Possible involvement of inhibin in the interrelationship between numbers of antral follicles and peripheral FSH concentrations in female rats

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Summary. The possible dependence of peripheral concentrations of FSH on a non-steroidal, ovarian factor, was studied in adult female rats. Increases in FSH levels during the periovulatory period were not correlated with decreases of steroid concentrations, and administration of steroids did not result in a reduction of FSH levels to basal values. However, a negative correlation between FSH levels and numbers of large follicles (volume ≥ 200 × 10^4 μm^3) was demonstrated, and injection of steroid-free bovine follicular fluid, which contains inhibin-like activity, suppressed FSH levels to basal values.

These results suggest that an ovarian, inhibin-like factor is involved in the fast regulation of FSH concentrations in the periovulatory period, and that this inhibin-mediated control of FSH might play a role in the regulation of the number of follicles maturing in female rats.

Introduction

Campbell & Schwartz (1977) provided evidence that in adult female rats the control of pituitary secretion of follicle-stimulating hormone (FSH) may differ from that of luteinizing hormone (LH). Secretion of FSH, but not of LH, is greatly enhanced about 5–12 h after unilateral ovariectomy (Welschen & Dullaart, 1974; Campbell, Schwartz & Gorski-Firlit, 1977; Butcher, 1977; Welschen, Dullaart & de Jong, 1978), or, in intact rats, immediately following the ovulatory surge of LH release (Gay, Midgley & Niswender, 1970). Evidence is accumulating that in such circumstances the rise of FSH secretion is not dependent on a drop in peripheral levels of ovarian steroid hormones. In studies on steroid levels after unilateral ovariectomy there was no decrease of oestradiol levels during the first 24 h (Campbell et al., 1977; Butcher, 1977; Welschen et al., 1978). Only Chappel & Barraclough (1977) found decreased oestradiol levels after ovariectomy in pro-oestrus in 4-day cyclic rats. However, replacement therapy with oestradiol (Chappel & Barraclough, 1977; Butcher, 1977) was ineffective in preventing the acute rise in FSH levels after unilateral ovariectomy, whereas antiserum against oestradiol-17β injected into intact rats did not provoke such a rise (Butcher, 1977). Peripheral levels of progesterone were decreased immediately after unilateral ovariectomy, but adrenalectomy, resulting in decreased oestradiol and progesterone levels (Campbell et al., 1977), did not induce an acute rise in FSH secretion.

In 1932, McCullagh postulated that a non-steroidal, specifically FSH-suppressing substance, "inhibin", is secreted by the male gonad. There is considerable evidence for the existence of inhibin in male animals (see for reviews Baker et al., 1976; Setchell, Davies & Main, 1977;
Animals. Adult female rats of a Wistar strain (R-Amsterdam) were kept under controlled conditions of light (light period 05:00–19:00 h) and temperature (22–24°C) and received standard dry pellets and tap water ad libitum. Daily vaginal smears were taken and only rats with 2 consecutive 5-day cycles before the cycle of treatment were used.

Follicular fluid. Cow ovaries were obtained at a local slaughterhouse, and follicular fluid was aspirated from follicles of 10–20 mm diameter immediately after collection of the ovaries. The bovine follicular fluid was stirred with charcoal (50 mg/ml) at 21 ± 1°C for 60 min and centrifuged at 10,000 g for 30 min. After treatment, concentrations (ng/ml) of oestradiol-17β (0.04) and progesterone (0.33) were <1% of the original concentrations (103 and 150 respectively).

Bovine plasma was used as control fluid because it has a protein composition similar to that of follicular fluid (Caravaglios & Cilotti, 1957). Injections of bovine follicular fluid and bovine plasma were given intraperitoneally.

Experiments. All operations, injections and blood collections were performed under light ether anaesthesia. Animals were bled by puncture of the ophthalmic venous plexus. At least 6 rats were used in all groups of control and experimental animals unless otherwise stated. Details of the experiments are given in 'Results'.

Hormone determinations. Serum FSH and LH concentrations were estimated by radioimmunoassay (RIA) as described previously (Welschen et al., 1975). All results are expressed in terms of NIAMDD-rat-FSH-RP1 and NIAMDD-rat-LH-RP1. Inter-assay variability (coefficients of variation) was 16% for FSH and 14% for LH.

Oestradiol, progesterone and testosterone were assayed by radioimmunoassay as described by de Jong, Hey & van der Molen (1973), de Jong, Baird & van der Molen (1974) and Verjans, Cooke, de Jong, de Jong & van der Molen (1973). Results were corrected for recovery after extraction and chromatography on LH-20 microcolumns. The latter technique was used for the assay of oestradiol and testosterone. The detection limits of the various assays, defined as the blank + twice the standard deviation at the level of the blank, were 5 (oestradiol) and 10 (progesterone and testosterone) pg/tube. Interassay variability, calculated from repeated results of pooled plasma samples, varied between 10 and 15%. The main cross-reacting steroids in these assays were dihydrotestosterone (60%, expressed as (mass of ‘measured steroid’ suppressing %B to 50% of B0/mass of cross-reacting steroid causing the same suppression) × 100%) in the testosterone assay; oestrone (3%) and oestriol (5%) in the oestradiol assay and 11β-hydroxyprogesterone (17%) in the progesterone assay.

Follicle counts. Follicles were counted in ovarian sections of 5 μm after routine histological procedures (fixation in Bouin's fluid, staining with haematoxylin and eosin). Follicles were classified using the method of Boling, Blandau, Soderwall & Young (1941), as slightly modified...
by Welschen (1973). The volume classes correspond with a mean follicle diameter and with the stage numbers used by Mandl & Zuckerman (1952) approximately in the following way: 200–499 × 10⁴ μm³ with 350–450 μm and stages 3 and 4; ≥500 × 10⁴ μm³ with ≥450 μm and stages 5 to 8.

Statistics. For statistical analysis of results the Student's t test or the Wilcoxon two-sample test were used. A difference was considered as statistically significant when the double-tail probability was <0.05.

Results

The interrelationship between inhibin-like activity and ovarian steroid hormones on the one hand, and gonadotrophin levels and numbers of antral follicles during pro-oestrus and oestrus on the other were studied in 2 experiments to investigate the effect of bovine follicular fluid on the secondary rise of FSH at pro-oestrus.

Experiment 1

Rats were killed and bled at 4-h intervals between 12:00 h on the day of pro-oestrus and 16:00 h on the day of oestrus. Serum and plasma were used for FSH, LH, oestradiol, 

Text-fig. 1. Peripheral levels (mean ± s.e.m.) of FSH, LH, oestradiol-17β (E₂), progesterone (P) and testosterone (T) and numbers of follicles ≥200 × 10⁴ μm³ in adult female rats (N = 5) between 12:00 h on the day of pro-oestrus and 16:00 h on the day of oestrus.
progesterone and testosterone determinations and ovaries were fixed in Bouin's fluid for follicle counts. The results are given in Text-fig. 1. All hormone concentrations followed the well-known patterns (see Gay & Tomacari, 1974; Nequin, Alvarez & Schwartz, 1975), although the absolute values of testosterone appeared considerably lower than those reported by Gay & Tomacari (1974). Ovulation apparently occurred in all follicles \( \geq 500 \times 10^4 \text{mm}^3 \) between 00:00 and 04:00 h on the day of oestrus. A new group of follicles with a well-developed antrum (size class \( \geq 200 \times 10^4 \text{mm}^3 \)) was observed from 04:00 h on the day of oestrus onwards. During the period between 12:00 h on the day of pro-oestrus and 16:00 h on the day of oestrus there was a significant negative correlation between mean numbers of follicles in this size class and mean peripheral FSH levels \( r = -0.65, n = 8, P < 0.05 \).

**Experiment 2**

At 14:00 h on the day of pro-oestrus the rats received 500 \( \mu \)l bovine follicular fluid or plasma/100 g body weight or 3 silicone-tube implants that were empty or contained oestradiol, progesterone or testosterone. The lengths and outer diameter of the tubings were 5 and 1.4 mm for oestradiol; 5 cm and 3.8 mm for progesterone; and 5 and 3.8 mm for testosterone. Oestradiol and testosterone were mixed 1:10 with cholesterol (w/w). The tubings were placed in phosphate-buffered saline, pH 7.2, overnight before implantation. The rats were bled and killed at 04:00 h and 12:00 h on the day of oestrus for hormone determinations and follicle counts. Of

<table>
<thead>
<tr>
<th>Treatment at 14:00 h on day of pro-oestrus</th>
<th>Time of autopsy (h) on day of oestrus</th>
<th>No. of rats</th>
<th>Hormone conc. (ng/ml)</th>
<th>No. of follicles ( \geq 200 \times 10^4 \text{mm}^3 ) per ovary</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (3 empty tubes + bovine plasma)</td>
<td>04:00</td>
<td>8</td>
<td>394 ± 29</td>
<td>82 ± 4</td>
</tr>
<tr>
<td></td>
<td>12:00</td>
<td>8</td>
<td>274 ± 19</td>
<td>45 ± 6</td>
</tr>
<tr>
<td>Steroid implants* (see text)</td>
<td>04:00</td>
<td>7</td>
<td>328 ± 13</td>
<td>41 ± 5*</td>
</tr>
<tr>
<td></td>
<td>12:00</td>
<td>8</td>
<td>249 ± 25</td>
<td>32 ± 11</td>
</tr>
<tr>
<td>Bovine follicular fluid (500 ( \mu )/100 g body wt)</td>
<td>04:00</td>
<td>7</td>
<td>54 ± 2*</td>
<td>52 ± 14</td>
</tr>
<tr>
<td></td>
<td>12:00</td>
<td>7</td>
<td>136 ± 17*</td>
<td>52 ± 8</td>
</tr>
</tbody>
</table>

† Mean of 2 determinations in pooled plasma of control rats and implant-bearing rats at 04:00 h at oestrus: 220 and 3750 pg testosterone/ml; not determined and 831 pg oestradiol/ml; and 12 and 53 ng progesterone/ml respectively.

* \( P < 0.05 \) compared with corresponding control values.

**Table 2. Influence of administration of testosterone by means of an implant placed subcutaneously or on the surface of each ovary on mean ± s.e.m. plasma concentrations of FSH and numbers of antral follicles at early oestrus**

<table>
<thead>
<tr>
<th>Treatment at 14:00 h on day of pro-oestrus</th>
<th>No. of rats</th>
<th>At autopsy at 16:00 h on day of oestrus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>FSH (ng/ml)</td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>188 ± 8</td>
</tr>
<tr>
<td>Implant, subcutaneous</td>
<td>9</td>
<td>282 ± 18*</td>
</tr>
<tr>
<td>Implant, on ovarian surface</td>
<td>9</td>
<td>559 ± 29*</td>
</tr>
</tbody>
</table>

Mean of 2 determinations of testosterone concentrations (pg/ml) in pooled plasma at 04:00 h on day of oestrus was 720 for controls, 3050 for s.c. implants and 3140 for implants on the ovarian surface.

* \( P < 0.05 \) compared with values in both other groups.
these treatments only injection of bovine follicular fluid (Table 1) resulted in a significant suppression of FSH levels at 04:00 h and 12:00 h on the day of oestrus and in decreased numbers of follicles with a volume of $200-500 \times 10^3 \, \mu\text{m}^3$, but there was no effect on the occurrence of ovulation or on LH levels.

Silicone tubes containing testosterone (length: 4 mm, o.d. 1.4 mm) were implanted either inside the bursa ovarica on the ovarian surface or subcutaneously. The position of the implants was confirmed at autopsy. Implants resulted in peripheral testosterone concentrations of about 4 times those found in control rats (Table 2); the implants on the ovarian surface produced significantly increased FSH levels at 16:00 h on the day of oestrus, and significantly decreased numbers of antral follicles. Implants placed s.c. induced only a marginal increase of serum FSH levels.

**Discussion**

The present results support the hypothesis that in the female rat the ovaries can exert an inhibitory feed-back on the pituitary secretion of FSH via inhibin-like activity during the periovulatory period. The inverse relationship between FSH levels and numbers of follicles of volume $\geq 200 \times 10^3 \, \mu\text{m}^3$ suggests that these follicles secrete inhibin-like activity. Furthermore, antral follicles seem to limit their own number by the inhibin-feedback on FSH secretion.

In the adult female rat injection of inhibin-like material in a dose that causes maximal suppression of FSH levels appears to exert short-term effects on FSH secretion only (de Jong, Welschen, Hermans, Smith & van der Molen, 1978; DePaolo, Wise, Anderson, Barraclough & Channing, 1979; DePaolo, Hirshfield, Anderson, Barraclough & Channing, 1979). Probably inhibin-like activity is inactivated and removed from its target organ within a few hours. If so, changes in inhibin secretion from the ovary may be expected to be followed with a short latency by short-lived changes in FSH secretion.

Since inhibin-like activity has been defined as a factor which specifically suppresses FSH levels (de Jong, 1979) whereas steroid hormones (depending on the dose and time of treatment) influence both FSH and LH secretion, the involvement of inhibin-like activity in diverging changes of FSH and LH levels was studied in the present experiments. In the rat the second phase of FSH release about the time of ovulation occurs in the absence of a high level of LH release (Gay et al., 1970). This second phase of FSH release can be prevented by injection of antiserum to testosterone (Gay & Tomacari, 1974), and was explained as reflecting a facilitatory effect of endogenous testosterone on FSH secretion. The second phase of FSH release can also be prevented by injection of steroid-free follicular fluid (Schwartz & Channing, 1977). The results of the present experiments confirm these latter data. In addition, they demonstrate that increased levels of oestradiol, progesterone and testosterone cannot prevent the second phase of the FSH surge. Thus, if this second phase is induced by diminished inhibitory feedback from the ovary on FSH secretion, it must be due to a relative lack of ovarian release of inhibin-like activity and not to a lack of steroid hormones. Schwartz & Channing (1977) suggest that after a pro-oestrus or an artificial LH stimulus, the oocyte–follicular complex is altered, resulting in secretion of decreased amounts and/or chemically altered inhibin. The second phase of FSH release, thus induced, recruits—as the present experiment shows—a new cohort of follicles of $\geq 200 \times 10^3 \, \mu\text{m}^3$ in size (see also Schwartz, 1969; Welschen & Dullaart, 1976). Furthermore, this cohort of antral follicles, once recruited, seems to suppress FSH secretion to baseline values.

The data from the testosterone-implant experiment seem to confirm the above observations: a subcutaneous implant did not result in decreased numbers of follicles and did not prolong the second phase of FSH release, whereas implants on the ovarian surface did. Androgens produced in the ovary in response to hCG have been shown to act locally to inhibit the effect of oestrogen on follicular growth (Louvet, Harman, Schreiber & Ross, 1975). It therefore seems likely that...
testosterone implants on the ovary, instead of exerting a direct facilitatory effect on FSH release, have inhibited the growth of follicles to the antral stage and thus the secretion of sufficiently large amounts of inhibin to suppress FSH to baseline values. Assuming that this really is the case, the decreased FSH levels found by Gay & Tomacari (1974) after injection of antiserum against testosterone might reflect increased follicle growth and a greater inhibin feedback rather than a reduced direct stimulatory effect of testosterone on FSH release, although such a stimulatory effect has been shown to exist in certain circumstances (Drouin & Labrie, 1976; Juneja, Motta, Vasconi & Martini, 1977; Labrie et al., 1978).

The results of experiments with unilaterally ovariectomized rats also strongly suggest that in other circumstances regulation of FSH levels may occur via inhibin-like activity secreted by antral follicles (Welschen et al., 1978). For comparison these data have been reproduced in Text-fig. 2 and the similarity of the temporal relationship between levels of FSH, LH, steroid hormones and numbers of follicles in (pro)oestrous and in unilaterally ovariectomized rats (Text-figs 1 and 2) is clear. In both cases a decrease of high concentrations of FSH to basal levels is inversely correlated with increasing numbers of healthy follicles $\geq 200 \times 10^4 \, \mu m^3$ to about 25/animal (correlation between mean numbers of follicles and mean peripheral FSH levels in unilaterally ovariectomized rats: $r = -0.81$, $n = 8$, $P < 0.05$). In normal cyclic rats this number of 25 follicles/animal is never exceeded; smaller numbers (about 15/rat) are only found during the last days of the cycle. At that time, the mean diameter is greatly increased, resulting in

Text-fig. 2. Peripheral levels of FSH, LH, progesterone (mean ± s.e.m. for 5–6 samples) and oestradiol-17β (mean of 2–3 determinations in pooled plasma) and mean numbers of antral follicles $\geq 200 \times 10^4 \, \mu m^3$ in adult female rats 0–28 h after unilateral ovariectomy at 09:00 h on Day 2 of dioestrus. Throughout the experimental period, FSH and LH values in control animals ranged between 50 and 100 ng FSH/ml and between 10 and 30 ng LH/ml (Welschen et al., 1978).
a total number of granulosa cells similar to that present in 25 small follicles during the first days of the cycle (for data on granulosa cell numbers see Hage, Groen-Klevant & Welschen, 1978). This suggests that inhibin is produced predominantly by granulosa cells of antral follicles in vivo, as has been shown by Erickson & Hsueh (1978) with in-vitro experiments, and is also supported by data of Welschen et al. (1977) and of Becker, Klupp, Epstein, Seidl & Lunenfeld (1977) who showed that, at least in the cow, medium-sized and large antral follicles contain the highest concentration of inhibin-like activity. The inhibin-feedback mechanism may therefore be involved in the regulation of the number of follicles $\geq 200 \times 10^5 \, \mu m^3$ in size which are to ovulate or to become atretic during each cycle.

A similar control might also exist in the period characterized by the first growth of antral follicles, from Day 21 of life onwards. During this period, the pituitary gland becomes sensitive to inhibin-like activity as present in bovine follicular fluid (Hermans, van Leeuwen, Debets & de Jong, 1980). Levels of FSH, LH, oestriadiol and progesterone and the number of follicles $\geq 200 \times 10^5 \, \mu m^3$, as reported by various workers, reveal time relationships as shown in Text-fig. 3. After the dramatic fall in FSH levels around Day 20, there is a further decrease of FSH levels between 22 and 35 days of age. This decrease is not paralleled by increase of oestradiol levels and there is only a marginal increase of progesterone levels. Since injection of a steroid combination was

Text-fig. 3. Peripheral levels of FSH and LH (Ojeda & Ramirez, 1972; Döhler & Wuttke, 1975; Meijs-Roelofs, Uilenbroek, Osman, & Welschen, 1973a) and of oestradiol-17β (Meijs-Roelofs, Uilenbroek, de Jong & Welschen, 1973b; Döhler & Wuttke, 1975) and progesterone (Meijs-Roelofs, de Greef & Uilenbroek, 1975; Döhler & Wuttke, 1975) and numbers of follicles $\geq 200 \times 10^5 \, \mu m^3$ (Meijs-Roelofs et al., 1973a; and unpublished studies) in intact female rats between 17 and 35 days of age.
only effective in suppressing FSH levels at 25 days of age and not at later ages (Hermans et al., 1980), the involvement of another factor in FSH regulation seems more likely. That this factor might be inhibin-like activity is suggested by the finding that the decrease of FSH levels finds its mirror image in the increase of the number of antral follicles (Text-fig. 3) and the finding that a rapid rise of FSH levels after ovariectomy occurs from 25 days of age onwards. This increase can be prevented by injection of steroid-free bovine follicular fluid (Hermans et al., 1980).

In combination, the present data seem to suggest that from the age of 25–35 days onwards the regulation of FSH secretion is (at least partly) under the control of ovarian inhibin secretion. Inhibin might be produced by the granulosa cells of antral follicles. The data suggest that in late prepubertal and adult female rats a number of granulosa cells, as present in about 25 antral follicles in the size range of 200–500 × 10^3 µm^3 or in smaller numbers of larger follicles, produce amounts of inhibin sufficient to maintain peripheral FSH concentrations at about 100 ng/ml. This level of FSH seems sufficient to keep the number of healthy granulosa cells fairly constant. Any decrease in the number of inhibin-producing granulosa cells (or follicles) results in an enhanced pituitary secretion of FSH during at least 5–20 h after the decrease. The increased FSH levels then seem to stimulate granulosa cell proliferation to such an extent that the critical number required for inhibin levels sufficient to reduce FSH concentrations to basal values is reached.

References


Ovarian regulation of FSH in rats


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