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Inhibin A and B secretion in mouse preantral follicle culture

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Conditioned media from single mouse ovarian follicles cultured from the early preantral stage up to complete maturity were analysed for different immunoreactive inhibin forms. The inhibin assays measured (i) α-specific inhibin, as represented by a mix of 32 and and 57 kDa inhibins, inhibin precursors, α-subunit and its precursors; (ii) dimeric inhibin A; and (iii) dimeric inhibin B. The validity of these assays for the measurement of mouse inhibin was established. All forms of inhibin were secreted in culture media from the preantral follicle stage onwards. Inhibin B was the most sensitive marker for proliferation of early stage follicles, while inhibin A secretion became predominant at later stages, when antral-like cavities were formed in granulosa cell masses. Supplementation of standard culture medium with recombinant follicle stimulating hormone appeared to be the predominant regulator of inhibin secretion; addition of recombinant luteinizing hormone throughout the culture period did not cause any major shifts in the expression of dimeric inhibin or α-specific inhibin forms. In the absence of theca cells during isolation and culture (as reflected by absence of oestrogen secretion), follicles grew at a reduced rate, and produced lower inhibin concentration in conditioned medium. These data suggest (i) that monitoring of dimeric inhibins can provide useful markers of the growth and differentiation of cultured follicles and (ii) that dimeric inhibins A and B are secreted at an earlier stage *in vitro* than *in vivo*.

Key words: gonadotrophins/granulosa cell/mouse/oocyte/ovary

Introduction

Inhibins and activins form a complex mixture of intraovarian regulators together with other growth factors with which they share significant structural homologies of the β-subunits. These latter include transforming growth factor-β (TGFβ) and Müllerian inhibiting substance (MIS). Inhibins are dimers composed of an α-subunit associated with one of two β-chains (A or B), whereas activins are homo/heterodimers of two closely related β-subunits (Vale *et al*., 1990).

In prepubertal mice, inhibin α-subunit transcripts were present in ovaries from 13 day old animals, but not in 3 day old mice, suggesting that accumulation of the transcripts begins in stages immediately preceding antrum formation (Tone *et al*., 1990). The inhibin α-subunit gene was found to be expressed in mouse ovaries at all stages of maturation, including primary to tertiary follicles. In rats, Woodruff *et al*. (1988) found only very few transcripts in granulosa cells of preantral follicle stages. Inhibin α-subunit mRNA was also reported in thecal and stromal cells in rat ovaries (Jih *et al*., 1993). Inhibin β_A-subunit mRNA was not expressed until the follicles reached the antral stage (Meunier *et al*., 1988). Follicular atresia decreased β_A- and β_B-subunit expression, but α-subunit mRNA remained present. Earlier studies have shown the expression and the basal and pregnant mare's serum gonadotrophin (PMSG)-stimulated secretion of bioactive and immunoreactive inhibin in immature (Rivier and Vale, 1987) adult rats (Hasegawa *et al*., 1989). During follicular development, both α- inhibin and β_A-inhibin are specifically expressed in granulosa cells of developing rat follicles (Woodruff *et al*., 1988, 1990).

Inhibin is present in the circulation and in follicular fluid in a variety of molecular forms.

The combination of monoclonal antibodies raised to synthetic peptide sequences of each subunit within the format of two-site enzyme-linked immunosorbent assays (ELISA) and a profound sample pretreatment procedure using methionine oxidation and sodium dodecyl sulphate heat treatment at 100°C provided the required sensitivity and specificity to assess the different molecular forms in present biological fluids (Knight and Muttukrishna, 1994; Groome *et al*., 1996).

Using more specific immunometric assays for total α-inhibin and inhibin A ($\alpha\beta_A$-dimer) developed by Groome (1991), secretion of both substances was detected in serum of immature female rats in response to PMSG injection. These new reagents have also been used to confirm the relevance of inhibin A in the gonadotrophic control of ovarian function (Fahy *et al*., 1995).

Inhibin secretion in the rat ovary is under positive control of follicle stimulating hormone (FSH) and luteinizing hormone (LH) via cAMP mediation (Bicsak *et al*., 1986). Following luteinization, the concentration of inhibin mRNA remains low in the newly formed corpus luteum (Meunier *et al*., 1988).

The role of inhibin in mediating local ovarian events has been documented. Woodruff *et al*. (1993) described specific binding sites on granulosa and thecal cells in rats, supporting an autocrine/paracrine role for inhibins. An autocrine action of inhibin on oestradiol production by rat granulosa cells has been described (Ying *et al*., 1986). Inhibin exerts paracrine effects by a strong stimulatory effect on LH-induced androgen production by rat theca cells (Hillier *et al*., 1991). Finally the presence of inhibin forms might play a physiological role in regulating oocyte maturation. O *et al*. (1989) observed inhibin-

mediated inhibition of meiotic maturation by suppression of germinal vesicle breakdown (GVBD) in rat oocytes, while activin A was reported to accelerate GVBD in immature rat oocytes *in vitro* (Itoh *et al*., 1990). In follicle culture, the balance between activins and inhibins might play an important role in determining oocyte maturity. Studies therefore should be carried out using the more specific antibodies recently made available.

Concentrations of inhibin B dimer, specifically secreted during the early stages of follicular growth, have not yet been reported from the early preantral to antral stages. The availability of a specific $\alpha\beta_B$-inhibin dimer assay opens new possibilities of studying the regulatory function of this inhibin form (Groome and O'Brien, 1993). Although the human inhibin A and B assays had been successfully used in rats (Fahy *et al*., 1995; Woodruff *et al*., 1996) we have also validated these assay formats for use on conditioned media from mouse preantral follicle cultures (Cortvrindt *et al*., 1996).

Our aim was to study the secretion profile of the inhibin forms during in-vitro follicle culture under the influence of different gonadotrophins using specific monoclonal ELISA.

Materials and methods

Mice

F1 hybrids (C57 Bl×CBAca), housed and bred according to the national standards for animal care, were used after cervical dislocation. For these experiments approval was obtained from the Ethical Committee of our Institution.

Isolation of tissues

Ovaries from immature (14 day old) mice were removed immediately after death, in sterile conditions, and were dissected under a binocular microscope. Only primary follicles belonging to the class 3b, based on the Pedersen classification (Pedersen and Peters, 1968), with a mean diameter of 100–130 μm (as measured under the inverted microscope using an eyepiece graticule) were considered for in-vitro culture under standardized conditions as previously described (Cortvrindt *et al*., 1996).

Culture media and physical conditions

Follicles were cultured for 12 days in either 20 μl culture droplets covered by mineral oil in 60 mm culture plates or in a 50 μl culture volume in a 96-well half-Area (A/2) plate (Corning, Costar, Acton, MA, USA) covered by 30 μl of oil. The conditioned medium was sampled every 2 days (plates) or 3 days (wells).

The culture medium consisted of αMEM supplemented by transferrin, insulin, recombinant FSH (Cortvrindt *et al*., 1997) and/or recombinant LH (see experimental set-up for further details) and 5% heat-inactivated fetal calf serum (FCS). Follicles were cultured at 37°C, 100% humidification in 5% CO_2 in air.

Sampling procedure

Each 20 μl plate culture droplet contained a single follicle. 10 μl of medium was removed from each droplet on days 4, 6, 8, 10 and 12. The aliquots from one plate in which all follicles were cultured under similar conditions were pooled providing that the follicle showed a normal growth pattern as described previously (Cortvrindt *et al*., 1996). When follicles were cultured in 50 μl wells, 20 μl of conditioned media was removed on days 3, 6, 9 and 12, 10 μl of which was immediately diluted in 50 μl inhibin-free serum (Biosource, Fleurus, Belgium). This enabled quantification of the secretory products of a single follicle throughout its entire growth phase under well defined conditions. Media samples were stored immediately at –20°C for not longer than 2 months and all samples were thawed once and assayed using the same batch of reagents.

Culture characterization procedures

The plated follicles were assayed on day 1 after isolation under an inverted microscope (Nikon, Tokyo, Japan) at a magnification ×400 to ascertain follicular intactness, presence of theca cells and to measure the diameter of follicle and oocyte. Differentiation and growth were recorded on each day of medium sampling from day 1 up to day 12 of culture, using a classification procedure described previously (Cortvrindt *et al*., 1996).

Assays

17β-Oestradiol and progesterone concentrations were measured in the conditioned media using radioimmunoassays which were validated as described previously (Cortvrindt *et al*., 1996).

Inhibin was measured using an α-specific assay EASIA from Biosource (ex-Medgenix, Fleurus, Belgium). This assay is not specific for inhibin dimer but cross-reacts with 32 and 57 kDa inhibin, possibly other inhibin precursors, and α-subunit and its precursors. Samples were not pretreated but were assayed after dilution (1/121 for media up to day 6 and 1/451 for media from day 8 to day 12 of culture) in inhibin-free serum (Biosource, Fleurus, Belgium). The sensitivity of this assay as claimed by the manufacturer was ~40 pg/ml and intra- and inter-run coefficients of variation (CV) were respectively <3% and <9%. The standards were purified from human follicular fluid and 1 Medgenix Unit (MU) is equivalent to 400 pg of recombinant-$\alpha\beta_A$ preparation from Genentech (CA, USA).

The assays for inhibin A and inhibin B dimers were purchased from Serotec Ltd (Oxford, UK). Media samples were pretreated according to the manufacturer's instructions and following the advice of Professor Nigel Groome (Oxford Brookes University, Oxford, UK). Briefly, medium was diluted in FCS according to the amount of inhibin present. From this dilution, 100 μl was added to 50 μl of an SDS solution (6%) and boiled for 3 min. Subsequently the H_2O_2 solution (6%) was added and the mixture was incubated for 30 min. 80 μl of this was then used for each ELISA assay. The initial concentration of inhibins in each sample was calculated by considering the dilution which yielded a result located in the most precise regions of the standard curves. The analytical sensitivities for inhibin A and B kits were <10 pg/ml and <15 pg/ml respectively; the precision was <7% for both methods.

Both assays were validated for use in this mouse follicle culture system. Firstly, the culture medium supplemented by 5% FCS was measured for its content of both inhibin forms, but provided signals below the detection limit of the method. The 'inhibin-free' diluent (Biosource) used to dilute the 10 μl samples collected from individual culture wells also did not contain any significant amounts of inhibin A or B.

Suitability of the Serotec assays for measuring mouse inhibin A and B dimers was investigated, since the standard materials used in both kits were extracts from human follicular fluid which were calibrated against recombinant human 32 kDa inhibin A and B forms. As previously reported (McConnell *et al*., 1996) the antibodies in the Serotec kits were able to detect bovine, ovine, porcine, mouse and rat inhibin A in serum. These dose-response curves were obtained from different media collected from individual mouse follicles on day 12 of the culture period, at which inhibin concentrations were expected to be high. The samples were serially diluted in FCS (from

the same batch as that used in culture) to obtain dilutions ranging from 1/8 to 1/775 and inhibin concentrations were measured with both ELISA.

Calibration-curve parameters were calculated using the four-parameter logistic function. Parallelism of the dilution curves was evaluated by analysis of variance (*F*-test). Assay curves were judged parallel if the *P* value for the test of the sum of squares associated with parallelism was > 0.05.

Experimental set-up

Two different set-ups were used to study inhibin production and regulation throughout culture.

(i) Follicles were cultured individually in 50 µl volume wells. Forty follicles completed a 12 day culture period by releasing a mature oocyte and, of these, 11 were selected for longitudinal inhibin A and B measurement, on culture days 3, 6, 9 and 12. As the oestradiol concentrations of these follicles were known, five follicles were selected with a clear theca-cell layer which demonstrated oestradiol production and six follicles were selected with no visualized theca or oestradiol production. The aim of this was to compare total α-specific inhibin, inhibin A and B secretion profiles for the complete in-vitro maturation cycle.

(ii) Follicles were cultured individually in 20 µl volume droplets at 20 droplets per culture dish. The pooled aliquots obtained per plate

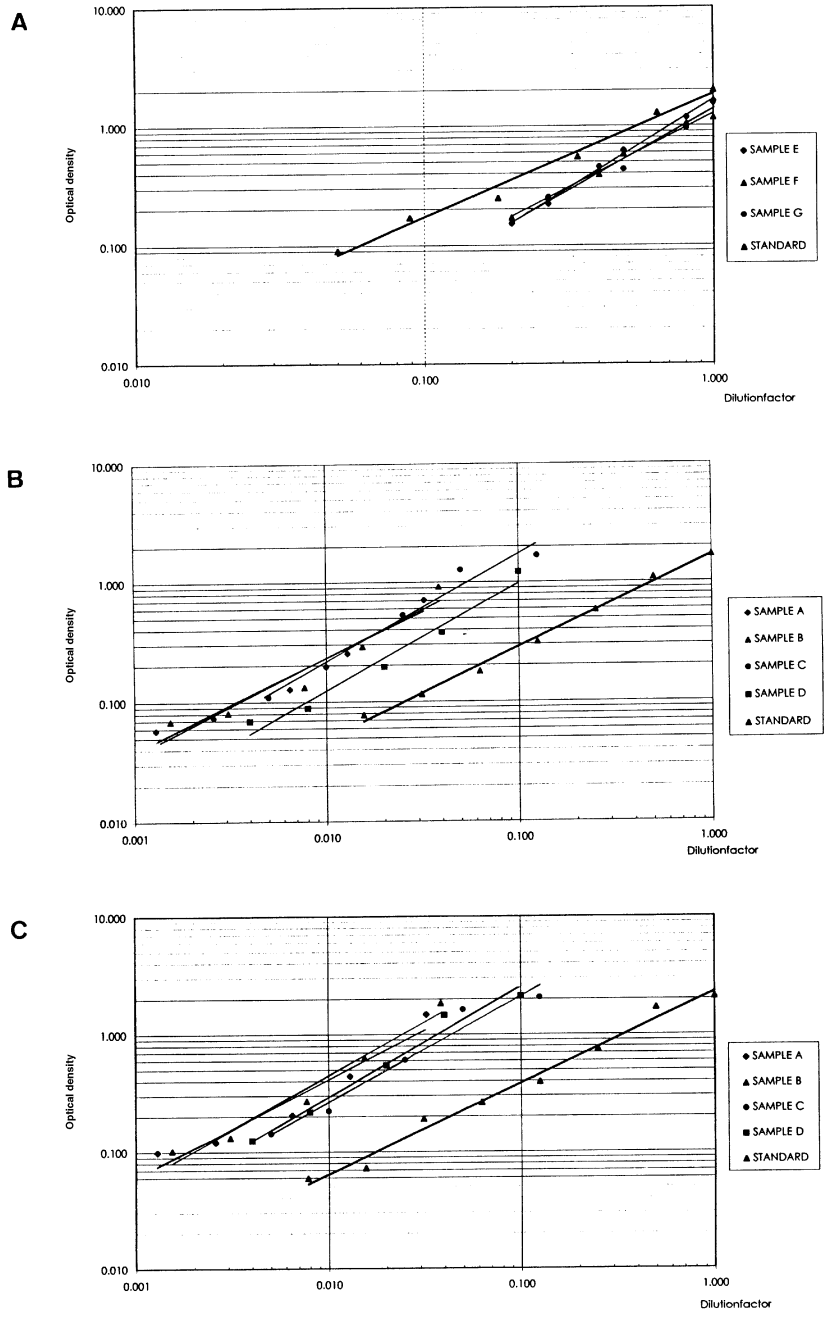

Figure 1. Serial dilutions of different samples (A–G) of mouse follicle culture medium were compared with a human standard preparation. The optical density (*y*-axis) is plotted against the dilution factor (*x*-axis). (**A**) α-Specific inhibin assay. (**B**) Inhibin B dimer assay. (**C**) Inhibin A dimer assay.

from culture days 4, 6, 8, 10 and 12 were analysed for total α-specific inhibin, inhibin A and B. The effect of gonadotrophin supplementation on production of the three inhibin forms was also examined: (a) FSH (one plate), (b) LH (two plates), (c) FSH + LH (two plates).

Recombinant FSH (Gonal-F®; Ares-Serono, Geneva, Switzerland) was used at a final concentration of 100 mIU/ml and recombinant LH (L-HADI®; Ares-Serono) at a final concentration of 100 mIU/ml. These final gonadotrophin concentrations were continuously provided during the entire 12 day culture period. All gonadotrophin-containing media were freshly prepared at the start of the experiment, kept at 4°C and aliquots for replenishments (every 2 days) were carefully pre-equilibrated at 37°C in the gassed incubator.

Results

Specificity of the different inhibin assays for mouse inhibin

As shown in Figure 1 dilutions of the conditioned media from mouse follicle culture resulted in log dose–log response points whose regression lines were parallel to the standard curve obtained using inhibin extracted from human follicular fluid. The slopes of the power regression lines varied between 0.84 and 0.94 for inhibin A; 0.78 and 0.90 for inhibin B and 1.24 and 1.48 for α-specific inhibin. The correlation coefficients (r^2) of the regressions ranged from 0.92 to 0.99.

All samples were assayed at two dilutions (1/10 and 1/40), so that results could be recalculated from the response closest to the ED_{50}. Results from mouse follicle culture media are expressed in pg/ml of human inhibin for inhibin dimers and in Medgenix Units for α-specific inhibin.

Conditioned media (n = 34) were also analysed at two dilutions (1/10 and 1/40) for each sample for α-specific inhibin, inhibin A and inhibin B. Figure 2 illustrates the relationship between these parameters, using the Passing–Bablok regression lines (Passing and Bablok, 1983). Linear regression was performed after logarithmic transformation of the data: the correlation coefficient was 0.97 between α-specific inhibin A and the equation: log inhibin A = 1.56 log α-inhibin – 0.84. The relation between inhibin B dimer and α-specific inhibin was log inhibin B = 1.15 log α-inhibin + 0.60 and r = 0.89. Inhibin dimer concentrations were at least ~10 times lower than the inhibin concentrations detected by the less specific α-inhibin assay.

Inhibin A and B dimer secretion by single preantral follicles during in-vitro culture

Follicles were selected for culture on the basis of follicular diameter (100–130 μm) and number of cuboidal granulosa cell layers (two). However, follicle growth within this class was different for follicles smaller or larger than 115 μm. The smallest follicles proliferated more slowly under the present culture conditions and did not completely differentiate into antral follicles (Figure 3).

Despite the fact that we aimed to plate follicles with some theca/interstitial cells attached, some cultures appeared to be theca-free when inspected on day 1. Absence of theca cells was confirmed *a posteriori* by the absence of oestrogen output by these follicles. Granulosa cells from theca-free follicles proliferated normally though at a slower rate and all follicles

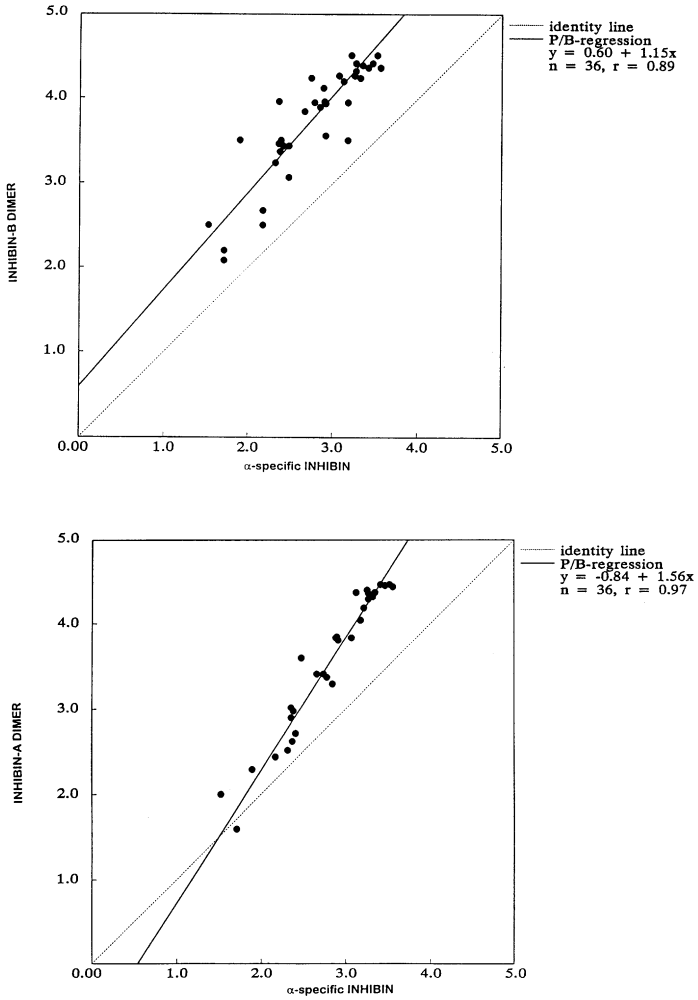

Figure 2. Linear regression performed on results obtained from measurement of inhibin B dimer and α-specific inhibin, $y = 1.15x + 0.60$, $r = 0.89$ (top panel), and on inhibin A dimer and α-specific inhibin, $y = 1.56x - 0.84$, $r = 0.97$ (lower panel).

selected for analysis of their conditioned media had fully grown oocytes which resumed meiosis under an HCG stimulus given on day 12. This reduced growth rate was reflected by lower inhibin A and B concentrations at different time points during culture. The general proportion of both inhibin forms was not changed.

As can be seen in Figure 4, inhibin B was present at concentrations well above the sensitivity of the assay in the culture media of those class 3b follicles which were >115 μm diameter at the start of culture. Follicles ⩽115 μm gave rise to dimeric inhibin B values just above the level of sensitivity of the assay. Inhibin A was present in conditioned media from the first 3 day culture period at a concentration of 20–85 pg/ml which appeared to be unrelated to the initial follicular diameter. The concentration of both inhibin dimers measured in the medium of the eleven follicles which were monitored every 3 days increased as culture progressed. When the ratio of inhibin B to inhibin A was examined it became clear that, as growth and differentiation progressed, inhibin A was preferentially secreted towards the end of culture (Figure 4, bottom panel). Figure 5 shows that for small follicles

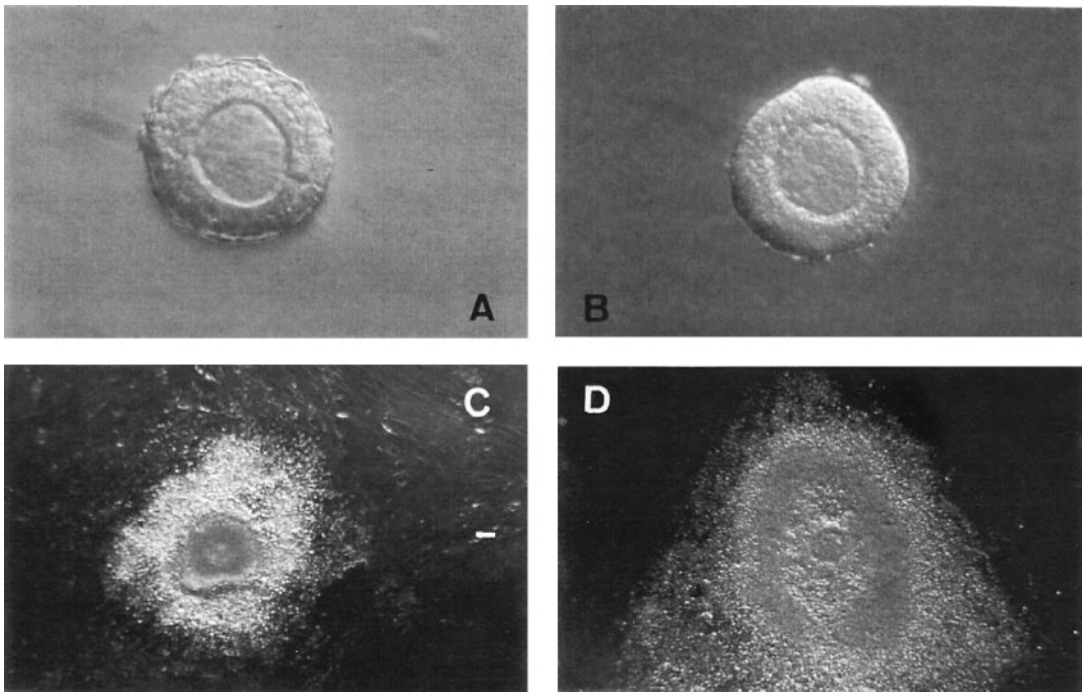

Figure 3. Follicles on day 1 of culture (original magnification ×200). (**A**) Early preantral follicle containing a theca cell layer. (**B**) Early preantral follicle without theca cells attached. Follicles on day 12 of culture (original magnification ×50). (**C**) Follicle which has not completely differentiated. (**D**) Follicle with completely differentiated granulosa cells and large antral cavity.

(⩽115 μm) the increase in inhibin B concentration was much more prominent than that of inhibin A. Results were presented for both inhibin forms as ratios (inhibin B/inhibin A) of the increases from the previous sample day, e.g. ratio day 6/ratio day 3. This calculation was performed for days 6, 9 and 12. For small follicles the inhibin B increase on day 6 was between 504 and 1346% of the value of day 3, while the increase for inhibin A dimer in the same samples was between 10 and 222%.

Follicles which formed clear antral-like cavities from day 9 onwards demonstrated a further increase in inhibin B concentration by a factor of 2–3, while those that remained less differentiated (see Figure 3) showed an increased value by a factor of 3–5.

In contrast to inhibin B, the concentration of inhibin A values showed its largest increase in follicles which commenced antral-like cavity formation between days 6 and 9: concentrations on day 9 had increased by between 579 and 1899%. During the last 3 days of culture, inhibin A concentrations continued to increase by a factor of 3–6 in antral-like follicles, while inhibin B concentrations rose by a factor of only 2–3. Thus although concentrations of both dimeric forms increased with progressing culture time, the inhibin B increase was greater in small and undifferentiated follicles, while inhibin A increases were more pronounced in larger follicles.

Secretion profiles of inhibin forms in response to different gonadotrophin conditions

Samples were obtained only from the medium of those follicles demonstrating granulosa cell growth. Follicles cultured in medium with no gonadotrophin supplement did not survive the 12-day culture period; during the first days there were no signs of growth and after day 6 the follicles disintegrated and showed morphological signs of atresia. The corresponding media had α-inhibin concentrations ⩽15 MU/l throughout the culture period. Cultures supplemented with 100 mIU/ml FSH (combined or not with 100 mIU/ml LH) demonstrated the highest inhibin concentrations over the entire 12 day culture period. All inhibin forms showed a similar trend: at day 10–12 a plateau was reached. In the absence of FSH in the culture medium (containing only LH), continued follicle growth was observed in at least half of the population of follicles. In this condition, the number of mitoses was reduced and the follicles maintained their intact 'follicular' shape much longer. Therefore cultures supplemented with only 100 mIU/ml LH had much lower specific inhibin dimer concentrations on days 4 and 6, but tended to catch up on day 8. Inhibin A concentrations in 'LH only'-supplemented cultures showed continuously increasing values up to culture day 12.

Discussion

Up to now, regulation of inhibin synthesis and secretion by granulosa cells has been studied using in-situ hybridization and fairly non-specific immunoassays (see editorial by Bhasin and de Kretser, 1996). Recently, Groome *et al*. (1993) have generated a series of specific monoclonal antibodies enabling the establishment of sandwich assays which now permit the reliable and specific measurement of inhibin A and inhibin B. Inhibin production by in-vivo murine granulosa cells is dependent upon their stage of cytodifferentiation. The factors which stimulate inhibin production are androgens, oestrogens, prostaglandins, transforming growth factor-β (TGFβ) and insulin-like growth factor-I (IGF-I) (Zhiwen *et al*., 1987; Lapolt *et al*., 1989; Erämaa and Ritvos, 1996); those which inhibit

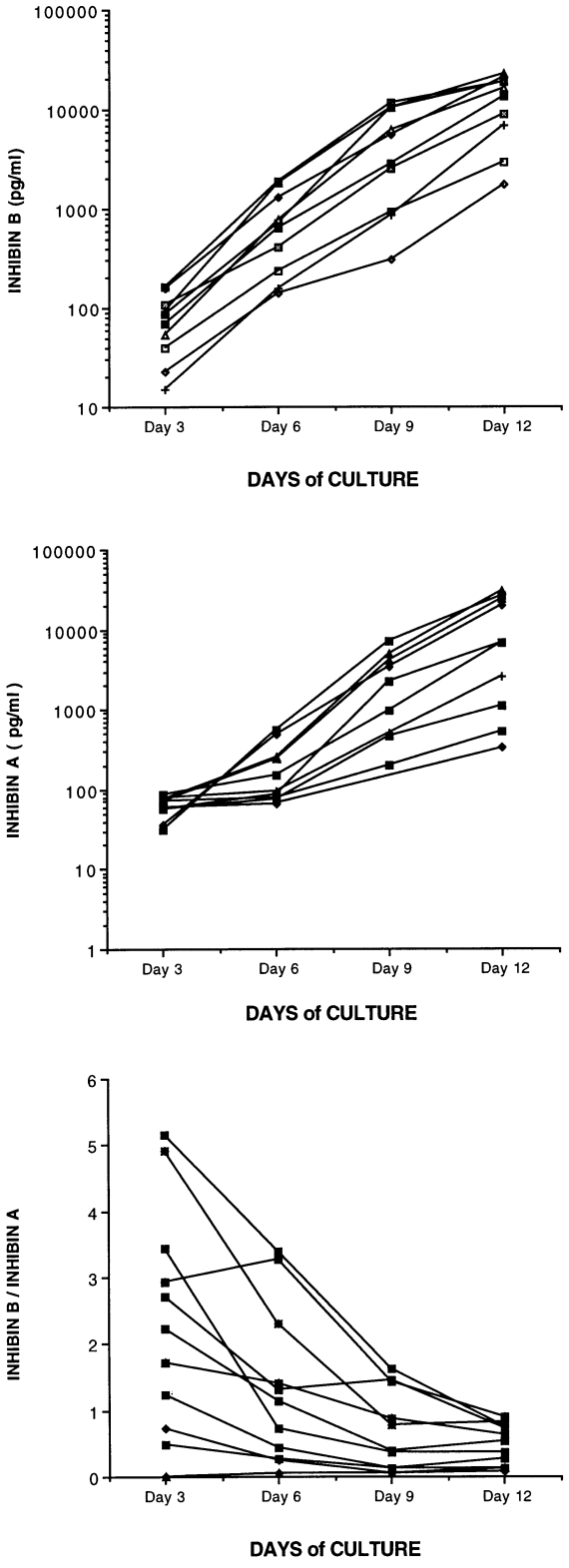

Figure 4. The concentrations of inhibin A dimers and inhibin B dimers in the media from 11 follicles cultured for 12 days.

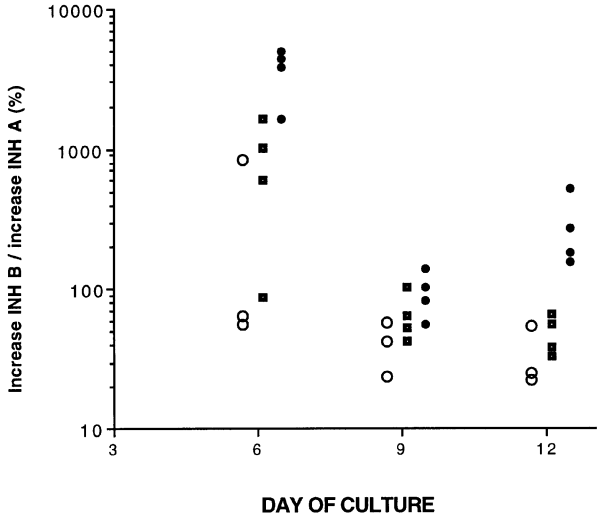

Figure 5. Ratio of the increase in inhibin (INH) B to inhibin A dimers as a percentage of the concentration on the previous sampling day. An example of the calculation for day 6: [(conc. INHB day 6 – conc. INHB day 3)/conc. INHB day 3] $\times 100 =$ Δ INHB (%). The same calculation was performed for Δ INHA (%). The points plotted for each follicle are Δ INHB/Δ INHA. Closed circles (●) give the values for the smallest follicles ($\leq$115 μm) at plating and which did not become antral. Squares (■) represent values from follicles ($>$115 μm) which did not form antral-like cavities. Open circles (○) are from follicles ($>$115 μm) which formed antral-like cavities from day 9 onwards.

inhibin production include epidermal growth factor (EGF) (Zhang *et al*., 1987; Lapolt *et al*., 1990).

In addition to their well established role at the pituitary level (inhibition of FSH synthesis), inhibins have less well defined paracrine and autocrine roles. It was demonstrated in rats that inhibin produced by the granulosa cells interacted at the theca-cell level with LH, IGF-I and possibly also with other locally produced factors which regulate theca-cell-derived aromatizable androgens (Hillier *et al*., 1991). Experiments on isolated preantral rat follicles indicated that granulosa-derived inhibin promoted thecal androgen synthesis and, consequently, oestrogen synthesis (Smyth *et al*., 1994). An autocrine action by inhibin was suggested by experiments in primates which demonstrated its ability to counteract activin stimulation of FSH-induced aromatase (Miro and Hillier, 1992). The observed secretion patterns of inhibin A and B in this granulosa-cell-enclosed oocyte culture might be relevant, as experimental evidence suggests that an earlier or more intense expression of inhibin-β subunit may be linked to progressive growth during early preantral stages. Inhibin B might thus be an important marker in selecting recruitable follicles, in monitoring the initial growth phase in early-stage follicles and in optimizing culture medium composition for small follicles. The inhibin β_A-subunit, being expressed predominantly in later-stage follicles (antral stages), may be a good marker for adequate differentiation of cultured follicular units.

Characterization of the secretion pattern and the regulation of inhibin forms under defined culture conditions might be relevant to the manipulation of the follicular environmental 'climate'. By modifying culture conditions, the complex interplay between inhibin, activin and follistatin may be driven in the direction of either oestrogenization or luteinization, depending on which is optimal for the oocyte.

A first step in this project used an inhibin sandwich assay to measure a mix of 32 and 57 kDa inhibin and inhibin α-subunit, and their precursor forms (Cortvrindt *et al*., 1996).

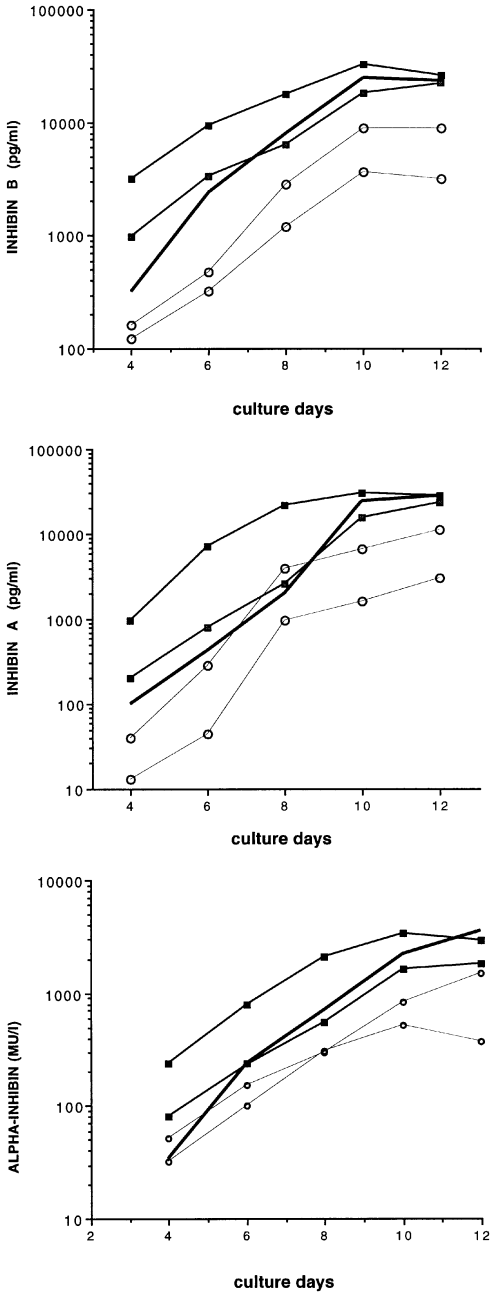

Figure 6. Increases in inhibin A and B dimer and α-inhibin in cultures treated with gonadotrophin supplements [follicle stimulating hormone (FSH): line without symbols; luteinizing hormone (LH): open circles; FSH plus LH: closed squares].

With this rather non-specific assay, we demonstrated increasing inhibin concentrations which paralleled granulosa cell proliferation *in vitro*. Maximum production of inhibin was reached between days 10 and 12 of culture, i.e. at the time at which the granulosa mass reached its maximum number of cells and terminal differentiation. Although this assay was a useful tool in monitoring granulosa cell proliferation *in vitro*, its relative insensitivity restricted its usefulness to monitor the earliest growth phase of cultured follicles, before day 6. Furthermore, it may be questioned whether this assay, which cross-reacts with inactive inhibin forms, can give us valuable information on the fine regulation of the bioactive dimeric inhibin forms.

The advantages of assaying specific dimeric inhibin A and B forms were evaluated after determining the adequacy of the assays to measure mouse inhibin in follicle culture medium. Previously, these assays had been validated for mouse serum inhibin measurements (Kananen *et al*., 1996). Parallelism of response curves after dilution of conditioned media from different single-follicle cultures indicated that these specific assays could be used in our mouse follicle culture experiments.

Individual two-granulosa-cell-layered follicles isolated from unstimulated ovaries were cultured in a small volume of medium (20 µl in Petri dishes or 50 µl in individual wells of surface-reduced microtitre plates). Hence, it was possible to measure reliably both inhibin forms in growing follicles from day 3 of culture onwards using Professor Groome's very sensitive ELISA. At this culture time the absolute number of granulosa cells per follicle ranged from 400 to 1000 cells. Production of inhibin A and B by single follicles was monitored longitudinally. During the initial culture period, up to day 6, inhibin B values increased more dramatically than inhibin A values in follicles which had been preselected for normal growth. During growth, inhibin B as well as inhibin A values continued to rise, but as differentiation increased (antral-like cavity formation and morphological signs of luteinization of follicle cells), the rise of inhibin A became more pronounced. These observations demonstrate for the first time that, in an in-vitro follicle culture system, secretion of the two bioactive inhibin forms is initiated from the preantral stages.

Meunier *et al*. (1988) reported the very early expression of the inhibin α-subunit, but no expression of the β_A- or β_B-subunit prior to antral cavity formation in rat follicle maturation *in vivo*. Woodruff *et al*. (1988) also reported only very few transcripts of the inhibin α-subunit gene in granulosa cells of preantral-follicle stages in rat ovaries, while the inhibin α-subunit gene was expressed in mouse follicles from the primary to the tertiary stage.

Measurements of inhibin A and B dimer mass in conditioned media suggested differences in regulation of inhibin expression *in vitro* compared to *in vivo*, as from the very early (preantral) stage onwards the two dimeric forms were secreted in considerable quantities. There was concordance with in-vivo data in terms of the differential expression of the two dimeric forms. The presence of stimulatory factors in the culture medium or removal of the follicle unit from an inhibitory intra-ovarian influence might precipitate inhibin gene expression.

The regulation of inhibin B seemed to be largely uninfluenced by the presence of thecal cells within the culture droplet. Follicles plated without theca cells (as visualized under the inverted microscope) demonstrated no oestrogen production but showed a progressive increase in inhibin B and A concentration in the conditioned medium. In theca-free follicles, inhibin A production throughout the culture period remained lower than in 'intact' follicles, reflecting a decreased differentiation towards the end of culture. Although oocyte morphology and fertilizing capacity were apparently not affected in theca-free unoestrogenized follicles, results are still too preliminary for us to make hard statements on this issue. It is well established that theca-cell-derived substances in addition to androgen precursors can enhance granulosa-cell growth and differenti-

ation, and inhibins might in their turn play an important role as paracrine stimulation factors on the theca cells (Hillier *et al*., 1991; Smyth *et al*., 1994).

As shown by Woodruff *et al*. (1989), follicles on their way to becoming atretic express little or no inhibin mRNA levels. Perhaps these proteins could also be used as early markers for follicular 'health' during in-vitro growth, but this aspect was not investigated in this study and requires further experimental work. This hypothesis has recently been tested in infertile women who were superovulated prior to IVF or ICSI (Seifer *et al*., 1997). The patients with a low inhibin B concentration on cycle day 3 demonstrated a poor folliculogenesis and were less likely to conceive a clinical pregnancy. This study suggested that reduced inhibin B concentrations might reflect both a decreased follicle number and quality.

Effects of recombinant gonadotrophin supplements on the synthesis of the different inhibin forms was analysed in pools of media which were obtained from individual follicles exposed to a similar gonadotrophin within one culture dish. Presence of FSH from the start of culture induced a large secretion of all inhibin forms. LH on its own did not enhance granulosa cell mitoses directly from the moment of its addition, but was a survival factor, probably through its binding to thecal LH receptors. Inhibin secretion from rLH-supplemented cultures increased more slowly, but the final differentiation of the granulosa cells was probably not hampered since in both gonadotrophin supplementation conditions a similar proportion of all the inhibin secreted was dimeric inhibin A. There was no relationship between oestradiol and/or progesterone concentrations secreted in conditioned medium and either of the inhibin forms.

In conclusion, dimeric inhibin A and B are secreted by cultured early preantral mouse follicles. Inhibin B is the most sensitive marker of granulosa cell growth in the early follicular preantral stages. Towards the end of the culture period, when antral-like cavities form, inhibin A secretion predominates over that of inhibin B. These results suggest earlier expression of dimeric inhibins when follicles are grown outside the ovary. FSH is the predominant regulator of inhibin secretion *in vitro*. The ratio of inhibin A to inhibin B mainly reflects follicular differentiation of granulosa cells. Addition of rLH to follicle cultures had no great impact on inhibin secretion patterns. Ratios of inhibin A to inhibin B in conditioned medium from follicles reflected granulosa cell proliferation and differentiation.

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