Modulation of the effects of FSH, androstenedione, epidermal growth factor (EGF) and insulin-like growth factor I (IGF-I) on bovine granulosa cells by GCIF, a growth-inhibitory factor of low molecular mass from bovine follicular fluid

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A granulosa cell-inhibitory factor (GCIF) of low molecular mass from bovine follicular fluid inhibits the proliferation of bovine granulosa cells in vitro and the growth of large follicles in rats in vivo. The present study examined the ability of GCIF to modulate the effects of FSH, epidermal growth factor (EGF), insulin-like growth factor I (IGF-I) and androstenedione on the proliferation of bovine granulosa cells and on aromatase activity in vitro. Granulosa cell proliferation was assayed by counting haemocytometric cells and by measuring the incorporation of \[^{3}H\]thymidine into acid-precipitable material. Assay of aromatase activity was based on the conversion of \[^{3}H\]androstenedione to \[^{3}H\]H2O. FSH, androstenedione, EGF and IGF-I all stimulated (P < 0.01) granulosa cell proliferation; however, the addition of GCIF reduced (P < 0.01) cell proliferation in their presence. In the case of EGF, the addition of GCIF almost abolished the stimulatory response. FSH and IGF-I, but not EGF, stimulated (P < 0.01) aromatase activity of granulosa cells. The stimulatory effect of IGF-I was decreased by GCIF. The inhibitory effects of GCIF indicate that it may play a significant role in regulating the effects of intraovarian growth factors on granulosa cells and the growth of follicles.

Introduction

Replication of granulosa cells is dependent on appropriate gonadotrophic and steroid hormone stimuli. FSH and oestradiol are known to cause a synergistic enhancement of granulosa cell replication and aromatase activity in vivo and in vitro (Hammond et al., 1982; Hseuh, 1986). However, the actions of these hormones are now thought to be modified by locally produced factors in the ovary. As the significance of these putative intraovarian regulators becomes increasingly recognised, much attention is being focused on the role of the peptide growth factors, epidermal growth factor (EGF) and insulin-like growth factors (IGFs) (Adashi et al., 1985). These growth factors are among the most potent stimulators of granulosa cell growth in vitro (Gospodarowicz and Bialecki, 1979). In vivo granulosa cells are known to have receptors for both EGF (Brigstock et al., 1989) and IGF-I (Kotsuji et al., 1990), although the precise role of these growth factors in vivo is, as yet, not fully understood (Drioncourt, 1991). The ability of granulosa cells of individual follicles to respond to these hormones in vivo could play a significant role in determining the fate of these follicles. Therefore any factors that modulate the effects of these hormones on granulosa cells could play a role in the selection of a dominant follicle.

Hynes et al. (1996) reported the partial purification of a granulosa cell inhibitory factor (GCIF) of low molecular mass from bovine follicular fluid that inhibited granulosa cell proliferation in vitro. In this paper we report on the ability of GCIF to modulate the effects of FSH, androstenedione, EGF and IGF-I on bovine granulosa cells in vitro.

Materials and Methods

Culture of granulosa cells

Granulosa cells were harvested from follicles of diameter 2–10 mm (medium follicles) and cultured as described by Hynes et al. (1996). After an initial preculture period of 24 h, spent culture medium was removed from all wells and was replaced by fresh culture medium (2 ml per well) containing a reduced fetal calf serum (FCS) concentration (1%). Growth factors being investigated were then added randomly at different concentrations to all wells. In all cases in which growth factors were added to culture wells, equivalent amounts of the vehicle used to dissolve them were added to the control wells. The granulosa cells were then cultured for a further 24 h, and then they were harvested and cell proliferation was monitored either by counting haemocytometric cells or by measuring the incorporation of \[^{3}H\]thymidine (Amersham

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International plc, Amersham, Bucks) into acid-precipitable material (Hynes et al., 1996).

**Experiment 1: investigation of the ability of GCIF to modulate the effects of FSH and androstenedione on granulosa cell proliferation in vitro**

This factorial (3 × 3 × 2) experiment involved three concentrations of GCIF (0, 100 and 1000 ng peptide equivalent material ml⁻¹), three concentrations of FSH (0, 30 and 60 ng ml⁻¹) and two concentrations of androstenedione (0 and 30 ng ml⁻¹). The GCIF material was a low molecular mass fraction of bovine follicular fluid partially purified by gel filtration chromatography as described by Hynes et al. (1996) and the amount of peptide equivalent material was determined by absorbance measurement; at 260 and 280 nm for concentrations > 1 mg ml⁻¹ (Johnstone and Thorpe, 1982) and at 205 nm for concentrations < 1 mg ml⁻¹ (Scopes, 1974). The FSH used (a gift from S. Leibo, San Antonio, Texas) contained 1.9 Armour FSH units mg⁻¹ and had LH activity removed by column chromatography (Donaldson and Ward, 1985). The androstenedione was obtained from Steraloids (Croydon, Surrey).

**Experiment 2: investigation of the ability of GCIF to modulate the effects of EGF and IGF-I on granulosa cell proliferation in vitro**

The ability of GCIF (0, 10, 100 and 1000 ng peptide equivalent material ml⁻¹) to inhibit cell proliferation stimulated by EGF (10 ng ml⁻¹) or IGF-I (10 ng ml⁻¹) was examined. Control treatments without GCIF, EGF or IGF-I were also used. The concentrations of EGF and IGF-I chosen were those shown to have the greatest effect on granulosa cell proliferation in preliminary experiments. The IGF-I was obtained from Gibco Geigy (CH-1556 St Aubin) and the EGF from Sigma (Poole).

**Experiment 3: investigation of the ability of GCIF to modulate the effects of FSH, EGF and IGF-I on granulosa cell aromatase activity in vitro**

The effects of FSH and IGF-I were tested in a 2 × 2 × 2 factorial experiment with two concentrations of FSH (0, 50 ng ml⁻¹), two concentrations of IGF-I (0, 50 ng ml⁻¹) and two concentrations of GCIF (0, 10 ng ml⁻¹). The effect of EGF was also tested in a separate 4 × 2 factorial experiment with four concentrations of EGF (0, 0.5, 5 and 50 ng ml⁻¹) and two concentrations of GCIF (0, 10 ng ml⁻¹). Cell aromatase activity was quantified by the conversion of [³H]androstenedione to [³H]estradiol (Gore-Langton and Dorrington, 1981; Garzo and Dorrington, 1984). Granulosa cells were initially cultured for 3 days in an androstenedione-free McCoy's 5A medium supplemented with 5% FCS, and 1% of each of the following solutions, 50 000 iu penicillin ml⁻¹, 50 mg streptomycin ml⁻¹, 0.25 mg fungizone ml⁻¹, 29.2 mg l-glutamine ml⁻¹ and a 50 × MEM non-essential amino acids solution without glutamine (all from GibcoBRL, Life Technologies, Paisley) with and without FSH and with and without IGF-I. After culture for 72 h, spent medium was discarded and the cells washed twice with sterile 0.9% (w/v) saline. The cells were then re-incubated for an additional 8 h in the presence and absence of GCIF and 2 μCi [³H]estradiol (Amersham International plc). The culture medium from each well was then extracted with 5 volumes of chloroform for 10 min. The aqueous phase was removed and treated with an equal volume of solution of dextran-activated charcoal (dextran 0.2%, charcoal 2.5%) for 10 min at 4°C. After centrifugation for 30 min at 800 g, 0.5 ml samples were taken from each supernatant and added to 5 ml scintillation cocktail and counted in a scintillation counter for 5 min.

### Statistical analyses

Data from all experiments were analysed by analysis of variance (Steel and Torrie, 1960) followed, where appropriate, by a two-tailed Dunnett’s test (Steel and Torrie, 1960) to compare each treatment with controls. Where the treatment × week (that is, blocks) interaction term was significant, a revised analysis of variance table was constructed, in which a pooled treatment × week interaction term was used as the corrected error term to test for treatment effects.

### Results

**Experiment 1: modulation by GCIF of the effects of FSH and androstenedione on granulosa cell proliferation in vitro**

Both FSH and androstenedione stimulated (P < 0.01) bovine granulosa cell proliferation in vitro (Fig. 1). There was an interaction (P < 0.01) between FSH and androstenedione, as can be seen from the synergistic action of 60 ng FSH ml⁻¹ and 30 ng androstenedione ml⁻¹ on granulosa cell proliferation. The addition of GCIF to granulosa cell cultures inhibited (P < 0.01) granulosa cell proliferation both in the presence and...
IGF-I and androstenedione absence

Experiment GCIF

Significant

Values measured factor.

cases. There was a significant stimulatory effect ($P < 0.01$) of FSH and inhibitory effect ($P < 0.01$) of GCIF on cell proliferation.

addition, respectively.

Values are means ± SEM based on 18 replicates. There was a significant stimulatory effect ($P < 0.01$) of androstenedione and inhibitory effect ($P < 0.01$) of GCIF on cell proliferation. There was also a significant interaction of androstenedione and GCIF ($P < 0.01$).

absence of FSH (Fig. 2) or androstenedione (Fig. 3). However, GCIF did not totally abolish the stimulatory effects of FSH or androstenedione on granulosa cell proliferation (Figs 2 and 3).

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Experiment 2: modulation by GCIF of the effects of EGF and IGF-I on granulosa cell proliferation in vitro

Addition of GCIF at all concentrations tested (10, 100, and 1000 ng ml$^{-1}$) inhibited ($P < 0.01$) the stimulatory effect of 10 ng EGF ml$^{-1}$ and, at a concentration of 10 ng ml$^{-1}$, reduced cell proliferation as measured by cell counting below that seen in the 0 control treatment without GCIF and EGF (Fig. 4). GCIF also inhibited ($P < 0.01$) the stimulatory effect of 10 ng IGF-I ml$^{-1}$ on granulosa cell proliferation but did not totally abolish the stimulatory effect of IGF-I at any of the concentrations tested (Fig. 5).

Experiment 3: modulation by GCIF of the effects of FSH, EGF and IGF-I on granulosa cell aromatase activity in vitro

EGF did not affect aromatase activity in these experiments (data not shown) but FSH and IGF-I separately, and in combination, markedly increased ($P < 0.01$) activity (Fig. 6). There was a significant interaction ($P < 0.01$) between FSH and IGF-I which was due to the failure of their stimulatory effects to be additive.

The stimulatory effect of IGF-I was markedly decreased ($P < 0.01$) by GCIF but it is clear (Fig. 6) that GCIF only marginally reduced the stimulatory effect of FSH. The significant ($P < 0.01$) interaction between GCIF and IGF-I is due to the fact that in these experiments GCIF only marginally inhibited aromatase activity in cells without IGF-I but markedly inhibited the aromatase activity stimulated by IGF-I (Fig. 6).

Discussion

We have previously reported (Hynes et al., 1996) that the follicular fluid from bovine follicles contains a non-steroidal granulosa cell inhibitory factor, GCIF, of low molecular mass, that inhibits the proliferation in vitro of granulosa cells collected from small and medium sized bovine follicles. In the study

Fig. 2. Effect of different concentrations of granulosa cell-inhibitory factor (GCIF) on the proliferation of bovine granulosa cells in the presence of different concentrations of FSH (□, 0; ■, 30 and ■, 60 ng ml$^{-1}$) as measured by $[^{3}H]$thymidine incorporation into acid-precipitable material. Values are means ± SEM based on 12 replicates. There was a significant stimulatory effect ($P < 0.01$) of FSH and inhibitory effect ($P < 0.01$) of GCIF on cell proliferation.

Fig. 3. Effect of different concentrations of granulosa cell-inhibitory factor (GCIF) on the proliferation of bovine granulosa cells in the absence (□) and presence of androstenedione (■, 30 ng ml$^{-1}$) as measured by $[^{3}H]$thymidine incorporation into acid-precipitable material. Values are means ± SEM based on 18 replicates. There was a significant stimulatory effect ($P < 0.01$) of androstenedione and inhibitory effect ($P < 0.01$) of GCIF on cell proliferation. There was also a significant interaction of androstenedione and GCIF ($P < 0.01$).

Fig. 4. Effect of different concentrations of granulosa cell-inhibitory factor (GCIF) in the presence and absence of epidermal growth factor (EGF) on the proliferation of bovine granulosa cells as measured by haemocytometric counting (□) and $[^{3}H]$thymidine incorporation into acid-precipitable material (■). Values are means ± SEM based on six replicates for both haemocytometric counts and $[^{3}H]$thymidine incorporation. *Significantly different ($P < 0.01$) from treatment with no GCIF and 10 ng EGF ml$^{-1}$.

Fig. 5. EGF (10 ng ml$^{-1}$)
reported here, the ability of GCIF to modify the effects of FSH, androstenedione, IGF-I and EGF on granulosa cell proliferation and aromatase activity was investigated.

The results of the experiments presented show that GCIF significantly inhibited proliferation of granulosa cells even in the presence of stimulation by FSH, androstenedione, EGF and IGF-I. GCIF also inhibited the stimulatory effect of IGF-I on aromatase activity of granulosa cells but had little effect on the stimulatory effect of FSH on aromatase activity. Overall, the results indicate that GCIF activity may play a major role in regulating granulosa cell function.

Even in the presence of GCIF there was always some stimulatory effect of FSH, androstenedione, and IGF-I on proliferation of granulosa cells. In contrast, GCIF (10 ng ml⁻¹) reduced EGF-stimulated cell proliferation as measured by cell counting below that seen in the control without GCIF and EGF. However, there was some evidence for residual EGF stimulation in that the reduction in cell proliferation due to GCIF was not as great as if EGF were not present.

During folliculogenesis, granulosa cells undergo repeated cell division and also profound changes in their hormonal responsiveness. Changes in the capacity of granulosa cells to metabolise androgens to oestrogens has been suggested to be a physiologically significant mechanism for determining the maturational fate of individual follicles (Kigawa et al., 1986). Treatment of granulosa cells with FSH, the major stimulator of follicular maturation, induces high concentrations of aromatase enzymes which convert androgens to oestrogens (Jia et al., 1986). Oestrogens in turn increase granulosa cell proliferation (Driancourt, 1991). In the work presented here, treatment with FSH and androstenedione in combination markedly stimulated proliferation of granulosa cells. The significant interaction between FSH and androstenedione may have been due to the stimulation of oestadiol formation from androstenedione by FSH and a consequent increase in oestrogen-stimulated cell division. It is, however, unlikely that the major effect of GCIF in inhibiting the FSH-stimulation of proliferation of granulosa cells is mediated via the inhibition of aromatase activity and oestrogen synthesis since GCIF had only a very slight effect on the FSH-stimulated increase in aromatase activity.

In addition to the central role of gonadotrophins such as FSH, in proliferation and differentiation of granulosa cells, other non-steroidal growth factors are known to be important (reviewed by Tonetta and DiZerega, 1989). EGF is one of the most potent stimulators of granulosa cell proliferation (May et al., 1988). Recent data has suggested that EGF is present in follicular fluid and that it is secreted locally by follicular cells (Westergaard and Yding-Andersen, 1989). The data reported here show that the stimulatory effect of EGF on replication of granulosa cells was dose-dependent with maximum stimulation occurring at a concentration of 10 ng ml⁻¹ and this stimulation was almost abolished by 10 ng GCIF ml⁻¹. Since EGF did not stimulate increased aromatase activity, the inhibitory effect of GCIF on EGF-stimulated proliferation of granulosa cells cannot be mediated by a change in aromatase activity.

In the first stage of follicle development, proliferation of granulosa cells is the predominant activity while in the second stage differentiation and aromatase activity are predominant features (Gougeon, 1982; Garzo and Dorrington, 1984). The interaction between IGF-I and EGF may be involved in defining these stages. Since EGF is known to stimulate the proliferation of granulosa cells (May et al., 1988) but not aromatase activity (Hseuh et al., 1981), GCIF could thus be involved in regulating the activity of EGF during the early follicular stage by preventing the stimulation of granulosa cell proliferation by EGF and hence the subsequent development of the follicle. This hypothesis is in agreement with our previous results showing

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Fig. 5. Effect of different concentrations of granulosa cell-inhibitory factor (GCIF) on the proliferation of bovine granulosa cells in the absence and presence of insulin-like growth factor I (IGF-I) as measured by [³H]thymidine incorporation into acid-precipitable material. Values are means ± SEM based on six replicates. *Significantly different (P < 0.05) from the treatment with no GCIF and 10 ng IGF-I ml⁻¹.

Fig. 6. Effect of granulosa cell-inhibitory factor (GCIF) (□, no GCIF; ■, 100 ng GCIF ml⁻¹) in modulating the effects of FSH (50 ng ml⁻¹), insulin-like growth factor I (IGF-I) (50 ng ml⁻¹) and FSH plus IGF-I on aromatase activity of bovine granulosa cells. Values are means ± SEM based on eight replicates. There were significant stimulatory effects of FSH and IGF-I (P < 0.01) and an inhibitory effect of GCIF (P < 0.01) on aromatase activity. There were significant interactions between GCIF and IGF-I, and FSH and IGF-I (P < 0.01).

that administration of GCIF to female rats inhibited the formation of large follicles (Hynes et al., 1996).

Although GCIF was also found to inhibit the activity of IGF-I, it did not, at any concentration, reduce the proliferation of granulosa cells in the presence of IGF-I to basal unstimulated values. Its inhibitory activity in the presence of IGF-I was of a similar percentage magnitude to that exerted by it in the absence of IGF-I. This finding suggests that GCIF is inhibiting normal granulosa cell growth by a mechanism that does not involve direct interaction with IGF-I.

Further investigation of the site and mode of action of GCIF must await its purification and structural identification.

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