Effects of gonadotrophins on progesterone production by isolated granulosa cells of the adenohipophysectomized domestic fowl (*Gallus domesticus*)

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Summary. Ovine LH and ovine FSH stimulated progesterone production in granulosa cells isolated from the F_1 , F_2 and F_3 follicles of hypophysectomized and control (sham-operation) hens when they were collected 6 h after operation, but the steroidogenic response to LH was greater for granulosa cells from hypophysectomized hens. At 15 h after operation progesterone production by granulosa cells was stimulated by LH in all 3 follicle types of control hens, but only in the F_1 follicles of hypophysectomized hens. The response to FSH at 15 h was similar for control and hypophysectomized hens. The time after hypophysectomy therefore appears to affect the LH-stimulated progesterone production by granulosa cells of the F_2 and F_3 follicles.

Introduction

The ovary of the domestic hen contains a hierarchy of yolk-filled follicles. The steroid content of the follicular wall as well as the steroidogenic profile of the three largest follicles changes as the follicles mature (Shahabi, Norton & Nalbandov, 1975; Imai & Nalbandov, 1978). The largest follicle secretes large amounts of progesterone, whereas the second and third largest follicles produce predominantly testosterone and oestradiol, respectively. Huang & Nalbandov (1979), measuring steroid secretion by isolated granulosa and theca cells, found that the granulosa layer is the major site for progesterone production while the theca layer is the major source of oestradiol.

While steroidogenesis by the granulosa cells of hens in response to the addition of exogenous gonadotrophins has been examined extensively (Huang & Nalbandov, 1979; Scanes & Fagioli, 1980; Culbert, Hardie, Wells & Gilbert, 1980; Zakár & Hertelendy, 1980), no similar studies have been done on the granulosa cells of the adenohipophysectomized hen. Therefore, the present study was carried out to examine the effect of exogenous gonadotrophins on progesterone production by isolated granulosa cells from F_1 , F_2 and F_3 follicles of adenohipophysectomized hens.

Materials and Methods

Hormones and reagents. Ovine LH (NIAMDD-oLH-24) and ovine FSH (NIAMDD-oFSH-15) were generously provided by Dr S. Raiti, National Pituitary Agency, Baltimore, MD, U.S.A. Stock solutions (1 mg/ml) of dissolved hormones were divided into small aliquots to avoid repeated thawing and freezing and stored at -20°C . Dilutions to required concentrations were made shortly before the experiment using TCM 199 (Nakarai Chemicals Ltd, Kyoto, Japan). Progesterone, bovine serum albumin fraction V, collagenase type I, penicillin-G and streptomycin sulphate were purchased from Sigma Co. (St Louis, MO, U.S.A.). 2,5-Diphenyloxazole (PPO), 2,2'-*p*-phenylenebis(5-phenyloxazole) (POPOP) and Triton X-100 were purchased from Nakarai Chemicals Ltd, Kyoto Japan.

Animals. White Leghorn hens with regular laying sequences of 6 or more eggs were used. The birds, caged individually, were provided with food and water *ad libitum*. The lighting regimen was 14 h light/24 h with lights on at 06:00 h. Time of oviposition was recorded daily to permit accurate prediction of ovulation.

Adenohypophysectomy. Light anaesthesia was achieved by i.v. injection of pentobarbitone sodium (15 mg/bird). The anterior lobes of pituitaries were removed by using a stereotaxic instrument, as described by Tanaka & Nobukuni (1977), 15 h before the expected time of oviposition of the 2nd or 3rd egg in the laying sequence. Control birds received the same operation but the anterior lobes of pituitaries were not removed. The birds were killed by decapitation 6 or 15 h after operation. We confirmed by sham-operations that our method does not have any effect on normal oviposition and ovulation times.

Preparation and incubation of granulosa cells. The birds had not already ovulated before they were killed. The F_1 , F_2 and F_3 follicles were collected, placed individually into ice-cold saline (0.9% NaCl) and the granulosa cell layer was isolated. The isolated membrana granulosa was washed with saline and minced. The minced preparations from 4 hens were transferred into a plastic vial containing 2 ml TCM 199 with collagenase (500 i.u./ml). The vial was incubated in a shaker with 95% O_2 and 5% CO_2 at 41°C for 10 min. After incubation, the contents were centrifuged at 600 *g* at 4°C for 7 min. The pellet was resuspended and washed 4 times with 3 ml TCM 199. The cell suspension, after final washing, was filtered through two layers of lens paper (Eastman Kodak Co., Rochester, NY, U.S.A.) to eliminate clumps of cells. Cell viability was determined before and after incubation by the trypan dye exclusion method and was above 90%. The cell suspension was then diluted with incubation medium to a final concentration of 10^4 or 10^5 cells/ml. In all experiments, the incubation medium used was TCM 199 containing 0.4% bovine serum albumin, penicillin-G (50 i.u./ml) and streptomycin sulphate (50 µg/ml). Samples (1 ml) of the cell suspension were incubated with or without the hormone being tested (1.0 ml/tube) at 41°C for 4 h in an humidified atmosphere of 95% O_2 and 5% CO_2 . At the end of incubation, the tubes containing the cell suspensions were stored at -20°C. All solutions used were sterilized by filtration with a 0.22 µm Millipore filters (Millipore Corp., Massachusetts, U.S.A.), and all equipment was autoclaved (120°C, 15 min).

Before setting up the radioimmunoassay for progesterone, the thawed and mixed contents of the tubes were diluted with assay buffer and appropriate aliquants were transferred to borosilicate RIA tubes. Values therefore represent total progesterone production (medium + cells).

Radioimmunoassay. Progesterone RIA was performed according to the method of Abraham, Swerdloff, Tulchinsky & Odell (1971). [$1,2,6,7\text{-}^3H$]progesterone (sp. act., 112.0 Ci/mmol) was obtained from New England Nuclear Company (Boston, MA, U.S.A.) and used without further purification. Rabbit antiserum against progesterone was purchased from Teikoku-zoki Co. (Tokyo, Japan). Radioactivity was counted by using a Packard Liquid Scintillation System (TRI-CARB 460CD). The counting efficiency for tritium was 49%.

Cross-reactivity of the progesterone antiserum with other major steroids was as follows: pregnenolone, 1.46%; 20α -dihydroprogesterone, 6.73%; testosterone, 0.17%; oestradiol-17 β , 0.06%. The sensitivity of the assay was 60 pg/ml. Intra- and inter-assay coefficients of variation were 5.8% - 6.3% ($n = 5$) and 7.5% respectively. Further validation of the progesterone RIA was indicated by the parallelism of the regression lines obtained by assaying increasing amounts of progesterone standard and increasing volumes of samples.

Statistical analysis. Student's unpaired *t* test was used to assess the statistical significance of differences between means.

Results

Follicles collected 6 h after operation

Ovine LH stimulated progesterone production in granulosa cells isolated from F_1 , F_2 and F_3 follicles of control and hypophysectomized birds (Text-fig. 1a). The ratio of the maximum

progesterone production with LH to basal progesterone production without LH for cells from F_1 , F_2 and F_3 follicles were 2.9, 2.9 and 1.6 for control birds and 14.3, 6.3 and 4.0 for hypophysectomized birds, respectively. Therefore, the steroidogenic response to LH was greater for granulosa cells from hypophysectomized birds than that from control birds in all 3 types of follicle.

Text-fig. 1. Effect of ovine LH on progesterone production in granulosa cells isolated from the three largest follicles of hens at (a) 6 h and (b) 15 h after sham-operation (controls) and hypophysectomy. Values are mean $\pm$ s.e.m. for 5 culture tubes. * $P < 0.05$, ** $P < 0.01$ compared to the progesterone production at zero dose of LH for each follicle type.

Text-fig. 2. Effect of ovine FSH on progesterone production in granulosa cells isolated from the three largest follicles of hens at (a) 6 h and (b) 15 h after sham-operation (controls) and hypophysectomy. Values are mean $\pm$ s.e.m. for 5 culture tubes. * P < 0.05, ** P < 0.01 compared to the progesterone production at zero dose of FSH for each follicle type.

Ovine FSH stimulated progesterone production similarly in granulosa cells isolated from F₁, F₂ and F₃ follicles of control and hypophysectomized birds (Text-fig. 2a).

Follicles collected 15 h after operation

Ovine LH stimulated progesterone production in granulosa cells isolated from F₁, F₂ and F₃ follicles of control birds (Text fig. 1b) and the values of maximal/basal progesterone production were 3.1, 2.8 and 6.6, respectively. However, in the hypophysectomized birds LH stimulated progesterone production in cells from F₁ follicles (maximal/basal production = 5.3) but was not effective in cells from F₂ follicles (maximal/basal production = 1.0). For F₃ follicles, only the highest dose of LH had a significant effect and the maximal/basal production ratio (3.6) was less than that for F₃ follicles from control birds.

Ovine FSH stimulated progesterone production similarly in granulosa cells isolated from F₁, F₂ and F₃ follicles of control and hypophysectomized birds (Text-fig. 2b).

Discussion

It has been shown that ovine and bovine LH are potent stimulants of progesterone production in chicken granulosa cells (Hammond, Todd & Hertelendy, 1978, 1980; Zakár & Hertelendy, 1980). Hammond, Burke & Hertelendy (1981) studied the progesterone release in response to ovine and turkey LH and FSH in chicken granulosa cells isolated from ovarian follicles of different maturational stages and suggested that FSH-elicited progesterone secretion in granulosa cells from mature follicles was entirely due to contamination with LH. Hammond *et al.* (1981) considered that, by the last day before ovulation, the steroidogenic response to FSH completely disappears and the cells are solely under the regulatory influence of LH. However, our results show that ovine FSH can also stimulate progesterone production in chicken granulosa cells isolated from the 3 largest follicles. Scanes & Fagioli (1980) have reported that mammalian FSH induced increases in progesterone secretion in hens. The stimulatory effect of ovine FSH on progesterone production by granulosa cells may not be due to the LH contamination of FSH because the FSH preparation used in this study (NIAMDD-FSH-S15) has only 2% contamination of LH by weight. Nevertheless, a synergistic effect of this small amount of LH contamination with FSH cannot be excluded.

Adenohypophysectomy appears to affect LH-stimulated progesterone production by isolated granulosa cells 6 h after operation by sensitizing the F₂ and F₃ follicle cells to LH stimulation, although by 15 h after operation the granulosa cells of F₂ and F₃ follicles have lost their ability to respond to LH stimulation. Alternatively, differences between hypophysectomized and control birds in their response to exogenous LH at 15 h after operation could have been due to sensitization of follicles in the sham-operated hens following exposure to preovulatory changes in gonadotrophin secretion rather than to a desensitization of follicles in the hypophysectomized hens.

Although the precise mechanisms remains to be explored, our results appear to suggest that the sensitivity of the granulosa cells to LH may be involved in the mechanism of multiple ovulation after hypophysectomy reported by Opel & Nalbandov (1961) and Ogawa, Tojo & Kajimoto (1976).

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