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Endometrial expression of leptin receptor and members of the growth hormone–Insulin-like growth factor system throughout the estrous cycle in heifers

C. Sosa^a, M. Carriquiry^b, C. Chalar^c, D. Crespi^d, C. Sanguinetti^c, D. Cavestany^d, A. Meikle^{e,*}

^a Animal Production and Food Science, Veterinary Faculty, C/Miguel Servet 177, Zaragoza 50013, Spain

^b Animal and Pastures Production, Agronomy Faculty, Uruguay

^c Biochemistry, Science Faculty, Uruguay

^d Animal Reproduction, Uruguay

^e Laboratory of Nuclear Techniques, Veterinary Faculty, Lasplaces 1550, Montevideo 11600, Uruguay

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ABSTRACT

The growth hormone (GH)–insulin-like growth factor (IGF) system is expressed in bovine uterus during the estrous cycle and early pregnancy and is acknowledged to play an important role in regulating the development of the embryo and uterus. The leptin receptor (LEPR) is also expressed in the bovine uterus although it is not known whether its expression varies during the estrous cycle. In this study, the expression of the IGF-I and -II, the type 1 IGF receptor (IGF-1R), GH receptor (GHR) and LEPR transcripts was determined on endometrial transcervical biopsies collected on days 0 (estrus), 5, 12 and 19 of the cow estrous cycle ($n = 8$). The expression of mRNA was determined by RT real time PCR using ribosomal protein L19 as a housekeeping gene. It has been demonstrated for the first time that LEPR mRNA is expressed in the bovine uterus throughout the estrous cycle and that it presents a cycle-dependent variation, with higher levels observed during the luteal phase. The expression of IGF-I mRNA was greatest at estrus and day 5 (100%), and decreased on days 12 and 19 to 47% and 35% of the initial values. IGF-II mRNA increased on day 12 and decreased sharply thereafter (to one-third of day 12 values). Interestingly, IGF-1R showed the same pattern as IGF-II: increased 50% on day 12 compared to values at estrus and presented a sharp decrease on day 19. The expression of GHR transcript was greatest at estrus and on day 5 and progressively decreased thereafter. These results show that the GH–IGF system components are distinctively regulated during the estrous cycle suggesting that modulation of the IGF system may influence uterine activity during this period. The increase in the uterine sensitivity to IGFs during the late luteal phase – as demonstrated by the increased IGF-1R expression – concomitant with the increased IGF-II mRNA expression may reinforce the role of IGF-II during early pregnancy. Moreover, leptin is also likely to play roles during early embryo development.

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1. Introduction

The growth hormone (GH)–insulin-like growth factor (IGF) system is involved in reproductive processes that are critical for the establishment of pregnancy. The GH–IGF system consists of GH and its receptor (GHR), IGF-I, IGF-II, its receptors and IGF binding proteins (IGFBPs). The actions

* Corresponding author. Tel.: +598 2 6223106; fax: +598 2 6223106.
E-mail address: anamei@adinet.com.uy (A. Meikle).

of IGFs are primarily mediated by the type 1 IGF receptor (IGF-1R), although it exhibits a hierarchy of binding affinity which favors IGF-I (Thissen et al., 1994; Jones and Clemmons, 1995). In cattle, the IGF system has been implicated in cellular proliferation and differentiation of both the uterus and the embryo before and during implantation (Keller et al., 1998). Both IGFs are believed to play roles as mitogens and/or apoptotic survival factors during early bovine development (Byrne et al., 2002). Growth hormone and IGF-I and -II can stimulate embryonic development *in vitro* in cattle (Byrne et al., 2002; Moreira et al., 2002). Indeed, it has been demonstrated that GHR and IGF-1R are expressed in the endometrium and in ruminant embryos (Watson et al., 1992; Stevenson et al., 1994; Heap et al., 1996; Kolle et al., 1998).

Most of the circulating IGF-I and IGF-BPs are synthesized in the liver under the stimulus of GH (Thissen et al., 1994). Nevertheless, it has been demonstrated that IGFs are also produced locally in the reproductive tract of ruminants (cows, Geisert et al., 1991; sheep, Stevenson et al., 1994). Despite the presence of GHR mRNA in the bovine oviduct and uterus, no apparent effect of GH treatment on IGF-I expression was found (Kirby et al., 1996). On the other hand, treatment with bovine somatotropin affected endometrial gene expression, but it was not determined whether it was a direct action through endometrial GHR or indirect through hepatic IGF-I enhanced expression (Guzeloglu et al., 2004). In spite of the conflicting findings regarding the role of GH-GHR on the regulation of IGF-I, it is accepted that ovarian steroids regulate endometrial IGF-I expression in several species (Murphy and Ghahary, 1990; Simmen et al., 1992; Sahlin et al., 1994; Wathes et al., 1998). However, no studies were found on the physiologic variation of the endometrial sensitivity to GH and IGFs (i.e., receptor concentration) during the estrous cycle.

We have previously characterized endometrial gene expression of IGF-I, estrogen receptor α (ER α) and progesterone receptor (PR) mRNA and their relation to physiological circulating levels of ovarian steroids during the bovine estrous cycle (Meikle et al., 2001). Endometrial IGF-I mRNA concentrations were greater at estrus, coinciding with results from other groups (Robinson et al., 2000; Rhoads et al., 2008) and in association with high estradiol levels which could have induced cellular proliferation (Sahlin, 1998; Wathes et al., 1998). High IGF-I contents in the uterine fluid were found in cows and it was suggested to be due to enhanced transport from serum in response to estrogen stimulation (Geisert et al., 1991), but reports cited above also suggest that at least in part, the IGF-I increase in the uterine fluid is locally derived (e.g., secreted by the endometrium). Total GHR have also been reported to be increased at estrus in dairy cows (Rhoads et al., 2008), and it is known that GH actions are regulated by sex steroids (Chowen et al., 2004). However, to the best of our knowledge there are no reports on the temporal association of endometrial GHR, IGF-I, IGF-II and IGF-1R gene expression during the estrous cycle in ruminants.

Leptin is mainly produced by the adipose tissue in mammals and it is involved with the regulation of appetite (Chilliard et al., 2001), although the demonstration of its expression and its receptor expression in many other tis-

ues has led to the concept that it is a hormone with multi-systemic action (Chelikani et al., 2003). The presence of leptin and its receptor (LEPR) in the reproductive tissues has involved it in the reproductive processes and it has been postulated that it may serve as a mediator between the metabolic status and the reproductive function (Clarke and Henry, 1999). Specifically, the genes for the truncated (short; LEPRa) and the full-length (long; LEPRb) isoforms of the receptor are present in the uterus of heifers (Thorn et al., 2007). Moreover, it has been demonstrated that they are down-regulated after treatment with estradiol (Thorn et al., 2007) suggesting a role for leptin at the uterine level as has been reported in humans and mouse (Alfer et al., 2000; Gonzalez et al., 2000; Kawamura et al., 2002). Nevertheless, we have found no reports on LEPR gene expression throughout the bovine estrous cycle. Information on the uterine sensitivity to leptin during the bovine estrous cycle is necessary in order to understand the possible roles of leptin on the uterine environment.

Thus, the aim of this study was to monitor the mRNA expression of LEPR and of the GH-IGF system in endometrial biopsies throughout the estrous cycle in cattle. We have chosen to perform endometrial sampling by transcervical biopsies since it allows the study of different factors on the same experimental unit across time, with no repercussions to the animal reproductive performance (Meikle et al., 2001).

2. Materials and methods

2.1. Experimental design

Animal experimentation was approved by the Animal Experimentation Committee of the University of Uruguay, Uruguay. Ten Holstein heifers of (mean \pm S.E.M.) 24 ± 1 months of age were synchronized with estrus with an injection of 800 μ g of delprostenate (Glandinex, Universal Lab, Montevideo, Uruguay). Eight heifers that showed standing estrus on the same day (day 0) were selected for the study. The cows were grazing on improved pastures and supplemented with a mixture of concentrates and corn silage. Blood samples for hormone determination were taken every second day by jugular venipuncture using vacutainer tubes from day 0 to day 22. Samples were centrifuged within 1 h and plasma was stored at -20°C until hormone assays were performed. Endometrial samples 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 less than 10 min, and samples were stored at -80°C for subsequent analyses.

2.2. Hormone determinations

Progesterone was determined in all samples by solid-phase radioimmunoassay using a commercial kit (Coat-a-count, Diagnostic Products, Los Angeles, CA, USA) as previously validated for bovine (Toribio et al., 1994). The sensitivity of the assay was 0.04 nmol/L. All samples were determined in the same assay. The intra-assay coefficients of variation for low (2.2 nmol/L), medium (12.0 nmol/L) and

Table 1

Primer sequences and expected amplicon sizes used for real-time RT-PCR assays.

Gene	Accession #		Primer sequence	Length (bp)
LEPR	U83512.1	F	AGCGCGGAAGATAAAAACTGC	101
		R	GTTTGCACCTGTTGCTGAAAA	
IGF-I	NM_001077828	F	CCAGACAGGAATCGTGGATG	89
		R	ACTTGGCGGGCTTGAGAG	
IGF-II	*	F	GACCGCGGCTTCTACTTCAG	205
		R	AAGAACTGCCACGGGTAT	
IGF-1R	*	F	TTAAATGGCCAGAACCTGAG	314
		R	ATTATAACCAAGCCTCCAC	
GHR	NM_176608	F	TTCTGGGAATCCTAAATTCACCAA	91
		R	CTGTAACCTGTGATTAGCCCCATCT	
RPL19	AY158223	F	CCCAATGAGACCAATGAAATC	119
		R	CAGCCATCTTTGATCAGCTT	

The studied gene products are: leptin receptor (LEPR); insulin-like growth factor type I/II (IGF-I/II) and type 1 receptor (IGF-1R); growth hormone receptor (GHR) and ribosomal protein L19 (RPL19).

* Multiple species primers. Referenced primer sequences: IGF-I and GHR (Wu et al., 2004); IGF-II and IGF-1R (Pfaffl et al., 2002); RPL19 (Chen et al., 2006).

high (31.8 nmol/L) controls were 7%, 2% and 3%, respectively.

2.3. RNA isolation and reverse transcription

Total RNA from endometrial biopsies was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA) followed by precipitation with lithium chloride to remove inhibitors of cDNA synthesis and by DNase-treatment with DNA-Free™ Kit (Ambion, Austin, TX, USA) to remove contaminating DNA (Naderi et al., 2004). RNA concentration was determined by measuring absorbance at 260 nm, the purity of all RNA isolates was assessed from 260/280 absorbance ratio and the integrity by electrophoresis (1% agarose gel). All samples presented $A_{260/280}$ ratios between 1.8 and 2.0. For each sample, cDNA was synthesized (in a single run) by reverse transcription using the SuperScript III First-Strand Synthesis System Kit (Invitrogen, Carlsbad, CA, USA) with random primers and 1 µg total RNA added as a template.

2.4. Quantitative real time PCR

Detail of the primers used is presented in Table 1. Primers sequences for amplification of IGF-I, IGF-II, IGF-1R and total GHR were obtained from the literature (Pfaffl et al., 2002; Wu et al., 2004). Primers for total LEPR were designed based on bovine nucleotide sequences available from NCBI (<http://www.ncbi.nlm.nih.gov/>). Expression level of ribosomal protein L19 (RPL19) was used as an endogenous control, using the primer sequences of Chen et al. (2006). Ribosomal protein L19 has been used before as an endogenous control in tissues from ruminants (Chen et al., 2006; Sosa et al., 2009). Its expression was determined in the samples of this study and it proved a good housekeeping gene since there was no variation across days of the cycle (Fig. 1). Real time PCR was performed as described by Sosa et al. (2009). The PCR reactions consisted of 10 µl SYBR® Green mastermix (QuantiMix EASY SYG kit, Biotools B&M Labs, Madrid, Spain), equal amounts (500 nM) of forward and reverse primers (Operon Biotechnologies GmbH; Cologne, Germany), and 3 µl diluted cDNA (1:7.5 in RNase/DNase-free water) in a final volume of 20 µl. Samples were analyzed in duplicate in a 72-disk

Rotor-Gene™ 6000 (Corbett Life Sciences, Sydney, Australia). Amplification conditions were 10 min at 95 °C and 40 cycles of 15 s at 95 °C, 60 s at 60 °C, and 20 s at 72 °C. After each PCR reaction, the corresponding dissociation curves were analyzed to ensure that the desired amplicon was being detected and to discard contaminating DNA or primer dimers. Total RNA from endometrial tissue collected previously was analyzed in triplicate in each run to be used as the basis for comparative expression results (calibrator). Each run of real time PCR included samples from all days of the cycle to minimize run-to-run variations. The first cycle during which the fluorescence was significantly greater than a fluorescence threshold (C_T) was determined for each gene and sample. To standardize quantitative gene expression measurements for differences in cellular input, RNA quality and RT efficiency among samples, data for gene expression were analyzed by the $2^{-\Delta\Delta C_T}$ method which implies normalization to an endogenous control ($\Delta C_T = [C_T(\text{target gene}) - C_T(\text{RPL19})]$) and to the calibrator ($\Delta\Delta C_T = \Delta C_T - \Delta C_T(\text{calibrator})$) (Livak and Schmittgen, 2001). Titration curves (plot of log input cDNA versus the C_T) were performed for each target and control gene to validate the experiment and calculate the efficiency of the assay. Efficiency (E) of cDNA replication was calculated based on the slope of the plot, according to the formula $\%E = (10^{-1/\text{slope}} - 1) \times 100$ (Rutledge and Cote, 2003).

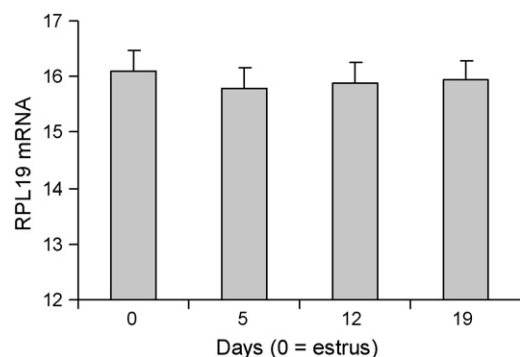


Fig. 1. Mean C_T values for RPL19 mRNA in the uterus of Holstein heifers on days 0 (estrus), 5, 12 and 19 of the estrous cycle. Data are presented as least square means \pm S.E.M.

2.5. Statistical analysis

All statistical analyses were carried out using the Statistical Analysis System (SAS Institute Inc., Cary, NC, USA). Data were analyzed in a randomized block design using a mixed model with repeated measures analysis to consider the correlation between sequential observations on the same animal (Littell et al., 2000). Day of the cycle was the fixed effect and for gene expression data, RNA extraction batch and PCR run were included in the model as random effects. Pearson correlation coefficient was used to describe relationships between the levels of expression of the different genes. Data are presented as least square means \pm pooled standard errors. The level of significance was considered to be $P \leq 0.05$.

3. Results

The cycle length was (mean \pm S.E.M.) 20.4 ± 0.4 days. All animals exhibited normal luteal activity as evidenced by progesterone plasma profiles. As expected, there was an effect of day of the cycle on plasma progesterone levels ($P < 0.0001$). Progesterone concentrations rose progressively from day 0, presented maximum levels on day 14 and then decreased to reach basal levels again on days 20–22.

The efficiency of quantification was 96% for RPL19 and 109% for LEPR mRNA. The expression of LEPR mRNA was demonstrated in all the days studied, and an effect of day

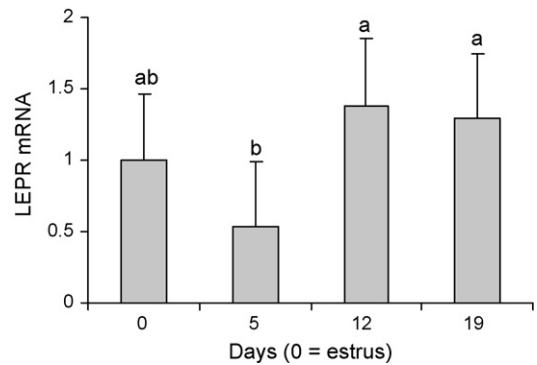


Fig. 2. Levels of expression (normalized against RPL19) of LEPR transcripts on days 0 (estrus), 5, 12 and 19 of the estrous cycle in Holstein heifers. Values are the least square means \pm pooled S.E.M. Different letters indicate statistical differences within the same variable, $P \leq 0.05$.

was observed ($P < 0.05$; Fig. 2). The expression was higher on days 12 and 19 than on day 5 ($P < 0.05$), and there was almost a 2-fold increase from day 5 to day 12 ($P < 0.01$).

Quantification efficiencies for IGF-I, IGF-II, IGF-1R and GHR mRNA were 96%, 105%, 98% and 110%, respectively. There was an effect ($P < 0.05$) of day of the cycle on the expression of IGF-I, IGF-1R and GHR transcripts, and a trend ($P = 0.065$) was observed for IGF-II mRNA. Data on the expression of these transcripts is presented in Fig. 3.

The expression of IGF-I mRNA was greater ($P < 0.05$) in the follicular phase and early luteal phase (days 0 and 5)

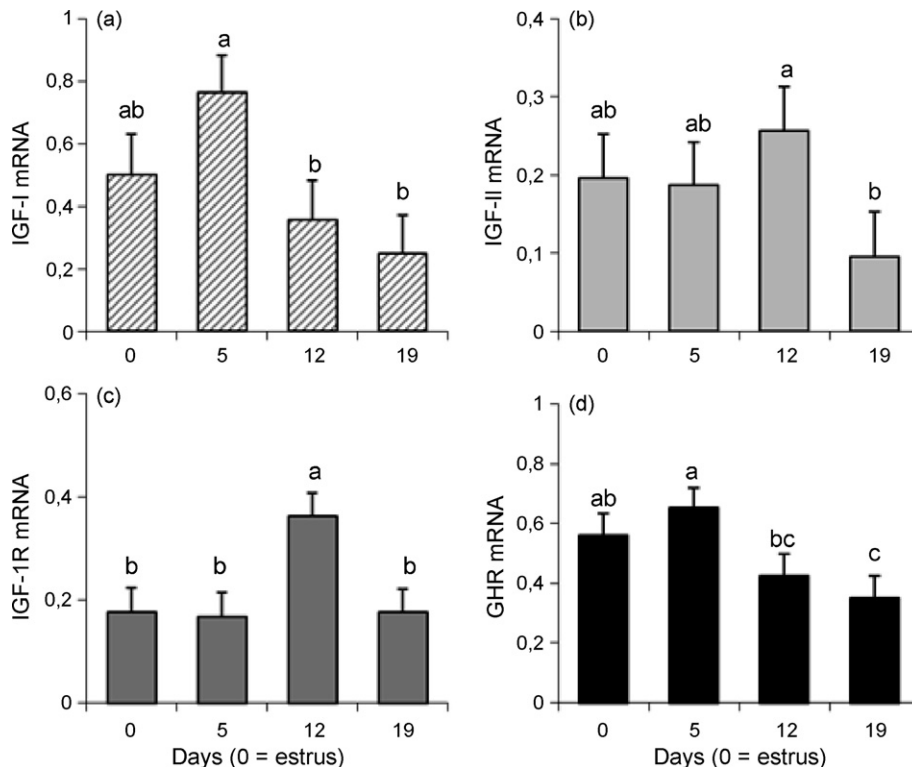


Fig. 3. Levels of expression (normalized against RPL19) of endometrial IGF-I (a), IGF-II (b), IGF-1R (c) and GHR (d) transcripts on days 0 (estrus), 5, 12 and 19 of the estrous cycle in Holstein heifers. Values are the least square means \pm pooled S.E.M. Different letters indicate statistical differences within the same variable, $P < 0.05$.

than on days 12 and 19. The amount of mRNA reached a maximum on day 5, and decreased on days 12 and 19 to 47% and 35% of peak values. IGF-II mRNA levels were similar on days 0, 5 and 12 but a significant ($P < 0.05$) reduction was observed on day 19.

The expression of IGF-1R transcript exhibited an increase ($P < 0.05$) on day 12 to return to the initial levels by day 19. Gene expression of GHR paralleled that of IGF-I, with maximum expression on day 5 and lower ($P < 0.05$) levels on days 12 and 19. However, in the case of GHR mRNA, the values reached on day 19 were lower ($P < 0.05$) than those on day 0.

The gene expression of IGF-II was highly and positively correlated with the expression of IGF-1R mRNA ($r = 0.73$, $P < 0.0001$, $n = 27$) as well as with IGF-I mRNA ($r = 0.50$, $P < 0.05$, $n = 26$). The expression of LEPR transcript was highly and negatively correlated with IGF-I mRNA ($r = -0.46$, $P < 0.05$, $n = 26$), and positively correlated with IGF-1R mRNA ($r = 0.59$, $P < 0.01$, $n = 27$).

4. Discussion

To our knowledge, this is the first report describing simultaneous changes in endometrial IGF-I, IGF-II, IGF-1R and GHR gene expression levels during the bovine estrous cycle, and the first evidence of changes in the expression of LEPR mRNA at different moments of the estrous cycle in the bovine reproductive tract.

The presence of the leptin/LEPR system has been demonstrated in the uterus of rodents, women, gilts and cows (Gonzalez et al., 2000; Yoon et al., 2005; Bogacka et al., 2006; Thorn et al., 2007). The novelty of the present study is the demonstration that the expression of the leptin receptor gene varies during the estrous cycle indicating a possible involvement of sexual steroid hormones in its regulation. Receptor expression was lower during the early luteal phase than in the mid-late luteal phase consistent with the estradiol downregulation of total LEPR observed in the endometrium of prepubertal dairy heifers (Thorn et al., 2007). Similarly, in the bovine ovary greatest expression of LEPR mRNA was found in the smallest follicles than in the bigger ones, which have higher estradiol content (Sarkar et al., 2010). The high uterine sensitivity to leptin on days 12 and 19 could suggest leptin actions on the preparation of the uterine environment for a possible pregnancy. Indeed, the role of leptin in implantation is strongly suggested by the fact that its receptors are preferentially expressed in the implantation sites rather than in the rest of the uterus in mice (Yoon et al., 2005).

The endometrial expression of IGF-I transcript was greater on days 0 and 5 than in the luteal phase, agreeing with previous results from our group using a different technique (Meikle et al., 2001). Results also agree with data by Robinson et al. (2000) and Rhoads et al. (2008) since they found the highest IGF-I expression at estrus. In the ovine uterus and oviduct, high levels were also found at estrus (Stevenson et al., 1994; Stevenson and Wathes, 1996). In the cow oviduct, a peak in IGF-I expression was reported around the ovulatory period (Schmidt et al., 1994). Independently of expression levels during the luteal phase, the high IGF-I gene expression at estrus seems

to be a common feature in ruminants both in oviducts and uterus. In fact, local production of IGF-I in the reproductive tract is stimulated by estrogenic action, most likely to mediate estrogen-driven cellular proliferation (Sahlin, 1998; Wathes et al., 1998; Meikle et al., 2001; Moyano and Rotwein, 2004). The marked loss of IGF-I mRNA observed in the middle of the luteal phase suggests that progesterone may inhibit IGF-I mRNA directly or indirectly via suppression of estrogen receptors. Although the importance of local synthesis of IGF-I is undeniable, the reproductive tract also receives the polypeptide through the general circulation. Plasma concentrations of IGF-I are increased at estrus in sheep (Sosa et al., 2009). High estrous IGF-I concentrations were found in the uterine fluid of cows and was suggested to be an estrogen-dependent enhanced transport from serum (Geisert et al., 1991). On the other hand, Bilby et al. (2006) reported that IGF-I in the uterine fluid was not correlated with its plasma levels and Rhoads et al. (2008) found no parallelism in the pattern of expression of hepatic and uterine IGF-I in cows. According to present and previous evidence, the IGF-I increase in the uterine fluid at estrus seems to have an important, finely regulated contribution from endometrial synthesis.

Overall, the expression of mRNA encoding IGF-II was relatively constant along the estrous cycle with maximum levels in the mid-luteal phase (day 12). However, a remarkable reduction (66%) was observed from days 12 to 19. This pattern of expression may reflect a putative stimulus of progesterone, which was high in the mid-luteal phase and declining by day 19. Indeed, it has been demonstrated in pigs that uterine IGF-II gene expression is regulated by estradiol and progesterone (Simmen et al., 1990). On the other hand, no difference was found in IGF-II levels in the uterine fluid between days 13 and 18 of the estrous cycle of cows (Geisert et al., 1991), which seems to be in contradiction with IGF-II mRNA expression found in those days in the present study. However, endometrial transcript expression does not necessarily reflect the protein content in the uterine fluid.

It has been suggested that IGF-1R is constitutively expressed in the bovine uterus (Robinson et al., 2000). This is in conflict with our results since there was a clear day effect on IGF-1R mRNA levels, with a 100% increase on day 12 to return to estrus levels on day 19, although the discrepancy is probably attributable to the different methodologies used for mRNA determination. The pattern of expression of IGF-1R mRNA was similar to that of IGF-II, which could imply a common regulating mechanism, i.e., modulation by estrogen and progesterone. It is interesting that the IGF-1R gene increases at the same time that IGF-I expression decreases and IGF-II expression increases. Type 1 receptor presents greater affinity for IGF-I than for IGF-II (Thissen et al., 1994), and the varying expression of IGF-1R gene could imply a compensatory mechanism to modulate both IGFs action.

The day of the estrous cycle also affected the expression of GHR, with greater levels at estrus and day 5 than on the late luteal phase (days 12 and 19). The lack of difference between days 0 and 5 is consistent with data from other authors (Rhoads et al., 2008). On the other hand, these results suggest, as for other components of the soma-

tropic axis, a regulation dependent on the estrous cycle. Indeed, there is a great body of information supporting the regulatory effects of sex steroids on GH secretion and actions (Chowen et al., 2004). IGF-I showed a similar pattern of expression as GHR, whereas IGF-II did not. This is consistent with a different stimulus for the production of both IGFs since the synthesis of IGF-I is much more GH-dependent than that of IGF-II (Thissen et al., 1994). A greater uterine sensitivity to GH could lead to greater expression of IGF-I, probably to mediate GH actions on the uterine function. Then, the uterine physiology will be under the combined and individual influence of estradiol, IGF-I and GH (acting through GHR) that would lead to cellular growth and proliferation and increased secretory activity to prepare the uterus for a possible pregnancy. Indeed, it has been demonstrated that treatment with bovine somatotropin increases plasma IGF-I concentrations, conceptus length and pregnancy rates in cattle (Moreira et al., 2001; Bilby et al., 2006).

Interpretation of the functions and relationships of the different components of the GH-IGF axis in the uterus is complex. They are not only subject of other hormones regulation (i.e., sexual steroid hormones) but are also modulated by other proteins such as the IGF binding proteins, which coordinate and regulate the biological activity of IGFs (Jones and Clemmons, 1995; Wathes et al., 1998). Moreover, IGF-II actions are also modulated by type 2 IGF receptor, which seems to have an inhibitory effect since it complexes with IGF-II and facilitates its lysosomal degradation (Brown et al., 2009), although some actions of IGF-II have been demonstrated to be mediated by this receptor type (Pantaleon et al., 2003).

If fertilization occurred, the hormonal scenario within the uterus would be the same as in a cyclic animal until around day 17 where the corpus luteum is rescued. The divergent gene expression of IGF-I and IGF-II observed in the present study suggests a preferential autocrine/paracrine role for IGF-I in the early stage of embryonic development, whereas IGF-II may be more involved in later stages of pregnancy. Interestingly, the increase of IGF-II mRNA observed in the present study (day 12) coincides with a moment in which the embryo has already hatched from the zona pellucida and expansion of trophoblast begins. Indeed, it has been speculated that IGF-II might serve as a differentiative agent during embryogenesis (Pantaleon et al., 2003).

In summary, the current study described for the first time the cyclic variations of the expression of the leptin receptor gene and monitored the integrated gene expression of important components of the GH-IGF system throughout the estrous cycle in heifers. These results are consistent with a regulatory action of sexual steroid hormones on the gene expression of LEPR, IGF-I and -II, IGF-1R and GHR.

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