Effect of ovine LH on the progesterone content of the granulosa cells in preovulatory follicles of the domestic fowl (*Gallus domesticus*)

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Summary. Laying hens were injected i.v. with a single dose of ovine LH (100 µg) 6–8 h after a known ovulation, i.e. when plasma LH concentrations are low. Groups of 4 birds were killed at 15 min intervals up to 1 h after injection and the complete granulosa was obtained from 4 or 5 of the largest preovulatory follicles of each bird for progesterone measurement by g.l.c. Compared with the saline-injected controls which had negligible levels of progesterone, granulosa progesterone increased in all the LH-treated birds. The maximum response (mean progesterone content 1966 ng/follicle) occurred 45 min after injection but there was a significant decline by 60 min. It is concluded that the granulosa cells provide most of the preovulatory plasma progesterone increase.

Introduction

The modern light hybrid fowl is almost a daily ovulator. To maintain the output of eggs, the ovary contains a hierarchy of developing follicles measuring <1 to 37 mm in diameter. Concomitant with this follicular growth, the ovary secretes steroid hormones into the blood stream, thus affecting most of the reproductive processes (Gilbert, 1971). Under the influence of gonadotrophins, mainly LH, there is a rise in plasma steroids from about 6 to 2 h before ovulation (Furr, Bonney, England & Cunningham, 1973; Shodono, Nakamura, Tanabe & Wakabayashi, 1975). Shahabi, Norton & Nalbandov (1975b) and Williams & Sharp (1978a, b) have emphasized the role of the largest preovulatory follicle in the secretion of the progesterone in this preovulatory phase. However, the ovary contains several types of steroidogenic cells, those of the stroma and those associated with the actual follicle, i.e. the theca and granulosa cells. Our anatomical and analytical studies (Gilbert, Evans, Perry & Davidson, 1977; Dick, Culbert, Wells, Gilbert & Davidson, 1978) have shown that the usual practice of slitting the follicle to remove the yolk before analysis would undoubtedly result in the loss of most of the granulosa cells and so the contribution of the granulosa cells to ovarian steroidogenesis in the fowl has probably been underestimated, but the extent of the discrepancy is unknown. Recent studies (Huang, Kao & Nalbandov, 1979; Wells, Gilbert & Culbert, 1980) have shown that the granulosa cells *in vitro* are able to produce progesterone, but there is no evidence for their activity *in vivo*.

This paper describes the effect of intravenous injection of ovine LH on the progesterone content of the granulosa cells obtained from the larger preovulatory follicles. The work complements that of Shahabi, Bahr & Nalbandov (1975a) and Imai & Nalbandov (1978) who measured the progesterone content of the thecal tissue in the follicular envelope after a single injection of LH.

Materials and Methods

Animals

The 21 laying hens (Shaver 288 type) used in these experiments were selected on the basis of egg records and oviposition times from a pool of 60 birds kept as previously described (Gilbert, Davidson & Wells, 1978). About 6–8 h after a predicted ovulation, each of the 16 experimental birds was weighed and injected intravenously with ovine LH (NIH-LH-S19 100 µg in 300 µl 0.15 M-NaCl). At 15 min intervals up to 1 h after injection, 4 birds at each time were killed by overdose with pentobarbitone sodium, 2 ml, i.v. (Expiral: Abbott Laboratories, Kent). All birds had a lightly calcified egg in the shell gland. Four or five of the largest preovulatory follicles were collected from each ovary and weighed. Each follicle was slit, allowing the yolk to fall into 0.15 M-NaCl. The granulosa cells were separated from the yolk as a unicellular layer contained between the basal lamina and the perivitelline membrane (Gilbert *et al.*, 1977) and this tissue was stored at -18°C until analysed. The predicted time of ovulation of each follicle (16, 40, 64, 88 and 112 h) was estimated from an examination of the bird's pattern of ovipositing times, together with a comparison of the mass of each follicle and that of the ovum in the calcified egg recovered from the shell gland.

The 5 control birds, selected in a similar way, were injected with 0.15 M-NaCl (300 µl) and killed only at 45 min after injection because Williams & Sharp (1978a) showed that plasma concentrations of progesterone in saline-injected birds did not change over a 90 min period.

Analysis of tissue

The granulosa tissue was homogenized with water (2.5 ml) containing 1517 Bq [1,2,6,7(n)- ^3H]progesterone (sp. act. 3.145 kBq/pmol; Radiochemical Centre, Amersham). The homogenate was extracted twice with dichloromethane (30 ml). The combined extract was evaporated down to 1 ml and chromatographed on a column of Florisil (8 cm, 6 mm diam.; 100–120 U.S. Mesh). The chromatogram was developed with 25 ml diethyl ether followed by 30 ml ether:methanol (70:30 v/v). The latter fraction contained the progesterone and was evaporated to dryness. The residue was dissolved in 25 µl acetone from which 5 µl were taken for measurement of radioactivity to estimate procedural losses. 5β-Pregnane-3,20-dione (1 µg; internal standard) was added to the remaining solution. Portions of this mixture were subjected to gas-liquid chromatography (g.l.c.) on OV 210 (1.7% w/w) coated on Chromsorb G (100–120 U.S. mesh; 1.1 m; 4 mm) at 277°C ; using a Pye 104 Chromatograph with argon as the carrier gas and a flame ionization detector. The areas of the separated constituents were measured by a 308 Computing Integrator (L.D.C., Shannon). Progesterone in the sample was estimated from the ratio of its peak area to that of the internal standard. The mass response ratio of progesterone to internal standard was determined with each batch of samples analysed, about 8 each day.

Specificity and sensitivity of the g.l.c. method

Attempts to perform g.l.c. analyses of crude extracts of granulosa cells were unsuccessful because of gross interference from the cholesterol present. A simple chromatographic step on a Florisil column (Culbert & Wells, 1973) produced a fraction which was sufficiently purified for g.l.c. Table 1 shows that progesterone was well separated from other naturally occurring steroid hormones or related compounds on OV 210. During these experiments, apart from progesterone and cholesterol, none of the steroids listed in Table 1 was detected in tissue extracts. 5β-Pregnane-3,20-dione was not separated from its 5α-epimer on OV 210. Occasionally an unidentified peak of retention time 21 min was observed in extracts but this substance did not interfere with the measurement of progesterone in tissue. The mean $\pm$ s.e.m. recovery of [^3H]progesterone from LH injected birds was $56.7 \pm 1.7\%$ in 57 analyses; with tissue from saline

injected birds, the recovery was lower ($34 \pm 3.6\%$, $n = 15$) because there was a greater loss through lack of carrier steroid.

Table 1. G.l.c. of various steroids on OV 210 at 277°C

Steroid	Retention time (min)
Cholesterol	7.5
Testosterone	10.7
20 β -Hydroxypregn-4-ene-3-one	12.5
5 β -Pregnane-3,20-dione	13.4
20 α -Hydroxypregn-4-ene-3-one	13.6
Androstenedione	15.9
Progesterone	20.2

The minimum amount of progesterone which was measurable by electronic integration of the peak was 66 ng (coefficient of variation 13%). This represents about 100 ng in the sample, allowing for losses of about 40% during the extraction and clean up stages. It was possible to detect, but not measure, 20–50 ng progesterone as a small peak on the baseline. Although not as sensitive as radioimmunoassay, the g.l.c. method is more adaptable for tissue analysis in that a small aliquot of the sample can be tested first. This trial may be used to assess whether dilution or concentration is necessary to bring the progesterone peak within the dynamic range of the detector within 30 min of the first injection.

Statistical analysis

During the 60 min after LH injection, the progesterone content of individual granulosa preparations varied considerably between 100 and 6000 ng. To normalize the variances for comparison of the means at each sampling time, the individual values were converted to natural logarithms before conducting an analysis of variance. Standard errors of these means (s.e.m.) were calculated from the residual mean squares. Means were compared by Student's *t* test. The 95% confidence limits for the geometric means were calculated using their s.e.m. and *t* values for the appropriate number of degrees of freedom and then converted to the arithmetic scale.

Results

Granulosa progesterone content after LH injection

Due to a shortage of ovine LH, dose-response studies were not carried out. However in preliminary studies, 4 birds were injected with 25 μ g LH (mean dose 13.7 ± 0.3 μ g/kg body weight). The granulosa tissue of one follicle contained 100 ng progesterone whereas that from the other 15 follicles analysed contained less than the minimum detectable quantity of progesterone, i.e. 20–50 ng. These results were taken to imply that a threshold dose of ovine LH was required to initiate a response by the granulosa cells *in vivo* 6–8 h after ovulation when plasma LH is at a low concentration (Furr *et al.*, 1973; Williams & Sharp, 1978a). To ensure that a measurable response was attained, an arbitrary amount of 100 μ g ovine LH was therefore chosen. The mean dose of LH for the 16 experimental birds used in the current study was 50.8 μ g/kg, about 2.5 times that used by Williams & Sharp (1978a).

The fowl ovary contains only one preovulatory follicle at each particular stage of maturity. Since individual birds showed a considerable variation in the granulosa response to the dose of ovine LH, statistical comparisons of the progesterone content in the granulosa of follicles of a particular age were not meaningful because the tissue from 4 birds was examined at each 15-min

interval. Analysis of variance showed that the major source of variation was between sampling times (with a mean square of 18.0754 (natural logarithms), d.f. = 3) as opposed to that between follicles (with a mean square of 0.7728, d.f. = 53): the variance ratio (F) was 23.4 and $P < 0.001$. The results for the follicles examined in this study are shown in Table 2.

Table 2. Progesterone content of follicular granulosa collected from the ovaries of laying hens at 15 min intervals after a single i.v. injection of ovine LH (100 µg)

Time after injection (min)	Progesterone content (ng/follicle)*		
	Geometric means $\pm$ s.e.m. (no. of follicles) (natural logarithm scale)	Geometric means (converted to arithmetic scale)	95% Confidence limits
15	^a 6.7259 $\pm$ 0.2651 (11)	836	463–1510
30	^a 6.6723 $\pm$ 0.2349 (14)	790	475–1313
45	^b 7.5838 $\pm$ 0.2198 (16)	1966	1231–3141
60	^c 5.0324 $\pm$ 0.2198 (16)	153	96–245

Means with different superscripts are significantly different ($P < 0.05$).

* The tissues from 4 birds were analysed for each time. The follicles ranged in maturity from 16 to 112 h before their estimated ovulation.

Granulosa obtained from the 4 or 5 largest follicles dissected from ovaries 12 h after the pre-ovulatory LH surge in birds injected with saline contained negligible amounts of progesterone, but within 15 min of an LH injection into the blood stream, there was at least a 5-fold rise in the granulosa progesterone levels, increasing during the next 30 min to over 2000 ng/follicle.

The granulosa tissue from follicles of the control birds contained only negligible levels of progesterone. Only one follicle in 15 examined had a progesterone content (169 ng) greater than the detectable limit.

The difference in mass between the four largest follicles of the LH-treated birds was approximately 3 g/day, e.g. for 16 h versus 40 h follicles from the same bird, the mean ($\pm$ s.e.m.) difference was 2.62 ± 0.34 g ($n = 13$; paired t test $P < 0.001$), for 40 h versus 64 h, 3.15 ± 0.32 g ($n = 8$; $P < 0.001$) and for 64 h versus 88 h follicles, 3.20 ± 0.39 g ($n = 9$; $P < 0.001$).

Discussion

The impetus to carry out these studies on the effect of LH on granulosa cells *in vivo* came from related work on the secretion of progesterone by fowl granulosa cells *in vitro* (Huang *et al.*, 1979; Wells *et al.*, 1980). Cells collected from follicles estimated to be ovulating 1–5, 24 and 48 h later responded similarly to exogenous ovine LH with increased secretion of progesterone. Ovine LH elevated progesterone concentration in blood plasma of the fowl for a period of at least 90 min after the injection (Shahabi *et al.*, 1975a; Williams & Sharp, 1978a; Imai & Nalbandov, 1978). Williams & Sharp (1978a) demonstrated that the response occurred irrespective of whether a bird was in the middle of an egg sequence or on a pause day. Previous studies of the follicular response to LH showed that the theca of the largest follicle contained 200–300 ng progesterone (300–400 ng/g) 30–45 min after treatment (Shahabi *et al.*, 1975a; Imai & Nalbandov, 1978). Since the tissues studied by these authors would not have contained many granulosa cells (see 'Introduction'), it is not surprising that the present results gave markedly higher values (>2000 ng progesterone/follicle).

We suggest that the granulosa cells of the large preovulatory follicles are the ovarian source of the preovulatory surge of progesterone during the ovulatory cycle of the hen (Wells *et al.*, 1979). Although the granulosa content at any time does not reflect the actual rate of progester-

one secretion, the sharp drop in granulosa progesterone levels between 45 and 60 min after LH injection in our study was surprising. There was a net fall of 1812 ng (mean) in the granulosa of each follicle, representing a decrease of 384 pmol/min. Possibly the slower decay in the plasma progesterone concentration observed by Shahabi *et al.* (1975a) and Williams & Sharp (1978a) after an LH pulse may be a consequence of the buffering effect of other tissue pools on the metabolic clearance.

Besides confirming the steroidogenic activity of LH previously demonstrated *in vitro* (Wells *et al.*, 1980), the similarity of the response of the 4 largest preovulatory follicles to exogenous LH casts doubt on the assumption that the potential of a follicle to ovulate is determined mainly by its ability to secrete progesterone in the presence of increased concentrations of LH (Etches & Cunningham, 1976; see also Williams & Sharp, 1978b) since if this were so, all the larger follicles should ovulate synchronously. On the other hand, after injecting birds with LH, Shahabi *et al.* (1975a) and Imai & Nalbandov (1978) showed that the largest follicle had lower oestrogen concentration than the next two follicles in the ovarian hierarchy. A similar pattern of increasing oestrogen concentration with decreasing follicle size was observed during the preovulatory phase of the daily cycle of the hen (Shahabi *et al.*, 1975b). Perhaps a decline in the aromatizing activity of the theca of the largest follicle, coupled with an increasing progesterone secretion by its granulosa in response to LH, is the primary factor in determining whether the hen follicle is ready to ovulate.

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