Effects of gonadotrophins, sex steroids and adenohipophysectomy on the activities of proteolytic enzymes in the ovarian follicle wall of the domestic fowl (*Gallus domesticus*)

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Summary. LH and progesterone added to cultures of the follicle wall of hens increased the total activity of neutral and acid proteases and collagenase and the increase was greater for follicle tissues from the stigma region. Adenohipophysectomy of hens resulted in the decreased activities of neutral and acid proteases and collagenase in the first and second largest follicles.

Introduction

Gonadotrophins, cyclic AMP, progesterone, oestrogens, prostaglandins, and presumably ascorbic acid appear to have specified roles in the ovulatory process (Espey, 1974, 1980; Rondell, 1974). However, a definite sequence of biochemical events which can account for the disruption of the follicle wall is not known. Schochet (1916) first suggested that proteolytic enzymes weaken the follicle wall by digesting the theca folliculi in the region of the stigma. Moricard & Gothie (1946) revived Schochet's hypothesis with tenuous evidence that gonadotrophins induce the secretion of proteolytic enzymes that digest the follicular wall to weaken it for rupture. Espey & Coons (1976) suggested that the decomposition of follicular connective tissue during ovulation is caused by proteolytic enzymes which have properties similar to other mammalian collagenases.

Very few reports are available on the biochemical changes which occur in the follicle wall of birds in relation to the ovulation (Nakamura & Nakajo, 1980; Fujii, Tojo & Koga, 1981a; Fujii, Tojo, Koga, Fukunaga & Sugimoto, 1981b; Tojo, Fujii & Ogawa, 1982). Therefore, a study was conducted to investigate the effects of LH, FSH, progesterone and oestradiol-17 β as well as the effect of adenohipophysectomy on the activities of proteolytic enzymes in the ovarian follicle wall of the hen.

Materials and Methods

Materials

LH (NIAMDD-oLH-24) and FSH (NIAMDD-oFSH-15) were supplied from NIADDK, NIH. Progesterone (No. 24614), trypsin (bovine pancreas type 3) and soybean trypsin inhibitor (Type I-S) were obtained from Sigma Chemical Co. (St Louis, Missouri, U.S.A.). Oestradiol-17 β and casein (No. 2242) were purchased from Merck (Darmstadt, West Germany). Haemoglobin (substrate powder) was from Worthington Biochemicals Corp. (Freehold, New Jersey, U.S.A.). Synthetic DNP-peptide, DNP-Pro-Leu-Gly-Ile-Ala-Gly-Arg-NH₂, was from the Protein Research Foundation (Osaka, Japan). All the other reagents were of analytical grade.

Experiment 1

Animals. White Leghorn hens which had regular clutches of at least 4 consecutive eggs were used. The birds were kept in individual cages, were exposed to 14 h light/24 h (06:00–20:00 h) and provided with food and water *ad libitum*.

Organ culture. Ovarian follicles were collected aseptically from hens killed by decapitation 2 h before the expected ovulation of the 2nd or 3rd egg in the laying sequence. The wall of the largest follicle, separated from the yolk and stratum granulosum, was classified into 2 regions, stigma and non-stigma. The separated follicle walls were cleaned with TCM 199 (Nakarai Chemicals, Ltd, Kyoto, Japan) and cut into small pieces (3×5 mm) according to the method of Rondell (1974). The culture method was a modification of that of Fujihara & Shiino (1983). Briefly, one piece of follicle wall (3×5 mm) without stratum granulosum was placed on a small piece ($5-7$ mm²) of lens paper (Eastern Kodak Co., Rochester, New York, U.S.A.) which was attached to a cellulose sponge raft ($5-7$ mm²) in a small polystyrene culture dish (35×10 mm; Miles Lab. Inc., Elkhart, Indiana, U.S.A.) containing TCM 199 with or without the hormone being tested (1.0 ml/dish). The tissues were then incubated at 41°C for 5 h in a humidified atmosphere of 95% O₂ and 5% CO₂.

After incubation, the dishes were immediately cooled in an ice bath, and the media were stored at -20°C . The tissues were homogenized with 1.0 ml 0.01 M-CaCl₂ containing 0.25% Triton X-100 and stored at -20°C . The media and homogenates were used within 3 days for enzyme assay. All solutions used were sterilized by filtration with 0.22 μm Millipore filters (Millipore Corp., Massachusetts, U.S.A.) and all equipment was autoclaved (120°C, 15 min), except for the polystyrene culture dishes which had been sterilized with u.v. radiation.

Experiment 2

Animals. White Leghorn hens which had regular clutches of at least 5 consecutive eggs were used. The birds were housed in the same environment as in Exp. 1.

Adenohypophysectomy. The anterior lobes of pituitaries were removed by using a stereotaxic instrument as described by Tanaka & Nobukuni (1977) 15–16 h before the expected time of ovulation. Control birds received the same operation but the anterior lobes of the pituitaries were not removed. At 30 min before the expected time of ovulation (immediately after oviposition) the birds were killed by decapitation.

Sample preparation. The first (F₁), second (F₂) and third (F₃) largest follicles were collected immediately after death and the yolk and stratum granulosum were removed. Each follicle wall was weighed, minced and homogenized with 5 volumes of 0.01 M-CaCl₂ containing 0.25% Triton X-100. The homogenate was centrifuged at 2000 *g* for 20 min at 4°C. After washing the precipitate with a small volume of the same solution, the suspension was centrifuged again and the supernatant was combined and diluted to 5 ml total volume. The precipitate was suspended in 0.1 M-Tris-HCl to give a final volume of 5 ml/follicle. The preparation was stored at -20°C and used within 3 days for enzyme assay. The supernatant of the homogenized follicle tissue was used for the assay of acid proteinase (EC 3.4.4). The activity of neutral proteinase (EC 3.4.4) and collagenase (EC 3.4.24.3) was measured in suspensions of the precipitate.

Enzyme assays

The activities of neutral and acid proteinases were measured according to the method described by Fujii *et al.* (1981b) and casein or haemoglobin were used as the substrate at pH 7.0 and 4.0, respectively. The assay media were incubated at 41°C for 40 min. The reaction was stopped by adding 5% (w/v) trichloroacetic acid. After centrifugation at 1500 *g* for 30 min, the absorbance of the supernatant was measured at 280 nm. One unit is defined as the amount of enzyme that catalyses the formation of 1 nmol tyrosine/min under the assay conditions.

The measurement of collagenase activity was as reported by Fujii *et al.* (1981a) and determined at pH 8.0 by using a synthetic peptide, DNP-Pro-Leu-Gly-Ile-Ala-Gly-Arg-NH₂ as a substrate. The medium was incubated at 41°C for 4 h. The reaction was stopped by addition of 1 N-HCl and then ethyl acetate was added. After centrifugation at 1500 *g* for 30 min, the absorbance of the supernatant was determined at 365 nm. The activity was expressed as nmol DNP-tripeptides released/h (DNP unit), using the molar extinction coefficient, 1.76×10^4 at 365 nm.

Statistical analysis

Student's unpaired *t* test was used to assess the statistical significance of difference between means of control and experimental groups.

Results

The results are shown in Table 1. LH caused an increase in neutral proteinase activity in the non-stigma and stigma parts of the follicle wall, but acid proteinase and collagenase activities were increased only in the stigma area.

FSH had no significant effects.

Progesterone increased the activity of acid proteinase and collagenase in both parts of the follicle wall but only the highest dose affected the neutral proteinase activity in the stigma area. Oestradiol-17 β caused increased activity of acid proteinase in both parts of the follicle wall.

Table 1. Effect of ovine LH, ovine FSH, progesterone and oestradiol-17 β on the activities of enzymes in stigma and non-stigma parts of the follicle wall of the hen

Hormone		Total enzymic activity					
		Non-stigma			Stigma		
		0 (control)	2 ng/ml	20 ng/ml	0 (control)	2 ng/ml	20 ng/ml
LH	Neutral proteinase	284 $\pm$ 10	345 $\pm$ 9**	349 $\pm$ 12**	299 $\pm$ 12	375 $\pm$ 17*	417 $\pm$ 26**
	Acid proteinase	257 $\pm$ 18	286 $\pm$ 16	296 $\pm$ 12	276 $\pm$ 10	325 $\pm$ 12*	376 $\pm$ 21**
	Collagenase	19 $\pm$ 1	22 $\pm$ 1	26 $\pm$ 2	19 $\pm$ 1	32 $\pm$ 3**	40 $\pm$ 1**
FSH	Neutral proteinase	295 $\pm$ 7	297 $\pm$ 4	300 $\pm$ 11	302 $\pm$ 6	302 $\pm$ 9	334 $\pm$ 12
	Acid proteinase	239 $\pm$ 19	238 $\pm$ 22	254 $\pm$ 30	250 $\pm$ 24	262 $\pm$ 18	288 $\pm$ 20
	Collagenase	17 $\pm$ 1	18 $\pm$ 1	17 $\pm$ 1	17 $\pm$ 1	19 $\pm$ 1	19 $\pm$ 1
Progesterone	Neutral proteinase	233 $\pm$ 5	247 $\pm$ 5	255 $\pm$ 8	233 $\pm$ 5	264 $\pm$ 12	268 $\pm$ 3**
	Acid proteinase	215 $\pm$ 12	314 $\pm$ 27*	327 $\pm$ 18**	222 $\pm$ 12	282 $\pm$ 6**	320 $\pm$ 18**
	Collagenase	13 $\pm$ 1	20 $\pm$ 1**	23 $\pm$ 1**	16 $\pm$ 1	25 $\pm$ 1**	30 $\pm$ 2**
Oestradiol-17 β	Neutral proteinase	207 $\pm$ 9	212 $\pm$ 5	203 $\pm$ 2	230 $\pm$ 9	223 $\pm$ 14	233 $\pm$ 11
	Acid proteinase	194 $\pm$ 4	254 $\pm$ 19*	291 $\pm$ 9**	193 $\pm$ 9	302 $\pm$ 19**	301 $\pm$ 7**
	Collagenase	16 $\pm$ 2	22 $\pm$ 3	21 $\pm$ 2	17 $\pm$ 1	21 $\pm$ 2	21 $\pm$ 3

Values are mean $\pm$ s.e.m. for 4 culture dishes and represent the sum of the enzyme activity in the medium and in the cellular homogenate.

P* < 0.05, *P* < 0.01 compared with control value.

Table 2. Effect of adenohipophysectomy on the activities of enzymes in the follicle wall of the hen

Follicle	Activity of enzyme (units/100 mg wet tissue)					
	Control (N = 5)			Hypophysectomy (N = 4)		
	Neutral proteinase	Acid proteinase	Collagenase	Neutral proteinase	Acid proteinase	Collagenase
F ₁	884 ± 40	696 ± 29	62.5 ± 1.6	471 ± 21**	550 ± 32*	51.4 ± 1.5**
F ₂	595 ± 15	600 ± 27	39.9 ± 1.8	397 ± 21**	491 ± 23*	27.4 ± 2.5**
F ₃	511 ± 28	569 ± 30	39.2 ± 5.7	471 ± 32	512 ± 69	25.8 ± 3.4

Values are mean ± s.e.m.

P* < 0.05, *P* < 0.01 compared with the control value for that follicle type.

Adenohipophysectomized birds showed decreased activities of all 3 enzymes in the F₁ and F₂ follicles but values in the F₃ follicles were not significantly different from those of control birds (Table 2).

Discussion

Wright (1961) postulated that the role of gonadotrophins in the ovulation process is simply to induce the synthesis of ovarian steroids, probably specifically progesterone, which in turn is responsible for follicular rupture and the extrusion of the ovum. Rondell (1974) suggested that the follicular reaction to the formation of an LH-receptor complex has at least 2 discrete steps: the stimulation of steroid synthesis by the LH, and the activation of an ovulatory enzyme by the steroid released. Our present data of LH- but not FSH-stimulated increases in neutral and acid proteinases and collagenase activities in the hen's ovarian follicle wall (without the stratum granulosa) appear to support Rondell's (1974) hypothesis. However, there is no evidence that thecal cells have LH receptors in the hen. In addition, the significance of the thecal cells in follicular steroidogenesis is not clear. Huang, Kao & Nalbandov (1979) reported that thecal cells could not synthesize progesterone or oestradiol. Therefore, to determine conclusively the action of LH on the follicle wall, the following investigations are necessary: (1) examination for the existence of LH receptors on the thecal cells of hens; and (2) examination of the significance of thecal cells in follicular steroidogenesis of hens.

The stimulatory effects of progesterone on neutral and acid proteinase and collagenase activities of the cultured follicle wall emphasize the importance of this steroid in the ovulatory process reported by Rondell (1974) and Espey (1974, 1980).

Although there are some reports which have examined the effect of sex steroids on the ovarian collagenolytic activity in mammals (Rondell, 1974; Hunzicker-Dunn, Jungman & Birnbaumer, 1979), there is no record of the effect of sex steroids on the activity of collagenase in birds. Our present results suggest that there is a stimulatory effect of progesterone on the activity of collagenase in the follicle wall of hens.

The effects of adenohipophysectomy on the activities of follicular enzymes in the fowl have not been examined. Our present finding of decreased activities of collagenase and neutral and acid proteinase in the 1st and 2nd but not the 3rd largest follicles from hypophysectomized hens appears to suggest that gonadotrophin, presumably LH, may activate the synthesis of follicular enzymes in the more mature follicles.

There are various reports on the mechanism controlling the last step of ovulation in the hen. The digestion of stigma tissue with proteolytic enzymes may be essential in the process of rupture (Nakajo, Zakaria & Imai, 1973; Yoshimura & Fujii, 1981; Fujii *et al.*, 1981a, b; Tojo *et al.*, 1982).

Our present results, in conjunction with those of others, suggest that LH and progesterone may be concerned in the enzymic mechanism controlling the ovulation in the hen.

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