Ovulation rate, follicle population and FSH levels in cyclic rats after administration of an inhibin-neutralizing antiserum

H. J. Sander, P. Kramer*, E. C. M. van Leeuwen*, W. A. van Cappellen*, H. M. A. Meijs-Roelofs* and F. H. De Jong*

Departments of Anatomy and *Endocrinology and Reproduction, Medical Faculty, Erasmus University, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands

REVISED MANUSCRIPT RECEIVED 12 February 1991

ABSTRACT

Ovulation rate, follicle growth, serum FSH and oestradiol concentrations were studied after a single intraperitoneal injection of inhibin antiserum in 5-day-cyclic rats. Control rats received (non-immune) serum from castrated sheep or saline. Rats were injected at 10.00 h on dioestrus-1 (D1), i.e. the day following the day of oestrus, or at 17.00 h on dioestrus-2 (D2). The ovaries were excised at necropsy 48 h after injection, or at first or second oestrus after injection. After routine histology fresh corpora lutea were counted and/or differential follicle counts were made.

Results from rats injected with either (non-immune) serum from castrated sheep or with saline were not different and were therefore combined to form the control group. The activity of inhibin-neutralizing antibodies in the circulation of antiserum-treated rats was reduced by approximately 39% between 8 h and second oestrus after injection, as determined by the binding of purified bioactive radioiodinated 31 kDa bovine inhibin.

Rats were injected on D1 and killed at first oestrus. The number of fresh corpora lutea was significantly higher in antiserum-treated rats than in controls (13.9 ± 0.4 vs 11.8 ± 0.4; P < 0.05). Other rats injected on D1 were killed either 48 h or at the second oestrus after injection. Blood was collected 8, 16, 24 and 48 h and at first and second oestrus after injection. At 48 h after injection differential follicle counts showed that the ovaries of antiserum-treated rats contained approximately 32 more healthy follicles and 11 fewer atretic follicles than controls (both P < 0.05 vs control; data for follicles with volume > 100 x 105 µm³ and diameter > 260 µm). The ovaries of the antiserum-treated group collected at second oestrus contained more corpora lutea than controls (17.5 ± 0.5 vs 13.6 ± 0.4; P < 0.001). Serum FSH levels at 8, 16, 24 and 48 h after antiserum injection were elevated (P < 0.05). Overall oestradiol levels in antiserum-treated rats were increased from 8 to 24 h and at first oestrus (P < 0.05) as compared with control rats. Further rats were injected on D2 and necropsied at first or second oestrus which caused ovulation rate to almost double at first oestrus (antiserum 23.7 ± 1.4 vs control 12.4 ± 0.4; P < 0.01), while at second oestrus there was no difference between antiserum-treated and control rats.

The rise in FSH level after injection of antiserum on D1 caused follicle recruitment in addition to that normally occurring on the morning of oestrus (36 h earlier) and reduced atresia, resulting in a moderately increased ovulation rate on the first and second oestrus after injection. If the interval between antiserum injection and the next oestrus was shortened (injection on D2), ovulation rate was doubled, while on the next oestrus (second) there was no difference compared with controls. It is concluded that inhibin is progressively involved in the control of follicle growth and ovulation rate via its effect on serum FSH levels during the oestrus cycle of the rat.

Journal of Endocrinology (1991) 130, 297–303

INTRODUCTION

The relationship between follicle recruitment and follicle growth on the one hand and serum follicle-stimulating hormone (FSH) levels on the other has been well established in rodents (Greenwald, 1962; Schwartz, 1974; Welschen & Dullaart, 1976; Greenwald & Terranova, 1988), as has the
stimulatory effect of administration of FSH and FSH-containing preparations on the numbers of eggs shed at ovulation (Weifenbach, 1965; Richards, 1980). FSH also stimulates ovarian inhibin production and secretion (Burger, Carson, Davis & Zhiwen, 1987; Hasegawa, Miyamoto, Igarashi et al. 1987). Furthermore, inhibin was shown to be an important, granulosa cell-derived suppressor of pituitary FSH secretion (Schwartz & Channing, 1977; De Jong, Sander, Ultee-van Gessel & van der Molen, 1985; De Jong, 1988; Ying, 1988; Vale, Rivier, Hsueh et al. 1988). Finally, circulating inhibin and FSH levels were shown to have an inverse relationship during the oestrous cycle in the rat (Watanabe, Taya & Sasamoto, 1990). These observations indicate that the number of large antral follicles in the ovaries, the ovarian content and the circulating level of inhibin and the circulating level of FSH are functionally interrelated. Such a relationship can play a major role in the regulation of the number of follicles that ovulate at the end of a cycle.

After passive immunization against inhibin of 4-day-cyclic rats during the pro-oestrous/oestrous period (Rivier, Rivier & Vale, 1986) or during the dioestrus period (Rivier & Vale, 1989; Culler & Negro-Villar, 1989), a selective increase in mean plasma FSH levels was found. Furthermore, the ovulation rate at the first oestrous after immunization was doubled in 4-day-cyclic rats passively immunized on dioestrous-1 or -2 (Rivier & Vale, 1989). Immunization on pro-oestrous caused an elevated ovulation rate only at the second oestrous after injection (Rivier & Vale, 1989). However, changes in ovarian follicle populations following immunoneutralization of inhibin have not yet been reported.

The present study was undertaken to investigate the changes in follicle populations after immunoneutralization of inhibin in more detail. Secondly, ovulation rates at the first and second oestrus after immunization were determined. Data from a pilot experiment have been reported earlier (De Jong, Grootenhuis, Sander et al. 1987).

MATERIALS AND METHODS

Animals

Female rats of a Wistar strain (R-Amsterdam) with regular 5-day oestrous cycles were used. They were kept in conditions of controlled temperature (23 ± 2 °C) and light (lights on from 05.00 to 19.00 h) and allowed free access to standard dry pellets and tap water. Adult litter-mates were divided equally among the treatment groups. Vaginal smears were taken every morning and rats were used only after at least two successive 5-day oestrous cycles.

Antiserum

The inhibin antiserum used in this study was the same as that used in late-pubertal females in the accompanying paper (Sander, Meijs-Roelofs, van Leeuwen et al. 1991). In a parallel experiment (Sander et al. 1991), prepubertal rats were injected with antiserum, (non-immune) serum from castrated sheep, with their respective immunoglobulin (IgG) fractions, or with saline (0-9% (w/v) NaCl). No differences in effect were seen between groups receiving sera or IgG fractions, nor between groups receiving castrated serum or saline. This ruled out non-specific effects of sheep serum. Thus, also in the present study, results from animals injected with (non-immune) serum from castrated sheep or saline were combined and are labelled 'control'. The presence of inhibin-neutralizing antibodies in the circulation of antiserum-treated and control rats was verified by estimating the amount of radiiodinated 31kDa inhibin (McLachlan, Robertson, Burger & DeKretser, 1986) bound by blood samples obtained at 8, 16, 24 and 48 h and at first and second oestrus after injection, as present after second antibody precipitation with rabbit anti-sheep antiserum.

Treatment

Intraperitoneal injections with 0.5 ml/100 g body weight on dioestrous-1 (D1, day following the day of oestrus), sampling of blood by puncture of the ophthalmic venous plexus and necropsy were performed under light ether anaesthesia between 09.00 and 10.00 h; on dioestrous-2 (D2) the injections were given at 17.00 h.

At necropsy on the first or second oestrus or at 48 h after injection, the ovaries were excised, weighed, fixed in Bouin’s fluid, serially sectioned and stained with haematoxylin and eosin. Ovulation rate at first or second oestrus was determined by counting the number of fresh corpora lutea in the sections. Differential counts of antral, healthy and early atretic follicles in ovaries 48 h after injection were made according to Meijs-Roelofs, Osman & Kramer (1982). Volume classes and diameters for antral follicles were defined as follows: class 1, 100–200 × 10³ μm³ and 260–340 μm; class 2, 200–350 × 10³ μm³ and 340–405 μm; class 3, 350–500 × 10³ μm³ and 405–460 μm; class 4, 500–1000 × 10³ μm³ and 460–580 μm; class 5, >1000 × 10³ μm³ and >580 μm. Atretic follicles were counted only if they were at an early stage of atresia, i.e. atresia in progress for 0–24 h, oocyte with nucleus present, and focal or widespread pyknosis in the granulosa cell layer (Osman, 1985).
In experiment 1 animals were injected on D1 and necropsied at first oestrus (control, \( n = 16 \); antiserum-treated, \( n = 9 \)) when the number of fresh corpora lutea was counted.

In experiment 2 rats were necropsied after 48 h or at second oestrus after injection on D1 and follicles (48 h) or corpora lutea (second oestrus) were counted. In the 48-h group, blood was sampled 16 and 48 h after injection (or D3, i.e. the day between D2 and pro-oestrus; \( n = 7, 8 \)). In the group at second oestrus, blood was sampled in subseries either at 8 h (\( n = 6, 5 \)) or at 24 h (\( n = 6, 7 \)), and then in all animals at first oestrus (\( n = 12, 10 \)) and at second oestrus (\( n = 12, 10 \); necropsy). After centrifugation, serum was separated and stored at \(-20^\circ C\) until estimation of hormone levels.

In experiment 3 a group of rats was injected at 17.00 h on D2 and necropsied at first oestrus (\( n = 6, 6 \)) or second oestrus (\( n = 5, 5 \)) when fresh corpora lutea were counted histologically.

**Hormone determinations**

Concentrations of FSH in serum were estimated in duplicate by radioimmunoassay (RIA) with antiovine FSH as antiserum and rat FSH as tracer, and expressed as \( \mu g \) NIADDK-rat FSH-RP-1/litre, as described by Welschen, Osman, Dullaart et al. (1975) and modified by Sander, Meij-Roelofs, van Leeuwen et al. (1986). Within and between assay variations were 8\% and 17\% respectively, the detection limit of the assay being 8 \( \mu g/l \). Oestradiol was estimated by RIA in serum using kits provided by Diagnostic Products Corporation (Los Angeles, CA, U.S.A.); within and between assay variations were <15\% and <19\% respectively at levels below 100 pmol/l.

**Statistical procedures**

Experimental data are presented as means \( \pm \) S.E.M. Analysis of variance (ANOVA) was performed to detect overall differences. Student’s \( t \)-test was used to detect day-to-day differences within and between series. A difference was considered significant if the double-tail probability \( (P) \) was <0-05.

**RESULTS**

Ovarian weights 48 h after injection were not different between antiserum-treated and control rats (antiserum-treated, 72.2±0.2 mg, \( n = 8 \); control, 70.7±2.3 mg, \( n = 7 \)). Ovarian weights and ovulation rates at first and second oestrus are given in Table 1. In rats treated with antiserum on D1, the number of corpora lutea was higher at first oestrus \( (P<0.05) \) and both ovarian weight and number of corpora lutea were higher than in control rats at second oestrus \( (P<0.05) \). In rats injected with antiserum on D2 the number of corpora lutea had nearly doubled and ovarian weight was higher compared with control rats (both \( P<0.001 \)) at first oestrus. Ovarian weight was not different in the groups, whereas ovulation rates were not significantly affected at second oestrus \( (P=0.1) \).

The clearance of antiserum was approximately 39\% over the experimental period (i.e. 10 days). Binding of \(^{125}\text{I}\)-labelled 31 kDa inhibin by inhibin-neutralizing

**TABLE 1. Ovarian weight and number of fresh corpora lutea in antiserum-treated or control rats. Mean \( \pm \) S.E.M. weight of both ovaries and mean \( \pm \) S.E.M. number of fresh corpora lutea per treatment group are shown**

<table>
<thead>
<tr>
<th>Day and time</th>
<th>Treatment</th>
<th>No. of rats</th>
<th>Ovarian weight (mg)</th>
<th>Corpora lutea</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1 10.00 h</td>
<td>Control</td>
<td>1-OE 16</td>
<td>71.2±0.4</td>
<td>11.8±0.4</td>
</tr>
<tr>
<td></td>
<td>Antiserum</td>
<td>1-OE 9</td>
<td>75.2±3.3</td>
<td>13.9±0.4*</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>2-OE 12</td>
<td>84.3±2.0</td>
<td>13.6±0.4</td>
</tr>
<tr>
<td></td>
<td>Antiserum</td>
<td>2-OE 11</td>
<td>100.3±2.6*</td>
<td>17.5±0.5*</td>
</tr>
<tr>
<td>D2 17.00 h</td>
<td>Control</td>
<td>1-OE 6</td>
<td>75.4±1.4</td>
<td>12.4±0.4</td>
</tr>
<tr>
<td></td>
<td>Antiserum</td>
<td>1-OE 6</td>
<td>87.8±2.2**</td>
<td>23.7±1.4**</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>2-OE 5</td>
<td>78.7±2.6</td>
<td>12.8±0.6</td>
</tr>
<tr>
<td></td>
<td>Antiserum</td>
<td>2-OE 5</td>
<td>82.9±1.7</td>
<td>14.8±0.5</td>
</tr>
</tbody>
</table>

\( *P<0.05, **P<0.001 \) vs control (Student’s \( t \)-test).

5-day-cyclic rats were treated with inhibin antiserum or control on dioestrus-1 (D1 = day following oestrus) or dioestrus-2 (D2) and killed at first (1-OE) or second oestrus (2-OE) after injection.
Figure 1. Mean ± S.E.M. numbers of healthy (H) and of early atretic (A) antral follicles with a volume > 100 x 10⁶ μm³ (or diameter > 260 μm) in the ovaries of 5-day-cyclic rats injected on dioestrus-1 with inhibin antiserum (solid bars, n = 8), or their controls (open bars, n = 7) and killed 48 h after injection. (a) Total numbers of follicles with a volume > 100 x 10⁶ μm³. In (b) healthy and in (c) atretic follicles are subdivided into five volume classes (x 10⁶ μm³): class 1, 100–199; class 2, 200–349; class 3, 350–499; class 4, 500–999; class 5, > 1000. Early atretic follicles had an intact oocyte nucleus and focal or widespread pyknosis in the granulosa cell layer.

*P < 0·05 compared with controls (Student's t-test).

Antibodies in the circulation of antiserum-treated rats at 8, 16, 24, 48 h and at first and second oestrus after injection were present in volume classes 2, 3 and 4 (all P < 0·05). The number of follicles in class 5 was reduced (P < 0·05) in the antiserum-treated rats. In ovaries of antiserum-treated animals fewer small and more large atretic follicles were seen than in those of controls; the difference being significant only for atretic follicles in classes 1 and 4. Thus, the total number of follicles in the ovaries of antiserum-treated rats was significantly larger than in control rats (Fig. 1a, healthy + atretic, P < 0·01).

In antiserum-treated and control rats, serum levels of FSH showed a significant time-dependent variation (Fig. 2; P < 0·001); the variation being significantly (P < 0·001) different between the antiserum-treated and the control group. In the antiserum-treated group, 8 h after injection the serum level of FSH had increased (P < 0·01) to 680 ± 64 μg/l compared with 192 ± 6 μg/l in the control group. After, 16, 24 and 48 h the serum level of FSH in the antiserum-treated group remained elevated (P < 0·05) above the levels found in the control group. At first and second oestrus the levels of FSH were not different between groups.

Serum levels of oestradiol also showed a significant, time-dependent variation (P < 0·01; Fig. 3); the time-dependent variation in the antiserum-treated group was significantly (P < 0·01) different from that in the control group. At 24 h and at first oestrus the oestradiol level in the antiserum-treated group was significantly (P < 0·01) higher than in the control group.

Discussion

The antiserum used in this study was shown by van Dijk, Steenbergen, Gielen & De Jong (1986) to
neutralize the ability of follicular fluid to suppress FSH secretion by pituitary cells in vitro. We now report in-vivo effects of this antiserum in cyclic rats, including increased levels of FSH and oestradiol and increased ovulation rate. For the first time the effect of inhibin antiserum on ovarian follicular populations was investigated.

Increased levels of FSH are seen within 8 h of i.p. antiserum injection on D1 in cyclic rats, comparable with those seen at 8 h after unilateral ovariectomy (Welschen, Dullaart & De Jong, 1978; Hermans, van Leeuwen, Debets & De Jong, 1980). Ovulation rates at first and second oestrus following injection on D1 were increased. After antiserum injection on D2 ovulation rate was increased at first oestrus, while at second oestrus no significant increase was found.

It is probable that the profound changes in follicle growth after antiserum injection were caused by immunoneutralization of inhibin activity and the subsequent increase of circulating FSH levels. In a previous study in pubertal rats, a negative correlation between ovarian inhibin content and the mean serum FSH concentrations was seen, as well as a positive correlation between the total volume of large antral follicles (vol. > 350 x 10^5 µm^3) and the ovarian inhibin content (Sander et al. 1986). It was therefore concluded that, in pubertal rats, inhibin keeps serum FSH at a low concentration, which serves to maintain the number of ovulatory follicles within the normal range (Sander et al. 1986). In the present report, 32 more healthy antral follicles and 11 fewer atretic follicles were found in rats 48 h after an injection of antiserum compared with control, indicating that about 21 new follicles were recruited in the rats injected with antiserum in this 48-h period. The elevated serum FSH levels presumably also induced the increase in the number of medium and large (class 3–5) atretic follicles seen at 48 h, but local ovarian factors different from inhibin may also have been involved in this (Ying, 1988; Tonetta & DiZerega, 1989; Tsafiri, Vale & Hsueh, 1989). The number of potentially ovulable follicles (classes 3–5) present at 48 h after inhibin neutralization on D1, as reported here, was found to be 205% of control values. This figure is in agreement with the doubling in ovulation rate at first oestrus after injection on D2 (17.00 h) in our 5-day-cyclic rats and with the doubled ovulation rate at first oestrus reported by Rivier & Vale (1989) in 4-day-cyclic rats injected intravenously with an inhibin-neutralizing antiserum at D1 or D2 (10.00 h). Rivier & Vale (1989)
did not find a significant increase in ovulation rate at second oestrus. They suggested that raised serum FSH may have increased recruitment of follicles and/or decreased total atresia of preovulatory follicles, as now shown by us.

The smaller increase in ovulation rate after antiserum injection on D1 observed by us at first oestrus (18%), compared with that found by Rivier & Vale (1989) (115%), must presumably be attributed to the difference in cycle length. If injected at 17.00 h on D2, our 5-day-cyclic rats had also almost doubled their ovulation rate at first oestrus. This indicates that the time-interval between antiserum injection—and, therefore, of FSH-induced additional recruitment of new preovulatory antral follicles—and the moment of ovulation is critical in determining the extent of atresia among the cohort of growing preovulatory follicles and, thus, for ovulation rate. Similarly, in prepubertal female rats we found that a progressively higher ovulation rate resulted when antiserum injection was given nearer to ovulation within the last 5 days before the day of first ovulation (Sander et al. 1991). In both cyclic and pubertal (Sander et al. 1991) rats the optimal time for induction of increased ovulation rate by antiserum injection is approximately 3 days before the expected day of next ovulation.

The number of fast growing, ovulable follicles present in the ovaries of 5-day-cyclic rats at 48 h after antiserum injection on D1 was apparently greatly reduced before ovulation on the next oestrus through atresia of growing follicles. In the study of Rivier & Vale (1989) the net result of increased numbers of growing follicles and ovulation rates was reduced later because of abnormally high postnatal mortality rates.

The increased ovulation rate found at second oestrus after antiserum injection on D1 (128% of control) and the tendency to an increased ovulation rate seen after injection on D2 suggest a more long-term disruption of the normal control mechanism between FSH and inhibin and follicle growth after immunoneutralization of inhibin at this stage of the cycle. The finding that antiserum activity was still present at second oestrus supports this suggestion. Alternatively, the increased ovulation rate at second oestrus may be explained in part by the assumption of Schwartz (1974), as recently substantiated by Osman (1985) and D’Agostino, Woodruff, Mayo & Schwartz (1989), that follicles destined to ovulate (but not yet morphologically recognizable as such) are already recruited during the preceding cycle.

A fourfold increase in serum FSH level at 8 h after antiserum injection has been reported by Rivier et al. (1986), Sander (1988), Culler & Negro-Vilar (1989) and Rivier & Vale (1989). Levels of FSH remained elevated for at least 48 h, suggesting ongoing reduced inhibin activity, in agreement with antiserum activity measured in the circulation throughout the experimental period.

The higher serum oestradiol level during the first 24 h in the antiserum-treated group may be explained by assuming supplementary oestradiol release by additional healthy antral follicles, as present in the antiserum-treated group at 48 h after injection and by the increased stimulation by FSH. The increased oestradiol levels at first oestrus cannot yet be satisfactorily explained.

In conclusion a single injection of an inhibin-neutralizing antiserum is an effective approach for further elucidating the role(s) of inhibin in the regulation of follicle dynamics during the oestrous cycle by way of its control of serum FSH levels. Neutralization of peripheral inhibin provokes a series of events consisting of elevation of serum FSH levels, prevention of atresia and recruitment of additional follicles resulting in increased ovulation rate. An essential role of inhibin in ovarian physiology, notably in the control of follicle growth, through the regulation of peripheral FSH levels, was demonstrated.

ACKNOWLEDGEMENTS

The authors wish to thank Dr J. Moll for his comments on the manuscript. They are grateful to the NIADDK, Bethesda, MD, U.S.A. for the gonadotrophins used in the radioimmunoassay.

REFERENCES


Welschen, R. & Dullaart, J. (1976). Administration of antiserum against ovine follicle-stimulating hormone or ovine luteinizing hormone at pro-oestrus in the rat: effects on follicular development during the oncoming cycle. Journal of Endocrinology 70, 301–306.

