Exit of inhibin-like bioactivity from
gonadotropin-stimulated prepubertal pig ovaries

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Abstract. Inhibin-like bioactivity (IBA) was assessed by bioassay in the follicular fluid, ovarian venous plasma, and peripheral plasma of immature gilts in which follicular development had been synchronized by administration of pregnant mare serum gonadotropin (PMSG) and hCG. IBA was detectable in the pooled follicular fluid of control animals, but the concentration as well as the total content increased markedly with the growth of the follicle induced by PMSG. About 36–72 h after PMSG treatment, IBA was found consistently in the venous plasma of both ovaries, although in controls as well as after ovulation (i.e. +48 h after hCG) IBA was not detected. The amount of IBA exiting the ovary by way of venous circulation was only a small fraction (<1%) of the total activity calculated to be in each ovary at a given time. IBA was undetectable in the peripheral plasma at any sampling time. On the basis of these results we could speculate that other routes of entry of inhibin into peripheral circulation may exist in the immature pig and/or that the predominant action of IBA might be within the follicle itself.

Inhibin belongs to a class of novel protein and peptide factor(s) of the gonads in which there is current interest in understanding their biochemistry and physiological significance (Sairam & Atkinson 1984). The selective suppression of secretion of FSH by the pituitary is regarded as one of the principal actions of ovarian inhibin (De Jong & Sharpe 1976; Schwartz 1983). This protein is a product of granulosa cells and is secreted into the follicular fluid (Erickson & Hsueh 1978; Henderson & Franchimont 1981). Inhibin-like bioactivity (IBA) is stimulated by gonadotropin administration and significant amounts of it have been shown to appear in the peripheral circulation of pregnant mare serum gonadotropin (PMSG) primed immature rats (Lee et al. 1981), mice and rabbits (Lee 1984). Gonadotropin treatment increases inhibin in human follicular fluid (Channing et al. 1984). Inhibin levels also increase in large healthy follicles during spontaneous cycles as reported for ovine (Tsonis et al. 1983), bovine (Henderson et al. 1984), and pig (Van de Wiel et al. 1983). As pig ovarian inhibin is a glycoprotein (Sairam et al. 1984; Mason et al. 1985), the nature and extent of glycosylation or other processing could be influenced by follicular development and domestic animals such as pigs provide a good model to explore such patterns. Recent studies with FSH and LH wherein proper glycosylation of the subunits are critical for hormonal activity, may be cited as an example of post-translational modifications of hormonal activity (Sairam & Bhargavi 1985). The object of the present investigation was to analyze the changes in IBA in the ovarian follicular fluid of pigs in which the development of a large number of follicles were precisely synchronized by PMSG/hCG treatment. Previous work (Ainsworth et al. 1980) has shown that the development, maturation and function of the porcine ovarian follicle are controlled by gonadotropin stimulation. We have measured by in vitro bioassays using dispersed
pituitary cell cultures, IBA in follicular fluid, ovarian venous plasma (OVP), and peripheral plasma (PP) of pigs treated with PMSG and hCG. A preliminary report of this work has appeared recently (Sairam & Downey 1984).

Materials and Methods

Design of the experiment and animals
Synchronization of follicular development was done essentially according to the schedule of Ainsworth et al. (1980), except that the dose of PMSG was increased to 1000 IU. Fifteen prepubertal gilts (60–65 kg) were randomly allotted to one of five groups (A–E). Each animal received hormones (PMSG/hCG) according to the following protocol.

1000 IU PMSG

\[ \begin{array}{c|c|c|c|c|c}
  -72 h & -36 h & 0 h & +36 h & +48 h & \text{Sampling time} \\
  \hline
  500 IU hCG & A & B & C & D & E
\end{array} \]

All the animals except the first control group (A) received 1000 IU of PMSG. Seventy-two hours later (0 h with respect to hCG), two groups (D and E) were also treated with 500 IU of hCG. At time intervals as indicated, the gilts were anaesthetized and mid-ventral laparotomy was performed for exteriorization of both ovaries. Ovarian (left and right) venous blood was collected and the ovaries were removed for separation of follicles according to size (4–6, 7–8 and 10 mm). Follicular fluid was aspirated from individual follicles of all groups except control, where it was possible to collect only a pool from follicles of \( \leq 2 \) mm size. A peripheral blood sample was also collected from the jugular vein of each animal at the time of surgery. The plasma and clear (centrifuged) follicular fluids were stored frozen at \(-20^\circ\text{C}\) until bioassayed for IBA. A total of 129 samples were thus collected for assessment.

Inhibin bioassay
The IBA in the samples was assessed by their ability to suppress FSH secretion by rat anterior pituitary cells in culture (Channing et al. 1981), as adopted in our laboratory (Sairam et al. 1984). Briefly, dissected anterior pituitaries of 34-day-old male rats were minced and digested for 20 min with a mixture of collagenase, DNAse (Worthington, NJ) and dispase (Boehringer Mannheim, Montreal, Canada). The dispersed cells were washed and suspended in Dulbecco’s modified Eagles medium (Gibco, Long Island, NY) containing 7.5% foetal calf serum and plated in 0.5 ml on poly-L-lysine coated multiwell plates. Following attachment for 72 h, the cells were incubated in the presence of \( 10^{-8} \) mol/l oestradiol-17\( \beta \) and \( 10^{-6} \) mol/l progesterone in 7.5% foetal calf serum. In agreement with Channing et al. (1981), the inclusion of steroids served to elevate basal FSH secretion from these male rat pituitary cells under our culture conditions. A stable laboratory standard of pig follicular fluid inhibin was dissolved in medium containing 1% bovine serum albumin (Sigma, St. Louis, MO) and used at 5–6 doses in triplicate (Sairam et al. 1984). At least 3 dilutions of the test samples were also assayed at each time in triplicates. After 48 h of incubation, the medium was analyzed for FSH content by radioimmunoassay using kits supplied by the National Pituitary and Hormone Program (NIADDK, Bethesda, MD). Inhibin potencies were calculated by parallel line bioassay estimates (Finney 1964) and are expressed in terms of our laboratory standard (Sairam et al. 1984). The index of precision of the bioassay was 0.16–0.2 and inter-assay variation of the standard preparation was 6.4% (\( N = 25 \)). The assay sensitivity was 0.08 unit per well and anything below this was considered undetectable or not significant. One unit of activity was defined as that amount of our standard (12.4 ± 0.8 µg, \( N = 25 \)) required to reduce basal FSH secretion by 50%. The inhibin potency of our laboratory standard was estimated to be 0.16 ± 0.02 (\( N = 6 \)) times that of the rete testis fluid (RTF) preparation provided by Dr G. Baker of Australia and based on the protein content stated on the vial. This has been included as a point of validation but was not used in expressing our results.

Results
The estimated size, number and volume of follicles in the five different groups are shown in Table 1. The response induced by hormonal treatment is similar to that reported by Ainsworth et al. (1980).

In order to evaluate IBA in the raw fluids, it was necessary to validate the assay under our conditions. As shown in Fig. 1, both follicular fluid and ovarian venous plasma (taken from groups B and C) at various dilutions inhibited FSH secretion from the rat pituitary cells in a manner parallel to the standard. The maximal final concentration of progesterone and oestradiol-17\( \beta \) in the different volumes of follicular fluids included in the inhibin bioassay was estimated to be \( 10^{-6} \) and \( 1.5 \times 10^{-8} \) mol/l, respectively, on the basis of previous data (Ainsworth et al. 1980). These concentrations were not very different from the exogenous addi-

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Table 1.
Estimate of size and volume of follicles induced by gonadotropin treatment in immature pigs

<table>
<thead>
<tr>
<th>Treatment and animal</th>
<th>Left ovary</th>
<th>Right ovary</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average diameter (mm) and (number)</td>
<td>Volume of follicle (µl)/volume of follicular fluid per ovary (ml)</td>
</tr>
<tr>
<td>Control 36 h</td>
<td>(3) ≤ 2</td>
<td>4.2/N.C.</td>
</tr>
<tr>
<td>post-PMSG 72 h</td>
<td>(1) 5 (28)</td>
<td>66/1.83</td>
</tr>
<tr>
<td></td>
<td>(2) 5 (30)</td>
<td>66/1.97</td>
</tr>
<tr>
<td></td>
<td>(3) 7 (31)</td>
<td>180/5.58</td>
</tr>
<tr>
<td>post-PMSG 36 h³</td>
<td>(1) 8 (11)</td>
<td>268/2.42</td>
</tr>
<tr>
<td></td>
<td>(2) 6 (22)</td>
<td>113/2.49</td>
</tr>
<tr>
<td></td>
<td>(3) 6 (15)</td>
<td>113/1.70</td>
</tr>
<tr>
<td>post-hCG 48 h</td>
<td>(1) 8 (21)</td>
<td>268/5.63</td>
</tr>
<tr>
<td></td>
<td>(2) 8 (24)</td>
<td>268/6.43</td>
</tr>
</tbody>
</table>

1 The total number of small follicles in the control group were not determined. Hence, volume of the follicular fluid per ovary could not be calculated (N.C.).

2 Volume of each follicle was calculated assuming a spherical shape and using the estimated average diameter.

3 No samples were collected from the third animal in this group, as it had not responded to PMSG treatment.

In addition to these two steroids included in the assay medium. Moreover, in the presence of 10⁻⁶ mol/l progesterone and 1.5 × 10⁻⁸ mol/l of E₂, castrated pig serum did not decrease basal FSH secretion. This permitted the use of raw fluids in the assay without any attempts to remove these and other steroids. On the basis of our previous data (Sairam et al. 1982) we felt that charcoal treatment of follicular fluid may lead to variable losses in inhibin bioactivity and thereby introduce some uncertainty.

IBA was present in the follicles at all stages, including the control group of immature gilts. The mean IBA content of individual follicles selected at random from either the left or right ovary was no different from those obtained using pooled fluid from each ovary of the animals within the same group (Table 2). The concentration in the follicular fluid, however, rose dramatically following PMSG treatment (Table 2). About 36 h after PMSG, when follicular size had significantly increased, the IBA concentration was more than doubled (P < 0.01 groups A and B) and was highest in group C, 72 h following PMSG injections (i.e. 0 h hCG). From 36 to 72 h post-PMSG, there was approx 33% increase in IBA concentration, coincident with slightly larger follicles. Following hCG treatment, the IBA concentration decreased (P < 0.05 group C vs D), although the size of the follicle was still increasing (group D). Because of the increased follicular volume, the total activity per ovary in group D remained the same as in group C. In the last group, IBA was present only in the few non-ovulated follicles.
Comparison of the effects of our laboratory standard of PFF inhibin protein(s) (●—●), ovarian venous plasma (□—□), and follicular fluid (▲—▲) of immature pigs, on FSH release by cultured rat pituitary cells. The secretion of 32.9 ± 0.9 ng per well (N = 8) of iodination grade rat FSH (NIH) in control cells was considered 100% for calculation of inhibitory responses. Corresponding amounts of standard or test materials had no direct effect in the rFSH radioimmunoassay. Bars show mean ± SEM.

<table>
<thead>
<tr>
<th>Group</th>
<th>Status</th>
<th>IBA units/ml</th>
<th>Estimated content per ovary</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ovarian venous plasma</td>
<td>Follicular fluid (individual)</td>
</tr>
<tr>
<td>A</td>
<td>Control</td>
<td>N.D.</td>
<td>N.C.</td>
</tr>
<tr>
<td>B</td>
<td>36 h post-PMSG</td>
<td>127 ± 25 (5)</td>
<td>3918 ± 335 (10)</td>
</tr>
<tr>
<td>C</td>
<td>72 h post-PMSG</td>
<td>84 ± 3 (4)</td>
<td>5370 ± 437 (11)</td>
</tr>
<tr>
<td></td>
<td>(0 h hCG)</td>
<td>15–161 (2)</td>
<td>3092 ± 279 (8)</td>
</tr>
<tr>
<td>D</td>
<td>+36 h post-hCG</td>
<td>N.D.</td>
<td>N.C.</td>
</tr>
<tr>
<td>E</td>
<td>+48 h hCG</td>
<td>N.D.</td>
<td>N.C.</td>
</tr>
</tbody>
</table>

IBA was estimated by bioassay in pituitary cell cultures and expressed in terms of a lyophilized laboratory standard of pooled pig follicular fluid (with 1 unit being equivalent to 12.4 ± 0.8 µg, N = 25 assays). Mean ± SEM. N.D. = not detectable (i.e. below sensitivity of our assay); N.C. = not calculated. Number in parentheses indicates number of samples (mean ± SEM). Estimated content per ovary is calculated on the basis of average volume of follicular fluid in the group. In group E, low IBA was found in the few non-ovulated follicles.
Discussion

On the basis of considerable research experience with this model in studying phenomena such as intrafollicular steroid and prostaglandin production as well as oocyte maturation, we believe that the PMSG/hCG treated pig is a representative of the pubertal situation (Ainsworth et al. 1980; Tsang et al. 1985). This prompted us to employ the same protocol to evaluate the dynamics of inhibin-like bioactivity with respect to follicular growth and examine its appearance in circulation in pigs.

A surprising observation of this study was that only a very small amount of IBA was detectable in the ovarian venous plasma (left or right). It was below the detection limit of the assay in groups A and E. The levels in OVP became detectable only in groups B, C, D, in which there were a large number of follicles at a stage of active secretion. From the data in Table 2, we have estimated that only a very small percentage (about 2–5 expressed on a ml basis or <1% of the total ovarian content of IBA) exits the ovary at a given time. The total amount exiting would also be related to the ovarian venous flow which was not measured in this study. We should also note here that some mixing of the ovarian and uterine blood is most likely due to the vascular anatomy of the pig. As a result of this, there may be some dilution of the ovarian venous plasma. Nevertheless, the output by this route would still remain small. This gets further diluted extensively in the peripheral plasma, where it was undetectable in all groups. Only in group B was there some evidence for activity, but the low activity precluded reliable estimates. These will have to await the development, in future, of more sensitive and validated radioimmunoassays, which can provide a true measure of bioactive inhibin levels. Immunoassays which cannot discriminate between active or inactive inhibin forms, if existing, would be of little value in such experiments.

In terms of the dynamics of IBA secretion, the pig model as seen from the present data, is clearly different from that of the immature rat model, wherein Lee et al. (1982) were able to demonstrate activity in the peripheral plasma and estimate half-life of the order of about 30 min following PMSG treatment. In two other polytocous animals, e.g. mouse and rabbit, Lee (1984) observed the appearance of inhibin in circulation after hormone (PMSG) treatment. However, Findlay et al. (1986) were unable to detect IBA in the ovarian or jugular venous plasma of sheep injected with PMSG, an observation similar to that reported here. The vast differences in the total ovarian IBA and the concentration in the venous plasma (Table 2) of the pig raise several important questions. First, is entry by way of venous plasma a major contributor to the endocrine effect of inhibin on FSH secretion? In this regard, Findlay et al. (1986) detected significant IBA in ovarian lymph, but not venous plasma of PMSG primed ewes and suggested entry into the lymphatic system as a possible means of exit from the ovary. However, this possibility was not examined in the present study. Secondly, could glycosylation or other form of active secretory mechanism be functioning to regulate entry of a 32000 Dalton protein such as inhibin (Ling et al. 1985)? Finally, if only a small amount of actively synthesized IBA ever leaves the ovary for its peripheral site of action at the pituitary and/or the hypothalamic level, then its major function could be autocrine or paracrine in nature. In this case, these functions may be of greater physiological significance than the preferential inhibition of gonadotropin secretion. Purified porcine inhibins have been shown to modulate FSH inducible aromatase activity in immature oestrogen-treated hypophysectomized rats (Ying et al. 1986). Animal model such as that used in the present study may be useful in analyzing these and other effects in a homologous system. Such studies are presently under way.

Acknowledgments

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References


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