Evidence for a gonadal nonsteroidal factor that specifically inhibits release of luteinizing hormone in ewes

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Summary. Bilaterally ovariectomized ewes were used to investigate the effect of systemic administration (i.v.) of charcoal-treated aqueous luteal extracts from ovine corpora lutea on plasma concentrations of pituitary gonadotrophins. Jugular blood samples were taken every 15 min at least 5 h before (control period) and 5 h after (treatment period) injection. In Expt 1, the administration of luteal extract from corpora lutea of days 70–76 of pregnancy, but not of the extract prepared from muscular tissue, resulted in a significant decrease of mean concentrations of luteinizing hormone (LH) ($P < 0.02$) and frequency of LH pulses ($P < 0.01$). Plasma follicle-stimulating hormone (FSH) concentrations were not affected by injections of either extract. These findings provide the first demonstration of the presence of a nonsteroidal factor in the corpus luteum of midpregnancy that selectively suppresses the secretion of LH. In Expt 2, mean concentrations of LH and FSH and frequency of LH pulses were unaffected by injections of luteal extracts from ovine corpora lutea of days 10–12 of the oestrous cycle or day 15 of pregnancy. These data suggest that some factor(s), probably from the fetoplacental endocrine unit, is required to ensure the production of a significant quantity of the luteal LH-inhibiting factor after day 15 of pregnancy. In Expt 3, treatment of luteal extract from corpora lutea of day 70 of pregnancy with proteolytic enzymes destroyed the LH-inhibiting activity, suggesting the proteic nature of the luteal LH-inhibiting factor. In Expt 4, plasma concentrations of LH were not affected by injection of charcoal-treated extract prepared from fetal cotyledonary tissue of days 110–120 of pregnancy suggesting that the LH-inhibiting factor exclusively originates from the corpus luteum during pregnancy.

These experiments provide the first direct evidence for the existence of a potent nonsteroidal factor of luteal origin that specifically inhibits pulsatile secretion of LH, without influencing FSH release in female animals. We propose the term LH-release-inhibiting factor (LH-RIF) to describe this activity.

Keywords: ewe; corpus luteum; luteinizing hormone; follicle-stimulating hormone; LH-release-inhibiting factor

Introduction

During pregnancy, the maintenance of luteal function in most mammals depends on a complex relationship between the fetoplacental unit, the ovaries and the hypophysis (for review, see Denamur, 1968; Moor, 1968). One important function of the corpus luteum is the synthesis and secretion of progesterone, which is required to establish and maintain pregnancy. In some species, ovariectomy can be performed during early (sheep, macaque and man) or late (cat, guinea-pig, cow and horse) pregnancy without inducing abortion (for review, see Ryan, 1969). In these species,

during the period when the fetoplacental endocrine unit assumes a predominant role in progesterone secretion, the physiological importance of the presence of the functional corpus luteum is unknown. In sheep, while the corpus luteum is not necessary for maintenance of pregnancy during the last two-thirds (Casida & Warwick, 1945; Neher & Zarrow, 1954; Denamur & Martinet, 1955), it affects the recruitment of the primordial follicles systemically (Al-Gubory & Martinet, 1986; Al-Gubory *et al.*, 1987) and locally (Al-Gubory & Martinet, 1987). It has also been demonstrated that surgical removal of the corpus luteum at day 70 of pregnancy has no subsequent effect on plasma concentrations of progesterone and follicle-stimulating hormone (FSH), but results in an increase in the frequency of pulses of luteinizing hormone (LH) release (Al-Gubory *et al.*, 1989a). The frequency of LH pulses increases more rapidly during the early *post-partum* period in ewes from which the corpus luteum was removed at midpregnancy than in intact ewes (Al-Gubory *et al.*, 1989b). These two reports provide the first evidence that the ovine corpus luteum of pregnancy produces a factor, other than progesterone, that specifically inhibits release of LH. The present experiments investigated the presence of this compound in ovine corpus luteum and its nature.

Materials and Methods

Experimental animals and management

Adult ewes of the Préalpes-du-Sud breed were bilaterally ovariectomized 1–4 months before treatment. They were housed under conditions of natural daylength and temperature, fed straw and hay daily and had free access to mineral licks and water. Since the presence of rams affects the frequency of the LH pulses in ewes (Martin *et al.*, 1983), the ewes were isolated from rams throughout the experiment.

Preparation of extracts of ovine corpora lutea, placental and muscular tissue

At midpregnancy (days 70–76), corpora lutea were surgically removed as described by Al-Gubory *et al.* (1989a), immediately frozen in liquid nitrogen and stored at -20°C until used. Corpora lutea of early (day 15) and late (days 110–120) pregnancy and of the oestrous cycle (days 10–12), fetal cotyledons of days 110–120 of pregnancy and muscle were collected at the local abattoir, frozen in liquid nitrogen and stored at -20°C . On the day of injection of ovariectomized ewes, corpora lutea, cotyledons or muscle were thawed, minced and homogenized in ice-cold saline ($0.154\text{ mol NaCl l}^{-1}$). The homogenate was stirred for 20 min and centrifuged for 30 min at $20\,000\text{ g}$. The supernatant was stirred with dextran-coated charcoal (5 mg dextran and 50 mg charcoal in 1 ml extract) for 15 min and centrifuged for 30 min at $20\,000\text{ g}$. The resulting supernatant was extracted again with dextran-coated charcoal (1 mg dextran and 10 mg charcoal in 1 ml) for 15 min, centrifuged as already described and then filtered through a $0.45\text{ }\mu\text{m}$ Millipore filter. All operations were conducted at 4°C . After charcoal treatments, 99.7% of the luteal and placental progesterone had been removed.

Animal treatments

In a pilot trial (August 1987), a single i.v. injection of charcoal-treated aqueous luteal extract (6 ml) from four corpora lutea of day 70 of pregnancy caused a decrease in plasma LH concentration for at least 5 h, with a maximum decrease at about 4 h after treatment in ewes that had been ovariectomized 4 months earlier (Fig. 1). On the basis of results of this experiment, the conditions used in four other experiments were a single i.v. injection of 6 ml luteal extract per ewe (equivalent to four corpora lutea) and blood sampling for at least 5 h after treatment.

In the four experiments, blood samples were taken from the jugular vein into evacuated heparinized tubes every 15 min for 10–12 h beginning at 09:00 h. Injections were given after a control bleeding period of 5–6 h. Samples were kept at 4°C before centrifugation (3000 g) for 30 min. Plasma was stored at -20°C until the hormone concentrations were assayed.

Experiment 1. This experiment examined the effect of administration of aqueous extracts from corpora lutea of days 70–76 of pregnancy on the secretion of LH and FSH in ten ewes. At day 30 after ovariectomy (8 April 1988), blood samples were taken from each ewe every 15 min for 11 h beginning at 09:00 h. At day 40 after ovariectomy (18 April), five ewes received luteal tissue extract and the other five ewes were injected with 6 ml muscular tissue extract (equivalent to the weight of four corpora lutea).

Experiment 2. This experiment investigated the effect of administration of aqueous extracts from cyclic (days 10–12) or early pregnancy (day 15) corpora lutea on the secretion of LH and FSH, in the ten ovariectomized ewes used in Expt 1. At day 70 after ovariectomy (18 May), ewes were sampled for 11 h and given extracts of cyclic ($n=3$), early

Fig. 1 Effects of i.v. injection of 6 ml saline (0.9% NaCl) (a) charcoal-treated aqueous luteal extracts (6 ml) from one (b), two (c) or four (d) corpora lutea of day 70 of pregnancy, on plasma concentrations of luteinizing hormone (LH) in eight ovariectomized ewes (two ewes per group). Data are expressed as percentage of control (0 h) and each line refers to an individual ewe.

pregnancy ($n=2$) corpora lutea or extracts of muscular tissue ($n=5$). Eight days later (day 78 after ovariectomy), the five ewes that received luteal tissue extracts were treated with muscular tissue extracts, whereas the five ewes that received the muscular extracts were treated with luteal extract from cyclic ($n=3$) or early pregnancy ($n=2$) corpora lutea.

Experiment 3. This experiment tested the effect of administration of protease-treated extract from corpora lutea of midpregnancy on LH secretion in four ovariectomized ewes. Before the injection, charcoal-treated extract from corpora lutea of day 70 of pregnancy was incubated at 37°C for 3 h with proteinase K (200 $\mu\text{g ml}^{-1}$) and then for 3 h with pronase (200 $\mu\text{g ml}^{-1}$). At day 40 after ovariectomy (29 November 1989), each ewe received 6 ml of this extract.

Experiment 4. The effect of aqueous extracts from corpora lutea or fetal cotyledons of days 110–120 of pregnancy on LH secretion was investigated. At day 35 after ovariectomy (November 1990), five ewes received luteal tissue extract and the other five ewes were injected with cotyledonary tissue extract.

Assay of LH and FSH

All samples from the same experiment were run in duplicate and assayed separately for each hormone. Plasma concentrations of LH were measured by a double-antibody radioimmunoassay as described by Pelletier *et al.* (1982) using NIH-LH-S1 as standard. The intra-assay coefficient of variation was <6% and the limit of sensitivity was 0.2 ng ml^{-1} . FSH concentrations were measured by the double-antibody radioimmunoassay of Blanc & Poirier (1979) with NIH-FSH-S3 as standard. The intra-assay coefficient of variation was <3% and the limit of sensitivity was 2 ng ml^{-1} .

Data analysis

The PULSAR ALGORITHMIC PROGRAM developed by Merriam & Wachter (1982) for the study of pulsatile hormone secretion was used to calculate the basal concentrations, the frequency and the amplitude of gonadotrophin episodic release (pulses) in individual profiles of the serial sampling period. The G values, or number of assay coefficients of

variation by which a pulse must exceed the base line, were 3.00, 2.26, 1.56, 1.50 and 1.20 for pulses with one to five consecutive increased points, respectively. Samples collected before injection were considered as the control period for each ewe. The effects of injection within each group were assessed using the general linear models (GLM) procedures of the statistical analysis system (SAS, 1987) for repeated measures.

Results

Experiment 1

The 11 h profiles (09:00–20:00 h) of LH and FSH in four representative ovariectomized ewes (Fig. 2) show that, as expected, plasma concentrations of LH fluctuated in an episodic manner. During the sampling period, 11.4 LH pulses (range 9–13) were identified, which occurred every 45 min (range 30–75 min). During this period, neither LH pulse frequency nor FSH concentrations changed with hour of sampling.

Fig. 2. Concentrations of luteinizing hormone (LH, ●) and follicle-stimulating hormone (FSH, ○) in plasma samples collected from the jugular vein for 15 min over an 11-h sampling period in four ovariectomized ewes. Note scale of FSH in (a).

Peripheral concentrations of LH were markedly reduced after injection of charcoal-treated extracts from corpora lutea of days 70–76 of pregnancy, but not from muscular tissue (Fig. 3). Luteal extracts significantly ($P < 0.02$) lowered mean LH concentrations from 17.3 ± 2.6 to 12.2 ± 1.3 ng ml⁻¹. The frequency of LH pulses was significantly ($P < 0.01$) different before and after treatment (5.8 ± 0.2 versus 2.2 ± 0.6 per 5.5 h), but the amplitude of LH pulses before

Fig. 3. Concentrations of luteinizing hormone (LH, ●) and follicle-stimulating hormone (FSH, ○) in plasma samples collected from the jugular vein for 15 min over an 11-h sampling period in four ovariectomized ewes injected (i.v.) with charcoal-treated luteal tissue extract from corpora lutea of days 70–76 of pregnancy (a and b), or charcoal-treated muscular tissue extract (c and d). Time of injection is indicated by the arrow. Note scale of FSH in (a).

($7.6 \pm 0.6 \text{ ng ml}^{-1}$) and after ($7.1 \pm 1.3 \text{ ng ml}^{-1}$) treatment with luteal extract was not significantly different. Plasma FSH concentrations were not affected by injections of luteal or muscular tissue extracts (Fig. 3).

Experiment 2

Injection of luteal tissue extracts from corpora lutea of days 10–12 of the oestrous cycle or day 15 of pregnancy did not produce changes in pulsatile LH release, nor did muscular tissue extract (Fig. 4), except in one of the four ewes treated with extract from corpora lutea of day 15 of pregnancy (Fig. 4e). The mean plasma LH concentrations before and after injection of luteal tissue extracts of days 10–12 of the oestrous cycle (17.2 ± 1.1 versus $17.9 \pm 1.2 \text{ ng ml}^{-1}$) or day 15 of pregnancy (18.8 ± 2.7 versus $17.9 \pm 2.1 \text{ ng ml}^{-1}$) and muscular tissue extract (18.3 ± 1.6 versus $17.6 \pm 1.4 \text{ ng ml}^{-1}$) were not significantly different. Plasma concentrations of FSH were not affected by any of the above treatments (Fig. 4).

Experiment 3

Peripheral concentration of LH was not affected after injection of protease-treated extracts from corpora lutea of day 70 of pregnancy (Fig. 5). No differences were found between the

Fig. 4. Concentrations of luteinizing hormone (LH, ●) and follicle-stimulating hormone (FSH, ○) plasma samples collected from the jugular vein for 15 min over an 11-h sampling period in individual ovariectomized ewes injected (i.v.) with charcoal-treated muscular tissue extract (a and b), charcoal-treated luteal tissue extracts from corpora lutea of days 10–12 of the oestrous cycle (c and d), or day 15 of pregnancy (e and f). Time of injection is indicated by the arrow.

pre-treatment and the post-treatment periods in the frequency (6.5 ± 0.6 versus 6.0 ± 0.6 per 6 h) or the amplitude (3.9 ± 1.0 versus 3.2 ± 0.6 ng ml⁻¹) of LH pulses.

Experiment 4

There was great variability in the LH response among ewes treated with extracts from corpora lutea of days 110–120 of pregnancy (Fig. 6). Two of the five ewes showed a response to this treatment (Fig. 6a,c). No significant differences were found before and after treatment in the frequency (5.8 ± 0.8 versus 5.8 ± 0.9 per 5 h) or the amplitude (3.7 ± 0.7 versus 2.9 ± 0.5 ng ml⁻¹) of LH pulses. Pulsatile LH release was not affected in ewes that received extracts from fetal cotyledons of days 110–120 of pregnancy (Fig. 6b,d,f).

Fig. 5. Concentrations of luteinizing hormone (LH) in plasma samples collected from the jugular vein for 15 min over a 10-h sampling period in individual ovariectomized ewes injected (i.v.) with protease-treated luteal extract from corpora lutea of day 70 of pregnancy. Time of injection is indicated by the arrow.

Discussion

In ewes, plasma concentrations of LH and frequency and amplitude of LH pulses were low and relatively stable between days 60 and 140 of pregnancy (Al-Gubory *et al.*, 1989a, b) and increased markedly after surgical removal of the corpora lutea at day 70 of pregnancy (Al-Gubory *et al.*, 1989a). Under these experimental conditions, peripheral concentrations of FSH were not affected. Since the ovine placenta is the main source of progesterone during the last two-thirds of pregnancy (Linzell & Heap, 1968; Moore *et al.*, 1972), plasma concentrations of this steroid are not markedly affected after bilateral ovariectomy (Beal *et al.*, 1986) or corpus luteum excision (Al-Gubory *et al.*, 1989a) performed at midpregnancy. Pulsatile LH release increased more rapidly after parturition in ewes from which corpora lutea were removed at day 70 of pregnancy than in intact ewes, whereas peripheral concentrations of progesterone could not be detected during the first 20 days *post partum* in either group of ewes (Al-Gubory *et al.*, 1989b). Our findings (Al-Gubory *et al.*, 1989a, b) constitute the first demonstration that the corpus luteum of pregnancy secretes a factor, other than progesterone, that contributes selectively to the inhibition of pulsatile LH secretion. The results of the present experiments confirm these observations and provide a clear demonstration of the presence of a nonsteroidal factor in the ovine corpus luteum of pregnancy. The degree of inhibition of luteal extract from corpora lutea of day 15 (Expt 2), days 70–76 (Expt 1) or days 110–120 (Expt 4) of pregnancy on the secretion of LH was widely different (i.e. 1/4, 5/5, 2/5 ewes, respectively, showed a clear response). These results could be of significant physiological value and may reflect differences in luteal content of the LH-inhibiting factor. However, this interpretation is still far from complete and needs additional investigation. Nevertheless, compared with luteal extract from

Fig. 6. Concentrations of luteinizing hormone (LH) in plasma samples collected from the jugular vein for 15 min over a 10-h sampling period in individual ovariectomized ewes injected (i.v.) with charcoal-treated extracts of corpora lutea (a, c and e), or fetal cotyledons (b, d and f) of days 110–120 of pregnancy. Time of injection is indicated by the arrow.

corpora lutea of days 110–120, the failure of injection of extract from placental tissue of the same stage of pregnancy to affect the secretion of LH in any of the five treated ewes could be taken as an argument in favour of the hypothesis that the LH-inhibiting factor is exclusively of luteal origin. Mean concentrations of LH and frequency of LH pulses were unaffected in ewes that received luteal extracts from corpora lutea of days 10–12 of the oestrous cycle (Expt 2). These findings suggest that some message, still unknown, is required for the production of large quantities of the luteal LH-inhibiting factor and is probably delivered from the fetoplacental unit. Treatment of luteal extracts from corpora lutea of day 70 of pregnancy with proteolytic enzymes (proteinase K and pronase) destroyed the luteal LH-inhibiting activity. It appears, therefore, that the luteal LH-inhibiting factor is proteic in nature.

There is now considerable experimental evidence that ovarian follicular fluid from various species, including sheep, contains several nonsteroidal factors, in particular inhibin, follistatin and

activin, that selectively affect the synthesis and secretion of FSH (for review see Ying, 1988; De Krester & Robertson, 1989) by direct action on the pituitary gland (Carroll *et al.*, 1989). In ovariectomized ewes, various studies have shown that only supraphysiological doses of ovine or bovine follicular fluid administered 2–3 times day⁻¹ for at least 2–3 days can reduce plasma LH concentrations (Cummins *et al.*, 1983; Findlay *et al.*, 1985; Clarke *et al.*, 1986; Martin *et al.*, 1986, 1987, 1988; McNatty *et al.*, 1989) suggesting that inhibin or other follicular fluid proteins may play a role in the control of secretion of LH. However, Knight & Castillo (1988) failed to show any effect of high doses of follicular fluid on spontaneous LH release or induced by exogenous gonadotrophin-releasing hormone in ovariectomized ewes. It has also been demonstrated, by using frequent portal and jugular blood sampling schedules, that administration of follicular fluid into ovariectomized ewes had no effect on pulsatile release of LH (Knight & Castillo, 1988; Li *et al.*, 1989) and gonadotrophin-releasing hormone (Li *et al.*, 1989). Since peripheral injection of highly purified inhibin selectively suppressed plasma FSH concentrations, without affecting plasma concentrations of LH in ovariectomized rats (Ying *et al.*, 1987), ewes (Findlay *et al.*, 1987) and heifers (Beard *et al.*, 1990), it would appear unlikely that inhibin contributes to the regulation of LH secretion. These results are in agreement with those reported by Lumpkin *et al.* (1981), who showed that injection of purified inhibin preparation into the third ventricle of conscious orchidectomized rats specifically depressed plasma FSH concentrations, within 4 h of injection, with no effect on LH release. Under our experimental conditions, a single i.v. injection of charcoal-treated extract from corpora lutea of midpregnancy selectively suppressed pulsatile LH release, without influencing FSH secretion in ovariectomized ewes. These results therefore suggest that the factor (LH-inhibiting factor) in the corpus luteum of pregnancy is distinct from inhibin, at least in terms of its biological activity.

There is considerable evidence that follicular oestradiol is directly involved in the luteolytic process in ewes (Stormshak *et al.*, 1969; Hawk & Bolt, 1970; Karsch *et al.*, 1970; Ginther, 1971; Howland *et al.*, 1971; Denamur & Kann, 1973; Barcikowski *et al.*, 1974). The role of oestradiol in luteolysis appears to be via the induction of uterine oxytocin receptors (McCracken *et al.*, 1984; Hixon & Flint, 1987) and oxytocin then stimulates the secretion of the uterine prostaglandin F_{2α} (Fairclough *et al.*, 1984; Sheldrick & Flint, 1986). During follicular development, thecal cells respond to LH stimulation leading to production of androstenedione, which is then converted to oestrogen by granulosa cells (for review, see Ireland, 1987). It has been demonstrated that the increased follicular androstenedione and oestradiol secretion is due to repeated stimulation by episodic LH release at the time of luteal regression (Baird *et al.*, 1981; Campbell *et al.*, 1990a, b). The suppression of pulsatile LH release could be one pathway that leads to the maintenance of corpus luteum activity. It is thus quite likely that the physiological significance of secretion of the luteal LH-inhibiting factor lies in the control of episodic LH release and successful maintenance of pregnancy.

The present report provides the first experimental evidence for the existence of a gonadal protein that selectively inhibits the secretion of LH, but not of FSH, in female animals. The term LH-release-inhibiting factor (LH-RIF) is proposed to describe this activity, which is distinct from that of highly purified inhibin (Findlay *et al.*, 1987; Ying *et al.*, 1987; Beard *et al.*, 1990) and recombinant inhibin (Rivier *et al.*, 1991), which specifically suppresses FSH secretion without affecting LH release.

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