Introduction

Although pituitary-derived LH is the primary regulator of corpus luteum function, it is apparent that other factors of extra- and intraovarian origin have the potential to modulate the luteal response to gonadotrophins. The insulin-like growth factor (IGF) system plays a central role in these interactions and studies in vitro have shown that both IGF-I and IGF-II have wide ranging effects on luteal function (Giudice, 1992; Spicer and Echternkamp, 1995).

The formation of the corpus luteum in response to the preovulatory LH surge involves changes in cellular morphology and ultrastructure, and key alterations in steroid hormone synthesis (Smith et al., 1994). After the LH surge, production by the ovulatory follicle of oestradiol and androstenedione ceases and progesterone synthesis increases. This marked shift in steroidogenesis is the result of changes in the tissue content of steroidogenic enzymes and factors involved in the provision of steroid precursor (Rodgers et al., 1986, 1987; Couet et al., 1990; Voss and Fortune, 1993).

IGF-I increases the binding capacity of the LH receptor in rat granulosa cells luteinized in culture, acting in synergy with FSH (Adashi et al., 1985). The increase in number of receptors is accompanied by increased sensitivity to LH and potentiation of its luteotrophic effects. In addition, IGF-I has direct stimulatory effects on key components of the steroidogenic pathway, and increased progesterone secretion has been demonstrated in the luteal tissue of rats (Talavera and Menon, 1991), sheep (Khan-Dawood et al., 1994), pigs (Yuan and Lucy, 1996), rabbits (Constantino et al., 1991), humans (Devoto et al., 1995) and cattle (McArdle and Holtorf, 1989; Sauerwein et al., 1992). IGF-I has potent actions on sterol metabolism that include amplification of steroidogenic acute regulatory protein (StAR) (Balasubramanian et al., 1997) and the delivery and utilization of steroid precursors (Veldhuis, 1989). The key steroidogenic enzymes, cholesterol side-chain cleavage (P450ccc) and 3β-hydroxysteroid dehydrogenase (3β-HSD) are also targets of IGF action demonstrated by both increased gene expression and enzyme activity in response to IGF-I stimulation in rats (Magoffin et al., 1990; Magoffin and Weitsman, 1993; deMoura et al., 1997).

Many studies have focused on the potential roles of IGF-I...
in ovarian function. However, the regulatory effects of IGF-II have not been studied to the same extent, possibly because IGF-II has been considered to exert its effects mainly prenatally (Hossner et al., 1997). Recent results have indicated that IGF-II is the major ligand of the IGF system in the bovine follicle (Armstrong et al., 2000). Moreover, IGF-II has been shown to increase progesterone production by luteal cells in vitro (Sauerwein et al., 1992) and detailed studies in pig granulosa cells have demonstrated potent stimulatory effects of IGF-II on progesterone biosynthesis facilitated mainly by increased sterol delivery via increased lipoprotein binding, internalization and utilization, and P450scc enzyme activity (Garmey et al., 1993).

Studies in vitro strongly indicate a role for IGF-I or IGF-II in regulating the acquisition and maintenance of differentiated function in the corpus luteum. However, the IGFs may have additional actions within the ovary, particularly within the corpus luteum, which extend beyond the regulation of steroidogenesis to include angiogenesis and apoptosis. The vascularization of the developing corpus luteum increases significantly to support tissue growth and steroid production, in addition to cellular differentiation of luteal cells. The primary regulators of ovarian angiogenesis appear to belong to the vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) families (Reynolds and Redmer, 1998), although IGF-I has also been implicated in neovascularization occurring in response to injury (Hansson et al., 1989), in some disease states (Vialletes et al., 1994) and in the classical angiogenic model of the rabbit cornea (Grant et al., 1993). Immunohistochemical studies also indicate a role for IGF-II in the regulation of luteal vasculature (Amselgruber et al., 1994).

The corpus luteum is a transient endocrine gland, which must undergo functional and structural regression in the absence of pregnancy. Morphological and biochemical events indicative of apoptotic cell death have been associated with this controlled demise (Zeleznik et al., 1989; Sawyer et al., 1990; Juengel et al., 1993). The interaction of the IGF receptor with IGF-I or IGF-II protects different types of cell, including ovarian cells (Chun et al., 1994), from apoptosis (Harrington et al., 1994; Singleton et al., 1996; Parrizas and LeRoith, 1997).

As part of studies aimed at understanding the role of the IGF system in regulating corpus luteum function, the present study investigated the temporal and spatial changes in the expression of the mRNAs encoding IGF-I, IGF-II and the type 1 IGF receptor during the growth and regression of the bovine corpus luteum.

Materials and Methods

Animals

The oestrous cycles of mature Holstein and Friesian dairy cows (n = 9) were synchronized using an intravaginal progesterone-releasing device (PRID) 1.55 g progesterone, 10 mg oestradiol benzoate; Sanofi Animal Health Ltd, Watford) over 12 days. Luteolysis was induced by prostaglandin analogue (Estramate, 500 µg; Coopers Animal Health Ltd, Crewe) administered i.m. on the day before removal of the intravaginal progesterone-releasing device. Ovaries were collected on days 5, 10 or 15 after the onset of oestrus in the subsequent oestrous cycle (n = 3 per group). An additional three animals were treated with the prostaglandin analogue and the ovaries were collected 48 h later to study corpora lutea undergoing regression. After ovariectomy, all corpora lutea were excised, divided into blocks, frozen in liquid nitrogen and stored at –80°C before cryostat sectioning or RNA extraction. Sections from ovaries collected for follicular studies (Armstrong et al., 1998) were also subjected to in situ hybridization.

RNA extraction

Total cellular RNA was prepared from frozen luteal pieces by homogenization in 4 mol guanidine isothiocyanate l⁻¹ and phenol: chloroform extraction (Chomczynski and Sacchi, 1987). The pellet containing RNA was dissolved in diethylpyrocarbonate (DEPC)-treated water and stored in aliquots at –70°C. RNA was quantified by spectrophotometric absorbance at 260 nm, and the degree of protein contamination was assessed by the ratio of absorbance at 260 and 280 nm. The A260:A280 ratio was > 1.8 for all RNA samples. RNA integrity was assessed by examining the 28S and 18S ribosomal RNA bands on ethidium bromide-stained agarose-formaldehyde gels. Total cellular RNA was used as the template for subsequent cDNA synthesis.

RNA probes

Homologous bovine IGF-II and type 1 IGF receptor probes were prepared after RT–PCR from total luteal RNA. First strand cDNA synthesis was carried out using Superscript™ II reverse transcriptase (Life Technologies Ltd, Paisley) (Armstrong et al., 1998). IGF-II mRNA transcripts were amplified using a 21-mer upstream primer (5'-TCTGTGCGGGGAGCTGGT-3') and a 22-mer downstream primer (5'-AGTCTCCAGCAGGGCCAGTGTCG-3'). The amplified product corresponds to position 7–160 of a bovine IGF-II cDNA (Brown et al., 1990). Type 1 IGF receptor mRNA transcripts were amplified using a 20-mer upstream primer (5'-CCAAGCTTACCCGGCTCAAC-3') and a 19-mer downstream primer (5'-TTTACAAGCCTCCCAC-3'). The amplified product is homologous to a partial bovine type 1 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). Samples were heated to 94°C for 5 min and subsequently amplified for 30 cycles. Each cycle comprised 30 s denaturation at 94°C, 30 s annealing at 65°C (IGF-II) or 60°C (type 1 IGF receptor), followed by 30 s extension at 72°C. The extension phase of the last cycle was increased to 5 min. Reverse transcriptase blanks, RNA blanks and PCR blanks (no cDNA) were included in each analysis. PCR products were subsequently visualized by electrophoresis on 4% (w/v) agarose gels (NuSieve GTG agarose; Flowgen Instruments Limited, Lichfield). PCR products of the expected size were purified...
and subcloned into pGEM-T (Promega Limited, Southampton) using standard procedures. Restriction enzyme digestion confirmed successful ligation and the integrity and orientation of the cDNA inserts were assessed by dideoxy DNA sequencing using the chain termination method. Ribonuclease protection assays were performed using the homologous bovine riboprobes for the type 1 IGF receptor and IGF-II in samples of bovine luteal RNA, and produced RNase-protected hybrids of the expected sizes of 189 and 153 bases, respectively. A plasmid containing a pig IGF-I probe was a gift from M. Lucy (University of Missouri) and corresponded to a 472 bp cDNA for IGF-I (Yuan et al., 1996).

Constructs were linearized by restriction digestion to generate sense and antisense templates and incubated with proteinase K (0.2 mg ml⁻¹) to minimize RNase contamination before phenol–chloroform–isoamyl alcohol extraction and ethanol precipitation. Linearized templates were dissolved before phenol–chloroform–isoamyl alcohol extraction and ethanol precipitation. Antisense and sense RNA probes were transcribed from linearized cDNA templates using a standard transcription protocol (Promega). The RNA probes were labelled with [³⁵S]-UTP (Amersham Life Science Ltd, Bucks). After transcription, the DNA template was removed by incubation with DNase I (1 U µg⁻¹ template) (Promega) and unincorporated label was removed by centrifugation on Sephadex G-50 spin columns (CP Laboratories, Hertfordshire).

In situ hybridization

Frozen sections (14 µm) of ovarian tissue were dehydrated, fixed and probed with [³⁵S]-labelled IGF-I, IGF-II and type 1 IGF receptor riboprobes according to the method described by Yuan et al. (1996) with two modifications as follows: (i) after dehydration, sections were delipidated in chloroform (5 min); and (ii) riboprobes were diluted in hybridization buffer to give 500 000 c.p.m. per 50 µl aliquot (1 × 10⁷ c.p.m. ml⁻¹). After incubation overnight, sections were exposed to a number of washes as described by Yuan et al. (1996) and 3% mercaptoethanol (0.1%, v/v) was added to the washes after RNase treatment.

Sections were dehydrated through a graded series of alcohols, air dried and dipped in autoradiographic emulsion (K2; Ilford Ltd, Mobberley) and exposed for 3 weeks at 4°C. After exposure, sections were developed (D-19; Kodak, IBI Ltd, Cambridge) and fixed (Hypam fix; Ilford Ltd) before counterstaining in haematoxylin and eosin. Sections were mounted (DPX mountant, R.A Lamb, London) and examined by bright and dark field microscopy.

Image analysis

Antisense RNA probes for IGF-I, IGF-II and the type 1 receptor were each hybridized to two serial sections per corpus luteum or ovary. The sense probe for each mRNA species was applied to a further serial section. The intensity of the in situ hybridization signal was quantified using an NIH-Image analysis system (NIH, Bethesda, MD). Four fields of view were chosen at random within each slide and the number of pixels occupied by silver grains was counted. The results were presented as a percentage of the total number of pixels within the defined area and antisense signals were compared with their respective sense (background) signals.

Statistical analysis

In situ hybridization data were analysed using a split-plot ANOVA (Genstat 4.1) to determine the effect of stage of the oestrous cycle (treatment factor) on mRNA expression. Measurements made within slides were nested within cows for use as the blocking factor. Significant differences among time points were tested using least significant differences with a pooled standard error of the difference (SED) determined by ANOVA. Differences between stages were considered significant when P < 0.05.

Results

Expression of IGF-I mRNA

mRNA encoding IGF-I was expressed in corpora lutea at all time points studied. Expression of IGF-I mRNA was low and widespread, but increased towards the periphery and in lines radiating through the corpus luteum (Fig. 1). The exact identity of these cells could not be determined, although they were not clearly associated with any major structural component of the corpus luteum, for example blood vessels. Concentrations of IGF-I mRNA varied significantly with the day of the oestrous cycle (p < 0.05) (Fig. 2a). Steady-state IGF-I mRNA concentrations were significantly higher on day 15 than on day 10, and the IGF-I expression observed in the regressing corpus luteum 48 h after prostaglandin administration was significantly greater than IGF-I concentrations detected in the early and mid-luteal phase (days 5 and 10).

Expression of IGF-II mRNA

mRNA encoding IGF-II was present in corpora lutea at all time points studied. IGF-II mRNA was localized to a subset of steroiogenic luteal cells and was also present in cells associated with blood vessels of the corpus luteum (Fig. 3). Quantitative analysis showed no significant effect of day of the oestrous cycle on expression of IGF-II mRNA (Fig. 2b).

Expression of type 1 IGF receptor mRNA

At all time points studied, corpora lutea expressed mRNA encoding the type 1 IGF receptor. The hybridization signal was widespread and the pattern of hybridization indicated that expression was localized to both small and large luteal cells (Fig. 4). Expression was absent from peripheral (stromal or capsular) regions and some large blood vessels. Statistical
analysis revealed no significant changes in expression of mRNA encoding the type 1 IGF receptor throughout the luteal phase (Fig. 2c).

**Discussion**

The present study describes the temporal and spatial changes in mRNAs encoding IGF-I and -II and the type 1 IGF receptor during growth and regression of the bovine corpus luteum. It extends the current understanding of the IGF system in the bovine corpus luteum and is the first detailed report of the localization of mRNAs encoding IGF-I, IGF-II and the type 1 IGF receptor throughout the luteal phase.

The expression of mRNA encoding IGF-I, IGF-II and type 1 IGF receptor showed distinct spatial patterns of expression within the bovine corpus luteum. Expression of IGF-I mRNA was low throughout the corpus luteum, but increased
IGF expression in the bovine corpus luteum

In addition, follicular patterns of expression of mRNA encoding IGF-I, IGF-II and the type 1 IGF receptor are consistent with the observations of Armstrong et al. (2000).

IGF-I and IGF-II have the ability to regulate ovarian function, and the expression of mRNA and protein components of the IGF system within the ovaries of a range of species has led to the hypothesis that the IGF system can exert its influence at the autocrine and paracrine levels. Although studies of the local production of components of the IGF system are less complete for the corpus luteum than the follicle, the bovine corpus luteum has been shown to express IGF-I mRNA (Einspanier et al., 1990; Vandehaar et al., 1995; Kirby et al., 1996), and IGF-I and -II proteins have also been localized in the bovine corpus luteum (Amselgruber et al., 1994). Moreover, binding that is characteristic of the type 1 IGF receptor has been demonstrated in the corpus luteum throughout the luteal phase (Sauerwein et al., 1992).

mRNA encoding IGF-I, IGF-II and the type 1 IGF receptor has been localized in the bovine corpus luteum at uncharacterized stages of the oestrous cycle (Perks et al., 1999). The present study extends the understanding of the role of the IGF system in bovine luteal function by the use of corpora lutea collected at precise time points throughout the luteal phase.

In the present study, mRNA encoding IGF-I was demonstrated in the corpus luteum throughout the oestrous cycle. However, the temporal changes in expression of IGF-I mRNA in the ruminant corpus luteum remain controversial. A study of the presence of IGF-I mRNA in the bovine corpus luteum by northern blot hybridization demonstrated low expression in the early luteal phase (days 1–5), which increased from day 6 to day 11 and reached a maximum on days 12–17, before decreasing rapidly at the time of natural luteolysis (days 18–21) (Einspanier et al., 1990). In contrast, in the present study, IGF-I mRNA concentrations were low in the early and mid-luteal phase (days 5 and 10), increased significantly between day 10 and day 15, and reached a maximum after prostaglandin-induced luteal regression. The increase in IGF-I mRNA between early and mid-luteal groups observed by Einspanier et al. (1990) is not supported by the results of the present study. Differences in methodology may partly explain differences in IGF-I mRNA expression patterns, as analysis of IGF-I mRNA expression by northern blot hybridization is complicated by the similar size of major luteal IGF-I mRNA transcript(s) (3–5 kb) and the 28S ribosomal RNA (4.7 kb). The differences in IGF-I mRNA expression observed between the present study and that of Einspanier et al. (1990) might also be due to differences in tissue collection. The early luteal phase group of Einspanier et al. (1990) were corpora lutea at 1–5 days after ovulation as determined by macroscopic observations, and therefore probably included younger corpora lutea than were used in the present study in which an oestrous synchronization regimen was used to collect luteal tissue of a known age. If luteal IGF-I mRNA concentrations are low before day 5 this would not be reflected by the first time point of the present study, but would result in an increase between the early and mid-luteal groups in the study by Einspanier et al. (1990). Indeed, luteal IGF-I mRNA concentrations were shown to increase between day 3 and

towards the periphery and in lines radiating through the corpus luteum. In contrast, mRNA encoding IGF-II was found in a subset of steroidogenic luteal cells and also in association with luteal blood vessels. mRNA encoding type 1 IGF receptor was widely expressed throughout the corpus luteum in a pattern indicative of expression in small and large luteal cells, but was absent from peripheral regions of the corpus luteum and some large blood vessels. Although mRNA encoding IGF-I, IGF-II and the type 1 IGF receptor differ spatially within the bovine corpus luteum, only IGF-I mRNA showed significant temporal changes in expression.

Fig. 2. Quantitative analysis of insulin-like growth factor I (IGF-I) (a), IGF-II (b) and the type 1 IGF receptor (c) mRNA expression in bovine luteal tissue on days 5, 10 and 15 of the oestrous cycle, and after exogenous prostaglandin (after PG). Luteal time points with different letters are significantly different ($P < 0.05$). Bars represent pooled standard error of the difference (SED). mRNA expression is expressed as the percentage of the total number of pixels occupied by silver grains within a defined area.
Fig. 3. Bovine ovarian sections (14 μm) were probed with an insulin-like growth factor II (IGF-II) antisense (a–h) or sense (i,j) riboprobe, and the same fields of view are shown under light field (a,c,e, g,i) and dark field (b,d,f,h,j) illumination. Localization of mRNA encoding IGF-II is shown in a typical corpus luteum at three different magnifications (a–f). Follicular expression is shown in theca tissue of a small follicle (g,h). The asterisk in (c) shows a blood vessel. G: granulosa; T: theca cells. Scale bars represent 180 μm (a,b,g,h), 90 μm (c,d,i,j) and 45 μm (e,f).
day 6 of the ovine oestrous cycle (Juengel et al., 1997), although in other studies limited variation throughout the ovine oestrous cycle was observed (Perks et al., 1995).

An important regulatory role for IGF-I in the corpus luteum is indicated by IGF-I-stimulated progesterone release in vitro, and is supported by positive immunostaining for IGF-I in both large and small steroidogenic luteal cells (Amselgruber et al., 1994). The present study confirmed the expression of IGF-I mRNA in the bovine corpus luteum, which indicates that IGF-I may act as an autocrine and paracrine regulator of luteal function. The relationship between increasing IGF-I mRNA and peak progesterone production (Ireland et al., 1980; Fields and Fields, 1996) implies a physiological significance for locally produced IGF-I within the bovine corpus luteum. Increased IGF-I expression during periods of high metabolic activity may

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**Fig. 4.** Bovine ovarian sections (14 μm) were probed with a type 1 insulin-like growth factor (IGF) receptor antisense (a–f) or sense (g,h) riboprobe, and the same fields of view are shown under light field (a,c,e,g) and darkfield (b,d,f,h) illumination. Localization of mRNA encoding the type 1 IGF receptor is shown in the same corpus luteum at two magnifications (a–d) and in luteal tissue adjacent to a small follicle (e,f). CL: corpus luteum; G: granulosa cells; T: theca cells. Scale bars represent 450 μm (a,b), 180 μm (c,f) and 90 μm (c,d,g,h).
regressive changes will have occurred (Juengel et al., 1993). As indicated in ewes (Perks et al., 1997) was not associated with any significant alteration in the expression of IGF-I mRNA for up to 24 h after administration of PGF2α. Furthermore, the expression of IGF-I mRNA was higher in the corpus albicans than in the corpus luteum of ewes (Perks et al., 1995) and increased twofold after prostaglandin-induced luteal regression in pregnant rats (Tamada et al., 1995). The demonstration of a rapid decrease in IGF-I expression and content on days 18–21 of the oestrous cycle (Einspanier et al., 1990) indicates that bovine luteolysis is associated with reduced IGF-I action. Since numerous stimulatory effects of IGF-I have been shown in ovarian cells in vitro (Giudice, 1992; Spicer and Echternkamp, 1995), the reduction of IGF-I expression in vivo might be expected to influence luteal function both directly, and indirectly by modulating the trophic support of LH, therefore contributing to the demise of the corpus luteum. In addition, decreased IGF-I concentrations and the subsequent abrogation of type 1 IGF receptor activation may also influence apoptotic cascades. However, studies to date have not addressed the potential of reduced IGF-I stimulation as a proximal cause of luteal regression and cell death.

In the present study maximum IGF-I mRNA expression was observed 48 h after exogenous prostaglandin administration, at which time both functional and structural regressive changes will have occurred (Juengel et al., 1993). As indicated in ewes (Perks et al., 1995) the high expression of IGF-I mRNA may be due to immune cell activity during the cellular destruction and phagocytosis associated with structural luteal regression (Pate and Townsend, 1994; Penny et al., 1998). IGF-I is produced by immune cells (Rappolee et al., 1988; Baxter et al., 1991), it affects immunoregulation extensively (Renier et al., 1996) and stimulates wound healing (Suh et al., 1992). Identification of the cellular source of IGF-I mRNA expression in the regressing corpus luteum will further the understanding of the role of IGF-I in tissue remodelling.

The demonstration of local ovarian IGF-I mRNA production in other studies has favoured IGF-I rather than IGF-II as an autocrine and paracrine intra-luteal regulator. The present study demonstrates that the bovine corpus luteum is a site of high IGF-II mRNA expression, which is in agreement with results from large bovine corpora lutea at undefined stages (Perks et al., 1999). Although in the present study no change in expression of IGF-II mRNA was detected throughout the luteal phase, the results highlight the importance of locally produced IGF-II and indicate a major role in luteal function. However, it remains unclear whether the ratio of IGF-I:IGF-II has an important influence on luteal function or whether the growth factors serve different but crucial functions in different types of cell.

The intense hybridization for IGF-II mRNA in the luteal vasculature is in agreement with immunohistochemical observations (Amselgruber et al., 1994) that localized IGF-II protein to perivascular cells. The site of IGF-II mRNA expression was not fully determined in the present study. However, the hybridization signal was not limited to endothelial cells lining the vessels, and pericytes, fibroblasts or smooth muscle cells may contribute to IGF-II mRNA expression. Whether the association of IGF-II with blood vessels reflects a role for IGF-II in controlling angiogenesis, luteal blood flow or interactions between vascular and steroidogenic cells requires further investigation. Before the present study, the interest in the potential role of IGFs in the corpus luteum was based primarily on the ability of IGFs to stimulate luteal steroid biosynthesis. However, for IGF-II in particular, additional functions and targets should now be considered.

The IGFs exert their effects by interacting with cell surface receptors. The type 1 IGF receptor is activated by both IGF-I and IGF-II binding. The presence of the type 1 IGF receptor within the bovine corpus luteum has been indicated by competitive binding characteristics, as well as by the ability of IGF-I (and to a lesser degree IGF-II) to modulate luteal function. The expression of mRNA encoding the type 1 IGF receptor throughout the luteal phase, the results highlight the importance of locally produced IGF-II and indicate a major role in luteal function. However, it remains unclear whether the ratio of IGF-I:IGF-II has an important influence on luteal function or whether the growth factors serve different but crucial functions in different types of cell.

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receptors will be necessary to increase the understanding of the roles of IGF-I and IGF-II in regulating bovine corpus luteum function.

In conclusion, the present study (i) describes the temporal and spatial patterns of IGF-I, IGF-II and type 1 IGF receptor mRNA expression in the bovine corpus luteum throughout the luteal phase; (ii) demonstrates that the bovine corpus luteum is a site of IGF production and reception; and (iii) highlights the potential importance of IGF-II, in addition to IGF-I, in bovine luteal function.

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