Measurement of inhibin-A ($\alpha\beta$ A dimer) during the oestrous cycle, after manipulation of ovarian activity and during pregnancy in ewes

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A new two-site ELISA was validated for ovine plasma and used to measure circulating inhibin-A concentrations during a synchronized oestrous cycle in four ewes and throughout pregnancy in six ewes. Inhibin A concentrations were also determined in four ewes during chronic treatment with a GnRH agonist and after subsequent exposure to pregnant mares' serum gonadotrophin (PMSG) to stimulate ovarian follicular development. Concentrations of FSH, LH, oestradiol and progesterone were determined by radioimmunoassay. The detection limit of the inhibin-A ELISA was approximately 50 pg ml⁻¹ and no significant crossreaction was observed with a range of related molecules including activin-A, inhibin-B, activin-B, follistatin and α_2 -macroglobulin. Inhibin-A concentrations were below the detection limit in plasma from hypophysectomized and ovariectomized ewes. During the oestrous cycle, plasma inhibin-A concentrations (approximately 0.3–0.4 ng ml⁻¹) did not vary during the follicular phase whereas plasma oestradiol increased approximately tenfold. After the preovulatory LH/FSH surge, inhibin-A fell to a nadir (approximately 0.15 ng ml⁻¹) coincident with the peak of the postovulatory FSH rise. During the next 2 days, FSH concentrations fell to basal values as inhibin-A concentrations increased ($P < 0.05$) to a peak (approximately 0.5 ng ml⁻¹) 3 days after the preovulatory LH/FSH surge. Over the following 3 days, FSH values increased again ($P < 0.05$) as inhibin-A concentrations fell to approximately 0.25 ng ml⁻¹ ($P < 0.05$). Chronic GnRH agonist treatment suppressed FSH concentrations by about 50%, while inhibin-A and oestradiol concentrations fell below detection limits. Within 2 days after the PMSG injection, concentrations of inhibin-A (approximately 4.5 ng ml⁻¹) and oestradiol (approximately 20 pg ml⁻¹) had increased to very high values, while FSH concentrations had been reduced by a further 50%. Plasma concentrations of inhibin-A and FSH were similar to those in nonpregnant ewes during the first 60 days of gestation, but inhibin-A values fell markedly (sevenfold; $P < 0.01$) between days 60 and 90, coincident with a twofold decrease in FSH ($P < 0.05$). Inhibin A and FSH concentrations remained low for the remainder of gestation and were positively correlated throughout pregnancy ($r = 0.48$; $P < 0.005$). These observations support an endocrine feedback role for ovarian inhibin-A and oestradiol in controlling the secondary (post-ovulatory) FSH surge in ewes, but indicate that an increase in oestradiol is responsible for the characteristic reduction in FSH during the early to mid-follicular phase. The reduced secretion of FSH from mid- to late pregnancy cannot be attributed to increased inhibin-A secretion by the feto-placental unit, but most likely reflects increased steroid secretion from this source.

Introduction

The relative importance of inhibins as gonadal feedback regulators of pituitary FSH secretion in sheep has been difficult to evaluate, mainly due to the unavailability of specific and

sensitive immunoassays applicable to peripheral serum or plasma from this species. Despite this lack of information on circulating inhibin concentrations in sheep, immunoneutralization studies (Mann *et al.*, 1989, 1990; Wrathall *et al.*, 1990; Campbell *et al.*, 1995) and experiments involving the administration of exogenous inhibin (McNeilly, 1984; Findlay *et al.*, 1987; Knight and Castillo, 1988; Martin *et al.*, 1988) support an endocrine role for ovarian inhibin in modulating FSH secretion.

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Two isoforms of inhibin have been identified, referred to as inhibin A and inhibin B. Both are disulfide-linked dimers that share a common α subunit, but have distinct β subunits, termed β A and β B, respectively (Ying, 1988; Baird and Smith, 1993). Although free α or β subunits have no intrinsic inhibin-like biological activity in terms of pituitary FSH suppression, the gonads synthesize and secrete an excess of free α subunit (Knight, 1996), potentially confounding the interpretation of inhibin measurements made using 'first generation' inhibin radioimmunoassays (for example, McNeilly *et al.*, 1989; Campbell *et al.*, 1990; Findlay *et al.*, 1990) that used antibodies directed against the α subunit and which were therefore unable to discriminate between the free α subunit, inhibin-A and inhibin-B. Recently, a new generation of highly sensitive and specific, two-site inhibin immunoassays has been developed, based on the use of monoclonal antibodies against defined epitopes on the human α , β A and β B inhibin subunits. These assays have been used extensively to quantitate circulating inhibin-A and -B in humans (Groome *et al.*, 1994, 1996; Muttukrishna *et al.*, 1994, 1995). However, they have proved to be unsuitable for plasma inhibin measurement in sheep and cattle owing to the poor crossreactivity of the α subunit monoclonal antibody used (clone R1) with ovine or bovine inhibin-A (N. P. Groome and P. G. Knight, unpublished).

In an attempt to overcome this problem, a new monoclonal antibody was generated against a more conserved region of the mature α C subunit. As anticipated, use of this antibody in a two-site ELISA format provided an inhibin-A assay of broader species specificity. The objective of the present study was to validate this assay for ovine plasma in order to allow the measurement of circulating concentrations of inhibin-A in ewes in various endocrinological states, including the normal oestrous cycle, after manipulation of ovarian function and during pregnancy, and to relate these to the profiles of other reproductive hormones.

Materials and Methods

Animals and sample collection

Inhibin-A during the oestrous cycle. During the mid-breeding season, four crossbred (Mule) ewes were treated with intra-vaginal progestagen sponges (Chronogest; Intervet UK Ltd, Cambridge) for 12 days to synchronize oestrus. Blood samples (4 ml) were collected via indwelling jugular vein catheters every 12 h for 20 days starting 3 days before withdrawal of the progestagen sponges. Additional blood samples were collected every 2 h over 5 days, beginning 12 h before progestagen withdrawal, in order to define hormone profiles in the peri-ovulatory period more clearly.

Inhibin-A after manipulation of ovarian function with GnRH agonist and PMSG. Four non-synchronized, crossbred ewes received a subcutaneous slow-release implant containing 3.6 mg of the superactive GnRH agonist Zoladex (a gift from Zeneca, Macclesfield, Cheshire) to suppress gonadotrophin secretion and inhibit ovarian activity (Knight and Castillo, 1988). Three weeks later, a second implant was inserted, and after a further 3 weeks, all ewes received an i.m. injection of

2000 iu of pregnant mares' serum gonadotrophin (PMSG) (Folligon; Intervet, Boxmeer) to stimulate follicular development. Blood was collected from a jugular vein every 1–2 weeks from the time of placing the first GnRH agonist implant and every 12 h for 48 h from the time of PMSG administration.

Inhibin-A during pregnancy. Blood samples were obtained by venepuncture from seven groups of pregnant Scottish Blackface ewes ($n = 5$ –6 per group) before slaughter at 35, 40, 60, 90, 110, 125 and 135 days of gestation. The samples were collected initially as part of another study (Sinclair *et al.*, 1996).

Inhibin-A ELISA

Preparation and biotinylation of inhibin α monoclonal antibody. A new monoclonal antibody (mAb) to the mature α C inhibin subunit was made by immunizing Bacillus Calmette–Guerin (BCG)-primed hypogonadal mice with a synthetic peptide corresponding to residues 1–32 of the human α C subunit coupled to tuberculin-purified protein derivative as described by Groome and Lawrence (1991). The immunization schedule and splenocyte–myeloma fusion protocol followed were similar to those reported previously (Groome and Lawrence, 1991; Groome *et al.*, 1996). Hybridoma fusion supernatants were screened using microplates coated with purified 32 kDa bovine inhibin following a standard ELISA procedure (Groome *et al.*, 1990; Groome and Lawrence, 1991). Positive clones were expanded and recloned in methyl cellulose and one clone (173 29 H2) identified as an IgG₁-secretor was selected for use in the present assay since further screening revealed high affinity binding to both bovine 32 kDa inhibin and human 32 kDa inhibin. This clone was grown to produce ascites fluid in nude mice and the IgG was purified by standard protein G chromatography and biotinylated as described by Groome *et al.* (1990).

Assay procedure. Serial dilutions of purified 32 kDa bovine inhibin-A (assay standard; 1.56–100 pg per 100 μ l), pooled ovine follicular fluid (1:5000–1:40 000 dilution) and ovine plasma samples (neat to 1:8 dilution) were prepared using hypophysectomized ewe plasma (kindly provided by H. Buttle, Institute of Grassland and Environmental Research, Hurley, Berks) as diluent to compensate for any matrix effect of ovine plasma in the assay. No endogenous inhibin-A immuno-reactivity was detectable in this plasma pool or in plasma from an ovariectomized ewe. Aliquots (100 μ l) of diluted standards and test samples were mixed with an equal volume of ELISA buffer (10% (w/v) BSA, 5% (v/v) Triton X-100, 0.15 mol NaCl l⁻¹ in 0.1 mol Tris–HCl buffer l⁻¹, pH 7.5) before adding 50 μ l distilled water containing 6% (v/v) hydrogen peroxide (Sigma UK Ltd, Poole, Dorset) to oxidize methionine residues on inhibin and enhance reactivity with E4 mAb (Knight and Muttukrishna, 1994). Tubes were mixed and incubated for 30 min at room temperature before transferring duplicate 100 μ l aliquots to E4-coated microplates (Groome and O'Brien, 1993) which were placed in a humid box and incubated overnight on a microplate shaker (Wellmix, Denley Instruments, Billingham, West Sussex). Plates were washed (15 cycles) before adding 50 μ l ELISA buffer containing

approximately 25 ng biotinylated α subunit specific mAb (mAb 173 29 H2; $0.5 \mu\text{g ml}^{-1}$) to each well. After incubating for 1 h at room temperature on a microplate shaker, plates were washed (15 cycles) and $50 \mu\text{l}$ ELISA diluent containing streptavidin-alkaline phosphatase conjugate (Dako, High Wycombe, Bucks; $1:10^5$ dilution) was added to each well. After a further 1 h incubation on a microplate shaker, plates were washed thoroughly (15 cycles) and bound alkaline phosphatase was quantitated using a commercially available ELISA amplification kit (Immuno Select ELISA Amplification System; GIBCO BRL, Uxbridge, Middlesex) used according to the supplier's instructions. Absorbance at 492 nm was read on a microplate reader (MR700; Dynatec Laboratories, Billingham, Sussex) and data processed by immunoassay curve-fitting software (Riacalc, Pharmacia, Milton Keynes, Bucks). Crossreactivities of related molecules were assessed and recovery experiments were performed.

Gonadotrophin and steroid assays

Gonadotrophins. Plasma concentrations of FSH were determined using a homologous ovine FSH radioimmunoassay kit provided by NIDDK (Bethesda, MD) as reported previously (Knight and Castillo, 1988; Wrathall *et al.*, 1990). The detection limit of the assay, expressed as the amount of FSH standard (NIDDK-oFSH-RP1) required to inhibit the binding of the iodinated tracer (NIDDK-oFSH-I1) to the antibody (NIDDK-anti-oFSH-I; final dilution $1:16\ 000$) by 20% was $0.025 \text{ ng per tube}$. Mean intra- and interassay coefficients of variation (CV) were 4% and 7%, respectively. Plasma concentrations of LH were determined as described previously (Knight and Castillo, 1988) using NIH-LH-S16 as standard. The limit of detection was 0.1 ng per tube and mean intra- and interassay CVs were 8 and 11%, respectively.

Steroids. Concentrations of oestradiol in plasma samples were determined using a commercial human oestradiol radioimmunoassay kit (Biostat, Stockport) modified and validated for ovine plasma as described by Tannetta *et al.* (1997). Intra-assay and interassay CVs were 12% and 21%, respectively, and the detection limit of the assay was 0.25 pg per tube (equivalent to 0.5 pg ml^{-1} ovine plasma). Concentrations of progesterone in plasma were measured by direct ELISA as described previously (Sauer *et al.*, 1986; Wrathall *et al.*, 1990). The detection limit was 0.17 ng ml^{-1} and intra- and inter-plate CVs were $< 10\%$.

Statistical analysis

Repeated-measures analysis of variance (ANOVA) and post-hoc Fisher's PLSD test for individual pairwise comparisons were used to determine whether plasma hormone concentrations varied significantly during the oestrous cycle. The same tests were used to analyse the changes in plasma hormones after GnRH-agonist treatment and subsequent PMSG injection. Pregnancy data were evaluated by factorial ANOVA and post-hoc PLSD tests. In each study, simple regression analysis was also used to investigate relationships between concentrations of different hormones.

Fig. 1. Representative dose-response curves for the 32 kDa bovine inhibin-A standard (○), ovine follicular fluid (FF) (●) and two ovine plasma pools (□, ■) in the inhibin-A ELISA. Values are means of duplicate determinations.

Results

Validation of the inhibin-A ELISA for sheep plasma

Serial dilutions of pooled ovine follicular fluid and ovine serum gave ELISA response curves parallel to the purified 32 kDa bovine inhibin standard (Fig. 1). Neither recombinant human (rh) activin-A, rh activin-B, rh inhibin-B (gifts from Genentech Inc., South San Francisco, CA) at 25 ng ml^{-1} , bovine inhibin α subunit (pro- αC ; Knight *et al.*, 1989) at 100 ng ml^{-1} , rh follistatin-288 (a gift from NIDDK, Baltimore, MD) at 100 ng ml^{-1} nor α_2 -macroglobulin (Calbiochem, Nottingham) at $500 \mu\text{g ml}^{-1}$ showed any significant crossreaction in the ELISA (all $< 0.1\%$ relative to the bovine inhibin-A standard). Recovery of bovine inhibin standard ($50 \text{ pg per } 100 \mu\text{l}$) added to individual ovine plasma samples before assay was $91 \pm 8\%$ ($n = 8$). The detection limit was approximately 50 pg ml^{-1} ovine plasma and within- and between-plate CVs were $< 10\%$.

Inhibin-A during the oestrous cycle

Mean serum profiles of inhibin-A, FSH, LH and progesterone based on samples taken every 12 h throughout a synchronized oestrous cycle of four ewes are shown (Fig. 2a–c). The corresponding hormone profiles (inhibin-A, LH, FSH, oestradiol) determined from the samples taken every 2 h for 5 days over the periovulatory period are also shown (Fig. 2d–e). Plasma oestradiol was only assayed in every third sample (that is, at intervals of 6 h) during this period of more frequent sampling. Plasma concentrations of FSH, LH, oestradiol and progesterone showed the expected pattern of change during the cycle with a preovulatory surge in LH and FSH occurring 50 h after withdrawal of the progestogen insert. In contrast to

Fig. 2. Mean ($\pm$ SEM) concentrations of (a) LH ($\circ$) and progesterone ($\bullet$), (b) FSH, (c) inhibin-A, (d) FSH ($\bullet$) and inhibin-A ($\circ$) and (e) LH ($\blacksquare$) and oestradiol ($\square$) in peripheral plasma of four ewes during a progestagen ($\square$)-synchronized oestrous cycle. (a–c) show data based on blood samples taken twice a day for 20 days; while (d–e) show data based on the more frequent sampling (every 2 h) during the periovulatory period (corresponding to the black bar in (c)). Note that oestradiol measurements were restricted to the periovulatory period. ($\square$) denotes the presence of an intravaginal progestagen-containing sponge and the vertical dashed line in (a–c) indicates the peak of the preovulatory LH surge. * $P < 0.05$.

plasma oestradiol, inhibin-A showed no consistent pattern of change in response to progestagen withdrawal and did not increase significantly during the follicular phase (Fig. 2d,e). However, from the time of the preovulatory LH/FSH surge, inhibin-A concentrations fell steadily (approximately 50%; $P < 0.01$) to reach a nadir 22 h later coincident with the peak of the secondary FSH surge (Fig. 2d). During the next 36 h, inhibin-A increased progressively (approximately twofold; $P < 0.01$) and this was accompanied by a progressive 70% reduction in plasma FSH ($P < 0.01$). Over the next 3 days, FSH increased again by about 100% ($P < 0.01$) (Fig. 2b) concomitant with an approximate fall of 90% ($P < 0.05$) in inhibin-A (Fig. 2c). When all plasma samples collected from the four ewes across the cycle are considered, inhibin-A showed a weak inverse correlation with FSH ($r = -0.21$; $P < 0.001$; $n = 416$), but not with LH ($r = -0.05$; $P = 0.36$; $n = 416$).

Inhibin-A after manipulation of ovarian function with GnRH agonist and PMSG

Chronic exposure of ewes to the GnRH agonist was associated with a reduction in plasma concentrations of FSH, inhibin-A and oestradiol, the last two falling to undetectable values (Fig. 3). Administration of PMSG to reinstate follicle development promoted a marked increase in plasma inhibin-A ($P < 0.001$) and oestradiol ($P < 0.001$), while FSH concentrations were reduced further ($P < 0.01$). Concentrations of inhibin-A (approximately 4.5 ng ml^{-1}) and oestradiol (approximately 21 pg ml^{-1}) observed 48 h after PMSG administration were about 15-fold and 3.5-fold higher than corresponding values reached in the normal preovulatory period. Plasma inhibin-A and oestradiol were positively correlated ($r = 0.95$; $P < 0.0001$). Plasma FSH was negatively correlated

Fig. 3. Changes in mean ($\pm$ SEM) concentrations of inhibin-A ($\square$), oestradiol ($\bullet$) and FSH ($\blacksquare$) in peripheral plasma of four ewes in response to chronic GnRH-agonist treatment ($\square$) and subsequent injection of a superovulatory dose of pregnant mares' serum gonadotrophin (PMSG) (2000 iu).

Fig. 4. Mean ($\pm$ SEM) concentrations of inhibin-A ($\bullet$), FSH ($\circ$) and progesterone ($\square$) in peripheral plasma of ewes (five to six ewes per time-point) sampled between days 35 and 135 of gestation. Means without a common letter are significantly ($P < 0.05$) different.

with both inhibin-A ($r = -0.47$; $P < 0.01$) and oestradiol ($r = -0.55$, $P < 0.01$).

Inhibin-A during pregnancy

Between days 35–60 of gestation, plasma concentrations of inhibin-A, FSH and progesterone were similar to those observed in nonpregnant ewes during the luteal phase (Fig. 4).

Plasma progesterone increased steadily from about 5 ng ml^{-1} on day 35 to about 18 ng ml^{-1} on day 135 of gestation. Between days 60 and 90 of gestation, plasma inhibin-A fell about sevenfold ($P < 0.01$) while FSH fell about twofold ($P < 0.05$). Both hormones remained at basal values for the rest of gestation. Plasma progesterone was negatively correlated with inhibin-A ($r = -0.53$, $P < 0.001$) and FSH ($r = -0.51$, $P < 0.001$) during pregnancy, while inhibin-A and FSH were positively correlated ($r = 0.48$; $P < 0.01$).

Discussion

It has been possible, by incorporating a new biotinylated 'detector antibody' (clone 173 29 H2; anti- α C¹⁻³²) into an established amplified two-site ELISA for human inhibin-A (Groome *et al.*, 1994; Muttukrishna *et al.*, 1994), to generate an assay with improved crossreactivity with bovine/ovine inhibin-A. As reported here, the assay is satisfactory in terms of its reproducibility, accuracy and specificity and, although sensitivity is not as high as that of the original inhibin-A ELISA, it can measure inhibin-A concentrations in peripheral blood of female sheep in a variety of physiological states. Plasma concentrations of inhibin-A in cyclic heifers are considerably lower than in sheep and therefore, in its present form, the assay is not sufficiently sensitive for use in cattle.

mRNAs for both β A and β B subunits are expressed in ovine granulosa cells (Engelhardt *et al.*, 1993). It is therefore likely that inhibin-B is also synthesized and secreted by the sheep ovary, perhaps in a differential manner to inhibin-A, as indicated by recent studies in humans (Groome *et al.*, 1996) and rats (Woodruff *et al.*, 1996). However, when assayed using the same inhibin-B ELISA (Groome *et al.*, 1996) inhibin-B concentrations in pooled ovine follicular fluid ($105 \pm 16 \text{ ng ml}^{-1}$, $n = 3$ pools) were some 50–100 times lower than inhibin-A concentrations (S. A. Feist and P. G. Knight, unpublished observations). Moreover, inhibin-B concentrations in plasma pools from cyclic, ovariectomized, PMSG-treated and pregnant ewes were found to be below the assay detection limit ($< 20 \text{ pg ml}^{-1}$, expressed in terms of hr inhibin-B standard). Since inhibin-B has never been isolated from sheep follicular fluid and is thus unavailable for testing, the possibility that poor crossreaction of sheep inhibin-B in the human inhibin-B ELISA accounts for the very low values in sheep follicular fluid and plasma cannot be excluded. Despite this uncertainty, the present balance of evidence suggests that inhibin-A is the major isoform synthesized and secreted by the sheep ovary. So far, our attempts to introduce the new broad specificity anti- α C monoclonal into the existing inhibin-B ELISA (Groome *et al.*, 1996) have not been successful.

Contrary to expectations based on findings during the human menstrual cycle (Groome *et al.*, 1994; Muttukrishna *et al.*, 1994) and the rat oestrous cycle (Fahy *et al.*, 1995; Woodruff *et al.*, 1996), plasma inhibin-A concentrations, as measured by specific ELISA, did not increase during the follicular phase in ewes, despite the occurrence of an approximate tenfold increase in plasma oestradiol derived from growing preovulatory follicle(s). As suggested previously (Tsonis *et al.*, 1988; McNeilly *et al.*, 1989), this result indicates that the characteristic fall in FSH during the early follicular phase is due mainly to an increased negative feedback action of follicular oestradiol, rather than an increase in inhibin feedback. It also reinforces earlier evidence that follicles, other than large oestrogen-active ones, produce appreciable amounts of inhibin and contribute significantly to concentrations present in the peripheral circulation (Tsonis *et al.*, 1983; Mann *et al.*, 1992; Engelhardt *et al.*, 1993). Immediately after the preovulatory LH/FSH surge, plasma inhibin-A values fell steeply to reach a nadir coincident with the peak of the postovulatory FSH surge. Findlay *et al.* (1990) also observed a fall in plasma ir-inhibin at this time while, in contrast, others (McNeilly *et al.*, 1989;

Campbell *et al.*, 1990) reported an increase after the preovulatory LH surge. The present observation suggests that, as in rats (Hasegawa *et al.*, 1989; Fahy *et al.*, 1995), the postovulatory FSH surge in ewes is generated, at least in part, by a reduction in inhibin feedback. However, an abrupt reduction in plasma oestradiol began 2–6 h before the preovulatory LH/FSH peak and preceded the fall in inhibin-A by 6–8 h. Given that both inhibin-A and oestradiol fell to basal values after the preovulatory LH/FSH surge and that the ovine pituitary is highly sensitive to the negative feedback effects of inhibin and oestradiol (Martin *et al.*, 1988; Muttukrishna and Knight, 1990), it is likely that a reduction in concentrations of both hormones contributes to the generation of the postovulatory FSH rise required to initiate the FSH-dependent growth of a new cohort of follicles (Ginther *et al.*, 1995).

Previous studies of circulating concentrations of ir-inhibin in ewes using α subunit-directed radioimmunoassays have generated conflicting results with regard to the pattern of secretion during the oestrous cycle (McNeilly *et al.*, 1989; Campbell *et al.*, 1990; Findlay *et al.*, 1990). None of these is wholly consistent with the present findings obtained using a specific inhibin-A assay. It is likely that these discrepancies can be accounted for mainly by the inherent limitations of the 'first generation' radioimmunoassays used in earlier studies, although other differences between studies (for example, breed, experimental design, sampling method) could also contribute to the variable findings. In addition, differences between ewes, in the follicle population present at the time of luteal regression, would be expected to generate additional variation in inhibin and oestradiol secretory profiles. In the absence of information on ovarian follicular dynamics in the ewes used in the present study, the observed changes in peripheral hormone concentrations cannot be directly associated to the growth of discrete follicle waves as recently identified in sheep (Ginther *et al.*, 1995; Souza *et al.*, 1996).

Chronic exposure of ewes to a superactive GnRH agonist to suppress gonadotrophin secretion and arrest follicular development (Knight and Castillo, 1988; Pictou *et al.*, 1990) reduced plasma inhibin-A and oestradiol to undetectable concentrations (as observed in ovariectomized or hypophysectomized ewes), supporting the view that ovarian follicles are the principal source of inhibin-A in the peripheral circulation. This finding is consistent with those of Findlay *et al.* (1990) who observed a reduction in ir-inhibin and oestradiol in ovarian vein plasma of ewes after cauterization of visible follicles on the ipsilateral ovary. As anticipated, restoration of follicle growth, by administration of a superovulatory dose of PMSG, in the ewes treated with the GnRH-agonist promoted a rapid and parallel increase in plasma inhibin-A and oestradiol. Increases in ir-inhibin secretion in ewes after exogenous FSH treatment have been reported previously (McNeilly *et al.*, 1989; McNatty *et al.*, 1993); however, the present data confirm that a substantial increase in follicular production of the α - β A dimer occurs.

The changes observed in plasma inhibin-A in the pregnant ewes in the present study support previous findings obtained using the 'Monash' inhibin radioimmunoassay (Findlay *et al.*, 1991). Inhibin-A concentrations were similar to those in nonpregnant animals until day 60 of gestation, after which they fell about sevenfold, to remain at basal values until at least day 135 of gestation (last time-point studied). This finding contrasts

with human pregnancy (Muttukrishna *et al.*, 1995), during which circulating inhibin-A concentrations are greatly increased, particularly during the third trimester when the foeto-placental unit is the predominant source of inhibin-A reaching the maternal circulation (Petraglia *et al.*, 1994). Our observation that plasma FSH also fell significantly between days 60–90, and was positively correlated with inhibin-A, confirms that any reciprocal relationship between inhibin and FSH is lost during pregnancy in ewes (Jenkin *et al.*, 1995). This loss is presumably a result of the combined negative feedback action of oestrogens and progesterone (Phillips *et al.*, 1988) that are secreted in increasing amounts by the developing foeto-placental unit (Challis, 1971; Findlay *et al.*, 1991; Jenkin *et al.*, 1995).

In conclusion, the development and validation of a new inhibin-A ELISA with broader species specificity than the original assay (Groome *et al.*, 1994) has enabled us to clarify the relationship between circulating inhibin-A and other reproductive hormones during the ovine oestrous cycle, pregnancy, and after ovarian manipulation. This assay should prove useful for further endocrinological studies in sheep, although the challenge remains to develop a comparable assay capable of detecting inhibin-B in this species.

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