Effect of pregnancy and progesterone concentration on expression of genes encoding for transporters or secreted proteins in the bovine endometrium

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Effect of pregnancy and progesterone concentration on expression of genes encoding for transporters or secreted proteins in the bovine endometrium. Physiol Genomics 41: 53–62, 2010. First published December 8, 2009; doi:10.1152/physieg.00162.2009.—The objective of this study was to determine the temporal and spatial expression patterns of genes encoding transporters, as well as selected secreted proteins that may be regulated by progesterone (P4) and/or the presence of the conceptus in the bovine endometrium. Estrus-synchronized beef heifers were randomly assigned to either: 1) pregnant, high P4; 2) pregnant, normal P4; 3) cyclic, high P4; or 4) cyclic, normal P4. Uteri were collected on days 5, 7, 13, and 16 of the estrous cycle or pregnancy. Localization of mRNAs for ANPEP, CTGF, LPL, LTF, and SLC5A1 in the uteri was determined by radioactive in situ hybridization, and expression quantified in the endometria by quantitative real-time PCR. ANPEP localized to luminal (LE) and superficial glandular (sGE) epithelia of all heifers on days 5 and 7 only. SLC5A1 mRNA was detected in the LE and sGE on days 13 and 16 in all heifers, and expression increased on day 16 in pregnant groups. CTGF localized weakly to the LE and GE on days 5 and 7 but increased on days 13 and 16 with an increase (P < 0.05) in CTGF expression in high P4 (day 7) and pregnant heifers (day 16). Both LPL and LTF localized to the GE only on days 5 and 7. In conclusion we have characterized the temporal expression pattern of these genes and modulation of their transcript abundance by P4 (CTGF, LPL) and/or the conceptus (CTGF, SLC5A1) likely modifies the uterine microenvironment, enhancing histotroph composition and contributing to advanced conceptus elongation.

THE PREIMPLANTATION PERIOD in cattle is the period during which most embryonic loss occurs (9), highlighting the fact that the conceptus-maternal dialogue is of critical importance in maintaining pregnancy prior to maternal recognition of pregnancy (MRP), which, in cattle, occurs around day 16 of pregnancy. A significant body of evidence supports the concept that, once in the uterus, the developing embryo relies on uterine histotroph for its development, i.e., secretions primarily derived from the uterine glands (12, 13, 15, 16). Sheep studies utilizing the uterine gland knockout ewe model demonstrated the absolute requirement for secretions from the uterine glands for normal development of the conceptus and, in particular, for posthatching conceptus elongation from spherical to tubular and filamentous forms. When neonatal ewes are exposed to progestins, uterine gland development is permanently ablated and the conceptuses will not elongate past the tubular to early filamentous stage of development. In addition, elongation of ruminant conceptus has not been achieved in vitro (1, 4), reinforcing the importance of the in vivo uterine environment.

Recent studies in sheep have shown components of histotroph, such as glucose and amino acids, vary in expression and abundance according to day of the estrous cycle and pregnancy but only increase significantly in pregnant ewes between days 10 and 16 of pregnancy (12, 16). Moreover, changes in expression of mRNAs and proteins involved in transport of glucose and amino acids in histotroph are regulated temporally during early pregnancy by progesterone (P4) and/or conceptus products such as interferon tau (IFNT) (12, 13, 15).

The benefit of increased circulating concentrations of P4 in the immediate postconception period on advancing conceptus development and increasing embryo survival has been demonstrated in both beef and dairy cattle (6, 9, 17, 24, 31) as well as sheep (27). In an effort to understand the mechanisms involved in this process, using a large-scale microarray approach, we recently described changes that occur in the endometrial transcriptome in the presence of elevated P4 at key check-points during the estrous cycle and early pregnancy (11). Our results indicate that these P4-induced changes are key to the advancement in conceptus development observed when circulating concentrations of P4 are high (6, 27). Among genes in endometria of heifers that are regulated by P4 are those that encode for transport proteins, as well as selected secreted proteins, which may be crucial in determining the composition of histotroph upon which the embry/conceptus is dependent for growth and development. Therefore, the objectives of this study were to: 1) determine temporal and cell-specific changes in expression of such genes in bovine uteri; 2) quantify abundance of mRNA transcripts of these genes; and 3) determine if changes in circulating concentrations of P4 and/or the presence of a conceptus alters expression of these genes and contributes to advanced development of the conceptus.

MATERIALS AND METHODS

Animals and Treatments

All experimental procedures involving animals were licensed by the Department of Health and Children, Ireland, in accordance with the Cruelty to Animals Act (Ireland 1897) and the European Community Directive 86/609/EC. All procedures were sanctioned by the University College Dublin, Ireland Animals Research Ethics Committee.

The experimental design has been described previously (6). Briefly, 263 cross-bred beef heifers were synchronized to estrus by insertion of a controlled internal drug release (CIDR) (1.94 g P4; InterAg, Hamilton, New Zealand). Three days before CIDR removal all heifers received a 15 mg intramuscular injection of a prostaglandin F2α analog (PG, Estrumate, Shering-Plough Animal Health, Hertfordshire, UK). Two-thirds (n = 140) of the heifers in standing estrus were

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inseminated, and one-third \((n = 70)\) were left as noninseminated cyclic controls. Approximately one-half of the heifers in both groups received a progesterone intravaginal releasing device (PRID, 1.55 g P4; CEVA Animal Health, Chesham, UK) on day 3 to increase circulating concentrations of P4. This generated four treatment groups: 1) pregnant, high P4 (PH); 2) pregnant, normal P4 (PN); 3) cyclic, high P4 (CH); and 4) cyclic, normal P4 (CN). Uteri were recovered at slaughter from heifers on either day 5, 7, 13, or 16 of pregnancy or the estrous cycle, corresponding in pregnant heifers to the 8- to 16-cell stage, blastocyst stage, initiation of elongation of conceptus, and MRP stages, respectively. At slaughter the reproductive tract of all heifers was flushed using 20 ml of phosphate-buffered saline supplemented with 5% fetal calf serum (Sigma, Dublin, Ireland). Whole uterine cross sections were taken from the uterine horn ipsilateral to the corpus luteum of each heifer and immersed in a 10% formalin solution for 24 h. Strips of endometria (~300 mg) were recovered from the midsection of the ipsilateral horn. These were immersed in 1:5 wt/vol RNEAlater (Sigma, Dublin, Ireland), transported to the laboratory on ice, and left at 4°C for 24 h. Excess RNEAlater was removed, and all samples were transferred to RNase/ DNase-free tubes for storage at −80°C prior to RNA extraction. Five heifers per treatment per time point were used for mRNA localization and quantification \((n = 80)\) in total. For the PH and PN heifers, only those with an appropriately developed embryo/conceptus \((i.e., at the correct stage for age)\) were used in the study.

In Situ Hybridization Analysis

We reported that a large number of genes are temporally regulated between days 7 and 13 of the estrous cycle and early pregnancy and that the alteration in the timing and duration of these genes contribute to advanced conceptus development on day 13 and 16 of pregnancy \((11)\). One way in which these genes may contribute to conceptus development is by altering the composition of uterine histotroph. We therefore chose to further characterize five genes that encode for secreted proteins or proteins involved in transport of nutrients \((i.e., potentially contributing to histotroph composition). Localization of mRNAs for alanyl \((\text{membrane})\) aminopeptidase \((\text{ANPEP})\), connective tissue growth factor \((\text{CTGF})\), lipoprotein lipase \((\text{LPL})\), lactotransferrin \((\text{LTF})\), and solute carrier family 5 \((\text{sodium/glucose cotransporter})\), member 1 \((\text{SLC5A1})\) was determined by radioactive in situ hybridization as previously described \((30)\). In brief, 5 μm cross sections of the ipsilateral uterine horn were cut from paraffin-embedded tissue, deparaffinized, rehydrated, and deproteinated. Antisense or sense cRNA probes, generated from linearized deparaffinized, rehydrated, and deproteinated. Antisense or sense cRNA probes, generated from linearized

Table 1. Full name, abbreviation, accession number, and primer sequences for all genes used in quantitative RT-PCR analysis of gene expression in Bos taurus

<table>
<thead>
<tr>
<th>Accession Number</th>
<th>Entrez Gene Symbol</th>
<th>Gene Name</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Concentration, nMol</th>
</tr>
</thead>
<tbody>
<tr>
<td>AV067592</td>
<td>ACTB</td>
<td>actin, β</td>
<td>CCGCATGATAGATGATATGGC</td>
<td>AAGGCCGCTTTGAGAT</td>
<td>300/300</td>
</tr>
<tr>
<td>NM_001075144</td>
<td>ANPEP</td>
<td>alanyl ((\text{membrane})) aminopeptidase</td>
<td>ATCCGAGTGGTCGTGATTCTC</td>
<td>TCTGTATAGCCAAAGGTGTGCA</td>
<td>300/300</td>
</tr>
<tr>
<td>NM_174030</td>
<td>CTGF</td>
<td>connective tissue growth factor</td>
<td>CGTGTCAGCGCTAAAAGATG</td>
<td>TCCGCTCTGTCAGATGCTCT</td>
<td>300/300</td>
</tr>
<tr>
<td>NM_001075120</td>
<td>LPL</td>
<td>lipoprotein lipase</td>
<td>CAGGGTGAAGTCCGGAATCCA</td>
<td>GAAAGTGGCTTCGTTAGGGTAA</td>
<td>300/300</td>
</tr>
<tr>
<td>NM_180998</td>
<td>LTF</td>
<td>lactotransferrin</td>
<td>GAAGTCTAGACCTGGGCTGTA</td>
<td>CAGTTTCTGAGGTTTGCTGCAAG</td>
<td>300/300</td>
</tr>
<tr>
<td>NM_174606</td>
<td>SLC5A1</td>
<td>solute carrier family 5 ((\text{sodium/glucose cotransporter})), member 1</td>
<td>CTGGTTTGGCTTTCCAGGAA</td>
<td>GTGTCACAGTGGGATGCACT</td>
<td>300/300</td>
</tr>
</tbody>
</table>

Primer concentrations and sequence information for the primers are given in the 5’ to 3’ direction.
ANPEP mRNA Expression and Localization

Gene expression. ANPEP in CN heifers increased significantly from days 5 to 7 and then decreased to undetectable levels on days 13 and 16 in cyclic heifers (P < 0.05, Fig. 1A). Cyclic heifers with high P4 displayed similar expression profiles. Pregnancy status had no effect on ANPEP expression, irrespective of the P4 status, and mirrored the expression patterns in cyclic heifers. Consequently, there was no day-by-pregnancy status effect on expression of ANPEP (P > 0.05).

Localization. In both pregnant and cyclic heifers, irrespective of P4 status, ANPEP localized to the luminal (LE) and superficial glandular (sGE) epithelia on days 5 and 7 (Fig. 1B, Supplementary Fig. S1A) but was not detectable in the deep glandular epithelium (GE) or stromal cells. Furthermore, ANPEP was not detected in any of the cells of the uterus on days 13 or 16, irrespective of treatment group of the heifer.

CTGF mRNA Expression and Localization

Gene expression. CTGF expression in cyclic heifers with normal P4 (CN) increased from day 7 to day 13 (Fig. 2A, P < 0.05), with expression remaining stable between days 13 and 16. In CH heifers, the temporal expression of CTGF was the same as that for CN heifers; however, when CTGF expression was compared between CN and CH heifers on a specific day of the estrous cycle, expression was higher in CH animals on day 7 (P < 0.05). In pregnant heifers, expression of CTGF was similar between days 5 and 7 but increased on days 13 and 16 (P < 0.05). Moreover, PH heifers had greater CTGF expression on day 7 than PN heifers and CTGF increased in all pregnant heifers on day 16 compared with cyclic heifers (P < 0.05).

Localization. CTGF mRNA localized weakly to the LE and GE on days 5 and 7 in cyclic heifers (Fig. 2B, Supplementary Fig. S1B) but increased in GE on days 13 and 16 in both CN and CH heifers. In pregnant heifers, CTGF localization to LE and GE was similar to that for cyclic heifers; however, intensity of CTGF mRNA localization was greater in LE and sGE for PH compared with PN heifers.

LPL mRNA Expression and Localization

Gene expression. A temporal comparison of LPL revealed a significant decrease in expression in CN and CH heifers between days 7 and 13, which was maintained to day 16 (Fig. 3A, P < 0.05). However, CH heifers had higher expression values than CN heifers on day 5 of the estrous cycle (P < 0.05). LPL expression in pregnant heifers followed a similar pattern as for cyclic heifers with the exception that in pregnant heifers with normal P4 LPL expression increased from day 5 to day 7. Moreover, the decrease on day 13 compared with day 7 (P < 0.05) was greater in PH than PN heifers (P < 0.05).

Localization. In cyclic heifers, LPL localized to the GE on days 5 and 7, was weakly detectable on day 13, but was not detected on day 16 in any cell type in the uterus (Fig. 3B, Supplementary Fig. S1C). Localization of LPL was similar for pregnant heifers except that it also localized to LE on days 5 and 7. There was no effect (P > 0.05) of elevated P4 on LPL localization in any treatment group.

LTF mRNA Expression and Localization

Gene expression. LTF expression in CN heifers increased between days 5 and 7 then decreased to minimal levels on day 13, which were maintained to day 16 (Fig. 4A, P < 0.05). For CH heifers, LTF expression did not change as the estrous cycle progressed, and there was no effect of P4 on LTF expression. In PN heifers, LTF expression increased between days 5 and 7 and decreased on day 13 (P < 0.05), whereas LTF only exhibited a change in expression between day 7 and day 13 in PH heifers (P < 0.05).

Localization. LTF (Fig. 4B, Supplementary Fig. S1D) was limited to the GE in all heifers on days 5 and 7 but was not detectable on either days 13 or 16 of the estrous cycle or pregnancy and did not differ due to P4 status of the heifers.

SLC5A1 mRNA Expression and Localization

Gene expression. In cyclic heifers, SLC5A1 expression was low on days 5 and 7 but increased on day 13 and day 16 (Fig. 5A, P < 0.05) independent of P4 status. For pregnant heifers, SLC5A1 expression increased from days 7 to 16 but was not different between days 13 and 16 for PN and PH heifers. However, SLC5A1 mRNA was more abundant in pregnant compared with cyclic heifers on day 16 (P < 0.05).

Localization. SLC5A1 mRNA was not detectable on days 5 and 7 of the estrous cycle (Fig. 5B, Supplementary Fig. S1E). However, on days 13 and 16, SLC5A1 mRNA was detected in LE and sGE independent of pregnancy and P4 status.

DISCUSSION

Insertion of a PRID device on day 3 postestrus results in a rapid rise in P4 concentrations with physiological ranges; we reported previously that such elevated P4 between day 3 and 7 of the estrous cycle/early pregnancy advances the temporal pattern of gene expression, which, in part, may be associated with the significant advancement of conceptus elongation on day 13 and 16 (6, 11). The aim of this study was to ascertain the temporal and spatial expression patterns of genes encoding glucose and lipid transporters, as well as selected secreted proteins regulated by P4 and/or the presence of the conceptus in bovine uteri, which may contribute to advanced conceptus elongation. Overall the localization of genes in the uterus tends to inform their function (reviewed by Ref. 2). The localization of ANPEP, CTGF, LPL, LTF, and SLC5A1 to the GE indicates that these genes may function predominantly to contribute to histotroph composition. However, the localization of ANPEP and LPL to the LE, during the early stages of the estrous cycle/pregnancy may indicate that they function as a barrier to attachment and that the downregulation of this gene between day 7 and 13 may be required for implantation to occur. Conversely localization of CTGF and SLC5A1 to the LE may implicate these genes in enhancing the attachment process of the conceptus trophectoderm.

ANPEP, a membrane-bound zinc-dependent peptidase, cleaves neutral amino acids from the amino terminal of peptides (reviewed by Ref. 29). Previous reports have indicated that ANPEP protein is more abundant in human endometrial stromal cells during the secretory (follicular phase) compared with the proliferative phase (luteal phase) of the menstrual cycle (28). However, the present study demonstrates that there...
Fig. 1: A: Quantitative real-time (Q-RT)-PCR analysis of alanyl (membrane) aminopeptidase (ANPEP) gene expression relative to β-actin (ACTB, the normalizer) in cattle for: cyclic, normal P4 (CN, black bars); cyclic, high P4 (CH, white bars); pregnant, normal P4 (PN, light gray bars); and pregnant, high P4 (PH, dark gray bars). Expression values are presented as means ± SE (n = 5 per treatment group per day). *Significant differences among treatment groups on a specific day. Letters A–D, significant differences within a treatment group due to effects of day (P < 0.05).

B: Representative slides for pregnant heifers with normal P4 at each stage of pregnancy for ANPEP mRNA as seen under light and dark fields of the microscope. P4, progesterone. To view representative slides for all treatments and time points see Supplementary Fig. S1A.
was no relationship between elevated or normal P4 on ANPEP expression in the bovine uterus. If ANPEP has a role to play in contributing neutral amino acids to the histotroph it is not dependent on P4 for this function in cattle, although ANPEP may play different roles in endometria of different species. This alternate function is not unprecedented. For example, LGALS15 regulates conceptus growth and elongation only in sheep and goats, but not in cattle where it is not expressed (22, 27).

LPL, which codes for an enzyme involved in lipid metabolism and transport, exhibits an expression pattern similar to that for ANPEP in the bovine uterus. These lipoproteins prevent lipid loss into the circulation and are also involved in the
delivery of lipids such as triacylglycerol (TAG) to target tissues (reviewed by Ref. 25). Utilization of TAG up to the blastocyst stage of development in cattle has been documented in vitro (10). Localization of LPL to the LE and sGE on days 5 and 7 suggests that TAG, transported into the uterine lumen by LPL, functions as an energy source during early embryonic development before glucose is available as an energy source at the blastocyst stage. Moreover, in heifers with elevated P4, peak LPL expression occurs earlier and its downregulation on day 13 is accelerated and points toward a role for LPL-mediated alterations in histotroph that are advantageous for blastocyst and conceptus growth and development.

We and others have shown that up to the blastocyst stage, TAG may be a source of energy for the developing embryo before the switch to utilization of glucose as an energy source from the blastocyst stage onward (10, 11). Further evidence for
this potential temporal switch in energy source for the developing embryo is based on the expression pattern of a facilitative glucose transporter in the bovine endometrium. Members of the SLC family have been characterized in uteri of a number of species during early pregnancy (12, 21, 34). SLC5A1, a member of the solute carrier family of proteins, is a facilitative glucose transporter (19). In ewes, SLC5A1 is localized to the LE and sGE and its expression increases as pregnancy progresses (12). Additionally, expression of SLC5A1 mRNA expression is induced by P4 and further stimulated by IFNT produced by the conceptus between days 10 and 21 of pregnancy with localization transitioning to the GE enhancing the
capacity of the endometrium to transport glucose as implantation and placentation progress (12). Results of the present study are the first to characterize the localization and expression patterns for SLC5A1 in bovine uterus during the estrous cycle and early pregnancy and to suggest that glucose transport into the uterine histotroph occurs at a time coincident with the ability of the conceptus to utilize glucose as its energy source. Moreover, secretion of IFNT by the developing conceptus stimulates SLC5A1 expression in the ewe (12), and in the present study we see an increase in its expression concomitant with the time when the conceptus produces IFNT. This indicates that glucose transport into the uterine luminal histotroph

Fig. 5. A: Q-RT-PCR analysis of solute carrier 5 (sodium/glucose cotransporter), member 1 (SLC5A1) gene expression relative to ACTB (the normalizer) in cattle for: CN (black bars), CH (white bars), PN (light gray bars), and PH (dark gray bars). Expression values are presented as means ± SE (n = 5 per treatment group per day). *Significant differences among treatment groups on a specific day. Letters A–D, significant differences within a treatment group due to effects of day (P < 0.05). B: representative slides for pregnant heifers with normal P4 at each stage of pregnancy for SLC5A1 mRNA as seen under light and dark fields of the microscope. To view representative slides for all treatments and time points see Supplementary Fig. S1E.
is maintained in pregnant, but not cyclic heifers in which there is no developing conceptus to nourish.

LTF, a glycoprotein secreted by epithelial cells in a variety of mammalian species, has many functions including iron homeostasis and modulation of both the innate and adaptive immune systems (reviewed by Ref. 18). Its functional role during early pregnancy has not yet been elucidated; however, in mice, Ltf is present in uterine flushings at all stages of the estrus cycle and is localized to the uterine LE and GE with greatest expression during pre-estrus and estrus stages (26). Its expression is also induced by estrogen (7) although P4 does not alter Ltf expression in uteri of mice (23). Localization of LTF to uterine GE in cattle indicates it may be secreted into the uterine lumen on days 5 and 7 of pregnancy or the estrus cycle to modulate immune cell functions. Moreover, analysis of the temporal changes in LTF indicated its expression was high in a relatively low P4 environment (day 7, zona enclosed blastocyst) but decreased to basal levels when P4 increased (day 13, ovoid conceptus hatched from the zona pellucida), and this decrease was more apparent on day 13 in heifers with elevated P4. This suggests that any role for LTF is restricted to the preimplantation period of pregnancy and that its expression is limited to the period of expression of receptors for P4 in uterine epithelia that downregulate earlier in response to high P4 (29).

CTGF, a cysteine-rich protein and a member of the CNN family of proteins, is expressed in multiple tissues where it has different functions (3, 8), including stimulation of proliferation, migration, and adhesion of cells, all important for conceptus development and implantation in mammals (8). With regard to its role in the uterus, CTGF has been localized to uterine LE and GE in an number of species during the luteal phase of the estrous and menstrual cycle (32, 33) with increased expression during early pregnancy in mice (up to day 3.5; Ref. 32) and cattle (day 18; Ref. 20). Moreover, CTGF is a secreted protein found in uterine luminal flushings of both pigs and mice (5, 32). With this evidence, in conjunction with results of the present study, we propose that secreted CTGF from uterine LE and GE of cattle plays a role in cell proliferation, migration, and/or adhesion of conceptus trophoectoderm during early pregnancy. In addition, elevated P4 increases and IFNT further stimulates CTGF expression, which likely promotes advanced conceptus elongation in heifers with high P4 (6).

The results of the present study are the first to quantify and localize expression of ANPEP, LPL, LTF, CTGF, and SLC5A1 mRNAs in bovine uterus during the estrus cycle and early pregnancy. Of the genes studied only CTGF and LPL were affected by elevated P4 concentrations, indicating that these genes are the most likely candidates that contribute to advanced conceptus development. That ANPEP, LPL, CTGF, and SLC5A1 are affected by the presence of an embryonic/conceptus indicates these genes may modulate the endometrial surface to enhance trophoectoderm attachment. In conclusion, we submit that modulation of the temporal expression pattern of these genes and their transcript abundance by P4 (CTGF, LPL) and/or conceptus-derived factors including IFNT (CTGF, SLC5A1) modifies the uterine microenvironment by altering uterine histotroph composition and receptivity to implantation.

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DISCLOSURES
The authors have nothing to declare.

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