Effect of progesterone on the GnRH-induced secretion of oestradiol and androstenedione from the autotransplanted ovary of the anoestrous ewe

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Two experiments were conducted during the anoestrous period in Border Leicester × Merino ewes with ovarian autotransplants to study the effects of a single injection of 20 mg progesterone on follicular steroid secretion. The aim of these experiments was to determine whether pretreatment with a 20 mg intramuscular injection of progesterone could reduce GnRH-induced ovarian steroid secretion in anoestrous ewes. In both experiments, an injection of 150 ng GnRH induced an LH pulse in all ewes with a maximum concentration 10 min (the first post-injection sample) after injection. Oestradiol and androstenedione secretion increased progressively after the GnRH-induced LH pulse and reached maximum rates of secretion between 60 and 90 min before decreasing slowly to pre-injection rates at 150 min. There were no differences in the pattern of secretion of oestradiol (measured in both experiments) or androstenedione (measured only in Expt 2). In Expt 1, the injection of progesterone 72 h before the challenge with GnRH had no effect on the maximum rate of oestradiol secretion from the autotransplanted ovary. However, in Expt 2, when progesterone was given either 36 or 60 h before GnRH, there was a significant suppression in the maximum rate of secretion of both oestradiol and androstenedione between 60 and 90 min after GnRH injection. These data show that pretreatment of anoestrous sheep with progesterone can suppress LH-stimulated steroid secretion from the ovary and indicate that progesterone may have a direct effect on oestrogenic follicles in sheep.

Introduction

The sudden introduction of rams into a flock of anoestrous ewes will induce ovulation in the ewes, a phenomenon known as the 'ram effect' and which has been extensively reviewed (Knight, 1983; Martin *et al.*, 1986; Signoret, 1980; Signoret *et al.*, 1984). The ram effect is widely used to advance the onset of the breeding season of sheep in France (Cognié *et al.*, 1980) and Australia (Pearce and Oldham, 1984). The introduction of rams leads to a rapid increase in the frequency of pulsatile LH release in ewes which, in turn, leads to the stimulation of oestradiol secretion from a developing follicle (for review, see Martin *et al.*, 1986). An LH surge, ovulation and the formation of a corpus luteum then occur (Martin *et al.*, 1986). However, ovulation is usually not accompanied by oestrus and the corpus luteum that forms invariably regresses prematurely (Oldham and Martin, 1978), resulting in a short oestrous cycle, typically of 5–7 days. Premature regression of the corpus luteum does not occur in

hysterectomized ewes, indicating that the premature release of a luteolytic amount of uterine prostaglandin $F_{2\alpha}$ is responsible for the short cycle phenomenon (Southee *et al.*, 1988; Hunter *et al.*, 1989).

If the introduction of rams is preceded by a 12 day period of pretreatment with a progestagen, ovulation is accompanied by oestrus and the ensuing corpus luteum functions normally (Oldham *et al.*, 1980, 1985; Pearce *et al.*, 1985). The period of progestagen pretreatment can be reduced to a single i.m. injection of 20 mg progesterone (Cognié *et al.*, 1982; Lindsay *et al.*, 1984; Hunter *et al.*, 1986) but, under these circumstances, ovulation is not accompanied by oestrus. The mechanism of action of progesterone on the developing ovulatory follicle(s) is not fully understood and it has been suggested that the prevention of the short cycles associated with the ram effect is mediated by a direct action of progesterone on the developing ovulatory follicles (Martin *et al.*, 1986). The two experiments reported in this paper were carried out to determine whether pretreatment with a single i.m. injection of 20 mg progesterone was able to reduce GnRH-induced ovarian steroid secretion in anoestrous ewes at a time when progesterone was no longer present in the circulation. For these experiments, the ovarian autotransplant model of Goding *et al.* (1967) was used. Anoestrous ewes

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with an ovarian autotransplant were pretreated with a single injection of 20 mg progesterone before the induction of an LH pulse by a rapid i.v. injection with a small dose (150 ng) of GnRH. The secretion rates of oestradiol (Expt 1) and of oestradiol and androstenedione (Expt 2) were determined in response to a GnRH-induced LH pulse.

Materials and Methods

Experimental animals

Two experiments were conducted a year apart in November (late spring), which is the normal period of anoestrus in the Border Leicester Merino crossbred sheep used in these experiments. The ewes were injected i.m. twice, 21 days apart, with 125 µg synthetic prostaglandin (PG; Estrumate; ICI, Sydney) and then monitored for the presence of functional luteal tissue (by determining the concentration of progesterone in jugular venous blood collected by jugular venepuncture three times per week) to ensure they were anoestrus and that their ovaries contained no luteal tissue. Ewes were considered anoestrus if the progesterone concentration remained below 0.5 ng ml⁻¹ for > 1 week (that is, in ≥ 4 blood samples). The ewes were cannulated when all experimental animals were in a confirmed state of anoestrus, and the experiment commenced the following day.

Experimental design

In Expt 1, eight Border Leicester × Merino ewes with ovarian autotransplants prepared at least 12 months before (Goding *et al.*, 1967) were allocated randomly to one of two groups: control (*n* = 4) and progesterone-treated (*n* = 4). Progesterone (20 mg in 2 ml peanut oil) was administered by i.m. injection 72 h before assessment of follicular steroid secretion using a GnRH function test; control ewes were injected i.m. with 2 ml peanut oil at the same time.

In Expt 2, twelve Border Leicester × Merino ewes with ovarian autotransplants prepared at least 12 months before (Goding *et al.*, 1967) were allocated randomly to one of three groups: control (*n* = 4), progesterone-treated 60 h before GnRH (*n* = 4) and progesterone-treated 36 h before GnRH (*n* = 4). Progesterone (20 mg in 2 ml peanut oil) was administered by i.m. injection; control ewes were injected i.m. with 2 ml peanut oil 36 h before the GnRH function test.

The jugular vein draining the transplanted ovary and the contralateral jugular vein were cannulated (Downing, 1994). Cannulation was carried out after an injection of a local anaesthetic (Xylocaine; Astra Pharmaceuticals, North Ryde, NSW) into the skin surrounding the cannulation site (Downing, 1994).

GnRH function test

Gonadotrophin-releasing hormone (GnRH) was injected i.v. at time 0 (150 ng in 2 ml sterile saline) via the jugular

venous catheter contralateral to the transplant. Samples of both ovarian (5 ml from the jugular vein draining the transplanted ovary) and peripheral (3 ml from the contralateral jugular vein) venous blood were taken every 10 min from 30 min before until 150 min after GnRH administration. The GnRH function test was carried out twice in each group of ewes. In the first test, GnRH was injected 3 h before progesterone pretreatment. The GnRH test was then repeated 72 h after the injection of progesterone (treated group) or oil (control group). Some changes were made to the experimental protocol for Expt 2. The sampling period was extended to commence 60 min before and continued until 180 min after GnRH injection and the pretreatment GnRH test was not included.

Radioimmunoassays

The jugular venous concentration of LH was determined using a validated radioimmunoassay (Downing *et al.*, 1995). The LH antiserum (R29) was raised in rabbits against NIH-oLH-S19 and showed <0.1% crossreactivity with oFSH, oLH-α subunit, prolactin or bTSH (McNeilly *et al.*, 1986). The range of the assay was 0.5–47.9 ng ml⁻¹ with a sensitivity of 0.2 ng ml⁻¹. The intra- and interassay coefficients of variation for a quality control of medium concentration were 3.4 and 8.1%, respectively.

The concentrations of androstenedione and oestradiol in ovarian venous plasma and of progesterone in jugular venous plasma were determined by radioimmunoassays carried out after solvent extraction (Scaramuzzi *et al.*, 1975; Downing *et al.*, 1995). The antisera for androstenedione (2119), oestradiol (727) and progesterone (7943) were each raised in sheep against androstenedione-7-human serum albumin (HSA), oestradiol-6-BSA and progesterone-11-BSA, respectively. The crossreactivities of each of the three steroid antisera have been reported (Campbell *et al.*, 1990). The sensitivities (minimum detectable mass) of the androstenedione, oestradiol and progesterone radioimmunoassays were 4.0, 2.7 and 2.5 pg per tube, respectively. The intra- and inter-assay coefficients of variation for androstenedione, oestradiol and progesterone were 10.1% and 12.5%, 3.2% and 11.9%, and 3.7% and 12.9%, respectively, for quality controls of medium range concentration.

The volume of each ovarian venous blood sample, the time to collect the sample and the packed cell volume were recorded and used to calculate the rate of secretion of androstenedione and oestradiol (Goding *et al.*, 1967; Collett *et al.*, 1973).

Statistical analyses

Differences between treatment groups and within treatment groups (time effects) were tested by a repeated measures (split-plot) analysis of variance using the CLR ANOVA computer program on an Apple Macintosh computer (Clear Lake Research Incorporated, Houston, TX).

Fig. 1. The mean ($\pm$ SEM) jugular venous concentration of LH and ovarian secretion rate of oestradiol in two groups of anoestrous ewes injected i.v. with 150 ng GnRH at time 0, 3 h before ($\blacksquare$) and 72 h after ($\circ$) pretreatment with a single i.m. injection of 20 mg progesterone in oil (b,d) or vehicle alone (a,c).

Results

In both experiments, all ewes were confirmed as anoestrous before the start of the experiment.

Experiment 1

The injection of progesterone 72 h before a GnRH challenge had no detectable effect on the LH response to GnRH or on the pattern of the LH-induced secretion of oestradiol (Fig. 1) when compared with either the pre-treatment response in the treated group of ewes or to the post-treatment response in the control group of sheep. The maximum concentration of LH was not different and occurred at the same time (10 min after GnRH injection) in both groups of ewes at both times. The peak concentrations (mean $\pm$ SEM) were: control ewes, 5.60 ± 1.62 and 9.05 ± 2.26 ; progesterone treated ewes, 9.05 ± 2.50 and 6.41 ± 1.99 ng ml⁻¹, pretreatment and post-treatment, respectively.

Neither the general pattern of the LH-induced secretion of oestradiol (Fig. 1) or the maximum secretion of oestradiol were affected by pretreatment with progesterone. The secretion rate of oestradiol increased by 20 min after GnRH treatment, reached and maintained maximum rates of

secretion from 40 to 90 min after GnRH treatment before decreasing slowly towards pretreatment values. These values were still decreasing when the sampling ended at 150 min after GnRH treatment (Fig. 1). There were no significant differences between or within treatment and control groups.

Experiment 2

The injection of progesterone 60 and 36 h before a GnRH challenge had no detectable effect on the LH response to GnRH (Fig. 2a). The maximum concentration of LH was not significantly different (Table 1) and occurred at the same time (10 min after GnRH injection) in all three groups of ewes.

The qualitative pattern of the LH-induced secretion of oestradiol (Fig. 2b) was not altered by pretreatment with progesterone. The secretion rate of oestradiol increased 20 min after GnRH injection and reached peak rates between 40 and 90 min after GnRH injection; it then decreased, reaching pretreatment values by 170 min after GnRH treatment (Fig. 2b). However, there were some quantitative differences between the control and treatment groups. The secretion rate of oestradiol in ewes pretreated with progesterone was not significantly different from that of the control ewes in the

Fig. 2. The mean ($\pm$ SEM) jugular venous concentration of LH (a), the ovarian secretion rate of oestradiol (b), and the secretion rate of androstenedione (c) in three groups of anoestrous ewes injected i.v. with 150 ng GnRH at time 0. The ewes were treated with a single i.m. injection of 20 mg progesterone 36 ($\circ$) or 60 ($\bullet$) h before GnRH injection. The control group ($\blacksquare$) was injected with the oil vehicle alone 36 h before GnRH injection.

period before the injection of GnRH. However, after the injection of GnRH, the secretion rate of oestradiol was significantly ($P < 0.001$ and $P < 0.01$ for progesterone treatment 36 and 60 h before GnRH, respectively) lower than it was in control ewes by 20 min after GnRH. These significant differences persisted until 120 min after GnRH (Fig. 2b). The secretion rate of oestradiol in treated ewes also decreased to pretreatment rates and, by 160 min, the secretion rate of oestradiol in treated ewes was not different from that of control ewes.

The qualitative pattern of the LH-induced secretion of androstenedione (Fig. 2c) was not altered by pretreatment with progesterone. The secretion rate of androstenedione increased 20 min after GnRH injection and reached peak rates between 40 and 90 min after GnRH injection; it then decreased, reaching pretreatment values by 170 min after GnRH treatment (Fig. 2c). However, there were some quantitative differences between the control and treatment groups. The secretion rate of androstenedione in ewes pretreated with progesterone was not significantly different from that of the control ewes in the period before the injection of GnRH. However, after the injection of GnRH, the secretion rate of androstenedione was significantly ($P < 0.001$ and $P < 0.01$ for progesterone treatment 36 and 60 h before GnRH, respectively) lower than in control ewes by 20 min after GnRH. These significant differences persisted until 120 min after GnRH (Fig. 2b). The secretion rate of androstenedione in treated ewes also decreased to pretreatment rates and, by 150 min, the secretion rate of androstenedione in treated ewes was not different from that in control ewes.

Discussion

Pretreatment with progesterone 36 or 60 h before a GnRH function test was able to reduce GnRH-induced, LH-stimulated ovarian secretion of androstenedione and oestradiol. The effect of pretreatment with progesterone within 60 h was a suppression of the steroidogenic response to an LH pulse induced by an i.v. GnRH challenge of 150 ng and the effect was not due to a reduced LH pulse amplitude.

This effect is probably not the result of alterations in gonadotrophin secretion in response to progesterone pretreatment because there were no significant changes in GnRH-induced LH pulse amplitude as a result of progesterone pretreatment (Table 1). Furthermore, endogenous LH pulsatility is already suppressed during anoestrus (Scaramuzzi and Baird, 1977), and the LH pulse frequency is not further reduced by progesterone (Martin *et al.*, 1983). There is little evidence to support the suggestion that pretreatment with progesterone exerts an inhibitory feedback effect on FSH secretion (Saba *et al.*, 1975), and a single injection of GnRH has no short-term effect on FSH secretion (Clarke *et al.*, 1986).

Another explanation of these data is that progesterone has a direct action on the follicle (Martin *et al.*, 1986). Pretreatment of anoestrous sheep with a single injection of progesterone has a number of demonstrated actions. In the present study, it inhibited follicular steroid secretion when given 36 or 60 h earlier. The ram-induced LH surge can be

Table 1. The mean ($\pm$ SEM) maximum concentration of LH in jugular venous plasma induced by the intravenous injection of 150 ng GnRH in anoestrous ewes pretreated with 20 mg progesterone or with the oil injection vehicle either 36 or 60 h previously

Group	Maximum LH concentration (ng ml ⁻¹)
Control (oil injected; n = 4)	7.35 $\pm$ 1.69
Treated (progesterone 36 h before; n = 4)	5.05 $\pm$ 1.43
Treated (progesterone 60 h before; n = 4)	5.83 $\pm$ 1.34

There are no significant differences among groups.

delayed by approximately 40 h if progesterone is given 24 h before the introduction of rams (Pearce *et al.*, 1985). Another effect is to prevent premature luteal regression or short cycles. Short cycles can be prevented if progesterone pretreatment is given 72 h before the introduction of the ram, whereas progesterone at this time has no effect on the timing of the LH surge (Pearce *et al.*, 1987) or on steroid secretion from the ovary. Progesterone pretreatment prevents the premature secretion of a luteolytic amount of prostaglandin F_{2α} from the endometrium (Southee *et al.*, 1988) and the short cycles associated with ram-induced ovulations. These differences in timing suggest that progesterone pretreatment of anoestrous ewes has separate actions on the follicle and on the endometrium.

After a single i.m. injection of progesterone, the blood plasma concentration increases to peak values by 4–6 h and then decreases to basal concentrations by 12 h (Yuthasastrakosol *et al.*, 1974). Progesterone is cleared rapidly from the body with a biological half-life of 3.3 h (Stupnicki *et al.*, 1969) so that virtually all the progesterone is cleared within 24 h of injection. Therefore, the effect of pretreatment with progesterone described in the present study is on the follicle population present at the time of injection. However, it is not known what happens to these follicles over the 36 or 60 h between pretreatment with progesterone and the GnRH test. Follicle growth in sheep is continuous, even in anoestrus (Souza *et al.*, 1996) and, in its final stages, it assumes a wave-like pattern with the emergence of a dominant follicle from a cohort of similar sized, but subordinate, follicles. Each wave lasts around 5–7 days and is accompanied by similar waves of secretion of FSH and oestradiol (Souza *et al.*, 1997). During anoestrus, the waves of hormone secretion are less well defined, presumably because the frequency of endogenous LH pulses is too low to maintain high oestradiol secretion from the dominant follicle (Souza *et al.*, 1996). Therefore, the steroid secretory responses observed after a GnRH-induced LH pulse may be from an oestrogenic follicle present at the time of progesterone pretreatment, or from a new oestrogenic follicle that has emerged from the pool of small, growing non-oestrogenic follicles as a result of progesterone pretreatment.

The maximum secretion of both oestradiol and androstenedione in response to a GnRH-induced LH pulse were suppressed by progesterone pretreatment. This finding

indicates that the action of progesterone may have been on theca cells, the principal source of ovarian androstenedione (Baird and Scaramuzzi, 1976). The suppression of oestradiol is probably an indirect effect of reduced substrate availability. Therefore, it is suggested that progesterone acts on the sheep antral follicles to suppress LH-induced androgen synthesis by theca cells. Although this is an attractive hypothesis, it is also possible that progesterone pretreatment reduces LH pulse frequency and that the reduced background LH pulse frequency at the time of the GnRH tests is responsible for the diminished androgen response to the GnRH-induced LH pulse. Further research is required to investigate this hypothesis. Progesterone receptors have been identified in follicles from a number of species. The progesterone receptor is present in primate theca cells from small antral follicles (Hild-Petito *et al.*, 1988) and in the granulosa cells of preovulatory follicles from rats, rabbits and pigs (Iwai *et al.*, 1991a,b; Park-Sarge and Mayo, 1991; Natraj and Richards, 1993). On the basis of the published evidence, it is highly likely that the sheep follicle also contains progesterone receptors.

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