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IN VITRO STUDIES ON STEROIDOGENESIS IN THE PRESENCE OF PREGNENOLONE AS PRECURSORS BY THE FOLLICULAR TISSUE OF THE DOMESTIC FOWL (*GALLUS DOMESTICUS*)

TAKAO NAKAMURA, MIKI FUNABASHI and YUICHI TANABE*
Department of Animal Science and Technology, Faculty of Agriculture, Gifu University, Yanagido, Gifu 501-11, Japan

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Summary—Chicken granulosa and theca cells were isolated from F1 and F4–6 follicles 2–4 h before ovulation, and the amounts of progesterone, testosterone and oestradiol released in the medium during incubation for 3 h, in the presence or absence of pregnenolone as a percursor and stimulatory drugs or inhibitory drugs, were measured. Progesterone synthesis by granulosa cells was stimulated with oLH or theophylline. Much more progesterone was synthesized when prenenolone was added to the medium. The amount of testosterone produced by the granulosa cells was similar to that produced by the theca cells. The production of testosterone was increased by the addition of oLH or theophylline. Oestradiol synthesis by F4–6 follicles was higher than by F1 follicles, and it was higher in the theca cells than in the granulosa cells. The addition of oLH or theophylline increased oestradiol synthesis in the theca cells and the granulosa cells of F4–6 follicles. The results indicate that oestradiol can be produced from pregnenolone by the theca cells alone. It is possible, however, that the theca cells also take in the precursors for the production of oestradiol from the granulosa cells.

INTRODUCTION

Ovarian steroid hormones have been implicated in the control of various reproductive processes of avian species. Many works have been published concerning production [1–3], metabolism [4, 5] and secretion [6, 7] of ovarian steroid hormones of the chicken (*Gallus domesticus*). In this species, the principle cellular source of progesterone appears to be the granulosa cells of the largest follicle (F1) [1, 8], while oestradiol is produced by the thecal layer of the smaller follicles [9]. A two-cell model for steroidogenesis in the ovarian follicle has been proposed, and suggests that the granulosa cells are responsible for producing the precursor, progesterone, which is further metabolized by the thecal layer to testosterone and to oestradiol. Further, it is noted that progesterone was synthesized not only in granulosa cells of F1 but also in those of smaller follicles when mammalian LH was added to the incubation medium [1, 2].

The present study was carried out to elucidate the effects of the addition of 3β-hydroxy-5-pregnen-20-one (pregnenolone), and stimulating or inhibiting drugs for steroidogenesis, on progesterone, testosterone and oestradiol production by isolated granulosa and theca cells of the hierarchical follicles of the hen, and also to determine whether the two-cell model for steroidogenesis applies to chicken follicles.

EXPERIMENTAL

Fifty laying White Leghorn hens (Babcock B. 300V), 13 months of age, with regular sequences of at least 6 eggs, were used. They were caged individually under a 14 h light: 10 h dark (lights on 0500–1900 h) photoperiodic condition, and had free access to feed and water. The hens were killed after the second clutch by cervical dislocation 2–4 h before the expected ovulation. The largest (F1) and the 4th–6th largest (F4–6) follicles were removed. All the follicles from all 50 hens were pooled. The granulosa and theca membranes were isolated and prepared following the methods of Gilbert *et al.* [10] and Huang and Nalbandov [8], and were weighed and dispersed by incubation with 0.4% collagenase (Sigma, St Louis, Mo.) in 1.0 ml incubation buffer (HEPES containing 0.4% D-glucose and

*To whom correspondence should be addressed.

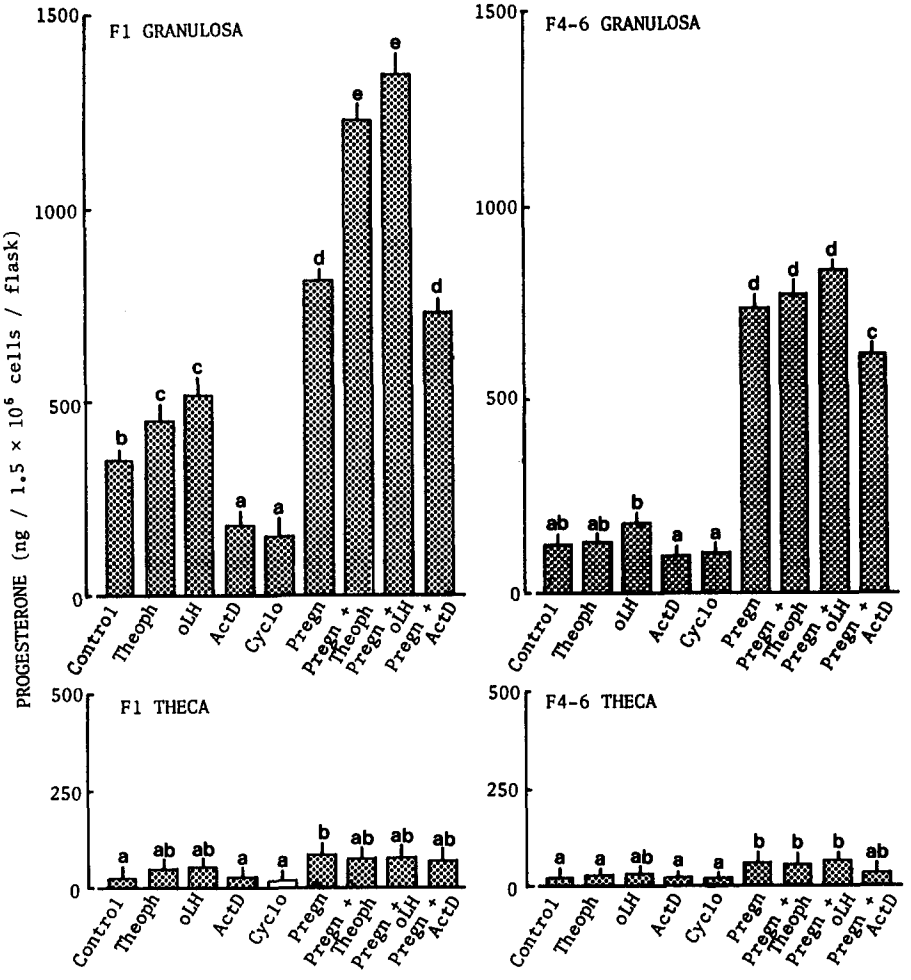

Fig. 1. The effect of oLH, theophylline(Theoph), actinomycin D(Act D), cyclohexamide(Cyclo) and pregnenolone(Pregn) on *in vitro* progesterone production by follicular granulosa and theca cells of the largest follicle (F1) and the 4th–6th largest follicles (F4–6) of the hen after 180 min incubation. All values represent the mean of the four flasks with SE. The means of treatment groups with different letters differ significantly ($P < 0.05$) from each other by Duncan's multiple range test.

0.1% bovine serum albumin, pH 7.2) in a glass tube. During digestion of the tissue, the content was frequently aspirated and expelled with a Pasteur pipette. After 5 min incubation at 37°C, the cells were collected by centrifugation at 800 *g* for 5 min. The precipitate was then washed three times with 2 ml incubation buffer in order to remove the collagenase. The concentration of cells was determined by counting on a Toma–Zeiss hemocytometer and was adjusted to 1.5×10^6/ml by dilution with Romanoff's avian Ringer's albumin buffer (RRA, containing 0.4% D-glucose and 0.5% bovine serum albumin, pH 7.2). The suspension of dispersed granulosa cells and theca cells (1 ml/flask) was incubated in RRA containing 0.25% bovine serum albumin and 0.2% glucose in a shaking water bath with vehicle, ovine LH (oLH, S-14, NIH; 5.0 μg/flask) and pregnenolone (1.0 μg/flask) for 3 h, at 40°C in a 95% O_2:CO_2 atmosphere. In some experiments, the incubation buffer contained 10 μg/ml theophylline (Sigma), 20 μg/ml actinomycin D (AI 410, Sigma) or 20 μg/ml cyclohexamide (Sigma). The incubation was terminated by quick freezing at −80°C, and the tubes were kept at the same temperature until the radioimmunoassay of steroids was performed. Progesterone, testosterone and oestradiol concentrations in the media were measured by radioimmunoassay according to the method of Tanabe *et al.* [11]. Rabbit antiprogesterone-3-(*O*-carboxymethyl)-oxime-BSA serum was obtained from Teikokuzoki, Tokyo. Rabbit antitestosterone-3-(*O*-carboxymethyl)-oxime-BSA serum (HAC-AA61-02-RBP81) and antioestradiol-6-(*O*-carboxymethyl)-oxime-BSA serum (HAC-AA64-02-RBP79) were obtained from

Hormone Assay Centre, Institute of Endocrinology (Gunma University, Maebashi, Japan). [1,2,6,7,16,17-^{3}H]Progesterone (sp. act. 117 Ci/mmol, Amersham), [1,2,6,7,16,17-^{3}H]testosterone (sp. act. 114 Ci/mmol, New England Nuclear) were used as antigens in radioimmunoassays. The antiprogesterone serum cross-reacted with progesterone, pregnenolone, 17α-hydroxy-4-pregnene-3,20-dione (17α-hydroxyprogesterone), 11-deoxycorticosterone, testosterone, and oestradiol at 100, 1.46, 0.89, 6.60, 0.17, and <0.06% levels, respectively. The antitestosterone serum cross-reacted with testosterone, pregnenolone, progesterone, 17α-hydroxyprogesterone, 4-androstene-3,17-dione (androstenedione), and oestradiol at 100, <0.01, <0.01, <0.01, <0.04 and <0.01% levels, respectively. The antioestradiol serum cross-reacted with oestradiol, oestrone, oestriol, testosterone, androstenedione, progesterone, and pregnenolone at 100, <0.10, <0.10, 0.29, 0.45, <0.08, and <0.08% levels, respectively. The overall intra-assay coefficient of variance was 9.4% for progesterone, 6.9% for testosterone and 5.4% for oestradiol. All data were expressed as the mean of 4 flasks $\pm$ SE. The minimal detectable levels of progesterone, testosterone and oestradiol, respectively, are 5, 3 and 3 pg per 0.2 ml. Significant differences among treatments, those of F1 and F4–6, and those of granulosa and theca cells were evaluated by Duncan's multiple range test [12]. The results were expressed in ng of progesterone per 1.5×10^6 cells, pg of testosterone per 1.5×10^6 cells and pg of oestradiol per 1.5×10^6 cells.

RESULTS

The amounts of progesterone produced by the follicular granulosa and theca cells of the F1 and of F4–6 are shown in Fig. 1. Progesterone production was much higher in F1 than in F4–6 without pregnenolone added in the medium, and significantly higher in the granulosa cells than in the theca cells. Progesterone production by granulosa cells was stimulated by oLH or theophylline in F1. Significantly more progesterone was produced by the granulosa cells of F1 or F4–6 when pregnenolone was added to the medium at the dose of 1 μg per flask. The addition of the precursor to the medium containing oLH or theophylline further promoted the production of progesterone by F1 but not by F4–6. The addition of either actinomycin D or cycloheximide inhibited progesterone production by both F1 and F4–6.

The theca cells had 3β-hydroxy-Δ^5-steroid dehydrogenase activity catalyzing of progesterone from pregnenolone. Low but still detectable amounts of progesterone were produced by theca cells from both F1 and F4–6 collected at 2–4 h before ovulation.

The amounts of testosterone produced by follicular granulosa and theca cells of F1 and of F4–6 are shown in Fig. 2. Testosterone production by granulosa cells from F1 and F4–6 was stimulated by both oLH and theophylline. Significantly more testosterone was produced when pregnenolone was added to the medium. The addition of either oLH or theophylline to the medium containing pregnenolone further promoted the production of testosterone by F1 and F4–6. The addition of either actinomycin D or cyclohexamide inhibited testosterone production in both F1 and F4–6.

The amount of testosterone produced by the theca cells was similar to that produced by the granulosa cells. The production by both F1 and F4–6 was significantly stimulated by the addition of oLH or theophylline in comparison to the control. Much more testosterone was produced by both F1 and F4–6 theca cells when pregnenolone was added to the medium.

The amounts of oestradiol produced by the granulosa and theca cells of the F1 and of F4–6 are shown in Fig. 3. Oestradiol production by F4–6 was higher than that by F1 ($P < 0.05$). It was higher in the theca cells than in the granulosa cells in both F1 and F4–6 ($P < 0.05$). Maximum production of oestradiol was obtained in the theca cells of F4–6 obtained 2–4 h before ovulation. The addition of either oLH or theophylline to the medium increased oestradiol production in the granulosa and theca cells of F4–6 obtained 2–4 h before ovulation. The addition of either oLH or theophylline to the medium containing pregnenolone increased oestradiol production in the theca cells of F4–6. The addition of either antinomycin D or cyclohexamide inhibited oestradiol production.

The granulosa cells had a little aromatase enzyme system activity catalyzing of C_{19} steroid to oestrogen. Lower oestradiol production was observed in the granulosa cells of F1 collected 2–4 h before ovulation in comparison with the theca cells. The production of oestradiol by the granulosa cells of F1 was not stimulated by oLH or theophylline in the condition.

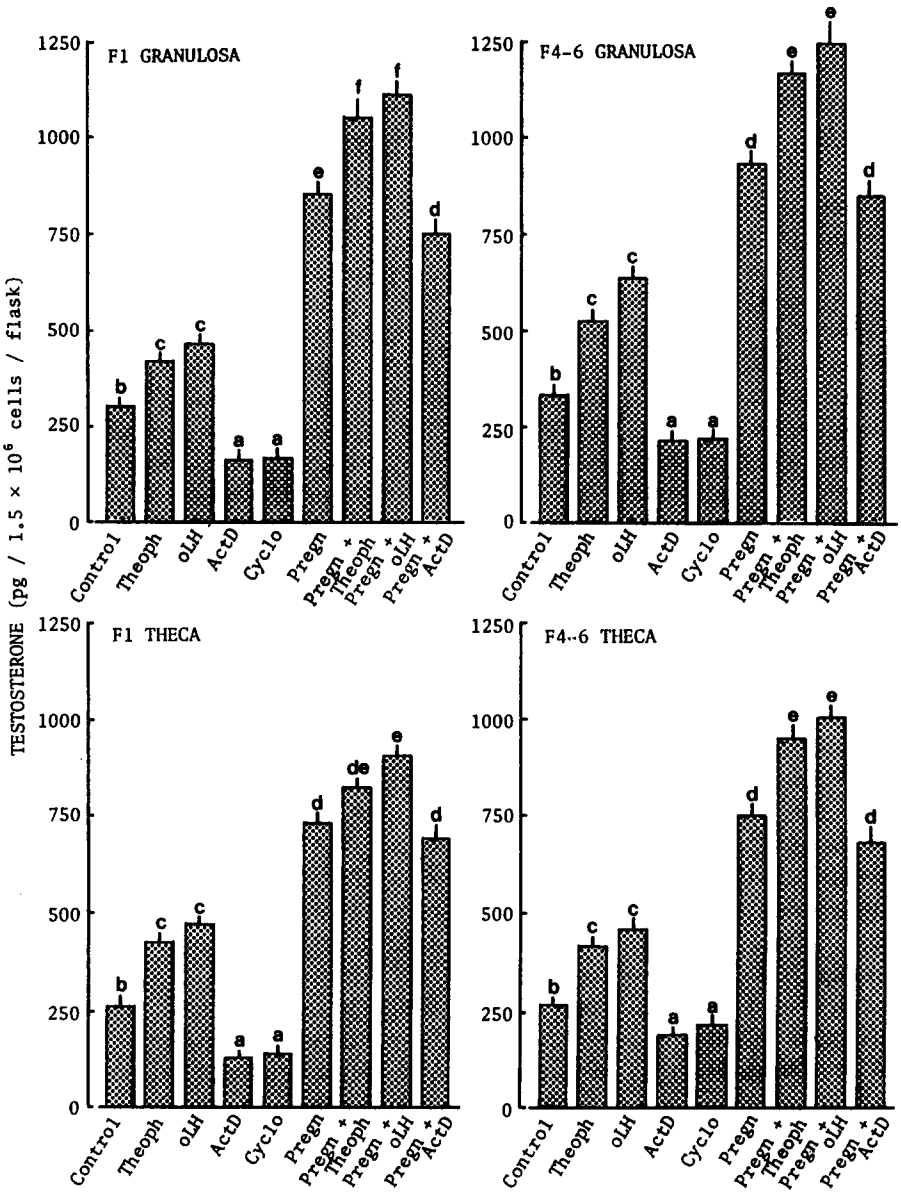

Fig. 2. The effect of oLH, theophylline(Theoph), actinomycin D(Act D), cyclohexamide(Cyclo) and pregnenolone(Pregn) on *in vitro* testosterone production by follicular granulosa and theca cells of the largest follicle (F1) and the 4th–6th largest follicles (F4–6) of the hen after 180 min incubation. All values represent the mean of the four flasks and the SE. The means of treatment groups with different letters differ significantly ($P < 0.05$) from each other by Duncan's multiple range test.

DISCUSSION

The results demonstrate that progesterone was mainly produced in the granulosa cells of F1 follicles *in vitro* and the production of progesterone was greatly increased when the granulosa cells from either F1 or F4–6 were incubated in the medium with exogenous pregnenolone.

In the chicken, it was reported that high cholesterol C_{20}–C_{22} lyase activity was present in the granulosa cells of F1 [13]. Cholesterol C_{20}–C_{22} lyase is activated by theophylline, an inhibitor of phosphodiesterase, showing that adenyl cyclase–cAMP systems are involved in this reaction. The granulosa cells of preovulatory follicles of the chicken contain 3β-hydroxy-Δ^5-steroid dehydrogenase, and the activity of this enzyme was higher in F1 than in F2 follicles [14]. The present study showed that the granulosa cells of F1 reacted to oLH or theophylline by increasing the production of progesterone, but the amount produced was much less than that produced by the addition of pregnenolone with or without oLH and theophylline in the medium.

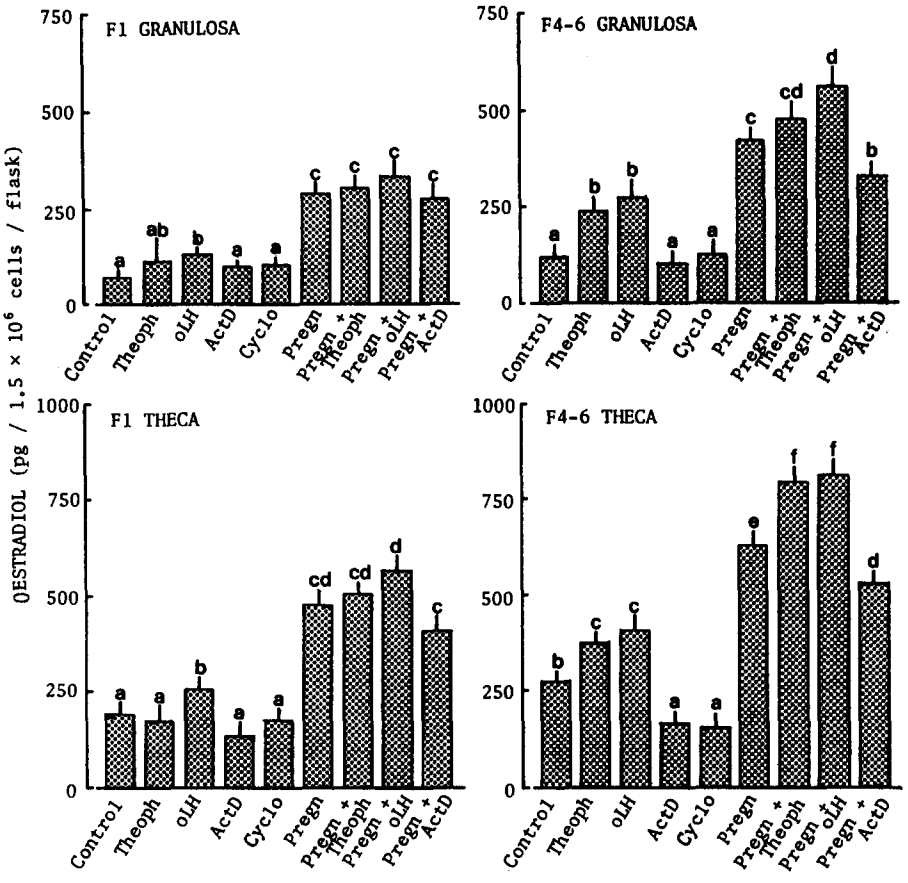

Fig. 3. The effect of oLH, theophylline(Theoph), actinomycin D(Act D), cyclohexamide(Cyclo) and pregnenolone(Pregn) on *in vitro* oestradiol production by follicular granulosa and theca cells of the largest follicle (F1) and the 4th–6th largest follicles (F4–6) of the hen after 180 min incubation. All values represent the mean of the four flasks with SE. The means of treatment groups with different letters differ significantly ($P < 0.05$) from each other by Duncan's multiple range test.

The production of progesterone by the theca cells was low and showed no marked reaction to oLH or theophylline even in the presence of a sufficient amount of pregnenolone. From this result, it is suggested that the main sites of action of gonadotropin responsible for the production of progesterone reside in the activation of cholesterol C_{20}–C_{22} lyase, regulating the process of pregnenolone synthesis, and/or reside in the control of the turnover rate of progesterone to other steroids such as testosterone and oestradiol. Furthermore, the activity of 3β-hydroxy-Δ^5-steroid dehydrogenase is high in the granulosa cells, and the enzyme is not considered to be as the rate limiting one for converting pregnenolone to progesterone.

No difference in testosterone production by the chicken follicles was observed between F1 and F4–6 follicles, and between granulosa cells and theca cells. However, testosterone production was much lower than progesterone production in the granulosa cells. It is suggested that this is due to the activity of 17α-hydroxylase, 17α-hydroxylase activity may be suppressed to regulate the production of testosterone both in granulosa cells and theca cells, being most severely suppressed in the granulosa cells of F1 at preovulatory stage [14]. The level of testosterone in the plasma of laying hens showed two peaks, at 18–22 h and at 4–6 h before ovulation [15]. Since the peak 4–6 h before ovulation disappeared in hens which failed to ovulate, the peak production of testosterone in follicles may be under the control of the LH surge [16].

Oestradiol is produced from progesterone via testosterone by the catalytic action of aromatase enzyme system in the chicken ovulatory follicle [17]. From the results of this experiment, it was shown that oestradiol was mainly produced in the theca cells of F4–6 follicles. Etches *et al.* [9] reported that oestradiol was detected at a higher concentration in the venous blood of the small follicles than in that of the larger follicles. In the present study, the theca cells of small follicles

showed a positive reaction to oLH or theophylline by increasing the production of oestradiol, and the effect of gonadotropin became more pronounced when pregnenolone were added into medium. The concentration of oestradiol in the plasma of the laying hen showed two peaks during the ovulatory cycle, and the second peak, 4 h before ovulation, coincided with the peaks of LH and progesterone [18]. The author's unpublished data show that no distinct production patterns between 20–21 h and 2–4 h before ovulation were observed, except in production of progesterone by granulosa cells of F1 where the production of progesterone increased.

Although oestradiol was produced mainly in the theca cells, the granulosa cells could also produce it to some extent and theca cells could produce testosterone and oestradiol from pregnenolone. Therefore, it is suggested that the "2-cell model" [1] with respect to the production mechanism of oestradiol can be applied in a loose sense in the chicken. At present, it is still unknown whether theca cells can take up precursors for the production of oestradiol from granulosa cells.

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