Autoradiographic analysis of changes in ovarian binding of FSH and hCG during induced follicular atresia in the hamster

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Summary. Immediately after hypophysectomy, 30 i.u. PMSG were injected s.c. and 3 days later an antiserum to PMSG was injected i.p. Groups of hamsters were killed at 0, 24, 48 and 72 h after PMSG antiserum. The ovaries were prepared for topical autoradiography and the numbers of silver grains in different ovarian compartments were counted. The numbers of binding sites for ^{125}I -labelled FSH in the granulosa cells of the antral follicles dropped sharply to 33, 14 and 5% of that at 0 h at 24, 48 and 72 h respectively. Binding of ^{125}I -labelled hCG to granulosa cells declined more slowly, being 47, 27 and 24%, respectively. Binding of ^{125}I -labelled hCG to thecal and interstitial cells was unaffected. Compared to other models of atresia, the changes in gonadotrophin binding observed in this model occur at an accelerated rate because of the acute deprivation of PMSG.

Introduction

The majority of ovarian follicles are destined to become atretic at some phase of their life cycle but because of the random nature of follicular death it is difficult to analyse the associated steroidogenic, biochemical and morphological changes. Accordingly, several animal models have been proposed which provide a more definite time scale for the inception of atresia: blockade of ovulation in pro-oestrous animals by administration of barbiturates (rat: Braw & Tsafiriri, 1980a; hamster: Terranova, 1980); hypophysectomy of cyclic animals with large antral follicles in the ovaries (Talbert, Meyer & McShan, 1951); injection of immature animals with PMSG and evaluation of the subsequent temporal changes in the ovaries (mouse: Byskov, 1979; rat: Braw & Tsafiriri, 1980b).

A model developed in this laboratory (Greenwald, 1963, 1973) which gives a very precise start for the onset of follicular atresia involves the administration of PMSG to hypophysectomized hamsters followed 72 h later by an i.p. injection of an antiserum to PMSG. This results in acute deprivation of gonadotrophins to the extent that within 1 h serum oestradiol levels have fallen from 1030 pg/ml to 461 pg/ml (Bill & Greenwald, 1981).

Atresia has been postulated to be caused by a decrease in sensitivity to gonadotrophins (Richards & Midgley, 1976) and a decrease in gonadotrophin receptors occurs in rat follicles with advancing atresia (Uilenbroek, Woutersen & van der Schoot, 1980). In the hamster, cyclic nucleotides decrease with time in atretic antral follicles and this may be related to changes in the numbers or properties of gonadotrophin receptors (Hubbard & Greenwald, 1981).

The purpose of the present study was to quantify the changes occurring in binding sites for gonadotrophins in this hamster model of atresia by topical autoradiography (Bortolussi, Marini

& Reolon, 1979) which involves the application of radioiodinated FSH and hCG to fixed frozen sections of ovary. The latter hormone was used instead of LH because both hormones share the same receptors but hCG is less damaged by iodination and is more stable.

Materials and Methods

Female hamsters (*Mesocricetus auratus*) weighing 80–120 g were maintained in lighting of 14 h light : 10 h dark (lights on at 05:00 h). The day of ovulation (Day 1) was detected by the copious vaginal discharge. The animals underwent a minimum of 3 cycles before treatment. They were hypophysectomized via a parapharyngeal approach on the morning (09:00–10:00 h) of Day 1 of the cycle. Immediately after surgery, 30 i.u. PMSG were injected s.c. On Day 4, 100 μ l antiserum to PMSG was injected i.p. at 09:00 h. The antiserum to PMSG was raised in rabbits by Dr D. C. Johnson and has been previously characterized for the hamster by Bill & Greenwald (1981). A control group (Group 0) consisted of hypophysectomized PMSG-treated hamsters which received no antiserum and were killed on the morning of Day 4 at 09:00 h. The other animals were killed 24, 48 or 72 h after the antiserum injection (Groups 1, 2 and 3, respectively). In a second set of experiments to test the effectiveness of PMSG in sustaining follicular development beyond Day 4, animals were treated as in Groups 0 and 1 and a third group was treated with 100 μ l normal rabbit serum at 09:00 h on Day 4 and killed 24 h later.

Tissue processing. The ovaries were placed in cryoembedding medium and frozen over liquid nitrogen. Sections were cut at 10 μ m in a freezing microtome kept at -20°C . The sections were fixed in picric acid–paraformaldehyde fixative and washed in 0.01 M-phosphate buffered saline (PBS, pH 7.0), air dried and stored in -20°C .

Iodination. The hormones for iodination were FSH (NIAMDD-rat-FSH-I-4) and hCG (Calbiochem, LaJolla, California), 10 605 i.u./mg. The hormones were iodinated by the chloramine-T method, a modification of the procedure of Butt (1972). Sephadex G-75 was used to separate the labelled hormone from free ^{125}I . For competitive tests, the unlabelled hormones used were NIAMDD-rat-FSH-RP1 and hCG (Calbiochem, 2000 i.u./mg). The specific activities for the labelled hormones were approximately 33.25 $\mu\text{Ci}/\mu\text{g}$ protein and 51.2 $\mu\text{Ci}/\mu\text{g}$ protein for FSH and hCG, respectively. The specific activities for hormones used in the second group of experiments (Table 2) were 31.13 $\mu\text{Ci}/\mu\text{g}$ protein for FSH and 18.34 $\mu\text{Ci}/\mu\text{g}$ protein for hCG.

Preparation of slides. The sections were incubated for 1 h in moist chambers with 25 μ l of labelled hormones diluted in 0.01 M-PBS with 1% egg white to give concentrations of FSH and hCG of approximately 6 and 4 ng/ml, respectively. The counts for FSH applied to slides were 1.0×10^4 counts/25 μ l/min and for hCG 1.2×10^4 counts/25 μ l/min. For the second set of experiments the counts applied for FSH and hCG were 1.2×10^4 counts/25 μ l/min, the concentration of hormones being 4 ng FSH/ml and 12 ng hCG/ml. These concentrations were chosen because previous studies in our laboratory have demonstrated that these values were optimal for quantitative studies under our experimental conditions. To check the specificity of binding (Midgley, 1973), competitive tests were performed on parallel sections by adding an excess of the unlabelled homologous (rat FSH for labelled rat FSH and unlabelled hCG for labelled hCG) or heterologous (rat FSH for labelled hCG and hCG for labelled rat FSH) hormone. After incubation the slides were washed, fixed in PBS–3% glutaraldehyde and dipped in Kodak NTB 2 nuclear track emulsion (Eastman Kodak, Rochester, New York) and exposed at $0-4^{\circ}\text{C}$ for 40 h. A separate group of slides was exposed for 9 days. After development, the sections were stained with nuclear fast red stain and saturated alcoholic picric acid and mounted in permount.

Quantification. Photomicrographs were taken at random from each slide of adjacent sections exposed for 40 h and silver grains were counted. Both ovaries from 3 animals were used for each group. From each animal 3 large antral follicles were selected for photography. For thecal and

PLATE 1

All slides were exposed for 9 days before developing.

Fig. 1. Autoradiograph showing binding of ^{125}I -labelled FSH to granulosa cells of an antral follicle before injection of PMSG antiserum (time zero). $\times 185$.

Figs 2–4. Binding of ^{125}I -labelled FSH to granulosa cells of ovarian follicles 24 (Fig. 2), 48 (Fig. 3) and 72 (Fig. 4) h after the injection of antiserum to PMSG. $\times 185$.

PLATE 2

Fig. 5. Autoradiograph showing binding of ^{125}I -labelled hCG to granulosa cells of an antral follicle and to the interstitium at time zero. $\times 185$.

Figs 6–8. Binding of ^{125}I -labelled hCG to the granulosa cells and the interstitium at 24 (Fig. 6), 48 (Fig. 7) and 72 (Fig. 8) h after induction of atresia. $\times 185$.

interstitial compartments two areas were selected per animal and photographed. For the second set of experiments 7–9 follicles per animal were counted. The granulosa cells and interstitium were photographed at $\times 250$ while the theca was filmed at $\times 900$ magnification. As shrinkage of granulosa cells occurs with atresia, the grains were counted and expressed per cell whereas for thecal and interstitial cells the grains were expressed as per 100 mm^2 . The areas on the photomicrographs were measured on a digitizer (software was supplied by Ralph L. Smith Computer Center). Values obtained in equivalent areas of sections where competitive tests were performed with excess homologous hormones were assumed to correspond to non-specific binding plus background values.

Statistics. To compare the values for Groups 0, 1, 2 and 3 and those for groups in the second set of experiments, a one-way analysis of variance followed by Duncan's new multiple range test was performed. To compare counts from sections incubated with excess unlabelled heterologous hormone and those incubated only with labelled hormone a paired *t* test was performed. The level of significance was considered to be $P < 0.05$ or lower.

Results

Qualitative changes in binding of iodinated gonadotrophins to the ovary

Tissue sections incubated with ^{125}I -labelled FSH or ^{125}I -labelled hCG revealed highly specific binding to different tissue compartments which was almost completely inhibited by excess of unlabelled homologous hormone but not significantly affected by unlabelled heterologous hormone, thus indicating the high specificity of the binding.

General pattern of gonadotrophin uptake

In slides exposed for 9 days, sections incubated with ^{125}I -labelled FSH revealed localization of silver grains over the granulosa cells of the antral follicles. Theca and interstitium did not show any appreciable number of silver grains. As shown in Pl. 1, Fig. 1, the number of silver grains was very high in Group 0, dropped sharply in Group 1 and steadily in Groups 2 and 3 (Pl. 1, Figs 2–4). The number of silver grains in Group 2 was less than that in Group 1. ^{125}I -labelled hCG bound to the granulosa cells of antral follicles, the thecal cells and interstitial cells. There was a drop in the number of silver grains in the granulosa cells comparable to that seen for binding of ^{125}I -labelled FSH (see Pl. 2, Figs 5–8).

The distribution of silver grains over the interstitial and thecal cells did not differ significantly during the 72 h period (Pl. 2, Figs 2 and 3).

Quantitative aspects of binding after induced follicular atresia

The decline in grains/granulosa cell was much more precipitous for FSH than for hCG. There were no significant differences in the binding of hCG to thecal or interstitial cells over the 72-h time span of induced atresia (Table 1). There was no significant difference in grain counts between animals killed at time 0 and animals treated with NRS and killed 24 h later, thus showing the ability of PMSG to maintain follicular growth beyond Day 4. Animals treated with PMSG antiserum on Day 4 and killed 24 h later showed significant differences in counts of silver grains (Table 2).

The difference in the number of silver grains per cell in the two sets of experiments involving hCG (Tables 1 and 2) is attributable to the difference in specific activity of the labelled hormone (see 'Materials and Methods').

Table 1. Binding of ^{125}I -labelled FSH and ^{125}I -labelled hCG to different ovarian cell types during induced follicular atresia in hamsters

Cell type	No. of animals	Hormone	Time killed after PMSG antiserum (h)	Counts (mean $\pm$ s.e.m.)
				<i>Grains/cell</i>
Granulosa cells	3	FSH	0	13.55 $\pm$ 1.69
			24	4.20 $\pm$ 0.45*
			48	1.74 $\pm$ 0.12*
			72	0.62 $\pm$ 0.22*†
Granulosa cells	3	hCG	0	12.71 $\pm$ 1.00
			24	5.95 $\pm$ 1.25*
			48	3.48 $\pm$ 0.15*
			72	2.99 $\pm$ 1.00*†
				<i>Grains/100 mm²</i>
Interstitial cells	3	hCG	0	12.49 $\pm$ 2.05
			24	8.93 $\pm$ 1.04
			48	13.18 $\pm$ 1.36
			72	8.58 $\pm$ 1.28
Thecal cells	3	hCG	0	1.96 $\pm$ 1.00
			24	1.19 $\pm$ 0.21
			48	2.38 $\pm$ 0.37
			72	1.98 $\pm$ 0.92

* Significantly different from the 0 h value, $P < 0.01$.† Significantly different from the value at 24 h, $P < 0.05$.**Table 2.** Binding of ^{125}I -labelled FSH and ^{125}I -labelled hCG to granulosa cells in hamsters treated with PMSG, normal rabbit serum (NRS) and antiserum to PMSG

Treatment	No. of animals	Time after treatment (h)	Hormone	Grains/granulosa cell (mean $\pm$ s.e.m.)
PMSG	4	0	FSH	15.37 $\pm$ 1.41
NRS	4	24	FSH	13.24 $\pm$ 1.69
PMSG antiserum	3	24	FSH	4.27 $\pm$ 1.52*
PMSG	4	0	hCG	5.02 $\pm$ 0.32
NRS	3	24	hCG	4.06 $\pm$ 1.37
PMSG antiserum	3	24	hCG	0.72 $\pm$ 0.32*

* Significantly different from the values for the other two groups, $P < 0.05$.

Discussion

After the induction of follicular atresia by PMSG antiserum, the binding of labelled FSH and hCG to granulosa cells dropped sharply during the first 24 h and decreased further at 48 and 72 h. However, the binding of ^{125}I -labelled hCG to thecal and interstitial cells remained relatively constant throughout the 72-h period studied. In advanced stages of atresia there is loss of LH and FSH binding in the ewe (Carson, Findlay, Burger & Trounson, 1979) and loss of LH binding in immature rats (Peluso, Steger & Hafez, 1977).

Similarly, in a model of atresia based on postponing ovulation in pro-oestrous rats by daily administration of pentobarbitone sodium, binding to granulosa cells of atretic follicles of FSH and hCG decreases during a 4-day period; there was also no appreciable change in the binding of

hCG to thecal cells over this period (Uilenbroek *et al.*, 1980). In the present study, because of the acute deprivation of gonadotrophin support of the follicle, the changes in gonadotrophin binding sites occur very quickly.

Hubbard & Greenwald (1981) incubated antral follicles at various times after atresia was induced in hamsters by treatment with PMSG antiserum. Oestradiol and testosterone levels dropped significantly in antral follicles and medium within 2 h whereas progesterone levels remained elevated even at 72 h. This was associated with significant cell death of granulosa cells within 24 h as evidenced by a significant decline in DNA per follicle and ovarian histology; on the other hand, the theca was histologically normal but showed no signs of hypertrophy. The present finding that labelled hCG binding to thecal cells was unaffected for as long as 72 h after the induction of atresia suggests that the theca is the source of the prolonged synthesis of progesterone noted by Hubbard & Greenwald (1981).

Both FSH and LH stimulate synthesis of 3'5'-cyclic AMP in antral follicles in the hamster (Makris & Ryan, 1978). The decrease in binding of ^{125}I -labelled FSH and ^{125}I -labelled hCG to the follicle as atresia progresses (Table 1) could lead to the decrease in production of follicular cyclic nucleotides observed in this model by Hubbard & Greenwald (1981). The presence of hCG receptors in large quantities in the interstitium even at 72 h may account for the fact that hamster ovaries deprived of gonadotrophins by hypophysectomy produce *in vitro* significant quantities of progesterone which can be further stimulated by LH (Taya & Greenwald, 1980).

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