Modulatory effects of gonadotrophins and insulin-like growth factor on the secretion of inhibin A and progesterone by granulosa cells from chicken preovulatory (F1–F3) follicles

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The aim of this study was to compare the actions and interactions of gonadotrophins (LH and FSH) and an analogue of insulin-like growth factor I (LR3-IGF-I) on the secretion of inhibin A, inhibin B and progesterone by cultured chicken granulosa cells derived from the three largest (F1–F3) follicles of the preovulatory hierarchy. Treatment with LH or FSH promoted marked dose- ($P < 0.0001$) and time- ($P < 0.0001$) dependent increases in both inhibin A and progesterone secretion, with the magnitude of response (< 15 -fold compared with basal) increasing over time in culture. Concentrations of inhibin B were below the detection limit in all samples. Initially, F1 cells were more LH-responsive than were F3 cells in terms of progesterone secretion ($P < 0.02$) but this difference between follicles decreased over time in culture. In contrast, LH-induced inhibin A secretion tended to be highest from F3 cells, although this was not significant. Cells from F3 follicles were consistently more FSH-responsive than F1 cells in terms of both progesterone ($P < 0.01$) and inhibin A ($P < 0.02$) secretion. Initially, F1 cells were more responsive to LR3-IGF-I than were F3 cells

in terms of progesterone secretion ($P < 0.001$) but were less responsive in terms of inhibin A secretion ($P < 0.001$). Again, these inter-follicle differences decreased over time in culture (not significant on day 3 of treatment). Co-treatment experiments showed that LR3-IGF-I enhanced both LH- and FSH-induced secretion of inhibin A and progesterone in a time- ($P < 0.001$) and follicle- ($P < 0.001$) dependent way. Initially, F1 cells showed highest LR3-IGF-I enhancement of LH-induced inhibin A and progesterone secretion; in contrast, F3 cells showed the highest LR3-IGF-I enhancement of FSH-induced inhibin A and progesterone secretion. These inter-follicle differences persisted over time in the case of FSH-induced hormone responses but not in the case of LH-induced responses, even though the relative degree of LR3-IGF-I enhancement increased markedly over time. Collectively, these data support a positive role for IGF-I, presumably of thecal origin, as an amplifier of gonadotrophin action on granulosa cell inhibin A and progesterone production by preovulatory chicken follicles.

Introduction

The functional left ovary of a domestic hen typically contains between five and nine yolk-filled preovulatory follicles arranged in a distinct size hierarchy. The largest follicle, designated F1 (approximately 35 mm in diameter), is first to ovulate, followed by the second largest follicle (F2) approximately 24–26 h later and this continues down the hierarchy. This ordered arrangement of preovulatory follicles makes the hen an excellent model for the study of factors regulating the later stages of folliculogenesis. The production of steroids by granulosa and theca (interna and externa) layers of hen hierarchical follicles has been studied extensively. Granulosa tissue of F1–F4 preovulatory follicles contains significantly more progesterone than does

theca tissue (Etches and Duke, 1984; Kato *et al.*, 1995). Conversely, thecal tissue contains significantly more testosterone and oestradiol (Kato *et al.*, 1995). A three-cell model of steroidogenesis in avian follicles that takes the differential steroidogenic activities of the theca externa, theca interna and granulosa cell layers into account has been proposed by Porter *et al.* (1989).

There is increasing evidence, mainly from mammalian species, to support local autocrine/paracrine roles of various growth factors, including insulin-like growth factors (IGF-I and II), transforming growth factor β (TGF β) superfamily members (for example, inhibins and activins) and epidermal growth factor (EGF)-related peptides (including TGF α) in modulation of ovarian follicle function and steroidogenesis (Adashi *et al.*, 1992; Knight and Glistler, 2001). For example, IGF-I, activin A and EGF/TGF α are expressed in rat follicles: IGF-I and activin A augment FSH-dependent processes, such as inhibin production and

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steroidogenesis, in cultured rat granulosa cells (Adashi *et al.*, 1992; Xiao *et al.*, 1992), whereas EGF/TGF α suppresses inhibin production and steroidogenesis (Hsueh *et al.*, 1981; Glister *et al.*, 2001). Chicken granulosa cells express receptors for IGF-I (Roberts *et al.*, 1994) and EGF/TGF α (Onagbesan *et al.*, 1994) but there have been relatively few studies to investigate the potential actions of such growth factors on granulosa cell proliferation and steroidogenesis in birds (Peddie *et al.*, 1994; Onagbesan and Peddie, 1995; Onagbesan *et al.*, 1999a,b, 2000).

Likewise, there have been only a few attempts to study the factors regulating the production of inhibin-related peptides from avian granulosa cells, despite their well-established roles in mammalian species. Vanmontfort *et al.* (1992) cultured granulosa cells from hen preovulatory (F1–F4) follicles and found that secretion of immunoreactive inhibin was highest from F4 follicles and lowest in F1 follicles. Secretion of immunoreactive inhibin was stimulated by both LH and FSH. However, the inhibin radioimmunoassay used in the study of Vanmontfort *et al.* (1992) has limited specificity and crossreacts extensively (100%) with free inhibin α -subunit forms. The high concentrations of inhibin α -subunit present in hen plasma (Lovell *et al.*, 2000a, 2001), cockerel plasma (Lovell *et al.*, 2000b) and F1 preovulatory follicle granulosa cell extract (Lovell *et al.*, 1998) signal the need for caution in interpreting earlier findings based on the use of 'first generation' inhibin radioimmunoassays lacking specificity for the $\alpha\beta$ dimeric molecule. In chicken preovulatory follicles, inhibin A, like progesterone, is produced selectively by granulosa rather than theca cells (Lovell *et al.*, 2000c, 2001), with maximal concentrations in the F1 granulosa layer (Lovell *et al.*, 1998, 2000a, 2001), which also shows maximal expression of inhibin/activin β_A subunit mRNA (Chen and Johnson, 1996a; Safi *et al.*, 1998). In contrast, theca layers of F1–F4 follicles contain significantly more activin A than do corresponding granulosa layers (Lovell *et al.*, 1998, 2000c, 2001).

The aims of the present study were to use a hen granulosa cell culture system in combination with highly specific inhibin A and inhibin B immunoassays to: (i) compare the effects of LH and FSH on the secretion of dimeric inhibins and progesterone by granulosa cells from F1, F2 and F3 preovulatory follicles; and (ii) investigate the effects of an IGF-I analogue on basal, LH-induced and FSH-induced secretion of dimeric inhibins and progesterone.

Materials and Methods

Experimental animals

Laying hens (ISA brown) towards the end of their first year of laying were caged individually and maintained under a standard long-day photoschedule of 16 h light:8 h dark, at an ambient temperature of 21–23°C. Food and water were freely available. Daily oviposition times were recorded and used to predict the timing of oviposition.

Isolation of granulosa cells

Hens ($n = 5$ –6 per culture experiment) were killed by cervical dislocation approximately 4 h after ovulation of a mid-sequence egg. The three largest preovulatory follicles (F1–F3) of the ovarian hierarchy were removed and placed immediately in sterile Hank's balanced salt solution (HBSS; Gibco-BRL, Uxbridge). Granulosa and theca layers were separated in sterile conditions in a laminar flow cabinet (Gilbert *et al.*, 1977).

In brief, granulosa layers derived from a given hierarchical follicle position in different hens were combined (creating separate F1, F2 and F3 pools) and transferred to digestion medium (medium 199 with 25 mmol Hepes l^{-1} , 0.1% (w/v) collagenase (type IV), 0.01% (v/v) trypsin inhibitor (type I-S), 0.3% (w/v) BSA (fraction V), 0.01% (v/v) L-glutamine and 1% (v/v) antibiotic stock solution (5000 U penicillin ml^{-1} and 5000 U streptomycin ml^{-1} ; all from Sigma UK Ltd, Poole). Approximately 2 ml of digestion medium was used per 500 mg tissue. Tissues were aspirated 30 times with a sterile Pasteur pipette (Alpha Laboratories Ltd, Eastleigh), incubated for 30 min at 37°C and then re-aspirated 30 times. Cells were retrieved by centrifugation at 400 g for 8 min at room temperature. The supernatant was decanted; the pellet was resuspended in 10 ml fresh incubation medium (medium 199 with 25 mmol Hepes l^{-1} , 0.01% (v/v) L-glutamine and 1% (v/v) antibiotic solution) and centrifuged at 400 g for 8 min at room temperature. The supernatant was decanted and the pellet was dispersed in 2 ml fresh plating medium (incubation medium containing 2% (v/v) charcoal-stripped fetal calf serum; Sigma UK Ltd). The number of viable cells was estimated by trypan blue exclusion (Sigma UK Ltd). Isolated granulosa cells were diluted in plating medium and divided into aliquots in 24-well plates (Falcon 3047; Becton Dickinson Labware, Franklin Lakes, NJ) at 3×10^5 viable cells per well. The cells were maintained for 24 h at 39°C. Unattached cells were aspirated off and the attached cells were washed three times with 1 ml serum-free incubation medium. All further incubations were done under serum-free conditions. Incubation buffer (1 ml) and test treatments were added to appropriated wells. The cells were incubated for 24 h and conditioned media were aspirated and stored at -20°C . The media were replenished every 24 h and the test treatments were also added at these times. At termination of culture, the media were removed and the monolayers were washed three times with PBS (Oxoid Ltd, Basingstoke). The plated cells were sonicated in PBS containing 1% (w/v) BSA (protease free), 1% (v/v) Triton-X100 (Sigma UK Ltd) and 0.1% (w/v) sodium azide (Fisher Scientific UK Ltd, Loughborough; 200 μl per well), and the resultant suspension was stored at -20°C .

Treatments

All treatments were prepared as stock solutions: oLH (NIDDK-oLH-25) and oFSH (NIDDK-oFSH-19-SIAPH) were prepared at 10 $\mu\text{g ml}^{-1}$ in HBSS containing 0.3% (v/v) BSA

Fig. 1. Effect of LH on the secretion of (a,c,e) inhibin A and (b,d,f) progesterone by cultured hen granulosa cells derived from F1 (▨), F2 (■) and F3 (□) preovulatory follicles. Data for three consecutive 24 h incubations are shown: (a,b): 24–48 h; (c,d) 48–72 h; and (e,f): 72–96 h. Values are mean $\pm$ SEM ($n = 3$ replicate cultures). Results of ANOVA are indicated on each panel.

(fraction V); and long R3 IGF-I (LR3-IGF-I; Sigma UK Ltd) was prepared at 0.1 mg ml⁻¹. Before culture, the treatments were diluted in HBSS containing 0.3% (v/v) BSA to a $\times 40$ treatment concentration and filter-sterilized using a 0.2 μ m Millipore filter (FlowPore D; ICN Biomedicals Ltd, Basingstoke). Treatments were added to respective wells to give final concentrations of 1, 10 and 100 ng ml⁻¹ for oLH and oFSH, and 1 and 10 ng ml⁻¹ for IGF-I. LR3-IGF-I, a recombinant analogue of human IGF-I, was used in preference to the native molecule as it does not interact with IGF binding proteins.

Immunoassays

Cell-conditioned media were assayed for inhibin A and inhibin B concentrations by specific two-site ELISAs that use monoclonal antibodies raised against synthetic peptide fragments of the human α -, β_A - and β_B -subunits (Muttukrishna *et al.*, 1994; Groome *et al.*, 1996). These assays have been validated previously for domestic fowl as described by Lovell *et al.* (1998, 2000b). Recombinant human inhibin A and inhibin B were used as assay standards. The detection limits of the inhibin A and inhibin B ELISAs were 2 and 15 pg ml⁻¹, respectively. The recoveries of known amounts of exogenous inhibin A or inhibin B standard were quantitative (94–108%). Intra- and inter-plate coefficients of variation were $< 10\%$. Progesterone was determined by direct radioimmunoassay as described by Wrathall and Knight (1995). The total cellular DNA content of each well at the end of the culture was determined by fluorimetric assay (Labarca and Paigen, 1980), allowing hormone

secretion to be expressed on a per μ g DNA basis, thereby normalizing for cell number.

Statistical analysis

ANOVA was used to evaluate the effects of single and combined treatments on hormone secretion and DNA content, and to make comparisons between cells from F1, F2 and F3 follicles. Hormone release data for each of the three successive periods of culture were analysed separately. In a given culture experiment, treatments were tested using triplicate wells and each experiment was repeated in three independent cultures. Values are mean $\pm$ SEM based on three independent cultures.

Results

The viability of freshly dispersed cells was always $> 90\%$. Cells were cultured for a maximum of 4 days (24 h plating plus 72 h treatment) and hormone concentrations were determined in conditioned media samples corresponding to 24–48 h, 48–72 h and 72–96 h of culture, hereafter referred to as treatment days 1, 2 and 3, respectively. Concentrations of inhibin A and progesterone in conditioned media were easily measurable throughout the study; concentrations of inhibin B were below the assay detection limit.

Basal secretion of inhibin A and progesterone from F1 to F3 granulosa cells

Basal secretion of both inhibin A and progesterone in F1, F2 and F3 cultures decreased significantly ($P < 0.05$)

Fig. 2. Effect of FSH on the secretion of (a,c,e) inhibin A and (b,d,f) progesterone by cultured hen granulosa cells derived from F1 (▨), F2 (■) and F3 (□) preovulatory follicles. Data for three consecutive 24 h incubations are shown: (a,b): 24–48 h; (c,d) 48–72 h; and (e,f): 72–96 h. Values are mean $\pm$ SEM ($n = 3$ replicate cultures). Results of ANOVA are indicated on each panel.

between day 1 and day 2 of culture, and remained low during the final day of culture (Fig. 1). A comparable decrease was observed in residual cellular content of inhibin A (data not shown).

Effect of LH on inhibin A and progesterone secretion from F1 to F3 granulosa cells

Treatment with LH elicited significant dose-dependent increases ($P < 0.001$) in inhibin A secretion in F1–F3 granulosa cell cultures (Fig. 1a). The magnitude of the response, relative to basal inhibin A secretion, increased progressively over the 3 days of treatment (approximate fourfold increase on day 1 compared with approximate 15-fold increase on day 3). Two-way ANOVA of results for each day of treatment showed a significant ($P < 0.05$) effect of follicle position on inhibin A secretion: LH-induced inhibin A secretion was slightly higher from F3 granulosa cells than from F1 granulosa cells.

LH treatment also elicited marked dose-dependent increases in progesterone secretion in F1–F3 granulosa cell cultures, with the magnitude of the response increasing over time in culture (approximate sixfold increase on day 1 compared with approximate 15-fold increase on day 3) (Fig. 1b). In contrast to inhibin A secretion, LH-induced progesterone secretion tended to be higher in F1 than in F2 and F3 granulosa cells (only significant on day 1).

Effect of FSH on inhibin A and progesterone secretion from F1 to F3 granulosa cells

As with LH, treatment with FSH elicited significant dose-dependent increases ($P < 0.001$) in inhibin A secretion in F1–F3 granulosa cell cultures (Fig. 2a). The magnitude of the response, relative to basal inhibin A secretion, increased progressively over the 3 days of treatment (approximate fourfold increase on day 1 compared with approximate 12-fold increase on day 3). Two-way ANOVA of results for each day of treatment showed a highly significant ($P < 0.0001$) effect of follicle position on inhibin A secretion and a significant follicle position $\times$ FSH treatment interaction ($P < 0.05$): FSH-induced inhibin A secretion was highest from F3 granulosa cells, intermediate from F2 cells and lowest from F1 cells.

FSH treatment also promoted dose-dependent increases in progesterone secretion in F1–F3 granulosa cell cultures (Fig. 2b). Relative to basal secretion, the magnitude of the response was less than for inhibin A and only increased slightly over the 3 days of treatment. However, two-way ANOVA of results for each day of treatment showed a significant ($P < 0.005$) effect of follicle position on progesterone secretion and a significant follicle position $\times$ FSH treatment interaction ($P < 0.01$). As observed for inhibin A secretion, FSH-induced progesterone secretion was inversely related to follicle position, being highest from F3 granulosa cells.

Fig. 3. Effect of long R3 insulin-like growth factor I (LR3-IGF-I) on the secretion of (a,c,e) inhibin A and (b,d,f) progesterone by cultured hen granulosa cells derived from F1 (▨), F2 (■) and F3 (□) preovulatory follicles. Data for three consecutive 24 h incubations are shown: (a,b): 24–48 h; (c,d) 48–72 h; and (e,f): 72–96 h. Values are mean $\pm$ SEM ($n = 3$ replicate cultures). Results of ANOVA are indicated on each panel.

Relationship between secreted and cellular inhibin A contents

A selection of matched conditioned media and cell lysate samples from F1, F2 and F3 granulosa cell cultures terminated after 1, 2 and 3 days of treatment ($\pm$ LH or FSH), were analysed for inhibin A. A significant positive relationship ($r = 0.805$, $P < 0.001$) was observed between secreted (y) and cellular (x) inhibin A content (relative to DNA), as represented by the following regression equation: $y = 14.419x + 0.003$. Residual cellular content of inhibin A represented $< 10\%$ of the total inhibin A present in each well.

Effects of LR3-IGF-I analogue on inhibin A and progesterone secretion from F1 to F3 granulosa cells

Considering the results for all three follicle classes, treatment with IGF-I only significantly ($P < 0.005$) enhanced inhibin A secretion during all three culture periods, although the response was most significant on day 1 of treatment (Fig. 3a). Two-way ANOVA of day 1 data showed a significant ($P < 0.001$) effect of follicle position and a significant ($P < 0.001$) follicle class $\times$ IGF-I treatment interaction, with cells from the F3 follicle being most responsive to IGF-I and cells from F1 follicles being least responsive. This difference was not apparent on days 2 and 3 of treatment.

Overall, IGF-I had a modest stimulatory effect on progesterone secretion at all three time points (Fig. 3b). In

contrast to inhibin A secretion, IGF-I-induced progesterone secretion was highest from F1 granulosa cells, with F3 granulosa cells showing little or no response at any time point.

Modulatory effect of LR3-IGF-I analogue on LH-induced secretion of inhibin A and progesterone from F1 to F3 granulosa cells

Co-treatment with IGF-I resulted in a significant time-dependent enhancement of LH-induced inhibin A secretion that became clearly evident during days 2 and 3 of treatment (Fig. 4a). During day 1 of treatment, the inhibin A response to co-treatment ranged from being approximately additive (in F1 cells) to being no different from the responses to either factor alone (in F3 cells). This difference in responsiveness of F1, F2 and F3 cells is supported by the ANOVA, which indicated a significant effect of follicle position, as well as significant follicle position $\times$ treatment interactions. On days 2 and 3 of treatment, a synergistic effect of IGF-I on LH-induced inhibin A secretion was clearly evident. Relative to the inhibin A response elicited by 10 ng LH ml⁻¹ only, co-treatment with 10 ng IGF-I ml⁻¹ promoted a further three- to fourfold increase ($P < 0.001$) on both days. However, the response was not uniform among cells from different follicle positions: in relative terms, F2 cells showed a greater response to co-treatment than did either F1 or F3 cells, this difference being most marked on day 3 ($P < 0.0001$).

Fig. 4. Effects of LH and long R3 insulin-like growth factor I (LR3-IGF-I), alone and in combination, on the secretion of (a,c,e) inhibin A and (b,d,f) progesterone by cultured hen granulosa cells derived from F1 (▨), F2 (■) and F3 (□) preovulatory follicles. Data for three consecutive 24 h incubations are shown: (a,b) 24–48 h; (c,d) 48–72 h; and (e,f) 72–96 h. Values are mean $\pm$ SEM ($n = 3$ replicate cultures). Results of ANOVA are indicated on each panel.

Co-treatment with IGF-I also enhanced LH-induced progesterone secretion but, in contrast to inhibin A secretion, a marked synergistic effect was evident in F1 cells from day 1 of treatment onwards (fivefold enhancement of LH-induced progesterone secretion; $P < 0.001$). This response to IGF-I was much lower in F2 cells and absent in F3 cells (ANOVA: follicle position $\times$ LH $\times$ IGF-I interaction, $P < 0.001$). On days 2 and 3, IGF-I also had a marked synergistic effect on LH-induced progesterone secretion (ANOVA: LH $\times$ IGF-I interaction, $P < 0.001$); similar enhancement was observed for all three follicle positions (ANOVA: follicle position $\times$ LH $\times$ IGF-I interaction, not significant).

Modulatory effect of LR3-IGF-I analogue on FSH-induced secretion of inhibin A and progesterone from F1 to F3 granulosa cells

Co-treatment with IGF-I also enhanced FSH-induced inhibin A secretion in a time- and follicle-dependent way (Fig. 5a). During day 1 of treatment, the response to the two

factors was additive in F1 and F2 granulosa cells but much less than additive in F3 cells. On days 2 and 3, a clear synergistic effect of IGF-I on FSH-induced inhibin A secretion was evident in F3 granulosa cells (approximate threefold enhancement relative to FSH only, $P < 0.001$) but not in F1 granulosa cells, in which the response to both treatments was barely additive (ANOVA: follicle position $\times$ FSH $\times$ IGF-I treatment interaction, $P < 0.001$).

Overall, co-treatment with IGF-I did not significantly modify FSH-induced progesterone secretion during day 1 or 2 of treatment (ANOVA: FSH $\times$ IGF-I treatment interaction, not significant on either day). However, IGF-I tended to enhance FSH-induced progesterone secretion in F3 granulosa cells and this effect was significant by day 3 of treatment (approximate threefold enhancement, $P < 0.001$).

Effect of gonadotrophins and LR3-IGF-I analogue on cell proliferation

Treatment of cells from F1, F2 and F3 follicles with either LH or FSH alone had no effect on number of cells, as

Fig. 5. Effects of FSH and long R3 insulin-like growth factor I (LR3-IGF-I), alone and in combination, on the secretion of (a,c,e) inhibin A and (b,d,f) progesterone by cultured hen granulosa cells derived from F1 (▨), F2 (■) and F3 (□) preovulatory follicles. Data for three consecutive 24 h incubations are shown: (a,b): 24–48 h; (c,d) 48–72 h; and (e,f): 72–96 h. Values are mean $\pm$ SEM ($n = 3$ replicate cultures). Results of ANOVA are indicated on each panel.

reflected by cellular DNA content measured at the end of the culture period. In contrast, LR3-IGF-I either alone, or in the presence of LH or FSH, promoted a small but significant increase in DNA content (approximate 25% increase, $P < 0.001$) in cells from all three follicle classes (data not shown).

Discussion

To our knowledge, this study is the first to attempt to quantify the secretion of specific inhibin dimers (inhibins A and B) from cultured avian granulosa cells and to relate this to steroid (progesterone) secretion. Unfortunately, it was only possible to measure inhibin A and progesterone secretion, as inhibin B concentrations in cell-conditioned media were below the detection limits of the assay. The main findings were: (i) under basal conditions, granulosa cells from F1, F2 and F3 preovulatory follicles secreted similar amounts of inhibin A throughout the culture period; (ii) inhibin A secretion was responsive to stimulation by both LH and FSH in a time- and follicle-dependent way; (iii)

inhibin A secretion was positively correlated with residual cellular inhibin A content; and (iv) IGF-I stimulated inhibin A secretion and enhanced the secretory response to both LH and FSH in a time- and follicle-dependent way.

In general, the differences in basal and gonadotrophin-induced progesterone secretion by F1, F2 and F3 granulosa cells observed in the present study are in accordance with previous reports (Hammond *et al.*, 1981; Asem and Hertelendy, 1985; Nakamura *et al.*, 1991; Onagbesan *et al.*, 1999b, 2000). However, inter-follicle differences in basal and LH-induced progesterone secretion were less pronounced than in other studies and were confined to day 1 of treatment. This difference is probably because the hens were killed about 4 h after ovulation of the previous F1 follicle in the present study, rather than about 12 h after ovulation as reported in most other studies. Thus, the F1 follicles from which granulosa cells were harvested in the present study had only recently acquired F1 status. This time point was selected on the basis of our previous finding (Lovell *et al.*, 1998) that the *ex vivo* tissue content of inhibin A in the F1 granulosa layer was already some 20-fold higher

than in the corresponding F2 granulosa layer at this time. Given this, it was surprising to find that granulosa cells isolated from F1, F2 and F3 follicles secreted similar amounts of inhibin A after 2, 3 or 4 days of *in vitro* culture; moreover, residual cellular contents of inhibin A after 4 days of culture were similar in F1, F2 and F3 granulosa cells. These apparent discrepancies between *ex vivo* tissue contents of inhibin A (Lovell *et al.*, 1998) and *in vivo* rates of inhibin A secretion or cell content, as measured in the present study, presumably reflect the failure of the culture system to reproduce adequately the environment under which granulosa cells operate *in vivo*. In particular, the fact that the granulosa cells are deprived of paracrine factors arising from the surrounding theca layer (such as IGF-I and activin) may perturb their function *in vitro*.

Nonetheless, differential inhibin A and progesterone responses to gonadotrophins were evident between follicle categories, with F3 granulosa cells being more sensitive to FSH than were F1 granulosa cells; conversely, F1 granulosa cells were generally more sensitive to LH than were F3 granulosa cells. A decrease in FSH responsiveness as follicles move up the preovulatory hierarchy is consistent with previous observations: You *et al.* (1996) reported a significant reduction in expression of FSH receptor mRNA in granulosa cells from F3 to F1 follicles, which corresponded to a decreased FSH receptor binding, without a change in receptor affinity (Ritzhaupt and Bahr, 1987). Calvo and Bahr (1983) also found higher FSH-stimulated adenylyl cyclase activity in granulosa cells from smaller preovulatory follicles.

Although LH-stimulated progesterone secretion during the day 1 of treatment increased during follicular development (F1 > F2 > F3) there was no significant difference between F1 and F3 granulosa cells in LH-stimulated inhibin A secretion at this time. Granulosa cell LH receptor mRNA expression is similar in F3 to F1 follicles (Johnson *et al.*, 1996). Moreover, at the stage of the ovulatory cycle at which granulosa cells were collected in the present study (approximately 4 h after ovulation of the previous F1 follicle), Calvo *et al.* (1981) found no inter-follicle difference in basal and LH-stimulated granulosa cell adenylyl cyclase activity; increased responsiveness of the adenylyl cyclase system to LH was not demonstrated in the F1 and F2 granulosa layers until 12 h before the expected time of ovulation of the F1 follicle (some 8 h later than the time of follicle collection in the present study). Consistent with our findings, Davis *et al.* (1999) reported that both LH and FSH increase expression of inhibin- α subunit mRNA and inhibin- α protein by cultured hen granulosa cells. These authors also noted a requirement for gonadotrophin to maintain expression of inhibin- α mRNA *in vitro*, with concentrations decreasing to barely detectable values after 24–48 h of culture in the absence of LH or FSH. This requirement is in accordance with our observation of a significant reduction in basal inhibin A secretion from F1, F2 and F3 granulosa cell cultures after 48 h of culture. A comparable decrease in residual cell content of inhibin A, consistent with a reduced

rate of inhibin- α mRNA expression, translation and post-translational processing to form dimeric inhibin A, was also observed in the present study. The strong positive correlation between cellular and secreted inhibin A ($r = 0.805$, $P < 0.001$), and the finding that > 90% of total inhibin A was present in cell-conditioned media, indicate that newly synthesized inhibin A dimer is not stored in significant amounts but is secreted rapidly.

Although Davis *et al.* (1999) demonstrated that gonadotrophins increase mRNA expression and secretion of inhibin- α in preovulatory granulosa cells, Chen and Johnson (1996a) estimated expression of F1 granulosa α -subunit mRNA to be seven-times higher than for the corresponding β_A -subunit. It has also been shown that F1 preovulatory follicles (Lovell *et al.*, 1998) and hen plasma (Lovell *et al.*, 2000a, 2001) contain an excess of inhibin- α compared with dimeric inhibin A. These observations indicate that β_A -subunit expression or production may be a key factor controlling inhibin A production. In support of this view, Chen and Johnson (1996b) found that administration of a large dose of LH to laying hens (to mimic the preovulatory LH surge) reduced expression of β_A -subunit mRNA in F1 granulosa cells, although it did not alter expression of inhibin- α mRNA. Moreover, during the hen ovulatory cycle, plasma inhibin A reached a maximum at the time of the LH surge and, thereafter, concentrations were reduced (Lovell *et al.*, 2000a).

Secretion of dimeric inhibins has recently been investigated in rat (Lanuza *et al.*, 1999) and bovine (Boudjemaa *et al.*, 2000; Glister *et al.*, 2001) granulosa cells, human granulosa-lutein cells (Vanttinen *et al.*, 2000) and mouse preantral follicles (Smitz and Cortvrindt, 1999). Increased expression of inhibin/activin α - and β_A -subunit mRNA in mammals has been demonstrated in response to FSH (Su and Hsueh, 1994) or cAMP analogues (Tuuri *et al.*, 1996). Although both genes were stimulated by cAMP, expression of inhibin- α mRNA was stimulated preferentially at low cAMP concentration, whereas expression of inhibin/activin β_A -subunit mRNA required a high cAMP concentration. Expression of inhibin/activin β_A -subunit gene was also enhanced more rapidly (approximately 3 h) than expression of inhibin- α (24–48 h) (Tuuri *et al.*, 1996). Mammalian granulosa cells, like avian granulosa cells, synthesize excess inhibin- α (Bicsak *et al.*, 1988; Knight *et al.*, 1989, 1991; Lovell *et al.*, 1998); however, all the monomeric inhibin- α forms isolated to date lack classical inhibin-like activity, as they do not have a suppressive effect on pituitary FSH secretion. Schneyer *et al.* (1991) demonstrated that full-length inhibin- α precursors can compete with FSH for FSH receptor binding. Therefore, the possibility of an autocrine or paracrine action of endogenous inhibin- α precursor in modulating granulosa cell responsiveness to FSH during these *in vitro* studies cannot be eliminated.

IGF-I and IGF-II are both expressed in the ovary of domestic hens (Roberts *et al.*, 1994; Armstrong and Hogg, 1996). Theca expression of IGF-I mRNA increased during

follicular development from follicles 2–6 mm in diameter to the F1 stage, whereas granulosa expression of IGF-I mRNA could not be detected (Armstrong and Hogg, 1996). As expression of IGF receptor mRNA has also been reported in preovulatory follicle granulosa cells (Armstrong and Hogg, 1996), we investigated the putative role of IGF-I in regulating basal and gonadotrophin-induced inhibin A and progesterone secretion by granulosa cells.

In the present study, IGF-I analogue enhanced basal, LH- and FSH-stimulated inhibin A and progesterone secretion in a follicle position- (F1–F3) and time-dependent way. The magnitude of IGF-I enhancement of LH-stimulated progesterone and inhibin A secretion during the first 24 h of treatment was greater for F1 follicles than for F3 follicles, indicating that, in F1 follicles, theca-derived IGF-I may exert a paracrine action to sensitize granulosa cells to LH and, thus, maximize secretion of progesterone and inhibin A at about the time of the preovulatory LH surge. In this context, Lovell *et al.* (2000a) reported maximum inhibin A concentrations in peripheral blood of hens coincident with the preovulatory LH and progesterone increase. IGF-I also enhanced FSH-stimulated secretion of inhibin A by granulosa cells; however, in absolute terms, inhibin A secretion was significantly higher for F3 than for F1 granulosa cells. This presumably reflects a decrease in FSH sensitivity as follicle maturation proceeds, as IGF enhancement of FSH-induced progesterone secretion was also highest for F3 cells and lowest for F1 cells. Comparable effects of LH, FSH and IGF-I on progesterone secretion have been reported previously in cultures of granulosa cells from hen preovulatory follicles (Onagbesan and Peddie, 1995; Onagbesan *et al.*, 1999b, 2000) but the present study is the first in which these observations have been extended to inhibin A secretion. A modulatory action of IGF-I on basal and FSH-stimulated immunoreactive inhibin and steroid secretion has been documented in rat (Bicsak *et al.*, 1986), pig (Michel *et al.*, 1991) and bovine (Glister *et al.*, 2001) granulosa cell cultures.

In conclusion, as in mammals, inhibin A secretion by ovarian granulosa cells of hens is regulated by a combination of endocrine and paracrine/autocrine factors. Our findings support a positive role for theca-derived IGF-I acting both alone, and in combination with LH and FSH, to upregulate granulosa cell steroidogenesis and inhibin A production by preovulatory follicles.

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