***In vitro* bioactivity of gonadotrophin surge attenuating factor is not affected by an antibody to human inhibin**

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The effect of anti-inhibin antibodies on gonadotrophin surge attenuating/inhibiting factor (GnSAF/GnSIF) and its effect on gonadotrophin secretion in a pituitary cell bioassay were determined by culturing rat pituitary cells in a serum-free medium to which inhibin and a partially purified preparation of gonadotrophin surge inhibiting factor (GnSIF) were added. The samples were treated with an anti-inhibin antiserum and basal and GnRH-stimulated FSH and LH secretion were measured by radioimmunoassay of the culture supernatant. Inhibin had a dose-dependent inhibitory effect on both basal and GnRH-stimulated FSH secretion and also on GnRH-stimulated LH secretion. Anti-inhibin antibody blocked the inhibition of FSH secretion, except at the highest two doses of human inhibin (1.25 ng ml⁻¹, $P = 0.04$; 2.5 ng ml⁻¹, $P = 0.0004$). The GnSIF preparation inhibited basal and GnRH-stimulated FSH and GnRH-stimulated LH secretion and was not affected by the anti-inhibin antibody. The novel hormone, GnSIF, is different from inhibin.

Introduction

Ovarian peptide hormones have important effects on pituitary gonadotrophin secretion. Inhibin has been shown to have effects, at different concentrations and in different conditions, on basal secretion of FSH and on both GnRH-stimulated FSH and LH secretion (Burger, 1992). A number of groups have proposed the existence of an inhibitory factor, different from inhibin, that is produced by ovarian follicles and suppresses GnRH-stimulated LH, and possibly also FSH, secretion. This factor has been named gonadotrophin surge inhibiting (Sopelak and Hodgen, 1984) or attenuating (Messinis and Templeton, 1989) factor (GnSIF/GnSAF).

A 37 kDa monomeric polypeptide with GnSIF bioactivity, purified from a rat Sertoli cell culture was found to inhibit both GnRH-stimulated LH and basal FSH secretion (Tio *et al.*, 1994). Other groups are attempting to purify GnSAF from pig (Danforth *et al.*, 1987; Danforth and Cheng, 1993) and human (Fowler *et al.*, 1990) follicular fluid. The hypothesis is that GnSAF antagonizes the priming of the pituitary gland by GnRH and raises its threshold to the action of GnRH, preventing a premature LH surge. The surge may then occur when high serum oestradiol concentrations increase the sensitivity of the pituitary to GnRH, so overriding the inhibitory factor. If secretion of the putative GnSAF/GnSIF is deficient, hypersecretion of LH might result, as seen in women with the polycystic ovary syndrome (PCOS) (Balen and Jacobs, 1991).

The aim of this study was to test the hypothesis that inhibin and GnSAF are different by examining the differential effects of

inhibin and a highly purified sample of pig follicular fluid, which contained only GnSAF/GnSIF bioactivity (Danforth and Cheng, 1993), and to observe the effect of anti-inhibin antibodies on both of these activities.

Materials and Methods

The methodology for the pituitary cell bioassay was described by Balen *et al.* (1995). Anterior pituitary glands were obtained from adult female (200–250 g) Wistar rats. The animals were killed by CO₂ anaesthesia and asphyxiation and, after removal of the pituitary gland, the posterior lobe was dissected free and discarded. The anterior lobes were trypsinized in a solution containing 0.5% (w/v) trypsin (Sigma, Poole) and 0.1% (w/v) DNase (Sigma) and the pituitary cells were then mechanically dispersed using a 5 ml plastic pipette. The number of dead cells was assessed by Trypan blue exclusion and the preparation was used only if the cell viability was greater than 95%.

The suspension of cells was dispensed into 24-well culture plates (Falcon 3047 plates, Becton Dickinson, NJ) in a final concentration of 4×10^5 cells ml⁻¹ per well. A serum-free medium was used consisting of Dulbecco's modified essential medium/Ham's F-12 medium (DME/F-12, Sigma) plus a combination of 10 mg insulin ml⁻¹ (Sigma) and 10 mg transferrin ml⁻¹ (Sigma). The medium was supplemented with 0.1% (w/v) albumin, 1.2 g sodium bicarbonate l⁻¹, 10^5 units l⁻¹ (100 mg l⁻¹ penicillin, 100 mg l⁻¹ streptomycin and 2 mg amphotericin l⁻¹) (Sigma).

The pituitary cells were incubated at 37°C in an atmosphere of 100% humidity and 5% CO₂. The culture medium was buffered with bicarbonate to maintain a pH of between 7.2 and

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7.6. The cells were incubated for 24 h, after which samples were added for 48 h (basal culture) and then 10^{-8} mol GnRH l^{-1} was added for 4 h. Samples were added randomly to quadruplicate wells. The 48 h basal culture was used for measurement of FSH secretion and the effects of FSH-inhibitory factors, as negligible amounts of LH are secreted in the absence of GnRH (Balen *et al.*, 1995). The 4 h GnRH stimulation was used for assay of FSH and LH secretion and the effects of FSH- and LH-inhibitory factors. The supernatants were frozen immediately at -20°C and stored until the gonadotrophin concentrations were measured.

Radioimmunoassay of rat gonadotrophins

Materials for the radioimmunoassay of rat LH and FSH were kindly supplied by the National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), Baltimore. The reference preparations were NIDDK-rFSH-RP-2 and NIDDK-rLH-RP-3. The sensitivity of the FSH assay was 3.0 ng ml^{-1} and the sensitivity of the LH assay was 0.8 ng ml^{-1} . Intra- and interassay coefficients of variation were calculated using internal standard preparations as controls; they were, respectively, 8.7% and 7.9% for FSH and 6.9% and 8.8% for LH.

Inhibin dose-response

Two different preparations of inhibin (pig and human) were used because the anti-inhibin antibody was raised against human inhibin and the preparation of GnSIF was purified from pig follicular fluid. The pig preparation was the First International Standard for Porcine Inhibin (Code 86/690) (Gaines Das *et al.*, 1992) in doses of 0.2, 2.0, 6.25, 12.5, 20, 25, 50, 100 and 200 iu ml^{-1} . The second preparation was recombinantly derived 32 kDa human inhibin A, which was an NIBSC ampouled preparation, with a nominal content of $5\text{ }\mu\text{g}$ per ampoule. In the assay system used, $1\text{ }\mu\text{g}$ had a nominal activity equivalent to 40 000 units of pig inhibin (86/690) and so doses of 0.15625, 0.3125, 0.625, 1.25, 2.5 and 5 ng ml^{-1} were given. This conversion factor was used in all of the experiments in this study, pending the definitive International Standard unit for human inhibin.

Inhibin immunoprecipitation with ovine anti-inhibin antibody

Immunoprecipitation experiments were performed using ovine anti-human 1-23 alpha inhibin antibody (Y33), kindly provided by A. McNeilly (Medical Research Council Reproductive Biology Unit, Edinburgh). Non-immunized normal sheep serum (NSS) was also provided as a control. Immunoprecipitation was carried out using Protein-G sepharose (Sigma). The anti-inhibin antibody and the normal sheep serum were incubated with the Protein-G sepharose in a 1:1 ratio (sample (ml):Protein-G sepharose (mg)). The incubation was performed overnight at 4°C . After washing with buffered medium (three times), the Protein-G sepharose-anti-inhibin antibody complex and Protein-G sepharose-NSS were incubated with blank medium, human follicular fluid, pig inhibin (50 iu ml^{-1}) and recombinant human inhibin at four concen-

trations (0.3125, 0.625, 1.25 and 2.5 ng ml^{-1}). The absorbed inhibin was removed by pelleting the Protein-G sepharose-anti-inhibin antibody complex by centrifugation (400 g, 20 min).

A highly purified form of GnSIF was kindly provided by D. Danforth (Ohio State University). The GnSIF preparation was derived from pig follicular fluid (pFF) via a number of purification steps including charcoal extraction, heparin-sepharose, Q-sepharose, Mono-S, hydroxylapatite and gel permeation chromatography (Danforth and Cheng, 1993). This preparation of GnSIF gives two protein bands on electrophoresis (Danforth and Cheng, 1993), of which only one has GnSIF bioactivity, a molecular mass of about 71 kDa and a novel amino-terminal sequence (D. R. Danforth, personal communication). The GnSIF preparation was added at doses of 2, 4 and $8\text{ }\mu\text{g ml}^{-1}$. As the GnSIF had been purified from pig follicular fluid, pig inhibin (50 iu ml^{-1}) was used as an additional control in these experiments.

Statistical analysis

The mean ($\pm$ SEM) was calculated for each group of replicated samples. The ED_{50} value was calculated as the dose at which 50% inhibition was achieved compared with the controls. One-way analysis of variance was used to compare the differences between the means of the samples and a value of $P < 0.05$ was considered to be significant. The analyses were performed using the Minitab statistical package (Minitab Inc., New York).

Results

Pig inhibin (1st I.S., NIBSC 86/690) had a dose-dependent inhibitory effect on both basal and GnRH-stimulated FSH secretion and a less marked effect on GnRH-stimulated LH secretion (Fig. 1). At least 6.25 iu ml^{-1} and 12.5 iu ml^{-1} , respectively, were required to obtain inhibition of basal and GnRH-stimulated FSH secretion. The ED_{50} value for basal FSH was $11\text{ iu inhibin ml}^{-1}$ and, for GnRH-stimulated FSH, 17 iu ml^{-1} . The ED_{50} value was not achieved for either basal or GnRH-stimulated LH secretion, although the ED_{25} value for the latter was 40 iu ml^{-1} . There was a similar effect of recombinant 32 kDa human inhibin A on FSH secretion, but its effect on LH secretion was less pronounced (Fig. 2). The ED_{50} value for basal FSH was $0.8\text{ ng inhibin ml}^{-1}$ and, for GnRH-stimulated FSH, 3.9 ng ml^{-1} . The ED_{50} value was again not achieved for either basal or GnRH-stimulated LH secretion, and the ED_{25} value for the latter was 4.5 ng ml^{-1} .

Two separate experiments were performed using the preparation of GnSIF, with similar results. The data from one of these experiments are presented here. There was significant inhibition of basal FSH secretion by recombinantly derived human (0.313, 0.625, 1.25 and 2.5 ng ml^{-1}) and purified pig (50 iu ml^{-1}) inhibin (Fig. 3). The four doses of human inhibin inhibited basal FSH secretion by 61.6%, 75.4%, 75.7% and to below the sensitivity of the radioimmunoassay, respectively, and the single dose of pig inhibin achieved 75% inhibition. Anti-inhibin antibody completely blocked the inhibition of FSH secretion, except at the highest two doses of human inhibin

Fig. 1. Dose-response curve of cultured rat pituitary cells to pig inhibin, illustrating the effects of inhibin on (a) basal (○) and GnRH-stimulated (●) FSH secretion, and (b) basal (○) and GnRH-stimulated (●) LH secretion. Basal secretion was measured over 48 h followed by 4 h stimulation with 10^{-8} mol GnRH l⁻¹. ED₅₀ value for basal FSH was 11 iu inhibin ml⁻¹, for GnRH-stimulated FSH, 17 iu ml⁻¹. The ED₅₀ value was not achieved for either basal or GnRH-stimulated LH secretion, although the ED₂₅ value for the latter was 40 iu ml⁻¹.

Fig. 2. Dose-response curve of cultured rat pituitary cells to recombinant human inhibin, illustrating the effects of inhibin on (a) basal (○) and GnRH-stimulated (●) FSH secretion, and (b) basal (○) and GnRH-stimulated (●) LH secretion. Basal secretion was measured over 48 h followed by 4 h stimulation with 10^{-8} mol GnRH l⁻¹. The ED₅₀ value for basal FSH was 0.8 ng inhibin ml⁻¹, for GnRH-stimulated FSH, 3.9 ng inhibin ml⁻¹. The ED₅₀ value was not achieved for either basal or GnRH-stimulated LH secretion and the ED₂₅ value for the latter was 4.5 ng ml⁻¹.

(1.25 ng ml⁻¹, $P=0.04$; 2.5 ng ml⁻¹, $P=0.0004$, compared with control). However, there was a significant effect of the anti-inhibin antibody on all doses of human inhibin (0.313 ng ml⁻¹, $P=0.0001$; 0.625 ng ml⁻¹, $P<0.0001$; 1.25 ng ml⁻¹, $P=0.002$ and 2.5 ng ml⁻¹, $P<0.0001$) and the pig inhibin ($P<0.0001$). With respect to GnRH-stimulated FSH secretion, the preparation was more sensitive to lower doses of inhibin

(data not presented) and maximal suppression was achieved with 0.3 ng ml⁻¹; the anti-inhibin antibody affected all doses of inhibin. The four doses of human inhibin (0.313, 0.625, 1.25 and 2.5 ng ml⁻¹) inhibited GnRH-stimulated FSH secretion by 51.0%, 51.6%, 47.8% and 50.3%, respectively, and the single dose of pig inhibin achieved 52.2% inhibition. There was a significant effect of the anti-inhibin antibody on all doses of

Fig. 3. The effect of recombinantly derived human inhibin, pig inhibin and gonadotrophin surge inhibiting factor (GnSIF) on (a) basal and (b) GnRH-stimulated FSH secretion and (c) basal and (d) GnRH-stimulated LH secretion. Pig inhibin was used as a control as the GnSIF was derived from pig follicular fluid. The samples were added untreated (□) and after treatment with an anti-inhibin antibody (■). *Significantly different from control; + significant inhibition of inhibin effect by anti-inhibin antibody.

human inhibin (0.313 ng ml⁻¹, $P=0.001$; 0.625 ng ml⁻¹, $P=0.0003$; 1.25 ng ml⁻¹, $P=0.0003$ and 2.5 ng ml⁻¹, $P=0.0008$) and on the pig inhibin ($P=0.014$). Basal LH secretion was suppressed by pig inhibin but not by human inhibin (Fig. 3). Treatment with anti-inhibin antibody reversed the effect of pig inhibin and caused a slight stimulation at some doses, possibly owing to a nonspecific protein effect. GnRH-stimulated LH secretion was inhibited by both the pig inhibin (17.1% inhibition) and the highest two doses of human inhibin (1.25 ng ml⁻¹, 23.2% and 2.5 ng ml⁻¹, 35.1% inhibition) and these effects were lost after treatment with anti-inhibin antibody (pig inhibin, $P=0.014$; human inhibin 1.25 ng ml⁻¹ and 2.5 ng ml⁻¹, $P<0.0005$).

The GnSIF preparation inhibited basal FSH (8 µg ml⁻¹, $P=0.0003$; 4 µg ml⁻¹, $P=0.003$; 2 µg ml⁻¹, $P=0.015$ compared with control) with an ED₅₀ value of 8 µg ml⁻¹. These effects of GnSIF were not affected by the anti-inhibin. Only the highest dose of GnSIF (8 µg ml⁻¹) inhibited GnRH-stimulated FSH secretion ($P=0.003$) and the ED₅₀ value was 7 µg ml⁻¹. GnSIF had no significant effect on basal LH secretion but significantly inhibited GnRH-stimulated LH secretion at 4 µg ml⁻¹ and 8 µg ml⁻¹ ($P=0.0033$ and $P=0.00047$, respectively). The anti-inhibin antibody did not remove the inhibitory effects of GnSIF in these experiments.

Discussion

An anti-inhibin antibody was used to determine the differential effects of inhibin and a partially purified preparation of GnSIF on gonadotrophin secretion by a rat pituitary bioassay.

Because the GnSIF was purified from pig follicular fluid and an anti-human inhibin antibody was used, both pig and human inhibin were used in the bioassay. There is considerable cross-species conservation of the inhibin molecule and both pig and human inhibin have biological effects on the secretion of FSH by rat pituitary cells (Gaines Das *et al.*, 1992; Rivier *et al.*, 1991). The First International Standard preparation for pig inhibin (NIBSC 86/690) was used in some of the experiments and in others an NIBSC-ampouled preparation of recombinant DNA derived human inhibin was used.

In rats treated with FSH, the preovulatory gonadotrophin surge of FSH and LH is inhibited; this effect is reversed by the administration of an anti-inhibin antibody (Culler, 1992). However, the same anti-inhibin antibody does not affect GnSIF bioactivity in human serum and follicular fluid (Byrne *et al.*, 1995; Fowler *et al.*, 1995). Furthermore, the purified preparation of GnSIF from a Sertoli cell culture (Tio *et al.*, 1994) inhibits both GnRH-stimulated LH and basal FSH release.

In the study reported here the highly purified preparation of GnSIF had a dose-dependent inhibitory effect on GnRH-stimulated FSH and LH secretion. In addition, there was an inhibitory effect on basal FSH secretion, in the absence of any signs of toxicity. The inhibition of basal FSH is contrary to the findings of other workers (D. R. Danforth, personal communication); this may be due to different bioassay and experimental designs. Although the original hypothesis was that the effects of GnSIF are on GnRH-stimulated gonadotrophin secretion, others have occasionally observed such an inhibitory effect on basal FSH secretion (Tio *et al.*, 1994, P. Fowler, personal communication). A possible explanation is that other inhibitors of basal FSH secretion, such as follistatin,

are present in the partially purified preparations that have been used, although none was detected in the GnSIF that was added in the above experiments (D. R. Danforth, personal communication).

The precise actions of GnSAF/GnSIF will not be known until the protein is completely purified and its properties examined thoroughly. It seems reasonable to postulate that inhibin and other non-steroidal hormones have effects at different stages of the oestrous cycle and at different serum concentrations, and are involved in the fine-tuning of pituitary secretion of gonadotrophins.

Anti-human inhibin antibody immunoneutralized the effects of both human and pig inhibin. There is considerable similarity between the structure of the α - and β -chain within and between species (Forage *et al.*, 1987). The sequences of the α -chain show 85% similarity between cattle, humans and pigs. Even greater conservation is seen with the β_A -subunit; there is a single amino acid difference in sheep molecules from those of cattle, pigs, rats and humans (Forage *et al.*, 1987). Where it is present, the β_B -subunit shows over 85% conservation of structure. Therefore, it is not surprising that the anti-human inhibin antibody recognizes pig inhibin. The observation that the anti-inhibin antibody did not inhibit the bioactivity of GnSIF demonstrates that the putative pig GnSIF is different from inhibin and confirms the observations of Byrne *et al.* (1995) and Fowler *et al.* (1995). In the study reported here a preparation of almost completely purified GnSIF was added to the bioassay, and FSH and LH secretion were inhibited. The effects of GnSIF were not inhibited by the anti-inhibin antibody, demonstrating that pig GnSIF is different from inhibin.

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