Estrogen Primes Cultured Granulosa Cells of the Hen for Progesterone Production in Response to LH

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Granulosa cells isolated from the largest preovulatory follicle of the ovary of the hen were cultured in a defined serum-free medium for 4 days, and the effect of 17β -estradiol on basal and LH-stimulated progesterone production was examined. When the cells were cultured with estrogen or LH for 4 hours on day 4 of culture, progesterone production was stimulated by LH but not by estrogen. When the cells were cultured with estrogen together with LH, progesterone production in response to LH did not change, but significantly increased when precultured with estrogen for 48 hours prior to the 4-h culture with LH on day 4 of culture. A similar effect of the preculture with estrogen was also found in the cells of the second and the third largest preovulatory follicles. The results suggest that estrogen primes the hen granulosa cells to increase the responsiveness to LH for progesterone production.

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Key words : progesterone production, estrogen, granulosa cells, monolayer culture, LH.

Introduction

Granulosa cells of ovarian follicles of the hen produce progesterone¹⁾ in response to LH²⁻⁵⁾, the production being highest in the largest follicle^{1,3,5,6)}. The cells produce little estrogen⁷⁻¹⁰⁾, but have estrogen receptors in their nuclei¹¹⁾. The existence of the receptors suggests a certain action of estrogen on the cells. The present experiments were performed to elucidate whether estrogen affects basal and LH-stimulated progesterone production by hen granulosa cells.

Materials and Methods

Birds and collection of follicles

Hens used were a commercial breed (Dekalb Excellink), 24 months of age, weighting 1.8 to 2.0 kg and laying 4-5 eggs in a sequence. They were caged individually under a light regime of 14 h light and 10 h darkness with feed and water provided *ad libitum*. They were killed 2-3 h after oviposition of the first or the second egg in the sequence, i.e., approximately 22-24 h before ovulation of the second or third ovum of the sequence, and the largest preovulatory follicle (F₁) was immediately excised from the ovary. In another experiment, the second (F₂) and the third (F₃) largest follicles were also obtained together with F₁.

Hormones and reagents

The chicken LH, CP-2, was kindly donated by Drs. Y. HASEGAWA and M. IGARASHI (Gunma University, Japan). This preparation was biologically and immunologically as potent as the chicken LH, IRC-2 (Gunma), which was purified by HATTORI and WAKABAYASHI¹²⁾.

Progesterone and 17 β -estradiol were obtained from Sigma Chemical Co. (St. Louis, MO) and 1, 2, 6, 7- H^3 -progesterone (107.3 Ci/mmol) was purchased from Amersham Co. (Buckinghamshire, England). Progesterone anti-serum (rabbit, progesterone-3-oxime-BSA) was a generous gift by Drs. Y. HASEGAWA and M. IGARASHI. McCoy's 5a medium without serum and Ham's F-12 medium were purchased from Gibco Life Technologies Inc. (Grand island, NY). Corticosterone, bovine serum albumin (BSA : Fraction V), bovine transferrin and collagenase (Type 2) were obtained from Sigma Chemical Co. and porcine insulin was obtained from Biochemical Technologies Inc. (Stoughton, MA).

Cell dispersion and culture

Granulosa layers were isolated from the excised follicles according to the method of GILBERT *et al.*¹³⁾. After washing with HEPES buffer (25 mM Hepes, 137 mM NaCl, 5 mM $CaCl_2$, 0.7 mM $Na_2HPO_4 \cdot 12 H_2O$, 10 mM glucose and 360 μM $CaCl_2$, pH 7.4), the layer was placed in 10 ml HEPES buffer containing 0.08% collagenase in a 50 ml plastic tube, and then incubated for 5 min at 37°C while shaking (120 cycle/min). After the incubation, the dispersion was aspirated and expelled for approximately 30 sec with a siliconized Pasteur pipette, and centrifuged (250 $\times$ g, 15°C, 5 min). The pellet was redispersed in 10 ml HEPES buffer, gently stirred and filtered through a nylon gauze (62 μm mesh) into a sterile plastic tube, and centrifuged (250 $\times$ g, 5 min, 4°C). The cell pellet was washed twice with 20 ml HEPES buffer and again washed with 20 ml serum-free medium consisting of a 1 : 1 mixture of McCoy's 5a medium and Ham's F-12 medium supplemented with insulin (20 mU/ml), transferrin (5 $\mu g/ml$), corticosterone (20 ng/ml), BSA (4 mg/ml), Hepes (10 mM), L-glutamine (100 $\mu g/ml$), penicillin (50 U/ml) and streptomycin sulphate (5 $\mu g/ml$). The final washed pellet was suspended in a known volume of the serum-free medium, and the number of cells was counted on an hemocytometer following trypan blue exclusion. Based on the count, the cell suspension was diluted to a final concentration of 1.5×10^6 viable cells/ml with the serum-free medium. All tools were sterilized either with a high temperature sterilizer (160–180°C, 90 min) or with an autoclave (1.2 barometric pressure, 120°C, 20 min.). Liquids were sterilized by filtration (pore size : 0.22 μm).

The cell suspension, 200 μl , containing 3×10^5 viable cells and 200 μl of the serum-free medium was placed in 24-well multiwells (Nunc Inter. Med) and cultured at 38°C under a water saturated atmosphere of 5% CO_2 and 95% air for 4 days. Medium was changed every 2 days. Following the culture for 4 days, the cells were normal in appearance under a microscope.

Experiments

(1) Culture with LH alone : on day 4 of culture, the medium was replaced by the fresh one containing various amounts of the chicken LH (2–64 ng/well) and cultured

Table 1. Progesterone production in cultured granulosa cells of the hen in response to LH

LH (ng/well)	Progesterone (ng/well/4 h)
0	6.23 ± 0.65* a**
2	17.17 ± 0.60 a
4	46.65 ± 3.22 b
8	101.69 ± 15.18 c
16	149.75 ± 2.26 d
32	193.76 ± 5.89 e
64	177.93 ± 11.12 de

On day 4 of culture, the granulosa cells were cultured for 4 h in media containing LH at various concentrations.

* Mean ± SE of 6 determinations.

** Means bearing different letters are significantly different ($P < 0.01$) by Duncan's multiple range test.

for 4 h.

(2) Culture with estrogen alone : on day 4 of culture, the medium was replaced by the fresh one containing 17β -estradiol at various concentrations (10^{-10} – 10^{-6} M) and cultured for 4 h.

(3) Culture with estrogen and LH added together : on day 4 of culture, the medium was replaced by the fresh one containing 17β -estradiol at various concentrations (10^{-10} – 10^{-6} M) together with an effective dose of LH (40 ng/well) and cultured for 4 h.

(4) Preculture with estrogen and then culture with LH : on day 2 of culture, the medium was replaced by the fresh one containing 17β -estradiol at various concentrations (10^{-10} – 10^{-6} M) and cultured for 48 h (up to day 4). Then, the medium was replaced again by the fresh one containing LH (40 ng/well) and recultured for 4 h.

(5) Culture of cells of three hierarchical follicles (preculture with estrogen and then culture with LH) : granulosa cells obtained from F_1 , F_2 and F_3 were cultured according to the manner described above. On day 2, the medium was replaced by the fresh one containing 17β -estradiol (10^{-8} M) and cultured for 48 h. After the culture, the medium was replaced again by the fresh one containing LH (40 ng/well) and recultured for 4 h.

After the above cultures, media were collected, and stored at -20°C until assay for progesterone.

Progesterone assay

Progesterone concentrations in the media were measured without extraction by a routine radioimmunoassay. For the separation of bound and unbound steroids, a dextran-coated charcoal absorption method was used. All samples were measured in duplicate in a single assay to eliminate the variation between assays. The sensitivity of the assay system was 2.5 pg/tube. The intra-assay variation was 6.6%.

Statistical analyses

Duncan's multiple range test was used for the significance of difference between means of different groups.

Results*Effect of chicken LH alone*

When the hen granulosa cells were cultured with the chicken LH for 4 h, the progesterone concentration in the medium increased linearly as the dose of LH increased from 4 to 32 ng/well, and then remained at a maximum level (Table 1).

Effect of estrogen alone

When the cells were cultured for 4 h in the presence of estrogen alone at various concentrations, no significant change in the progesterone concentration was observed (Table 2).

Effect of estrogen on LH-stimulation

Cells cultured for 4 h with various concentrations of estrogen together with a maximum dose of LH did not produce more progesterone than cells cultured with LH alone (Table 2).

Effect of estrogen pretreatment

When the cells were precultured with estrogen at various concentrations for 48 h from day 2 and then cultured in the absence of LH for 4 h, the progesterone concentration in the media did not change significantly (Table 3). However, when the cells were cultured in the presence of LH after the preculture with estrogen, the progesterone concentration in the media in response to LH increased more in the groups

Table 2. Progesterone production in cultured granulosa cells treated with 17β -estradiol alone or simultaneously with LH

E_2 (M)	Progesterone (ng/well/4 h)			
	E_2 alone		E_2 + LH	
			(6.71 $\pm$ 0.28) ^a	
0	6.74 $\pm$ 0.20*	a**	224.90 $\pm$ 12.12*	a**
10^{-10}	6.81 $\pm$ 0.36	a	212.10 $\pm$ 9.04	a
10^{-9}	5.87 $\pm$ 0.46	a	226.38 $\pm$ 7.74	a
10^{-8}	7.12 $\pm$ 0.33	a	219.62 $\pm$ 9.65	a
10^{-7}	6.78 $\pm$ 0.48	a	238.22 $\pm$ 6.36	a
10^{-6}	6.97 $\pm$ 0.61	a	251.40 $\pm$ 18.59	a

On day 4 of culture, the granulosa cells were cultured for 4 h in media containing 17β -estradiol (E_2) at various concentrations without or with LH (40 ng/well).

^a Media containing neither E_2 nor LH (control for the culture with E_2 + LH).

* Mean $\pm$ SE of 6 determinations.

** Mean bearing the same letter within column are not significantly different ($P < 0.05$) by Duncan's multiple range test.

of pretreatment with estrogen than in the group of pretreatment without estrogen, and the increase was greater as the estrogen dose increased.

Effect of estrogen pretreatment on the cells of hierarchial follicles

A greater increase in the progesterone concentration in response to LH was also found in the cells of F₂ and F₃ as in those of F₁ when the granulosa cells were precultured with estrogen than when without estrogen (Table 4).

Table 3. Progesterone production in cultured granulosa cells pretreated with 17 β -estradiol and then treated with or without LH

Pretreated E ₂ (M)	Progesterone (ng/well/4 h)	
	Not treated with LH	Treated with LH
		(6.64 $\pm$ 0.52) ^a
0	6.56 $\pm$ 0.53 a ^{**}	237.10 $\pm$ 19.75 a ^{**}
10 ⁻¹⁰	5.80 $\pm$ 0.25 a	303.41 $\pm$ 5.49 b
10 ⁻⁹	6.00 $\pm$ 0.78 a	322.98 $\pm$ 12.24 bc
10 ⁻⁸	5.73 $\pm$ 0.52 a	355.45 $\pm$ 16.04 bc
10 ⁻⁷	5.27 $\pm$ 0.03 a	366.60 $\pm$ 17.76 c
10 ⁻⁶	6.30 $\pm$ 0.27 a	375.54 $\pm$ 26.74 c

From day 2 of culture, the granulosa cells were cultured in media containing 17 β -estradiol (E₂) at various concentrations for 48 hours. After the pretreatment, the cells were washed and then recultured for 4 h in media with or without LH (40 ng).

^a Not pretreated with E₂ and cultured in media not containing LH (control for the treatment with LH).

* Mean $\pm$ SE of 6 determinations.

** Means bearing different letters within column are significantly different ($P < 0.01$) by Duncan's multiple range test.

Table 4. Progesterone production in cultured granulosa cells of different hierarchial preovulatory follicles pretreated or not pretreated with E₂ and treated or not treated with LH

Pretreat ment	Treat ment	Progesterone (ng/well/4 h)		
		F ₁	F ₂	F ₃
None	None	7.67 $\pm$ 0.56* a	4.46 $\pm$ 0.01* a	1.07 $\pm$ 0.06* a ^{**}
E ₂	None	11.56 $\pm$ 0.28 a	5.92 $\pm$ 0.16 a	2.92 $\pm$ 0.29 a
None	LH	188.90 $\pm$ 11.60 b	180.31 $\pm$ 4.38 b	17.45 $\pm$ 0.59 b
E ₂	LH	279.61 $\pm$ 7.12 c	248.93 $\pm$ 7.88 c	76.36 $\pm$ 1.34 c

From day 2 of culture, granulosa cells were cultured with or without 17 β -estradiol (E₂: 10 M) for 48 h. After the culture, the cells were washed and then recultured in the presence or absence of LH (40 ng/well) for 4 h.

F₁, F₂, F₃: the largest, the second largest and the third largest follicle, respectively.

* Mean $\pm$ SE of 6 determinations.

** Means bearing different letters within column are significantly different ($P < 0.01$) by Duncan's multiple range test.

Discussion

The hen granulosa cells cultured in a serum-free medium responded to chicken LH in a dose-dependent manner for the production of progesterone, and a maximum response was shown at around 32 ng/well (Table 1). Although the addition of 17 β -estradiol in the medium for 4 h simultaneously with LH (40 ng/well) which may cause the maximum response did not affect the progesterone production (Table 2), pretreatment with the estrogen for 48 h caused a significant increase in the progesterone production in response to LH stimulation (Table 3), suggesting a priming action of estrogen for the LH-stimulatable progesterone production. The action of estrogen may be dependent on LH receptor formation, since the preculture with estrogen was required more than at least 4 h. Although the progesterone production was less in the smaller follicles than in the largest follicle (Table 4) as in the earlier findings^{1,3,5,6)}, pretreatment with estrogen for 48 h caused similar effects in the cells of these smaller follicles. This may probably due to a more binding sites of estrogen receptors in the cells of the smaller follicles¹¹⁾.

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ニワトリの卵胞顆粒層細胞におけるプロジェステロン生産に 及ぼすエストロジェンの影響

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ニワトリの卵胞顆粒層細胞にエストロジェンのレセプターが存在することから、エストロジェンが顆粒層細胞に何らかの影響を及ぼしていると思われるが、エストロジェンが顆粒層細胞におけるプロジェステロン (P_4) 生産に影響を与えているか否かは未だ十分に検討されていない。そこで本実験では、排卵前 22~24 時間の卵胞から単離し無血清培養液で単層法により培養したニワトリの顆粒層細胞において、エストロジェンがプロジェステロン生産に影響を与えるか否かを検討した。

培養 4 日目に、LH 又はエストラジオール-17 β (E_2) を含む培養液に交換して 4 時間培養したところ、 P_4 生産は LH によって刺激されたが、 E_2 によって刺激されなかった。又、LH と同時に E_2 を添加しても、その 4 時間後で P_4 生産に関する E_2 添加の影響は認められなかった。しかしながら、培養 2 日目から 4 日目にかけて

E_2 を含む培養液で 48 時間培養して、その後に LH を含む培養液に交換して 4 時間培養したところ、LH による P_4 生産は E_2 の前処理により有意に増加した。このような E_2 による効果は最大卵胞においてのみならず、大きさが 2 番目と 3 番目の卵胞における顆粒層細胞においても認められた。

以上の結果から、ニワトリの顆粒層細胞において、エストロジェン単独ではほとんど P_4 生産に影響を与えないが、予めエストロジェンで前処理することによりエストロジェンが LH による P_4 生産に対する反応性を高めることが明らかとなった。

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キーワード : プロジェステロン生産, エストロジェン, 顆粒層細胞, 単層培養, LH