Effects of insulin-like growth factor I and interactions with transforming growth factor α and LH on proliferation of chicken granulosa cells and production of progesterone in culture

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The effects of insulin-like growth factor I (IGF-I), alone, and in combination with LH or transforming growth factor α (TGF- α), on replication and progesterone production by cultured avian granulosa cells obtained from the three largest (F1–F3) follicles were studied. IGF-I and TGF- α stimulated proliferation of granulosa cells in a dose-dependent manner, and responsiveness decreased as the cells matured. IGF-I stimulated progesterone production from granulosa cells of all the follicles with no change in ED₅₀ value during follicular maturation; however, the maximum response was from cells derived from F1 follicles. IGF-I plus LH had an additive effect on progesterone production by cells from all follicles. In contrast, TGF- α inhibited basal and LH- and IGF-I-stimulated progesterone production. These data show that IGF-I and TGF- α may interact with each other during granulosa cell maturation, such that efficacy of IGF-I increases, while that of TGF- α decreases before ovulation. Furthermore, both growth factors interact with LH, either to enhance or inhibit progesterone production by granulosa cells. However, LH, IGF-I and TGF- α combine to stimulate proliferation of granulosa cells.

Introduction

The importance of gonadotrophins in regulating steroidogenesis in ovarian follicular granulosa cells of birds is well established. During follicular maturation, the granulosa tissue increases in size with a progressive increase in sensitivity to gonadotrophins (Asem and Hertelendy, 1985; Etches, 1990; Johnson, 1990). However, the role of growth factors as local paracrine and autocrine regulators in granulosa cells is less well characterized (Peddie *et al.*, 1993). In contrast, there is substantial evidence of the role of insulin-like growth factor I (IGF-I) (Adashi *et al.*, 1985a, 1992) and transforming-growth factor α (TGF- α) (Adashi *et al.*, 1987; May *et al.*, 1990) as local modulators of ovarian function in mammals. IGF-I causes proliferation of granulosa cells and amplifies the effects of FSH on differentiation of granulosa cells and the synthesis of progesterone and oestrogen (Adashi *et al.*, 1985b, 1988a; Hutchinson *et al.*, 1988; Schams *et al.*, 1988). In the thecal interstitial cells, LH/hCG-stimulated androgen production is enhanced by IGF-I (Cara and Rosenfeld, 1988; Hernandez *et al.*, 1988). The IGF-I gene is expressed in the granulosa itself in rat and pig ovaries (Hsu and Hammond, 1987; Mondschein and Hammond, 1988; Oliver *et al.*, 1989 and Hernandez *et al.*, 1989), while IGF-I receptors have been localized in both granulosa and thecal cells (Davoren *et al.*, 1986; Adashi *et al.*, 1988b; Hernandez *et al.*, 1990). In chickens, the IGF-I gene is localized within the granulosa and

thecal cells (Roberts *et al.*, 1994). However, the effects of IGF-I on proliferation of granulosa cells and production of progesterone have not been extensively reported. Recent reports have shown that chicken granulosa and thecal cells contain IGF-I receptors and that IGF-I stimulates DNA synthesis in both cell types (Huybrechts *et al.*, 1991; Roberts *et al.*, 1994), whereas IGF-I stimulates production of progesterone by the granulosa cells *in vitro* (Peddie *et al.*, 1993; Williams *et al.*, 1994). Similarly, receptors for epidermal growth factor (EGF) and the ligands EGF and TGF- α were localized in the granulosa and theca (Onagbesan *et al.*, 1994a) where they modulate cell proliferation and steroid production (Onagbesan *et al.*, 1994b; Peddie *et al.*, 1994). EGF/TGF- α are thought to be produced in thecal cell in mammals (Skinner *et al.*, 1987; Lobb *et al.*, 1989) and also possibly in birds (Peddie *et al.*, 1994) and cause proliferation of granulosa cells and modulation of steroid synthesis.

The aim of the present study was to determine the effects of IGF-I on steroid production and replication of granulosa cells from the three largest follicles F1–F3. It is inevitable that increased numbers of granulosa cells during follicular growth will contribute to the total follicular production of progesterone, but modulation of the responses of individual cells to LH by potential paracrine factors may also contribute substantially to the overall progesterone production. We, therefore, also investigated the potential for modulation of LH responses by IGF-I and TGF- α , and the possibility of interactions between IGF-I and TGF- α in regulating net progesterone production and of granulosa cell responses.

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Materials and Methods

Animals

Commercial ISA Brown hens, aged 25–35 weeks, were used for this study. Hens were kept in floor pens under a photoperiod of 14 h light:10 h dark and fed on layers' diet and water *ad libitum*. Egg laying was recorded daily for each hen and hens laying 5–6 egg sequences were selected for experiments. Hens were killed by cervical dislocation 12–14 h before the next expected ovulation. The ovaries were removed and placed in warmed (37°C) phenol-free Hanks' balanced salt solution (HBSS).

Reagents

Medium 199 (M199) with Earle's salts, phenol-free HBSS, antibiotic–antimycotic solution (PSA), BSA, L-glutamine, sodium bicarbonate, calcium- and magnesium-free HBSS, Hepes, trypsin–EDTA, Trypan blue, bisbenzamide, calf thymus DNA, transferrin, selenium and collagenase were obtained from Sigma Chemicals (Poole, Dorset). Fetal calf serum (FCS) was from Imperial Laboratories, (Andover, Hants), and recombinant human IGF-I and recombinant human TGF- α were purchased from Bachem Ltd (Saffron Walden, Essex). Ovine LH was a gift from S. Raiti (NIADDK, Baltimore, MD).

Preparation and culture of granulosa cells

The first (F1), second (F2) and third (F3) largest follicles in the ovary were removed and granulosa cells were prepared from each category of follicle separately as described by Peddie *et al.* (1994). Two or three birds were used for each experiment and granulosa cells from the same rank of follicles were pooled. Cell density was determined by measuring DNA in an aliquot using the method reported by Labarca and Paigen (1980) in which 1 μ g DNA corresponds to approximately 1×10^6 cells. Cell viabilities were assessed by the Trypan blue method and found to be greater than 95%.

Cells were seeded at 0.2 μ g DNA per well in 24-well plastic plates and cultured in M199 supplemented with 2 mmol glutamine l^{-1} , 1% (v/v) PSA and 5% (v/v) charcoal-stripped FCS for 48 h in a humidified atmosphere of 5% CO₂ in air at 37°C. The medium was discarded and cells washed with phenol-free HBSS and then cultured for a further 48 h in serum-free M199 supplemented with glutamine, sodium bicarbonate, 0.1% BSA, PSA, 6.25 μ g transferrin ml^{-1} , 5 ng selenium ml^{-1} and various concentrations (0–100 ng ml^{-1}) of IGF-I with or without LH (20 ng ml^{-1}). TGF- α (0–100 ng ml^{-1}), with or without LH, was applied or the effects of combinations of IGF-I and TGF- α (with or without LH) were investigated, at doses defined in Figs 6 and 7. The total incubation volume was always 500 μ l per well. At the end of the second 48 h incubation period, culture media were collected and stored at –20°C until assayed for progesterone. In another experiment, the cultures were washed twice with phenol-free HBSS and exposed for 3 h to a further LH challenge. For this, fresh M199 (500 μ l) containing 0.5% (w/v) BSA, with or without 20 ng LH ml^{-1} , was added and the cells

incubated for 3 h at 37°C in a humidified atmosphere of 5% CO₂ in air. Media were collected again and stored at –20°C for progesterone assay.

DNA content of wells

Parallel plates were set up as described above to determine the DNA content of wells during and after the culture period. At the end of the second 48 h of culture, media were discarded and monolayer cultures washed with phenol-free HBSS. Cell viabilities were again checked before cells were disaggregated with 200 μ l trypsin–EDTA in Ca²⁺-free 2 mol NaCl-phosphate l^{-1} buffer, followed by sonication. Viabilities were greater than 99% in all cell cultures. The DNA in aliquots of the lysate was determined by the method of Labarca and Paigen (1980) using calf thymus DNA as standard and bisbenzamide as the fluorogen.

Hormone assay

Progesterone was determined in the medium, without prior extraction, by radioimmunoassay as described by Brain *et al.* (1988). The minimum sensitivity of the assay was 0.01 pmol per tube. The intra- and interassay coefficients of variation were 5.08% and 9.5%, respectively.

Statistical analyses

Results are expressed as means $\pm$ SEM of four experiments, each with four replicates. DNA content of wells is expressed as μ g per well. All statistical analyses on progesterone data were transformed and expressed as pmol μ g^{–1} DNA per well to account for changes in number of cells between follicles during the culture period. Differences between groups were compared by ANOVA followed by Duncan's multiple-range test where applicable. Two-way ANOVA was applied to test interactions between hormones where necessary and *P* values < 0.05 were considered significant.

Results

Effects of IGF-I, TGF- α or LH on proliferation of granulosa cells

IGF-I caused a marked increase in DNA content of culture wells (Fig. 1). The maximum effective dose of IGF-I was 12.5 ng ml^{-1} . The F3 granulosa cell cultures showed the greatest response compared with the F2 and F1 cultures (50% increase at maximum effective dose (ED) compared with 35% for the F2 and 25% for the F1 granulosa). The calculated ED₅₀ values for IGF-I on the proliferation of cells from the three follicles was 2.00 ng ml^{-1} . Data on the effect of LH (20 ng ml^{-1}), IGF-I (3.13 ng ml^{-1}) and TGF- α (0.078 ng ml^{-1}) alone, or in combination, on accumulated DNA per well are summarized (Fig. 2). At this dose, IGF-I had no significant effect, by itself, on replication by the F1 granulosa cells; TGF- α had a small, but significant, effect (*P* < 0.05), while a combination of TGF- α and IGF-I had no extra effect on the total DNA content

Fig. 1. Dose-dependent effects of insulin-like growth factor I (IGF-I) on accumulated DNA in chicken ovarian granulosa cells cultured from F1 (□), F2 (△) or F3 (○) follicles for 48 h in serum-free medium. Values are means $\pm$ SEM of four experiments. *Significantly different from respective control for each follicle size ($P < 0.05$).

of the wells. While neither LH nor IGF-I alone stimulated DNA accumulation in cells from the largest most mature follicle, LH and IGF-I together had a significant proliferative effect ($P < 0.05$). In contrast, the less mature cells were responsive to the mitogenic effects of IGF-I; 3.13 ng IGF-I ml^{-1} caused a significant increase in DNA, as did 0.078 ng TGF- α ml^{-1} and together the growth factors showed increasing efficacy in less mature granulosa. LH had no significant effect by itself on granulosa from F2 and F3, but combined with IGF-I, it significantly potentiated the effect of IGF-I ($P < 0.05$). Thus, proliferative responses to both growth factors is greatest in the least mature cells, $F3 > F2 > F1$ ($P < 0.05$).

Effect of IGF-I on progesterone production

Data on progesterone production are expressed as pmol μg^{-1} DNA (Fig. 3), since the effects of IGF-I on proliferation were substantial and markedly influenced the interpretation of the raw data. The data show a dose-dependent stimulation of progesterone by IGF-I that reached a peak at a concentration of 25 ng IGF-I ml^{-1} . At this dose, granulosa cells cultured from F1 follicles showed a significantly greater increase in basal progesterone production compared with cells from F2 and F3 cells ($P < 0.001$). The calculated ED_{50} values for progesterone production by the three follicles was 4.60 ng IGF-I ml^{-1} .

The response to a 3 h LH challenge of cells previously cultured for 48 h with IGF-I in serum-free medium is shown (Fig. 4). Culturing granulosa cells with IGF-I, whilst enhancing basal progesterone production, also altered the ability of LH to subsequently cause an incremental increase in progesterone production. Basal and LH-stimulated progesterone production increased with increasing doses of IGF-I to a maximum dose of 12.5 ng ml^{-1} . Previous exposure to IGF-I potentiated the response of the F1, F2 and F3 cells to LH, and increased progesterone production by $200 \pm 26.4\%$, $43 \pm 6.20\%$ and $5 \pm 1.02\%$, respectively, at the maximum effective dose. However, the ED_{50} values for basal and LH-stimulated progesterone production under these short-term incubations were similar for all three follicle groups (4.60 ng ml^{-1}).

Fig. 2. Effects of insulin-like growth factor I (IGF-I) (3.13 ng ml^{-1}), LH (20 ng ml^{-1}) and transforming growth factor α (TGF- α) (0.078 ng ml^{-1}) alone or in combination, on accumulated DNA per well in chicken ovarian granulosa cells from (a) F1, (b) F2 and (c) F3 follicles cultured for 48 h in serum-free medium. Cultures were established in Medium 199 containing 5% (v/v) serum for 48 h, washed twice with HBSS and then cultured for a further 48 h in serum-free Medium 199 with IGF-I, TGF- α , LH or combinations of hormone and growth factors added simultaneously. Values are means $\pm$ SEM of four experiments. Different letters above columns indicate significant differences between treatments within each individual follicle category ($P < 0.05$).

Fig. 3. Effect of insulin-like growth factor I (IGF-I) on progesterone accumulation in culture medium ($\text{pmol } \mu\text{g}^{-1} \text{ DNA}$) by chicken granulosa cells from F1 ($\square$), F2 ($\triangle$) and F3 ($\circ$) follicles cultured for 48 h in serum-free medium. Data are means $\pm$ SEM of progesterone production during the second 48 h period of culture ($n=4$). *Significantly different from respective control for each individual follicle size ($P < 0.05$).

The effect of culturing cells for a second 48 h period in serum-free medium with 20 ng LH ml^{-1} in combination with various concentrations of IGF-I was also investigated. The results of progesterone accumulation in the medium is shown for F3 granulosa cells only (Fig. 5). Culturing cells in the presence of IGF-I enhanced progesterone accumulation in the medium as before, with an ED_{50} value of 4.60 ng ml^{-1} . The addition of LH alone caused $95 \pm 10.8\%$ increase in progesterone accumulation and this basal production of progesterone was significantly potentiated by IGF-I ($P < 0.05$). Again, the effect of IGF-I was dependent on the dose and follicle size.

Interactions of IGF-I with LH and TGF- α on progesterone production

The dose-dependent effects of TGF- α on progesterone accumulation in the medium during the second 48 h period of culture in serum-free medium for F3 granulosa cells are shown (Fig. 6). TGF- α inhibited progesterone production by up to 75% at the maximum effective dose of 10.1 ng ml^{-1} . The effects of culturing cells in the presence of 20 ng LH ml^{-1} , $3.13 \text{ ng IGF-I ml}^{-1}$, and $0.078 \text{ ng TGF-}\alpha \text{ ml}^{-1}$ and combi-

Fig. 4. Progesterone production ($\text{pmol } \mu\text{g DNA}^{-1}$), after a 3 h LH challenge, by chicken granulosa cells previously cultured for 48 h with increasing amounts of insulin-like growth factor I (IGF-I) (0 – 100 ng ml^{-1}) in serum-free medium. Granulosa cells from (a) F1, (b) F2 and (c) F3 follicles were established and then cultured with IGF-I for 48 h; the culture media were discarded and cells were washed twice with HBSS and then incubated with (hatched) or without ($\square$) LH (20 ng ml^{-1}) for a further 3 h. Data are means $\pm$ SEM ($n=4$) of progesterone production during the 3 h incubation. *Significantly different from cultures without LH treatment at each dose of IGF-I ($P < 0.05$).

nations of hormone and growth factors on progesterone accumulation in the medium during the second 48 h period of culture in serum-free medium are summarized (Fig. 7). LH or IGF-I alone stimulated progesterone production in all three follicle categories and IGF-I and LH together had a significantly greater ($P < 0.05$) effect than either one alone; the combined

Fig. 5. Effects of insulin-like growth factor I (IGF-I) and LH on progesterone production by chicken F3 granulosa cells cultured for 48 h with combinations of IGF-I (0–100 ng ml⁻¹) and LH (20 ng ml⁻¹) in serum-free medium. Granulosa cells from F3 follicles were established, washed and then cultured for a further 48 h in serum-free medium with IGF-I only (□) or with IGF-I and LH (▨) added to the culture simultaneously. Data are means $\pm$ SEM of accumulated progesterone during the 48 h of culture ($n = 4$). *Significantly different cultures with IGF-I only present ($P < 0.05$).

Fig. 6. Dose-dependent inhibitory effect of transforming growth factor α (TGF- α) on progesterone production by chicken F3 granulosa cells cultured for 48 h in serum-free medium. Granulosa cultures were established as previously described, washed twice and then cultured for a further 48 h in serum-free Medium 199 containing different concentrations of TGF- α (0–100 ng ml⁻¹). Data are means $\pm$ SEM of progesterone accumulated in the medium during the 48 h culture period. *Significantly different from the control ($P < 0.05$).

effects were additive in F2 and F3 but summated in F1. TGF- α inhibited basal progesterone production in the F2 and F3 follicles, and the effect of either LH or IGF-I alone or in combination in all three follicle categories. The inhibitory effect of 0.078 ng TGF- α ml⁻¹ on the combined effect of LH and IGF-I appeared to be greatest in the F3 group, with a reduction of about $50 \pm 8.20\%$; whereas in F1 group, this dose

Fig. 7. Effects of insulin-like growth factor I (IGF-I) (3.13 ng ml⁻¹), LH (20 ng ml⁻¹) and transforming growth factor α (TGF- α) (0.078 ng ml⁻¹) on progesterone accumulation in the culture medium by chicken (a) F1, (b) F2 and (c) F3 granulosa cells cultured for 48 h in serum-free medium. Cultures were established, washed twice in HBSS, and then cultured for a further 48 h in serum-free Medium 199 containing the growth factors or LH alone, or in combination, added simultaneously. Data are means $\pm$ SEM of progesterone accumulated during the 48 h culture period. Different letters above columns indicate significant differences ($P < 0.05$) between treatments within each follicle category. ** $P < 0.01$ synergistic effect between IGF-I and LH.

caused only a $20 \pm 2.25\%$ reduction in LH/IGF-I-stimulated progesterone production.

Discussion

The effects of LH on avian granulosa cells are well documented (Johnson, 1990), although the effects of LH or FSH on the proliferation of granulosa cells are less well researched (Yoshimura and Tamura, 1988; Roberts *et al.*, 1994). However, the differences in sensitivity of the follicles to these hormones during maturation suggest that additional intra-ovarian modulatory systems exist. Current opinion, from mammalian work, suggests that putative intra-ovarian regulators, including the IGFs and EGF-like peptide family, are engaged in subtle modulation of the growth and function of ovarian cells. We reported the effects of EGF on the proliferation of and progesterone production from cultured granulosa cells of chickens (Peddie *et al.*, 1994). However, in contrast to mammals, the effects of the IGFs in birds remain to be clarified, although mRNA encoding IGF-I is expressed in the granulosa and theca of chicken ovaries (Roberts *et al.*, 1994). Roberts *et al.* (1994) also showed that IGF-I stimulated [^3H]thymidine incorporation into both the granulosa and thecal cells in culture but no data on the effects on progesterone production were given. We recently reported that IGF-I stimulated progesterone production by cultured granulosa cells, but at that time the influence of proliferation on net progesterone production was not incorporated (Williams *et al.*, 1994). In contrast, the results of the present study show that while IGF-I stimulates both proliferation and progesterone production by cultured avian granulosa cells, these effects are independent of each other, and that although the ED_{50} value of IGF-I did not alter during follicular maturation, the total amount of progesterone secreted per cell increased markedly as the follicle matured. However, the effects of IGF-I on proliferation were greatest in the F3 cells, with no changes in ED_{50} value and maximum response. It has been suggested that IGF-I-enhanced progesterone production from the granulosa and theca of mammals is due to stimulation of P450 scc and the 3β -hydroxysteroid dehydrogenase expression (Adashi *et al.*, 1986; Veldhuis *et al.*, 1986; Magoffin and Weistman, 1993). Since the activities of these enzymes also increase during follicular maturation in chicken cells (Davidson *et al.*, 1979; Armstrong, 1982), IGF-I may exert its effect on progesterone production via these enzymes. This is consistent with the lack of effect on ED_{50} value but greater maximal progesterone production with IGF-I. However, the interactions between IGF-I and LH which further enhanced progesterone production may be more complex since in rats gonadotrophins were found to enhance IGF-I binding to granulosa cells and IGF-I can stimulate an increase in the expression of LH/FSH receptors (Adashi *et al.*, 1986).

It has been suggested that EGF-like peptides may have a role in the avian ovary (Pulley and Marrone, 1986; Yoshimura and Tamura, 1988). EGF inhibits progesterone production by chicken granulosa cells (Pulley and Marrone, 1986; Peddie *et al.*, 1994), while stimulating cell proliferation (Yoshimura and Tamura, 1988; Peddie *et al.*, 1994). Here, we have demonstrated that TGF- α , a peptide with considerable similarity to EGF, also inhibits basal and LH-stimulated

progesterone production by cultured chicken granulosa cells. Tilly and Johnson (1990) reported an inhibition of progesterone production by freshly dispersed granulosa cells; however, they did not compare changes in responsiveness during follicular maturation and they did not examine effects on proliferation. Our data also show that TGF- α can inhibit IGF-I-stimulated progesterone production and that the sensitivity of the cultured cells to TGF- α greatly exceeds that to IGF-I. TGF- α also stimulates proliferation of granulosa cells with an additive effect when in combination with IGF-I. Thus TGF- α and EGF have similar actions in granulosa cells, although the ED_{50} value for TGF- α (0.20 ng ml^{-1}) is 100 times lower than that of EGF (20 ng ml^{-1}) (Peddie *et al.*, 1994). This difference probably reflects the difference in binding affinity of TGF- α ($K_d = 0.9 \times 10^{-9} \text{ mol l}^{-1}$) and EGF ($K_d = 2.6 \times 10^{-7} \text{ mol l}^{-1}$) for the avian EGF-receptor (Lax *et al.*, 1989).

The study reported here clearly demonstrates that any one growth factor acting alone is unlikely to be responsible for regulating any one granulosa cell function. We have previously shown that the sensitivity of the granulosa to EGF diminishes as the follicle matures, thus allowing for increased production of progesterone as the follicle matures (Peddie *et al.*, 1994). The study reported here shows that increasing efficacy of IGF-I during follicular maturation also leads to increased progesterone production. It would seem, therefore, that a balance between these two growth factors may regulate responses to LH and account for the increasing responsiveness of the cells to LH before the LH surge. However, the two growth factors also interact with LH to stimulate cell proliferation. Thus, successive LH surges before ovulation contribute to the growth and maturation of the smaller follicles. Hence, during follicular maturation, increasing numbers of granulosa cells and their increasing sensitivity to LH account for the increased production of progesterone from the preovulatory follicles. It remains to be determined whether thecal production of TGF- α , or synthesis of IGF-I and its binding proteins, are also modulated during follicular maturation, but alterations in intra-ovarian production or activity of these growth factors are additional factors which will contribute to the regulation of ovarian progesterone production, by granulosa cells.

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