Effect of oestrogen and an LH-RH agonist on the release of gonadotrophins in ovariectomized ewes deprived of LH-RH

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Summary. Endogenous LH-RH in ewes was inhibited by active immunization or by injection of LH-RH antiserum. Plasma levels of LH and FSH were elevated in 3 ovariectomized control ewes but low in 3 LH-RH immunized ovariectomized ewes. Oestradiol benzoate (50 μg i.m.) caused a marked rise in LH concentrations in control ewes but not in the immunized ewes. In the immunized ewes the low plasma levels of FSH decreased even further 8–36 h after injection of oestrogen, indicating a direct inhibitory action of the steroid on the pituitary. Both groups responded to the oestrogen injection by a rise in plasma levels of prolactin and by exhibiting normal oestrous behaviour.

When the control ewes were again challenged with oestradiol benzoate and, after 10 h, given an i.v. injection of 75 ml antiserum to LH-RH, the LH surge was abolished in one animal and reduced in another. These experiments indicate that the continued presence of LH-RH is necessary for the occurrence of the oestrogen-induced LH surge in the ewe.

Administration of a stimulatory analogue of LH-RH released LH and FSH in control and immunized ewes but the responsiveness to further injections at intervals of 3 h decreased, particularly for FSH.

Introduction

The way in which the negative feedback effect of oestrogen on the release of luteinizing hormone (LH) can change to positive feedback and bring about the preovulatory LH surge is still not clear. It has not been established whether the LH surge is preceded by a surge of luteinizing hormone-releasing hormone (LH-RH), composed of an increase in the number and/or amplitude of LH-RH pulses, or whether the rising oestrogen concentrations in the blood are the primary cause of the LH surge by a direct action on the pituitary, with LH-RH secretion remaining constant. There is also no reason why these mechanisms might not act together, with some differences in their relative importance amongst species. Increased levels of LH-RH in the hypophysial portal blood are associated with the LH surge in the rat (Sarkar, Chiappa, Fink & Sherwood, 1976), but the changes in LH-RH secretion in other species during the induced LH surge before ovulation in other species are unknown because of the problem of obtaining portal blood.

One experimental approach to this problem is to inhibit endogenous LH-RH immunologically, by active immunization or by injection of LH-RH antiserum. Injection of LH-RH antiserum to rats and hamsters at 12:00 h on the day of pro-oestrus prevents the peovulatory
LH surge (Koch, Chobieng, Zor, Fridkin & Lindner, 1973; de la Cruz, Arimura, de la Cruz & Schally, 1976), and to chickens blocks the progesterone-induced LH surge (Fraser & Sharp, 1978). In marked contrast, LH-RH antiserum was without effect on the oestrogen-induced LH surge in the rhesus monkey (McCormack, Plant, Hess & Knobil, 1977). Active immunization against LH-RH prevents ovulation in the rat (Fraser & Baker, 1978), sheep (Clarke, Fraser & McNeilly, 1978; Jeffcoate, Foster & Crighton, 1978), marmoset monkey (Hodges & Hearn, 1977) and stump-tailed macaque (H. M. Fraser, unpublished), but the mechanisms causing this have not been studied.

We have therefore investigated the effect of immunological inhibition of endogenous LH-RH on the ability of oestrogen to induce an LH surge in ovariectomized ewes. We have also attempted to restore pituitary function in actively immunized animals by administering a stimulatory analogue of LH-RH which is immunologically different from LH-RH, and which releases gonadotrophins, after a single injection, from the pituitaries of rats (Fraser & Sandow, 1977) and sheep (Clarke et al., 1978) immunized against LH-RH.

Materials and Methods

Animals

Three Scottish Blackface ewes were immunized in August 1976 with LH-RH conjugated to bovine serum albumin (BSA) by carbodiimide, and 3 control ewes were immunized against BSA alone (Clarke et al., 1978). Booster immunizations in Freund's incomplete adjuvant were given 3, 4, 5 and 7 months later. Four months after immunization, all the ewes were ovariectomized and when blood samples were taken 3 weeks later the levels of LH and FSH were elevated in the controls but not in the ewes immunized against LH-RH (Clarke et al., 1978).

Effect of oestradiol benzoate on gonadotrophin secretion and oestrous behaviour

In January 1977, 3 weeks after ovariectomy, all ewes were treated with progestagen pessaries (Cronolone: Searle) for 12 days. An i.m. injection of 50 µg oestradiol benzoate (Intervet, Organon Laboratories Ltd) in 1 ml arachis oil was given 2 days after removal of the pessary and the ewes were run in a pen with a raddled vasectomized ram. The ewes were observed every 2 h to detect the onset of heat. Blood samples were taken by jugular venepuncture every 4 h until the oestrogen injection then every 2 h for a further 36 h.

In February 1978 the effect of injecting antiserum to LH-RH on the oestrogen-induced LH surge in the 3 control ovariectomized ewes was investigated. Blood samples were taken from an indwelling jugular catheter inserted 24 h before starting the experiment and the animals were kept in individual crates. Progestagen pessaries were not given and the animals were not tested for oestrous behaviour. In a control test an injection of 50 µg oestradiol benzoate induced an LH surge in all 3 ewes. The experiment was repeated 2 weeks later, except that the ewes were given an i.v. injection of 75 ml LH-RH antiserum (taken from Ewe 39) 10 h after administration of the oestradiol benzoate, i.e. 2–8 h before the expected time of the LH surge. The specificity of the antiserum was similar to that described previously (Lincoln & Fraser, 1979) but the antibody titre was three times higher, being 1 : 105 000.

Effect of an LH-RH agonist on gonadotrophin release

Different dose regimens of (d-Serine-t-butyl8,des-Glycine-NH210) LH-RH ethylamide (LH-RH agonist: Hoechst A.G.) were given to assess the ability of the pituitaries of the control and LH-RH immunized ewes to respond to exogenous LH-RH. An indwelling jugular venous catheter was inserted and the animals caged individually, 24 h before starting the experiment.
Blood samples were taken at 20-min intervals for 2 h before treatment. A series of LH-RH agonist injections were given between February and May 1977 as follows: (1) a single injection of 5 μg (February), (2) 8 injections of 1 μg at intervals of 3 h (beginning of March), (3) 2 injections of 100 ng 3 h apart (end of March) and (4) 8 injections of 20 ng every 3 h (May). All injections were given i.v. in 1 ml saline (9 g NaCl/l).

Collection of pituitaries

In April 1978, 11 months after the agonist experiments the immunized ewes were given a final booster immunization, and 2 weeks later all the animals were killed. After pentobarbitone sodium anaesthesia, the jugular vein and carotid artery were cut, the animals bled out and the pituitary gland rapidly removed. The anterior pituitary gland was dissected free of other tissue, weighed, and bisected. One half was placed in Bouin’s fixative, and after sectioning, stained with Alcian blue-PAS-orange G. The remainder of the anterior pituitary was weighed before homogenization in 5 ml 0-1 m-phosphate buffer, pH 7-4, containing 0·2% BSA. After centrifugation the supernatant was stored at −20°C until required for assay of LH, FSH and prolactin.

Radioimmunoassays

The concentration of LH was determined by radioimmunoassay (Martensz, Baird, Scaramuzzi & Van Look, 1976) of duplicate 200 μl (LH-RH immunized ewes) or 50 μl (control ewes) quantities of plasma and results were expressed in terms of ng NIH-LH-S14/ml. The sensitivity of the assay was 0·3 ng/ml and the intra- and inter-assay coefficients of variation were 8 and 10% respectively. FSH was measured in duplicate quantities of 150 μl (all ewes) and 50 μl (some control samples) using the radioimmunoassay described by McNeilly, McNeilly, Walton & Cunningham (1976) and results were expressed as ng NIH-FSH-S10/ml. Assay sensitivity was 20 ng/ml with intra- and inter-assay coefficients of variation being 9 and 12% respectively. Prolactin was measured in duplicate quantities of 30 μl plasma by radioimmunoassay (McNeilly & Andrews, 1974) and results were expressed in terms of ng NIH-PRL-S6/ml. This assay had a sensitivity of 0·05 ng/ml and intra- and inter-assay coefficients of variation of 8 and 11% respectively. LH-RH antibody titre was assessed as before (Clarke et al., 1978) and expressed as the initial dilution binding 33% of 125I-labelled LH-RH.

Statistical analysis

Differences in endogenous levels of LH, FSH and prolactin were compared using a 2-factor analysis of variance with replication. Changes in concentrations of FSH after treatment with oestriadiol benzoate and LH-RH agonist were analysed by analysis of variance without replication.

Results

Throughout the period of study the immunization against LH-RH was successful in inhibiting the action of the endogenous hormone since plasma gonadotrophin levels were consistently low (<0·8 ng LH/ml, 30–100 ng FSH/ml) in all 3 treated animals, while values in control animals were markedly elevated (e.g. see Text-figs 1 and 3). LH-RH antibody levels during the experiments were high in 2 ewes and relatively low in the remaining animal (Table 1).
Table 1. Hormone content of anterior pituitary glands and range of LH-RH antibody titre during the experimental period in ovariectomized ewes immunized against LH-RH

<table>
<thead>
<tr>
<th>Group</th>
<th>Ewe No.</th>
<th>LH (pg/gland)</th>
<th>FSH (pg/gland)</th>
<th>Prolactin (mg/gland)</th>
<th>LH-RH antibody titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>59</td>
<td>4064</td>
<td>691</td>
<td>5934</td>
<td></td>
</tr>
<tr>
<td></td>
<td>66</td>
<td>4606</td>
<td>500</td>
<td>2808</td>
<td></td>
</tr>
<tr>
<td></td>
<td>242</td>
<td>1525</td>
<td>386</td>
<td>8286</td>
<td></td>
</tr>
<tr>
<td>LH-RH</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>immunized</td>
<td>23</td>
<td>32</td>
<td>42</td>
<td>3824</td>
<td>1:5000–1:12 800</td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>380</td>
<td>120</td>
<td>1452</td>
<td>1:800–1:1600</td>
</tr>
<tr>
<td></td>
<td>39</td>
<td>35</td>
<td>27</td>
<td>3987</td>
<td>1:17 000–1:105 000</td>
</tr>
</tbody>
</table>

Effect of exogenous oestrogen on gonadotrophin secretion and oestrous behaviour

In the control ewes the administration of oestrogen was followed by a decline in LH lasting 12 h but after 16–24 h a marked rise in LH concentrations occurred. In contrast, in all the LH-RH immunized ewes LH levels remained at <0.8 ng/ml throughout (Text-fig. 1).

Text-fig. 1. Plasma levels of LH, FSH and prolactin in (a) 3 control ovariectomized ewes and (b) 3 ovariectomized ewes immunized against LH-RH after the i.m. injection of 50 μg oestradiol benzoate (arrow). Note the difference (x 10) in the scale for (a) and (b).
There was a tendency for plasma FSH concentrations to decline during the last 8 h of the sampling period in the control ewes but no clear-cut response was evident. In all 3 LH-RH immunized ewes there was gradual decline in FSH levels, beginning 4 h after the oestrogen injection, and plasma FSH values at 8–36 h were significantly \((P < 0.001)\) lower than preinjection levels (Text-fig. 1).

Preinjection concentrations of prolactin were significantly \((P < 0.01)\) higher in the immunized ewes, than in the control but oestrogen injection resulted in a prolonged rise, beginning 12–20 h after the injection, in both groups (Text-fig. 1).

Oestrous behaviour appeared normal in all ewes and occurred 19 ± 2 h (controls) and 15 ± 10 h (immunized) (mean ± s.d.) after the oestrogen injection.

Injection of antiserum to LH-RH completely blocked the oestrogen-induced LH rise in one ewe and severely reduced it in another (Text-fig. 2). In the remaining animal the LH rise had not occurred by 17 h after the oestrogen injection when sampling was stopped; during the control test in this animal the plasma LH levels had been markedly elevated (39 ng/ml) by 16 h. The FSH response was unaltered in all 3 ewes.

**Text-fig. 2.** Plasma levels of LH in 2 ovariectomized ewes after an i.m. injection of 50 μg oestradiol benzoate (OB) during a control test (O) and after injecting 75 ml antiserum to LH-RH (○).

**Effect of LH-RH agonist on gonadotrophin release**

Before the agonist injections, LH and FSH concentrations were elevated in the control ewes but very low in the immunized animals.

**Schedule 1.** The injection of 5 μg LH-RH agonist induced a rapid release of LH with peak values being obtained 1–2 h later in both groups (Text-fig. 3). Concentrations in the LH-RH immunized ewes were much lower although the pattern of release appeared similar. The magnitude of the response in the immunized ewes was related to antibody titre, being highest (39 ng/ml) in the animal with the lowest titre and 10 ng/ml in the other 2 ewes. FSH levels also rose in both groups, with the increase in the LH-RH immunized ewes being less than that in the controls (Text-fig. 3).

Plasma levels of prolactin were not affected by the agonist. During the 6-h study period they were significantly higher \((P < 0.001)\) in the immunized animals, the mean ± s.e.m. values being 38 ± 9·3, 42 ± 13 and 49 ± 9 ng/ml in the LH-RH immunized ewes and 27 ± 8, 32 ± 9 and 32 ± 16 ng/ml in the controls.
Text-fig. 3. Plasma levels of LH and FSH (mean ± s.e.m.) in 3 control ovariectomized ewes (a, b) and 3 ovariectomized ewes immunized against LH-RH (c, d) following a single injection of 5 µg LH-RH agonist (Schedule 1) (a, c) or 8 injections of 1 µg LH-RH agonist at 3 h intervals (Schedule 2) (b, d). Note the difference for LH (× 10) and FSH (× 5) in the scale for (a, b) and (c, d).
Schedule 2. When 1 μg LH-RH agonist was injected on 8 occasions 3 h apart the control ewes showed a marked rise in LH levels in response to the first injection but there was a progressive diminution of response to each subsequent injection (Text-fig. 3). The LH-RH immunized ewes also responded most to the first injection, LH levels in the animal with a low titre reaching 23 ng/ml with highest levels in the other animals being 7 and 9 ng/ml. After subsequent injections plasma LH values were considerably lower and after the fifth injection there was very little response (Text-fig. 3).

There was a rise in plasma FSH levels in both groups, but the response was less sustained than that for LH, and after treatment for 15 h (i.e. after the 5th injection) the FSH levels were significantly lower \( (P < 0.001) \) in both groups than the preinjection levels (Text-fig. 3).

Schedule 3. LH concentrations in the immunized ewes rose from 0·6 ± 0·1 ng/ml to a maximum of 3·1 ± 0·3 ng/ml (mean ± s.e.m.) after the first injection of 100 ng LH-RH agonist, but the response to the second injection was reduced, levels rising to only 1·8 ± 0·2 ng/ml. Concentrations of FSH rose from 56 ± 9 to a maximum of 94 ± 17 ng/ml after the first injection and there was no clearly defined rise after the second injection. Control ewes were not studied.

Schedule 4. It was impossible to distinguish any response of the control ewes to the 8 injections of 20 ng LH-RH agonist. In Ewes 27 and 39 (immunized) this treatment caused LH levels to rise from 0·5 to 0·8–1·6 ng/ml after each injection but there was no enhancement of responsiveness. In the remaining LH-RH immunized ewe this dose of agonist was without effect on LH levels. In all 3 LH-RH immunized ewes plasma FSH values seemed unaffected by the treatment and remained below 70 ng/ml. At this time of year (May) the normal seasonal rise in prolactin levels had occurred in both groups of ewes and concentrations (mean ± s.e.m.) during this study period were 91 ± 28, 109 ± 26, 121 ± 26 ng/ml in the controls and 95 ± 30, 93 ± 33 and 106 ± 36 ng/ml in the LH-RH immunized ewes. These values were not significantly different.

Pituitary hormone contents and histology

Pituitary contents of LH and FSH were considerably reduced in the LH-RH immunized ewes, appearing inversely proportional to antibody titre, while pituitary contents of prolactin were similar in both groups (Table 1). Histological examination of the pituitaries revealed that acidophils were the predominant cell type in all animals. Identifiable gonadotroph cells were clearly fewer in number in pituitaries from the LH-RH immunized ewes and appeared small and shrunken compared to those of controls.

Discussion

The presence of circulating antibodies to LH-RH in ovariectomized ewes inhibits the action of the endogenous hormone as judged by the low levels of LH and FSH in the blood and by their reduced content in the anterior pituitary gland. A very small amount of LH-RH probably reaches the gonadotroph cells, particularly if antibody production is low, as illustrated by the fact that pituitary content of LH and FSH was highest in the ewe with the lowest antibody titre. Studies of rats with different LH-RH antibody titres have given similar results (Fraser & Baker, 1978). The presence of small amounts of biologically active LH-RH may also explain why some FSH is detectable in the plasma of LH-RH immunized ewes. Nevertheless, in these ewes the action of LH-RH is effectively blocked and as the source of the ovarian steroid hormones is also absent they provide an in-vivo system with which to study the ability of oestrogen and LH-RH agonists to stimulate the secretion of LH and FSH independently of endogenous LH-RH secretion. This study has established that the ability of oestrogen to induce an LH surge is
completely abolished in ewes immunized against LH-RH, showing that LH-RH is necessary for the LH surge to occur.

It was also important to establish how much, if any, gonadotrophin the pituitaries of these LH-RH immunized animals could release following exogenous LH-RH stimulation. This was done by injecting an agonist of LH-RH which is effective in small amounts and is not inhibited by the circulating LH-RH antibodies (Fraser & Sandow, 1977; Clarke et al., 1978; Jeffcoate et al., 1978). A comparison of Text-figs 1 and 3 shows that in control ewes a dose of 1 μg LH-RH agonist can produce an LH rise equivalent to that stimulated by an oestrogen test. Although the response of the LH-RH immunized ewes was much less it nevertheless showed that the pituitaries of these animals could release LH and FSH but that oestrogen alone could not exert any stimulatory gonadotrophin-releasing action on the pituitary.

When interpreting these findings to assess the role of LH-RH it must be remembered that the pituitaries of these animals had been deprived of the normal priming effect of endogenous LH-RH, which is important in contributing to the increased responsiveness to oestrogen, and their capacity to synthesize new hormone may therefore be impaired. These differences were avoided by carrying out the experiment in the control ewes and inhibiting LH-RH by injecting antiserum, but not until 10 h after the oestrogen injection. When oestrogen was allowed to act on a normal pituitary receiving LH-RH priming, the inhibition of LH-RH just before the expected LH surge completely abolished positive feedback in one animal and severely reduced it in another. Subsequent studies have confirmed the abolition of the LH surge in 5 intact ewes treated with oestrogen and an LH-RH antiserum (H. M. Fraser & A. S. McNeilly, unpublished observations). These results are similar to the inhibitory effects of injecting antibodies to LH-RH on the LH surge in the rat (Koch et al., 1973), hamster (de la Cruz et al., 1976) and fowl (Fraser & Sharp, 1978), but are in marked contrast to the lack of effect in the rhesus monkey, in which administration of antibodies both before and after oestrogen injection failed to abolish the LH surge (McCormack et al., 1977).

The usefulness of animals actively immunized against LH-RH would be enhanced if it were possible to induce normal synthesis and release of LH and FSH by exogenous releasing hormone. This might be attempted by injecting massive doses of LH-RH, but most of this would be inhibited by the circulating antibody (see Lincoln & Fraser, 1979). The use of an LH-RH agonist has the advantage that it is highly potent and can by-pass the circulating antibody because of immunochemical differences from LH-RH (Fraser & Sandow, 1977). However, the present attempts to stimulate pituitary activity by repeated injections of 20 ng–1 μg LH-RH agonist resulted in a decreased pituitary responsiveness in the control and immunized ewes, as has been found for higher doses of agonist in rams (Fraser & Lincoln, 1980), monkeys (Fraser, Laird & Blakeley, 1980) and women (Dericks-Tan, Hamer & Taubert, 1977). In the present study, using low doses, it was clear that a 3-h interval is not long enough for the gonadotroph cells to recover from the exposure to agonist.

In the LH-RH immunized ewes the administration of oestrogen caused the already low levels of FSH in the blood to decrease even further within a few hours. Studies of sheep pituitaries in vitro have shown that oestradiol-17β can have a direct inhibitory action on the secretion of FSH (Miller, Knight, Grimek & Gorski, 1977). Since, in LH-RH immunized animals, the pituitary is constantly deprived of LH-RH stimulation, the present results in vivo provide further evidence of a negative feedback action of oestrogen directly on the pituitary gonadotroph cells.

In the LH-RH immunized ewes a single injection of 1 or 5 μg LH-RH agonist gave a particularly rapid release of FSH which was slightly more pronounced than that of LH when compared with controls. Both gonadotrophins were readily available for release despite the lack of previous exposure to endogenous LH-RH. However, the FSH response soon became negligible in all the animals, and levels fell below preinjection values in both groups after the fifth agonist injection. This indicates that the mechanisms involved in release of additional stores of FSH are rapidly affected by repeated exposure to LH-RH agonist.
Prolactin concentrations were higher in the LH-RH immunized ewes than in controls during the months of January and February but this difference disappeared with the seasonal influences which normally induce high levels of prolactin (Walton, McNeilly, McNeilly & Cunningham, 1977). An elevation in prolactin levels in these ewes before ovariectomy has been described previously (Clarke et al., 1978), but the reason is unclear. It may be connected with the fact that in these animals all aspects of negative feedback have been removed by the absence of the ovaries and by the absence of elevated gonadotrophin levels. If this results in an increased output of LH-RH this might decrease hypothalamic turnover and output of dopamine, thus increasing prolactin secretion (McNeilly, 1980). Administration of LH-RH agonist was without effect on prolactin secretion while the injection of oestrogen resulted in a clear rise in prolactin levels in both groups. This effect of oestrogen is probably brought about by its ability to block dopamine receptors on the prolactin-secreting cells (Labrie et al., 1978).

We thank Dr K. McLaren and Dr G. E. Turner for advice on pituitary histology; Mr D. Patterson, Miss D. M. Blakeley and Miss B. A. Archibald for expert technical assistance; Dr S. Dombev (Hoechst, U.K.) for gifts of LH-RH and the agonist; and NIAMDD for radio-immunoassay materials.

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Received 21 July 1980