Relationship between follicle size and gonadotrophin surge attenuating factor (GnSAF) bioactivity during spontaneous cycles in women

Paul A. Fowler1,5, Tarja Sorsa1,2, William J. Harris2, Philip G. Knight3 and Helen D. Mason4

1Department of Obstetrics and Gynaecology, 2Department of Molecular and Cell Biology, University of Aberdeen, Aberdeen, 3School of Animal and Microbial Sciences, University of Reading, Reading and Departments of 4Obstetrics and Gynaecology and Physiology, St George’s Hospital Medical School, London, UK

5To whom correspondence should be addressed. E-mail: p.a.fowler@abdn.ac.uk

BACKGROUND: We have previously demonstrated that follicles ≤11 mm diameter from women undergoing IVF contain higher concentrations of gonadotrophin surge attenuating factor (GnSAF) bioactivity than large follicles from the same ovaries. METHODS: To determine whether this finding is relevant to spontaneous cycles, follicular fluid aspirated from 37 follicles between 3 and 25 mm in diameter from 14 pairs of ovaries from regularly cycling women undergoing total abdominal hysterectomy and bilateral salpingoophorectomy for benign gynaecological disease was pooled into size categories (3–4, 5–6, 7–8, 9–10, 11–12, 14–15, 18 and 25 mm). These pools were bioassayed for GnSAF and inhibin-A, inhibin-B and activin-A concentrations were determined.

RESULTS: Follicles of 5–6 mm diameter contained the highest concentrations of GnSAF bioactivity (reducing GnRH-induced LH secretion to 38±8% of control, P<0.001), while those of 25 mm diameter contained one quarter of this concentration (reducing GnRH-induced LH secretion to 72±2% of control, P<0.05). GnSAF bioactivity was closely related to follicle size (r = –0.836, P<0.01), but not to inhibin-A, inhibin-B or activin-A concentrations.

CONCLUSIONS: The finding that small follicles contain high concentrations of GnSAF bioactivity, which fall as folliculogenesis progresses during spontaneous cycles, support the hypothesis that GnSAF has a role in preventing the premature onset of the LH surge in women.

Key words: follicle size/GnRH/GnSAF/LH/spontaneous cycle

Introduction

Gonadotrophin surge-attenuating factor/inhibiting factor (GnSAF) has the specific biological effect of reducing pituitary responsiveness to gonadotrophin-releasing hormone (GnRH) without affecting constitutive LH or FSH secretion. The production of GnSAF by the ovary in various species is stimulated by FSH both in vivo and in vitro (Fowler and Templeton, 1996). Despite its obvious potential in reproductive technologies, GnSAF remains enigmatic: three different putative GnSAF amino acid sequences have been published (Tio et al., 1994; Danforth and Cheng, 1995; Pappa et al., 1999) and to date none has been confirmed as GnSAF.

We have previously demonstrated that GnSAF bioactivity is not an artefact of ovarian stimulation protocols since it is also found in spontaneous cycle follicular fluid (Fowler et al., 1995) and serum (Byrne et al., 1993). Although we know that small follicles contain much greater concentrations of GnSAF bioactivity than large follicles (Fowler et al., 1994), the latter data were based upon follicular fluid aspirated from IVF follicles and might possibly be an artefact of the stimulation regimes and not an aspect of normal ovarian physiology. While data from unstimulated pigs and cows show that GnSAF activity is present in follicular fluid, and in pigs declined with increasing size beyond a peak of activity in follicles between 7 and 8 mm diameter (Koppenaal et al., 1992; Kita et al., 1994), it is important to confirm such findings in humans. For GnSAF to have a role in the timing of the LH surge, its production during the follicular phase would need to be regulated and co-ordinate with folliculogenesis.

In this paper we present evidence that in the spontaneously cycling women, GnSAF bioactivity is inversely related to follicle size, with direct implications for the role of GnSAF in the regulation of the ovarian cycle.

Materials and methods

Follicular fluid from unstimulated ovaries

Ovaries were obtained from women undergoing oophorectomy for non-ovarian gynaecological pathology. Approval for the current study was granted by the local ethics committees of the hospitals concerned and each woman gave informed consent. Details of menstrual cycle history (cycle length, duration of menses) were obtained prior to
surgery. Samples were only collected from women giving a history of regular cycles and surgery was performed at random stages of the cycle. Following inspection of the tissue by a pathologist, ovaries were transported to the laboratory on ice.

Individual follicles were microscopically dissected intact from the surrounding stroma in 14 pairs of ovaries. Patient details are shown in Table I. Follicles were dissected cleanly from adhering stromal tissue to allow for accurate assessment of diameter using callipers. The fluid was then aspirated with a fine gauge needle and centrifuged to remove cellular debris. Samples were then frozen immediately prior to use. A total of 35 follicles were collected, covering the range 3–25 mm. To produce adequate volumes for bioassay, hormone measurement and samples reliably representative of each size range, fluids were pooled into eight groups according to size as shown in Table II. Follicular fluid from between two and 10 follicles was pooled and desalted into sterile distilled water (1.5 ml follicular fluid:2 ml sterile distilled water through a 5 ml HiTrap Desalting™ column; Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK). Subsequently 1 ml aliquots of this follicular fluid were stored at −20°C and used as a GnSAF bioactivity quality control (QC) follicular fluid, producing a 40–60% reduction in GnRH-induced LH at 50 µl/well doses in the GnSAF bioassay) in all bioassays performed as part of the present study. The follicular fluid would have been discarded if it had not been used in the present study.

**GnSAF bioassay**

Adult female Sprague–Dawley rats (10–14 weeks old) were maintained under a constant 12 h light:12 h dark, 22°C environment with access to food and water ad libitum. For each cell culture 15 rats, selected at random during the oestrous cycle, were killed by CO₂ exposure followed by cervical dislocation. Dispersion and culture of the pituitary cells was carried out as described in Fowler et al. (Fowler et al., 1994) and only preparations with >75% viability of dispersed cells were used for bioassay. Primary pituitary cell cultures were at 30,000 viable cells/200 µl culture medium per well in the inner 60 wells of 96-well culture plates. The outer 36 wells contained 200 µl of culture medium only. 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. (Fowler et al., 1994)).

All experiments were then carried out on quadruplicate wells as follows: 200 µl of fresh SFDM was added, together with the treatments made up to 25 µl with SFDM. All the culture plates contained at least 12 control wells receiving SFDM only. After 24-h incubation with the test substances, the medium was replaced and the wells were then treated with 0.1 µmol/l GnRH (Fertagyl, Intervet UK Ltd, Cambridge, UK) in 50 µl of SFDM. In all dishes, eight wells previously exposed to SFDM received GnRH alone while four wells previously exposed to SFDM received 50 µl of SFDM instead of the 50 µl of GnRH challenge. These acted as controls for the magnitude of the GnRH response. Cultures were terminated after 4 h incubation by collecting the media, which was stored at −20°C for subsequent measurement of GnRH-induced LH as an index of GnSAF bioactivity. The QC follicular fluid was added to each bioassay at 1, 5 and 25 µl/well, in at least four wells/dose/separate culture, to act as a GnSAF quality control.

**Hormone assays**

Concentrations of LH in cell-conditioned media from rat anterior pituitary cell cultures were determined using a homologous time-resolved fluoro-immunoassay (DELFIA) in which rat LH was labelled with europium instead of 125I and the assays were performed in microtitre plates. In other respects, the assay closely followed our existing rat radioimmunoassay (Fowler et al., 1994). Sensitivity and intra-assay and inter-assay coefficient of variation (CV) values were: 0.2 ng LH/ml (NIDDK-rLH-RP3) using NIDDK-anti-rLH-S11 and 5.4 and 7.9% respectively. Activin-A was measured using a two-site ELISA previously described (Knight et al., 1996; Muttukrishna et al., 1996) in which follistatin does not interfere. This assay has a sensitivity of 50 ng recombinant human activin-A (gift from Genentech Inc., San Francisco, CA, USA) and mean intra- and inter-assay CVs of 5.0 and 9.1% respectively. The recombinant human activin-A is equipotent with the proposed candidate WHO 91/626 Reference Reagent for human activin-A. Inhibin-A concentrations

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Table II. Characteristics of follicle pools

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<tr>
<th>Follicle pool size</th>
<th>No. follicles</th>
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<td>3 + 4</td>
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PMS = premenstrual syndrome; CA = carcinoma.
were determined with a two-site ELISA previously described (Muttukrishna et al., 1994, 1995) with a sensitivity of 2 ng recombinant human inhibin-A/L (a gift from Dr M. Rose, NIBSC, Potters Bar, Herts, UK) and mean intra- and inter-assay CVs of 3.7 and 9.5% respectively. Inhibin-B was measured using a two-site ELISA previously described (Lockwood et al., 1996; Muttukrishna et al., 1997) with a sensitivity of 12 ng recombinant inhibin-B/L (gift from Genentech Inc.) and intra- and inter-assay CVs of 6.2 and 9.5% respectively.

**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 μmol/l GnRH. The differences between treatment groups and dose-responses were assessed using two-way analysis of variance (ANOVA) of the combined bioassay data from two separate bioassays. Differences between treatments and controls were tested by Dunnet’s post hoc test and between treatments by the Bonferroni-Dunn post hoc test. Median effective doses (ED50) for GnSAF bioactivity were calculated from the dose-response curves by polynomial regression equations fitted separately for each dose-response curve. In this study, the ED50 is defined as the volume (μl) of QC follicular fluid/well dose required to produce 50% of its maximum suppression of GnRH-induced LH secretion in the relevant matching pairs of bioassays. To convert the ED50 values from an inverse relationship with bioactivity (the smaller the ED50 value the greater the GnSAF bioactivity) to positive values (>1), which allows more direct comparison with other hormone titres, arbitrary units of GnSAF were calculated as follows: [(1/ED50 for serum pool or fraction/ED50 QC hFF in matching cultures)]×10.

Relationships between variables were analysed by simple linear correlation with significance established using Fischer’s Z statistic. The analyses were performed using the Statview 5 programme (Abacus Concepts Inc., Berkley, CA, USA). All results are presented as means ± SEM.

**Results**

**Relationship between follicle size and GnSAF bioactivity**

The greatest suppressive effect was exerted by follicular fluid from follicles of 5 + 6 mm diameter, which reduced GnRH-induced LH secretion to 38 ± 8% of control (control = culture medium + GnRH only: P < 0.001, Figure 1). This suppression was greater than that achieved by the QC follicular fluid and the follicular fluid from 25 mm follicles which reduced GnRH-induced LH secretion to 54 ± 8% (P < 0.01) and 72 ± 2% (P < 0.05) of control respectively. The dose-response curves were condensed into ED50 in which the smaller the value, the less the amount of follicular fluid required to exert half the maximal effect of the QC follicular fluid and therefore the greater the concentration of GnSAF bioactivity. These values are shown in Table III, which clearly demonstrates that small follicles, particularly those between 5 and 10 mm in diameter, contain higher follicular fluid concentrations of GnSAF. The lowest ED50 was for follicular fluid from follicles 5 + 6 mm in diameter which contained significantly higher concentrations of GnSAF bioactivity than both follicles of 11 mm or over and the QC follicular fluid from IVF cycles (P < 0.05–0.001). Since maximal inhibition of GnRH-induced LH secretion was related to follicle size, these data clearly show a size-related decrease in concentrations of GnSAF bioactivity in follicular fluid.

**Relationship between GnSAF bioactivity and inhibin or activin**

Neither activin-A, nor inhibin-B showed a significant correlation with GnSAF bioactivity (Figure 2a and c), unlike inhibin-A (Figure 2b). However, although this was statistically significant, marked clustering of the data was evident. Nevertheless, it was clear that high inhibin-A concentrations were found in the largest follicles with the lowest GnSAF bioactivity (Figure 2d). Furthermore, follicular fluid inhibin-A concentrations were positively correlated with follicle size (r = 0.793, P < 0.05), while neither inhibin-B and activin-A correlated significantly with follicle size (r = 0.220, NS and r = 0.157, NS respectively).

**Discussion**

This study demonstrates for the first time that human GnSAF concentrations are higher in follicular fluid from small follicles during the spontaneous human ovarian cycle. Furthermore, the data for inhibin-A, inhibin-B and activin-A indicates that they do not alter with follicle size in the same way as GnSAF and suggests therefore that they are unlikely to modulate GnSAF bioactivity.

The relationship between follicle size and GnSAF bioactivity is qualitatively similar to that demonstrated by us for IVF-derived follicular fluid (Fowler et al., 1994). Direct comparison between the latter and the current study cannot, however, be made: firstly, because the bioassay used has evolved and become considerably more sensitive and secondly, due to the nature of bioassays, it would be necessary to run both sets of follicular fluid in the same assay to obtain a valid comparison. Although the trends are essentially similar, the difference in GnSAF bioactivity between follicles <11 mm and >21 mm from IVF cycles appeared to be in the order of 200-fold. The current study and data on inhibins and activins (Fowler et al., 1994, 1995; Magoffin and Jakimiuk, 1998; Lau et al., 1999) demonstrate more modest changes in follicular fluid concentrations of these hormones in different sized follicles. It is likely, therefore, that our current findings of a 4-fold decrease in GnSAF bioactivity between 5–6 mm and 25 mm follicles from spontaneous cycles is more physiological than in the aforementioned IVF study (Fowler et al., 1994).

There has always been a concern that inhibin may account for some or all GnSAF bioactivity (Culler, 1992). In the current study, the lack of relationship between GnSAF bioactivity and inhibin-A, inhibin-B or activin-A in follicular fluid from this large range of follicle sizes is clear evidence that these hormones do not account for observed GnSAF bioactivity. This is in agreement with earlier findings, including our investigation of GnSAF bioactivity in follicular fluid aspirated during spontaneous cycle IVF (Fowler et al., 1995), studies utilizing inhibin antiserum (Byrne et al., 1995) and the study by Kita et al. (Kita et al., 1994) showing no relationship between inhibin and GnSAF in pig follicles of different sizes.
Indeed, as can be seen in Figure 2, inhibin-A appeared to increase with decreasing GnSAF bioactivity although this relationship was not linear due to the close clustering of the data at the lower end of the scale. The lack of a relationship between inhibin-B and GnSAF bioactivity in the different size follicles is more surprising. It is known that inhibin-B titres in the peripheral circulation are dependent upon the action of FSH: raising FSH increases inhibin-B titres in the peripheral circulation within 36 h (Burger et al., 1998). In contrast, circulating inhibin-B concentrations fall sharply following GnRH antagonist administration (which reduces gonadotrophin secretion) during the follicular phase (Welt et al., 1999). Similarly, GnSAF bioactivity is detectable in women within 8 h following FSH administration (Messinis et al., 1994). The
fact that follicular fluid concentrations of GnSAF and inhibin-B did not correlate therefore suggests differences in the regulation of GnSAF and inhibin-B production.

Circulating concentrations of GnSAF are probably a result of a variety of factors, including: (i) follicular fluid concentrations of GnSAF, (ii) numbers of follicles, (iii) sizes of the follicles, (iv) the volume:surface area ratios of the follicles, (v) permeability of the follicular basement membranes and (vi) degree of any active transport and diffusion of GnSAF out of the follicles. Unfortunately, very little is known about the dynamics of follicle:circulation transport. However, follicular fluid concentrations of some hormones correlate with circulating concentrations, as is the case with inhibin-B (Hall et al., 1999). Therefore, if our early pilot results showing peak GnSAF concentrations in the circulation during the early and mid-follicular phases (Byrne et al., 1993; Martínez et al., 2000) are physiological, then the follicular concentrations of GnSAF will be an important component in determining circulating GnSAF titres. Therefore, when the ovary contains a number of small healthy follicles, such as in the early to mid-follicular phase, circulating GnSAF will be maximal, but with the establishment of dominance and atresia of the subordinate follicles from the mid-follicular phase onwards, GnSAF titres will decline, reducing the ‘clamping’ effect of GnSAF on pituitary responsiveness to GnRH.

In conclusion, we have demonstrated that GnSAF bioactivity in the follicular fluid during spontaneous cycles is inversely related to follicle size, but not to inhibin or activin concentrations. These findings are in agreement with previous studies using follicular fluid collected during IVF cycles, and support the concept of a physiological role for GnSAF in the regulation of LH secretion during the follicular phase. Studies to determine the site of GnSAF production and the mode of regulation of GnSAF secretion by FSH are ongoing.

Acknowledgements

We are grateful to M.Fraser and P.Cunningham for their expert technical assistance. We thank the staff at the Biological Services Unit (University of Aberdeen) for maintaining the rats used in this study and Dr A.F.Parlow at NIDDK’s National Hormone and Pituitary Program (Torrance, CA, USA) and SAPU (Law Hospital, Carluke, Scotland) for hormone assay materials. We are grateful to the BBSRC for their financial support.

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Received on November 24, 2000; accepted on March 12, 2001