The cultured rodent follicle as a model for investigations of gonadotrophin surge-attenuating factor (GnSAF) production

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Abstract

Gonadotrophin surge-attenuating factor (GnSAF) bioactivity (the suppression of GnRH-induced but not basal LH and FSH secretion from pituitary gonadotrophs) is produced by granulosa cells *in vitro*. Previous studies to investigate this bioactivity used dispersed granulosa cells which lack some cell types and the structural components of the follicle *in vivo*. The aim of this study, therefore, was to investigate whether intact rodent follicle culture was a suitable model for the study of the production of GnSAF bioactivity, allowing GnSAF to be investigated in a more physiologically realistic environment while still retaining culture conditions from which, as with granulosa cell cultures, extraneous factors can be excluded. Follicles from 16-day-old rats and 21-day-old mice were cultured for 3–6 days in the presence or absence of FSH and/or LH. The follicle-conditioned medium, and matching samples of unconditioned culture medium were added to our established rat pituitary monolayer GnSAF bioassay. Both mouse and rat intact follicles produced GnSAF bioactivity, reducing GnRH-induced LH secretion significantly. GnSAF output from the mouse follicles was highest during days 1–3 of culture, when follicles were at an early antral stage of development, and fell on days 4–6 as the follicles grew to the mid antral stage. While the stimulatory effects of FSH on rat follicle GnSAF secretion was dose-dependent, LH alone did not increase GnSAF production. An antibody against human GnSAF blocked GnSAF bioactivity produced by rat follicles, and recognised proteins within the expected pI and molecular weight range for GnSAF in two-dimensional gels of rat follicle-conditioned medium, showing a good homology between rodent and human GnSAF proteins. In conclusion, the release of GnSAF bioactivity is principally from small follicles stimulated by FSH. Therefore, intact rodent follicle culture systems offer an excellent model for the investigation of factors controlling GnSAF production under relatively physiological conditions.

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Introduction

The putative ovarian hormone, gonadotrophin surge-attenuating factor (GnSAF) has the specific biological action of reducing pituitary secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in response to gonadotrophin-releasing hormone (GnRH). However, the factor remains to be conclusively identified (Fowler *et al.* 2003). GnSAF is secreted by granulosa cells (Fowler *et al.* 2002) and there is a large body of data demonstrating that FSH administration stimulates GnSAF production both *in vivo* (Messinis *et al.* 1993, 1994, Fowler & Price 1997) and *in vitro* (Fowler & Mason 2000). However, there are a number of concerns about both kinds of data. FSH treatment *in vivo* stimulates a considerable number of protein and gene changes within the ovary. Such changes in expression levels (e.g. in oestradiol and inhibin-B) can, in turn, exert effects at the level of the hypothalamus and pituitary, making it difficult to

determine whether any effect of FSH on GnSAF is direct or indirect. On the other hand, FSH administration to cultured granulosa cells, while excluding the possibility of indirect effects, stimulates protein synthesis in the absence of normal structural relationships between granulosa cells and between granulosa, oocyte and thecal cells. There is the potential, therefore, for the response of cultured granulosa cells to deviate from the normal response to such stimulation *in vivo*.

Two publications have questioned a direct link between FSH and GnSAF. In the first, Tio *et al.* (1998) immunoneutralised FSH in female rats and concluded that there was no difference in circulating GnSAF. In the second, Schuiling *et al.* (1999) found that FSH treatment in female rats suppressed oestradiol-augmented LH responses to GnRH without affecting GnRH self-priming, a definite aspect of GnSAF from *in vitro* pituitary models (Fowler & Templeton 1996). In both of these studies the interpretation of the

data is complex and debatable, due to multiple factors operating *in vivo*. What is required is an analysis of FSH actions on the production of GnSAF from a model system demonstrating normal function *in vitro*. The intact rodent follicle model (Spears *et al.* 1994, McGee *et al.* 2001, Murray *et al.* 2001, Spears *et al.* 2002) retains the cell–cell interactions and structural framework within which the granulosa cell normally functions.

The aim of this study was to investigate the relationship between follicle development, gonadotrophin exposure and GnSAF production in intact rodent follicle *in vitro* models. The mouse follicle model was initially investigated because follicular growth can be maintained to the late antral stage, allowing the production of GnSAF from both pre-antral and antral follicles to be investigated. However, this necessitates the presence of mouse serum in the culture medium which unfortunately renders it impossible to utilise a defined medium. Thus, the pre-antral rat follicle model was used to investigate gonadotrophin regulation of GnSAF production under highly controlled conditions. The fact that there are far fewer contaminating proteins in the culture medium makes the rat follicle culture model more suitable for the investigation of the regulation of GnSAF production.

Materials and Methods

Animals

Sprague-Dawley rats and C57Bl/6 × CBA/Ca F1 female mice were housed in an environmentally controlled room on a 12 h light:12 h darkness photoperiod. Animals were provided with food and water which were available *ad libitum*.

Follicle isolation and culture

Mice and rats were killed by cervical dislocation and their ovaries removed to watch-glasses containing Leibovitz L-15 medium (Gibco-BRL, Renfrew, UK) supplemented with 3 mg/ml BSA (Fraction V, Sigma, Poole, Dorset, UK). Individual pre-antral follicles (<200 µm in diameter) were manually dissected using fine needles, randomly allocated to microtitre plates and cultured at 37 °C in a water-saturated atmosphere of 5% CO₂/95% air mixture. Mouse follicles were placed in 'U'-shaped wells of microtitre plates (Iwaki, Osaka, Japan) containing 30 µl minimal essential medium (Gibco-BRL) overlaid with 75 µl silicone fluid (Gibco-BRL; Merck, Poole, Dorset, UK). Culture medium for the mouse follicles was supplemented with 5% mouse serum and 1 IU recombinant human FSH (hrFSH) per ml (Serono-Ares, Geneva, Switzerland). Follicles were moved to fresh medium daily. Rat follicles were placed in polycarbonate membrane-lined flat-bottomed wells in 150 µl minimal essential medium overlaid with 75 µl silicone fluid. Rat follicle culture medium was supplemented with 3 mg/ml BSA and ITS+ (insulin, 10 mg/l; transferrin,

5.5 mg/l; linoleic acid, 4.7 mg/l; selenium, 5 mg/l; all from Sigma-Aldrich Company Ltd, Poole, Dorset, UK). Fifty per cent of used medium was replaced with 50% fresh medium daily. hrFSH and/or recombinant human LH (hrLH) (Serono-Ares) were added as described below. A minimum of 12 follicles were cultured and assessed independently for every treatment in each follicle culture experiment.

Quality control follicular fluid

All protocols employing human subjects were given Joint Ethical Committee Approval at Aberdeen and patients all gave informed consent. Follicular fluid (hFF) was aspirated from follicles ≤18 mm in diameter from 40 women undergoing IVF in Aberdeen and pooled and desalted as previously described (Fowler *et al.* 2001). Subsequently, 500 µl aliquots of these hFF pools were stored at –20 °C and used as a GnSAF bioactivity quality control (QC), producing a 40–60% reduction in GnRH-induced LH at 25 µl/well, as previously described (Fowler *et al.* 2001). This preparation is subsequently referred to as QC hFF.

GnSAF bioassay

For each cell culture, 15 rats, selected at random during the oestrous cycle, were killed by stunning and cervical dislocation. Dispersion and culture of the pituitary cells was carried out as described in Fowler *et al.* (1994, 2002) and only preparations with >75% viability of dispersed cells were used for bioassay. Primary pituitary cells were cultured at 30 000 viable cells/200 µl culture medium per well in 96-well tissue culture plates. The cells were cultured under sterile conditions for 24 h at 37 °C in a water-saturated atmosphere of 5% CO₂/95% air mixture with serum-free defined culture medium (SFDM) as defined in Fowler *et al.* (1994). Media and non-attached cells were removed by aspiration and the wells were washed with 500 µl SFDM. All experiments were then carried out on quadruplicate wells as follows: 200 µl fresh SFDM were added, together with the treatments made up to 25 µl with SFDM. All the culture plates contained at least four control wells receiving SFDM only. After 24-h incubation with the test substances, the wells were washed with fresh medium and then treated with 0.1 µM GnRH (Fertagyl; Intervet UK Ltd, Cambridge, Cambs, UK). Cultures were terminated after 4-h incubation by collecting the media which were stored at –20 °C for determination of GnRH-induced LH and FSH secretion.

Dose-responses for assessment of bioactivities

Doses of 0, 1, 5, 25 µl/well of rodent follicle-conditioned medium and QC hFF, with and without incubation with GnSAF antiserum, were added randomly to 5 independent rat pituitary cell cultures in quadruplicate wells. Each culture dish contained 4 control wells receiving SFDM only and 4 wells receiving 25 µl SFDM instead of the 25 µl GnRH challenge. This enabled the calculation of LH

release in the absence of GnRH stimulation in order to ensure comparable GnRH responses from one culture to another. The QC hFF ensured that comparable detection of GnSAF bioactivity occurred across the different cultures. In all experiments, specific controls for each dose of gonadotrophin were samples of medium + gonadotrophin at the relevant doses, but without conditioning by rodent follicles. This design controlled for the exposure of the pituitary cells to FSH and LH where appropriate. Because the wells were washed prior to the GnRH challenge, the presence of LH or FSH during the treatment period could not artefactually elevate LH or FSH concentrations in the GnRH-treated samples.

Two-dimensional gel electrophoresis

Proteins in rat follicle-conditioned medium ($\pm$ FSH) were freeze-dried and resuspended at a ratio of 250 μ l unproteased conditioned medium per 100 μ l lysis buffer (0.01 M Tris-HCl, pH 7.4, 1 mM EDTA, 8 M urea, 0.05 M dithiothreitol (DTT), 10% (v/v) glycerol, 5% (v/v) NP40, 6% (w/v) pH 3–10 Resolyte (VWR International Ltd, Poole, Dorset, UK)). Following centrifugation at 11 000 *g*, the supernatant was stored at -70°C . Soluble proteins were analysed by two-dimensional gel electrophoresis (2-D gels) using a small format gel system (Cash *et al.* 1997) with immobilised pH gradient (IPG) gels for the first dimension separation. Briefly, 40 μ l rat follicle-conditioned medium ($\pm$ FSH) containing 100 μ g total protein were adjusted to a final volume of 125 μ l in IPG re-swelling buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.3% (w/v) DTT, 2% (w/v) pH 4–8 Resolyte). The proteins were separated in the first dimension using 7 cm, pH 4–7 IPG gel strips (Amersham Biosciences UK Ltd, Chalfont St Giles, Bucks, UK). The dehydrated IPG strips were re-hydrated overnight in the IPG re-swelling buffer containing the conditioned media (Rabilloud *et al.* 1994). Following their re-hydration, the IPG gel strips were electrophoresed on a Multiphor II apparatus (Amersham Biosciences UK Ltd), the IPG gels were then equilibrated in buffer containing 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 1% (w/v) DTT for 30 min followed by a second equilibration for 30 min in the same buffer + 2.5% (w/v) iodoacetamide. The equilibrated strips were overlayed onto 7 $\times$ 8 cm 10–15% gradient polyacrylamide slab gels and processed as described previously (Cash *et al.* 1999). Proteins were located by silver staining and Western blot. For the latter, gels were transferred onto 0.45 μ m nitrocellulose at 200 V for 75 min in transfer buffer (0.25 M Tris-HCl, 19.2 mM glycine, 0.01% SDS, 2% methanol), washed in blocking buffer containing 2% non-fat powdered milk in Tris-buffered saline (TBS) for 45 min then washed for 3 $\times$ 10 min in TBS containing 3 g Tris, 8 g NaCl, 0.2 g KCl/l, pH 8 prior to incubation with GnSAF polyclonal antibody (pAb) at room temperature for 90 min. The membranes were washed (3 $\times$ 10 min, TBS + Tween) and incubated with

alkaline phosphatase-conjugated IgG diluted in TBS for 90 min. Recognised proteins were directly visualised with 5-bromo-4-chloro-3-indolylphosphate with nitroblue dimethyl formamide (Sigma-Aldrich Company Ltd).

Hormone assays

Concentrations of LH in cell-conditioned media from rat anterior pituitary cell cultures were determined using a homologous rat time-resolved fluoro-immunoassay (DEL-FIA) with sensitivity and intra- and interassay coefficients of variation values of 0.2 ng LH/ml (NIDDK-rLH-RP3) using NIDDK-anti-rLH-S11 and 5.4% and 7.9% respectively.

Experiments

Experiment 1 was carried out on mouse follicles, using follicles dissected from 21-day-old mice. Five percent mouse serum was added to the medium. Subsequent experiments (experiments 2–5) were then carried out using a rat follicle culture model which supports follicle growth in the absence of serum (although BSA is still required in the culture medium). This allowed possible confounding effects of serum proteins on the culture system to be removed from the model. This culture system used follicles dissected from 12-day-old rats.

Experiment 1: effect of duration of mouse follicle culture on GnSAF production

Pre-antral mouse follicles were cultured for 6 days in culture medium. The medium was collected every 24 h and stored at -20°C for subsequent bioassay. The aim of this experiment was to investigate how the profile of GnSAF production changed as follicles grew, from less than 200 μ m to around 400 μ m in diameter (Spears *et al.* 1994, 1998, 2002, Murray *et al.* 2001).

Experiment 2: effect of duration of rat follicle culture on GnSAF production

Pre-antral rat follicles were cultured in the presence or absence of 5 IU FSH/ml for 3 days. The medium was collected daily and stored at -20°C for subsequent bioassay. The aim of this experiment was to establish the pattern of GnSAF production over time in the presence and absence of FSH.

Experiment 3: effect of gonadotrophins on GnSAF production by intact rat follicles

(a) Pre-antral rat follicles were cultured in the presence of 0, 0.5, 1, or 5 IU FSH/ml for 3 days. The medium from the last 24 h was collected and stored at -20°C for subsequent bioassay. The aim of this experiment was to investigate the FSH dose-response behaviour of rat follicles in terms of GnSAF production. (b) Pre-antral rat follicles were cultured in the presence of (i) no gonadotrophin,

(ii) 5 IU FSH/ml, (iii) 0.5 IU LH/ml, (iv) 5 IU LH/ml, (v) 0.5 IU LH/ml + 5 IU FSH/ml or (vi) 5 IU LH/ml + 5 IU FSH/ml, for 3 days. The medium from day 2 was collected and stored at -20°C for subsequent bioassay. The aim of this experiment was to investigate whether LH treatment affected GnSAF production both in the presence and absence of FSH.

Experiment 4: effect of anti-human GnSAF polyclonal antibody

In order to investigate whether the GnSAF bioactivity produced by the rodent follicles was homologous to that produced by human granulosa cells and detected in human serum and hFF, an antibody bioactivity-blocking experiment was carried out as follows. Both the rat polyclonal antibody (pAb) which previously blocked *in vitro* GnSAF bioactivity in human material (Fowler *et al.* 2002) and a rat pAb raised against whole serum from post-menopausal women (PMWS) and employed as a control antiserum in Fowler *et al.* (2002), were used. These antibodies were mixed gently at 1:1 in 100 μl aliquots with 2-day and 3-day rat follicle-conditioned medium (obtained from follicles cultured in the presence of 5 IU FSH/ml), for 1 h at 37°C in 24-well dishes on an orbital shaker. The incubation experiment was repeated twice, combined and the resulting samples were bioassayed in two separate monolayer cultures. If the bioactivity produced by the rodent follicles had good homology with human GnSAF then we predicted that it would reduce apparent GnSAF bioactivity in rodent follicle-conditioned medium while the PMWS antiserum would have no effect since post-menopausal women do not produce GnSAF.

Experiment 5: effect of FSH on the protein secretion profile of rat follicles

Six rat follicles were cultured for 3 days both with and without 5 IU FSH/ml. For each treatment the media were collected and concentrated from 20 ml to 1000 μl , desalted and dehydrated in a vacuum centrifuge. The two

residues were resuspended in 250 μl of 2-D lysis buffer and electrophoresed using 2-D gels as outlined above.

Statistical analysis

The *in vitro* pituitary cell responses are expressed as percentages of the relevant control gonadotrophin concentrations secreted from wells on the same culture dishes. These controls were either wells exposed to SFDM alone (basal secretion) or wells exposed to SFDM + 0.1 μM GnRH. The differences between treatment groups and dose-responses were assessed using two-way analysis of variance (ANOVA). Differences between treatments and controls were tested by Dunnett's *post hoc* test and between treatments by the Bonferroni–Dunn *post hoc* test. The analyses were performed using the Statview 5 programme (Abacus Concepts Inc., Berkeley, CA, USA). All results are presented as means $\pm$ S.E.M. Since all bioassays were carried out in quadruplicate wells using two separate cell cultures, n is stated as 2×4 . For the rodent follicle growth curves $n \geq 12$ individual follicles.

Results

Experiment 1: GnSAF production from intact mouse follicles

When the secretory outputs of mouse follicles were followed over a 6-day period, there was marked GnSAF bioactivity production for the first 3 days of culture ($P < 0.01$ – 0.001 ; Fig. 1), resulting in a marked drop in LH secretion at the 25 μl conditioned medium/well dose. GnSAF production was highest on day 1 with even the 1 μl conditioned medium/well dose significantly reducing GnRH-induced LH secretion ($P < 0.001$). Between days 4 and 6 of culture the stimulatory effect of the follicle-conditioned medium increased, with GnRH-induced LH secretion increased to up to $180 \pm 16\%$ of control, $P < 0.01$ at 5 μl /well. Thus during days 4–6 of culture, decreases in LH secretion were only induced by the highest dose of conditioned medium/well.

Figure 1 Intact murine follicles cultured for 6 days. GnSAF bioactivity production declines with the duration of culture from (a) day 1 to (f) day 6. The horizontal shaded bar denotes the mean $\pm$ S.E. range for GnRH-induced LH secretion in the presence of medium only. Data represent the mean of two different rat pituitary cell culture bioassays for each dose–response curve. Significance values are by ANOVA relative to controls (culture medium only), $**P < 0.01$, $***P < 0.001$. Data are shown as means $\pm$ S.E., $n = 2 \times 4$.

Experiment 2: GnSAF production from intact rat follicles

As previously documented (McGee *et al.* 1997, 2001), this serum-free culture system supported the growth of follicles in the presence of FSH, although no antral development was seen in this or in subsequent rat follicle culture experiments. FSH stimulated increased GnSAF production by rat follicles (Fig. 2). In the absence of FSH there was only detectable GnSAF production by the follicles on day 1 of culture ($P < 0.05$). In contrast, FSH treatment stimulated GnSAF production on both days 1 and 2 of culture ($P < 0.01$ and $P < 0.05$ respectively). Although there was a statistically significant decline ($P < 0.05$) in GnSAF

production as the follicles grew from around 170 to 180 μm in diameter, this difference was reflected in an 8% difference in the suppression in GnRH-induced LH secretion at the highest dose of conditioned medium. These findings show that GnSAF production by rat follicles occurs in the presence of FSH and suggest that this occurs primarily in smaller follicles, as in the mouse.

Experiment 3: effect of gonadotrophins on GnSAF production by intact rat follicles

Experiment 3a showed that the stimulatory effect of FSH on GnSAF bioactivity was dose-dependent, while no such effect was seen on follicle growth (Fig. 3). All FSH doses stimulated follicle growth over a 3-day period ($P < 0.001$). In the absence of FSH, follicle diameter fell slightly over the course of the culture ($P < 0.05$). GnSAF production on day 2 was stimulated most by 1 IU FSH/ml ($P < 0.001$), a significantly greater production than that of other doses ($P < 0.05$), although there was no difference between the growth rates of any of the follicles grown in the presence of FSH. In contrast, experiment 3b showed that LH alone (at both 0.5 and 5 IU/ml) had no significant effect on GnSAF secretion (Fig. 4a) and that these follicles did not grow over the culture period (Fig. 4b). The presence of FSH stimulated growth ($P < 0.01$), as in experiment 3a, although growth was unaffected by the additional presence of LH. Co-treatment of LH with 5 IU FSH/ml did, however, further increase GnSAF bioactivity significantly at the 5 μl conditioned medium/well dose ($P < 0.05$).

Experiment 4: nature of GnSAF secreted by intact rat follicles

Neither the pAb raised against GnSAF or the pAb raised against serum from post-menopausal women had any effect on GnRH-induced LH secretion in the absence of rat follicle-conditioned medium. When rat follicle-conditioned medium from both 2 and 3 days of culture was incubated with the two rat pAbs (Fowler *et al.* 2002), the pAb raised against human GnSAF completely blocked GnSAF bioactivity. In contrast, following incubation with the rat pAb raised against serum from post-menopausal women, GnSAF bioactivity remained unchanged ($P < 0.01$) (Fig. 5). This suggests that there is relatively close homology between rodent and human GnSAF.

Experiment 5: effect of FSH on the protein secretion profile of rat follicles

The production of proteins by the rat follicles was further investigated by comparing silver-stained 2-D gels of medium conditioned in the presence and absence of FSH (Fig. 6a,b). Since the culture medium had to be supplemented with BSA to support follicle health, heavily stained areas coinciding with the established position of serum albumin were obvious. Despite this, the FSH could

Figure 2 Intact rat follicles cultured in the presence or absence of 5 IU FSH/ml for three days. (a) Peak GnSAF bioactivity production was seen on day 1 of culture, coincident with (b) moderate follicular growth. The horizontal shaded bars denote (a) the mean $\pm$ S.E. range for GnRH-induced LH secretion in the presence of medium only or (b) the mean $\pm$ S.E. range for follicle diameter at the start of the experiment. Data represent the mean of two different rat pituitary cell culture bioassays for each dose-response curve. Significance values are by ANOVA relative to controls (culture medium only), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Data are shown as means $\pm$ S.E., $n = 2 \times 4$.

Figure 3 Intact rat follicle culture: pre-antral rat follicles were cultured in the presence of 0, 0.5, 1 or 5 IU FSH/ml for three days. (a) Peak GnSAF bioactivity production was seen by treatment with 1 IU FSH/ml as assessed on day 2 of culture, compared with similar effects of FSH on (b) follicle growth. The horizontal shaded bars denote (a) the mean ± S.E. range for GnRH-induced LH secretion in the presence of medium only or (b) the mean ± S.E. range for follicle diameter at the start of the experiment. Data represent the mean of two different rat pituitary cell culture bioassays for each dose-response curve. Significance values are by ANOVA relative to controls (culture medium only), ***P < 0.001. Data are shown as means ± S.E., $n = 2 \times 4$.

clearly be seen to both stimulate and inhibit a range of protein spots. When probed with the anti-human GnSAF pAb in a Western blot (Fig. 6c), several proteins in a band between 50 and 65 kDa and between 4.5 and 5.5 pH were stained – an area similar to that observed when this antiserum was used to Western blot human granulosa cell-conditioned medium (Fowler *et al.* 2002). These Western blot-recognised protein spots matched several less abundant spots that increased with FSH treatment but are not highlighted in Fig. 6b in order to retain clarity. Unfortunately, the protein yield was not sufficient for any of the up-regulated protein spots to be excised and subjected to peptide mass mapping.

Discussion

This study clearly shows that, in the *in vitro* intact rodent follicle model, GnSAF production is FSH dependent and is highest in early antral follicles. These findings closely match data for other species, in particular the human,

both *in vivo* and *in vitro* (for reviews see Fowler & Templeton 1996, Fowler *et al.* 2003).

Follicles produced GnSAF only if cultured in the presence of FSH, the presence of LH alone being insufficient to induce its production. Since FSH, but not LH, is required for antral follicle survival in mice (Spears *et al.* 1998), the lack of GnSAF production in the absence of FSH is likely to be due to the induction of granulosa cell apoptosis in these follicles (Baker *et al.* 2001). Similarly, McGee *et al.* (1997) demonstrated increased apoptosis in cultured pre-antral rat follicles (using the same culture method as in these experiments), while Wang *et al.* (2003) showed that FSH is essential to protect early antral rat follicles from apoptosis. While the inability of LH to induce GnSAF production in the absence of FSH could be an indirect effect due to atresia in these follicles, we also showed that LH was unable to stimulate increased GnSAF production in the presence of FSH. The follicles in these experiments remained at the pre-antral stage throughout the culture period, and it is possible that these follicles

Figure 4 Intact rat follicle culture: pre-antral rat follicles were cultured in the presence of various doses of gonadotrophins. (a) Moderate augmentation of GnSAF bioactivity production by combined LH and FSH treatment was seen on day 3 of culture, although the effects of LH on (b) follicle growth were slight. The horizontal shaded bars denote (a) the mean $\pm$ S.E. range for GnRH-induced LH secretion in the presence of medium only or (b) the mean $\pm$ S.E. range for follicle diameter at the start of the experiment. Data represent the mean of two different rat pituitary cell culture bioassays for each dose-response curve. Significance values are by ANOVA relative to controls (culture medium only), *** $P < 0.001$. Data are shown as means $\pm$ S.E., $n = 2 \times 4$.

do not yet express active LH receptors. Our findings, therefore, emphasise that FSH (but not LH) is required for GnSAF production by pre-antral follicles.

The difference in the biological effect of secreted proteins from small and large mouse follicles is interesting beyond the observation of decreasing GnSAF production as the follicles grow. The suppression of GnRH-induced LH secretion by proteins secreted from small follicles rather than large ones is further indirect evidence that GnSAF activity is not due to inhibin, since inhibin production increases as mouse follicle cultures progress (Smitz & Cortvrindt 1998).

The stimulatory effects of proteins from large murine follicles on GnRH-induced LH secretion may possibly be due to the combined effects of increasing activin and decreasing GnSAF bioactivities. The reasoning behind this is that, because activin secretion is known to increase as the follicles grow (Newton *et al.* 2002) and

since activin stimulates GnRH-induced LH secretion from rat pituitary cells (Weiss *et al.* 1993), conditioned medium from larger follicles would be more likely to stimulate GnRH-induced LH secretion. In the present study, therefore, the lower doses of conditioned medium might favour the action of activin bioactivity, causing an increase in GnRH-induced LH secretion. In contrast, at the higher doses of conditioned medium, even though GnSAF bioactivity is reduced, there might be sufficient GnSAF bioactivity present to counteract the stimulatory effects of activin, which could have already exerted its maximal effect at the lower dose of medium. Data in the human suggest that GnSAF production declines rapidly as the follicle grows past 10 mm in diameter in spontaneously cyclic women (Fowler *et al.* 2001) or 13 mm in diameter in women undergoing IVF treatment (Fowler *et al.* 1994). This represents a decline in GnSAF production post-dominance or following major waves of

Figure 5 Effects of intact rat follicle-conditioned medium collected on days 2 (a) and 3 (b) of culture are blocked following incubation of the conditioned media with anti-human GnSAF pAb (open circles) but not following incubation with anti-postmenopausal woman serum pAb (solid circles). The horizontal shaded bar denotes the mean $\pm$ S.E.M. for GnRH-induced LH secretion in the presence of medium only. Data represent the mean of two different rat pituitary cell culture bioassays for each dose-response curve. Significance values are by ANOVA relative to controls (culture medium only), *** $P < 0.001$. Data are shown as means $\pm$ S.E., $n = 2 \times 4$.

follicular development (Baerwald *et al.* 2003) in the human. In the rodent, while there is no evidence for follicular waves, the wave of atresia due to follicle dominance appears to occur around the mid antral stage of development (Baker *et al.* 2001), with the fall in GnSAF shown here occurring immediately after that. GnSAF production is, therefore, falling as follicles mature towards the pre-ovulatory stage, with a concomitant increase in the production of oestrogens (Spears *et al.* 1998). Taken together these data are suggestive of the likely role of GnSAF in the rodent. The fact that GnSAF secretion will be highest prior to follicular maturity indicates that the proposal by Fowler *et al.* (2003) that GnSAF acts to keep LH pulsatility in check is feasible for the rodent. Declining GnSAF production during the last phase of follicular maturation would represent a fall in the overall negative feedback at the level of the pituitary, concomitant with increasing oestradiol secretion and the resulting positive feedback effects on both hypothalamus and pituitary.

The suggestion by Schuiling *et al.* (1999) that FSH stimulates rat ovarian production of an oestradiol-antagonising factor rather than GnSAF is only one of the two most likely interpretations of their data. The present study clearly demonstrates that FSH treatment of rodent follicles stimulates production of GnSAF bioactivity, and that this bioactivity is completely inhibited by co-incubation with an antibody that blocks human GnSAF (Fowler *et al.* 2002). This finding also casts doubt on the interpretation of the effects of FSH immunoneutralisation that Tio *et al.* (1998) reported did not affect the indirectly determined GnSAF bioactivity in rats.

Figure 6 Stimulation of intact rat follicle protein production by *in vitro* treatment with (a) medium alone and (b) 5 IU FSH/ml as shown by 2-D gel electrophoresis. FSH-induced up-regulation of protein production is shown by single-lined boxes, down-regulation by double-lined boxes on the silver-stained gels. In both gels the heavily stained area includes serum albumin. Western blotting with anti-human GnSAF pAb (c) stained several proteins at pH 4.5–5.5, 50–65 kDa.

It was unfortunate that we were unable to recover sufficient protein to identify some of the proteins secreted in response to FSH treatment, especially those recognised by the anti-GnSAF antiserum. These proteins fell within the expected pI (5.5–5.8 pH) and molecular mass (60–70 kDa) ranges proposed for GnSAF (Fowler *et al.* 2003) and it would be reasonable to predict that one of them may have been GnSAF. However, this study has clearly demonstrated that the intact rodent follicle culture model is suitable for the investigation of both GnSAF and the regulation of GnSAF production. To investigate the GnSAF molecule itself, a scaling-up of the rat follicle cultures would be required in order to produce sufficient protein for identification using serum protein depletion steps and proteomic techniques. In contrast, with respect to the study of the regulation of GnSAF production, the ability to control steroidal, gonadotrophin and inhibin signalling in the intact follicles and then subsequently determine changes in GnSAF bioactivity would greatly enhance our understanding of the factors regulating GnSAF during the ovarian cycle.

To summarise, the intact rodent follicle culture model demonstrates that GnSAF production is positively regulated by FSH and preferentially produced by smaller follicles, matching existing GnSAF data for the human in particular.

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