The effect of LH, FSH and pregnant mares' serum gonadotrophin on ornithine decarboxylase activity in thecal and granulosa tissue during follicular growth and atresia in laying hens (*Gallus domesticus*)

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The effect of ovine LH, porcine FSH and pregnant mares' serum gonadotrophin (PMSG) on the activity of ornithine decarboxylase activity in theca and granulosa tissue during folliculogenesis in laying hens is described. The changes in the activity of ornithine decarboxylase induced by hormonal challenge was used to measure the sensitivity of the tissue to the hormone. Thecal tissue from small (< 6 mm) follicles showed a large increase in the activity of ornithine decarboxylase 3 h after treatment with LH, FSH and PMSG, *in vivo*, whereas ornithine decarboxylase activity in thecal tissue from large (> 8 mm) preovulatory follicles and atretic follicles did not respond to any of the hormonal treatments. Ornithine decarboxylase activity in granulosa tissue from the largest preovulatory follicle increased significantly 3 h after treatment with LH and PMSG *in vivo*; no effect was observed with FSH. Granulosa tissue from the third largest and fifth largest preovulatory follicles were refractory to the hormonal treatments. Basal activity of ornithine decarboxylase in granulosa tissue from preovulatory follicles increased as the follicles approached ovulation, whereas the activity in thecal tissue from the same follicles decreased. The difference in sensitivity of thecal tissue from small and large preovulatory follicles towards gonadotrophin treatment *in vivo* is correlated with the difference in the observed rate of atresia occurring within the two groups of follicles. Atresia is the common fate for small follicles, whereas it is a rare event for large preovulatory follicles under normal physiological conditions. It is suggested that the decrease in sensitivity of thecal tissue towards gonadotrophin during recruitment of small follicles into the preovulatory follicular hierarchy is one reason for the difference in the incidence of atresia occurring within the two populations of follicles.

Introduction

The ovaries of laying hens contain a hierarchy of large yellow follicles (> 8 mm), which allows a mature follicle to ovulate on successive days throughout a laying sequence (Fraps, 1955; Gilbert and Wood-Gush, 1979; Etches, 1990). The hierarchy is maintained by the recruitment of small follicles (< 8 mm) into the hierarchy (Gilbert *et al.*, 1983; Waddington *et al.*, 1985).

Small follicles (< 8 mm) are present in a graded-size sequence; fewer follicles are present in each size class as they increase in size. Small follicles are either recruited into the follicular hierarchy or, more commonly, they undergo atresia, eventually being reabsorbed into the ovary (Gilbert *et al.*, 1983). In laying hens, once a follicle is recruited into the hierarchy, it is committed to ovulate within 5–7 days, as atresia within this group of follicles is rare under normal physiological conditions (Gilbert *et al.*, 1981, 1983; Etches *et al.*, 1984).

The biochemical and physiological events involved in the control of avian follicular growth and the initiation of atresia

are not known. However, a central role for gonadotrophins is well established. In mammals, pregnant mares' serum gonadotrophin (PMSG) has been shown to promote the recruitment of antral follicles and to prevent some normal, small follicles from becoming atretic (Moor *et al.*, 1984; Greenwald and Terranova, 1988). In hens, treatment with PMSG and FSH *in vivo* increases the rate of recruitment of small follicles into the follicular hierarchy (Opel and Nalbandov, 1961). Atresia can be induced in small and large follicles from laying hens after treatment with an antiserum to partially purified chicken LH (Sharp *et al.*, 1978). Injections of LH alone can also induce atresia in large preovulatory follicles; the effectiveness of the treatment depends on the stage of the ovulatory cycle at which the hormone is given (Gilbert *et al.*, 1981). An early event associated with the development of atresia of small follicles in hens is a reduction in their sensitivity to LH and a concomitant loss in aromatase activity (Armstrong, 1985, 1986a; Wells *et al.*, 1985a). Gilbert *et al.* (1985) suggested that these early changes are a consequence of the disruption of the vascular system in the small follicles destined to become atretic. In the study reported here, the growth of ovarian follicles from domestic

fowls was monitored by measuring the interaction of gonadotrophins with granulosa and thecal tissue from follicles at various stages of development. The interaction of the gonadotrophin preparations with follicular tissue was monitored by measuring changes in the activity of the enzyme ornithine decarboxylase induced by the hormone treatment. Ornithine decarboxylase catalyses the rate limiting step in the biosynthesis of polyamines (putrescine, spermidine and spermine) (Pegg and McCann, 1982; Grillo, 1985), which play important roles in the structure and function of nucleic acids, including DNA replication, transcription and translational processes. Ornithine decarboxylase activity is correlated with the rate of tissue growth (Russell and Snyder, 1968; Janne *et al.*, 1978). Its activity in target tissues rises markedly in response to hormones, growth factors and tumour promoters (Pegg and McCann, 1982) and it has been used successfully as a marker for gonadotrophin sensitivity in previous studies concerned with the control of follicular growth in the fowl (Armstrong, 1986a, b, c).

The results of this investigation indicate that the sensitivity of follicular tissue towards gonadotrophins changes during folliculogenesis, and provides information regarding the involvement of these hormones in the control of avian ovarian follicular growth and atresia.

Materials and Methods

Animals

A flock of laying birds (ISA Browns; weight $\pm$ SEM = 2.20 ± 0.04 kg, $n = 56$) were housed individually in standard cages under a lighting regimen of 14 h light:10 h dark with free access to food and water. During the course of the experiment the ages of the birds ranged from 25 to 43 weeks of age and all laid regular egg sequences of five or more eggs. All cages were fitted with an automatic timing device measuring the time of oviposition to within 1 min.

Hormone preparations

Pregnant mares' serum gonadotrophin (PMSG; gonadotrophin-FSH, specific activity 3600 iu mg⁻¹ protein) was obtained from Paines Byrne Ltd (Greenford). Porcine FSH (NIH-P2) and ovine LH (NIH-H-25) were supplied by NIADK (NIH, Bethesda). The various doses of PMSG were made up in 300 μ l lactose solution (5 mg ml⁻¹), whereas FSH and LH were prepared in 100 μ l distilled water containing BSA (1%; w:v). All hormone preparations were stored at -20°C until required.

Chemicals

L-[1-¹⁴C]ornithine hydrochloride (Amersham International plc, Bucks) was stored at 4°C in ethanol (2%, w:v) at a specific activity of 2.04 GBq nmol⁻¹. D,L- α -difluoromethylornithine was a gift from Merrill Dow Research Institute (Strasbourg). All other chemicals were obtained from Sigma Chemical Co Ltd (Poole, Dorset) and were of Analar grade.

Gonadotrophin treatment and collection of tissue

Birds were injected into the wing vein between 11.00 and 12.00 h on the morning of an oviposition with either LH, FSH or PMSG. Control birds were injected with either 300 μ l lactose (5 mg ml⁻¹) or 100 μ l BSA (1%, w:v). All birds were killed 3 h after treatment by an overdose of sodium pentobarbitone (Expiral: Ceva Ltd, Watford, Herts) and the ovaries removed and rinsed in physiological saline. Thecal and granulosa tissue were collected from the largest (F1), third largest (F3) and fifth largest (F5) preovulatory follicles and homogenized individually in 5 ml Tris buffer (50 mmol l⁻¹; pH 8.2 at 2°C) containing dithiothreitol (5 mmol l⁻¹), EDTA (1 mmol l⁻¹) and pyridoxal phosphate (1 nmol l⁻¹) using a polytron homogenizer. The homogenates were centrifuged at 600 g for 10 min at 4°C and the supernatants stored overnight at 4°C before assaying for enzyme activity.

Thecal tissue was also collected from small (< 6 mm) normal, atretic and grossly atretic follicles. Atretic follicles were distinguished from normal follicles by either the presence of small haemorrhages on their surface or, in the case of grossly atretic follicles, by their deformed appearance (Gilbert, 1979; Gilbert *et al.*, 1983). The thecal tissue from small nonatretic follicles of the appropriate size (1–3 mm diameter follicles: $n = 8$ –10 per bird and 3–6 mm diameter follicles: $n = 6$ –10 per bird), atretic follicles ($n = 4$ –10 per bird) and grossly atretic follicles ($n = 3$ per bird) were pooled, according to type and homogenized in 5 ml homogenization medium. Before homogenization, the small follicles were slit with a scalpel and the yolk and adhering granulosa tissue gently squeezed out. Histological evidence indicated that the thecal preparations were free of contaminating granulosa cells (results not shown).

Enzyme assay

The activity of ornithine decarboxylase (L-ornithine carboxylase; EC 4.1.1.17) was measured as described by Armstrong (1986a). Briefly, this involved incubating the tissue homogenate (100 μ l) with 200 μ l of a solution containing pyridoxal phosphate (0.3 mmol l⁻¹) and [¹⁴C] ornithine (1.85 Bq; specific activity 2.04 GBq nmol⁻¹). The incubation was carried out at 37°C for 60 min in sealed plastic tubes containing filter-paper inserts soaked in methyl benzethonium hydroxide. At the end of the incubation, 100 μ l sulfuric acid (5.0 mmol l⁻¹) was injected into the tube and the incubation was continued for a further 60 min. Incubations were performed in triplicate and tissue blanks containing BSA (0.1%, w:v) and total blanks containing D,L- α -difluoromethylornithine (5 μ mol l⁻¹), an irreversible inhibitor of ornithine decarboxylase (Metcalfe *et al.*, 1978), were included. After incubations the filter-paper inserts were removed and radioactivity was measured in an LKB RackBeta II liquid scintillation counter using the external standard channels ratio mode.

Protein was measured by the method of Bradford (1976) using BSA as the protein standard and enzyme activities were expressed as pmol CO₂ released h⁻¹ mg⁻¹ protein.

Statistical analysis

After transformation to a logarithmic scale, the data were analysed by an analysis of variance using a split-plot design

Table 1. The effect of gonadotrophin treatments on thecal ornithine decarboxylase activity in ovarian follicles from laying hens

Treatment	Thecal ornithine decarboxylase activity (pmol CO ₂ h ⁻¹ mg ⁻¹ protein)				
	F1	F3	F5	SY	SW
Control	30.4 ± 10.9	61.9 ± 11.6	145.1 ± 33.9	55.3 ± 18.2	42.7 ± 14.4
LH (40 µg)	21.6 ± 11.2	55.2 ± 11.0	152.6 ± 27.3	165.2 ± 21.5*	139.7 ± 20.0*
FSH (500 µg)	27.2 ± 8.5	63.0 ± 17.1	138.8 ± 21.6	141.8 ± 18.9*	151.0 ± 18.7*
PMSG (300 iu)	23.2 ± 9.9	58.3 ± 21.0	127.1 ± 25.6	211.7 ± 38.5*	213.1 ± 61.4*

Results are expressed as means ± SEM (*n* = 5). F1, F3 and F5 represent the largest, third largest, and fifth largest preovulatory follicle, respectively. SY and SW represent small follicles 3–6 mm in diameter and 1–3 mm in diameter, respectively. *Significantly different (*P* < 0.001) from control. PMSG: pregnant mares' serum gonadotrophin.

Table 2. The effect of gonadotrophin treatments on granulosa ornithine decarboxylase activity in ovarian follicles from laying hens

Treatment	Granulosa ornithine decarboxylase activity (pmol CO ₂ h ⁻¹ mg ⁻¹ protein)		
	F1	F3	F5
Control	286.0 ± 70.7	137.6 ± 25.5	62.6 ± 24.2
LH (40 µg)	589.0 ± 89.1*	130.5 ± 31.1	60.7 ± 15.2
FSH (500 µg)	397.5 ± 125.8	142.9 ± 41.0	69.1 ± 24.2
PMSG (300 iu)	622.4 ± 102.4*	158.4 ± 48.1	52.2 ± 23.1

Results are expressed as means ± SEM (*n* = 5). F1, F3 and F5 represent the largest, third largest, and fifth largest, preovulatory follicle, respectively. *Significantly different (*P* < 0.001) from control. PMSG: pregnant mares' serum gonadotrophin.

(Steele and Torrie, 1980). Differences within tissues between controls and treatments were tested by the method of least significant differences using a pooled residual mean square.

Results

Effect of a single dose of ovine LH, porcine FSH and PMSG on ornithine decarboxylase activity in ovarian follicular tissue

All of the mammalian hormone preparations significantly increased ornithine decarboxylase activity in thecal tissue from small (< 6 mm) nonatretic follicles (Table 1). PMSG (300 iu) was the most effective treatment followed by ovine LH (40 µg) and then FSH (500 µg). When the enzyme activities were expressed as activity mg⁻¹ protein, no difference was apparent between the two sizes of small follicles (Table 1). However, when the enzyme activity was expressed on a per follicle basis (results not shown) the 1–3 mm diameter follicles had a lower basal activity than did the 3–6 mm diameter follicles, and they showed a larger relative increase in enzyme activity after hormone treatments. In contrast to the small (< 6 mm) follicles, ornithine decarboxylase activity in thecal tissue from the large preovulatory follicles (F1, F3 and F5) were not affected by the hormonal treatments (Table 1).

Ornithine decarboxylase activity in granulosa tissue from the largest preovulatory follicle (F1) was significantly higher after LH and PMSG treatments *in vivo* (Table 2). Although the

ornithine decarboxylase activity in F1 granulosa was also increased by the FSH treatment, the variance of the data was too large to detect any significant changes. The enzyme activity in granulosa tissue from the F3 and F5 follicles was unaffected by any of the hormone treatments.

In contrast to the basal activity of ornithine decarboxylase in granulosa tissue from preovulatory follicles, which increased as the follicles approached ovulation, the ornithine decarboxylase activity in thecal tissue from corresponding preovulatory follicles decreased (Tables 1 and 2).

Effect of various doses of PMSG on ornithine decarboxylase activity in ovarian follicular tissue

Thecal tissue from small follicles 1–3 mm in diameter was more sensitive to PMSG than was thecal tissue from follicles 3–6 mm in diameter (Table 3). The former was stimulated by a dose of 30 iu, whereas the latter showed no change in ornithine decarboxylase activity with this dose of PMSG. The minimum dose of PMSG that caused a significant increase in follicles 3–6 mm in diameter was 75 iu. Maximum activity in follicles of both of these sizes was observed with a dose of 300 iu.

Granulosa tissue showed an initial decrease in enzyme activity and a minimum was observed at 75 iu. Thereafter, activity increased, reaching a maximum at 300 iu PMSG. A dose of 150 iu PMSG had no effect on granulosa ornithine decarboxylase activity.

Table 3. Stimulation of ornithine decarboxylase activity by pregnant mares' serum gonadotrophin (PMSG) in granulosa tissue from the largest preovulatory follicle and thecal tissue from small follicles from laying hens

PMSG (iu)	Ornithine decarboxylase activity (pmol CO ₂ h ⁻¹ mg ⁻¹ protein)		
	Granulosa	Small yellow	Small white
0	175.7 ± 43.5	49.2 ± 6.5	39.2 ± 6.0
30	115.0 ± 21.3	47.0 ± 12.2	78.3 ± 25.1*
75	37.6 ± 11.8*	83.7 ± 12.0*	85.3 ± 4.6*
150	176.0 ± 49.0	107.1 ± 11.5*	94.8 ± 23.3*
300	1059 ± 104*	164.9 ± 38.6*	215.5 ± 60.4*
600	827.8 ± 280.3*	140.3 ± 6.3*	143.6 ± 25.2*

The results are expressed as means ± SEM (*n* = 4). Small yellow and small white represents follicles 3–6 mm and 1–3 mm in diameter, respectively. *Significantly different (*P* < 0.001) from control.

Ornithine decarboxylase activity in atretic follicles

Ovine LH (40 µg) and porcine FSH (500 µg) stimulated ornithine decarboxylase activity in both sizes of small follicle. In contrast, neither hormone treatment increased ornithine decarboxylase activity in the atretic, or grossly atretic follicles (Fig. 1). The basal activity of thecal ornithine decarboxylase decreased as atresia progressed.

Discussion

The activity of ornithine decarboxylase has been used as a marker for tissue growth and hormonal stimulation in a variety of experimental systems. The activity of ornithine decarboxylase increases under conditions of cell proliferation and differentiation (Marton and Morris, 1987; White *et al.*, 1987; Hirvonen, 1989). When a hormone or growth factor produces a response in its target tissue, there is a concomitant change in ornithine decarboxylase activity (Janne *et al.*, 1978).

The results reported here indicate that the effect of exogenous gonadotrophin on ornithine decarboxylase activity in thecal and granulosa tissue from avian ovarian follicles depends on the stage of growth and atresia. The large increase in thecal ornithine decarboxylase activity in small (< 6 mm) nonatretic follicles, 3 h after treatment, with either LH, FSH or PMSG is in marked contrast to the lack of change in thecal enzyme activity in large preovulatory follicles. In addition, small follicles become less sensitive to exogenous PMSG as they increase in size. The results are similar to the changes in thecal ornithine decarboxylase activity observed after the preovulatory increase in plasma LH concentrations (Armstrong, 1986a). Further evidence for a reduction in the sensitivity of thecal tissue from preovulatory follicles towards circulating gonadotrophins is the reduction in basal activity of ornithine decarboxylase in thecal tissue from follicles approaching ovulation. This decrease is mirrored by similar reductions in the activities of 3β-hydroxysteroid dehydrogenase and the aromatase enzyme system (Armstrong, 1982, 1984). It is concluded from these observations that, during normal follicular development *in vitro*, there is a general decrease in the response of thecal tissue towards gonadotrophins. The effect is particularly apparent

Fig. 1. The effect of gonadotrophin treatment on thecal ornithine decarboxylase activity (pmol CO₂ h⁻¹ mg⁻¹ protein) in small (< 6 mm) nonatretic, atretic and grossly atretic follicles from laying hens. (■) Control; (▨) LH treatment; (▩) FSH treatment. SY and SW represent small nonatretic follicles 3–6 mm and 1–3 mm in diameter, respectively. *Significantly different (*P* < 0.001) from controls. Values are means ± SEM (*n* = 4).

during the developmental period, when small follicles are recruited into the preovulatory follicular hierarchy.

Although significant binding of ¹²⁵I-labelled bovine FSH to thecal tissue from large preovulatory follicles has been observed (Etches and Cheng, 1981), the results presented here indicate that exogenous gonadotrophin has less effect on thecal tissue from large preovulatory follicles than from small (< 6 mm) follicles. In this respect, the observation that 8-bromo 3'5' cyclic AMP and forskolin become less effective in stimulating androstenedione production in thecal tissue from follicles approaching ovulation (Marrone and Hertelendy, 1985) is relevant and indicates that the lack of response of thecal tissue from large preovulatory follicles towards gonadotrophin treatment *in vivo* may be due to the development of a lesion(s) in some post-receptor binding system involved in the transmission of the signal from the hormone-receptor complex into the

thecal cells of the large preovulatory follicles. Superimposed on the reduced sensitivity of thecal ornithine decarboxylase activity towards exogenous gonadotrophin during the recruitment of small follicles into the hierarchy is the increase in basal activity of the enzyme during the same development period. The basal activity of the enzyme in thecal tissue from the F5 follicle approaches that observed in thecal tissue from small follicles fully stimulated by gonadotrophin. It is possible that upon recruitment of the follicle into the hierarchy, gonadotrophin remains bound to its receptor, thus maintaining a high basal enzyme activity, while preventing subsequent stimulation by exogenous hormone. In addition, locally acting growth factors, produced by the follicle during the recruitment process, are also likely to be involved in maintaining a high basal ornithine decarboxylase activity in thecal tissue at this time.

Juxtaposed with the reduction in gonadotrophin responsiveness of thecal tissue in small follicles, upon recruitment into the follicular hierarchy, is the increase, *in vivo*, in responsiveness of granulosa tissue towards LH and PMSG during the final phase of follicular development. The effect of PMSG on F1 granulosa tissue is probably due to the LH-like activity within the PMSG preparation (Stewart *et al.*, 1976), although a direct effect of FSH on F1 granulosa tissue cannot be excluded. Binding of ¹²⁵I-labelled FSH to granulosa tissue homogenates was observed by Etches and Cheng (1981), but their preparation contained granulosa cells from follicles at various stages of development and direct comparisons cannot be made with the present results.

The observations, presented here, indicate that only granulosa cells from the largest preovulatory follicle respond to exogenous gonadotrophin. A similar result was observed by measuring the changes in ornithine decarboxylase activity in granulosa tissue after the preovulatory LH surge (Armstrong, 1986a). Measurement of progesterone content of granulosa cells, after LH treatment *in vivo*, also indicated that only the F1 granulosa cells responded to exogenous gonadotrophin (Wells *et al.*, 1985b). These observations are in direct contrast to those of Hammond *et al.* (1981) and Asem and Hertelendy (1985), who demonstrated that progesterone production by granulosa cells from all the large preovulatory follicles was higher after incubation with LH *in vitro*; albeit the F1 granulosa cells were stimulated to a greater degree than granulosa cells from the less mature large follicles. Differences in the experimental design may be, at least, partly responsible for these differences, in that the present experiment and those described by Wells *et al.* (1985b) were performed *in vivo*, whereas those of Hammond *et al.* (1981) were carried out *in vitro* using dispersed granulosa cells. Taken together, these results indicate that, although granulosa cells from all the large preovulatory follicles have the capacity to respond to gonadotrophin in the intact follicle, *in vitro*, only granulosa tissue from the most mature follicle can do so. The avian granulosa cell develops through a complex sequence of events including an increase in their sensitivity towards LH (Hammond *et al.*, 1981), the development of a functionally coupled adenyl cyclase system, and the maturation of post-cyclic AMP steps (Asem and Hertelendy, 1985), including an increase in 3 β -hydroxysteroid dehydrogenase activity (Armstrong, 1982) and cholesterol-20,22-desmolase activity (Asem and Hertelendy, 1985). The results presented here indicate that there is another level of control on granulosa

function imposed by the intact follicle. In this case, it is possible that the access of gonadotrophin to the granulosa tissue of preovulatory follicles may, because of their lack of a direct blood supply, somehow modulate the supply of hormone to the granulosa cells of all but the most mature preovulatory follicle. It is also likely that growth factors play a role in this process. In mammals, a variety of growth factors have been shown to either potentiate or attenuate the response of granulosa cells towards gonadotrophins (Adashi and Rohan, 1992; Adashi *et al.*, 1992; Giudice, 1992) and a similar mechanism probably operates in hens.

The response of ornithine decarboxylase activity in granulosa tissue from F1 follicles towards PMSG *in vivo* shows clearly that granulosa tissue is significantly less sensitive to PMSG *in vivo* than is thecal tissue from small (< 6 mm) follicles. The cause of the reduction in the enzyme activity in granulosa tissue caused by the 75 iu PMSG is not known. However, FSH has been shown to decrease ornithine decarboxylase activity in Sertoli cells of rats (Madhubala *et al.*, 1987). Whatever the cause, the inhibition is overcome at higher doses of PMSG.

The results reported here indicate that an early event, associated with the onset of atresia in small follicles, is a loss in sensitivity towards circulating gonadotrophin, as indicated by the lack of response of thecal ornithine decarboxylase activity in atretic follicles to the hormone treatments. Whether this effect is due to the disruption of the blood supply to the atretic follicles, thus limiting the access of the hormone to its target tissue, or to a failure in some receptor-mediated event cannot be distinguished from these results.

In conclusion, the results of this investigation demonstrate that thecal tissue from small ovarian follicles is more sensitive to stimulation by gonadotrophins than is thecal tissue from large preovulatory follicles. This observation has relevance with respect to the development of atresia during folliculogenesis in hens. Atresia is the common fate for the majority of small (< 8 mm) follicles, whereas it is a rare event, under normal physiological conditions, for the population of large preovulatory ovarian follicles from birds laying regular egg sequences (Gilbert *et al.*, 1983). The growth of small follicles is critically dependent on plasma concentrations of FSH (Opel and Nalbandov, 1961). Any mechanism that reduces their sensitivity, or their exposure to hormonal stimuli will decrease their chances of recruitment into the follicular hierarchy (Armstrong, 1986c), and lead to atresia and eventual reabsorption back into the ovary. After recruitment into the hierarchy, however, thecal tissue becomes refractory towards FSH and this may explain why large preovulatory follicles do not normally become atretic.

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