Effect of active immunization against oestrogens on plasma gonadotrophins in the ewe and the response to synthetic oestrogen or LH

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Summary. Immunization against oestrogen resulted in elevated LH concentrations, in the form of a pulsatile release, which rose from 3 to as much as 41 ng/ml approximately every 1·5 h, even in the presence of high plasma progesterone concentrations. Elevated FSH concentrations showed only minor oscillations without consistent synchrony to the LH pulses. Injection of 250 µg stilboestrol did not abolish the LH pulses but in 2 out of 8 ewes FSH was initially lowered. Injection of 1 mg stilboestrol abolished the LH pulses within 11 h and decreased FSH values in 2 out of 4 ewes. Between 16 and 35 h after injection there was a large increase in LH and FSH concentrations. Thus, in the ewe, the tonic secretion of LH and FSH is controlled by a negative feedback action of oestrogen, and diethylstilboestrol will exert both positive and negative feedback effects on both gonadotrophins, depending upon dose. Infusion of LH was unable to alter the frequency or height of the LH pulses, thereby excluding regulation of the LH pulses by a short feedback mechanism.

Introduction

Many studies have provided ample evidence to indicate that the ovarian oestradiol secretion at the time of oestrus triggers the release of LH and FSH in the cyclic ewe by a positive feedback action (Goding *et al.*, 1969; Pant, Hopkinson & Fitzpatrick, 1977) and that the administration of oestradiol evokes a discharge of both gonadotrophins in the anoestrous ewe (Jonas *et al.*, 1973; Pant & Ward, 1974).

The demonstration of a marked rise in the plasma LH of ewes after ovariectomy provided indirect evidence for the negative feedback effect of gonadal steroid hormones on LH release (Niswender, Roche, Foster & Midgley, 1968; Roche, Foster, Karsch & Dziuk, 1970). Scaramuzzi, Tillson, Thorneycroft & Caldwell (1971) reported an initial negative feedback effect of oestradiol benzoate on plasma LH concentrations in ovariectomized ewes, and Brown, Cumming, Goding & Hearnshaw (1972) observed a fall in the elevated plasma LH concentrations of ovariectomized ewes following prolonged infusion of oestradiol.

In none of the above studies was the concentration of plasma FSH measured. The present study was therefore designed to gain further insight into both the negative and positive feedback effects of oestradiol on LH and FSH secretion in ewes which were actively immunized against oestrogen.

Materials and Methods

Animals

In September, 5 mature Clun Forest ewes were immunized against oestrogen with a conjugate of oestradiol-17 β -hemisuccinate with bovine serum albumin after each had exhibited one normal

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oestrous cycle. The immunization schedule consisted of three subcutaneous injections of 2 mg conjugate emulsified in 1 ml Freund's complete adjuvant at 21-day intervals (Days 0, 21 and 42), followed by similar 'booster' injections throughout the following year on Days 109, 127, 158, 218, 290, 340 and 376. Each experiment took place 2 weeks after an injection of the immunogen. Each ewe was tested with a vasectomized ram for signs of oestrus. The ewes were housed indoors in individual pens and indwelling jugular venous catheters were inserted 25 h before each sampling period.

Experimental design

Experiment 1. In January, on Day 123 after immunization began, the ewes were injected with 0.5 ml arachis oil and bled every 2 h for 36 h without further treatment. In this, and subsequent experiments, 5 ml blood were collected into heparinized syringes and centrifuged and the plasma was stored at -15°C until analysis.

Experiment 2. On Day 141, blood was collected every 15 min for 8 h from each ewe, except No. 73, to investigate the short-term changes in plasma gonadotrophin concentration.

Experiment 3. On Day 172 the ewes were injected i.m. with a pharmacological dose of stilboestrol dipropionate (May & Baker Ltd), 250 μg in 0.5 ml arachis oil, and bled every 2 h for 48 h.

Experiment 4. On Day 232 (April) the ewes were injected i.m. with 1 mg oestradiol-17 β (Koch Light) and bled every 2 h for 36 h.

Experiment 5. Three of the ewes (Nos 17, 78 and 80) were treated on Day 304 with 0.5 ml arachis oil i.m. followed 4 h later by an i.m. injection of 250 μg stilboestrol dipropionate. Blood was collected every 15 min for 12 h after the arachis oil injection.

Experiment 6. On Day 390 4 ewes (Nos Y5, 17, 70 and 80), were bled at 15-min intervals for 4 h then injected i.m. with 1 mg stilboestrol dipropionate in arachis oil (0 h). Blood sampling continued every 15 min for 4 h and every 2 h for the next 48 h with a 2-h period of 15-min sampling interspersed at 11, 23 and 35 h.

Experiment 7. On Day 354, blood was collected (control period) every 15 min for 4 h from 4 ewes (Nos Y5, 17, 78, 80) and was followed by an i.v. infusion of ovine LH (NIH-LH-S16) at a rate of 30 mg/h for 4 h. Blood sampling was continued every 15 min from a catheter in the contralateral jugular vein.

Hormone radioimmunoassays

Oestrogen. The potency of the oestrogen antisera was estimated on Days 123 and 232 after the start of immunization in a liquid-phase system (Pant *et al.*, 1977) and the results are given in Table 1. The inter- and intra-assay coefficients of variation were 12.6 and 9.7% respectively. The cross-reactions of the antisera from each ewe were estimated as described by Abraham (1969); the cross-reactions ranged from 58 to 77% for oestrone and from 49 to 69% for oestradiol-17 α .

Table 1. Dilutions of serum samples from ewes immunized against oestrogen which bound 50% of 10 000 c.p.m. of ^3H -labelled oestradiol

Ewe	Titre	
	Exp. 1	Exp. 4
73	1: 4 000	1:10 000
Y5	1:17 000	1:37 000
78	1:24 000	1:42 000
80	1:29 000	1:40 000
17	1:34 000	1:62 000

Progesterone. The first sample from each ewe in each experiment was analysed for progesterone by the unmodified method described and verified by Pant *et al.* (1977). The progesterone antibody was raised against progesterone-6 α -(carboxymethylene) thio-ether-bovine serum albumin and showed only 2% cross-reaction with 20 α -hydroxypregn-4-en-3-one and 4% with 17 α -hydroxyprogesterone. The sensitivity of the method was 25 pg/0.5 ml and the inter- and intra-assay coefficients of variation were 10.6 and 9.7% respectively.

LH and FSH. These hormones were measured in every plasma sample by the unmodified methods described and verified by Pant *et al.* (1977). The anti-ovine LH antibody (GDN-15) was raised in rabbits by Dr G. D. Niswender and had no significant cross-reaction with other pituitary hormones. Purified ovine LH (LER-1056-C2) was used as the 125 I-labelled preparation. The sensitivity of the method was < 200 pg/0.2 ml and the inter- and intra-assay coefficients of variation were 14.3 and 8.8% respectively. The plasma results are expressed in ng equivalents of NIH-LH-S16/ml. The anti-ovine FSH antibody was raised in rabbits by Dr J. C. Hendrick and had 30% cross-reaction with TSH and 5% with LH. Absorbing the antiserum with 500 ng ovine TSH and LH made no significant difference to the plasma results. Rat FSH (NIH-FSH-RP1) was used as the 125 I-labelled preparation. The sensitivity of the method was 10 ng/0.2 ml with inter- and intra-assay coefficients of variation of 7.8 and 7.0% respectively.

Results

Experiment 1

The gonadotrophin pattern obtained in all the ewes is exemplified by that of Ewe Y5 (Text-fig. 1a). The mean ($\pm$ s.e.m.) hormone values in all 5 ewes were 8.3 ± 0.99 ng LH/ml and 124 ± 6.02 ng FSH/ml and these were significantly higher (Student's *t* test, $P < 0.01$) than the values observed in intact ewes during anoestrus (2.1 ± 0.13 ng LH/ml and 37 ± 2.6 ng FSH/ml, $N = 4$) and during the luteal phase of the oestrous cycle (2.6 ± 0.09 ng LH/ml and 62 ± 2.8 ng FSH/ml, $N = 4$). The progesterone concentrations at the beginning of this experiment were 0.4, 13.6, 0.5, 5.5 and 0.9 ng/ml for Ewes Y5, 17, 73, 78 and 80 respectively.

Text-fig. 1. Plasma LH (o) and FSH (●) concentrations in Ewe Y5 after i.m. injection (arrow) of (a) arachis oil (Exp. 1) and (b) 250 μ g stilboestrol dipropionate (Exp. 3).

Experiment 2

Frequent blood sampling revealed a pulsatile pattern of plasma LH release (Text-fig. 2); there was an abrupt and significant rise (one-way analysis of variance, $P < 0.001$) in plasma LH from nadir to peak within a period of 15 min. The interval between one peak and the next varied within

and between ewes, ranging from 75 to 100 min. The magnitude of the peaks ranged from 8.6 to 41.5 ng/ml with baseline values between 3.9 and 5.1 ng/ml. In contrast the concentration of plasma FSH did not change in such a pulsatile manner but showed minor oscillations without any consistent synchrony with LH pulses. The differences between maximum and minimum values of FSH were not significant. Plasma concentrations of progesterone were 0.5, 10.1, 0.5 and 5.5 ng/ml for Ewes Y5, 17, 78 and 80 respectively at the start of sampling.

Text-fig. 2. LH (○) and FSH (●) concentrations in ewes immunized against oestrogen in plasma samples collected at 15-min intervals (Exp. 2).

Experiment 3

An example of the gonadotrophin pattern obtained from sampling every 2 h after injection of 250 µg stilboestrol dipropionate is shown in Text-fig. 1(b). There was initially a decrease in both LH and FSH concentrations but this was not significant ($P < 0.1$, Student's paired t test). Three of the 5 ewes also showed a marked elevation in plasma gonadotrophins beginning between 18 and 36 h (23.3 ± 6.7 , s.e.m.) after the injection of stilboestrol and reaching peak values of 82–130 (114 ± 13.1) ng LH/ml and 135–160 (148.3 ± 5.92) ng FSH/ml between 20 and 38 h. The rise lasted for 8 to 12 h (10 ± 0.92 h). The progesterone concentrations were 1.7, 0.1 and 0.4 ng/ml in the 3 responding ewes (Nos Y5, 73 and 78 respectively) and 7.3 and 5.0 ng/ml in Ewes 17 and 80.

Experiment 4

In contrast to the effect of the synthetic oestrogen, no decrease or later elevation in the circulating gonadotrophins was produced by injection of oestradiol-17β. The initial concentration of progesterone was 0.5, 10.1, 0.5, 0.6 and 5.5 ng/ml for Ewes Y5, 17, 73, 78 and 80 respectively.

Experiment 5

In the 8 h after the injection of 250 µg stilboestrol dipropionate in oil, the pulsatile LH discharge was not abolished, although, as shown in Table 2, there was a non-significant decrease in both the

Table 2. Mean $\pm$ s.d. plasma concentrations (ng/ml) of LH and FSH in oestrogen-immunized ewes before and after treatment with ovine LH or stilboestrol dipropionate

	Ewe Y5		Ewe 17		Ewe 78		Ewe 80	
	LH	FSH	LH	FSH	LH	FSH	LH	FSH
Experiment 5 (250 μ g stilboestrol)								
Before								
After (0 to +8 h)			10.9 $\pm$ 2.0	333.2 $\pm$ 13.4	6.9 $\pm$ 1.8	153.8 $\pm$ 6.6	6.4 $\pm$ 1.6	153.0 $\pm$ 6.4
			8.4 $\pm$ 1.6	276.1 $\pm$ 15.4*	5.6 $\pm$ 0.8	151.0 $\pm$ 6.8	5.0 $\pm$ 0.4	139.7 $\pm$ 7.0**
Experiment 6 (1 mg stilboestrol)								
Before	6.5 $\pm$ 1.4	295.9 $\pm$ 67.8	17.6 $\pm$ 3.2	599.0 $\pm$ 37.3	6.2 $\pm$ 2.0	57.5 $\pm$ 8.1	9.7 $\pm$ 2.2	455.0 $\pm$ 70.7
After: 0-2 h	6.4 $\pm$ 1.7	293.3 $\pm$ 59.1	16.4 $\pm$ 2.5	614.4 $\pm$ 33.1	6.8 $\pm$ 2.1	56.4 $\pm$ 4.4	9.8 $\pm$ 2.0	492.7 $\pm$ 76.4
3-5 h	4.3 $\pm$ 0.7*	237.7 $\pm$ 40.5	17.2 $\pm$ 1.8	658.3 $\pm$ 57.9	5.0 $\pm$ 0.6	46.7 $\pm$ 6.9	5.6 $\pm$ 0.7*	638.8 $\pm$ 70.8
11-13 h	4.0 $\pm$ 0.6*	115.2 $\pm$ 34.6*	10.3 $\pm$ 1.8*	641.1 $\pm$ 106.8	3.9 $\pm$ 1.1*	35.5 $\pm$ 5.2*	5.3 $\pm$ 0.7*	471.1 $\pm$ 61.1
23-25 h	41.5 $\pm$ 4.2	256.8 $\pm$ 69.5	11.8 $\pm$ 2.6	684.4 $\pm$ 76.0	48.9 $\pm$ 2.9	167.1 $\pm$ 13.3	14.8 $\pm$ 2.4	392.5 $\pm$ 34.7
35-37 h	7.9 $\pm$ 1.3	101.6 $\pm$ 23.3	11.5 $\pm$ 4.7	536.6 $\pm$ 78.6	6.3 $\pm$ 1.3	80.4 $\pm$ 11.9	5.3 $\pm$ 0.9	223.3 $\pm$ 69.3*
Experiment 7 (ovine LH)								
Before	5.5 $\pm$ 1.6		17.3 $\pm$ 4.6		7.4 $\pm$ 2.4		10.7 $\pm$ 5.3	
During infusion	16.7 $\pm$ 2.8		32.0 $\pm$ 7.9		36.4 $\pm$ 4.4		34.0 $\pm$ 5.4	

Significantly lower than pretreatment value (Student's *t* test), * *P* < 0.01, ** *P* < 0.02.

mean LH concentration and the standard deviation (Student's paired *t* test). The frequency of LH pulses was not affected. However, in Ewes 17 and 80 the mean plasma FSH concentration was significantly reduced ($P < 0.01$ and 0.02 respectively). Plasma concentrations of progesterone at the start of the experiment were 3.5, 1.1 and 0.6 ng/ml for Ewes 17, 78 and 80.

Experiment 6

For the first 3 h after injection of 1 mg stilboestrol dipropionate in oil, the plasma LH pulses persisted in all 4 ewes (Table 2) and their frequency was sustained. Thereafter the peak heights diminished and by 11 h after injection the pulses were absent and did not reappear until after a very large increase in LH, ranging between 14.8 and 48.9 ng/ml, in Ewes 80, Y5 and 78. This raised concentration was sustained in the 3 ewes between 16 and 35 h after injection; it was not detected at all in Ewe 17. In Ewes Y5 and 78 the plasma concentration of FSH was reduced by +3 h with a further decline over the next 8 h; in these two animals large increases in FSH also occurred concurrently with LH at about +24 h. Plasma FSH did not alter significantly throughout the experiment in Ewe 17 and was only significantly lower in Ewe 80 at +35 h. Initial plasma concentrations of progesterone were 0.1, 0.2, 0.1 and 0.1 ng/ml for Ewes Y5, 17, 78 and 80 respectively.

Experiment 7

Before infusion of LH there was a pulsatile variation in LH concentration as described in Exp. 2. During the LH infusion the increase in mean LH concentration ranged from 2-fold to 5-fold, in the 4 ewes compared to the control period (Table 2). However, the standard deviation did not decrease, i.e. the pulsatile release of LH was not diminished by the higher circulating LH concentrations. The frequency of the pulses was not affected by the LH infusion. The initial plasma progesterone concentrations were 0.4, 1.0, 2.1 and 1.6 ng/ml for Ewes Y5, 17, 78 and 80 respectively.

Discussion

Active immunization against oestrogen appears to remove the suppression on the hypothalamic-pituitary axis which is exerted, at least partly, by oestrogens. The pulsatile pattern of plasma LH observed in Exp. 2 indicates that the elevated mean concentration found during Exp. 1 was not due to a continuous high rate of secretion but represents an integration of discrete discharges of LH from the pituitary of varied but high magnitude. In this respect immunization against oestrogen mimics the castration effect reported in other species (ewe: Butler, Malvern, Willett & Bolt, 1972; Reeves, O'Donnell & Denorscia, 1972; Diekman & Malvern, 1973; monkey: Dierschke, Bhattacharya, Atkinson & Knobil, 1970).

Our results show that the upper part of the descending limb of the LH pulses has a steeper slope than does the lower end. The half-life of LH calculated from the regression estimates of the upper segment of 11 descending limbs averaged 28.3 ± 1.02 min and is similar to that previously reported (28.6 min) for intact ewes (Geschwind & Dewey, 1968). However, the half-life calculated from estimates of whole descending limbs was 45.5 min, suggesting a sustained rather than abrupt release of LH from the pituitary at each pulse. Alternatively, it is an indication that the LH pulses may be superimposed on a high but relatively constant secretion as suggested earlier for ovariectomized monkeys (Yamaji, Dierschke, Bhattacharya & Knobil, 1972). An assumption implicit in this suggestion is that immunization does not alter the metabolic clearance rate of LH.

The relatively constant pattern of FSH concentration without pulses could be due, at least in part, to the longer half-life of ovine FSH (72 min; Kragt & Cons, 1973), to a differential effect of active immunization against oestrogen on the response of pituitary gonadotrophins to the gonadotrophin releasing factor, or to there being two separate releasing mechanisms regulating the secretion of two gonadotrophins.

As expected, after injection of oestradiol-17 β there was no alteration in the pattern of gonado-

trophins, presumably because of neutralization of the exogenous hormone by the high titre of antibodies to oestrogen. The synthetic oestrogen, stilboestrol dipropionate, was chosen to investigate the effect of oestrogen replacement because it would not cross-react with the antibodies present. The lower dose (250 µg) did not have any significant short-term effect (+8 h) on the LH pulses in Exps 3 and 5, but the higher dose (1 mg) lowered the mean LH concentration significantly by +11 h in all 4 ewes, demonstrating that the negative feedback system was still operative in oestrogen-immunized ewes. However, from our limited results it is not possible to distinguish between the effects of dose and time. This latter relationship has been shown in ovariectomized ewes after treatment with oestradiol: an infusion of 0.1 µg/h in saline took 3–4 weeks to reduce LH concentrations to pre-ovariectomy values (Brown *et al.*, 1972), whereas a single injection of 25 µg oestradiol benzoate in alcohol or 100 µg oestradiol in oil reduced LH concentrations within 4 h (Davis & Borger, 1974; Howland & Palmer, 1973). In our experiments there was a variable response of plasma FSH to stilboestrol dipropionate. This could again have been a dose effect: only 2 of the 8 ewes treated with 250 µg and 2 of the 4 treated with 1 mg had significantly lower plasma FSH values by +8 h. The independence of the FSH decrease in concentration from that of LH could be due to a differential effect of oestrogen on the pituitary content and turnover times of the two gonadotrophins, or to the existence of a separate FSH releasing mechanism.

A positive feedback effect after synthetic oestrogen was also demonstrated in Exps 3 and 6. The LH concentration began to rise 16.6 ± 1.0 h after the injection of 1 mg stilboestrol dipropionate in oil to ewes immunized for 390 days (Exp. 6) compared to a rise 23.3 ± 6.9 h after 250 µg stilboestrol dipropionate in oil to ewes immunized for 172 days (Exp. 3). Reeves, Beck & Nett (1974) reported the same time interval to gonadotrophin surges after different doses of oestradiol in alcohol given to anoestrous ewes. However, Brown *et al.* (1972) reported different intervals in the response to exogenous oestradiol of ewes which had recently been ovariectomized than in those ovariectomized 120 days previously. These observations suggest that the difference in the time lag to the induced gonadotrophin surges found in Exps 3 and 6 could be due to an alteration in the sensitivity of the hypothalamic–pituitary axis during the absence of oestrogen and not simply to the different doses of stilboestrol dipropionate used.

The lack of positive response in the 2 ewes in Exp. 3 could be associated with the high plasma progesterone concentrations in these animals (7.3 and 5 ng/ml), because others (Pelletier & Signoret, 1969; Scaramuzzi *et al.* 1971; Pant & Ward, 1974) have reported that progesterone can block the stimulatory effect of oestrogen on gonadotrophin release. The positive response to oestrogen in one ewe (Y5), despite a plasma progesterone of 1.78 ng/ml, confirms our earlier conclusion (Pant & Ward, 1974) that the inhibitory effect of progesterone can be overcome by a high dose of oestrogen.

The immunized ewes did not exhibit behavioural oestrus during the breeding season. The progesterone concentrations showed considerable variation ranging from 0.55 to 13.6 ng/ml. High values were also observed in samples taken during the non-breeding season when values of < 0.2 ng/ml are expected in untreated ewes, but the pulsatile mode of LH secretion was maintained even in the presence of these high concentrations of plasma progesterone.

The inability of an infusion of ovine LH to abolish the pulsatile pattern of endogenous LH would suggest that a 'short' feedback mechanism of pituitary secretion on hypothalamic activity is not the regulatory mechanism causing the sudden decreases in plasma LH concentration. David, Fraschini & Martini (1966) showed that median eminence implants of LH in the rat lowered plasma LH values; possibly a high enough hypothalamic concentration of LH was not achieved by our infusion. However, the concept of a short-feedback mechanism is hard to correlate with the high concentration of LH at the nadir of a pulse compared to normal luteal phase concentrations. If a short feedback mechanism did exist, the basal concentration of LH could not rise in immunized or castrated animals.

The present results do not reveal the mechanism which regulates the pulsatile release of LH in immunized ewes. Intermittent signals from the central nervous system may trigger a periodic release of gonadotrophin releasing factor, as suggested from studies in ovariectomized monkeys in which pulsatile LH surges were abolished by α -adrenergic blocking agents (Bhattacharya, Dierschke, Yamaji & Knobil, 1972).

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