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Endometrial mRNA expression of oestrogen receptor α, progesterone receptor and insulin-like growth factor-I (IGF-I) throughout the bovine oestrous cycle

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Abstract

This study characterized endometrial expression of mRNAs of oestrogen and progesterone receptors (ER, PR) and insulin-like growth factor-I (IGF-I) during the oestrous cycle. Seven Holstein heifers that showed standing oestrus on the same day (day 0) were selected and blood samples for oestradiol (E2) and progesterone (P4) determinations by RIA were taken daily until day 23. Endometrial samples were taken by transcervical biopsies on days 0, 5, 12 and 19 for mRNA determination by solution hybridization. The highest endometrial mRNA levels of ERα and PR were observed at oestrus and a decline was observed already at day 5, which then decreased progressively at the end of the luteal phase. IGF-I mRNA levels were higher at day 0 and 5 than at day 12. At day 19, mRNA levels of ERα, PR and IGF-I were the lowest in heifers that were at the end of their luteal phase ($n = 4$), but were high again in heifers which P4 levels were basal ($n = 3$). The temporal changes in mRNA endometrial expression of ERα, PR and IGF-I and their relation to the changes in steroid concentrations during the bovine oestrus cycle are described. © 2001 Published by Elsevier Science B.V.

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1. Introduction

Studies on the uterine environment in several mammalian species have implicated a role of the insulin-like growth factor (IGF) system in cellular proliferation and differentiation of both the uterus and the embryo before and during implantation (Keller et al., 1998). This system includes IGF-I and IGF-II, their membrane receptors (type 1 and 2) and at least six high-affinity binding proteins (IGFBs) that regulate the bio-availability of the IGFs to their receptors and modulate their action at the cellular level (Wathes et al., 1998). An endocrine role for IGF-I, produced by the liver, has been widely accepted as a primary factor mediating the mitogenic effects of growth hormone (GH) (Schoenle et al., 1982). However, studies of IGF-I in different extra hepatic tissues have confirmed also a local production as well as paracrine and/or autocrine modes of action (Han et al., 1987; Murphy et al., 1987). In the bovine oviduct and uterus, GH receptor mRNA has been identified but no apparent effect of GH treatment on IGF-I expression was found (Kirby et al., 1996). Instead, ovarian steroids have been suggested as the regulators of endometrial IGF-I expression (Simmen et al., 1992).

Although data are conflicting between and within species, it is generally accepted that oestrogens stimulate endometrial and myometrial expression of IGF-I and that IGF-I is a mediator of oestrogen-induced proliferation of the uterus (Cann et al., 1998; Sahlin, 1998). IGF-I mRNA in the oviduct and endometrium peaked at oestrus in ewes, which may be important for promoting survival of spermatozoa and/or of the early embryo (Stevenson and Wathes, 1996). In pigs, uterine IGF-I mRNA levels are positively correlated with luteal P4 levels. These findings indicate that the regulation and function of IGF-I are species-specific. In cows, no cyclic changes of endometrial IGF-I mRNA could be demonstrated, but IGF-I content in the uterine lumen was highest at oestrus (Geisert et al., 1991). This lack of increase in the synthesis of endometrial IGF-I mRNA at oestrus in cattle as determined by Northern blot analysis, led researchers to suggest that the increase in luminal content of IGF-I represents an increased transport from the serum enhanced by oestrogen stimulation (Geisert et al., 1991). A later study performed in cows (Robinson et al., 2000) showed cyclic changes in IGF-I mRNA levels in sub-epithelial stroma as determined by in situ hybridization. Since Northern blot on RNA from total endometrium showed no variations in IGF-I mRNA levels (Geisert et al., 1991), Robinson et al. (2000) suggested that the cyclic regulation of IGF-I expression may be limited to the sub-epithelial stroma.

Oestrogen actions on the uterus during the oestrous cycle are mediated through interactions with its respective intracellular oestrogen receptor (ER). Oestrogens induce the synthesis of their own receptor as well as the progesterone receptor (PR) in several species; in contrast, during the luteal phase, P4 down-regulates ER and PR levels (Clark et al., 1992). Maximum uterine levels of ER and PR determined by binding assays were found in cows around oestrus while the lowest levels of receptors were found at diestrus (Zelinski et al., 1982; Meyer et al., 1988; Vesanen et al., 1988). Changes in ERα and PR concentrations at different stages of the oestrous cycle were also found by immunohistochemistry; maximum levels of ER in six different uterine layers were found at oestrus, but although PR levels followed a similar pattern, increases could only be demonstrated in luminal epithelium, deep glands and myometrium 2–5 days after oestrus (Wathes and Hamon, 1993). In agreement

with these findings, Robinson et al. (1998) reported the highest levels of endometrial ERα mRNA on day 0 and PR mRNA at days 0–8.

There are no reports on the temporal association of ERα, PR and IGF-I gene expression during the oestrous cycle in cattle. The aim of this study was to monitor the mRNA expression of ERα, PR and IGF-I in endometrial biopsies with the cyclic changes of gonadal steroids.

2. Materials and methods

2.1. Experimental design

The study was conducted in accordance with the International Guiding Principles for Biochemical Research Involving Animals as promulgated by the Society for the Study of Reproduction. Seven Holstein heifers of 26 months of age that were synchronized with an injection of 800 μg of delprostenate and showed standing oestrus on the same day (day 0) were selected. The heifers had a body weight (mean ± S.E.M.) of 452 ± 14 kg and a body condition score of 2.8 ± 0.3 (scale 1–5). The cows were grazing on improved pastures and supplemented with a mixture of concentrates and corn silage. Blood samples for hormone determination were taken daily by jugular venipuncture using vacutainer tubes from day 0 to day 23. Samples were centrifuged within 1 h and plasma was stored at −20°C until hormone assays were performed. Endometrial samples for determinations of mRNA levels of ERα, PR and IGF-I were taken by transcervical biopsies on days 0, 5, 12 and 19 as described previously (Basu et al., 1988). The total time from performance of endometrial biopsies to liquid nitrogen freezing was 10 min. In order to determine if biopsies interfered with normal reproductive physiology, heifers were inseminated in the first detected oestrus after the experiment and three out of four heifers with normal cycles became pregnant, but none of the heifers with short cycles. In the following oestrus two heifers with short cycles were inseminated again and became pregnant.

2.2. Hormone determinations

Progesterone was determined in all samples by a commercial kit (Coat-a-count, Diagnostic Products, Los Angeles, CA, USA) as previously validated for cattle (Toribio et al., 1994). The sensitivity of the assay was 0.04 nmol/l. The intra-assay coefficients of variation for low, medium and high controls were 2% (3.0 nmol/l), 3% (19.5 nmol/l) and 2% (52.4 nmol/l). The inter-assay coefficients of variation were 3% (3.0 nmol/l), 4% (19.5 nmol/l) and 4% (52.4 nmol/l).

All plasma samples were assayed for oestradiol-17β (E2) determination in duplicate by a ^{125}I RIA (Estradiol double antibody, KE2D, Diagnostic Products Co, Los Angeles, CA, USA) previously validated for bovine plasma (Sirois and Fortune, 1990). The detection limit was 4 pmol/l. The intra-assay coefficients of variation for low, medium and high controls were 21% (11 pmol/l), 8% (59 pmol/l) and 3% (106 pmol/l). The inter-assay coefficients of variation were 11% (11 pmol/l), 14% (59 pmol/l) and 2% (106 pmol/l).

2.3. mRNA determinations

Solution hybridization assays of specific mRNAs for ERα, PR and IGF-I were performed in endometrial samples as described previously (Persson et al., 1997). In short, total nucleic acids were prepared and the concentration of DNA in the TNA samples was measured fluorometrically. Probes were synthesized in vitro and radiolabeled with ^{35}S-UTP essentially as described by Melton et al. (1984). The probes used for ERα mRNA and PR mRNA determinations are derived from full-length cDNAs containing the whole open reading frame of the human oestrogen and PR respectively donated by Donald McDonnell, Duke University Medical Center, Durham, North Carolina. The probe for IGF-I mRNA determinations is derived from a 775 bp *Rsa*I–*Eco*RI fragment of the human IGF-I cDNA, which was given by Peter Rotwein, Washington University School of Medicine, St. Louis, MO, USA. Overnight incubation was performed at two different concentrations of the samples which were then treated with RNase to digest unhybridized RNA. Labeled hybrids were precipitated with trichloroacetic acid and collected on filters, radioactivity was determined in a liquid scintillation counter. All the samples from the experiment were determined in the same assay. Levels of ERα mRNA, PR mRNA and IGF-I mRNA are expressed as cpm in relation to DNA content (cpm/μg DNA).

2.4. Northern blotting

In order to validate the human probes used for the bovine tissue, RNA was prepared from freshly collected bovine endometrium by transcervical biopsies. One part of the biopsy was prepared immediately using the commercial kit "RNAgents Total RNA Isolation System Z5110" (Promega Corp., Madison, USA) while the remaining tissue was placed in RNAlater (Ambion Inc., Austin, Texas, USA) and prepared for RNA, in the same way as the fresh biopsy, after 2 weeks storage at 4°C. The Northern blotting was performed as previously described (Persson et al., 1997). In short, 10 μg RNA of the freshly prepared sample or 16 μg RNA of the RNAlater treated sample were loaded per well onto a 0.9% agarose-formaldehyde gel. Electrophoresis was performed at 25 V for 16 h. The RNA was transferred to Hybond-N filters (Amersham Pharmacia Biotech, Uppsala, Sweden) and following prehybridization for 4 h at 55°C, ^{32}P-UTP-labeled RNA probes were hybridised (10^6 cpm/ml) to filters at 60°C for 40 h. Filters were exposed to X-ray film at −70°C with intensifying screens. As unlabeled size marker, a RNA molecular weight marker I (Roche Molecular Biochemicals, Mannheim, Germany) was used.

2.5. Statistical analysis

All statistical analysis were carried out using the Statistical Analysis System (SAS Institute Inc., Cary, NC, USA 1989). Data are presented as least square means ± S.E. Data of mRNAs and hormone concentrations were analyzed by mixed procedure (SAS) and the statistical model included the effects of sampling day, group (normal cycle versus short cycle) and the interaction of between group and day. Short cyclic cows were defined as having less than 12 days of luteal activity determined by P4 levels significantly different from day 0 (day of oestrus). Analysis of mRNA contents were performed using the units

described (cpm/μg DNA), but data are presented as percentage of day 0 for all seven animals (oestrus = 100%). The level of significance is $P < 0.05$, except where otherwise specified.

3. Results

The cycle length was (mean$\pm$S.E.M.) 18.9$\pm$0.9 days. Animals were classified according to the duration of the cycle length; normal cycles (20.8 $\pm$ 0.3 days, $n = 4$, group NC) and shorter than normal cycles (16.3 $\pm$ 0.3 days, $n = 3$, group SC), (Fig. 1, upper and lower panels).

3.1. Progesterone and oestradiol determinations

Concentrations of P4 increased significantly on day 5 in NC heifers and remained high until day 19. In SC heifers, P4 concentrations increased significantly on day 6 and remained high until day 15. Progesterone (nmol/l) did not differ between groups at oestrus (day 0: 2.2 ± 1.1 for NC and 1.8 ± 1.9 for SC groups) or until day 9 and thereafter concentrations in the NC group were higher ($P = 0.03$). Maximum levels of P4 (nmol/l) differed between groups: 29.9$\pm$1.1 (day 15) in NC heifers versus 20.6$\pm$1.9 (day 12) in SC heifers. Maximum levels of E2 (pmol/l) were found at day 0 for group NC (31 $\pm$ 4, $n = 4$) and for group SC (30.7$\pm$6, $n = 3$). In NC heifers, E2 concentrations tended to increase again at day 6 (17$\pm$4) and a sustained significant increase was observed on days 20 to 22 (3 days average: 27 $\pm$ 4) coincident with the following oestrus. In SC heifers, a significant increase on days 16–18 (3 days average: 27 $\pm$ 6) and on days 20 to 21 (2 days average: 29 $\pm$ 6) could be demonstrated. There were no differences between E2 concentrations among groups.

3.2. Northern blot

In Fig. 2, the results from the hybridizations with the human derived probes to RNA from bovine endometrium are shown. The size of the ERα is 6800 base (b). The major transcript for PR mRNA is 4300 b and two weaker transcripts are approximately 2400 and 1500 b. There are also two transcripts larger than 7000 b. The IGF-I transcripts are of sizes 7500, 4400 (major), 1800 and 1200 b. Thus, the human derived probes cross reacts with the RNA from the bovine endometrium.

In addition, the difference in results between freshly prepared RNA and RNA prepared from tissue stored in RNAlater can be compared on the ERα blot (Fig. 2). The left lane represents RNA from RNAlater stored tissue and the right lane shows the RNA from freshly prepared tissue. Hence, RNAlater is a good choice when fresh preparation of tissue is impossible.

3.3. mRNA determinations

The mean mRNA values of all heifers at day 0 (day of oestrus) were considered as control values and data is referred as percentage of the control values. Mean $\pm$ S.E.M. of mRNA of ER, PR and IGF-I were (cpm/μg DNA) 395 $\pm$ 48, 569 $\pm$ 76 and 237 $\pm$ 34, respectively.
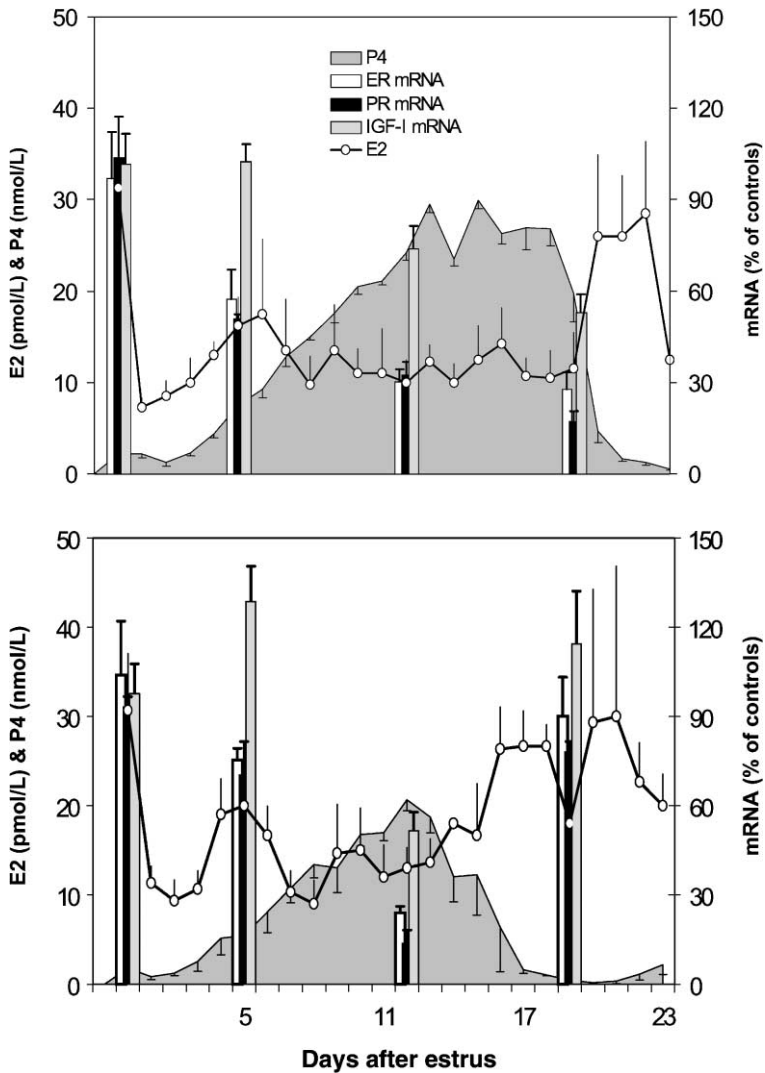
Fig. 1. Levels (least square mean ± S.E.M.) of mRNA (% of day 0 values) of oestrogen receptor α (ER mRNA), progesterone receptor (PR mRNA) and IGF-I (IGF-I mRNA) and oestradiol (E2), progesterone (P4), in heifers with a normal oestrous cycle length (upper panel, $n = 4$) and a short cycle length (lower panel, $n = 3$).

In the NC group, the highest mRNA levels of ERα, PR and IGF-I were observed at oestrus. Levels of ERα mRNA and PR mRNA declined on day 5, and continued to decrease until the end of the luteal phase (Fig. 1, upper panel; Table 1). Contents of ERα mRNA and PR mRNA had decreased four-fold at the end of the luteal phase. Endometrial IGF-I mRNA expression was similar on day 0 and day 5, but then decreased two-fold progressively until the end of the luteal phase.
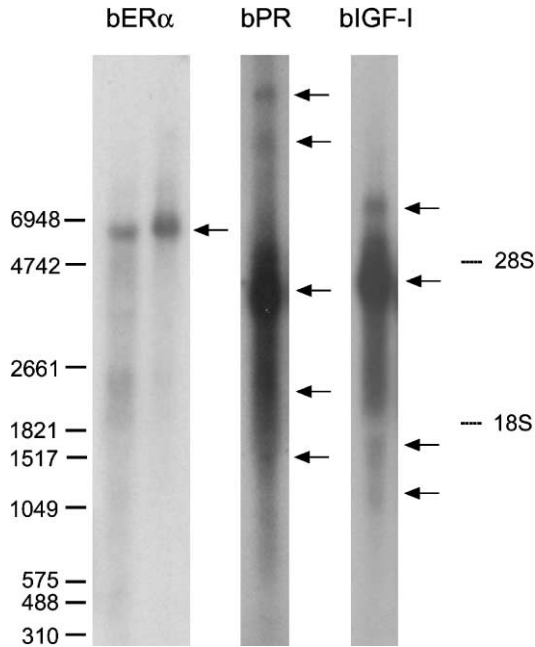
Fig. 2. Northern blot showing the human ERα, PR and IGF-I probes hybridized to the RNA from bovine en-dometrium. On the left side the size markers are shown and on the right side the locations of the 18S and 28S bands from the gel when stained with ethidiumbromide are shown. The arrows indicate the mRNA transcripts found for the different probes. Bovine ERα: the size of the mRNA transcript is approximately 6800 bases (b). bPR: the transcripts are approximately 4300 (major transcript), 2400, 1500 b, and two are much larger than the largest size marker. bIGF-I: the transcripts are approximately 7500, 4400 (major transcript), 1800 and 1200 b. The left lane on the ERα blot shows RNA prepared from tissue stored in RNAlater, and the right lane shows RNA prepared from fresh tissue. The ERα, PR and IGF-I films were exposed for 4, 7 and 1 days respectively.

Table 1
mRNA levels (least square means $\pm$ S.E.M., as percentage of control values) of oestrogen and progesterone receptors (ERα, PR) and IGF-I in heifers with normal and short cycle length[a]

mRNA	Days of the cycle	Normal cycle ($n = 4$)	Short cycle ($n = 3$)
ERα	0	97 ± 15 a	104 ± 18 a
	5	57 ± 10 b	75 ± 4 a
	12	30 ± 4 bc	24 ± 2 b
	19	28 ± 6 c	90 ± 13 a
PR	0	104 ± 13 a	93 ± 4 a
	5	51 ± 2 b	70 ± 11 b
	12	32 ± 4 c	14 ± 4 c
	19	17 ± 4 c	78 ± 3 b
IGF-I	0	102 ± 10 a	98 ± 10 a
	5	103 ± 5 a	129 ± 12 b
	12	74 ± 7 b	52 ± 6 c
	19	53 ± 6 b	114 ± 18 ab

[a] Different letters within column and specific mRNA differs $P < 0.05$.

In the SC group, a similar pattern of mRNAs expression during the oestrous cycle was observed (Fig. 1, lower panel; Table 1). The decrease observed at day 5 for ERα and PR mRNA was not so marked, and was not different for ERα mRNA. An increase in the IGF-I mRNA level could be seen in the SC group on day 5 (Table 1). At day 12, mRNA levels of ERα, PR and IGF-I had decreased. A restoration of all mRNA levels was seen at day 19, when the next oestrous cycle had already begun.

There was a significant interaction between the day of sampling and the group for ERα mRNA ($P < 0.03$), PR mRNA ($P < 0.0001$), IGF-I mRNA ($P < 0.002$). There were no differences in mRNA contents among groups on day 0, but on day 5, contents of PR mRNA were higher ($P = 0.036$) and tended to be higher for IGF-I mRNA ($P = 0.087$) in the SC group. On day 12, PR mRNA tended to be lower ($P = 0.076$) in the SC group. On day 19 all mRNA levels in the SC group differed from the NC group (in all cases $P < 0.001$).

4. Discussion

This is the first report describing simultaneous changes in endometrial IGF-I, ERα and PR mRNA levels and their relation to physiological circulating levels of ovarian steroids during the bovine oestrous cycle.

The length of the oestrous cycle was shorter in three of seven animals; this could be due to the repeated manipulation of the uterus or to the performance of the biopsies. In a recent study we have observed that there is a prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) release after uterine manipulation and/or uterine biopsy on day 17 of the cycle, but although P4 levels drop in the first hours, luteolysis does not occur and the cycle is not shortened (unpublished data). Nevertheless, biopsies were performed as early as day 0 and no tendency for an early luteolysis could be observed in agreement with a previous study (Basu et al., 1988). Most animals conceived in the first or second oestrus after the experiment, suggesting that the biopsies had no consequences on the following oestrous cycles. Subnormal luteal phases (short cycles and/or lower P4 production) occurs naturally around puberty in cows (Hunter, 1991), but we cannot demonstrate if the subnormal luteal phases observed were to occur naturally or were due to the uterine biopsy.

The sizes of the mRNAs for bovine ERα, PR and IGF-I are all in the range of published results from other mammals. We have not been able to find any sizes for bovine mRNAs of ERα and PR published before. The bovine ERα is approximately 6.8 kb, which is in agreement with the size described for ovine ERα (Ing et al., 1996). The PR transcripts are five of which two are larger than the largest size marker (6948 b) and could correspond to the 14 and 9.7–10.3 kb transcripts, which were found in the rat uterus (Kraus and Katzenellbogen, 1993). The major transcript is approximately 4.3 kb and there has been transcript sizes 4.3–4.5 found in human and rabbit tissues before (Nagai et al., 1994; Camacho et al., 1998). Smaller transcripts of 1.8 and 2.4 kb have also been described before (Kraus and Katzenellbogen, 1993; Nagai et al., 1994) which could correspond to the 1.5 and 2.4 kb transcripts found in this study. The IGF-I mRNAs are of sizes 7.5, 4.4, 1.7 and 1.2 kb, which are similar to the sizes published for mouse and human IGF-I mRNAs (Möller et al., 1991). Likewise, bovine IGF-I mRNA sizes of 7.5 kb (major transcript) and 1.0 kb (minor

transcript) have been described before (Geisert et al., 1991), although in the present study the 4.4 kb transcript was the major.

The highest levels of ERα and PR mRNAs were seen at oestrus (day 0), which is in agreement with previous determinations of mRNA of ERα and PR by in situ hybridization (Robinson et al., 1998). The results confirm the stimulatory effect of oestrogens on sex steroid receptor expression (Clark et al., 1992, Mann et al., 1999). The approximately three-fold decrease in the mRNA levels of ERα and PR from day 0 to day 12 is consistent with P4-induced down-regulation of its own and ERα expression. Results of the present study indicate that the endometrial PR mRNA level decreases before luteolysis is established. Zollers et al. (1993) suggested that the loss of PR might remove the inhibitory effect of P4 on either the release of $PGF_{2\alpha}$ or the endometrial synthesis of oxytocin receptors. The content of mRNA found agrees very well with the known regulation by ovarian steroids of their respective receptors in bovines (Meyer et al., 1988; Vesanen et al., 1988).

In this study we have shown that endometrial IGF-I mRNA concentrations are greater on day 0 and 5, which do not agree with a previous study where no cyclic variations were found (Geisert et al., 1991), possibly due to the difference in mRNA detection methodology. Results partially agree with data by Robinson et al. (2000) since they found the highest IGF-I expression in sub-epithelial stroma at oestrus, but do not agree with the decrease on day 2, and the subsequent increase on day 10 reported in that study. Differences can be due to the methodology used, but may also reflect that endometrial biopsies were collected over three estrous cycles and no hormone determinations were done to verify the phase of the oestrus cycle (Robinson et al., 2000). The activation of IGF-I expression associated in this study with high E2 levels may be an effect of E2-induced cellular proliferation of the uterus and oviduct (Sahlin, 1998; Wathes et al., 1998). At oestrus, high IGF-I contents in the uterine fluid were found in cows and was suggested to be an E2-dependent enhanced transport from the serum (Geisert et al., 1991). However, the present results suggest that, at least in part, the IGF-I increase in the uterine fluid is locally derived (e.g., secreted by the endometrium). The fact that the IGF-I mRNA level was high until day 5 suggests a role for IGF-I in the early stage of embryonic development. The marked loss of IGF-I mRNA observed in the middle of the luteal phase suggests that P4 may inhibit IGF-I mRNA directly or indirectly via suppression of ERα. Progesterone has been shown to enhance the E2-induced increase in uterine IGF-I mRNA levels in rats (Norstedt et al., 1989) and pigs (Simmen et al., 1990) whereas in other rat studies P4 inhibited uterine IGF-I expression (Croze et al., 1990; Murphy and Ghahary, 1990).

Differences in gene expression were found in heifers with short and normal cycles as early as day 5; higher mRNA levels of PR and IGF-I in the SC group. Results suggest that endometrium of SC heifers may have an altered gene expression during the early luteal phase which cannot be explained by gene expression at day 0 or by the circulating levels of steroids during days 0–5 (no differences between groups). An interesting finding was the more pronounced down-regulation in the transcription of these genes observed on day 12 in heifers with a short cycle, which was comparable with day 19 in heifers with a normal cycle. We have no obvious explanation for this, but if the lower PR mRNA levels at day 12 in the short cycle group reflect the actual content of the receptor protein, this could explain the loss of P4 dominance and the duration of the short cycle. Heifers that have short-lived corpora lutea and consequently short cycles are not capable of maintaining pregnancy. Progesterone

alters the uterine environment to allow the development of the embryo, and it has been reported that high progesterone levels are associated with high fertility (Thatcher et al., 1995; Mann et al., 1999).

5. Conclusion

In summary, this study describes the changes in endometrial expression of IGF-I, ERα and PR mRNA levels and their relation to physiological circulating levels of ovarian steroids during the bovine oestrous cycle. Results support the reported stimulatory effects of estrogens on ERα, PR and IGF-I endometrial expression as well as the inhibitory action of P4 on their expression.

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