Tissue concentration, mRNA expression and stimulation of IGF-I in luteal tissue during the oestrous cycle and pregnancy of cows*

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Summary. The expression of IGF-I in bovine luteal tissue was demonstrated by parallel measurement of IGF-I tissue concentration and its mRNA; highest synthesis was observed during Days 12–17 of the cycle and the first months of pregnancy. Tissue levels of IGF-I increased from Days 1–5 to Days 12–17 of the cycle followed by a rapid decrease at luteolysis; there was a continuous decline from early pregnancy until Months 6–9. Microdialysis perfusion experiments with corpora lutea *in vitro* at Days 8–11 of the cycle revealed a major effect: release of progesterone and oxytocin were highly stimulated in a dose-dependent manner. We suggest that IGF-I could be important in regulating the function of the bovine corpus luteum and may act in an autocrine/paracrine way.

Keywords: corpus luteum; IGF-I; expression; cattle

Introduction

The list of hormones and growth factors which have effects on the development and proliferation of ovarian tissue has grown considerably. The classical concept of endocrine control of ovarian function has been extended to paracrine and autocrine mechanisms and the local synthesis of growth factors in ovarian cells (IGF-I, TGF) has been reported (Skinner & Coffey, 1988; Oliver *et al.*, 1989). IGF-I especially appears to have important stimulatory effects on ovarian regulation: e.g. steroidogenesis in follicles (Spicer *et al.*, 1988), DNA synthesis and cell proliferation (May *et al.*, 1988) and stimulation of progesterone and oxytocin in bovine granulosa cells (Schams *et al.*, 1988), stimulation of aromatase activity (Adashi *et al.*, 1985) and amplification of FSH action (Davoren *et al.*, 1985; Adashi *et al.*, 1988) in human and rat granulosa cells. Recent data have been published about the stimulation and secretion of IGF-I in granulosa cells *in vitro* to examine differences during folliculogenesis (Hernandez *et al.*, 1989; Mondschein *et al.*, 1989). The aim of this study was to measure tissue concentrations of IGF-I and its mRNA in bovine corpus luteum during the oestrous cycle. A special perfusion technique *in vitro* was applied to demonstrate local effects.

Materials and Methods

The corpora lutea (CL) from mainly German Fleckvieh heifers were collected from a local slaughterhouse within 10–20 min of slaughter. The stage of the cycle was defined by macroscopic observation of the ovaries (follicles and corpus luteum) and the uterus (size, colour, consistency, connective tissue and mucus). The corpora lutea were divided into four groups during the oestrous cycle (Days 1–5, 6–11, 12–17, 18–21 after ovulation) and three groups during pregnancy (Months 1–2, 3–5, 6–9). IGF-I was extracted from 5 CL per group and the same tissue (i.e. 5 CL) was analysed for IGF-I mRNA by two independent experiments. If the cow was pregnant, the crown–rump length of the

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fetus was measured to evaluate the stage of pregnancy. Corpora lutea were separated immediately and stored in liquid nitrogen until extraction for RNA and IGF. For the incubation experiment, CL were washed with saline (0.154 M) at 38°C several times, then kept in Medium 199 (Sigma, Munich, FRG) at pH 7.2, 38°C containing 10 mmol NaHCO_3/l , Earle's salts, L-glutamine, 25 mmol Hepes/l, 0.5% (w/v) bovine serum albumin, 60 mg penicillin/l, 100 mg streptomycin/l, 2 mg amphotericin/l and 65 mg ascorbic acid/l (M199).

Hormone measurement. IGF-I in luteal tissue was measured by RIA after acid-ethanol extraction of the tissue. Tissue (0.2 g wet weight) was homogenized in a dismembrator (Braun, Melsungen, FRG) for 30 sec, cooled with liquid nitrogen and suspended in 3 volumes of 2 M-acetic acid/75 mM-NaCl. Extraction was done for 30 min at room temperature to separate IGF from its binding proteins (Daughaday *et al.*, 1980). After a brief centrifugation (10 min at 10 000 g) 50 μl of supernatant were extracted again and assayed by an IGF-I RIA. Validation of IGF-I tissue extraction was done by neutral gel filtration (40 $\times$ 1.6 cm column of Sephacryl 200: Pharmacia, Freiburg, FRG). Tissue extracts were neutralized, incubated for 1 h with labelled IGF-I and separated by gel chromatography. Fractions were measured for radioactivity in a gamma counter (LKB-Pharmacia, Freiburg, FRG). Distribution of endogenous IGF-I in the extracts was analysed by neutral gel filtration followed by determination of IGF-I in each fraction by RIA.

Recombinant human IGF-I (Amersham, Bucks, UK) was iodinated according to the lactoperoxidase method (Marchalonis, 1969). Synthetic IGF-I (kindly provided by the late C. H. Li, San Francisco, CA, USA) was used as a reference preparation. The antiserum from a rabbit was kindly supplied by P. Gluckman (Auckland, New Zealand) and showed no cross-reaction with related peptides such as insulin, proinsulin or IGF-II. Validation for tissue extraction was done by means of dilution curves and recovery experiments (92% recovery of labelled IGF-I). Separation of bound and free hormone was performed by a second antibody and polyethylene glycol (12%). Samples have been measured in duplicate. The intra-assay and inter-assay variations were <9% and <14% respectively.

Progesterone and oxytocin concentrations in the perfusate fractions were determined directly by enzyme-immunoassay (Prakash *et al.*, 1987) and radioimmunoassay (Schams, 1983), respectively.

RNA detection. Total RNA was isolated from luteal tissue by the guanidinium isothiocyanate method according to Maniatis *et al.* (1982). RNA northern blots were carried out on Hybond-ECL membrane (Amersham) after fractionation of total RNA in a 0.8% formaldehyde-agarose gel following the instructions of the manufacturer. The 542 bp cDNA coding for pig IGF-I (pIGF-I) has been published (Müller & Brem, 1990). Hybridization and detection of mRNA blots were done under stringent conditions according to the non-radioactive ECL gene detection system (Amersham).

Microdialysis perfusion system in vitro. Basically, the microdialysis method by Jarry *et al.* (1987) was used with modification for the in-vitro organ culture. In brief, the corpus luteum (Days 8–12 of the oestrous cycle) was cut with a scalpel to a 10–12 mm cube, and bisected. These two identical pieces were distributed to different IGF-I dose groups. Each luteal piece was penetrated by a 8–10 mm long dialysis capillary (Amicon Vitafiber 3S100; cut off M_r 100 000), with each end attached to Silastic tubings (i.d. 0.3 mm) by glue. The Silastic tubings were fixed on the face of the luteal piece by Histoacryl blau (B. Braun-Dexon GmbH, Spangenberg, FRG), so that the portion of dialysis capillary was held in the luteal piece. For the perfusion, one of the tubes was connected to a peristaltic pump, and the other was joined to the fraction collector. After connection to these systems, the luteal pieces penetrated by dialysis capillary were maintained in organ culture chambers (2070 Tube, Falcon, USA) filled with 50 ml M199, that were kept in a water bath at 38°C. Fresh M199 was dropped into the chamber at the rate of 15 ml/h from the top, and the medium flowed out at the same rate from the bottom driven by a peristaltic pump. During setting up the system, luteal pieces were always maintained in M199 at 38°C. Just after the start of the incubation, luteal pieces were perfused by Ringer's solution at a rate of 1 ml/10 min for 8 h. After 3 h preincubation, every 10-min perfusate fraction was collected for 5 h. Because of the great individual variations of hormone concentrations between luteal pieces, the mean progesterone or oxytocin concentrations of the first 6 fractions (1-h perfusion) were used for calculation of an individual baseline (100%). All hormone concentrations in fractions from each piece were then expressed as a percentage of individual baseline. This step allows comparison of the effect of IGF-I on secretion of hormone between different dose groups. Additionally the absolute concentrations (mean, range) are given. IGF-I (750 μg) was dissolved in 50 μl 1 M-HCl and diluted with Ringer's solution to a concentration of 100 $\mu\text{g}/\text{ml}$ (6.7 μM -HCl), and stored at -20°C as a stock solution. IGF was further diluted with Ringer's solution just before the start of the experiment to a final concentration (range of 0.2–10 $\mu\text{g}/\text{ml}$ for 30 min). Fractions were stored at -20°C until determination of progesterone and oxytocin. The diffusion capacity of the membrane was about 1% (0.5–2%) of infusate concentration as controlled by ^{125}I -labelled IGF-I.

Statistical analysis. Concentrations of IGF-I in the corpus luteum at different stages of the oestrous cycle and pregnancy were analysed by split-plot analysis of variance and Duncan's new multiple range test.

Results

IGF-I in cyclic corpus luteum

Concentrations of IGF-I in tissue of bovine corpus luteum changed during the oestrous cycle (Fig. 1a). Low amounts of IGF-I could be detected on Days 1–5 (227 ± 31 ng/g wet weight), higher

concentrations on Days 6–11 (465 ± 50 ng/g) and the maximum during the late luteal phase on Days 12–17 (1057 ± 269 ng/g) followed by a rapid decrease after luteolysis on Days 18–21 (172 ± 51 ng/g). Blood concentrations of IGF-I during the whole oestrous cycle showed a concentration of about 600 ± 180 ng/ml. Gel filtration experiments with tissue extracts demonstrated the presence of IGF-I binding proteins (large and small) in the luteal tissue. Striking differences of the binding capacity of mainly the small binding protein could not be detected during the cycle (data not shown). In the final extract for RIA most of the IGF-I was eluted in the fraction of M_r 7500, indicating removal of binding proteins. Using this technique we were able to exclude interfering binding proteins during the IGF RIA.

Fig. 1. Concentrations of IGF-I in bovine luteal tissue during (a) the oestrous cycle and (b) pregnancy. Values are mean $\pm$ s.e.m. for (a) 5–9 and (b) 5–6 CL and cows per group. Values with different superscripts differ significantly ($P < 0.01$).

Hybridization experiments with an IGF-I-specific cDNA (pIGF-I) yielded a main mRNA species of about 4 kb. Low IGF-I mRNA concentrations on Days 1–5 and 18–21 of the cycle were obvious (Fig. 2a), but higher values were present on Days 6–11 and were maximal on Days 12–17. The RNA hybridization experiments were done twice for each CL with various RNA preparations and showed similar results.

IGF-I in the corpus luteum during pregnancy

Values decreased significantly during pregnancy from the highest level of 812 ± 86 ng/g at the first month to 450 ± 14 ng/g during Months 3–5 to 146 ± 14 ng/g after 6 months of pregnancy (Fig. 1b). The northern blot experiment done with total RNA from the same CL demonstrated high expression of IGF-I mRNA during the first months of pregnancy, very little during Months 3–5 and none during Months 6–9 (Fig. 2b).

Effect of IGF-I on the secretion of luteal tissue

The baseline of perfusate fractions from luteal pieces (Days 8–12) varied between 0.41 and 5.37 ng/ml (mean 2.16 ng/ml) for progesterone and between 6.7 and 51.3 pg/ml (mean 20.6 pg/ml) for oxytocin. IGF-I stimulated the release of both progesterone and oxytocin (Fig. 3) in a dose-dependent manner from microdialysed luteal pieces *in vitro*. For progesterone, a release pattern of 2

Fig. 2. Concentrations of specific mRNA in bovine corpus luteum during (a) the oestrous cycle and (b) pregnancy. Northern hybridization with pIGF-I-cDNA was used and 30 µg total RNA per lane were separated by electrophoresis on a 0.8% formaldehyde-containing agarose gel. Detection was by the non-radioactive ECl system (Amersham, Bucks, UK).

or 3 waves was apparent after the first peak directly related to infusion of IGF-I, but no such pattern was seen for the release of oxytocin.

Discussion

We investigated in this study the distribution and function of the growth factor IGF-I in bovine luteal tissue. For the first time an expression of IGF-I in the bovine corpus luteum could be demonstrated by determining both IGF-I-specific mRNA and the peptide concentrations. Under our conditions, a main IGF-I RNA species, about 4 kb in length, could be detected and was comparable with liver RNA. Similar data have been reported for other species (van Neste *et al.*, 1988; Freed & Herington, 1989). Concentrations of IGF-I in this tissue correlated with the expression

Fig. 3. Dose-dependent release of (a) progesterone and (b) oxytocin from microdialysed bovine luteal tissue (Days 8–12 of the oestrous cycle) *in vitro* after stimulation with IGF-I (0.2, 1 and 10 µg/ml). The filled bars indicate IGF-I application. All values are shown as % of baseline (mean $\pm$ s.e.m.; $n = 5$ CL and cows per group) calculated from the first 6 fractions. Absolute concentrations ranged from 0.41 to 5.37 ng progesterone/ml (mean 2.16 ng/ml) and 6.7 to 51.3 pg oxytocin/ml (mean 20.6 pg/ml).

of mRNA. An accumulation of liver-derived IGF-I in the corpus luteum therefore seems to be unlikely. We used a pig IGF-I clone for the RNA hybridization experiments because evolutionarily conserved protein and RNA sequences for IGF-I have been reported for human, pig, rat and sheep (Bell *et al.*, 1986; Honegger & Humbel 1986; Rotwein, 1986; Lund *et al.*, 1986; Tavakkol *et al.*, 1988; Francis *et al.*, 1989). Moreover, a bovine IGF-I-cDNA has been isolated (Fotsis *et al.*, 1990) and an homology of 92% to the pig sequence is present.

Several recent observations have suggested IGF-I as a potential factor in the ovary and that it could act as a regulator of granulosa cell differentiation (Schams *et al.*, 1988; Holtorf *et al.*, 1989). The expression of IGF-I in rat ovarian tissue, especially granulosa cells, has been demonstrated by RNA techniques to be dependent on the developmental status (Carlsson *et al.*, 1989; Hernandez *et al.*, 1989; Oliver *et al.*, 1989). These reports suggest a major synthesis of IGF-I in granulosa cells of antral follicles and thereby a stimulation of proliferation and differentiation. Oliver *et al.* (1989) found that IGF-I expression could only be detected in granulosa cells but not in the corpus luteum of rats. In contrast to these observations, we were able to measure maximal expression of IGF-I in the bovine corpus luteum during the late luteal phase of the cycle and early pregnancy which is correlated with a high metabolic activity of this tissue. We have shown an immediate and dose-dependent release of progesterone and oxytocin after IGF-I by microdialysed luteal tissue during

that period. McArdle & Holtorf (1989) measured a similar response after long-term incubation of dispersed luteal cells. The difference in the effective time for response of luteal tissue between the two systems may suggest an important role of cell contact. Saturable, specific and high-affinity IGF-I receptors have been demonstrated on the granulosa cells of ewes (Monget *et al.*, 1989), women (Gates *et al.*, 1987) and rats (Adashi *et al.*, 1989). We assume that the similar presence of type I receptors on luteal cells and that the effect of IGF-I is mediated via membrane receptors. Perfusion with vehicle alone (Ringer and HCl) was ineffective and a non-specific effect can therefore be excluded. Sensitivity of luteal tissue to IGF-I is high after the mid-luteal phase. This parallels a high expression in the same tissue and seems to be independent of concentrations of IGF-I in blood. We therefore suggest an autocrine/paracrine function of IGF-I in the corpus luteum, especially during the mid- and late luteal phase of the cycle and during early pregnancy. Similar effects are postulated for human, rat and pig granulosa cell differentiation and growth (Mondschein *et al.*, 1989; Holly & Wass, 1989). Further work has to be done to elucidate the role of IGF-I in the ovary, especially in the corpus luteum. Interest should be focussed on IGF-I receptors and the localization of IGF-I synthesis in different luteal cells.

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