Differential regulation of pig theca cell steroidogenesis by LH, insulin-like growth factor I and granulosa cells in serum-free culture

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The regulation of pig theca cell steroidogenesis was studied by the development of a physiological serum-free culture system, which was subsequently extended to investigate potential theca–granulosa cell interactions. Theca cells were isolated from antral follicles 6–9 mm in diameter and the effects of plating density ($50\text{--}150 \times 10^3$ viable cells per well), LH ($0.01\text{--}1.0 \text{ ng ml}^{-1}$), Long R3 insulin-like growth factor I (IGF-I) ($10, 100 \text{ ng ml}^{-1}$) and insulin ($1, 10 \text{ ng ml}^{-1}$) on the number of cells and steroidogenesis were examined. The purity of the theca cell preparation was verified biochemically and histologically. Co-cultures contained 50×10^3 viable cells per well in granulosa to theca cell ratio of 4:1. Wells containing granulosa cells only were supplemented with 'physiological' doses of androstenedione or 100 ng ml^{-1} . Oestradiol production by co-cultures was compared with the sum of the oestradiol synthesized by granulosa and theca cells cultured separately. Oestradiol and androstenedione production continued throughout culture. High plating density decreased steroid production ($P < 0.01$). LH increased androstenedione ($P < 0.001$) and oestradiol ($P < 0.05$) synthesis and the sensitivity of the cells increased with time in culture. Oestradiol production was increased by $10 \text{ ng IGF-I ml}^{-1}$ ($P < 0.001$) but androstenedione required 100 ng ml^{-1} ($P < 0.001$). Co-cultures produced more oestradiol than the sum of oestradiol synthesized by theca and granulosa cells cultured separately ($P < 0.001$), irrespective of the androstenedione dose. This serum-free culture system for pig theca cells maintained *in vivo* steroidogenesis and gonadotrophin responsiveness. Thecal androstenedione and oestradiol production were differentially regulated and were primarily stimulated by LH and IGF-I, respectively. Theca–granulosa cell interactions stimulated oestradiol synthesis and this interaction was mediated by factors additional to the provision of thecal androgen substrate to granulosa cells.

Introduction

Although many studies have examined granulosa cell steroidogenesis and its regulation, few have considered the contribution made by theca cells. *In vitro* androgen production by theca cells has been investigated in several species (pigs: May *et al.*, 1992; Barbieri, 1994; cows: Demeter-Arlootto *et al.*, 1993; sheep: Campbell *et al.*, 1998). However, pig theca cells are of particular interest because in addition to androgen production, they are also capable of independent oestradiol synthesis (Evans *et al.*, 1981; Tsang *et al.*, 1985; Hunter *et al.*, 1994; Miller *et al.*, 1998) since they possess an active cytochrome P450 aromatase (P450_{arom}) (Lautinik *et al.*, 1994; Meduri *et al.*, 1996; Garrett and Guthrie, 1996, 1997). Studies in this laboratory demonstrated the presence in pig theca cells of P450_{arom} mRNA by northern blot analysis (Miller *et al.*, 1995) and the active P450_{arom} enzyme by

immunohistochemistry (Shores *et al.*, 1997). Precisely how thecal oestradiol production is regulated in pigs is unclear and progress has been hampered by the lack of a suitable cell culture system. Serum is commonly added to culture systems to provide the complex mixture of nutrients, hormones and growth factors required by the cells. Unfortunately, this means that the exact requirements of a particular type of cell are unknown. In addition, the use of serum can result in spontaneous luteinization of the ovarian cells (Engelhardt *et al.*, 1991; Conley *et al.*, 1994; Picton *et al.*, 1999) characterized by a loss of P450_{arom} activity and increased progesterone synthesis.

Accordingly, a serum-free system for the culture of pig granulosa cells was developed in this laboratory (Picton *et al.*, 1999) based on similar serum-free systems used for ruminants (Campbell *et al.*, 1996; Gutiérrez *et al.*, 1997a). In the pig system, granulosa cell P450_{arom} activity and thus oestradiol synthesis were maintained throughout the culture period. Granulosa cells cultured in this serum-free system remained round and formed clumps, whereas in the

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Received 9 August 1999.

presence of serum they became fibroblastic, forming monolayers on the plate surface. Therefore, morphologically, granulosa cells cultured under serum-free conditions were similar to their *in vivo* counterparts. It would be extremely valuable to develop a defined serum-free culture system for theca cells in which *in vivo* morphology, steroidogenesis and gonadotrophin responsiveness were maintained. This would allow investigation of the regulation of steroidogenesis by theca cells that maintain their follicular phase phenotype in culture.

The development of a defined serum-free culture system for pig theca cells, used in conjunction with the serum-free granulosa cell system, allows investigation of proposed theca-granulosa cell interactions using a co-culture that mimics *in vivo* conditions. An interaction between these types of cell is likely due to their close proximity and the need for the follicle to produce a co-ordinated response to a complex array of signals, such as gonadotrophins and growth factors. In the present study, the effect of theca-granulosa cell co-culture on oestradiol synthesis was investigated. Oestradiol was selected as an end point since it is the production of this hormone that ultimately triggers ovulation and because pig granulosa and theca cells are both capable of independent oestradiol production.

The aims of this study were: (i) to establish a physiologically relevant serum-free culture system for pig theca cells and to use it to study specific factors involved in the regulation of thecal steroidogenesis; and (ii) to establish a co-culture system to test the hypothesis that theca and granulosa cells interact, resulting in increased oestradiol production compared with the two types of cell cultured separately.

Materials and Methods

All chemicals were obtained from Sigma Chemical Company Ltd, Poole, unless otherwise stated.

Cell collection

Morphologically normal pig ovaries from sexually mature animals were obtained from a local abattoir and washed in pre-warmed (37°C) dissection medium (EMEM with 25 mmol HEPES l⁻¹ plus 100 iu penicillin ml⁻¹, 0.1 ng streptomycin ml⁻¹ and 50 µg gentamicin ml⁻¹ (GibcoBRL, Paisley)) with 10 mg amphotericin B ml⁻¹. Large, morphologically healthy (6–9 mm in diameter) follicles were dissected, the follicular fluid aspirated and the follicles were cut into four pieces in Dulbecco's PBS (DPBS) without calcium or magnesium. Granulosa cells were removed by gently scraping the inside of the follicle shells with a sterile plastic inoculation loop under a dissecting microscope followed by agitation through a plastic Pasteur pipette. The follicles were transferred to fresh DPBS and scraped again.

Granulosa cells were collected by centrifugation at 800 g for 10 min and washed twice in warm incubation medium (DMEM/Ham's F12 with 15 mmol HEPES l⁻¹ and NaHCO₃ plus 3 mmol L-glutamine l⁻¹, 100 iu penicillin ml⁻¹, 0.1 ng

streptomycin ml⁻¹, 2.5 µg transferrin ml⁻¹, 4 ng sodium selenite ml⁻¹ and 0.1% (w/v) BSA (fraction V cell culture grade)). The number of viable granulosa cells before the start of culture was assessed using trypan blue exclusion and was $54 \pm 5\%$.

After removal of granulosa cells, the theca layer was peeled away from the follicle shell using fine forceps under a dissecting microscope. Theca sheets from ≤ 20 follicles were dispersed by incubation in a 10 ml solution of 0.5% (w/v) collagenase type II and 0.1% (w/v) hyaluronidase type I-S in Hank's balanced salt solution with 20 mmol HEPES l⁻¹ and 5% fetal calf serum for a maximum of 30 min at 37°C in a shaking waterbath (Hunter and Armstrong, 1987; Ranson *et al.*, 1997). The dispersed cells were washed twice in warm incubation medium. The number of viable theca cells before the start of culture was assessed using trypan blue exclusion and was $91 \pm 7\%$.

For all cultures, the number of viable cells after 144 h was assessed by uptake of neutral red dye as described by Campbell *et al.* (1996) and Picton *et al.* (1999).

Assessment of the purity of the theca cell preparation

This procedure was based on the method of Kataoka *et al.* (1994) to confirm the purity of pig theca cell preparations. Both theca and granulosa cells were plated at 100×10^3 viable cells per well per 250 µl in 96-well plates (Nunc, Life Technologies Ltd). All wells contained incubation medium with 10 ng bovine insulin ml⁻¹, 100 ng Long R3 IGF-I ml⁻¹ (media grade synthetic IGF-I analogue Gropep Pty Ltd, Adelaide), 100 ng testosterone ml⁻¹ and 10% fetal calf serum. The amount of fetal calf serum was reduced to 2.5% from 48–144 h. FSH (USDA-pFSH-I-2; LH contamination < 1%) was added at 10 and 100 ng ml⁻¹. The medium was changed every 48 h for 144 h and the spent medium frozen for subsequent radioimmunoassay for progesterone.

Removal of granulosa cells was confirmed by histological examination of scraped follicle shells (6–9 mm follicles, $n = 35$). These were fixed in 4% (w/v) paraformaldehyde solution and embedded in paraffin wax. Sections were cut at 5 µm and stained by a standard haematoxylin and eosin protocol. Three random sections from each follicle were examined.

Theca cell culture

Theca cells were plated in serum-free medium at $50\text{--}150 \times 10^3$ viable cells per well per 250 µl with a range of LH (0.01–1.0 ng ml⁻¹; FSH contamination < 1%), insulin (1, 10 ng ml⁻¹) and Long R3 IGF-I (10, 100 ng ml⁻¹, subsequently referred to as IGF-I) doses. Each culture was carried out three times with four replicates per treatment in each culture. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air for 144 h; the medium was exchanged every 48 h. Only 175 µl of medium per well was exchanged slowly to reduce the disturbance of the cells. Spent medium was frozen at –20°C until assayed for progesterone, androstenedione and oestradiol.

Co-culture

A preliminary experiment was conducted to establish a suitable plating density for the co-cultures. Granulosa and theca cells were co-cultured in serum-free medium in a ratio of 4:1 at a total cell concentration of either 50×10^3 viable cells per well, that is 40×10^3 granulosa cells plus 10×10^3 theca cells, or 150×10^3 viable cells per well, that is 120×10^3 granulosa cells plus 30×10^3 theca cells. Control wells contained either 50 or 150×10^3 granulosa cells or 50 or 150×10^3 theca cells. All wells contained pre-warmed incubation medium plus 100 ng IGF-I ml⁻¹, 10 ng insulin ml⁻¹, 0.01 ng LH ml⁻¹ (USDA-pLH-B2) and 1 ng FSH ml⁻¹ (USDA-pFSH-I-2). Wells containing granulosa cells only were supplemented with 100 ng androstenedione ml⁻¹. Oestradiol production at 48 h only was measured.

After the preliminary experiment, co-cultures were established with a total cell concentration of 50×10^3 viable cells per well (40×10^3 granulosa cells plus 10×10^3 theca cells). Control wells contained 50×10^3 granulosa or 50×10^3 theca cells. Wells containing granulosa cells only received either a 'physiological' dose or 100 ng androstenedione ml⁻¹. The physiological dose was the estimated amount of androstenedione produced by 10×10^3 theca cells based on measurements taken during theca cell cultures. During the first 48 h, the dose was 5 ng per well, from 48–96 h 2 ng per well and from 96–144 h 1 ng per well. Wells containing theca cells only and co-culture wells received either no androstenedione or 100 ng ml⁻¹ was administered at all time points. Spent medium was collected every 48 h for 144 h and stored at -20°C before assay for oestradiol.

Oestradiol production by co-cultures was compared with the sum of oestradiol produced by granulosa and theca cells cultured alone. The sum of oestradiol production was calculated by the addition of 20% of the measured oestradiol from the theca cell control wells (20% is equivalent to 10×10^3 theca cells) to 80% of the measured oestradiol from granulosa cell control wells (80% is equivalent to 40×10^3 granulosa cells).

Assays

The concentrations of oestradiol, progesterone and androstenedione were measured in culture medium by radioimmunoassay as described by Grant *et al.* (1989) and Thomson *et al.* (1989). The limits of sensitivity and inter- and intra-assay coefficients of variation were, respectively, 1.1 pg per tube, 14.6% and 6.6% for oestradiol; 15.5 pg per tube, 14.2% and 9.5% for progesterone; and 1.6 pg per tube, 10.1% and 5.9% for androstenedione.

Statistical analysis

Hormone data from serum-free cultures were corrected for the residual media left in the wells when the media was exchanged at 48 and 96 h, as described by Campbell *et al.* (1996). The values were expressed as production per 1000 viable cells per 48 h. The number of viable cells at 48 h was

considered to be the initial plating density. At 144 h, the number of viable cells was that obtained using the neutral red dye method. The data were $\log(x + 1)$ transformed to remove heterogeneity of variance before ANOVA using the Genstat statistical package. The pooled variance was used to calculate the standard error of the difference (SED) between two means. Replicate cultures were treated as blocks and treatment effects were considered significant at $P < 0.05$. When indicated by ANOVA, individual treatment means were compared using Bonferroni's test.

Results

Assessment of the purity of the theca cell preparation

In the preliminary experiments, progesterone production during the final 48 h of culture was measured. FSH increased granulosa cell progesterone production in a dose-dependent manner. FSH doses of 0, 10 and 100 ng ml⁻¹ resulted in granulosa and theca cell log progesterone production of 1.47, 1.73 and 1.83 (SED 0.05, $P < 0.001$) and 1.84, 1.91 and 1.85 (SED 0.06) ng per 1000 viable cells, respectively. Theca cell progesterone output was therefore unaffected by FSH. Further confirmation of removal of granulosa cells was demonstrated by the histology of the scraped follicle shells (Fig. 1a,b).

Theca cell cultures

In the main experiments, dispersed theca cells rapidly formed clumps of rounded cells in culture (Fig. 1c) similar to those observed in serum-free granulosa cell cultures. Steroidogenesis continued throughout the culture period. Initial plating density had a profound effect on theca cell steroidogenesis (Table 1). Oestradiol output was influenced by plating density at 48 and 144 h ($P < 0.001$ at both time points), as was androstenedione synthesis ($P < 0.01$ at both time points). At 48 h, oestradiol production per 1000 viable cells was highest at a density of 50×10^3 viable cells per well. This density resulted in 33% more oestradiol than 75 and 100×10^3 viable cells and 80% more oestradiol than 150×10^3 viable cells ($P < 0.001$). After 144 h, 150×10^3 viable cells per well resulted in 50% lower oestradiol synthesis per cell than densities of 50, 75 and 100×10^3 viable cells ($P < 0.001$). Again, 50×10^3 viable cells per well produced the most oestradiol per 1000 cells.

At 48 h, androstenedione production was 2–4.5-fold higher at 50×10^3 viable cells than at other plating densities ($P < 0.001$). Densities of 75 and 100×10^3 viable cells were similar and both were at least twofold higher than 150×10^3 viable cells. Androstenedione production was 2.4 times higher at 75 than at 50 and 150×10^3 viable cells after 144 h in culture ($P < 0.01$). All other plating densities were similar.

At 48 h, oestradiol production was not altered by LH dose (Fig. 2a). After 144 h in culture, all LH doses increased oestradiol production by an average twofold above control values ($P < 0.05$; Fig. 2b).

Androstenedione production was stimulated by

Table 1. Effect of plating density ($50\text{--}150 \times 10^3$ viable cells per well) on pig theca cell steroidogenesis after 48 and 144 h in defined serum-free culture

Initial plating density ($\times 10^3$ viable cells)	Log oestradiol production (pg per 10^3 viable cells per 48 h)		Log androstenedione production (pg per 10^3 viable cells per 48 h)	
	48 h	144 h	48 h	144 h
50	1.61 ^a	1.54 ^a	2.48 ^a	2.69 ^a
75	1.52 ^b	1.48 ^a	2.19 ^b	3.11 ^b
100	1.48 ^b	1.51 ^a	2.11 ^b	2.76 ^{ab}
150	1.34 ^c	1.34 ^b	1.82 ^c	2.70 ^a
SED	0.03	0.04	0.05	0.14

Data were log transformed before ANOVA and values represent the mean of three separate cultures each containing four wells per treatment. Values in the same column with different superscripts are significantly different at $P < 0.05$.

increasing LH doses at all time points ($P < 0.001$; Fig. 2c,d). At 48 h, $0.5 \text{ ng LH ml}^{-1}$ was required to stimulate androstenedione production above control values; however, by 144 h the LH dose required to achieve an increase had decreased to 0.05 ng ml^{-1} . The magnitude of this increase also changed from 1.7-fold at 48 h to over fourfold at 144 h.

LH doses of 0.05 ng ml^{-1} or more increased progesterone production at 48 h ($P < 0.001$; Fig. 2e). LH at 1 ng ml^{-1} increased progesterone by more than 3.5-fold over control values. After 144 h in culture, LH dose no longer affected progesterone production (Fig. 2f).

IGF-I increased oestradiol and androstenedione production at 144 h ($P < 0.001$ for both hormones). Oestradiol output was increased by an IGF-I dose of 10 ng ml^{-1} ($P < 0.001$; Fig. 3a) but 100 ng ml^{-1} IGF-I was required to raise androstenedione above control values ($P < 0.001$; Fig. 3b).

Insulin did not influence oestradiol output (Fig. 3a) or androstenedione production (Fig. 3b). However, there was an interaction between IGF-I and insulin in terms of androstenedione production at 144 h ($P < 0.05$). The combination of $10 \text{ ng insulin ml}^{-1}$ with $100 \text{ ng IGF-I ml}^{-1}$ resulted in 12-fold more androstenedione than $10 \text{ ng insulin ml}^{-1}$ with $10 \text{ ng IGF-I ml}^{-1}$ ($P < 0.05$).

Co-cultures

The preliminary experiment revealed that oestradiol production by co-cultures was also influenced by initial plating density. The cells in low density co-cultures (total of 50×10^3 viable cells per well) produced almost twice as much oestradiol per cell compared with those in high density co-cultures (total of 150×10^3 viable cells per well) ($P < 0.001$; Fig. 4). Therefore, a plating density of 50×10^3 viable cells per well (10×10^3 viable theca cells plus 40×10^3 viable granulosa cells) was used for subsequent co-culture experiments.

Mixed co-cultures produced more oestradiol than the sum of separate theca and granulosa cell cultures ($P < 0.001$; Fig. 5). Androstenedione was added to wells containing granulosa cells only at a physiological dose (calculated to be the same as the amount of androstenedione produced by the

theca cells in the mixed wells, that is 10×10^3 viable cells per well) or at 100 ng ml^{-1} (the dose commonly used in previous co-culture studies). The higher androstenedione dose (100 ng ml^{-1}) was also added to mixed co-cultures and wells containing theca cells only to test whether any interaction occurred in the presence of additional androgen substrate. With either dose of androstenedione, co-cultures produced more oestradiol than the sum of separate cultures ($P < 0.001$). Addition of $100 \text{ ng androstenedione ml}^{-1}$ increased overall oestradiol concentrations ($P < 0.001$) but did not alter the occurrence of an interaction. Co-culture wells treated with the physiological dose had threefold higher oestradiol than the sum, whereas co-cultures treated with $100 \text{ ng androstenedione ml}^{-1}$ were 4.4-fold higher than the sum.

Discussion

In the present study, the purity of the theca cell preparation was confirmed biochemically using the response of ovarian cells to FSH. This experiment was conducted before the development of the serum-free culture system and was based on the validation method of Kataoka *et al.* (1994) using medium containing serum. Histological examination of scraped follicle shells confirmed the removal of granulosa cells. Purity is fundamental to ensuring any observed response to culture conditions is purely thecal in origin (Haney and Schomberg, 1981). Despite this, many reports do not mention any attempts to ensure purity in theca cell preparations (Morley *et al.*, 1989; Sirois *et al.*, 1991; Kotsuji and Tominaga, 1994; Armstrong *et al.*, 1996).

Theca sheets were digested to release individual cells for culture, but within 24 h these cells had formed clumps attached loosely to the plate by a flattened 'stalk'. The cells within these clumps were rounded, resembling the *in vivo* condition, and remained so throughout the culture period. Morphology may be a good indicator of function. An ultrastructural study of bovine granulosa cells (Gutiérrez *et al.*, 1997b) revealed that the round cells present in serum-free medium retained organelles characteristic of granulosa cells *in vivo* and continued to secrete oestradiol. Granulosa cells cultured with serum became flattened and ceased oestradiol production. This finding indicates that the theca cells within

Fig. 1. Large (6–9 mm in diameter) healthy pig follicles before (a) and after (b) the removal of granulosa cells. Sections were stained with haematoxylin and eosin. Comparison of the morphology of theca cells when cultured in serum-free medium (c) and with fetal calf serum (d) for 144 h. Theca cells were collected from large (6–9 mm in diameter) healthy pig follicles. Cells were stained with neutral red dye. Scale bars represent 20 μm.

the clumps would retain P450 17 α -hydroxylase (P450_{17 α}) and P450_{arom} activities, whereas the 'stalk' cells would lose them. Further work is required to confirm this observation.

Initial plating density was very important to steroid production. Precisely how density influences steroidogenesis is unclear, but Campbell *et al.* (1998) suggested that it may have a role in maintaining P450_{17 α} activity. The detrimental effects of high plating density indicate that the production of metabolic waste products may adversely affect steroidogenesis. The optimum density for pig theca cells in the present system is different from that of pig granulosa cells in a similar serum-free system (50 and 150 $\times 10^3$ viable cells

per well, respectively, Picton *et al.*, 1999). This difference may reflect observed differences in the numbers of each type of cell present within the follicle (Stoklosowa *et al.*, 1982; Kokia *et al.*, 1992) and was an important consideration in the development of a physiologically relevant co-culture system.

Theca cells were extremely sensitive to LH and the concentrations of LH used in this study are low compared with those used by other workers in theca cell culture (pigs: 250 ng ml⁻¹, Morley *et al.*, 1989, Engelhardt *et al.*, 1991; 25 ng ml⁻¹, Barbieri, 1994; 20 ng ml⁻¹, Caubo *et al.*, 1989; cows: 100 ng ml⁻¹, Bao *et al.*, 1995). Berndtson *et al.* (1995) suggested that high concentrations of gonadotrophins (32–256 ng ml⁻¹)

Fig. 2. The effect of LH dose on oestradiol (a,b), androstenedione (c,d) and progesterone (e,f) production by pig theca cells after 48 h (a,c,e) and 144 h (b,d,f) in defined serum-free culture. The data were log transformed before ANOVA and are expressed as production per 10^3 viable cells. Values are the mean of three separate cultures each containing four wells per treatment.

caused bovine ovarian cells to luteinize. Similarly, Armstrong *et al.* (1996) reported that LH doses ≥ 10 ng ml $^{-1}$ increased progesterone and decreased androstenedione production by ovine theca cells. Therefore, some earlier reports may be confounded by the high gonadotrophin concentrations causing the cells to luteinize. In the present study, progesterone output was on average 3.6-fold higher at

144 h than at 48 h. Campbell *et al.* (1998) also noted a fourfold increase in progesterone concentrations with time in culture using ovine theca cells and Picton *et al.* (1999) observed a sixfold increase in pig granulosa cell progesterone output over time. Despite the increase in progesterone concentrations, several factors show that in the present study the theca cells did not luteinize fully. Androstenedione and

Fig. 3. The effect of insulin-like growth factor I (IGF-I) and insulin doses on oestradiol (a) and androstenedione (b) production by pig theca cells after 144 h in defined serum-free culture. IGF-I doses: 0 (□), 10 (▨) and 100 ng ml⁻¹ (■). The data were log transformed before ANOVA and are expressed as production per 10³ viable cells. Values are the mean of three separate cultures each containing four wells per treatment.

oestradiol synthesis continued throughout culture and production remained responsive to LH. Oestradiol production by pig luteal cells *in vitro* is unresponsive to LH and hCG (Pitzel *et al.*, 1988). In addition, the cells maintained the same follicular phase morphology during culture. In the present study, after establishing that the theca cells did not luteinize in the serum-free culture system, the subsequent experiments concentrated on the influence of the additives on oestradiol and androstenedione synthesis.

Increasing LH dose stimulated androstenedione synthesis at all time points. In the growing follicle, theca cells under the influence of LH provide androgen substrate (Ryan, 1979; Evans *et al.*, 1981). In the current system, theca cells behaved as *in vivo* by responding to LH and producing androstenedione. The sensitivity of the cells to LH increased tenfold with time, indicating an increase in the number or sensitivity of thecal LH receptors over the culture period.

Fig. 4. Oestradiol production by pig granulosa and theca cells after 48 h in serum-free medium. Cells were plated in a ratio of granulosa to theca cells of 4:1 at a total density of either 50 or 150 × 10³ viable cells per well. The data were log transformed before ANOVA and are expressed as production per 10³ viable cells. Data are from a single preliminary experiment containing four wells per treatment. Co-culture (■), sum (□).

Fig. 5. Oestradiol production by pig granulosa and theca cells cultured separately (□) or as co-cultures (■) after 144 h in serum-free medium. The data were log transformed before ANOVA and are expressed as production per 10³ viable cells. Values are from three individual cultures each containing four wells per treatment.

However, oestradiol output was less sensitive to LH dose. At 48 h, oestradiol production was not significantly increased by LH. After 144 h, LH increased oestradiol synthesis but the response was not dose dependent. This finding shows that increased oestradiol production was not simply a result of increased androstenedione substrate availability. Furthermore, experiments using this culture system demonstrated that the addition of exogenous androstenedione did not increase thecal oestradiol output (E. Shores, unpublished).

The high levels of statistical significance resulting from analysis of the androstenedione data and the dose-dependent response indicate that LH plays a greater role in the regulation of androstenedione than oestradiol production. Furthermore, these observations imply that *in vivo*, the primary role of theca cells is androgen production, which is tightly regulated by LH. Oestradiol synthesis by theca cells is a secondary role, which does not warrant such strong LH control. Progesterone production at 48 h was increased by LH doses as low as 0.05 ng ml⁻¹. Doses of 0.1, 0.5 and 1.0 ng ml⁻¹ produced similar responses, which were on average 3.5-fold more than control values. Increased progesterone production may reflect increased demand for substrate for conversion to androstenedione by the theca cells.

IGF-I affected oestradiol and androstenedione output. However oestradiol synthesis was increased by a lower IGF-I dose (10 ng ml⁻¹) than androstenedione production (100 ng ml⁻¹). Therefore, IGF-I may act downstream of androstenedione production initially, possibly directly on P450_{arom}. Insulin alone did not influence oestradiol synthesis despite previous reports that insulin increased hormone production by pig theca cells *in vitro* (1 mg ml⁻¹, Morley *et al.*, 1989; 10–40 mg ml⁻¹, Stein *et al.*, 1990; 250 ng ml⁻¹, Barbieri, 1994). However, these studies used supraphysiological doses that were much higher than those in the present study. Nevertheless, in the current study, insulin acted synergistically with IGF-I (10 ng insulin ml⁻¹ plus 100 ng IGF-I ml⁻¹) to increase androstenedione production.

Within the follicle, the number of granulosa and theca cells are not equal. Stoklosowa *et al.* (1982) and Kokia *et al.* (1992) suggested a ratio of theca to granulosa cells of 1:4. *In vivo* estimates of the number of theca cells within a follicle are scarce. Downey and Driancourt (1994) reported a ratio of granulosa to theca cells of 2.5:1 in pig follicles. In the present study, total recovery rates per follicle revealed a ratio of theca to granulosa cells of 1:5. Importantly, the ratio adopted in the current experiments is more similar to *in vivo* conditions than the 1:1 ratio used in some earlier work (Tsang *et al.*, 1985).

Co-culture increased oestradiol synthesis twofold compared with the sum oestradiol produced after 48 h. However, after 144 h, the magnitude of the interactive stimulation of oestradiol synthesis had increased to approximately threefold. Stoklosowa *et al.* (1982) reported a similar increase in the difference in oestradiol production between co-cultures and cultures of a single type of cell (1.4-fold after 48 h, 2.5-fold after 144 h). This finding indicates that the cells become more sensitive to the factor or factors responsible for the interaction as time in culture progresses. Similarly, theca cells become increasingly sensitive to LH as time in culture progresses (see above; also ovine theca cells, Campbell *et al.*, 1998).

In the present study, androstenedione was added to the cells containing granulosa cells only at a dose similar to that produced by theca cells in co-cultures (1–5 ng per well). A dose of 100 ng ml⁻¹ (that is, 25 ng per well) was compared with the physiological doses to investigate whether higher doses of androgen changed the observed interaction. Addition of more androstenedione increased overall oestradiol production by the co-cultures but did not

influence the presence or size of the theca–granulosa cell interaction at 48 h. By 144 h, the higher androstenedione dose increased the size of the interactive increase in oestradiol output by co-cultures over the sum of individual cultures. This finding indicates increased sensitivity of the cells at the end of culture. Further work is required to identify the factor or factors responsible for the theca–granulosa cell interaction. The interaction may be mediated by growth factors produced by one or both types of cell. For example, granulosa-derived inhibin and IGF-I increase LH-stimulated androgen production by theca cells in rats (Zachow and Magoffin, 1995). The use of intraovarian factors means that the influences of the gonadotrophins can be modulated to achieve a wider range of more subtle effects than would be possible with just two messengers (Kotsuji and Tominaga, 1994; Armstrong and Webb, 1997).

Why should granulosa and theca cells interact? Evidence from other organs of the body shows that mesenchymal–epithelial cell interactions are very common, especially in those organs in which function follows cyclic patterns (Kotsuji and Tominaga, 1994; Parrott and Skinner, 1998). The ovary is an excellent example of such an organ with the mesenchymal-derived theca cells across a basement membrane from the epithelial-derived granulosa cells. The close proximity of granulosa and theca cells, together with the need to co-ordinate a cycle of growth and function implies a requirement for cell–cell communication. The current study demonstrates theca–granulosa cell interactions using a culture system designed to mimic closely *in vivo* conditions.

In conclusion, the present study demonstrates the development of a physiological serum-free culture system for pig theca cells in which *in vivo* morphology, steroidogenesis and gonadotrophin responsiveness are maintained. High initial plating density was detrimental to steroidogenesis. Theca cells were extremely sensitive to LH, becoming more sensitive with time in culture. Androstenedione production was regulated strongly by LH, whereas oestradiol synthesis was more sensitive to IGF-I dose. Theca and granulosa cells interacted in co-culture, resulting in increased oestradiol production in a system that mimicked *in vivo* conditions in terms of cell ratio and androgen availability. This culture system will enable further studies aimed at understanding the complex mechanisms involved in follicular development and steroidogenesis.

This work was supported by the BBSRC. Thanks are extended to D. G. Bolt, USDA, for the provision of pig gonadotrophins and to B. Cook for the androstenedione radioactive label.

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