Significance of oestradiol for follicular development in hypogonadotrophic immature rats treated with FSH and hCG

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Administration of hCG (0.5 i.u.) to immature female rats that were made hypogonadotrophic by injections of an LHRH-antagonist at 22, 24, 26 and 28 days of age and in which follicular development was induced by FSH (10 i.u., given from day 26 until day 29) stimulates follicles to grow to their preovulatory size with a low amount of atresia. To investigate whether this effect of hCG on follicular growth is due to stimulation of oestradiol production, intraovarian concentrations of oestradiol were suppressed by an aromatase inhibitor and oestradiol action was blocked by an oestrogen antagonist; both of these were administered from day 25 until day 29. By day 30 this treatment had resulted in an increase in the percentage of atretic follicles. In turn, oestradiol benzoate (100 µg or 1000 µg day⁻¹), given from day 26 until day 29 in rats treated with 10 i.u. FSH, resulted in (1) an increase in the total number of antral follicles, (2) an increase in follicular size and (3) a decrease in the percentage of atretic follicles. Although the administration of 1000 µg oestradiol benzoate can mimic the effects of 0.5 i.u. hCG on follicular growth to a large extent, the number of follicles with a diameter > 575 µm in rats treated with FSH plus oestrogen was smaller than that in rats treated with FSH plus hCG (10 ± 2 and 24 ± 2, respectively). Furthermore, a single injection of 10 i.u. hCG given on day 29 was used to induce ovulation; fewer ova were found in rats treated with FSH plus 1000 µg oestrogen than in rats treated with FSH plus hCG (15.2 ± 2.1 and 46.5 ± 1.6, respectively). From these studies, it is concluded that the effects of hCG on follicular growth and atresia can be largely attributed to the mitotic and anti-atretic effect of oestradiol. However, part of the effect of hCG cannot be explained on the basis of oestradiol action.

Introduction

The gonadotrophins FSH and LH play an essential role in stimulating follicular development. FSH receptors are exclusively present in granulosa cells and induce granulosa cell growth, antrum formation, the expression of gonadotrophin receptors and aromatase activity. LH receptors are present in thecal cells and stimulate androgen formation. These androgens can be converted to oestrogens by the aromatase activity present in the granulosa cells. Since oestrogen receptors are present in granulosa cells, oestrogen could have an autocrine effect in stimulating the further differentiation of granulosa cells (Richards, 1979). The administration of pure FSH (recombinant FSH) to immature rats, made hypogonadotrophic by hypophysectomy (Mannaerts et al., 1994) or by administration of an LHRH antagonist (Uilenbroek et al., 1996), or to hypogonadotrophic women (Schoot et al., 1992) can induce follicular development. These studies in rats showed that in the presence of hCG, lower doses of FSH could be used to induce full follicular development. A possible mechanism that makes the combined FSH and hCG treatment more effective in inducing follicular growth might be the increased synthesis of oestradiol. The present study was performed to investigate whether the effects of hCG on follicular development might be due to this production of oestradiol. Therefore, effects of oestradiol and hCG on follicular development were compared in immature rats made hypogonadotrophic with an LHRH agonist in which follicular development was stimulated by a low dose of FSH. Furthermore, the effects of hCG on follicular development were studied in rats in which the endogenous concentrations of oestradiol were suppressed by an aromatase inhibitor and in which the effect of oestradiol was blocked by an oestrogen antagonist.

Materials and Methods

Animals

Immature, locally bred, female Wistar rats were used. The animals were housed in a room in which the temperature was controlled at 20–23°C and lights were on from 05:00 h to 19:00 h.
Materials

LHRH antagonist (Org 30276), recombinant human FSH (Puregon) and hCG (Pregnyl) were supplied by Organon International b.v. (Oss). The characteristics of the LHRH antagonist have been described by Debeljuk and Schally (1986) and the FSH preparation by Mannaerts et al. (1991). The LHRH antagonist was dissolved in 0.9% (w/v) NaCl. FSH and hCG were dissolved in a phosphate buffer (pH 7.1–7.2) containing 0.1% (w/v) methylhydroxy-benzoate and 0.1% (w/v) albumin. The aromatase inhibitor (CGS 16949A) was a gift from Ciba-Geigy (Basel); its characteristics have been described by Häuser et al. (1989). CGS 16949A was dissolved in 0.9% NaCl containing 0.1% (w/v) gelatine. The oestrogen antagonist (ICI 182.780) was a gift from ICI Pharmaceuticals (Macclesfield); its characteristics have been described by Wakeling et al. (1991). ICI 182.780 was dissolved in ethanol:oil (1:19).

Experiments

Immature female rats were given s.c. injections of LHRH antagonist (500 μg per 100 g body weight) at 22, 24, 26 and 28 days of age. Rats were given FSH or FSH plus hCG on days 26, 27, 28 and 29. When the aromatase inhibitor or the oestrogen antagonist were used, daily injections were given starting at day 25. Injections of FSH, hCG or aromatase inhibitor were given s.c. twice a day at 09:00 h and 17:00 h. The total dose over the 4 days was 10 i.u. for FSH, 0.5 i.u. for hCG and 400 μg for aromatase inhibitor. The oestrogen antagonist was given s.c. at a dose of 500 μg day\(^{-1}\). To investigate whether the exogenous administration of oestradiol had the same effect as 0.5 i.u. hCG, rats treated with LHRH antagonist plus FSH were given 100 or 1000 μg oestradiol benzoate, the injections of which were given s.c. once a day on days 26, 27, 28 and 29. Pharmacological doses had to be given to obtain intraovarian concentrations of oestrogen similar to those found after FSH plus hCG.

The rats were killed at 30 days of age by ether anaesthesia after blood sampling by puncture of the ophthalmic venous plexus. Serum samples were stored at \(-20^\circ\text{C}\) until assayed for LH and prolactin. The ovaries and uterus were dissected out and weighed. One ovary from each animal was fixed in Bouin's fluid for histological examination; the other ovary was frozen in liquid nitrogen and stored at \(-80^\circ\text{C}\) until assayed for oestradiol and inhibin. Before hormone determination, this ovary was homogenized in 500 μl PBS containing 1% BSA and centrifuged at 30 000 g for 1 h. Oestradiol and inhibin were measured in the supernatant by radioimmunoassay.

In an additional experiment, the ovulatory response was measured in rats treated with FSH alone, in rats treated with FSH plus hCG and in rats treated with FSH plus oestradiol benzoate (100 μg or 1000 μg per day). An ovulatory dose of hCG (10 i.u.) was given on day 29 at 15:00 h and on day 30 the number of ova in the oviduct was counted.

Histology

For histological examination, the ovaries were embedded in paraffin and serial sections (thickness 10 μm) were stained with haematoxylin and eosin. Differential follicle counts were made according to the method described by Osman (1985). In one ovary from each animal, all antral follicles with a mean diameter > 275 μm were counted. Five follicle classes were distinguished on the basis of diameter: class 1 (275–350 μm), class 2 (351–400 μm), class 3 (401–450 μm), class 4 (451–575 μm) and class 5 (> 575 μm). Class 1 are small antral follicles, while classes 4 and 5 are follicles that can be induced to ovulate. The mean follicle diameter was calculated from the two perpendicular diameters in the section containing the oocyte nucleus. Degenerative changes were used as criteria for atresia in each counted follicle. Follicles were designated as early atretic when local or widespread pycnosis and cell shrinkage were observed in the granulosa wall, and as late atretic when the oocyte was degenerating.

Hormone assays

Oestradiol concentrations in ovaries were measured by a radioimmunoassay with prior extraction, as described by de Jong et al. (1973). The sensitivity was 25 pg per ovary and the intra- and interassay coefficients of variation were 8.2% and 6.3%, respectively. Oestradiol concentrations could not be measured in animals treated with the oestrogen antagonist, because this compound crossreacted by 0.7% in the oestradiol radioimmunoassay.

The concentration of inhibin in the ovaries was measured by radioimmunoassay (Robertson et al., 1988), using a bovine follicular fluid preparation with an arbitrary potency of 1 U μg\(^{-1}\) protein as a standard (Grootenhuis et al., 1989). The antibody was raised against a 32 kDa bovine follicular inhibin and crossreacts with free α subunits. The intra- and interassay coefficients of variation were 12% and 18%, respectively.

Serum concentrations of prolactin and LH were measured by double-antibody radioimmunoassay following the instructions provided by the NIDDK and were expressed as ng NIDDK-rat prolactin RP-3 or NIDDK-rat LH RP-2 ml\(^{-1}\), respectively. The intra- assay coefficients of variation for the prolactin and LH assay were 6% and 7%, respectively.

Statistical analysis

Results are expressed as means ± SEM. Statistical analysis consisted of one-way analysis of variance followed by Duncan’s multiple range test. A difference was considered significant if \(P\) was < 0.05 (two-tailed).

Results

Effect of hCG with and without aromatase inhibitor and oestrogen antagonist on FSH-induced follicular growth

The administration of 0.5 i.u. hCG to rats made hypogonadotrophic with an LHRH antagonist and in which follicular development was stimulated with 10 i.u. FSH resulted in a significant increase in ovarian and uterine weight and a significant increase in the amount of oestradiol and inhibin in the ovary compared with those in rats treated with LHRH antagonist plus FSH alone (Table 1). The simultaneous
administration of an aromatase inhibitor in rats treated with FSH plus hCG prevented the increase in ovarian and uterine weight induced by hCG and prevented the increase in oestriadiol and inhibin concentrations in the ovary. The combined administration of aromatase inhibitor and oestrogen antagonist to these rats resulted in a further decrease in ovarian and uterine weight and a further decrease in the ovarian content of inhibin. The total number of follicles and the percentage of early and late atretic follicles are also given in Table 1. The injection of hCG alone resulted in an increase in the total number of follicles, whereas the percentage of early atretic follicles decreased significantly. The simultaneous administration of hCG and the aromatase inhibitor suppressed the total number of follicles and resulted in a higher percentage of both early and late atretic follicles. The combined administration of the aromatase inhibitor and the oestrogen antagonist resulted in a lower percentage of early atretic follicles, but in a higher percentage of late atretic follicles compared with the animals receiving aromatase inhibitor without oestrogen antagonist. The distribution of healthy, early and late atretic follicles over the various size classes is depicted in Fig. 1. FSH alone resulted in the presence of follicles of all size classes except class 5. However, about 50% of follicles in all size classes were atretic. The combined treatment of FSH plus hCG resulted in a shift from small (classes 1 and 2) to large (classes 4 and 5) follicles and a decrease of atresia in all size classes. The additional administration of aromatase inhibitor with or without oestrogen antagonist resulted in an increase of atresia in all size classes. In particular, nearly all follicles with diameter > 400 μm were atretic.

Effect of oestrogen on FSH-induced follicular growth

Rats treated with LHRH antagonist plus FSH were given 100 μg or 1000 μg oestradiol benzoate daily to increase the concentration of oestradiol in the ovaries. Treatment for 4 days increased the amount of oestradiol and inhibin in the ovaries in a dose-dependent manner (Table 2). Uterine weight was already maximally stimulated after the administration of 100 μg oestradiol benzoate. Concentrations of prolactin in the serum were increased in the rats treated with oestradiol benzoate, while serum concentrations of LH were below the limit of detection in all three groups (< 0.1 ng ml⁻¹). Ovarian weight and the total number of antral follicles increased in a dose-dependent manner, while the percentage of atretic follicles declined with increasing amounts of oestradiol. The distribution of antral follicles over the various size classes is depicted in Fig. 2. Increasing amounts of oestradiol benzoate resulted in more and larger follicles and a decreased amount of atresia in every size class. The effect of 1000 μg oestradiol benzoate resembled that of 0.5 i.u. hCG (Fig. 1b), although the number of class 5 follicles was lower in the rats treated with oestradiol benzoate than in hCG-treated rats (10 ± 2 and 24 ± 2, respectively).

Ovulation response

The ovulation response in rats treated with FSH alone, in rats treated with FSH plus hCG and in rats treated with FSH plus 100 μg or 1000 μg oestradiol benzoate, was as follows: none of the eight rats treated with FSH ovulated, while all the rats treated with FSH and hCG ovulated (8 of 8, 47 ± 2 ova per ovulating rat). The ovulation response in rats treated with oestradiol benzoate was 18 ± 2 ova per ovulating rat (6 of 7) in rats treated with 100 μg oestradiol benzoate and 15 ± 2 ova per ovulating rats (7 of 7) in rats treated with 1000 μg oestradiol benzoate.

Discussion

Previous studies have shown that the administration of a low dose of hCG to FSH-treated immature rats results in a shift
from small to large antral follicles and a decrease in the incidence of atresia (Mannaerts et al., 1994; Uilenbroek et al., 1996). The aim of the present study was to investigate whether this folliculotrophic effect of hCG is due to its ability to stimulate oestradiol production. This hypothesis was tested by treating immature rats with an LHRH antagonist plus FSH and hCG, and oestrogen production was blocked by an aromatase inhibitor and residual oestrogen action was inhibited by an oestrogen antagonist. In addition, an attempt was made to mimic the effect of hCG by administration of oestradiol to rats treated with an LHRH antagonist plus FSH.

Table 2. Effect of injecting oestradiol benzoate (OB) into immature rats treated with FSH and an antagonist of luteinizing hormone releasing hormone

<table>
<thead>
<tr>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
</tr>
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<tbody>
<tr>
<td>FSH</td>
<td>FSH 100 µg OB</td>
<td>FSH 1000 µg OB</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovarian weight (mg)</td>
<td>26 ± 1</td>
<td>37 ± 1ab</td>
<td>56 ± 1b</td>
</tr>
<tr>
<td>Uterine weight (mg)</td>
<td>25 ± 1</td>
<td>140 ± 2a</td>
<td>147 ± 4b</td>
</tr>
<tr>
<td>Prolactin (ng ml⁻¹ serum)</td>
<td>18 ± 3</td>
<td>253 ± 39ab</td>
<td>210 ± 35</td>
</tr>
<tr>
<td>Oestradiol in ovary (pg per ovary)</td>
<td>23 ± 1</td>
<td>84 ± 5a</td>
<td>923 ± 77b</td>
</tr>
<tr>
<td>Inhibin in ovary (U per ovary)</td>
<td>537 ± 53</td>
<td>1232 ± 62ab</td>
<td>2583 ± 187b</td>
</tr>
<tr>
<td>Total number of follicles (&gt; 275 µm in diameter)</td>
<td>78 ± 5</td>
<td>108 ± 5a</td>
<td>136 ± 5b</td>
</tr>
<tr>
<td>Percentage of atretic follicles (&gt; 275 µm in diameter)</td>
<td>42 ± 2</td>
<td>33 ± 2a</td>
<td>11 ± 2b</td>
</tr>
</tbody>
</table>

Rats were given a single injection of an antagonist of luteinizing hormone releasing hormone on days 22, 24, 26 and 28 and FSH was given on days 26, 27, 28 and 29 twice daily at 09:00 h and 17:00 h (total dose of 10 i.u.). Oestradiol benzoate was given once daily on days 26, 27, 28 and 29. The rats were killed on day 30. Values are means ± SEM of six or seven rats.

abValues are significantly different (P < 0.05) from those of groups 1 and 2, respectively (ANOVA followed by Duncan’s multiple range test).
Effect of oestradiol and hCG on follicular growth

The additional administration of an oestrogen antagonist increased the rate of atresia even further. These results demonstrate that the effects of hCG in FSH-treated rats on follicle growth can be largely attributed to ovarian oestradiol production, and are in agreement with those of Selvaraj et al. (1994), who used the same aromatase inhibitor in rats treated with PMSG. However, these authors did not discriminate between various size classes of antral follicle. Although in our study hCG-stimulated oestradiol production was completely prevented by the aromatase inhibitor, there was still a difference in the distribution of follicles over the various size classes between animals treated with hCG plus aromatase inhibitor and oestrogen antagonist, and animals treated with FSH alone. In the animals treated with FSH alone, no late atretic class 4 follicles were present; however, in the animals treated with hCG plus aromatase inhibitor and oestrogen antagonist 7.6 ± 1.5 late atretic class 4 follicles were found. This finding indicates that although oestradiol production and action were prevented, hCG still had an effect on follicular growth.

The administration of oestradiol benzoate to FSH-treated rats increased the number of antral follicles and decreased the percentage of atretic follicles in all size classes, similar to results after administration of hCG. The effect on follicular growth and atresia is a direct effect of oestradiol benzoate and not due to an increase in the serum concentrations of LH, as LH was not detected in the serum in all groups. Although treatment with oestradiol benzoate could mimic the effect of hCG on follicular growth to a large extent, the number of class 5 follicles was lower in rats treated with FSH plus oestradiol benzoate than in rats treated with FSH plus hCG. Furthermore, the ovulatory response in rats treated with FSH plus oestradiol benzoate was significantly lower than the ovulatory response in rats treated with FSH plus hCG, indicating that not all effects of hCG on follicular growth are mediated by oestrogen. It is unlikely that more oestradiol benzoate needs to be given to obtain more class 5 follicles, since the intraovarian content of oestradiol after 1000 µg oestradiol benzoate (923 ± 77 pg per ovary) was already larger than that after 0.5 i.u. hCG (137 ± 19 pg per ovary). It is possible that the increased concentrations of prolactin after oestradiol administration might explain the lower number of class 5 follicles and the lower ovulation response, as it has been reported that high concentrations of prolactin can affect follicular activity (Uilenbroek et al., 1982). However, in that study high concentrations of prolactin did not affect follicular growth.

The mechanism by which oestrogen augments FSH action on follicular growth is by induction of gonadotrophin receptors (Ireland and Richards, 1978; Hsu et al., 1984). In the presence of oestrogen, low doses of FSH can induce full follicular development. However, oestrogens are not indispensable and high doses of FSH, in the absence of oestrogens, can have the same effect, although under these circumstances the granulosa cells have an abnormal appearance (Mannaerts et al., 1994). In addition, Whitelaw et al. (1992) reported that in hypophysectomized rats, FSH alone could induce the transcription of mRNAs encoding aromatase and the LH receptor, implying that oestrogen is not essential for this process. However, in hypophysectomized rats, the administration of oestrogen alone cannot induce the production of aromatase in the granulosa cells and increases only the number of large preantral follicles (Harman et al., 1975; Richards, 1979). The combined administration of FSH and oestrogen apparently is necessary for inducing the expression of FSH receptors in granulosa cells and LH receptors in granulosa and thecal cells.

In the present study, the ovarian content of inhibin was measured to study the functional activity of the follicles. Healthy large preovulatory follicles (classes 4 and 5) secrete large amounts of oestradiol and inhibin, in contrast to small and atretic follicles (van Cappellen et al., 1995). The amount of inhibin in the ovary is correlated with the presence of healthy large follicles. As reported by Uilenbroek et al. (1996),

![Fig. 2. Numbers of healthy (■), early atretic (□) and late atretic (■) antral follicles in various size classes (mean diameter of class 1: 275–350 µm; class 2: 351–400 µm; class 3: 401–450 µm; class 4: 451–575 µm; class 5: > 575 µm) present in one ovary of immature rats treated with an LHRH antagonist and (a) 10 i.u. FSH (b) 10 i.u. FSH plus 100 µg oestradiol benzoate and (c) 10 i.u. FSH plus 1000 µg oestradiol benzoate.](image-url)
the administration of hCG to FSH-treated rats increased inhibin production. The present study shows that oestrogen also increased the concentration of inhibin in FSH-treated rats. This finding confirms earlier observations that in hypophysectomized rats, FSH alone, hCG plus FSH, or oestradiol alone can increase inhibin production (Aloi et al., 1995). However, in this study, correlation with the induction of large follicles was not made.

As discussed above, in the absence of oestradiol synthesis and action, the pattern of follicular growth in rats treated with FSH plus hCG was still different from that in animals treated with FSH alone. Furthermore, administration of oestrogen to FSH-treated rats could not replace hCG entirely both in terms of the number of follicles and the ovulatory response. Therefore, it appears that not all effects of hCG on follicular growth are mediated by oestrogen, but that other mechanisms are involved such as a direct effect of hCG on the production of steroids or growth factors. It is known that in addition to oestrogen a number of locally produced growth factors can be involved in follicular growth such as insulin-like growth factor (Adashi, 1993), epidermal growth factor and transforming growth factor α (Mulheron and Schomberg, 1993). It has been suggested that the stimulatory effect of hCG on follicular growth is partly mediated by insulin-like growth factor I, since insulin-like growth factor binding protein 3 can partially reverse the anti-atretic effects of hCG (Chun et al., 1994).

In conclusion, the present study demonstrates that the stimulating effect of hCG on follicular growth in FSH-treated rats is largely the consequence of an increased oestradiol production. However, an additional effect of hCG on follicular growth is independent of oestrogen.

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