Role of Granulosa and Theca Cells on *in Vitro* Progesterone Production in Preovulatory Follicles of the Japanese Quail

Makoto MORI, Kaoru KOHMOOTO and Yoichi SHODA

Department of Animal Breeding, Faculty of Agriculture, University of
Tokyo, Bunkyo-ku, Tokyo 113, Japan

Introduction

The preovulatory follicles of the mature domestic birds are arranged in a distinct hierarchical order in the ovary. Presumably all preovulatory follicles are exposed to a daily surge of luteinizing hormone (LH), but only the largest follicle (F1) ovulates each day. This has been explained by the ability of progesterone synthesis of F1 in response to LH. In fact, progesterone contents in the follicle wall of F1, but not of the second largest (F2) or the third largest (F3) follicle, increased concomitantly with the raise of serum LH^{1,2)}. Even though the concentrations of LH were not significantly different among venous plasma of various sizes of follicles, only the venous plasma of F1 exhibited the preovulatory peak of progesterone³⁾. The ovulation was blocked by an injection of a steroid synthesis inhibitor, aminoglutethimide phosphate, and it could be overcome by an injection of progesterone⁴⁾. All of these observations support the idea that progesterone synthesis in the follicle wall of F1 after LH surge is associated with the ovulation.

From the *in vitro* incubation studies, the cellular source of progesterone in chicken follicles has been shown to be the granulosa cells⁵⁾. However, progesterone was synthesized not only by the granulosa cells of F1 but also by those of smaller follicles when LH was added to the incubation medium^{5,6,7)}. No systematic experiments have yet been done which allow one to explain the discrepancy between *in vivo* and *in vitro* studies. Therefore, the present study was conducted to elucidate the role of the granulosa cells and the theca cells isolated from various sizes of follicles for progesterone synthesis in the quail ovary.

Materials and Methods

Animals

Laying Japanese quail (*Coturnix coturnix japonica*), 9 to 18 weeks of age, were used in the experiments. The birds, caged individually, were provided with food and water *ad libitum*. The lighting schedule was 14L : 10D with lights on at 0500. Time of oviposition of all birds was checked daily at 0.5-hr intervals. The time of ovulation was predicted based on the time of oviposition of the previously ovulated follicle which usually occurred 15 to 30 min prior to the next ovulation⁸⁾.

Tissue preparation

The birds were killed with a guillotine. Three largest preovulatory follicles were collected at 9 to 10 hr before the expected time of ovulation of F1, when serum LH concentrations and follicular progesterone contents were low^{2,9)}, and placed in ice-cold physiological saline. For preparation of the intact follicle wall, deep cuts into the yolk mass were made with fine scissors along the direction of the stigma and at the right angle to it. The follicle wall was then washed carefully with a mild stream of saline from a Pasteur pipette in order to remove adherent yolk on the wall. Thus, approximately four equal pieces of the follicle wall which consisted of the theca and granulosa layers can be obtained without contamination of the yolk.

For preparation of granulosa cells, the granulosa layers were isolated according to the procedure of GILBERT *et al.*¹⁰⁾ as modified by Kotosai¹¹⁾ for quail, and were weighed and dispersed by incubating with 500 U/ml collagenase (Type I, Sigma, St. Louis, MO) in 1.0 ml of incubation buffer (Krebs-Ringer-HEPES containing 0.4% D-glucose and 0.1% bovine serum albumin, pH 7.4). During digestion in a polystyrene tube, the content was frequently aspirated and expelled with a Pasteur pipette. After 10 min of incubation at 37°C, the cells were collected by centrifugation at 800 × g for 3 min. The precipitate was then washed three times with 3 ml of the incubation buffer in order to remove collagenase. The final pellet derived from 10 mg wet weight of the original tissue was resuspended in 1.0 ml of the incubation buffer.

For preparation of the cell-free homogenates of theca layer, the theca layers were isolated according to the procedure of GILBERT *et al.*¹⁰⁾ and were homogenized in 50 volumes of the incubation buffer by using a motor-driven glass-glass homogenizer. The homogenates were centrifuged at 1,000 × g for 10 min at 4°C and the supernatant fluid was employed as the cell-free homogenates.

Incubation condition

A piece of follicle wall (8 to 20 mg wet weight) was incubated in 1.0 ml of the incubation buffer at 40°C in air with constant shaking. The suspension of dispersed granulosa cells derived from 1 mg wet weight of the tissue was also incubated in 1.0 ml of the incubation buffer at 40°C in air with constant shaking. In some experiments, the incubation buffer contained 2 µg/ml ovine LH (NIAMDD-oLH-23) or 1 mM N⁶, O^{2'}-dibutyryl adenosine 3':5'-cyclic monophosphate (dibutyryl cyclic AMP).

Radioimmunoassay of progesterone

Incubation was stopped by quick freezing at -80°C, and the tubes were kept at the same temperature until the radioimmunoassay of progesterone was performed. After thawing, whole contents were homogenized in a glass-glass homogenizer and steroids were extracted three times with 1.0 ml each of water-saturated diethyl ether. The pooled extract was then evaporated to dryness and subjected to the radioimmunoassay of progesterone without further purification. Progesterone antiserum was generously supplied by Dr. Michio Takahashi, Department of Veterinary Physiology, University of Tokyo, and [1, 2, 6, 7-³H (N)] progesterone was purchased from New England Nuclear Corp., Boston, MA, U.S.A. Protein was quantified by the method of Lowry *et al.*¹²⁾ using bovine serum albumin as the standard.

Results

In order to investigate effects of LH and dibutyryl cyclic AMP on *in vitro* progesterone synthesis in quail follicles, the follicle walls of F1, F2, and F3 were incubated with or without LH or dibutyryl cyclic AMP for 3 hr. Fig. 1 shows that only follicle wall of F1 responded to LH and dibutyryl cyclic AMP to produce progesterone. The follicle walls of F2 and F3 did not produce progesterone during 3 hr of incubation in the absence or the presence of LH or dibutyryl cyclic AMP. The follicle walls of F1, which contained initially more progesterone than those of F2 or F3, produced a significant amount of progesterone during 3 hr of incubation in the absence of LH or dibutyryl cyclic AMP. Addition of LH or dibutyryl cyclic AMP caused further increase in progesterone production.

Progesterone synthesis in the isolated granulosa cells of F1, F2 and F3 are shown in Fig. 2. In contrast to the similar experiment using the intact follicle walls, the isolated granulosa cells of F2 and F3 produced significant amounts of progesterone during 3 hr of incubation. However, in this case, progesterone production was observed in the control incubation and it was not significantly stimulated by the addition of LH or dibutyryl cyclic AMP. The responses of the granulosa cells of F1 to LH and dibutyryl cyclic AMP were not different from those of the follicle wall of F1.

Since the results shown in Figs. 1 and 2 strongly suggest that the granulosa cells of F2 and F3 have the ability of synthesizing progesterone, but the theca layer, another component of the follicle wall, may metabolize it, further experiments were carried out to delineate the differences of progesterone metabolic rates of the theca layers among the follicles. When the cell-free homogenates (720 μ g protein/ml) of the theca layers obtained from F1, F2, and F3 were incubated with progesterone (100 ng/ml) in the presence of NADPH, more than 90% of progesterone was metabolized after 3 hr (Table 1). When the concentration of progesterone was increased to 1 μ g/ml and the protein concentration of the cell-free homogenates

Fig. 1. Progesterone synthesis of the intact follicle walls of F1, F2 and F3 of the quail ovary. The follicle walls were incubated for 3 hr at 40°C in the presence or the absence of 2 μ g/ml ovine LH or 1 mM dibutyryl cyclic AMP (AMP). Values, expressed as ng/follicle, are geometric mean $\pm$ S.E. of four independent experiments.

* $P < 0.05$ against the control incubation in the absence of LH or dibutyryl cyclic AMP.

Fig. 2. Progesterone synthesis of the isolated granulosa cells of F1, F2, and F3 of the quail ovary. The granulosa cells were incubated for 3 hr at 40°C in the presence or the absence of 2 $\mu\text{g/ml}$ ovine LH or 1 mM dibutyryl cyclic AMP (AMP). Values, expressed as ng/follicle, are geometric mean $\pm$ S.E. of four independent experiments.

** $P < 0.01$ against the control incubation in the absence of LH or dibutyryl cyclic AMP.

Table 1. Decrease of progesterone by the cell-free homogenates of the theca layer

The cell-free homogenates of the theca layer were incubated with progesterone (100 ng/ml for column I, and 1 $\mu\text{g/ml}$ for column II) at 40°C in the presence or the absence of 268 μM NADPH. After 3 hr of incubation, progesterone was quantified by radioimmunoassay as described in Materials and Methods.

Addition to the incubation medium		Progesterone contents (ng/ml)	
Homogenates obtained from	Cofactor	I	II
—	—	109.2 $\pm$ 12.8 ^{a1)}	1038 $\pm$ 47 ^d
F 1 Theca layer (720 μg protein/ml)	—	88.9 $\pm$ 8.8 ^{a b}	
F 2 Theca layer (720 μg protein/ml)	—	83.4 $\pm$ 7.8 ^{a b}	
F 3 Theca layer (720 μg protein/ml)	—	77.6 $\pm$ 8.6 ^b	
F 1 Theca layer (720 μg protein/ml)	NADPH	6.4 $\pm$ 0.9 ^c	
F 2 Theca layer (720 μg protein/ml)	NADPH	1.6 $\pm$ 0.6 ^c	
F 3 Theca layer (720 μg protein/ml)	NADPH	1.4 $\pm$ 0.7 ^c	
F 1 Theca layer (72 μg protein/ml)	NADPH		929 $\pm$ 75 ^d
F 2 Theca layer (72 μg protein/ml)	NADPH		442 $\pm$ 47 ^e
F 3 Theca layer (72 μg pratein/ml)	NADPH		465 $\pm$ 20 ^e

1) mean $\pm$ S.E. (n=6) Means without a common superscript differ significantly ($P < 0.05$) from each other when analyzed by the Q method²⁰⁾.

of the theca layer was reduced to 72 $\mu\text{g/ml}$, more than 50% of progesterone was metabolized in the incubation of the cell-free homogenates of the theca layers of F2 and F3, while those of F1 did not metabolize progesterone appreciably (Table 1). Fig. 3 shows the time courses of the progesterone metabolism by the cell-free homogenates of the theca layers of F1 and F3. The rate of decrease of progesterone was faster in the incubation with the cell-free homogenates of the theca layer of F3 than those of F1.

In the next experiment, the cell-free homogenates of the theca layers were incubated

Fig. 3. Time course of decrease in progesterone by the cell-free homogenates of theca layer of the quail follicles. The cell-free homogenates (30 μ g protein/ml) of the theca layers of F1 (●) and F3 (▲) were incubated with 300 ng/ml of progesterone at 40°C in the presence of 268 μ M NADPH.

Table 2. Effects of the cell-free homogenates of the theca layer on LH-stimulated progesterone production by the granulosa cells of F1

The cell-free homogenates of the theca layer were incubated with the granulosa cells of F1 and 2 μ g/ml ovine LH at 40°C in the presence or the absence of 268 μ M NADPH. After 3 hr of incubation, progesterone was quantified by radioimmunoassay as described in Materials and Methods.

Addition to the incubation medium		Progesterone contents (ng/ml)
Homogenates obtained from	Cofactor	
—	—	92 $\pm$ 8.1 ^{d e 1)}
F1 Theca layer (720 μ g protein/ml)	—	144 $\pm$ 3.2 ^{a b}
F2 Theca layer (720 μ g protein/ml)	—	160 $\pm$ 8.2 ^a
F3 Theca layer (720 μ g protein/ml)	—	142 $\pm$ 1.5 ^{a b}
F1 Theca layer (72 μ g protein/ml)	—	98 $\pm$ 2.3 ^{c d}
F2 Theca layer (72 μ g protein/ml)	—	96 $\pm$ 5.5 ^{d e}
F3 Theca layer (72 μ g protein/ml)	—	99 $\pm$ 7.2 ^{c d}
F1 Theca layer (720 μ g protein/ml)	NADPH	123 $\pm$ 0.7 ^{b c}
F2 Theca layer (720 μ g protein/ml)	NADPH	108 $\pm$ 3.2 ^{c d}
F3 Theca layer (720 μ g protein/ml)	NADPH	70 $\pm$ 4.0 ^e
F1 Theca layer (720 μ g protein/ml)	NADPH	37 $\pm$ 5.1 ^f
F2 Theca layer (720 μ g protein/ml)	NADPH	22 $\pm$ 0.1 ^f
F3 Theca layer (720 μ g protein/ml)	NADPH	16 $\pm$ 1.1 ^f

1) mean $\pm$ S.E. (n=3)

Means without a common superscript differ significantly ($P < 0.05$) from each other when analyzed by the Q method²⁰⁾.

with the granulosa cells of F1 in the presence of LH, instead of progesterone as the source of the steroid. As shown in Table 2, the granulosa cells of F1 derived from 1 mg wet weight of the tissue produced 92 ng/ml of progesterone after 3 hr of incubation in the presence of LH. When the cell-free homogenates (72 μ g protein/ml) of the theca layers and NADPH were present during incubation, progesterone contents decreased. Moreover, this decrease was more prominent in the incubation with the theca later of less matured follicles, such as F2 and F3, than that of matured follicle. No such a decrease in progesterone con-

tents was observed in the incubation without NADPH. When much concentrated cell-free homogenates (720 μg protein/ml) of the theca layers were added to the incubation mixture together with NADPH, the amounts of progesterone after 3 hr of incubation were not decreased in the case of the theca layers of F1 and F2. Contrary, after the incubation with the cell-free homogenates of the theca layer of F1, progesterone contents increased. A slight decrease in progesterone was observed in the incubation with the cell-free homogenates of the theca layer of F3, but the degree of decrease was much smaller than in the incubation using more diluted cell-free homogenates. The increase in progesterone contents in the presence of the cell-free homogenates of the theca layers was markedly observed in the incubation without NADPH. In this case, however, no systematic tendencies in the effects were seen among the size of follicles.

Discussion

Our observations on the *in vitro* progesterone production in the intact follicle wall clearly demonstrate that only the follicle wall of F1 produces progesterone in response to LH and dibutyryl cyclic AMP. This is consistent with the *in vivo* findings of Doi *et al.*²⁾, who measured progesterone contents in the follicle wall of the Japanese quail at a 2-hr interval during the 24 hr of the ovulatory cycle. They observed that progesterone contents started to increase simultaneously with the increase in serum LH concentration and reached a peak 2 hr before the predicted ovulation of F1. Progesterone contents in the follicle walls of smaller follicles were always low and did not show any significant peak during the ovulatory cycle. A similar finding has been reported in the chicken follicle^{1,13,14)}. Moreover, the experiments of measurement of progesterone contents in follicular venous plasma during the ovulatory cycle of the hen also demonstrated that a preovulatory peak of progesterone exists only in the venous plasma from F1³⁾, although a peak of LH concentration was detected in the venous plasma of each follicle.

On the other hand, experiments on the *in vitro* progesterone production of the isolated granulosa cells showed that the cells from smaller follicles (F2 and F3) produced significant amounts of progesterone. However, these cells produced progesterone during 3 hr of incubation without exogenous LH or dibutyryl cyclic AMP, while the cells of F1 produced comparable amounts of progesterone to the intact follicle wall did in response to LH and dibutyryl cyclic AMP. Recently, CALBO and BAHR¹⁵⁾ reported that the adenylate cyclase activity in the chicken granulosa cells of the small follicles was enhanced by follicle-stimulating hormone (FSH). Serum FSH concentration during the ovulatory cycle of the hen showed a peak 11 to 14 hr before ovulation^{16,17)}. Since the granulosa cells used in the present experiments were isolated from birds 9 to 10 hr before the predicted ovulation of F1, the progesterone production in the cells of F2 and F3 during *in vitro* incubation without LH or dibutyryl cyclic AMP might be due to the endogenous FSH or cyclic AMP.

The reason why progesterone production was not detected in the incubation of the follicle walls of F2 and F3 in spite of the ability of the granulosa cells to produce progesterone was partly explained by the present investigation on the metabolism of progesterone in another component of the follicle wall, the theca layer. HUANG, KAO, and NALBANDOV⁶⁾ also

reported a depression in progesterone production of the granulosa cells by the co-incubation with the theca cells. As shown in Table 1 and Figure 3 in the present experiments, the cell-free homogenates of F2 and F3 have the ability to metabolize more progesterone than those of F1. Metabolic pattern of progesterone in avian follicles has not been well studied, but it was reported that the concentration of testosterone and estradiol-17 β in the follicle wall was higher in the small follicles than F1^{1,2,13,14)}. Indeed, WANG and BAHR¹⁸⁾ reported the *in vitro* secretion of estradiol-17 β by the theca cells of F2 and F3 of the hen. Therefore, progesterone synthesized in the granulosa cells of F2 and F3 may be transferred to the theca layer and used as substrates for testosterone and estradiol-17 β productions. NAKAMURA *et al.*¹⁹⁾ reported that the activity of 17 β -hydroxylase, one of the enzymes involved in the conversion of progesterone to testosterone, was higher in F2 than in F1.

The metabolism of progesterone may not be sole factor affected on the amount of progesterone production in the quail follicles. Unexpectedly, in the present study the addition of the cell-free homogenates of the theca layer augmented the progesterone production of the granulosa cells of F1 (Table 2). This is not due to contamination of serum nor the increased protein concentration in the incubation medium (data not shown). Further experiments on this augmenting factor are being carried out.

Summary

During 3 hr of incubation, the production of progesterone in the follicle wall from the largest preovulatory follicle (F1) of the Japanese quail was stimulated by ovine LH or dibutyryl cyclic AMP. The follicle walls from the second largest and the third largest follicles (F2 and F3) did not produce appreciable amounts of progesterone, while the granulosa cells isolated from these follicles produced significant amounts of progesterone. By incubating progesterone with the cell-free homogenates of the theca layer in the presence of NADPH, it was clarified that the metabolic rates of progesterone in the theca layer of F2 and F3 were higher than that of F1. These results suggested that in the quail follicles, progesterone is produced in the granulosa layer but the theca layers of the small follicles metabolize progesterone, so that only F1 secretes progesterone.

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ウズラ卵胞の顆粒膜と卵胞膜がプロゲステロン 生合成におよぼす役割

森 誠・河本 馨・正田 陽一

東京大学 農学部 家畜育種学教室
東京都文京区 千 113

排卵周期中の家禽の卵巣にはさまざまな大きさの卵胞が混在しているが、1日1個の排卵は最大卵胞(F1)からのみおこなわれる。F1はLHに反応してプロゲステロンを生成分泌することが知られているが、なぜF1のみがLHに反応し、その他の卵胞は反応しないのだろうか。本研究はこの疑問に答えるために、LHの影響を受ける前、すなわちF1の排卵予定時刻の9~10時間前にウズラを殺し、大きい順に3個の卵胞(F1, F2, F3)を摘出してそれらのプロゲステロン生合成能をインビトロで比較検討した。

まず卵胞からの卵黄のみを取り除いた卵胞壁をクレブス・リンガー・HEPES培養液中で40度、3時間インキュベートし、その後組織を培養液とともにホモゲナイズしてエーテルでプロゲステロンを抽出し、ラジオイムノアッセイで定量した。その結果、培養液に2 $\mu\text{g}/\text{ml}$ の羊LHまたは1 mMのジブチリルサイクリックAMPを添加すると、F1の卵胞壁のプロゲステロン生成量は顕著に増加した。一方F2やF3から得た卵胞壁ではこのような効果はみられず、プロゲステロン量はインキュベーション前とかわらなかった。卵胞壁から顆粒膜のみを単離し同様の実験をおこない、プロゲステロン生成量を卵胞あたりで比較したところ、F1では卵胞壁を用いた場合と結果は全く同じで、LHやジブチリルサイクリックAMPに反応してプロゲステロンを生成するのは顆粒膜

であることがわかった。しかし卵胞壁を用いた場合にはプロゲステロンの生成が認められなかったF2やF3では、顆粒膜細胞を用いるとプロゲステロンの生成が認められるようになった。ただしこの場合は、単に顆粒膜細胞を培養液中でインキュベートするだけでプロゲステロンの生成がおこり、LHやジブチリルサイクリックAMPを添加しても効果はなかった。

以上の結果は、F2やF3の顆粒膜細胞はプロゲステロンを生成しているのだが、卵胞壁のもう一つの構成因子である卵胞膜がそれを代謝してしまうためにこれらの卵胞ではあたかもプロゲステロンが生成されていないようにみえるのであろう事を強く示唆している。そこでF1, F2, F3の卵胞膜のホモジネートをNADPHの存在下でプロゲステロンとインキュベートすることによって代謝能を調べたところ、確かにF2やF3の卵胞膜はプロゲステロン代謝能がF1よりも高いことが明らかとなった。

F2やF3で生成されたプロゲステロンはおそらく卵胞膜でテストステロンやエストラジオールに代謝され、F1ではこれらのステロイドホルモンへの代謝が少いためにプロゲステロンのままで放出されていることが推察された。

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