Independence of progesterone blockade of the luteinizing hormone surge in ewes from opioid activity at naloxone-sensitive receptors

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Summary. Fifteen ovariectomized ewes were treated with implants (s.c.) creating circulating luteal progesterone concentrations of 1.6 ± 0.1 ng ml⁻¹ serum. Ten days later, progesterone implants were removed from five ewes which were then infused with saline for 64 h (0.154 mol NaCl l⁻¹, 20 ml h⁻¹, i.v.). Ewes with progesterone implants remaining were infused with saline ($n = 5$) or naloxone (0.5 mg kg⁻¹ h⁻¹, $n = 5$) in saline for 64 h. At 36 h of infusion, all ewes were injected with oestradiol (20 µg in 1 ml groundnut oil, i.m.). During the first 36 h of infusion, serum luteinizing hormone (LH) concentrations were similar in ewes infused with saline after progesterone withdrawal and ewes infused with naloxone, but with progesterone implants remaining (1.23 ± 0.11 and 1.28 ± 0.23 ng ml⁻¹ serum, respectively, mean $\pm$ SEM, $P > 0.05$). These values exceeded circulating LH concentrations during the first 36 h of saline infusion of ewes with progesterone implants remaining (0.59 ± 0.09 ng ml⁻¹ serum, $P < 0.05$). The data suggested that progesterone suppression of tonic LH secretion, before oestradiol injection, was completely antagonized by naloxone. After oestradiol injection, circulating LH concentrations decreased for about 10 h in ewes of all groups. A surge in circulating LH concentrations peaked 24 h after oestradiol injection in ewes infused with saline after progesterone withdrawal (8.16 ± 3.18 ng LH ml⁻¹ serum). At the same time, circulating LH concentrations in ewes infused with saline or naloxone, but with progesterone implants remaining, were similar to concentrations observed before injection (0.91 ± 0.27 and 1.35 ± 0.28 ng LH ml⁻¹ serum, respectively). The results suggested that naloxone antagonism of opioid-dependent progesterone suppression of LH secretion was not equivalent to progesterone withdrawal. It was concluded that progesterone blockade of oestradiol positive feedback on LH secretion in ewes does not depend upon endogenous opioid peptide activity at naloxone-sensitive receptors.

Keywords: opioids; luteinizing hormone surge; oestradiol; progesterone; naloxone; sheep

Introduction

Acute oestradiol administration has a biphasic effect on luteinizing hormone (LH) secretion in ovariectomized ewes (Scaramuzzi *et al.*, 1971; Coppings & Malven, 1976). Reduced LH secretion due to initial oestradiol negative feedback is followed 12–48 h later by a surge of LH secretion exceeding pretreatment concentrations, oestradiol positive feedback (Karsch *et al.*, 1980; Goodman *et al.*, 1981a, b). The surge of LH secretion following oestradiol administration to

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ovariectomized ewes is smaller in magnitude (Scaramuzzi *et al.*, 1971; Schillo *et al.*, 1985), but similar in time course, to the preovulatory gonadotrophin surge (Rawlings *et al.*, 1984). A similar increase in LH secretion follows progesterone withdrawal in ovariectomized ewes treated with progesterone and low, constant concentrations of oestradiol for 10 days (Rawlings *et al.*, 1984). However, oestradiol fails to exert positive feedback on LH secretion in ewes with circulating luteal progesterone concentrations (Jeffcoate *et al.*, 1984; Martin, 1984).

Most, perhaps all (Currie *et al.*, 1991a), progesterone suppression of tonic LH secretion in ewes depends upon opioid activity at naloxone-sensitive opioid receptors (Malven *et al.*, 1984; Brooks *et al.*, 1986a, b; Currie & Rawlings, 1987, 1989; Trout & Malven, 1987; Yang *et al.*, 1988). Reduced opioid activity may be one mechanism allowing increased LH secretion during the follicular phase and gonadotrophin surge (Knight *et al.*, 1990; Currie *et al.*, 1991b).

This study tested the hypothesis that progesterone blockade of oestradiol positive feedback on LH secretion in ewes depends upon opioid activity at naloxone-sensitive receptors. The hypothesis was tested by acute oestradiol administration during naloxone infusion of progesterone-implanted ovariectomized ewes. The trial was performed in ovariectomized, rather than intact, ewes, as naloxone does not antagonize the synergistic suppression of LH secretion by progesterone and oestradiol (Currie *et al.*, 1991a). Circulating progesterone and oestradiol can be detected in ewes during the luteal phase (Currie & Rawlings, 1987, 1989).

Materials and Methods

Animals

Fifteen Columbia ewes were housed in a drylot and fed pelleted lucerne (1.2 kg per ewe per day), with hay, mineralized salt and water provided *ad libitum*. The ewes were housed with marker-harnessed vasectomized rams and had regular oestrous cycles. The ewes were ovariectomized under halothane (Somnathane: Hoechst Inc., Montreal, Quebec, Canada) anaesthesia 2 weeks before the experiment.

Experimental treatments

The experiment was performed during the breeding season (early December). The ewes were divided into three groups of five and housed indoors in three pens (2.5 × 2.5 m). The trials were performed indoors, at 13°C, with lighting conditions simulating natural daylength. Fluorescent (890 lux) and incandescent red (<50 lux) light were used during normal hours of daylight and darkness, respectively. All of the ewes were treated with Silastic implants (s.c.) to create luteal progesterone concentrations (4-pregnene-3,20-dione: Sigma, St Louis, MO, USA) (Rawlings *et al.*, 1984). One day before infusions, vinyl catheters (i.d. 1.00 mm, o.d. 1.50 mm, SV-70; Dural Plastics and Engineering, Dural, NSW) were inserted into both jugular veins of each ewe. During infusions, ewes were housed side by side in 1.0 × 1.5 m carts, with pelleted lucerne, hay and water available. The ewes were randomly assigned to three groups.

Group 1

Ten days after insertion of progesterone implants, five ewes were infused with saline (0.154 mol NaCl l⁻¹, 20 ml h⁻¹) for 64 h. Infusions were via a multi-channel peristaltic pump (Cassette Pump, Manostat, New York, NY, USA) connected to the left jugular catheter with silicone rubber tubing (i.d. 2.38 mm, o.d. 3.97 mm; Manosil, Manostat). Ewes were injected with oestradiol (20 µg in 1 ml groundnut oil, i.m., β-oestradiol, Sigma, St Louis, MO, USA) at 36 h of infusion.

Group 2

Progesterone implants were removed 10 days after insertion. Five ewes were then infused with saline for 64 h. Ewes were injected with oestradiol (20 µg, i.m.) at 36 h of infusion.

Group 3

Ten days after insertion of progesterone implants, five ewes were infused with naloxone hydrochloride (0.5 mg kg⁻¹ h⁻¹; DuPont Pharmaceuticals, Garden City, NY, USA) in saline for 64 h. Ewes were injected with oestradiol (20 µg, i.m.) at 36 h of infusion.

Samples

Blood samples were collected by venepuncture, once a day for 10 days prior to infusion. Blood samples were collected via the right jugular catheter, once an hour throughout infusions. Samples were allowed to clot for 12 h at 20°C. Clots were removed and serum was centrifuged at 1500 *g* for 15 min. Serum was separated from remaining red blood cells and stored at -20°C until analysed.

LH assay

All serum samples collected during infusions were analysed for LH by radioimmunoassay (Rawlings *et al.*, 1984). Serum LH concentrations are expressed in terms of NIAMDD oLH-24. Intra- and interassay coefficients of variation (CVs) for an ovine reference serum with a mean of 2.60 ng LH ml⁻¹ were 4.1% and 6.0%, respectively, and 5.9% and 8.1%, respectively, for a reference serum of 1.48 ng LH ml⁻¹. Sensitivity of the LH assay, expressed as the lowest standard different from zero by unpaired *t*-test, was 0.1 ng ml⁻¹ serum (*P* < 0.05).

Steroid assays

Daily serum samples collected before infusions were analysed for progesterone and oestradiol by radioimmunoassay (Rawlings *et al.*, 1984; Jeffcoate *et al.*, 1984). Intra- and interassay CVs, for ovine reference sera with means of 10 and 16 pg oestradiol ml⁻¹ were both 11% and 17%, respectively. Sensitivity of the oestradiol assay was 1.0 pg ml⁻¹ serum (*P* < 0.05). Intra- and interassay CVs, respectively, for an ovine reference serum with a mean of 1.0 ng progesterone ml⁻¹ were 9.9% and 19%, respectively, and 8.5% and 15%, respectively, for a reference serum of 2.2 ng progesterone ml⁻¹. Sensitivity of the progesterone assay was 0.1 ng ml⁻¹ serum (*P* < 0.05).

Statistical analysis

Comparisons of circulating LH concentrations between groups were by one-way analysis of variance of (i) the mean of all hourly LH data from each ewe prior to oestradiol injection and (ii) the circulating LH concentration for each ewe 24 h after oestradiol injection. After a significant *F*-test, means were separated with Scheffe's test (GB-STAT 2.0, 1988, Dynamic Microsystems, Silver Springs, MD, USA).

Results

During the 10 days before infusion, ewes were treated in the same way. During this period, serum progesterone and oestradiol concentrations were 1.6 ± 0.1 ng ml⁻¹ and 3.2 ± 0.2 pg ml⁻¹, respectively. During the first 36 h of infusion, serum LH concentrations were similar in ewes infused with saline after progesterone withdrawal and in ewes infused with naloxone, but with progesterone implants remaining (1.23 ± 0.11 and 1.28 ± 0.23 ng LH ml⁻¹ serum, Groups 2 and 3, respectively, mean $\pm$ SEM, *P* > 0.05, Fig. 1). These values exceeded circulating LH concentrations during the first 36 h of saline infusion of ewes with progesterone implants remaining (Group 1, 0.59 ± 0.09 ng LH ml⁻¹ serum, *P* < 0.05). For approximately 10 h after oestradiol injection, mean circulating LH concentrations within each group were lower than those observed during the first 36 h of infusion. After this apparent decline, a surge in circulating LH concentrations, above pre-oestradiol injection concentrations (*P* < 0.05), was detected in ewes infused with saline following progesterone withdrawal (Group 2). Circulating LH concentrations in this group peaked 24 h after oestradiol injection (8.16 ± 3.18 ng LH ml⁻¹ serum). At the same time, circulating LH concentrations were less in ewes infused with saline (Group 1) or naloxone (Group 3), but with progesterone implants remaining (0.91 ± 0.27 and 1.35 ± 0.28 ng ml⁻¹ serum, respectively, *P* < 0.05).

Discussion

Oestradiol positive feedback is manifested as increased frequency of luteinizing-hormone-releasing hormone (LHRH) secretion from the hypothalamus (Schillo *et al.*, 1985; Clarke *et al.*, 1987) and

Fig. 1. Circulating luteinizing hormone concentrations (mean $\pm$ SEM) in ovariectomized ewes ($n = 5$ per group) treated for 10 days with implants (s.c.) creating circulating luteal progesterone concentrations and injected with 20 μ g oestradiol (i.m.) at 36 h of infusion (i.v.). (a) data before, and (b) data after oestradiol injection. Group 1: (□) saline infusion for 64 h, with progesterone implants remaining; group 2: (◆) saline infusion for 64 h after progesterone withdrawal; group 3: (○) naloxone infusion (0.5 mg kg⁻¹ h⁻¹) for 64 h with progesterone implants remaining.

increased pituitary responsiveness to LHRH (Coppings & Malven, 1976). Oestradiol directly increases pituitary responsiveness to LHRH (Reeves *et al.*, 1971; Jackson, 1975; Moss & Nett, 1980), reflecting increased LHRH receptor numbers and gonadotrophin synthesis (Landefeld *et al.*, 1985). Oestradiol fails to exert positive feedback on LH secretion in ewes with circulating luteal progesterone (Jeffcoate *et al.*, 1984; Martin, 1984). Progesterone has been shown to suppress LHRH pulsatility (Karsch *et al.*, 1987). However, physiological progesterone concentrations have little effect on pituitary responsiveness to LHRH (Goodman & Karsch, 1980). This suggests that

progesterone blockade of oestradiol positive feedback results from progesterone suppression of LHRH secretory frequency (Goodman & Karsch, 1980).

Morphine suppresses LH secretory frequency in ovariectomized ewes, mimicking effects of progesterone replacement (Currie & Rawlings, 1989; Currie *et al.*, 1991a). Naloxone enhances LH secretory frequency in progesterone-treated ovariectomized ewes and intact ewes during the luteal phase mimicking effects of progesterone withdrawal (Currie & Rawlings, 1987, 1989; Currie *et al.*, 1991a). Simultaneous infusion of ovariectomized ewes with morphine and naloxone does not affect LH secretion. This suggests that effects of morphine and naloxone on LH secretion are mediated at a common specific receptor (Currie *et al.*, 1991a).

Naloxone-reversible opioid suppression of LH secretion is reduced in ewes during the follicular phase (Brooks *et al.*, 1986b; Currie *et al.*, 1991b). This suggests that reduced opioid activity may be permissive to the preovulatory gonadotrophin surge (Currie *et al.*, 1991b). Hourly morphine administration throughout the follicular phase and oestrus delayed by 17 h, but failed to block, the gonadotrophin surge (Currie *et al.*, 1991b). Morphine and naloxone failed to affect the oestradiol-induced LH surge in anoestrous ewes (Horton & Clarke, 1988). However, acute administration of a potent enkephalin analogue, FK 33-824, to ewes during the follicular phase delayed ovulation by nearly 2 weeks (Brooks *et al.*, 1986b). FK 33-824 reduced total LH secreted during an oestradiol-stimulated gonadotrophin surge in anoestrous ewes, an effect partially reversed by naloxone (Knight *et al.*, 1990). However, FK 33-824 failed to block or delay the oestradiol-induced LH surge in intact or ovariectomized ewes during anoestrus (Knight *et al.*, 1990). Examination of opioid involvement in the gonadotrophin surge in ewes appears to be affected by season, recent ovarian steroid exposure and opiate analogue used.

In this study, tonic LH concentrations prior to oestradiol injection were similar ($P > 0.05$) in ewes infused with saline following progesterone withdrawal (Group 2) and ewes infused with naloxone, but with progesterone implants remaining (Group 3). These LH concentrations were greater ($P < 0.05$) than in ewes infused with saline, but with progesterone implants remaining (Group 1). This suggested that progesterone suppression of tonic LH secretion, before oestradiol injection, was antagonized by naloxone (Fig. 1). The results support the contention that a major portion of progesterone suppression of tonic LH secretion depends on opioid activity at naloxone-sensitive receptors (Currie *et al.*, 1991a).

After oestradiol injection at 36 h of infusion, LH suppression was observed in ewes of all groups (Fig. 1). A secondary positive response of LH secretion to oestradiol was observed in ewes from which progesterone was withdrawn (Group 2). Despite the antagonism of opioid-dependent progesterone suppression of tonic LH secretion by naloxone, oestradiol failed to exert positive feedback in ewes infused with saline or naloxone, but with progesterone remaining (Groups 1 and 3). This suggested that antagonism of opioid activity by naloxone was not equivalent to progesterone withdrawal. Opioid activity at naloxone-sensitive receptors may represent a component of progesterone blockade of oestradiol positive feedback, slowing LHRH pulse frequency. However, reduced opioid-dependent progesterone suppression of tonic LH secretion is not sufficient to permit oestradiol to induce a gonadotrophin surge. Progesterone blockade of oestradiol positive feedback may also involve nonopioid mechanisms or opioid activity at receptor types not sensitive to morphine (Currie *et al.*, 1991b). Alternatively, the dose of naloxone used was insufficient to overcome opioid-dependent progesterone blockade of oestradiol positive feedback.

Multiple opioid receptor types have been implicated in suppression of LH secretion, with opioids from all major opioid precursors reported to suppress circulating LH levels in rats (Wuster *et al.*, 1978) and men (Stubbs *et al.*, 1978; Reid *et al.*, 1981). Morphine and naloxone do not have identical affinities for all opioid receptor types. Morphine and naloxone bind with high affinity to μ_1 - and μ_2 -receptors (Lord *et al.*, 1977; Pasternak, 1986; Pasternak & Wood, 1986; Kazmi & Mishra, 1987). Enkephalins bind with high affinity to δ - and μ_1 -receptors (Lord *et al.*, 1977; Pasternak & Wood, 1986; Kazmi & Mishra, 1987). Naloxone also binds with high affinity to δ -receptors (Khatchaturian *et al.*, 1985). Evidence obtained in this and previous studies (Horton &

Clarke, 1988; Currie *et al.*, 1991b) suggests that reduced opioid activity at morphine or naloxone-sensitive receptors is not critical to the gonadotrophin surge in ewes. A lengthy delay to ovulation followed FK 33-824 administration to ewes during the follicular phase (Brooks *et al.*, 1986b). It is possible that progesterone blocks oestradiol positive feedback at an enkephalin-sensitive receptor that is not sensitive to naloxone. This may eliminate the involvement of opioid activity at μ_1 -, μ_2 - and δ -receptors in progesterone blockade of the gonadotrophin surge. Enkephalin blockade of the gonadotrophin surge may fail in anoestrus (Knight *et al.*, 1990) owing to an absence of recent ovarian progesterone exposure.

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