Effect of FSH on ovarian inhibin secretion in anoestrous ewes*

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Summary. In Exp. 1, 7 Finn–Merino ewes which had one ovary autotransplanted to a site in the neck had jugular and timed ovarian venous blood samples collected at 10-min intervals for 2 h before and 3 h after injection of 5 µg NIAMDD-oFSH-S16. In Exp. 2, 8 Finn–Merino ewes with ovarian autotransplants had jugular and timed ovarian venous blood samples collected at 15-min intervals for 2 h before and 12 h after bolus injection of 40 µg NIAMDD-oFSH-S16 and infusion of oFSH-S16 at 6 µg/min for 4 h. In Exp. 2 the follicular population of the ovary was assessed by real-time ultrasound at the beginning and end of the experimental period.

In both experiments the secretion rates of inhibin (1–3 ng/min) and oestradiol (0.5–8 ng/min) were similar to those observed during the luteal phase of the cycle in the breeding season, indicating significant follicular development in these animals. In Exp. 1 there was no change in the secretion of oestradiol or inhibin after the injection of FSH which resulted in a 25% increase ($P < 0.05$) in the concentration of FSH in plasma. Inhibin secretion was pulsatile but there was no difference in inhibin pulse frequency before (1.6 ± 0.2 pulses/h) or after (1.2 ± 0.5 pulses/h) injection of FSH. In Exp. 2 injection of FSH resulted in an increase ($P < 0.001$) in plasma concentrations of FSH in the sample taken 10 min after injection from a baseline of 1.2 ± 0.2 ng/ml to a peak of 10.6 ± 1.0 ng/ml (mean $\pm$ s.e.m.). FSH concentrations remained elevated over pre-treatment values for 10 h after the beginning of the treatment. The concentration of LH in jugular venous plasma increased ($P < 0.001$) after the bolus injection of FSH from a baseline of 1.4 ± 0.5 ng/ml to a peak of 6.7 ± 0.6 ng/ml. The ovarian rate of inhibin secretion did not change significantly following the injection and infusion of FSH. There was no difference in inhibin pulse frequency before (0.4 ± 0.2 pulses/h) or after (0.5 ± 0.1 pulses/h) injection of FSH. The rate of oestradiol secretion by the ovary increased ($P < 0.001$) after the injection of the FSH preparation, an effect that can be attributed to contamination of the FSH preparation with LH. Increasing jugular venous concentrations of FSH for 10 h had no effect on the ovarian follicle population.

We conclude that the ovarian secretion of inhibin is not acutely responsive to stimulation by FSH in anoestrous ewes.

Keywords: sheep; inhibin; FSH; ovary; anoestrus

Introduction

It has been clearly demonstrated that the ovarian secretion of inhibin increases following the infusion of follicle-stimulating hormone (FSH) or FSH-like gonadotrophins (sheep: bioactive

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inhibin: Tsonis *et al.*, 1988a; immunoactive inhibin: McNeilly *et al.*, 1989; rat: Lee *et al.*, 1981; man: McLachlan *et al.*, 1986a; Tsonis *et al.*, 1988b). It is unclear, however, whether this increase is due to stimulation of follicular growth or of the synthetic capacity of existing follicles.

Secretion of FSH by the pituitary is under the dual inhibitory control of oestradiol and inhibin (Martin *et al.*, 1988), which are synthesized by the granulosa cells of large dominant follicles (Baird & Scaramuzzi, 1976; Mann *et al.*, 1989; Campbell *et al.*, in press). Inhibin, however, is also secreted in appreciable quantities by large non-oestrogenic follicles and small follicles (Campbell *et al.*, in press). It is well established that oestradiol secretion is under the acute stimulatory control of luteinizing hormone (LH; Baird & McNeilly, 1981) but the factors controlling ovarian secretion of inhibin are not well understood. The ovarian secretion of inhibin is episodic (McNeilly & Baird, 1989; Campbell *et al.*, 1989, 1990a) although, unlike oestradiol, pulsatile inhibin secretion is not due to pulses of LH (McNeilly & Baird, 1989; Campbell *et al.*, 1989, 1990b). Although there are no pulses of FSH detectable in sheep (Findlay & Clarke, 1987), it is possible that fluctuations in FSH result in this episodic secretory pattern, as FSH has been shown to stimulate inhibin synthesis by granulosa cells in culture (Bicsak *et al.*, 1986; Suzuki *et al.*, 1987; Zhang *et al.*, 1988).

The purpose of the present experiment was to examine the effect of acute changes in peripheral FSH concentrations on the ovarian secretion of inhibin.

Materials and Methods

Experimental animals. Merino $\times$ Finnish Landrace ewes were kept under natural lighting conditions under cover in the Institute of Animal Physiology and Genetics Research, Edinburgh Field Station, Dryden, Roslin, Midlothian, UK. At least 6 years before the experiment, the animals had undergone surgery to remove the right ovary and autotransplant the left ovary and vascular pedicle to a site in the neck in order to facilitate the collection of ovarian venous blood. As the corpus luteum persists in ewes with ovarian autotransplants, 100 μ g cloprostenol, a potent analogue of prostaglandin F-2 α (Estrumate: ICI plc, Macclesfield, Cheshire, UK), was injected 12 days before the start of the experiment to ensure luteal regression. The anoestrous state of the animals was established by determining that the concentration of progesterone in jugular venous plasma was <1.5 nmol/l. On the day before the start of blood sampling both jugular veins were cannulated, the catheter on the side of the ovary being advanced so that its tip lay opposite the ovarian vein (Collett *et al.*, 1973). The animals in Exp. 2 had a second cannula inserted in the jugular vein, opposite the side anastomosed to the ovarian vein, to allow the infusion of FSH. The animals were placed in metabolism crates in a temperature-controlled room (11–15°C) during the period of blood sampling.

Experimental design. For Exp. 1, in June of 1988 samples of jugular (4 ml) and ovarian (5 or 10 ml) venous blood were collected at 10-min intervals for a total of 5 h from 7 ewes with ovarian autotransplants. After 2 h of blood sampling all ewes received a single injection of 5 μ g NIH-oFSH-S16 (20 times the biological activity of NIH-FSH-S1) in 0.9% (w/v) NaCl containing 1% normal sheep plasma (NSP).

For Exp. 2, in June of 1989 samples of jugular (4 ml) and ovarian (5 or 7.5 ml) venous blood were collected at 15-min intervals for a total of 14 h from 8 ewes with ovarian autotransplants. After 2 h of blood sampling all ewes received a bolus injection of 40 μ g NIH-oFSH-S16 in 0.9% (w/v) NaCl containing 1% NSP followed immediately by infusion of FSH (6 μ g/h NIH-oFSH-S16 in 0.9% (w/v) NaCl containing 1% NSP) for 4 h. The characteristics of the ovarian follicle population was assessed by real-time ultrasound before the start and at the end of blood sampling.

Ovarian scanning procedure. The ovarian follicle population was examined using a combined sector real-time ultrasound scanner (DSL 300) with a sector 7.5 MHz transducer probe (Diagnostic Sonar, Kirkton Campus, Livingston, UK). The area of skin over the ovary was clipped and the ovary scanned in both horizontal and vertical planes. The diameter of the antral cavity and position of all follicles greater than 2 mm in diameter in the medial/lateral, dorsal/ventral and cranial/caudal planes was noted.

Hormone assays. The blood was centrifuged at 4°C and the plasma stored at –20°C until analysis. Plasma concentrations of ovine LH and progesterone (McNeilly & Fraser, 1987; McNeilly *et al.*, 1986), oestradiol (Baird *et al.*, 1981), FSH (Campbell *et al.*, 1989) and 1-26 α -subunit of inhibin (McNeilly *et al.*, 1989) were determined using previously described radioimmunoassays. The sensitivity of the assays for LH, FSH, progesterone, oestradiol and inhibin were 0.2 ng (NIH-LH-S18)/ml, 0.3 ng (NIAMDD-FSH-RP1)/ml, 120 pg/ml (380 pmol/l) 20 pg/ml (73 pmol/l) and 35 pg/ml respectively. The intra- and inter-assay coefficients of variation were <15%. The ovarian blood flow was measured every hour by timing the collection of ovarian venous blood and the ovarian secretion rates of oestradiol and inhibin were calculated after correcting for the haematocrit (Collett *et al.*, 1973).

Statistical analysis. The parameters of pulsatile hormone secretion were determined using the Munro pulse analysis programme (Zaristow Software, Haddington, East Lothian, UK). The effect of time on the pattern and pulsatility

of hormone secretion were analysed by repeated sample analysis of variance. In Exp. 1, due to large between-animal variation, jugular FSH concentrations were expressed as a percentage of the mean levels before treatment, before analysis. Determination of the dimensions of the follicle revealed that they were often of irregular shape. The mean diameter of the follicle was determined from the 3 dimensions obtained by scanning and the follicles classified as large (≥ 4 mm in diameter) or small (< 4 mm in diameter). Comparisons of follicle populations before and after treatment was made by Student's paired *t* test.

Results

Experiment 1

Injection of FSH resulted in a 25% increase ($P < 0.05$) in plasma concentrations of FSH in the sample taken 10 min after injection from a baseline of 2.1 ± 0.2 ng/ml to a peak of 2.6 ± 0.2 ng/ml (mean $\pm$ s.e.m.; Fig. 1). The mean concentration of LH in jugular venous plasma did not change significantly over the period of blood sampling (Fig. 1). Endogenous pulses of LH, but not of FSH, were observed (Fig. 2).

Fig. 1. Experiment 1: the percentage change in FSH and concentrations of LH in jugular venous plasma and the ovarian secretion rate (SR) of inhibin and oestradiol in anoestrous Finn-Merino ewes with ovarian autotransplants ($N = 7$) which had both jugular and ovarian venous blood samples taken at 10-min intervals for 2 h before and 3 h after a single injection of $5 \mu\text{g}$ NIH-oFSH-S16 at time zero (dotted line). Values are means $\pm$ s.e.m.

Fig. 2. Experiment 1: hormone profile from an individual anoestrous ewe with an ovarian auto-transplant (OG10) showing the response in ovarian inhibin and oestradiol secretion rate (SR) to a single injection of 5 µg NIH-oFSH-S16 at time zero (dotted line). Note that ovarian inhibin secretion is episodic and that this ewe also had an endogenous pulses of LH during the first 2 h of blood sampling which resulted in an increase in oestradiol secretion.

There was no change in the ovarian secretion of either inhibin or oestradiol following injection of FSH (Figs 1 & 2). Inhibin secretion was pulsatile (Fig. 2) with an overall pulse interval, amplitude and nadir of 37.7 ± 2.7 min, 0.8 ± 0.2 ng/min and 1.5 ± 0.2 ng/min respectively. Pulses of inhibin, however, were not related to endogenous pulses of LH. In contrast, oestradiol secretion exhibited pulsatile responses to endogenous LH pulses (Fig. 2). There was no difference in pulses of inhibin before (1.6 ± 0.2 pulses/h) or after (1.2 ± 0.5 pulses/h) injection of FSH.

Experiment 2

Injection of FSH resulted in an increase ($P < 0.001$) in plasma concentrations of FSH in the sample taken 10 min after injection from a baseline of 1.2 ± 0.2 ng/ml to a peak of 10.6 ± 1.0 ng/ml (mean $\pm$ s.e.m.; Fig. 3). Within 1 h of the bolus injection FSH concentrations had fallen ($P < 0.01$) to 6–8 ng/ml where they remained for a further 1.5 h. Thereafter the concentration of FSH in jugular venous plasma fell to about 4–5 ng/ml for the remainder of the infusion. After the infusion of FSH was stopped FSH concentrations declined ($P < 0.001$) steadily to pre-treatment values within 10 h of the beginning of the treatment (Fig. 3). The concentration of LH in jugular venous plasma increased ($P < 0.001$) following the bolus injection of FSH from a baseline of 1.4 ± 0.5 ng/ml to a peak of 6.7 ± 0.6 ng/ml (mean $\pm$ s.e.m.; Fig. 3) within 10 min of injection. LH levels had returned to basal values within 95 min of the administration of the FSH bolus. The amplitude and duration of this LH pulse was similar to endogenous LH pulses (Fig. 4). Endogenous pulses of FSH were not observed (Fig. 4).

The secretion of inhibin by the ovary did not change significantly following the injection and infusion of FSH (Figs 3 & 4). Inhibin secretion was pulsatile (Fig. 4) with a pulse interval, amplitude and nadir of 59.3 ± 3.3 min, 2.9 ± 0.5 ng/min and 2.8 ± 0.5 ng/min respectively. There was

Fig. 3. Experiment 2: the mean concentration of FSH and LH in jugular venous plasma and the ovarian secretion rate (SR) of inhibin and oestradiol in anoestrous Finn-Merino ewes with ovarian autotransplants ($N = 8$) which had both jugular and ovarian venous blood samples taken at 15-min intervals for 2 h before and 12 h after a bolus injection of $40 \mu\text{g}$ NIH-oFSH-S16 at time zero (dotted line), followed immediately by an infusion of FSH ($6 \mu\text{g/h}$ NIH-oFSH-S16) for 4 h (hatched bar). Values are means $\pm$ s.e.m.

no difference in inhibin pulse frequency before (0.4 ± 0.2 pulses/h) or after (0.5 ± 0.1 pulses/h) injection of FSH. Pulses of inhibin were not related to endogenous pulses of LH (Fig. 4). The ovarian rate of oestradiol secretion increased ($P < 0.001$) following the injection of FSH preparation (Figs 3 & 4). The magnitude of this oestradiol response was similar to that seen to endogenous pulses of LH (Fig. 4).

Before the start of the experiment the ewes had a total follicle population of 2.0 ± 0.3 with 1.3 ± 0.4 large and 0.8 ± 0.4 small follicles/ewe. Increasing the jugular venous concentration of FSH had no significant effect on either the total number of follicles (2.8 ± 0.5 follicles/ewe) or the size distribution of the follicle population (large 1.5 ± 0.3 and small 1.3 ± 0.4 follicles/ewe).

Discussion

The results of this study demonstrate that neither modest (Exp. 1) nor large (Exp. 2) increases in concentrations of FSH in jugular venous blood affect the rate of ovarian inhibin secretion, suggesting that inhibin secretion is not under the acute control of FSH during anoestrus. Although the ewes in this study were anoestrous, both the oestradiol and inhibin secretion rates observed were similar to those observed in comparable ewes during the luteal phase of the breeding season (Campbell *et al.*, 1990c), indicating the presence of large oestrogenic follicles in the ovaries of these animals. The results of this experiment, together with our previous observation that inhibin

Fig. 4. Experiment 2: hormone profile from an individual anoestrous ewe with an ovarian autotransplant (2G20) showing the response in ovarian inhibin and oestradiol secretion rate (SR) to a bolus injection of 40 μ g NIH-oFSH-S16 at time zero (dotted line), followed immediately by an infusion of FSH (6 μ g/h NIH-oFSH-S16) for 4 h (hatched bar). Note that ovarian inhibin secretion is episodic and that this ewe also had an endogenous pulses of LH within 3 h of the injection of FSH which was of a magnitude similar to the exogenous pulse. The exogenously and endogenously induced pulses resulted in the pulsatile secretion of oestradiol.

secretion increases during the follicular phase when FSH concentrations are falling (Campbell *et al.*, 1990c), suggests that the secretion of inhibin by the ovary is not controlled acutely by FSH at any stage of the cycle. Further, it would also appear that the secretion of inhibin by the ovary is not responsive to LH, as injection of LH into anoestrous ewes (Campbell *et al.*, 1989) and removal of LH pulses during the follicular phase of the oestrous cycle by administration of a potent GnRH antagonist (Campbell *et al.*, 1990b) have no effect on the pattern of inhibin secretion. In addition, as observed in this and other studies (McNeilly & Baird, 1989; Campbell *et al.*, 1989, 1990a) there is little relationship between episodic LH and inhibin secretion.

The observation that ovarian inhibin secretion is not acutely responsive to stimulation by FSH is in contrast to the results of a number of in-vitro studies which have shown that FSH can stimulate inhibin synthesis by cultured granulosa cells (rat: Bicsak *et al.*, 1986; Suzuki *et al.*, 1987; Zhang *et al.*, 1988; cow: Henderson *et al.*, 1984; pig: Michel *et al.*, 1989; sheep: Campbell, 1989). It must be emphasized, however, that the present study examined the acute effects of FSH on ovarian inhibin secretion as distinct from inhibin synthesis by granulosa cells. Ovarian inhibin secretion can clearly be stimulated by long-term treatment with FSH (McNeilly *et al.*, 1989; Lee *et al.*, 1981; McLachlan *et al.*, 1986a; Tsonis *et al.*, 1988a, b) but these studies are confounded by the stimulatory action of FSH on follicular growth. The purified FSH used in the present experiments (NIH-oFSLH-S16) is of higher biopotency than that used by McNeilly *et al.* (1989) and has been shown to stimulate

follicular growth *in vivo* (Picton *et al.*, 1990) and to stimulate the synthesis of inhibin *in vitro* by cultured granulosa cells isolated from small follicles (Campbell, 1989). In addition, follicular growth in anoestrous ewes is readily stimulated by FSH (Wallace *et al.*, 1986). Further, the ovarian autotransplant model, in combination with the inhibin assay based on the 1-26 α subunit of inhibin, has previously been shown to detect changes in the ovarian secretion of inhibin resulting from changes in circulating FSH levels (McNeilly *et al.*, 1989; Baird *et al.*, 1990; Campbell *et al.*, 1990d). Recent reports of the presence of a high molecular weight (45 000) form of monomeric inhibin α -subunit in ovine follicular fluid (Knight *et al.*, 1989) may be the cause for some concern as this non-bioactive molecule cross-reacts with the antiserum used in this experiment (McNeilly *et al.*, 1989). The key unresolved issue is whether this subunit is secreted into the peripheral circulation in significant quantities. If this were the case then large discrepancies between bio- and immuno-activities would be expected. In a number of published studies we have demonstrated a consistent high correlation (r values = ~ 0.8) between bioactivity determined from the sheep pituitary cell assay (Tsonis *et al.*, 1986) and immuno-activity determined from the 1-26 α inhibin assay (McNeilly *et al.*, 1989; Mann *et al.*, 1989; Campbell *et al.*, 1989). In addition the 1-26 α assay has been shown to give comparable results (Campbell *et al.*, 1990c) in sheep to an inhibin assay based on intact inhibin (McLachlan *et al.*, 1986b). We therefore feel that the 1-26 α inhibin assay used in this study gives an accurate picture of the ovarian secretion of bioactive inhibin.

There are a number of possible explanations for the apparent difference between the findings of *in-vitro* studies that demonstrate a clear stimulatory effect of FSH on inhibin synthesis and the results of the current study which show that ovarian inhibin secretion is not acutely responsive to FSH. The first possibility is that the synthetic mechanisms resulting in the production of a mature inhibin molecule may require a much longer time than simpler molecules such as steroids, due to the possible need for post-translational processing. Recent studies indicate that it takes at least 6 h for FSH to stimulate the expression of mRNA encoding for the α -subunit of inhibin and to stimulate inhibin production by rat Sertoli cells in culture (Toebosch *et al.*, 1989). The second possibility is that an increase in inhibin synthesis by granulosa cells may not be reflected by an acute increase in ovarian inhibin secretion. Recent studies have shown that follicles containing granulosa cells with a low capacity to synthesize inhibin *in vitro* may contain antral fluid with appreciable concentrations of inhibin (Campbell *et al.*, in press). It is therefore possible that the control of inhibin release from the follicle into the circulation may include mechanisms controlling both the rate of synthesis by granulosa cells and the active transport of inhibin across the basement membrane. The third possibility is that the *in-vitro* models used may not represent the appropriate physiological situation. Many *in-vitro* studies (Bicsak *et al.*, 1986; Suzuki *et al.*, 1987; Zhang *et al.*, 1988) utilize granulosa cells from immature diethylstilboestrol-treated rats. In sheep, more than 80% (Mann *et al.*, 1989; Campbell *et al.*, in press) of inhibin secreted by the ovary comes from large follicles with a diameter greater than 4 mm, the granulosa cells of which are well differentiated. Granulosa cells from large follicles seem to be less sensitive to the actions of gonadotrophins (Hsueh *et al.*, 1984; Campbell, 1989; Hylka & diZerega, 1990) and this may explain the lack of effect of short-term increases in plasma FSH on inhibin secretion that were observed in the present study. *In-vitro* studies which have demonstrated an FSH-dependent increase in bioactive-inhibin secretion from granulosa cells isolated from large bovine (Henderson *et al.*, 1984) and porcine (Michel *et al.*, 1989) follicles, have involved long-term culture which could involve FSH-induced maturational changes in granulosa cell function. The results of the present study do not allow us to determine which of the above factors adequately explains why ovarian inhibin secretion is not acutely responsive to FSH. Profitable areas for future research in this area would include time-course studies on the effect of FSH on gene expression and inhibin production by sheep granulosa cells from large healthy follicles and determination of the mechanisms controlling the transport of inhibin across the basement membrane of the follicle.

While administration of moderate doses of FSH had no effect on oestradiol secretion, following the administration of a large bolus of FSH there was a marked increase in ovarian oestradiol

secretion (Fig. 3). While FSH may have caused this increase, it is more likely that it was due to contamination of the FSH preparation with LH as peripheral LH levels increased markedly following injection of the FSH bolus and it is well recognized that LH stimulates ovarian oestradiol secretion (Baird *et al.*, 1981; Campbell *et al.*, 1990a). As we have previously shown that exogenous LH has no effect on inhibin secretion in anoestrous ewes (Campbell *et al.*, 1989) the contamination of this FSH preparation with LH has little effect on the interpretation of the present study. This observation, however, has important implications for the interpretation of studies involving the use of similar FSH preparation *in vivo* and *in vitro*. In Exp. 2, the jugular venous concentrations of FSH began to decline before the end of the FSH infusion. While this decrease may be due to methodological problems, it is more likely to reflect a decrease in the release of endogenous FSH by the pituitary caused by the increase in oestradiol secretion observed after the bolus injection of the LH-contaminated FSH preparation.

We can conclude from this study that ovarian inhibin secretion, at least in anoestrous ewes, is not acutely responsive to stimulation by FSH, therefore suggesting that the increase in inhibin secretion observed following long-term infusion of FSH is due to the stimulation of follicular growth.

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