook, Bucks, and sodium dodecyl sulphate (SDS) was obtained from Serva, Heidelberg, Germany.

Animals

Virgin Wistar rats (180 g body weight) were bilaterally ovariectomized at oestrus (in later studies, ovariectomy at other stages of the cycle was found to give the same results). The rats were maintained on a lighting schedule of 00.05 hours to 19.30 hours without treatment for 30 days to deplete them of endogenous ovarian steroids.

Hormonal regimen

The hormonal regimen was started at the end of 30 days (body weight 250 g) with a subcutaneous injection of 5 mg progesterone and 1.0 µg oestradiol benzoate in 0.5 ml arachis oil to induce endometrial growth. Thereafter, 4.0 mg progesterone/rat/day was given subcutaneously in 0.2 ml arachis oil until the 10th day after the first hormone injection. At the end of the 10th day, a group of animals was given 0.2 µg oestradiol together with 4 mg progesterone. The oestrogen-treated animals were assigned to seven groups and each group was made up of four to six rats. The intraluminal flushings were collected sequentially 1, 6, 12, 18, 24, 30 and 48 hr after oestrogen treatment.

Collection of biological material

Animals were lightly anaesthetized with ether, decapitated and exsanguinated to minimize contamination of the uterine samples with serum. The uterine horns were dissected out, placed on a cold glass plate on filter paper moistened with saline, trimmed of connective tissue, and traces of blood on the external surface were removed by gentle rolling on the filter paper. Each horn was flushed with 0.05 ml of the appropriate sample buffer, and the material from the two horns was combined for one electrophoretic run in 0.1 ml sample buffer.

Disc-gel electrophoresis

Electrophoresis was carried out using a Shandon apparatus in gel tubes 11 cm x 0.58 cm.
Separation at alkaline pH. The biological material was collected at 4°C in the sample buffer of orthophosphoric acid-tris base, pH 6.9, containing 0.154 M-sodium chloride. The samples were centrifuged at 15,000 g at 4°C for 1 hr and stored at −20°C for not more than 2 weeks. The method employed was that described by Ornstein & Davis (1961). The running gel (8.5 cm) was 7.5% acrylamide made in tris-HCl buffer, pH 8.9. The stacking gel (0.1 cm) was 3.0% acrylamide in the sample buffer, pH 6.9. The reservoir buffer was tris-glycine, pH 8.3. Separation of proteins was carried out for 1.5 hr (running front to 8.0 cm) at a constant current of 3 mA/tube.

Separation of protein–sodium dodecyl sulphate complexes. The uterine horns were flushed with a sample buffer consisting of 0.1% SDS, 0.01 M-sodium phosphate buffer, 0.14 M-β-mercaptoethanol, 10% glycerol and 0.002% Bromophenol blue. Samples were collected at 0°C and centrifuged at this temperature for 1 hr. The supernatant was warmed at 65°C for 1 hr to form protein–SDS complexes and then stored for not more than 1 week at 4°C. The separation was highly reproducible with the method similar to that described by Shapiro, Vinuela & Maizel (1967). The 8.5-cm length of running gel was made of 7.5% acrylamide in 0.2 M-sodium phosphate buffer containing 0.1% SDS, pH 7.2. No stacking gel was used. The reservoir buffer was 0.1 M-sodium phosphate buffer and 0.2% SDS, pH 7.2. Electrophoresis was continued for 5 hr (running front to 8.0 cm) at a constant current of 8 mA/tube.

All gels were stained with Coomassie Brilliant Blue (0.25% CBB in methanol: acetic acid: water, 5:1:5 by vol.) overnight, and destained in a solvent made of 7.5% acetic acid and 5% methanol.

RESULTS

Separation of proteins at alkaline pH

In the secretions from the rats receiving progesterone alone, albumin was the predominant component (Pl. 1, Fig. 1a and Table 1). Following oestradiol treatment, albumin stained more intensely, and a new protein migrating just behind albumin was detected up to 6 hr after oestrogen (Pl. 1, Fig. 1b). Further changes were seen about 18 hr after oestradiol, marked by two intensely staining bands in the prealbumin region and a protein migrating in the post-transferrin region (Pl. 1, Figs 1f to 1h). Several faintly staining prealbumin bands were detected throughout the course of the experiments but their migratory properties and the timing of their appearance were variable.

Separation of protein–sodium dodecyl sulphate complexes

In the secretions from the rats treated with progesterone alone, most of the proteins present (Pl. 2, Fig. 2a and Table 1) were of low molecular weight (70,000 and less). The high molecular weight proteins were largely undetectable. Several of the proteins were evidently uterine-specific compared to plasma. One hour after oestradiol treatment, a protein band was detected in the molecular weight range of approximately 70,000 (Pl. 2, Fig. 2b) and was faintly detected at 6 hr. The other proteins remained largely unchanged. The 13- to 20-hr period was the second phase of the change with a marked shift towards the
secretion of the high molecular weight proteins not detected in the progesterone-treated animals. The small molecular weight proteins persisted but apparently stained less intensely (Table 1). The protein profiles at 13 to 20 hr were similar to those found on Day 5 of pregnancy (compare Pl. 2, Fig. 2d with Fig. 2i), the day of implantation in the rat. At 24 to 48 hr, there was a reversal to the secretion proteins found 1 hr after treatment with oestradiol. High molecular weight

Table 1. Summary of the changes noted in rat uterine secretion proteins by disc-gel electrophoresis, pH 8-9, after treatment with progesterone alone and at different times after oestrogen

<table>
<thead>
<tr>
<th>Protein components</th>
<th>Progesterone</th>
<th>1 hr</th>
<th>6 hr</th>
<th>12 hr</th>
<th>18 to 48 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Proalbumin</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Prealbumins (PreA, 1 and 2)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+PreA</td>
<td>+PreA 1 and 2</td>
</tr>
<tr>
<td>Post-transferrin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

The intensity of staining is marked with + signs.

Table 2. Summary of the protein-SDS complex separation of proteins of rat uterine secretions, after treatment with progesterone alone and sequential changes in the proteins after treatment with oestrogen

<table>
<thead>
<tr>
<th>Protein components</th>
<th>Progesterone</th>
<th>1 hr</th>
<th>6 hr</th>
<th>13 to 20 hr</th>
<th>24 to 48 hr</th>
<th>Day 5 of pregnancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Protein of mol. wt 70,000</td>
<td>-</td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Three sub-units (S1 to S3) of mol. wt 60,000 to 70,000</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>±</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Proteins of mol. wt 70,000 and less</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Proteins of mol. wt 70,000 and more</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

The changes are compared with the material from normal pregnant rats on Day 5 of pregnancy. The intensity of staining is marked with + signs.

proteins were absent but three proteins in the range of 60,000 to 70,000 mol. wt were present, which were not detected before or on Day 5 of pregnancy (Pl. 2, Fig. 2i).

DISCUSSION

The results with the protein-SDS separation proved to be highly reproducible and the technique was also more sensitive than the method of separation at alkaline pH (Shapiro, Vinuela & Maizel, 1967). The collection of the intra-
Fig. 1. Disc-gel electrophoresis of rat uterine fluid at pH 8.9 as outlined in 'Materials and Methods'. The samples were collected after progesterone (P) injections alone (Fig. 1a) or at different times after progesterone + oestrogen (POe) (Figs 1b to 1h). Rat plasma is included for reference. Albumin (Alb) is indicated in all gels, postalbumin (PA) at 1 to 6 hr after oestrogen (Figs 1b and 1c), prealbumin bands (PreA) from 12 to 48 hr after oestrogen (Figs 1d to 1h) and post-transferrin (PT) from 18 to 48 hr after oestrogen (Figs 1e to 1h). Anode is at the bottom of the tubes.
Fig. 2. Separation by disc-gel electrophoresis of protein-SDS complexes, of rat uterine fluid at pH 7.2. The samples were collected after progesterone (P) injections alone (Fig. 2a) or at different times after progesterone + oestrogen (POe) (Figs 2b to 2h). The rat plasma sample and the uterine secretion samples of Day 5 of normal pregnancy (Fig. 2i) were used as reference. The position of the migration of albumin (Alb) is indicated in all gels with reference to plasma albumin. The protein of mol. wt 70,000 is marked in Figs 2b to 2i (x) and the three proteins (S₁ to S₃) of mol. wt 60,000 to 70,000 are shown in Figs 2g and 2h. The proteins are divided into two groups: those with mol. wt > 70,000 and those of mol. wt < 70,000. The anode is at the bottom of the tubes.
luminal proteins at 0°C by the sample buffer containing SDS and β-mercapto-
ethanol is considered to be a reliable method for collecting the uterine secretion
proteins from this species. The study also shows that embryos in normal preg-
nant rats do not affect the uterine secretion patterns in a detectable way, as they
do in marsupials (Renfree, 1973). The embryos may however affect the tem-
poral aspects of the secretions.

The qualitative changes in the secretion proteins could be due to de novo
synthesis in the endometrium (or elsewhere) or to the selective secretion (and
modification) of serum proteins into the uterine lumen. The lack of high mole-
cular weight proteins (including the protein of mol. wt 70,000) in the females
given progesterone alone may signify the inability of the endometrium to
synthesize and secrete these proteins. Alternatively, all the proteins may be
present throughout the course of the experiments, and administration of
oestradiol affects the quantitative balance of the macromolecules. Thus,
oestradiol could act not only by the induction of unique proteins but also on the
selective secretion of serum macromolecules.

The analysis of uterine secretion proteins can be used to explain some
physiological aspects of implantation. The factor responsible for lysis of the zona
appears to be an oestrogen-dependent maternal protein. In ovariectomized
pregnant animals given oestrogen, the lysis of the shed zona occurs 24 hr after
the hormone treatment (Surani, 1975). The two prealbumin bands and a post-
transferrin band (Pl. 1, Figs 1e to 1h) and the three proteins of mol. wt 60,000 to
70,000 (Pl. 2, Figs 2f to 2h) detected at this time are unlikely to be the zona
lytic proteins as they are absent in the normal flushings from pregnant uteri
(Pl. 2, Fig. 2i). The proteins secreted earlier may, however, have this enzymatic
property.

The release of embryos from diapause and their attachment occurs in two
phases; resumption of RNA and protein synthesis within 1 hr after oestrogen
(Dass et al., 1969), followed by embryo attachment 24 hr later (Psychoyos,
1969). The secretion of the protein of mol. wt 70,000 is the major initial change
at 1 hr. The protein may regulate embryo metabolism in a ‘cascade-like’
manner, as suggested for the uterine key intermediary protein (Baulieu, Wira,
Milgrom & Raynaud-Jamet, 1972). The direct influence of oestrogen at the
level of the embryo for the release from diapause remains uncertain (Prasad,
Sar & Stumpf, 1974). The low molecular weight proteins in progesterone-
treated animals may inhibit embryo metabolism, but it is more likely that the
lack of high molecular weight proteins in the uterine lumen of these animals
leads to quiescence. A number of high molecular weight proteins are detected
13 to 20 hr after oestradiol treatment, i.e. shortly before embryo attachment.

Studies on mammalian cell lines show that environmental conditions revers-
isibly affect the growth of the cells. For example the presence of serum in the
medium stimulates growth, withdrawal or exhaustion of serum leads to transla-
tional inhibition (Hassel & Engelhardt, 1973) and brings about quiescence,
when cells arrest in the G1 phase of the cell cycle without loss of viability
(Pardee, 1974). Such a regulatory response occurs through a co-ordination of
unrelated biochemical events and has been called pleiotropic response (Hershko,
Mamont, Shields & Tomkins, 1971). Apart from serum, insulin (and adenosine
3':5'-cyclic monophosphate) may also be a pleiotypic modulator (Kram, Mamont & Tomkins, 1973). There is strong suggestive evidence that embryos display in a pleiotypic fashion in utero and in vitro, in response to proteins.

In vitro, normal embryos or those in diapause show increased nucleic acid and protein synthesis (Gwatkin, 1966; Ellem & Gwatkin, 1968; Psychoyos & Bitton-Casimiri, 1969) and glucose utilization (Menke & McLaren, 1970) when cultured in the presence of serum. Furthermore, the high molecular weight serum proteins are essential for giant cell transformation (Spindle & Pedersen, 1974).

In utero, the embryos in diapause are blocked in the G1 phase of the cell cycle (Sherman & Barlow, 1972; Surani, 1975) during the time when high molecular weight proteins are undetectable. The effects of maternal environment on the stimulation of embryo metabolism before implantation are reversible since transfer of normal blastocysts to animals experiencing 'delayed' implantation leads to a decline in protein synthesis (Weitlauf, 1969). Furthermore, an embryo is not fully committed to implant if implanted embryos with giant cells on the 6th day of pregnancy are transferred to mothers injected with progesterone alone, they will revert to a state of diapause until oestrogen is injected (Psychoyos, 1961). Cyclic AMP stimulates RNA synthesis in quiescent embryos (Mohla & Prasad, 1970) and induces implantation (Webb, 1975). It is suggested that the protein of mol. wt 70,000 detected 1 hr after oestrogen may be a pleiotypic modulator and the presence of high molecular weight proteins later (with the protein of mol. wt 70,000) results in giant cell transformation and embryo implantation. Embryos transferred 24 hr after oestradiol injection fail to implant (Psychoyos, 1969).

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REFERENCES


Uterine secretions and implantation in rats


