Inhibin in individual ovine follicles in relation to diameter and atresia

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Summary. Inhibin activity was measured by bioassay in follicular fluid of 99 individual ovine follicles ranging from 1.4 to 6.8 mm diameter (used to calculate volume) and in various stages of atresia. Treatment of samples before assay with charcoal concentrations of >1 mg/ml resulted in significant loss of inhibin activity.

The inhibin content of follicular fluid from individual follicles varied with follicular fluid volume but not with the degree of atresia, as assessed by morphological criteria. Inhibin concentration was not related to atresia, but was correlated with follicular fluid volume. However, aromatase activity in granulosa cells and oestradiol-17 β concentration of follicular fluid, considered to be good indices of atresia, were highly correlated with both inhibin content and concentration in follicles ≥ 3.5 mm diameter.

Inhibin in ovine follicular fluid shows marked variation between follicles and it is suggested that this reflects a combination of the number and activity of granulosa cells within the follicle and the exit rate of inhibin from the follicle.

Introduction

Inhibin is a non-steroidal gonadal hormone capable of selective suppression of FSH release from the pituitary gland (Main, Davies & Setchel, 1979). Follicular fluids from several species, including cow (de Jong & Sharpe, 1976; Welschen, Hermans, Dullaart & de Jong, 1977; Henderson & Franchimont, 1981), sheep (Scott, Quigg, Trounson, Tsonis & Findlay, 1980a), pig (Marder, Channing & Schwartz, 1977; Lorenzen, Dworkin, Schwartz & Channing, 1978), monkey (Channing, Anderson & Hodge, 1980) and man (Chari, Hopkinson, Daume & Sturm, 1979; Channing *et al.*, 1981), have been found to contain inhibin activity.

Data from the literature are contradictory concerning the levels of ovarian inhibin during follicular development, and the studies have usually been performed on pools of fluid rather than on fluid from individual follicles. It has been claimed that the concentration of inhibin decreases with growth of follicles in the pig (Lorenzen *et al.*, 1978) and the cow (Henderson & Franchimont, 1981). In contrast, Welschen *et al.* (1977) detected inhibin in small bovine follicles but measured higher concentrations in medium and large follicles. Relatively little is known about the relationship between inhibin and follicular atresia. One study of the human follicle (Channing *et al.*, 1981) and some preliminary data on sheep follicles (Scott *et al.*, 1980a) indicate a decrease in inhibin concentrations with atresia.

There is indirect evidence that ovarian follicular inhibin may be an important regulator of FSH in the ewe (Goodman, Pickover & Karsch, 1981; Cummins, O'Shea, Bindon, Lee & Findlay, 1983).

The aim of this study was to measure the content and concentration of inhibin in follicular fluid of individual sheep follicles and relate this to the diameter and stage of atresia of the follicle.

Materials and Methods

Inhibin bioassay

Inhibin activity was measured by an in-vitro pituitary cell culture system using FSH cell content as the end point (Scott, Burger & Quigg, 1980b). Unknowns were compared to a reference preparation derived from ovine testicular lymph protein (OTLP-6) which was assigned a potency of 1 U/mg (Eddie, Baker, Higginson & Hudson, 1979). The standard curve in quadruplicate and unknowns in triplicate were assayed in at least 3 dilutions to generate parallel FSH inhibition curves (Text-fig. 1). Ovine follicular fluid had no significant effects on the LH content of the pituitary cells (Text-fig. 1). Relative potencies were calculated for those samples that showed no significant departure from parallelism to the reference preparation, analysed by regression analysis and parallel line assay statistics (Borth, 1976).

Text-fig. 1. Effect of ovine follicular fluid on the FSH and LH content of pituitary cells.

Isolation of follicles

Sheep ovaries were obtained from a local abattoir, placed in 0.154 M-NaCl (saline) containing 50 U kanamycin (Sigma, Melbourne, Australia)/ml plus 0.1% (w/v) glucose (BDH Chemicals, Melbourne, Australia) and transported on ice to the laboratory. Individual follicles were dissected and classified according to diameter, measured by a calibrated eyepiece graticule to the nearest 0.5 mm or 0.1 mm (by taking two diameters perpendicular to each other and averaging). Only follicles ≥ 3.5 mm in which diameter was measured to the nearest 0.5 mm were used to determine inhibin content, whereas those measured to the nearest 0.1 mm were used to determine content and concentration. Since the protein content of follicular fluid measured by the method of Lowry, Rosebrough, Farr & Randall (1951) did not vary significantly with atresia in follicles < 3.5 mm diameter (Table 4), inhibin concentration in these follicles ($N = 47$) measured to the nearest 0.5 mm and 0.1 mm diameter was expressed as U/mg protein. In addition, inhibin concentration in follicular fluid was determined small follicles measured to the nearest 0.1 mm diameter. The volume of fluid (V) contained within each follicle was calculated from the diameter of the follicle (D) by the formula, $V = 0.52(D)^{2.7}$ (Carson, Findlay, Clarke & Burger, 1981). The stage of atresia

was assessed by morphological criteria (Carson, Findlay, Burger & Trounson, 1979), and graded I–V, follicles of class I being non-atretic and those of class V being terminally atretic.

Aromatase assay

The aromatase activity (pmol oestradiol-17 β /10⁶ viable granulosa cells/h) was measured *in vitro* by incubating 0.5–17.4 $\times$ 10⁴ granulosa cells for 1 h in the presence of 0.1 μ M-testosterone in medium 199 (Tsonis & Findlay, 1981). The oestradiol content of the media and of follicular fluid was measured (after extraction) by radioimmunoassay (WHO Matched Assay Reagents). The within- and between assay coefficients of variation for 33 consecutive oestradiol assays were < 15% (1.5–86 fmol/tube) and 10.7% (at 20 fmol/tube) respectively.

Experiment 1

The aim of this experiment was to determine the optimum concentration of charcoal which removed most of the steroids, without affecting inhibin activity in follicular fluid.

Four 5 mm follicles of stages I–III atresia were placed in 100 μ l Dulbecco phosphate-buffered saline (DPBS; CSL, Melbourne, Australia) and punctured several times with a sharp needle. Each sample was centrifuged (7000 *g* for 2 min) to remove tissue debris. Portions of the supernatant were further diluted and treated with various concentrations, up to 1.0 mg/ml, of charcoal coated with 0.01% dextran (Lee, McMaster, Quigg, Findlay & Leversha, 1981) for 30 min at 4°C and centrifuged as above. The supernatants were assayed for inhibin activity.

The ability of similar concentrations of charcoal to remove steroid from diluted follicular fluid was assessed by adding 8000 c.p.m. ¹²⁵I-labelled testosterone to duplicate aliquants of follicular fluid from 3 individual follicles of 3 mm diameter and at stage I or II of atresia and measuring the radioactivity remaining after charcoal treatment.

Because the values for inhibin in ovine follicular fluid from this experiment appeared to be much higher than those in our earlier study (Scott *et al.*, 1980a) and those reported by Henderson & Franchimont (1981) for cow follicles, we compared treatment of follicular fluid with 1 and 10 mg charcoal/ml. Cow and sheep ovaries were obtained, dissected and follicles classified as above. One small (2.2 mm) and one large (5.8 mm) ovine follicle and one small (3.4 mm) and one large (6.4 mm) bovine follicle were individually ruptured in 250 μ l medium 199, buffered with 20 mM-Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphuric acid; CSL) containing 50 U kanamycin/ml. Aliquants of 100 μ l of each sample were subjected to two charcoal regimens: (a) 1 mg/ml containing 0.01% (w/v) dextran for 10 min at room temperature, 30 min at 4°C and then centrifuged at 7000 *g* for 2 min (our method); or (b) 10 mg/ml containing 0.1% (w/v) dextran, mixed for 16 h at 4°C and centrifuged at 3000 *g* for 30 min (adapted from Henderson & Franchimont, 1981). The supernatants were serially diluted and assayed for inhibin activity.

Experiment 2

In this experiment, we measured inhibin content and concentration in follicular fluid from individual ovine follicles in relation to diameter and atresia. Sheep ovaries were obtained as described above. Individual follicles were hemisected in 200 μ l Medium 199 buffered with 20 mM-Hepes (containing 50 U kanamycin/ml), centrifuged (800 *g* for 5 min) and the supernatant stored at –15°C. Before assay, samples were treated with 0.01% dextran-coated charcoal at 1 mg/ml final concentration.

The volume of follicular fluid released into the medium was accounted for when calculating inhibin content and concentration. The data were transformed into logarithms and then analysed using an unpaired *t* test and ANOVA and Duncan's test. Follicles were divided into small (< 3.5 mm) and large ($\geq$ 3.5 mm) groups, using 3.5 mm diameter as a cut-off point because of functional changes in steroidogenesis and LH receptor number at this diameter (Carson *et al.*, 1979, 1981).

Results

Experiment 1

At charcoal concentrations ≥ 0.5 mg/ml, approximately 1% of ^{125}I -labelled testosterone remained in the diluted follicular fluid of 3 individual follicles (Table 1). The inhibin potencies and slopes of the dose-response curves for 4 different follicles were not significantly altered by the charcoal concentration (0–1 mg/ml) (Table 2). At least 10-fold more inhibin activity was measured in ovine and bovine follicular fluid after charcoal treatment of 1 mg/ml compared with 10 mg/ml (Table 3).

We therefore chose a charcoal concentration of 1 mg/ml to treat follicular fluid before measuring inhibin activity.

Table 1. The percentage of ^{125}I -labelled testosterone remaining after dextran-coated charcoal treatment of follicular fluid from 3 individual ovine follicles

Follicle diam. (mm)	Stage of atresia	Charcoal conc. (mg/ml)			
		0.125	0.25	0.5	1.0
3	I	4.8	2.2	1.1	1.1
3	I	5.3	1.6	1.1	1.0
3	II	3.8	1.3	0.8	0.8

Table 2. Inhibin activity (kU/ml) in follicular fluid of ovine follicles treated with various concentrations of dextran-coated charcoal

Follicle diam. (mm)	Stage of atresia	Charcoal conc. (mg/ml)		
		0	0.5	1.0
5	II	37.9 (21.5–64.6) {18.5}	38.1 (22.6–63.0) {18.9}	33.6 (19.8–57.0) {24.0}
5	I	63.4 (48.5–83.1) {20.1}	42.8 (32.7–55.9) {15.6}	60.8 (45.2–81.9) {20.4}
5	II	35.2 (27.0–46.3) {17.8}	45.4 (35.0–59.1) {16.0}	34.3 (25.6–46.3) {25.4}
5	III	31.1 (22.3–43.2) {15.7}	18.6 (12.4–27.6) {38.5}	32.7 (24.0–44.4) {18.0}

Index of precision (λ) = -0.15 .

Values represent inhibin potency (95% confidence limits) and numbers in braces are slope values.

Experiment 2

Inhibin and follicular diameter. Inhibin content varied markedly between and within follicles of the same diameter and ranged from 3 to 1140 U/follicle (Text-fig. 2a). There was a positive relationship ($\ln y = 1.09 \ln x + 1.85$) between inhibin content (y) and follicular fluid volume (x), ($r = 0.85$; d.f. = 98; $P < 0.001$). When these follicles were distinguished as large (≥ 3.5 mm) or small (< 3.5 mm), there was a significantly higher ($P < 0.001$) inhibin content (mean $\pm$ s.e.m.) in the large (448 ± 62 U/follicle, $N = 27$) than in the small follicles (61 ± 7 U/follicle; $N = 72$).

The inhibin concentration in follicular fluid was determined only in those follicles in which diameter was measured to the nearest 0.1 mm ($N = 43$), and not those measured to the nearest 0.5 mm, since the accuracy of measuring follicle diameter is critical in calculating follicular volume and

hence inhibin concentration. Inhibin concentration varied from 2 to 29 kU/ml and increased with follicular diameter and hence fluid volume ($\ln y = 0.24 \ln x + 1.47$) ($r = 0.35$; d.f. = 42; $P < 0.05$) (Text-fig. 2b). There was no significant difference ($P > 0.05$) in inhibin concentration (mean $\pm$ s.e.m.) between small (8.2 ± 1.2 kU/ml; $N = 23$) and large (12.3 ± 1.8 kU/ml; $N = 20$) follicles.

Table 3. A comparison of two methods of charcoal treatment of ovine and bovine follicular fluid on the inhibin concentration (kU/ml) (mean, 95% confidence limits; index of precision (λ) = -0.14)

Follicle type and diameter	Charcoal conc. (mg/ml)	
	1	10
Ovine, 2.2 mm	5.3 (4.0-7.1)	$\leq 1.2^a$
Ovine, 5.8 mm	17.0 (12.7-22.9)	1.6 (1.2-2.3)
Bovine, 3.4 mm	2.7 (2.2-3.3)	$\leq 0.4^b$
Bovine, 6.4 mm	5.6 (4.6-6.8)	0.5 (0.3-0.7)

^{a,b} These values were calculated, based on the sensitivity of the inhibin bioassay (0.1 U/well) and volume of follicular fluid (highest dose of follicular fluid used in culture).

Text-fig. 2. Relationship between inhibin content (a) and inhibin concentration (b) and follicular fluid volume (and follicular diameter) of individual ovine follicles. In (a), $\ln y = 1.09 \ln x + 1.85$; $r = 0.85$, $P < 0.001$ ($N = 99$). In (b), $\ln y = 0.24 \ln x + 1.47$; $r = 0.35$, $P < 0.05$ ($N = 43$).

Inhibin activity and atresia. There were no significant differences between inhibin content, inhibin concentration or protein content in follicular fluid of small follicles in the various stages of atresia (Table 4). In large follicles (≥ 3.5 mm), inhibin content was also not significantly different in the different stages of atresia (Table 5). Furthermore, inhibin concentration in follicular fluid from follicles in stages I and II (healthy or early in atresia) and follicles in stages III, IV and V (atretic) showed no significant difference in the small (< 3.5 mm) or large (≥ 3.5 mm) size groups (Table 6).

Table 4. Inhibin content, concentration and protein content in follicular fluid of small follicles (<3.5 mm) at various stages of atresia (see text)

	Stage of atresia					F value
	I	II	III	IV	V	
Inhibin content (U/follicle)	54 ± 18 (17)	60 ± 18 (18)	65 ± 13 (16)	37 ± 7 (9)	57 ± 16 (11)	F _{4,66} = 0.32
Inhibin conc. (U/mg protein)	201 ± 51 (10)	108 ± 29 (9)	146 ± 27 (10)	112 ± 21 (8)	106 ± 17 (10)	F _{4,42} = 1.64
Protein content (µg/follicle)	346 ± 92 (10)	411 ± 110 (9)	487 ± 69 (10)	384 ± 91 (8)	494 ± 93 (10)	F _{4,42} = 0.52

Values represent mean ± s.e.m. and numbers in parentheses refer to number of follicles.

Table 5. Inhibin content (U/follicle) in follicular fluid of large (≥3.5 mm) ovine follicles at various stages of atresia

Stage of atresia						F value
I	II	III	IV	V		
315 ± 113 (6)	601 ± 301 (3)	536 ± 116 (9)	464 ± 133 (6)	311 ± 97 (4)		F _{4,23} = 0.74

Values are mean ± s.e.m. for the no. of follicles in parentheses.

Table 6. The concentration of inhibin (kU/ml) in 'non-atretic' and 'atretic' small (<3.5 mm) and large ovine follicles (≥3.5 mm)

Follicle diam.	'Non-atretic'	'Atretic'	t values
Small (<3.5 mm)	8.8 ± 1.6 (16)	6.9 ± 1.0 (7)	0.763 (d.f. = 21)
Large (≥3.5 mm)	16.2 ± 3.5 (6)	10.6 ± 2.0 (14)	1.513 (d.f. = 18)

Values are mean ± s.e.m. for the no. of follicles in parentheses.

Oestrogen production has been used as an indicator of the health of the follicles (McNatty, Moore Smith, Osathanondh & Ryan, 1979; McNatty, 1981; Carson *et al.*, 1981). When aromatase activity in the granulosa cells (pmol oestradiol-17β/10⁶ viable cells/h) and oestradiol-17β concentration of the follicular fluid were measured, inhibin concentration (kU/ml) (*y*) and content (U/follicle) (*z*) were positively correlated with aromatase activity (*x*) in large follicles ($\ln y = 0.30 \ln x + 1.54$; $r = 0.74$; d.f. = 13; $P < 0.01$; $\ln z = 0.23 \ln x + 5.30$; $r = 0.71$; d.f. = 13; $P < 0.01$, respectively) but not in small follicles ($r = 0.09$; $r = 0.20$). There was also a positive correlation between inhibin (*y*) and oestradiol-17β (*x*) concentrations in fluid of large follicles ($\ln y = 0.35 \ln x + 1.22$; $r = 0.71$; d.f. = 13; $P < 0.01$), but not in small follicles ($r = 0.46$).

Discussion

We have shown that the inhibin content of follicular fluid from individual follicles varied with follicular fluid volume (and follicular diameter) but not with the degree of atresia assessed by morphological criteria. Inhibin concentration was not related to atresia, but was correlated with follicular fluid volume (and diameter). However, aromatase activity in granulosa cells or oestradiol-17 β concentration of follicular fluid, considered good indices of atresia (McNatty, 1981; Carson *et al.*, 1981), were highly correlated with both inhibin content and concentration, but only in large follicles (≥ 3.5 mm diam.).

It is difficult to compare the inhibin levels measured by us with many other reports because most studies have been on pooled follicular fluid and there is a lack of uniformity amongst inhibin standards and sample preparation, particularly treatment with charcoal. Concentrations of charcoal in excess of 1 mg/ml, millipore filtering and freeze drying can result in considerable loss of inhibin activity (V. W. K. Lee, unpublished observations). The values reported in this paper are up to 10-fold higher than some data in our preliminary communication on ovine follicular fluid pools (Scott *et al.*, 1980a) and in bovine follicular fluid (Henderson & Franchimont, 1981). These differences in inhibin activity can be attributed in part to the differences in charcoal treatments, as shown by the results in Table 3, and the dilution of inhibin before charcoal treatment (V. W. K. Lee, unpublished observations).

Actively growing sheep and human follicles are characterized by an exponential increase in the number of granulosa cells and a concomitant increase in antral fluid volume (McNatty 1981; Tsonis & Findlay, 1981). In the later stages of growth, antral fluid volume increases at a faster rate than granulosa cell number (McNatty, 1981; C. G. Tsonis, unpublished observations). Granulosa cells have been shown to be a source of inhibin in the follicle of the rat (Erickson & Hsueh, 1978), cow (Henderson & Franchimont, 1981), sheep (C. G. Tsonis, unpublished observations) and pig (Anderson, Shander, Channing & Barraclough, 1979). Therefore, assuming little change in inhibin production per granulosa cell, inhibin content could be expected to increase with the diameter of healthy growing follicles until the LH surge and luteinization, whereas inhibin concentration in sheep follicles might not alter so dramatically because of the increase in antral fluid volume.

There have been few reports in the literature concerning inhibin and its relation to atresia. Although in our preliminary study we noted a decrease in inhibin concentration in pools of ovine follicular fluid from large follicles judged by morphological criteria to be atretic (Scott *et al.*, 1980a) we were unable to confirm this observation when individual follicles were examined. This could be due to the difference in charcoal treatments, to the variability in inhibin content between follicles or to a lack of consistency in assessing atresia by morphological criteria. Because aromatase of granulosa cells is an early casualty in the atretic process (McNatty *et al.*, 1979; McNatty, 1981), measurement of this activity or its product, oestradiol-17 β in follicular fluid, has been promoted as a marker of atresia (McNatty, 1981). Channing *et al.* (1981) noted that inhibin concentrations in follicular fluid of individual human follicles decreased with the oestrogen/androgen ratio. We noted a similar change in inhibin content and concentration with both oestradiol-17 β concentration in follicular fluid and aromatase activity in granulosa cells, but only in large (≥ 3.5 mm) follicles. The lack of a similar relationship in small follicles could have been due to the fact that healthy, growing small follicles can be 'androgenic' because of relatively low aromatase activity and hence oestradiol content (Moor, Hay, Dott & Cran, 1978; Carson *et al.*, 1981). Therefore the use of details of oestrogen production for assessing follicular atresia may not be applicable to these small follicles. Channing *et al.* (1981) also failed to observe a correlation of oestrogen and inhibin content in follicular fluid of small human follicles. Resolution of this problem awaits estimates of inhibin production by granulosa cells of small atretic and non-atretic follicles.

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