Expression of mRNA encoding IGF-I, IGF-II and type 1 IGF receptor in bovine ovarian follicles

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Abstract

IGFs regulate gonadotrophin-stimulated proliferation and differentiation of granulosa and theca cells *in vitro*. However, the detailed pattern of mRNA expression of IGFs in bovine follicles remains controversial. The objectives of this study were therefore to describe the temporal and spatial pattern of expression of mRNA encoding IGF-I, IGF-II and the type 1 IGF receptor in bovine follicles *in vivo*. The expression of mRNA encoding IGF-II was detected in theca tissue from around the time of antrum formation up to and during the development of dominance. No IGF-II mRNA expression was detected in granulosa cells. In the majority of follicles we were unable to detect mRNA encoding IGF-I in either granulosa or theca tissue from follicles at any stage of development. Occasionally low amounts of mRNA encoding IGF-I were detected in the theca externa and connective tissue surrounding some follicles. Type 1 IGF receptor mRNA was detected in both granulosa and theca cells of preantral and antral follicles. Expression was greater in granulosa

tissue compared with theca tissue. We also measured IGF-I and -II mRNA in total RNA isolated from cultured granulosa and theca cells using reverse transcriptase PCR. In contrast to the *in vivo* results, IGF-II mRNA was detected in both granulosa and theca tissue. IGF-I mRNA was detected in theca tissue and in very low amounts in granulosa cells. Using a specific IGF-I RIA we were unable to detect IGF-I immunoreactivity in granulosa conditioned cell culture media. Using immunohistochemistry we detected IGF-I immunoreactivity in some blood vessels within the ovarian stroma. We conclude from these results that IGF-II is the principal intrafollicular IGF ligand regulating the growth of bovine antral follicles. In preantral follicles the expression of mRNA encoding type 1 IGF receptor but absence of endogenous IGF-I or -II mRNA expression, highlights a probable endocrine mechanism for the IGF regulation of preantral follicle growth.

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Introduction

Insulin-like growth factors (IGFs) are putative regulators of follicular growth and enhance the actions of gonadotrophins on the ovary (Adashi & Roban 1992, Guidice 1992, Armstrong & Webb 1997, Webb & Armstrong 1998). IGF-I stimulates granulosa and theca cell proliferation and differentiation *in vitro* in a number of mammalian species (Hammond *et al.* 1991, Adashi 1994, Spicer & Echternkamp 1995, Gutierrez *et al.* 1997a, Campbell *et al.* 1998). In contrast IGF-II has not been as extensively studied as IGF-I; however, its effects on the proliferation and differentiation of granulosa cells have been demonstrated in a number of species including the pig and sheep (Hammond *et al.* 1991, Campbell *et al.* 1995, Monget & Monniaux 1995).

The expression of mRNAs encoding IGF-I and -II in ovarian follicles is developmentally regulated in a species-specific manner (Armstrong & Webb 1997, Webb & Armstrong 1998). Although it has been clearly established that IGF-I is expressed in granulosa cells of rodents and pigs its expression in the ruminant remains controversial, with reports demonstrating both the presence (Spicer *et al.* 1993, Leewenberg *et al.* 1995, Spicer & Echternkamp 1995, Yuan *et al.* 1998) and absence (Perks *et al.* 1995, 1999, Armstrong *et al.* 1998a) of mRNA encoding IGF-I in both bovine and ovine granulosa cells. Similarly, although Spicer *et al.* (1993) detected IGF-I immunoreactivity in bovine granulosa cell conditioned medium we were unable to demonstrate the presence of immunoreactive IGF-I in bovine granulosa cell conditioned culture medium after the removal of endogenous

IGF-binding proteins (IGFBPs) by chromatography under acidic conditions (Gutierrez *et al.* 1997b). To shed light on this controversy and provide further information on the role of the IGF system in the bovine ovary we have examined the patterns of expression of mRNAs encoding both the IGFs and their receptor (type 1 IGF receptor) during folliculogenesis *in vivo*. We also examined the expression of IGF-I and -II mRNA in bovine granulosa and theca cells *in vitro*.

Materials and Methods

Materials

Cell culture material was obtained from GIBCO BRL Life Technologies Ltd, Paisley, Renfrewshire, UK. Recombinant human Long R3 IGF-I (LR3-IGF-I, media grade) and recombinant human IGF-I were purchased from Gropep Pty Ltd (Adelaide, SA, Australia). The US Department of Agriculture generously donated follicle-stimulating hormone (FSH) (USDA-bFSH-I-2; potency 854 IU/mg; no cross reactivity with USDA-bLH-B-5 using USDA-5 antiserum) and the National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases (Torrance, CA, USA) generously donated luteinizing hormone (LH) (NIDDK-oLH-S26; potency 2.3 IU/mg; FSH contamination <0.5% by weight).

Collection of tissue and characterization of follicles

Experiment 1 Ovaries ($n=6$) were obtained from six cows from the local abattoir. They were either processed directly for cell culture or divided into blocks. The diameters of follicles on the surface of the ovaries were measured before the blocks were snap frozen in liquid nitrogen and stored at -70°C until required for *in situ* hybridization. Follicles were classified as healthy, atretic or grossly atretic as described previously (Armstrong *et al.* 1998b). Expression of mRNAs encoding IGF-I and -II and type 1 IGF receptor was determined by *in situ* hybridization in 65 follicles from six ovaries. The follicle numbers were made up of 33 small (1–4 mm) follicles (18 healthy, 10 atretic and 5 grossly atretic), 34 medium (4–8 mm) follicles (8 healthy, 16 atretic and 10 grossly atretic) and 8 large (>8 mm) follicles (5 healthy, 2 atretic and 1 grossly atretic).

Experiment 2 Ovaries were collected from lactating Holstein–Friesian cows ($n=5$) during the luteal phase of the oestrous cycle (on days 14, 16 and 18 following natural oestrus) and during the follicular phase (on day 20 following natural oestrus and 2 days after the initiation of luteolysis by the prostaglandin F2 α analogue cloprostenol (Estrumate; Schering–Plough Animal Health, Welwyn Garden City, UK). Immediately following slaughter the reproductive tract was removed and transported to the

laboratory where ovaries were stored at 4°C until processing as for the previous experiment. Blocks containing the largest follicles (15–20 mm diameter) were analysed by *in situ* hybridization.

Granulosa and theca primary cell cultures

Granulosa and theca cells from medium-sized (4–8 mm) gonadotrophin-dependent follicles were isolated and pooled. Granulosa cells (3×10^6 viable cells) were plated in 10 ml culture medium containing testosterone ($0.1 \mu\text{mol/l}$) in 50 ml culture bottles for up to 96 h, with the medium changed after 48 h, as described previously (Gutierrez *et al.* 1997a, Armstrong *et al.* 1998b). Granulosa cell cultures were supplemented with insulin (10 ng/ml), LR3-IGF-I (10 ng/ml) and either FSH (0, 1 or 50 ng/ml) or LH (0 or 100 ng/ml).

After isolation of the granulosa cells, the remainder of the follicle wall was dispersed by enzymatic digestion. Isolated theca cells (3×10^6 viable cells) were cultured in DMEM:Hams F12 (1:1) media (10 ml) using 50 ml culture bottles in the presence of insulin (10 ng/ml), IGF-I (10 ng/ml) and LH (0 or 100 ng/ml) for 4 days (Campbell *et al.* 1998).

Reverse transcriptase PCR (RT-PCR)

RNA was isolated from granulosa and theca cells after 96 h of culture using RNeasy spin columns (Qiagen Ltd, Crawley, West Sussex, UK) according to the manufacturer's instructions. Concentrations were estimated by absorbance at 260 nm and the samples were stored in RNase-free TE buffer at -80°C until required. The A_{260}/A_{280} ratio for all RNA samples was >1.8 . First-strand cDNA synthesis was carried out using Superscript II reverse transcriptase (Life Technologies Ltd) (Armstrong *et al.* 1998b). The primers used for the amplification of the IGF-I and -II mRNA transcripts were as shown in Table 1 and span the coding region of their respective mRNAs. Analysis was carried out on three separate cell cultures. Samples were heated to 94°C for 3 min and amplified for 25 cycles (94°C for 1 min; 55°C for 1 min and 72°C for 1 min). The final 72°C incubation was continued for a further 4 min. Reverse transcriptase blanks, RNA blanks and PCR blanks (no cDNA products) were included in each analysis. Aliquots (5 μl) of each of the PCR reactions (total volume 20 μl) were analysed by electrophoresis on 4% agarose gels (NuSieve GTG Agarose; FMC Bioproducts, Rockland, ME, USA). Liver mRNA was included as a positive control and in all experiments reverse transcriptase blanks, RNA blanks and PCR blanks were negative. Identities of the PCR products were confirmed by restriction endonuclease digestion.

IGF-I and -II and type 1 IGF receptor probes

The homologous bovine IGF-II and type 1 IGF receptor probes were prepared following RT-PCR (see previous

Table 1 Primer pairs used for PCR amplification of bovine IGF-II and type I IGF receptor cDNAs

Gene	Size (bps)		Primer
IGF-I	196 ^a	Upstream primer	5'-CCTCTGCGGGGCTGAGTTGGT-3'
		Downstream primer	5'-CGACTTGGCGGGCTTGAGAGGC-3'
IGF-II	154 ^b	Upstream primer	5'-TCTGTGCGGCGGGGAGCTGGT-3'
		Downstream primer	5'-AGTCTCCAGCAGGGCCAGGTCG-3'
Type 1 IGF receptor	189 ^c	Upstream primer	5'-CCAAGCTAAACCGGCTCAAC-3'
		Downstream primer	5'-TTATTACCAAGCCTCCAC-3'

^aAmplified product corresponds to position 156–351 of a bovine IGF-I cDNA (Fotsis *et al.* 1990).

^bAmplified product corresponds to position 7–160 of a bovine IGF-II cDNA (Brown *et al.* 1990).

^cAmplified product is homologous to a partial bovine type I receptor cDNA clone (Sneyers *et al.* 1991) and corresponds to position 2717–2905 of a human type 1 IGF receptor cDNA (Abbot *et al.* 1992).

section) using the primers described in Table 1. Amplified products were cloned into pGEM-T (Promega Ltd, Southampton, UK) using standard procedures and sequenced to confirm their identity and orientation within the plasmid. The IGF-II construct was linearized with NcoI and SP6 RNA polymerase was used to generate the antisense probe. The sense probe was generated using T7 RNA polymerase after linearizing the plasmid with NsiI. The type 1 IGF receptor construct was linearized with NotI, and T7 RNA polymerase was used to generate the antisense probe. The sense probe was generated using SP6 RNA polymerase after linearizing the plasmid with NcoI.

In ribonuclease protection assays RNase-protected bands of size 153 and 189 nucleotides were obtained using the bovine IGF-II and type 1 IGF receptor probes respectively.

The IGF-I probe used for *in situ* hybridization was the same as that described by Yuan *et al.* (1998) and spanned the coding region (bases 25–496) of a cDNA described by Tavakkol *et al.* (1988).

In situ hybridization

Frozen sections (14 µm) were dehydrated, fixed and probed with ³⁵S-labelled riboprobes (Armstrong *et al.* 1998b). After the final high stringency wash the sections were dipped in autoradiographic K2 photographic emulsion (Ilford Limited, Mobberley, Cheshire, UK) and exposed for 3 weeks at 4 °C. Sections were then developed (Kodak D-19) and fixed (Ilford Hypam fixer) before staining in haematoxylin and eosin. The sections were finally mounted in DPX (R A Lamb, Sunbeam Road, London, UK) mountant before microscopic examination using both light- and darkfield illumination.

Image analysis

The intensity of the *in situ* hybridization signal was analysed using an NIH-Image analysis system (NIH, Bethesda, MD, USA). The number of graphic pixels occupied by silver grains (identified by a set grey threshold) within a defined area of the tissue section was

counted and presented as a percentage of the total pixel number within the defined area. Background hybridization intensity, measured with the sense RNA probes was subtracted from the measurements obtained with the antisense probes to give the final hybridization signal. Within each follicle three separate fields were analysed for each probe. There was no difference ($P>0.05$) in hybridization intensity obtained with antisense and sense RNA probes within a non-expressing region of a tissue section. Under the conditions described here the hybridization signal was proportional to the length of time the slides were exposed to photographic emulsion for up to 3 weeks.

IGF-I immunohistochemistry

Immunohistochemistry was carried out as described previously. The rabbit anti-human IGF-I antiserum (Armstrong *et al.* 1990) was diluted 1:500 prior to use. After probing with primary antibody the sections were washed and stained using an anti-rabbit IgG biotin-avidin, peroxidase kit (Sigma, Poole, Dorset, UK) according to the manufacturer's instructions.

Steroid and IGF-I RIAs

Androstenedione and progesterone were measured according to the method described by Corrie *et al.* (1981). Oestradiol-17β was also measured by RIA as described by Webb *et al.* (1985). IGF-I was measured by RIA (Gutierrez *et al.* 1997b), after acid gel filtration to remove IGFBPs, using a rabbit polyclonal antibody raised against human recombinant IGF-I (Armstrong *et al.* 1990). The sensitivity of the assay was 22 pg (two standard deviations from the minimum detectable level) and the intra- and interassay coefficients of variation were 13 and 8% respectively.

Statistical analysis

Steroid production by cell cultures is presented as mean ± S.E.M. (three cultures with two replicates). The effects of FSH or LH (fixed effects) were analysed using a

Figure 1 Lightfield (a, d and g) and darkfield (b, c, e, f, h and i) illumination of ovarian sections probed with an IGF-II antisense (b, e and h) and sense (c, f and i) riboprobe. IGF-II mRNA expression is shown in theca tissue of a small follicle around the time of antrum formation (a–c), in theca tissue of a 0.5 mm diameter antral follicle (d–f) and in theca tissue in a group of three follicles (2×1 mm and 25 mm in diameter) (g–i). The bar represents 180 μ m (a–f) and 450 μ m (g–i).

Figure 2 Lightfield (a) and darkfield (b and c) illumination of serial sections of two 0.4 mm diameter antral follicles probed with an IGF-II (b) and IGF-I (c) antisense riboprobe. The bar represents 180 μ m. t and g represent theca and granulosa respectively.

mixed model, allowing for variation due to culture and different pools of cells (random effects). The effect of follicle size and health status on the intensity of the *in situ* hybridization signal was determined by ANOVA.

Results

Expression of IGF-I and -II and type 1 IGF receptor mRNA in vivo

mRNA encoding IGF-II was expressed in theca tissue in follicles from around the stage of antrum development up to ovulation (Fig. 1). No expression was detected in granulosa tissue. There was no difference ($P>0.05$) in expression of IGF-II mRNA in small (1–4 mm), medium (4–8 mm) or large (>8 mm) healthy antral follicles. During the development of atresia there was a decrease ($P<0.001$) in the abundance of IGF-II mRNA in theca tissue (results not shown).

We were unable to detect mRNA encoding IGF-I in granulosa tissue in any of the follicles examined. Very low expression was detected in the theca externa of healthy antral follicles (Fig. 2). Low expression was also occasionally detected in connective tissue surrounding some follicles (results not shown).

Expression of mRNA encoding type 1 IGF receptor mRNA was detected in granulosa and theca cells from both preantral and antral follicles (Fig. 3). Expression was

greater in granulosa cells compared with theca tissue and decreased ($P<0.001$) during the development of atresia (results not shown). During antral follicle growth there was no difference ($P>0.05$) in the expression of mRNA encoding type 1 IGF receptor between small (1–4 mm), medium (4–8 mm) or large (>8 mm) healthy follicles. Type 1 IGF receptor mRNA was also detected in oocytes from preantral and antral follicles.

Expression of IGF-I and -II mRNA in large (>15 mm) follicles

The expression of mRNA encoding IGF-I and -II was measured in large follicles at defined stages of the oestrous cycle. The results presented in Fig. 4 shows the expression in a 19 mm diameter, dominant follicle on day 20 of the oestrous cycle. IGF-II mRNA was restricted to theca tissue. mRNA encoding IGF-I was not detected in either granulosa or theca tissue. Similar patterns of expression (results not shown) were observed with ovaries collected on day 14 (18 mm diameter; atretic follicle), day 16 (15 mm diameter; healthy follicle) and day 18 (15 mm diameter; healthy follicle) of the oestrous cycle as well as 48 h after prostaglandin treatment (15 mm diameter follicle).

Steroid production by cultured granulosa and theca cells

In granulosa cell cultures oestradiol production (Fig. 5a) was higher ($P<0.01$) after 4 days in the presence of 1 ng

Figure 3 Lightfield (a, d and g) and darkfield (b, c, e, f, h and i) illumination of ovarian sections probed with a type 1 IGF receptor antisense (b, e and h) and sense (c, f and i) riboprobe. IGF receptor mRNA expression is shown in granulosa cells of a group of primary follicles (a–c), in granulosa tissue, theca tissue and oocyte of a 0.3 mm diameter antral follicle (d–f) and in granulosa and theca tissue of a 15 mm diameter antral follicle (g–i). The bar represents 180 μ m (a–f) and 450 μ m (g–i). t and g represent theca and granulosa respectively.

Figure 4 Lightfield (a) and darkfield (b–d) illumination of serial sections of an ovulatory follicle (19 mm diameter) probed with an IGF-II (b) and IGF-I (c) antisense and IGF-I sense (d) riboprobe. The bar represents 180 μ m. T and G represent theca and granulosa respectively.

FSH/ml compared with cultures in the absence of FSH. In the presence of 50 ng FSH/ml, oestradiol concentrations were lower ($P<0.01$) relative to the culture incubated in the presence of 1 ng FSH/ml. In contrast 50 ng FSH/ml increased ($P<0.01$) the amount of progesterone in the culture medium (Fig. 5b). LH (100 ng/ml) increased ($P<0.05$) both oestradiol (Fig. 5c) and progesterone (Fig. 5d) production by granulosa cells after 4 days of culture and may in part be due to FSH contamination of the LH preparation (<0.5% by weight).

In theca cell cultures LH (100 ng/ml) increased ($P<0.01$) both androstenedione (Fig. 6a) and progesterone (Fig. 6b) production after 4 days of culture.

Expression of mRNA encoding IGF-I and -II in granulosa and theca cells in culture

mRNA encoding IGF-I and -II in the granulosa and theca cell cultures characterized in the previous section was detected by RT-PCR. Representative results from three

Figure 5 Oestradiol-17 β (a and c) and progesterone (b and d) production (ng/10⁶ cells per 48–96 h) by granulosa cells cultured in the presence of testosterone (0.1 μ mol/l), LR3-IGF-I (10 ng/ml), insulin (10 ng/ml) and FSH (0, 1 and 50 ng/ml) (a and b) or in the presence and absence of LH (c and d). The bar represents a pooled S.E.M. obtained from two replicate measurements of three cultures.

separate experiments are shown in Figs 7 and 8. Specific primers for IGF-I and -II amplified cDNAs from RNA isolated from theca cells (Figs 7 and 8 respectively) both before and after culture and in the presence or absence of LH (100 ng/ml). Using RNA isolated from granulosa cells, amplification of cDNA was observed with IGF-II specific primers (Fig. 8); however, only very low amounts of amplified cDNA was detected with the IGF-I specific primers (Fig. 7). FSH had no effect on the expression of mRNA encoding IGF-I or -II in the cultures described here (results not shown).

IGF-I production by granulosa cell cultures

We were unable to detect significant amounts of immunoreactive IGF-I in granulosa cell conditioned medium even after 10-fold concentration using Amicon–Centricon YM-3 membranes, molecular weight cut off

3×10^3 (Millipore UK Ltd, The Boulevard, Blackmore Lane, Watford, Hertfordshire, UK).

Immunohistochemical localization of IGF-I in the bovine ovary

Immunoreactive IGF-I was located in endothelial cells of the ovarian vasculature (Fig. 9). We were unable to detect staining in granulosa or theca cells throughout follicular development.

Discussion

The principal aim of this study was to describe the spatial and temporal changes in the expression of mRNAs encoding IGF-I, IGF-II and the type 1 IGF receptor in granulosa and theca tissue from follicles at different stages

Figure 6 Androstenedione (a) and progesterone (b) production (ng/10⁶cell per 48–96 h) by theca cells cultured in the presence of LR3-IGF-I (10 ng/ml) and insulin (10 ng/ml) in the presence and absence of LH (100 ng/ml). The bar represents a pooled S.E.M. obtained from two replicate measurements of three cultures.

of development in the bovine ovary. The work follows on from an earlier study describing IGFBP-2 and -4 mRNA expression in bovine follicles (Armstrong *et al.* 1998b) and together they provide a more comprehensive description of the anatomy of the bovine ovarian follicular IGF system.

Analysis of the expression pattern for mRNA encoding IGF-II indicated that this ligand was expressed in theca tissue in follicles from around the time of antrum formation up to ovulatory sized follicles. No expression of mRNA encoding IGF-II was detected in granulosa tissue at any stage of follicular development. This spatial distribution is similar to that described in sheep, rats and pigs (Armstrong & Webb 1997). However, the timing of the onset of IGF-II mRNA expression in developing follicles is earlier in cows (around the time of antrum formation) than in pigs (Yuan *et al.* 1996).

The factors involved in switching on theca IGF-II mRNA expression are unknown. Theca cells surround the granulosa cells prior to antrum formation and express LH receptor mRNA at this early stage of development (Xu *et al.* 1995a, Bao & Garverick 1998). In contrast, significant aromatase expression in granulosa cells only becomes apparent in follicles >4 mm in diameter. Expression of mRNA encoding steroidogenic enzymes (P450_{sc}, P450_{17αH}ase and P450_{lyase}) is first detected in theca tissue of preantral follicles and their expression increases during antrum formation (Xu *et al.* 1995b, Boa & Garverick 1998). The expression of mRNA encoding IGF-II in theca cells in early antral follicles can therefore be correlated with the acquisition of LH receptors on theca cell plasma membranes and their ability to produce androgen. Since IGF-I has been shown to increase LH receptor

Figure 7 Effect of 0 or 100 ng/ml of LH on IGF-I mRNA expression by bovine granulosa and theca cells at the start of culture (T₀) and after 96 h of culture. Hepatic mRNA was used as a positive control. M represents the marker lane, the size of the markers (nucleotide number) are shown on the right of the figure.

Figure 8 Effect of 0 or 100 ng/ml of LH on IGF-II mRNA expression by bovine granulosa and theca cells at the start of culture (T_0) and after 96 h of culture. Hepatic mRNA was used as a positive control. M represents the marker lane, the size of the markers (nucleotide number) are shown on the right of the figure.

mRNA expression in rat theca cells (Magoffin & Weitsman 1994) and LH receptor number in bovine theca cells (Stewart *et al.* 1995) it can be speculated that in ruminants *in vivo* IGF-II takes the place of IGF-I and regulates the timing of the onset of LH receptor expression in theca tissue via an autocrine mechanism.

IGF-I mRNA was detected in very low amounts in theca tissue from healthy antral follicles. The low amounts of mRNA encoding IGF-I detected in theca tissue is probably associated with the vascular endothelial cells within this tissue, a cell type that is known to express mRNA encoding components of the IGF system. Using immunohistochemistry we confirmed the presence of IGF-I immunoreactivity in the ovarian vasculature. Our *in situ* hybridization data failed to detect the presence of IGF-I mRNA in granulosa tissue at any stage of development. These data support the results of Perks *et al.* (1995, 1999) in sheep and cattle, but are at odds with those of Leewenburg *et al.* (1995) in sheep and Yuan *et al.* (1998) in cattle who have demonstrated the presence of mRNA encoding IGF-I in granulosa cells from both subordinate and dominant follicles. Previous work using bovine granulosa cell cultures (Spicer *et al.* 1993, Spicer & Echternkamp 1995) also detected IGF-I mRNA.

The differences in IGF-I mRNA expression patterns between laboratories has been explained previously by differences in the sensitivity of probes used in the different studies. In this case, however, the IGF-I probe used in the present study and that described by Yuan *et al.* (1998) was the same. Nevertheless, despite the differences in abundance of IGF-I mRNA detected between laboratories, the results of Yuan *et al.* (1998) also show that IGF-II mRNA expression is significantly greater than that of IGF-I and support our conclusion that IGF-II is the major intrafollicular IGF ligand regulating the growth of bovine antral follicles.

We further examined the expression of mRNAs encoding IGF-I and -II in bovine granulosa and theca cell cultures using RT-PCR. The detection of IGF-I mRNA in cultured theca cells is probably a reflection of the cellular heterogeneity of this tissue. The detection of IGF-II mRNA as well as low levels of IGF-I mRNA in total RNA extracts from granulosa cells may indicate contamination of this culture with theca cells. Alternatively luteinization of granulosa cells could switch on IGF-II mRNA expression. In support of this latter conclusion is our recent result demonstrating expression of IGF-II mRNA in a subset of large luteal cells in sections of early bovine corpora lutea (Woad *et al.* 1998). These *in vitro* results highlight the need for care when interpreting PCR results obtained using granulosa and theca cell cultures. Minor contamination with other cell types and/or the spontaneous luteinization of a proportion of the cultured cells can seriously distort the results from the actual situation observed *in vivo*.

Further evidence supporting our observation on the lack of IGF-I mRNA expression in bovine granulosa cells *in vivo* is the absence of IGF-I immunoreactivity in granulosa cell conditioned medium. This supports results from an earlier study that also failed to detect IGF-I immunoreactivity in granulosa cell culture medium (Gutierrez *et al.* 1997b). IGF-I production by granulosa cells has been reported (Spicer *et al.* 1993); however, in this case, the interpretation of the results were compromised because measurements were carried out on acid-ethanol extracted samples. We have recently shown that a similar procedure fails to remove all the IGF-BPs from granulosa cell conditioned media resulting in false positive results (Gutierrez *et al.* 1997b). Alternatively assay sensitivity may account for these discrepancies.

Type 1 IGF receptor mRNA was expressed in granulosa tissue from preantral and antral follicles.

Figure 9 Bovine ovarian sections incubated with an antibody raised against human recombinant IGF-I in the absence (a and b) and presence (c) of exogenous IGF-I. Bar represents 180 μm (a) and 450 μm (b and c).

Expression was also detected in theca tissue from antral follicles but this was always significantly less than in granulosa tissue from the same follicle. In addition, binding of IGF-I to preantral bovine follicles has been described by Wandji *et al.* (1992). Although the type 1 receptor binds IGF-II at a lower affinity than IGF-I we hypothesize that since IGF-II is the major intrafollicular IGF ligand, local concentrations of IGF-II within follicular tissue *in vivo* will be high enough to result in binding to the type 1 receptor and activate the second messenger system.

Type 1 IGF receptor expression was demonstrated in follicles of all sizes even though expression of IGF-II mRNA was not apparent in theca tissue until around the time of antrum formation. We propose, therefore, that preantral follicles are regulated by IGFs from the circulation, i.e. through endocrine mechanisms, whereas in antral follicles intrafollicular as well as endocrine mechanisms are probably involved in the IGF regulation of follicle development. Experiments described by Gong *et al.* (1993) provide evidence supporting an endocrine role for IGF-I for the regulation of follicle growth. The responsiveness of preantral follicles towards IGF-I has recently been demonstrated *in vitro* (Gutierrez *et al.* 1997c). In this case physiological concentrations of IGF-I in the culture medium increase the growth rate of preantral follicles. In the pig such an endocrine mechanism for the regulation of preantral follicle growth is not necessary since in this species granulosa cells of preantral follicles have been shown to express mRNA encoding IGF-I (Yuan *et al.* 1996).

Quantitative analysis of the expression of mRNA encoding IGF-II in theca and type 1 IGF receptor in bovine granulosa tissue indicated there were no significant changes in the level of their expression during follicle development after antrum formation. In contrast to these observations, Yuan *et al.* (1998) described a significant increase in IGF-II mRNA expression in dominant compared with subordinate follicles. This probably reflects differences in experimental design between the two studies in that the results described here were primarily obtained using abattoir material whereas Yuan *et al.* (1998) collected ovaries at precise times throughout the first wave of follicle growth after oestrus synchronization.

The results described here when combined with our previous study concerning the expression of mRNA encoding IGFBP-2 and -4 (Armstrong *et al.* 1998b) confirm the existence of a complete intrafollicular IGF system within bovine antral follicles, i.e. the ability to produce IGF ligand, the presence of receptors to mediate the biological actions of IGFs and the production of IGFBPs to regulate IGF bioavailability. These two studies have highlighted two major changes occurring within the ovarian IGF system during bovine follicle growth. The first is a switch from a total endocrine dependence towards IGFs in preantral follicles to a combination of intrafollicular and endocrine mechanisms in antral follicles. The second is an increase in bioavailability of IGFs in dominant follicles arising from a decrease in the expression of mRNA encoding IGFBP-2 (Armstrong *et al.* 1998b).

In conclusion, our results support the hypothesis that theca-derived IGF-II is the major intraovarian IGF ligand produced by bovine antral follicles. The expression of mRNA encoding type 1 IGF receptor in granulosa cells from preantral follicles before the developmental stage when IGF-II mRNA is expressed indicates an endocrine role for IGFs in regulating preantral follicle growth.

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