Expression and localization of relaxin in the ovary of the mare

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Immunoreactive, chromatographic and molecular techniques were used to study the expression of relaxin in mare ovaries at different stages of the oestrous cycle. Relaxin in follicular fluid ranged from 1.6 to 2.5, from 1.4 to 5.2, from 1.2 to 6.7 and from 1.0 to 3.5 ng ml⁻¹ in small (≤ 2 cm), medium ($> 2 \leq 3$ cm), medium-large ($> 3 \leq 4$ cm) and large (> 4 cm) follicles, respectively, and total content of fluid relaxin per follicle increased ($P < 0.05$) with follicular size. When subjected to reverse phase HPLC analysis, follicular fluid yielded absorbance profiles corresponding closely to those of purified relaxin, and immunoreactive peaks in follicular fluid fractions measured by radioimmunoassay matched peaks of the relaxin standard. While relaxin was localized immunocytochemically to granulosa and theca cells of preovulatory follicles, northern blot and reverse transcriptase-PCR followed by Southern blot analysis failed to detect a relaxin transcript in these tissues. A single relaxin transcript (428 bp) corresponding to mRNA encoding relaxin was identified in early, mid- and late stage corpora lutea but not in corpora haemorrhagica or albicantia. Northern blot analysis revealed a weakly expressed 1 kb transcript in total cellular RNA from mature corpora lutea. *In situ* hybridization studies localized the mRNA to the large luteal cells of mature corpora lutea and relaxin protein was detected by immunocytochemistry in the same tissue. This is the first report demonstrating relaxin in the equine ovary and its expression by luteal cells, thereby suggesting a role for relaxin in follicular or corpus luteum function in cyclic mares.

Introduction

Relaxin is a peptide hormone associated with pregnancy (Sherwood, 1994), but has also been shown to be present in the follicles of pigs (Bryant-Greenwood *et al.*, 1980; Matsumoto and Chamley, 1980) and in the human ovary (Noci *et al.*, 1983; Wathes *et al.*, 1986) during the cycle. The localization of relaxin in follicular structures led to the hypothesis that the hormone may have a paracrine role in the regulation of follicular function (Bryant-Greenwood, 1982). In pig ovarian follicles, relaxin has been localized to the theca tissue by immunoreactive (Evans *et al.*, 1983) and immunocytochemical (Bagnell *et al.*, 1987; Denning-Kendall *et al.*, 1989) studies. The theca interna has since been identified as the source of pig follicular relaxin by molecular analysis (Bagnell *et al.*, 1990a). In human preovulatory follicles, relaxin gene expression and immunoreactive relaxin studies have also identified the theca cells as the source of the hormone (Lee *et al.*, 1991). Relaxin has also been localized by immunocytochemical and molecular techniques in the corpus luteum of the cycle and pregnancy in pigs (Fields

and Fields 1985; Denning-Kendall *et al.*, 1989; Ali *et al.*, 1986; Bagnell *et al.*, 1989, 1990b, 1993) and in the human ovary (Ivell *et al.*, 1989; Stoelk *et al.*, 1991).

Since considerable tissue remodelling occurs in the mammalian ovary during follicular growth and ovulation, relaxin may be involved in the breakdown of the follicle wall through stimulation of the secretion of proteolytic enzymes such as plasminogen activator, proteoglycanase, and collagenase (Too *et al.*, 1984). Relaxin is known to soften collagenous tissues in the pelvic symphysis and the cervix (for review see Sherwood, 1994), and dermal fibroblasts are claimed to be targets of the hormone (Unemori and Amento, 1995). Relaxin has also been shown to modulate synthesis and secretion of procollagenase and collagen in human dermal fibroblasts (Unemori and Amento, 1990; Unemori *et al.*, 1992, 1993) and of collagens synthesized by rabbit chondrocytes (Bonaventure *et al.*, 1988). Relaxin has also been demonstrated to act as a growth factor in porcine follicles by increasing both theca and granulosa cell DNA synthesis and proliferation *in vitro* (Zhang and Bagnell, 1993, 1994; Ohleth and Bagnell, 1995).

The ovary of the mare presents a unique opportunity to study the possible intraovarian actions of relaxin in the process of follicular growth and ovulation. The mare is distinct among domestic animals in that the ovary has an inverted anatomy (cortex within medulla) with a tough tunica albuginea so that

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the unusually large preovulatory follicle (30–49 mm) must rupture through a thick wall of collagenous tissue at a specialized site known as the ovulation fossa (Stabenfeldt *et al.*, 1975; Ginther, 1992). Follicular rupture at the fossa is a distinct feature of ovulation in mares and probably requires specialized local mechanisms to ensure that the follicle migrates to, or grows toward, the fossa (Ginther, 1992). Little is known about the mechanisms associated with follicle migration and ovulation in mares, but they almost certainly involve softening or remodelling of the ovarian stroma to facilitate passage of the follicle to the fossa and thereby successful rupture. Equine relaxin has been purified and characterized by Stewart and Papkoff (1986). During pregnancy, the equine placenta is the primary source of the hormone (Stewart *et al.*, 1982) and placental trophoblast cells have been identified as the specific site of relaxin synthesis and secretion by Klonisch *et al.* (1995, 1997). Therefore, the aim of this study was to determine, using immunoreactive and molecular techniques, whether the equine ovary expresses relaxin during the ovarian cycle. We report here the identification and localization of equine relaxin in the preovulatory follicle and corpus luteum of the cyclic mare.

Materials and Methods

Reagents

All chemicals and molecular supplies were purchased from Sigma Chemicals (St Louis, MO) and Gibco-BRL Life Technologies (Burlington, ON), respectively. Purified equine relaxin and relaxin antisera (number 3150) raised in rabbit were generously provided by D. R. Stewart (University of California at Davis, CA). Non-immune rabbit serum was used as a control for relaxin antibody specificity and immunocytochemical studies were performed using a streptavidin–biotin system (Histostain kit[®]; Zymed Laboratories, San Francisco, CA).

Ovarian tissues

Ovaries from mature, cyclic mares were collected from a local abattoir (Barton Feeders, Owen Sound, ON) during the months of April–September or from the Ontario Veterinary College at necropsy. The reproductive history of the mares was unknown. Ovaries were transported to the laboratory on ice in Hanks' balanced salt solution with gentamicin ($5 \mu\text{g ml}^{-1}$) or snap-frozen in liquid nitrogen at the time of collection. Follicle size was determined (before freezing) by measuring the greatest distance between opposite follicle walls. Follicular fluid was aspirated from small (≤ 2 cm), medium ($> 2 \leq 3$ cm), medium–large ($> 3 \leq 4$) and large (> 4 cm) follicles and stored at -20°C . Healthy and atretic follicles were distinguished morphologically according to the criteria of Moor *et al.* (1978). For example, follicles with cloudy or blood-tinged fluid were excluded from the study. Stage of corpora lutea development was assessed by gross and microscopic morphological examination and described as follows: early (days 1–2 after ovulation), maturing (days 3–7), mature (days 9–11) and ageing (days 15–17) corpus luteum (Levine *et al.*, 1979; Ginther, 1992). Haemorrhagic corpora lutea were identified by the presence of a bloody clot, a distinct luteal papilla at the

ovulation fossa and having palpable characteristics similar to those of preovulatory follicles (Ginther, 1992), while later stages of luteal development were identified using the histological criteria of Levine *et al.* (1979). Tissue samples for immunocytochemistry were fixed (10% (v/v) formalin in PBS, pH 7.4) and embedded in paraffin wax, while samples for protein and molecular studies were snap-frozen upon recovery (less than 20 min after slaughter) and stored at -80°C .

Extraction and HPLC analysis of follicular fluid

Fluid samples were separated by HPLC chromatography using a modification of the method described by Stewart *et al.* (1991) to confirm that immunoreactive relaxin was equine relaxin in follicular fluid. Proteolysis was reduced by subjecting follicular fluid samples to octadecylsilica purification as described by Walsh and Niall (1980). Briefly, pooled or individual follicular fluid samples (20 ml) from follicles (2, 3, 4 or ≥ 5 cm diameter) were diluted with an equal volume of distilled, deionized water (DDH_2O) and filtered ($0.45 \mu\text{m}$ filter size). Diluted samples were passed through activated (with 10 ml methanol followed by 0.1% (v/v) trifluoroacetic acid (TFA)) C18 cartridges (two in series; Sep-Pak, Waters Associates, Mississauga, ON). Fractions were then eluted with increasing concentrations (23%, 40% and 80%) of acetonitrile in 0.1% (v/v) TFA. Eluted fractions were then evaporated to near dryness, redissolved in 10 ml DDH_2O and dialysed overnight (3 kDa cut-off). Samples were then lyophilized and resuspended in 250 μl of 0.1% (v/v) TFA. Aliquots (25 μl) of each fraction (23%, 40% and 80% acetonitrile eluates) were analysed on a Waters HPLC system using an 8 mm $\times$ 10 cm Waters Radial Pak (8NVC₁₈–4 μ) column with a linear gradient of 25–30% acetonitrile in 0.5% (v/v) TFA over 45 min. Fractions (1 ml) were collected at 1 min intervals (or pooled, 10–40 min), lyophilized and resuspended in 200 μl DDH_2O . Seventy microlitres of the suspension were diluted to a total volume of 350 μl with assay buffer of which 100 μl was taken, in triplicate, for relaxin radioimmunoassay. Chromatograms detected at 280 nm (Waters Model 441 UV detector) were retrieved and analysed using a Waters Maxima and Baseline 810 chromatography work station (Waters, Mississauga, ON). HPLC analysis was repeated at least twice per sample and representative profiles are shown.

Radioimmunoassay for equine relaxin

Follicular fluid samples were analysed directly by radioimmunoassay for relaxin content. Relaxin radioimmunoassay measurements were performed on follicular fluid samples and material eluting from HPLC columns by a homologous equine relaxin assay as described by Stewart (1986). Purified equine relaxin was labelled with tracer (^{125}I -labelled sodium iodide; Amersham, Oakville, ON) using the Chloramine-T method (Stewart, 1986). The antiserum (number 3150) was used at a dilution of 1:20 000, giving approximately 30% binding when using 20 000 counts tracer min^{-1} . Pregnant mare and gelding plasma were used as positive and negative controls. Sensitivity of the assay was 0.37 ng ml^{-1} and the intra- and interassay coefficients of variation were 7.5% and 14%, respectively.

Northern and Southern blot analyses

Northern and Southern blot analyses were performed as described by Klonisch *et al.* (1995). Briefly, total cellular RNA (tcRNA) was extracted from follicles (2, 3, 4 or ≥ 5 cm diameter), corpora lutea at early, middle and late stages of development, and corpora albicantia according to the method of Chomczynski and Sacchi (1987). Two or more structures from different animals were examined in each follicle and luteal category. For northern analysis, the quantity and purity of the tcRNA was determined spectrophotometrically at 260 and 280 nm and by ultraviolet light visualization of ethidium bromide-stained agarose-formaldehyde gels. Total cellular RNA (20 μ g) and molecular size marker (0.44–1.77 kb RNA ladder) were separated by electrophoresis in 1% (w/v) agarose-formaldehyde gels and transferred onto charged nylon membranes (Zeta-Probe; Bio-Rad, Mississauga, ON) by the downward alkaline blotting technique for 1.5 h (Chomczynski, 1992). After blotting, nylon membranes were bound by UV irradiation for 1 min, washed for 10 min in $4 \times$ SSC (20 $\times$ SSC, 3 mol NaCl l^{-1} , 0.3 mol CH_3COONa l^{-1} , pH 7.0) and dried for 2 h at 80°C. Membranes were either used immediately or sealed in hybridization bags and stored at 4°C. Total cellular RNA from mare placenta (120 days gestation) served as a positive control for equine mRNA encoding relaxin.

For Southern blots a 123 bp ladder was used as a marker and amplified cDNA products (follicular and luteal) were separated on a 2% (w/v) Tris glacial acetic acid-EDTA (TAE) agarose gel. The gel was then denatured for 30 min in 0.5 mol NaOH l^{-1} , 1.5 mol NaCl l^{-1} and then incubated for 15 min in blotting buffer before transfer onto nylon membrane as described for northern blotting. For both northern and Southern blotting, nylon membranes were prehybridized for 30 min at 70°C in Rapid Hyb-buffer (Amersham, Oakville, ON). Thereafter, the prehybridization solution was replaced with an equal volume of the same solution containing approximately 300 ng digoxigenin-labelled cRNA probe ml^{-1} (Klonisch *et al.*, 1995) and hybridized overnight at 70°C in a shaking water bath. Membranes were then washed 2×15 min in $2 \times$ SSC + 0.1% (w/v) SDS at room temperature, then twice in $0.5 \times$ SSC + 0.1% (w/v) SDS at 55°C for 20 min, respectively. The digoxigenin-labelled cRNA probe used in all hybridization studies was prepared according to the manufacturer's instructions (Boehringer-Mannheim, Laval, PQ) and the hybridization signals were visualized by immunocytochemistry and alkaline phosphatase staining. Northern and Southern blot analyses were repeated at least three times. Results were similar in all experiments and representative images for Southern blotting are presented.

Reverse transcriptase-PCR

DNase-digested tcRNA (10 μ g) from follicles and corpora lutea was reverse transcribed into cDNA by Superscript[™] reverse transcriptase (kit; Gibco-BRL) and either 1 μ l (500 ng ml^{-1}) of the reverse primer or oligo d(T_{12–18}) as primer. RT-PCR was performed with and without the addition of an RNaseH digestion step after first strand cDNA synthesis and before PCR (Yoon and Glawes, 1993).

For RT-PCR, 10% of the cDNA reaction was mixed in a final volume of 50 μ l with $1 \times$ Taq polymerase buffer (20 mmol Tris-HCl l^{-1} (pH 8.4) 50 mmol KCl l^{-1}), 2.5 mmol MgCl₂ l^{-1} , 1 μ l (500 ng ml^{-1}) the following set of degenerate, oligonucleotide primers: forward primer, 5'ATTAARGCM TGGGCGCGMGAATT3'; reverse primer, GTRCAACCC CARTARCARCATT3', 200 μ mol dNTP l^{-1} and 2.5 U Taq-polymerase. The reaction was overlayed with 50 μ l light mineral oil. The PCR conditions consisted of an initial denaturing step at 94°C for 5 min, followed by 40 cycles with 1 min denaturing at 94°C, 2 min annealing at 60°C, 2 min elongation at 72°C, and a final 10 min elongation at 72°C. Twenty per cent of these reaction mixtures (PCR amplification products) was run on a 2% (w/v) agarose gel in TAE buffer.

In situ hybridization

Frozen sections (8 μ m) of OCT-embedded (Miles Laboratories, Elkhardt, IN) ovarian tissue were mounted on slides coated with 2% (v/v) 3-aminopropyltri-ethoxy-silane (APTEx; Sigma, St Louis, MO) in acetone. Sections were fixed with 4% (w/v) paraformaldehyde in PBSM (0.2 mol phosphate buffered saline l^{-1} , 5 mmol MgCl₂ l^{-1}) for 15 min at room temperature, washed for 5 min in PBSM and stored in 70% ethanol at 4°C until further processing. The sections were rehydrated in PBSM for 10 min and then processed according to the method of Lewis and Wells (1992). Digoxigenin-labelled cRNA in the tissue sections was detected after overnight incubation with an anti-digoxigenin, alkaline phosphatase conjugated antibody (Boehringer Mannheim, Laval, PQ) at a dilution of 1:1000 in TNMT buffer (100 mmol Tris-HCl l^{-1} , (pH 7.5), 100 mmol NaCl l^{-1} , 2 mmol MgCl₂ l^{-1} , 0.05% (v/v) Triton X-100). After counterstaining with 0.2% (v/v) fast green (Hilbelink and Kaplan, 1978), the slides were mounted in glycerol gel and examined under bright-field microscopy.

Immunocytochemistry

Immunolocalization of equine relaxin in ovarian tissue was performed using the streptavidin-biotin immunoperoxidase system. Sections (10 μ m) of fixed, paraffin wax-embedded ovarian tissue were mounted on slides coated with 2% (v/v) APTEx and allowed to dry overnight in air. After deparaffinization and rehydration, tissue sections were pretreated with 0.5% (v/v) peroxide in methanol for 10 min, to inactivate endogenous peroxidase activity, and with 10% (v/v) normal goat serum (NGS) for 30 min, to saturate nonspecific binding sites. Tissue sections were then incubated with rabbit anti-horse relaxin antibody (number 3150, 1:500 in 1% (v/v) NGS in PBS l^{-1}) or normal rabbit serum (1:500 in 1% v/v NGS in PBS l^{-1}) for 30 min as a negative control. After three 2 min washes in PBS, sections were incubated with a biotinylated goat anti-rabbit IgG for 30 min. All incubations with serum and antibodies were performed in a moist chamber at room temperature. Peroxidase-dependent staining was demonstrated by incubation with 3,3'-diaminobenzidine (DAB) containing 0.03% (v/v) hydrogen peroxide in PBS for 3–5 min. After counterstaining with haematoxylin for 6 min, slides were mounted with glycerol gel and examined by bright-field microscopy. Near-term placenta was used as a positive control

Table 1. Relaxin content of mare ovarian follicular fluid from follicles of different sizes

Category (cm)	<i>n</i>	Concentration (ng ml ⁻¹)	Total amount (ng)	Follicle size (cm)	Fluid volume (ml)
Small (≤2)	9	2.1 ± 0.3 ^a	7.9 ± 1.3 ^a	1.9 ± 0.06 ^a	3.8 ± 0.1 ^a
Medium (>2 ≤3)	15	2.5 ± 0.3 ^a	18.6 ± 2.2 ^a	2.8 ± 0.06 ^b	7.4 ± 0.6 ^b
Medium-large (>3 ≤4)	13	3.1 ± 0.5 ^a	60.5 ± 9.7 ^b	3.8 ± 0.07 ^c	20.1 ± 1.4 ^c
Large (>4)	16	2.4 ± 0.3 ^a	91.5 ± 9.2 ^c	5.3 ± 0.2 ^d	37.6 ± 1.5 ^d

Values (mean ± SE) in a column that do not share a common superscript are significantly different ($P < 0.05$).

for *in situ* hybridization and immunocytochemistry staining studies (Klonisch *et al.*, 1995). Two or more structures from different animals were examined in each follicle and luteal category and staining was repeated on at least two separate occasions.

Statistical analysis

Relaxin content of follicular fluid is presented as the mean ± SE in ng ml⁻¹ or as the mean ± SE of the total follicular fluid content per follicle for each follicle size group. Statistical analysis was performed with the StatView 4.5 application (Abacus Concepts, Berkeley, CA) run on an Apple Macintosh computer (Apple Computer Inc., Cupertino, CA). Data were analysed by analysis of variance and tested for difference by Fisher's protected least square difference (PLSD) and Bonferroni-Dunn test for multiple comparisons.

Results

Relaxin content of follicular fluid

Follicular fluid from follicles of different sizes was analysed for relaxin content by radioimmunoassay directly (Table 1). While relaxin content of follicular fluid on a ng ml⁻¹ basis did not differ ($P > 0.05$) between small, medium, medium-large and large follicles, total follicular fluid relaxin content on a per follicle basis, increased ($P < 0.05$) with follicle size (Table 1). After extraction and elution from Sep-Pak columns with 23, 40 and 80% acetonitrile, HPLC absorbance profiles of follicular fluid revealed that the 40% acetonitrile fraction was similar to that of a purified equine relaxin standard at 30% (Fig. 1a-d). Radioimmunoassay analysis detected immunoreactive relaxin in fractions from a large follicle, eluting between 10 and 25 min, and these immunoreactive peaks corresponded closely to those of the chromatogram (Fig. 1d). However, there were immunoreactive peaks observed between 25 and 35 min that did not correspond to any absorbance peaks (data not shown). In one instance, serous fluid (1 ml total) was recovered from a corpus haemorrhagicum, and the relaxin content of fluid on a ng ml⁻¹ basis was approximately six times higher in the pooled HPLC eluate (10-40 min) of the 40% acetonitrile extract fraction than that observed in the same eluate of fluid

from a large follicle (approximately 5 cm; 10.7 ng ml⁻¹ and 1.8 ng ml⁻¹, respectively).

Northern blot analysis

Total cellular RNA from equine follicles (≥2 cm in size) and corpora lutea at various stages of luteal development were subjected to Northern blot analysis with a digoxigenin-labelled antisense cRNA probe for equine relaxin. A weak hybridization signal at 1 kb was detected with tcRNA from mid- (approximately day 9) and late stage corpora lutea (approximately day 14), while near-term placenta gave a strong signal as a positive control (data not shown). Follicle and corpus albicans tcRNA did not yield a detectable hybridization signal.

RT-PCR and Southern blot analysis

RT-PCR was used to amplify transcripts of mRNA encoding relaxin in follicles, corpora lutea and corpora albicantia specimens (Fig 2a). The presence of a single amplification product of approximately 428 bp, using primer sets corresponding to the A- and B-chain of equine relaxin, was detected in maturing (days 3-7, lanes 7-8), mature (days 9-11, lane 9) and ageing (days 15-17, lanes 10-11) corpora lutea. A weak signal was also detected in an early stage corpus luteum (1-2 days after ovulation, lane 6). No signal was evident in any of the follicular structures examined (lanes 2-5) or in the aged corpus albicans (lane 12). A strong signal was detected at 428 bp from mare placenta (approximately 120 days gestation, lane 13) which was included as a positive control (Klonisch *et al.*, 1995). No amplification product was detected in the absence of reverse transcriptase or when tcRNA was omitted from the reaction (data not shown). Southern blot analysis using a previously characterized clone for equine relaxin (Klonisch *et al.*, 1995) identified the PCR products amplified in Fig. 2a as fragments encoding equine relaxin (Fig. 2b).

For testing the hypothesis of local expression of mRNA encoding relaxin in large follicles, tcRNA was isolated from three large, intact follicles (5-6 cm) and processed for RT-PCR. No PCR product was evident after gel electrophoresis and no transcript could be detected upon Southern blot analysis from these follicles (data not shown).

Fig. 1. Reverse phase chromatograms of purified equine relaxin and follicular fluid from mare ovarian follicles. Absorbance (280 nm) profiles of (a) purified relaxin standard and (b–d) follicular fluid extracts from follicles about 2, 3, and 5.5 cm diameter, respectively, are presented. Profiles represent the 40% acetonitrile fraction eluted off Sep–Pak C18 columns. A linear gradient was run from 25% to 30% acetonitrile/0.5% trifluoroacetic acid (v/v) over 60 min with the 10–40 min segment displayed. Relaxin immunoreactive values (pg) corresponding to the major absorbance peaks observed in fluid from a large follicle are indicated in (d). Profiles shown in (b) and (c) are the results of fluid pooled from three or more follicles.

In situ hybridization

Digoxigenin-labelled sense and antisense cRNA probes specific for equine relaxin were used for *in situ* hybridization

studies (Klonisch *et al.*, 1995). Specific hybridization for mRNA encoding relaxin, observed in cryostat sections of corpora lutea from approximately 3–17 days after ovulation, was confined to single cells and restricted to luteal gland tissue (Fig. 3a). No

Fig. 2. A representative Southern blot of RT-PCR analysis of relaxin in mare ovarian structures. (a) Total cellular RNA, isolated from ovarian follicles and corpora lutea, was reverse transcribed and amplified using degenerate primers encoding equine relaxin. Lane 1: 123 base pair molecular mass ladder; lanes 2, 3, 4 and 5: 2, 3, 4 and > 5 cm follicles, respectively; lane 6: early corpus luteum; lanes 7–8: days 3–7 corpus luteum; lane 9: day 9–11 corpus luteum; lanes 10–11: days 15–17 corpus luteum; lane 12: aged corpus albicans; lane 13: equine placenta (day 120 of gestation). (b) The corresponding Southern blot was probed with a digoxigenin-labelled antisense cRNA derived from Bluescript clone containing a PCR fragment encoding equine relaxin.

labelling was observed in serial tissue sections using the digoxigenin-labelled sense probe (Fig. 3b). Sections of tissue from follicles, regressed corpora lutea and corpora albicantia did not show any staining for equine mRNA encoding relaxin.

Immunocytochemistry

The secretory product of the transcript localized in the corpus luteum and the immunoreactive product determined in follicular fluid were detected by subjecting sections of corpora lutea and follicles to immunocytochemistry using a rabbit anti-equine relaxin antibody (number 3150; Stewart, 1986). Discrete staining for immunoreactive relaxin was evident in both the theca interna and granulosa cells of mature follicles (Fig. 4a). Staining was most intense in cells of the mature corpus luteum (Fig. 4c). No staining was observed in the ovarian stromal tissue or in serial sections of follicles (Fig. 4b) or corpora lutea (Fig. 4d) when the antibody was substituted with non-immune rabbit serum.

Discussion

This study demonstrates for the first time the expression and localization of relaxin in the ovaries of cyclic mares. Immunoreactive relaxin was detected in fluid from developing and preovulatory follicles and HPLC analysis confirmed that relaxin profiles of follicular fluid from follicles of different sizes were consistent with that of purified relaxin. Stewart *et al.* (1991) identified several isoforms of equine relaxin in affinity-purified preparations after HPLC analysis. In agreement with these findings, we identified several peaks in follicular fluid samples that were immunoreactive and corresponded closely to the relaxin standard. Immunoreactive relaxin has been identified in the ovarian follicular fluid of nonpregnant pigs (Bryant-Greenwood *et al.*, 1980; Matsumoto and Chamley, 1980) and in the fluid of human ovarian follicles (Noci *et al.*, 1983; Wathes *et al.*, 1986). Although relaxin titres in equine follicular fluid were not markedly different among small, medium, medium-large and large follicles, total content increased with the size of the follicle.

Fig. 3. Localization of mRNA encoding relaxin in the mature corpus luteum (about day 9) during the oestrous cycle of the mare, demonstrated by *in situ* hybridization of a digoxigenin-labelled cRNA probe to equine relaxin. (a) Stained cells (arrows) indicating expression of mRNA encoding relaxin are scattered throughout the corpus luteum but are confined to luteal tissue. (b) Adjacent section showing the lack of specific labelling when hybridized with the sense probe. Scale bars represent 133 µm.

Northern analysis and RT-PCR amplification of total cellular RNA from follicles of various sizes failed to identify a signal for mRNA encoding relaxin. The possibility that localized expression of mRNA encoding relaxin was overlooked in follicles was excluded by processing intact, whole follicles (5–6 cm diameter) for RT-PCR. Since low amounts of mRNA encoding relaxin were expected in follicular tissue, samples were pre-treated with RNase after reverse transcriptase to improve PCR amplification of cDNA (Yoon and Glawes, 1993). These approaches, however, failed to produce PCR-amplification products. It is difficult to explain the immunoreactive presence of relaxin in the absence of our ability to detect message in the developing follicles of mares either by northern or Southern blot analysis or with *in situ* hybridization studies. In this study, we used degenerate, oligonucleotide primers (Klonisch *et al.*, 1995) which may not be as sensitive as more specific primers in detecting low copy expression of mRNA encoding relaxin. Non-radioactive labelled probes are less sensitive under low copy gene expression conditions (Bathgate *et al.*, 1996). In contrast, Bagnell *et al.* (1990a) were able to detect a 1 kb transcript of mRNA encoding relaxin in ovarian extracts of prepubertal pigs after gonadotrophin stimulation and from sows on day 13 of the oestrous cycle.

The precise source of ovarian follicular relaxin in mares is not clear. Although molecular approaches failed to identify the site of relaxin synthesis in the mare ovarian follicle, our

immunocytochemical studies localized the protein in both granulosa and theca cells of preovulatory follicles. This is in contrast to the situation in pigs, in which relaxin is found only in the theca cells of developing follicles. The source of relaxin in the pig follicle was determined initially to be the theca cells (Evans *et al.*, 1983) and this was confirmed subsequently by immunocytochemical (Bagnell *et al.*, 1987) and *in situ* hybridization studies (Bagnell *et al.*, 1990a). Whether relaxin is taken up by mare follicular cells from the follicular fluid is not clear. The application of more sensitive *in situ* hybridization techniques may answer this question.

It is possible, in the absence of detectable mRNA encoding relaxin, that the source of relaxin in mare ovarian follicles is the corpora lutea. Reverse transcriptase-PCR followed by Southern blot analyses revealed the presence of mRNA encoding relaxin in the mare corpus luteum and demonstrated that expression of message increased with luteal development, reaching maximal concentrations at about mid-dioestrus, then declining coincidentally with luteal regression. The earliest detectable message for relaxin was obtained from luteal structures consistent with about days 2–3 of dioestrus. No signal was evident in the postovulatory follicle or corpus haemorrhagicum, although nanogram quantities of relaxin could be measured in bloody, serous fluid recovered from the corpus haemorrhagicum. This pattern of relaxin gene expression is similar to that observed in pig corpora lutea during the

Fig. 4. Photomicrographs of immunocytochemical localization of relaxin in the follicle and corpus luteum of the cyclic mare. Formaldehyde-fixed, paraffin wax-embedded tissue sections (10 μ m) from a large (about 5.5 cm) follicle (a, b) and mature (mid-dioestrus) corpus luteum (c, d) were incubated with either a rabbit anti-equine relaxin antibody (a, c) or with non-immune rabbit serum (b, d) as controls. In the follicle (a), discrete but specific brown staining was observed in both the theca interna (T) and granulosa cell (G) layers, while no staining was evident in the ovarian stroma (St). In the corpus luteum (c), staining was much more pronounced in the luteal cells (Lc) but absent in the ovarian stroma (St) and blood vessels (Bv). No staining was evident with non-immune rabbit serum when used as a substitute to the primary (anti-equine relaxin) antibody. The brown colour stain is produced by the action of horseradish peroxidase on 3,3'-diaminobenzidine (DAB) substrate. Slides were counterstained with haematoxylin for contrast. Scale bars in (a) and (b) represent 64 μ m, and in (c) and (d) 80 μ m.

oestrous cycle (Bagnell *et al.*, 1993), in human luteal tissue during the menstrual cycle (Ivell *et al.*, 1989) and that of a relaxin-like factor gene in the bovine corpus luteum of the oestrous cycle (Bathgate *et al.*, 1996). These observations are reflected in the late appearance of relaxin in the utero-ovarian vein and peripheral plasma of pigs and humans (Kotwica *et al.*, 1991; Stewart *et al.*, 1995), usually several days after the preovulatory gonadotrophin surge. The precise timing of the appearance of relaxin in mare utero-ovarian vein blood after ovulation has yet to be determined, although analysis of peripheral blood failed to detect the hormone (Stewart and Stabenfeldt, 1981; Stewart *et al.*, 1992).

Northern analysis indicated the presence of a weak, 1 kb transcript signal in mature corpora lutea. This 1 kb transcript

for equine relaxin is consistent in size with that reported for both the pig (Bagnell *et al.*, 1990b) and human (Ivell *et al.*, 1989) corpus luteum. While relaxin message in the developing follicle and the early corpus luteum (corpus haemorrhagicum) were below the limit of detection under the present experimental conditions, *in situ* hybridization identified luteal cells of mature corpora lutea as the source of the hormone. The scattered appearance of positively labelled cells was confined to luteal tissue. In pigs, *in situ* hybridization analysis demonstrated that in mature, cyclic corpora lutea, message for relaxin was localized in large luteal cells (Bagnell *et al.*, 1993). In addition, there are several lines of evidence in pigs and humans indicating that ovarian relaxin gene expression and production are greatest during the luteal phase of the cycle and decline

after luteolysis (pig, Bagnell *et al.*, 1989, 1993; human, Ivell *et al.*, 1989; Stewart *et al.*, 1995). These reports are consistent with our observations in mares.

Immunocytochemical localization of relaxin in luteal cells confirmed our *in situ* hybridization studies. Immunostaining for relaxin was most intense in luteal cells of the mature corpus luteum. This is consistent with findings in other species (pig, Ali *et al.*, 1986; Bagnell *et al.*, 1989; Denning-Kendall *et al.*, 1989; human, Stoelk *et al.*, 1991), where intensity of staining increased with maturation of the corpus luteum (Ali *et al.*, 1986; Stoelk *et al.*, 1991) and staining was found predominantly in large luteal cells (Taylor *et al.*, 1987; Bagnell *et al.*, 1989).

A paracrine role for relaxin in the regulation of follicular development has been proposed (Bryant-Greenwood *et al.*, 1982). Owing to the unique morphology of the mare ovary (cortex within the medulla), the preovulatory follicle must migrate through dense connective tissue and rupture at a specialized site, the ovulation fossa (Ginther, 1992; Stabenfeldt *et al.*, 1975). Follicular rupture at the fossa is a distinct feature of ovulation in the mare, and the localization of relaxin in the mare ovary suggests that relaxin activity may be one of the mechanisms that facilitates follicle migration and ovulation. It has been suggested that relaxin is involved in follicular rupture (Evans *et al.*, 1983) and that it enhances collagenolysis in the follicular wall (Norström and Tjugum, 1986). Relaxin increases the release of plasminogen activator, plasminogen activator inhibitor, gelatinases, collagenase and proteoglycans, indicating a relaxin-induced effect on connective tissue remodelling (Too *et al.*, 1984; Hwang *et al.*, 1996). In support of this evidence, Brännström and MacLennan (1993) demonstrated that relaxin induces ovulations in the *in vitro* perfused rat ovary and that this process can be blocked by the use of a synthetic collagenase inhibitor (Butler *et al.*, 1991). Furthermore, Hwang *et al.* (1996) demonstrated that relaxin antibody administration partially suppressed gonadotrophin-induced ovulation in immature rats and that this effect could be reversed with relaxin treatment. Whether luteal relaxin is involved in the remodelling of ovarian stroma and facilitation of follicle migration and ovulation in mares remains to be investigated. The absence of luteal relaxin may explain, in part, why follicles do not ovulate readily in mares during the transition from the anovulatory to the ovulatory season.

The functional significance of relaxin expression in the equine corpus luteum remains to be elucidated. Relaxin may promote vascularization during the development of the corpus luteum into a new endocrine gland. Relaxin, in synergy with oestrogen and progesterone, has been shown to have angiogenic or vasodilatory properties in endometrial and mammary gland tissues (Hisaw *et al.*, 1967; Vaslienko *et al.*, 1986; Bani *et al.*, 1988, 1995). The spatial and temporal expression pattern of relaxin in the corpus luteum is similar to that of vascular endothelial growth factor (VEGF), a known promoter of angiogenesis (Klagsbrun and D'Amore 1991; Redmer and Reynolds, 1996). In the ovary, mRNA encoding VEGF is expressed predominantly in tissues that acquire new capillary networks, such as the luteal cells, and thus is thought to provide directional clues for the developing vasculature (Phillips *et al.*, 1990; Shweiki *et al.*, 1993). Whether relaxin facilitates the expression of VEGF in the mammalian ovary is currently unknown.

In summary, our findings of relaxin gene and protein expression in the corpus luteum suggest a role for relaxin in the ovaries of cyclic mares. Further studies are required to elucidate the functional significance of ovarian relaxin expression, and to map more precisely the onset of relaxin expression in the cyclic mare ovary using tissue from animals of known reproductive status.

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