EFFECTS OF HYPOPHYSECTOMY ON PROGESTERONE PRODUCTION IN THE FOLLICULAR GRANULOSA CELLS OF THE JAPANESE QUAIL

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Summary

In order to investigate the mechanism of regulation of progesterone production, quail were hypophysectomized at various times during the ovulation cycle, and granulosa cells were isolated from follicles 4 hr after the operation. They were incubated *in vitro* at 40°C with or without LH or dibutyryl cyclic AMP, and the amounts of progesterone produced during 3 hr of incubation were measured by radioimmunoassay. Hypophysectomy at 8 hr or 20 hr before the predicted time of ovulation caused a reduced responsiveness of F1 granulosa cells to exogenous LH or dibutyryl cyclic AMP. Although hypophysectomy at 24 hr before ovulation caused a slight reduction of responsiveness of F1 granulosa cells, the reduction of the progesterone production during the incubation without any stimuli was prominent by the sham operation. These results suggest that the presence of pituitary gland influences the ability of the granulosa cells to produce progesterone in response to LH or dibutyryl cyclic AMP.
(Key Words: Granulosa Cell, Follicle, LH, Cyclic AMP, Japanese Quail)

Introduction

The primary source of progesterone in the quail ovary is granulosa cells of the largest (F1) follicle (Mori et al., 1984; Asem et al., 1985). Granulosa cells of the second (F2) or the third (F3) largest follicles produce a significant amount of progesterone during *in vitro* incubation, but these cells do not respond to exogenous tropic hormones. Recently, we have clarified that granulosa cells acquire the responsiveness to luteinizing hormone (LH) or dibutyryl cyclic AMP (dbcAMP), an analog of intracellular messenger of LH, as soon as the follicle becomes the largest in the hierarchy (Mori and Kantou, 1987). The responsiveness of granulosa cells to LH or dbcAMP increased until 17 hr before ovulation, followed by a transient decrease. The degree of response increased again at 9-11 hr before ovulation and maximum stimulation was observed in the cells obtained within 5 hr prior to ovulation. Thus, the ability of granulosa cells to produce progesterone in response to tropic stimuli changes during follicular maturation.

Since it is well established that increases in plasma LH and progesterone concentrations are associated with ovulation in avian species (Etches, 1984), the acquisition of responsiveness of granulosa cells to LH is an important determinant of ovulation. In order to clarify the mechanism of regulation of the responsiveness of granulosa cells to LH or dbcAMP, we have observed the changes in *in vitro* progesterone production of granulosa cells isolated from quail which were hypophysectomized at various times during the ovulation cycle.

Materials and Methods

Laying Japanese quail of Jq-NIBS-WE strain were obtained from Nihon Institute of Biological

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Science (Kobuchizawa, Nagano, Japan). They were caged individually and had free access to food and water. The lighting schedule was 14L:10D with lights on at 0600. Accurate times of oviposition were recorded automatically for at least 2 weeks prior to the experiment. Ovulation in the mid-sequence was assumed to occur 15 to 30 min after oviposition of the previously ovulated ovum (Woodard and Mather, 1964; Opel, 1966).

The birds, selected on the basis of regular sequence lengths and times of oviposition within a sequence, were randomly assigned to treatment groups of 5 birds each. For the hypophysectomy or sham operation, a light anaesthesia was achieved by i.v. injection of pentobarbital Na (Nembutal, 40 mg/Kg body weight). The operation was performed 8, 20, or 24 hr before the expected time of ovulation with the aid of a stereotaxic instrument utilizing the transbuccal approach, as described by Tanaka and Nobukuni (1977). The birds were killed by decapitation 4 ± .25 hr after the operation. Intact birds were also killed at the equivalent time during the cycle, that is 4, 16, and 20 hr before ovulation.

The two largest preovulatory follicles, F1 and F2, were collected and placed in ice-cold physiological saline. Granulosa layers were isolated according to the procedure of Gilbert et al. (1977) as modified by Mori et al. (1984) for quail, 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). After 5 min of digestion at 37°C with gentle shaking, cells were collected by centrifugation at 800 x g for 3 min. The precipitate was then washed twice with 2 ml of the incubation buffer in order to remove collagenase. The final pellet derived from 1 mg wet weight of the original tissue was resuspended and incubated for 3 hr in 1.0 ml of the incubation buffer at 40°C with or without 2 μg/ml ovine LH (NIAMDD-oLH-25, generously provided by the National Hormone and Pituitary Program, NIADDK) or 1 mM $N^6,O^{2'}$-dibutyryladenosine 3':5'-cyclic monophosphate (Sigma, St. Louis, MO). The number of cells was counted using a hemocytometer.

Incubation was stopped by quick freezing at -20°C and the tubes were kept at the same temperature until radioimmunoassay of progesterone was performed. After thawing, steroids were extracted three times with 1.0 ml each of water-satured diethyl ether. The pooled extract was then evaporated to dryness and subjected to a validated radioimmunoassay of progesterone without further purification (Takahashi et al., 1980). Progesterone antiserum was generously supplied by Dr. Michio Takahashi, Department of Veterinary Physiology, University of Tokyo, and [1,2,6,7-^{3}H] progresterone (sp act, 101.0 Ci/mmol) was purchased from New England Nuclear Corp. (Boston, MA).

Prior to statistical analyses, amounts of progesterone produced per 10^5 cells during 3 hr of incubation were transformed to logarithms. The differences were tested by analysis of variance and, when significant, the means were separated using Duncan's multiple range test (Duncan, 1955).

Results

F1 Granulosa Cells.

Figure 1-A shows the effect of hypophysectomy at 8 hr before the expected time of ovulation on *in vitro* progesterone production in F1 granulosa cells. Granulosa cells obtained from intact and sham-operated birds responded to the addition of LH or dbcAMP ($P < .01$). After hypophysectomy, the responsiveness of granulosa cells to LH or dbcAMP had disappeared. A similar tendency was also found by hypophysectomy performed at 20 hr or 24 hr before ovulation (Figure 1-B and 1-C). Although the sham operation at 20 hr before ovulation caused a slight decrease in response to LH (control *vs* LH; $P < .01$ in intact group, $P < .05$ in sham-operated group), granulosa cells obtained from hypophysectomized birds completely lost the responsiveness to LH and dbcAMP. Hypophysectomy at 24 hr before ovulation caused a slight decrease in LH- and dbcAMP-stimulated progesterone production (control *vs* LH or dbcAMP; $P < .01$ in intact and sham operated groups, $P < .05$ in hypophysectomized group). When the amount of progesterone produced during the control incubation was compared between the operation groups, the reduced production was prominent by the sham operation.

F2 Granulosa Cells.

Also examined was the effect of hypophysectomy on *in vitro* progesterone production in F2 granulosa cells (figure 2). Consistent with our

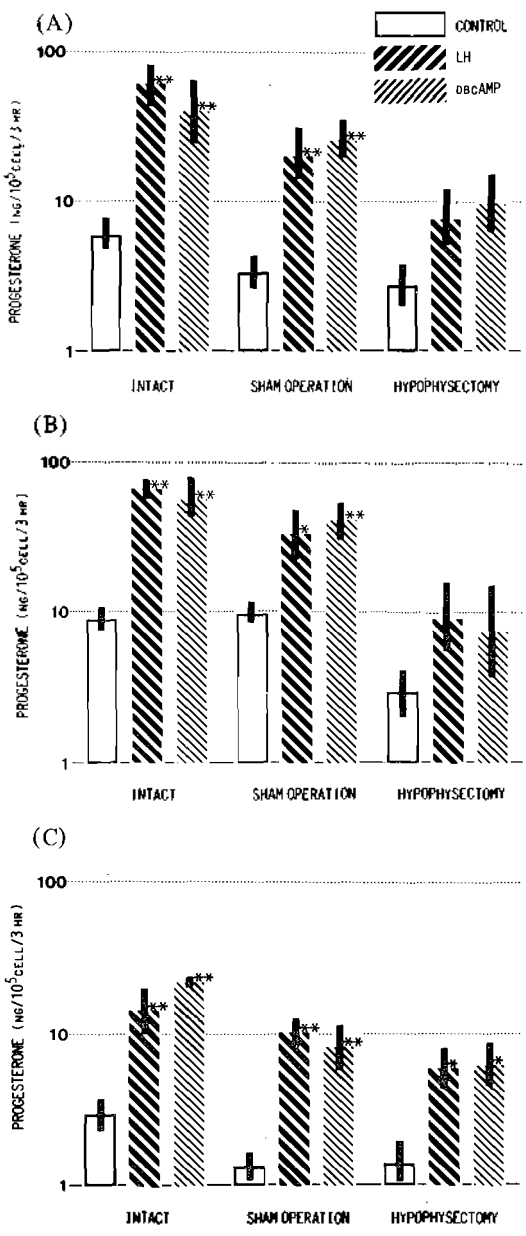

Figure 1. Effects of hypophysectomy on the *in vitro* progesterone production by F1 granulosa cells in the presence or absence of LH or dbcAMP. Birds were hypophysectomized or sham-operated at 8 hr(A), 20 hr(B), and 24 hr(C) before ovulation, and granulosa cells were obtained 4 hr after the operation. Values are mean ± S.E. (n=5). *P < .05 and ** P < .01 against the control incubation of the same treatment group.

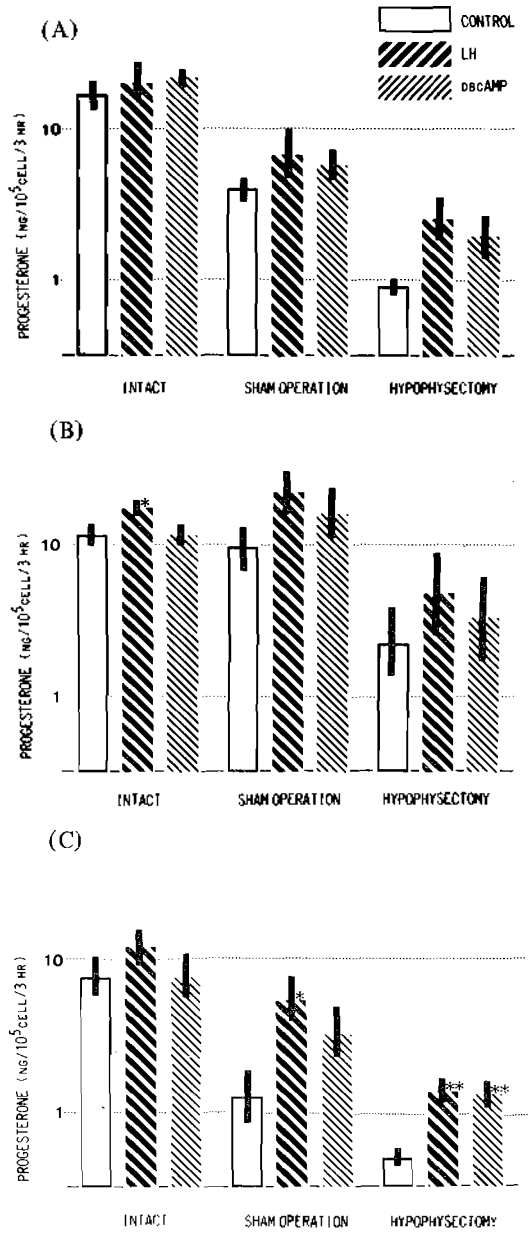

Figure 2. Effects of hypophysectomy on the *in vitro* progesterone production by F2 granulosa cells in the presence or absence of LH or dbcAMP. Birds were hypophysectomized or sham-operated at 8 hr(A), 20 hr(B), and 24 hr(C) before ovulation, and granulosa cells were obtained 4 hr after the operation. Values are mean ± S.E. (n=5). * P < .05 and ** P < .01 against the control incubation of the same treatment group.

previous findings (Mori and Kantou, 1987), F2 granulosa cells obtained from the intact birds killed at 8 hr or 24 hr prior to ovulation did not respond to the addition of LH or dbcAMP (figures 2-A and 2-C), and those obtained at 20 hr before ovulation showed only a slight increase in progesterone production by stimulation with LH (figure 2-B). Hypophysectomy at 8 hr and 20 hr before ovulation did not alter the responsiveness of the cells to LH or dbcAMP, although the amount of progesterone produced in the control incubation as well as in the incubation with LH or dbcAMP decreased. Contrary to the results of F1 granulosa cells, hypophysectomy at 24 hr before ovulation caused an appearance of responsiveness to LH and dbcAMP (Figure 2-C). Although the sham operation caused the appearance of responsiveness to LH ($P < .05$), a significant stimulation both by LH and dbcAMP was observed in the hypophysectomized group ($P < .01$). This is mainly due to the decrease in the amount of progesterone produced during the control incubation of the sham-operated and hypophysectomized groups (intact *vs* sham-operated or hypophysectomized groups; $P < .01$).

Discussion

Our studies on progesterone production in quail granulosa cells demonstrated that the presence of pituitary gland and/or the operation has an effect on *in vitro* responsiveness of the cells to tropic stimulus.

An elevated production of progesterone by stimulation of LH is the result of a series of biochemical reactions, many of which are not completely understood. However, it is generally accepted that the initial reaction of LH on granulosa cells is the binding to its receptor and activation of adenylate cyclase, which catalyzes the conversion of adenosine 5'-triphosphate to cyclic AMP (Hammond *et al.*, 1980; Asem and Hertelendy, 1985). If one accepts the hypothesis that pituitary hormone(s) regulate(s) the responsiveness of granulosa cells only at the step of activation of adenylate cyclase, then one could expect to find changes in the amount of progesterone produced by LH, but not by dbcAMP. However, the present investigations demonstrated that hypophysectomy caused a decrease in the responsiveness of F1 granulosa cells to LH as well as to dbcAMP. This result indicated that pituitary hormone(s) affect(s) the steroidogenic capacity of the cells at the step after the increase in intracellular cyclic AMP concentration.

Recent studies on human fetal and bovine adrenal cells have shown that the action of the adrenocorticotropic hormone involves the stimulation on transcription of cytochrome P-450$_{CSCC}$ in rat granulosa cells is regulated by a follicle stimulating hormone (FSH) at the transcriptional level. Since the activities of Δ5-3β-hydroxysteroid dehydrogenase and Δ4-Δ5 lyase in the granulosa cells do not change during the ovulation cycle in chicken (Marrone and Hertelendy, 1983), it is quite possible that pituitary hormone(s) regulate(s) the steroidogenic capacity of granulosa cells at the step of cholesterol side chain cleavage enzymes.

In the present investigation, there is no direct evidence for which hormone is involved in the regulation of the responsiveness of granulosa cells to tropic stimulus. However, it can be speculated from the time of hypophysectomy and the changes in plasma hormone levels during the ovulation cycle. Plasma LH level in laying quail begins to increase at 10 hr before ovulation and forms the preovulatory peak at 6 hr before ovulation (Doi et al., 1980). In addition to this peak, Gulati et al. (1981) observed a second small peak of plasma LH at 21 hr before ovulation. Although plasma FSH level has not been studied in quail, it was reported that the concentration of this hormone in chicken is high during the middle of the cycle, and low at the preovulatory peak of LH (Imai and Nalbandov, 1971). Moreover, Etches and Cheng (1981) reported that granulosa cells of chicken F1 contain few FSH receptor in comparison with those of the smaller follicles. Putting these results together, the reduction of the responsiveness of F1 granulosa cells by hypophysectomy might be due to the reduction of plasma LH concentration.

Hypophysectomy not only causes a reduction in plasma pituitary hormone levels, but may also cause a reduction in plasma steroid hormone levels. Injection of chicken anterior pituitary extract into laying hens at 14 hr before ovulation caused an increase in estradiol contents (Imai and Nalbandov, 1978), and injection of LH caused an increase in testosterone contents in small follicles (Shahabi et al., 1975). Moreover,

in vitro estradiol production by theca cells of the small follicles was reported to be stimulated by the addition of LH (Huang et al., 1979). Although we did not measure the plasma concentration of steroid hormones after hypophysectomy, it is probable that the concentration of these hormones is also depressed by hypophysectomy.

In summary, we have found that the responsiveness of progesterone production of granulosa cells to LH or dbcAMP is regulated by the pituitary hormone(s). Whether pituitary hormone(s) act(s) directly on granulosa cells or act(s) directly by increasing plasma testosterone or estradiol level is not clear from this study.

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Literature Cited

Asem, E. K. and F. Hertelendy. 1985. Influence of follicular maturation on luteinizing hormone-, cyclic 3′5′-adenosine monophosphate-, forskolin- and cholesterol-stimulated progesterone production in hen granulosa cells. Biol. Reprod. 32:257-268.

Asem, E., B. L. Marrone and F. Hertelendy. 1985. Steroidogenesis in ovarian cells of the Japanese quail (*Coturnix coturnix japonica*). Gen. Comp. Endocrinol. 60:353-360.

Doi, O., T. Takai, T. Nakamura and Y. Tanabe. 1980. Changes in the pituitary and plasma LH, plasma and follicular progesterone and estradiol, and plasma testosterone and estrone concentrations during ovulatory cycle of the quail (*Coturnix coturnix japonica*). Gen. Comp. Endocrinol. 41:156-163.

Duncan, D. B. 1955. Multiple range multiple F tests. Biometrics 11:1-24.

Etches, R. J. 1984. Maturation of ovarian follicles. Pages 51-73 *in:* Reproductive Biology of Poultry. F. J. Cunningham, P. E. Lake and D. Hewitt, ed. British Poultry Science Ltd., Longman Group, Harlow.

Etches, R. J. and K. W. Cheng. 1981. Changes in the plasma concentrations of luteinizing hormone, progesterone, oestradiol and testosterone and in the biding of follicle-stimulating hormone to the theca of follicles during the ovulation cycle of hen (*Gallus domesticus*). J. Endocrinol. 91:11-22.

Gilbert, A. B., A. J. Evans, M. M. Perry and M. H. Davidson. 1977. A method for separating the granulosa cells, the basal lamina and the theca of the preovulatory ovarian follicle of the domestic fowl (*Gallus domesticus*). J. Reprod. Fert. 50:179-181.

Goldring, N. B., J. M. Durica, J. Lifka, L. Hedin, S. L. Ratoosh, W. L. Miller, J. Orly and J. S. Richards. 1987. Cholesterol side-chain cleavage P450 messenger ribonucleic acid: Evidence for hormonal regulation in rat ovarian follicles and constitutive expression in corpora lutea. Endocrinology 120:1942-1950.

Gulati, D. P., T. Nakamura and Y. Tanabe. 1981. Diurnal variations in plasma LH, progesterone, testosterone, estradiol, and estrone in the Japanese quail. Poultry Sci. 60:668-673.

Hammond, R. W., H. Todd and F. Hertelendy. 1980. Effects of mammalian gonadotropins on progesterone release and cyclic nucleotide production by isolated avian granulosa cells. Gen. Comp. Endocrinol. 41:467-476.

Huang, E. S. R., K. J. Kao and A. V. Nalbandov. 1979. Synthesis of sex steroids by cellular components of chicken follicles. Biol. Reprod. 20:454-461.

Imai, K. and A. V. Nalbandov. 1971. Changes in FSH activity of anterior pituitary glands and of blood plasma during the laying cycle of the hen. Endocrinology 88:1465-1470.

Imai, K. and A. V. Nalbandov. 1978. Plasma and follicular steroid levels of laying hens after the administration of gonadotropins. Biol. Reprod. 19:779-784.

John, M. E., E. R. Simpson, M. R. Waterman and J. I. Mason. 1986. Regulation of cholesterol side-chain cleavage cytochrome P-450 gene expression in adrenal cells in monolayer culture. Mol. Cell. Endocrinol. 45:197-204.

Marrone, B. L. and F. Hertelendy. 1983. Steroid metabolism by avian ovarian cells during follicular maturation. Biol. Reprod. 29:953-962.

Mori, M. and T. Kantou. 1987. Changes in progesterone production in granulosa cells during the ovulatory cycle of the Japanese quail (*Coturnix coturnix japonica*). Gen. Comp. Endocrinol. 68:57-63.

Mori, M., K. Kohmoto and Y. Shoda. 1984. Role of granulosa and theca cells on *in vitro* progesterone production in preovulatory follicles of the Japanese quail. Japan. Poult. Sci. 21:206-214.

Opel, H. 1966. The timing of oviposition and ovulation in the quail (*Coturnix coturnix japonica*). Br. Poult. Sci. 7:29-38.

Shahabi, N. A., J. M. Bahr and A. V. Nalbandov. 1975. Effect of LH injection on plasma and follicular steroids in the chicken. Endocrinology 96:969-972.

Takahashi, M., N. Murakami, H. Naito and Y. Suzuki. 1980. Blockade of pseudopregnancy in the rat by treatment with antiprogesterone serum. Biol. Reprod. 22:423-429.

Tanaka, K. and K. Nobukuni. 1977. A stereotaxic device for hypophysectomy of the domestic fowl. Jan. J. Zootech. Sci. 48:532-537.

Woodard, A. E. and F. B. Mather. 1964. The timing of ovulation, movement of the ovum through the oviduct, pigmentation and shell deposition in Japanese quail (*Coturnix coturnix japonica*). Poultry Sci. 43: 1427-1432.