Radioreceptor and autoradiographic analysis of FSH, hCG and prolactin binding sites in primary to antral hamster follicles during the periovulatory period*

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Summary. As measured by radioreceptor assays, binding sites for FSH and prolactin were present at 09:00 h on the day of pro-oestrus in Stage 1–10 follicles (primary to antral) with prolactin receptors 3–6 times higher than FSH sites in Stages 1–3 (3 layers of granulosa cells). Specific binding sites for hCG were present in Stage 1 and 2 follicles (2 layers of granulosa cells) but thereafter their distribution was erratic and they were not consistently detectable until Stage 5, when thecal cells first appeared. Using topical autoradiography, specific binding for FSH was evident in Stage 1–4 follicles (4 layers granulosa cells) whereas specific hCG-binding was not. After the preovulatory gonadotrophin surges, by 21:00 h on pro-oestrus, FSH receptors declined in Stages 5–10, prolactin receptors fell in Stages 8 and 10 (small and large antral follicles) and hCG receptors were reduced in Stages 7 (start of antral cavity) to 10. On the morning of oestrus, for follicles from Stage 4 onwards, receptor numbers usually returned to levels found at 09:00 h on pro-oestrus. At oestrus, the few remaining Stage 10 follicles were all atretic and contained significantly reduced FSH and prolactin receptors but numbers of hCG binding sites comparable to those at 09:00 h of pro-oestrus. These results provide evidence of gonadotrophin receptors in small primary and secondary follicles which is consistent with increased DNA synthesis in small hamster follicles on the afternoon of pro-oestrus and on the morning and afternoon of oestrus. Periovulatory changes in gonadotrophin concentrations may therefore affect early stages of folliculogenesis.

Introduction

Several autoradiographic and biochemical studies provide convincing evidence for the presence of binding sites for FSH, LH/hCG and prolactin in large preantral and antral follicles of several species (Richards & Williams, 1976; Bortolussi *et al.*, 1977; Rajaniemi *et al.*, 1977; Ireland & Roche, 1983; Webb & England, 1982; Oxberry & Greenwald, 1982). However, for any species, information is unavailable about gonadotrophin binding to smaller preantral follicles. The periovulatory gonadotrophin surges in the hamster influence the incorporation of [³H]thymidine into DNA of small primary and secondary follicles (Roy & Greenwald, 1986a, b) and these findings suggest that small preantral follicles in the hamster possess specific receptors for gonadotrophins. To test this hypothesis, the concentration of FSH, hCG and prolactin receptors of follicles at defined stages of development were quantitatively and qualitatively determined during the pre- and post-ovulatory period. The study was made possible by the development of a technique to dissociate follicles from the hamster ovary enzymically and to sort them into 10 stages based on their diameter and histological criteria (Roy & Greenwald, 1985a).

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Materials and Methods

Isolation of follicles

Adult, golden hamsters maintained under 14 h light and 10 h dark were checked for at least 3 cycles to establish the regularity of the oestrous cycle (oestrus = Day 1, characterized by a copious vaginal discharge; pro-oestrus = Day 4). Hamsters were decapitated on Day 4, 09:00 h (6 h before the FSH and LH surges) and 21:00 h (6 h after the peak levels of FSH and LH) and on Day 1, 09:00 h (while the second FSH surge is in progress). Large preantral and antral follicles (Stages 6–10; Stage 6 = follicles with 7–8 layers of granulosa cells and theca; Stage 10 = preovulatory follicles) were dissected by hand under a stereomicroscope, cleaned and saved. The remaining ovarian fragments were digested with a mixture of collagenase, DNase and pronase for 20 min at 37°C to disperse small primary and secondary follicles as described earlier (Roy & Greenwald, 1985a). Follicles were classified into 10 stages: Stages 1 to 4 with 1–4 layers of granulosa cells and no theca; Stage 5 = a mixture of follicles with 5 layers of granulosa cells but no theca and with 6 layers of granulosa cells with the appearance of thecal cells; Stage 7 = early formation of antral cavity; Stages 8 to 10 = small to large antral follicles with well developed theca. Follicles from Stages 1–10 were sorted with micropipettes, counted and washed twice in cold assay buffer (50 mM-Tris buffer with 10 mM-CaCl₂ and 10 mM-MgCl₂, pH 7.2) and stored at –80°C until processed for receptor assays.

Preparation of follicles and assay of receptors

Ovine FSH (NIAMMD I-1), highly purified hCG (14 500 i.u./mg; Radioassay Labs, Inc, Carson, CA) and ovine prolactin (NIADDK I-1) were labelled with Na¹²⁵I (sp. act. 13.9 mCi/μg; Amersham, Arlington Heights, IL) by the lactoperoxidase method (Catt *et al.*, 1976) and characterized as described previously (Kim & Greenwald, 1985, 1986). In brief, 25 μl 0.5 mM-potassium phosphate buffer (pH 7.4 for gonadotrophins and pH 7.0 for prolactin), 4 μg FSH, hCG or prolactin and 60 i.u. lactoperoxidase (Sigma, St Louis, MO) were added to an unstoppered ice-cooled 1-ml serum vial. A stopper was then placed on the vial and 0.3 mCi Na¹²⁵I was injected into the vial with a Hamilton syringe. Iodination was initiated by addition of 10 μl of 1:80 000 dilution of 50% hydrogen peroxide (Fisher Scientific Co., Fair Lawn, NJ) into the vial. The reaction was allowed to proceed for 50 sec for FSH or hCG and 2 min for prolactin and was then terminated by injection of 300 μl 0.025 M-Tris buffer (pH 7.2 for gonadotrophins and pH 8.0 for prolactin). The reaction solution was then transferred to a 0.7 × 18 cm Sephadex G-100 column and eluted with Tris buffer to separate labelled hormone from free iodine.

A mixture of Day 3 and Day 4 homogenates of hamster ovaries was used for characterization of ¹²⁵I-labelled FSH and ¹²⁵I-labelled hCG while a 20 000 g crude liver membrane preparation from the Day-14 pregnant rat was used for ¹²⁵I-labelled prolactin. The specific activities for the iodinated FSH, hCG and prolactin were 63.1, 45.0, and 77.9 μCi/μg and the active fractions were 54%, 51% and 49%, respectively. Active fraction is defined as the percentage of labelled hormone which will specifically bind to its receptor.

The concentrations of receptors for FSH, hCG and prolactin were measured by radioreceptor analysis. Follicles in assay buffer at different stages of development were sonicated by a Kontes ultrasonic cell disruptor (5 W, 10–15 sec) and an homogenate volume of 250 μl for each stage was incubated with ¹²⁵I-labelled FSH, hCG and prolactin (10 000 c.p.m.) in the presence or absence of 1000-fold excess of unlabelled homologous hormones at 30°C for 6 h. Using a preparation of hamster follicular homogenate, the optimal temperature and time required to attain binding equilibrium was 30°C for 4–10 h (data not shown). Therefore, 6 h was selected as the incubation period. Nonspecific binding for labelled tracers was always <10% of total hormone. Free and bound hormones were separated by centrifugation (2000 g for 30 min). Samples of follicular sonicates (30–50 μl) were used to determine the DNA content by a microfluorometric method described previously (Roy & Greenwald, 1986a). Since isolation of follicles required the presence of bovine serum albumin (BSA) in the medium, no attempts were made to determine follicular protein content. Specific binding of hormones was expressed as fmol/μg DNA. For each time period, follicles were pooled from 6 animals and 4 replicates were collected and used in the same assay for each hormone.

Autoradiography of FSH, hCG and prolactin binding sites

Although the number of peptide hormone receptors in preantral follicles (Stages 1–4) was quantitated by biochemical procedures, the number of follicles required for a single determination was very large (e.g. 400 follicles for Stage 1) and the values were very low and frequently undetectable. Therefore, to validate further the results obtained by biochemical assays, autoradiographic techniques were used on follicles at Stages 1–4.

Follicles at Stages 1–4 were collected at 09:00 h on the day of pro-oestrus; rinsed well with assay buffer and incubated in 100 μl buffer at 37°C for 90 min in the presence of 20 000 c.p.m. of ¹²⁵I-labelled FSH, hCG or prolactin. Nonspecific binding was determined by incubating follicles in the presence of a 100-fold excess of unlabelled homologous hormone in addition to labelled peptides. Follicles were washed several times for 30 min, embedded in agar and sectioned at 6 μm. Tissue sections were coated with Kodak NTB2 nuclear track emulsion, exposed at 4°C for 10 days, developed in dektol (Kodak, Rochester, NY), and stained with nuclear fast red and alcoholic picric acid and photographed.

Statistics

Multivariate analysis of variance was used for data analysis and utilized Biomedical Data Package Program software. The Tukey test was applied for multiple comparisons. The level of statistical significance was at 1%.

Results

On the morning of pro-oestrus, Stage 10 follicles (the largest antral stage) had considerably more FSH receptors than did follicles at Stages 1–9 ($P < 0.01$) in which the levels were fairly constant (Fig. 1). FSH receptors were consistently present from Stage 2 onwards and in 2 of 4 replicates for Stage 1 (primary follicles). Stages 1 and 2 follicles showed hCG binding in all replicates but thereafter it was not until Stage 5 when low but detectable levels were always present, when adherent thecal cells first appear. It was not, however, until Stage 6 that appreciable hCG binding was detected. At 09:00 h on Day 4, Stage 10 follicles had the greatest concentration of hCG receptors ($P < 0.01$). On the other hand, prolactin showed specific binding sites at all stages with maximal concentrations in Stages 1, 2 and 10. Considerably greater amounts of prolactin than FSH receptors were present in Stage 1–3 follicles on the morning of Day 4.

Fig. 1. Concentrations of follicular FSH, hCG and prolactin receptors at different developmental stages during the periovulatory period (Day 4 = pro-oestrus; Day 1 = oestrus). The values are mean + s.e.m. of 4 replicates. The number in parentheses indicates number of replicates with detectable activity if less than 4; N = non-detectable for all 4 replicates. Stages of follicles are: S1–4, 1–4 layers of granulosa cells with no theca; S5, 5–6 layers of granulosa cells plus early thecal shell; S6, 7–8 layers of granulosa cells plus well developed theca; S7, early antral cavity; S8, small antral follicle; S9, intermediate antral follicle; S10, large antral follicle. Statistical significance is summarized in the text.

After the preovulatory surges of FSH and LH, the concentration of follicular receptors showed significant changes by Day 4, 21:00 h (Fig. 1). For Stages 1–3, FSH receptor numbers were virtually unchanged whereas a marked drop in binding sites was evident in Stages 5–8 ($P < 0.05$) and in Stage 10 ($P < 0.01$). Similarly, receptor concentration for hCG fell significantly in Stages 7–10 ($P < 0.01$). In contrast, prolactin receptors increased significantly in Stage 1 follicles, were unchanged in Stages 2–7 and dropped appreciably for Stages 8–10 ($P < 0.01$).

On the morning of oestrus (Day 1 of the cycle) FSH binding sites were reduced for Stages 1–3 but for Stages 6–8 the values returned to the levels found at 09:00 h of pro-oestrus. Large antral follicles (Stages 9 and 10) still present on Day 1 are all atretic (Greenwald, 1974); FSH receptors were still present in these anovulatory follicles, albeit in greatly reduced numbers. Receptors for hCG were absent or negligible in follicles of Stages 1–5 with an upward trend in numbers for Stages 7 and 8. Large atretic follicles contained substantial amounts of hCG receptors, in fact as many as on the morning of Day 4. Prolactin binding remained low in Stages 6 and 8 compared to the values found at 09:00 h, Day 4.

Although 400–600 follicles were pooled for each replicate for Stages 1–4, the results for FSH and hCG binding sites were equivocal, especially for the latter receptor. Therefore, topical autoradiography was used to localize FSH and hCG receptors on Stage 1–4 follicles; however, only autoradiographs of Stages 3 and 4 are shown in Figs 1–8. Definite FSH binding sites were demonstrable; few binding sites were present on Stage 1 follicles with a gradual increase with developmental age. Binding of ^{125}I -labelled FSH was considerably reduced in the presence of unlabelled FSH, indicating the specificity of the binding (Figs 2–5). In contrast, Stage 1–4 follicles showed no specific binding sites for hCG (Figs 6–9). Patchy prolactin binding was observed for Stage 1–4 follicles (not illustrated).

Discussion

Isolated hamster follicles with 1–4 layers of granulosa cells possess FSH and prolactin binding sites whereas hCG receptors only appear consistently, and at low levels, in follicles of Stage 5 or more, when they are first invested with thecal cells (Fig. 1). Previous autoradiographic studies have

Fig. 2. Autoradiograph of a Stage 3 follicle (3 layers of granulosa cells) exposed to ^{125}I -labelled FSH. Prominent binding of labelled hormone is evident.

Fig. 3. A Stage 3 follicle, incubated with ^{125}I -labelled FSH and 100-fold excess of unlabelled FSH. Very few silver grains are found over the follicular cells, indicating the specificity of the binding sites.

Fig. 4. A Stage 4 hamster follicle (4 layers of granulosa cells) exposed to labelled FSH showing heavy deposition of silver grains at the binding sites.

Fig. 5. Autoradiograph of a Stage 4 follicle showing almost complete inhibition of ^{125}I -labelled FSH binding to granulosa cells by unlabelled FSH.

Figs 6 & 7. Stage 3 follicle exposed to ^{125}I -labelled hCG without (Fig. 6) and with (Fig. 7) a 100-fold excess of unlabelled hCG. Note the lack of difference in the grain density between the two follicles.

Figs 8 & 9. Stage 4 hamster follicles incubated in the absence (Fig. 8) and presence (Fig. 9) of unlabelled hCG. The lack of difference in the grain density between autoradiographs confirms the absence of specific hCG binding sites in these follicles.

localized ^{125}I -labelled FSH to granulosa cells of all stages of follicular development in the hamster ovary with specific hCG binding to thecal cells and to mural granulosa cells of antral follicles (Oxberry & Greenwald, 1982). Similar findings have been reported for the rat ovary (Bortolussi *et al.*, 1977).

In the rat, the development of prolactin receptors in granulosa cells coincides with the formation of the antral cavity (Midgley, 1973). The present study provides the first convincing evidence for the presence of specific prolactin receptors in primary and small secondary follicles. In fact, Stage 1 and 2 follicles (with 1 and 2 layers of granulosa cells, respectively) possess considerably more prolactin than FSH receptors. The number of prolactin receptors declines after Stage 3 with a trend for increasing levels from Stage 6 and onwards (Fig. 1). This pattern, which is quite different from that of FSH and hCG, may arise from differences in turnover rates at different stages of folliculogenesis. With the appearance of a well formed thecal layer at Stage 6, prolactin receptor synthesis regains momentum with a distribution comparable to that of hCG binding sites (Oxberry & Greenwald, 1982). Since the number of small preantral follicles needed for a single run was very large (~400–600/stage), it was not feasible to use Scatchard analysis to characterize receptor kinetics. The constant levels of FSH receptors in Stages 1–9 and of hCG receptors in Stages 5–9 suggests a gradual increase in receptor numbers with follicular growth. Nevertheless, marked increases in FSH and hCG binding in preovulatory follicles (Stage 10) indicate that although cellular growth abates with maturity, receptor synthesis continues. For example, granulosa cells from large pig follicles bind more ^{125}I -labelled hCG than do those from small follicles (Channing & Kammerman, 1973).

In the hamster, FSH increases DNA replication in Stage 1–4 follicles (Roy & Greenwald, 1986c), labelling index in follicles with 2–3 layers of granulosa cells in the hypophysectomized animal (Chiras & Greenwald, 1978), recruitment of follicles at oestrus (Greenwald, 1974; Greenwald & Siegel, 1982), follicular growth in hypophysectomized animals (Kim & Greenwald, 1984) and in-vitro progesterone production by follicles of Stages 1–5 from oestrous animals (Roy & Greenwald, 1985b). LH, on the other hand, maintains the growth of large preantral and antral follicles in hypophysectomized hamsters (Kim & Greenwald, 1984) and stimulates in-vitro steroidogenesis by large preantral and antral follicles in cyclic hamsters (Terranova & Garza, 1983; Roy & Greenwald, 1986c). Binding sites for prolactin have been demonstrated by autoradiography in the granulosa, theca and interstitium (Rolland & Hammond, 1975; Oxberry & Greenwald, 1982, 1984). The exact role of prolactin receptors in follicles at smaller stages is unknown; however, our preliminary studies suggest a possible mitogenic role (Roy & Greenwald, 1986c).

In the present study, a drop in FSH receptor numbers occurred after the pro-oestrous LH and FSH surges for Stage 4 onwards; for hCG, however, significant differences were only observed for Stages 7–10. Down regulation of receptors by heterologous or homologous ligands is therefore not limited to the preovulatory follicles but is also demonstrable in smaller stages. By the next morning, receptor numbers usually reverted back to Day 1, 09:00 h levels, possibly due to the recruitment of new cohorts of follicles into each developmental stage.

The loss of prolactin receptors following the gonadotrophin surges was not marked until Stage 8. Significant loss of prolactin receptors was noticed in Stages 8 and 10. Oxberry & Greenwald (1984) previously reported that, in hamster ovarian follicles, preovulatory surges of gonadotrophins down regulate the number of prolactin receptors on antral follicles and the present results provide quantitative confirmation of their findings. On the morning of oestrus the large antral follicles (Stage 10) left over from the cohort that ovulated were all atretic and contained greatly reduced levels of FSH receptors but the level of hCG receptors was unchanged from the 09:00 h pro-oestrous value most probably because of the prolonged survival of the thecal layer after regressive changes appear in the granulosa compartment. It appears likely that these follicles failed to ovulate because they were already deficient in FSH receptors on the day of pro-oestrous and probably had fewer granulosa cells, secreted less oestrogen than normal and less progesterone after the pro-oestrous surge of LH.

Roy & Greenwald (1986a, b) have established that Stage 1–4 follicles of the hamster show a dramatic increase in DNA synthesis after the pro-oestrous surge of gonadotrophins and that follicles of Stages 1–8 at oestrus show similar sustained increases. In the present study, the presence of FSH and prolactin receptors on Stage 1–4 follicles therefore supports the hypothesis that changes in circulating concentrations of gonadotrophins can influence the development of small preantral follicles.

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