Expression of luteinizing hormone genes in bovine conceptuses

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RT-PCR analysis demonstrated that bovine conceptuses at days 16, 23 and 30 expressed LH- β -like and glycoprotein hormone α -like transcript sequences; adult kidney, liver and brain produced predominantly unspliced products. Sequencing of the LH- β -like fragment (from conceptuses at day 30) indicated complete homology with the published sequence. In addition, ribonuclease protection assay of RNA samples from bovine conceptuses at day 30 with a bovine LH- β probe revealed the presence of protected molecules that appeared to be full length. Northern blot analysis of total RNA from conceptuses at day 30 failed to demonstrate

the presence of LH- β or glycoprotein α subunit transcripts, whereas both transcripts were readily detected in adult pituitary RNA. Administration of hCG into the uterus of heifers from day 14 to day 16 of the oestrous cycle did not affect circulating progesterone concentrations, whereas the same dose increased progesterone concentrations ($P < 0.05$) when administered intravenously. These results indicate that the early bovine conceptus transcribes genes encoding LH- α and - β subunits, but at a level unlikely to be of physiological consequence.

Introduction

An important requirement for successful establishment of pregnancy in cattle is the extension of the lifespan of the corpus luteum to maintain progesterone secretion necessary for continued gestation. Maintenance of the corpus luteum is achieved by direct luteotrophic support from pituitary LH during the luteal phase, and from chorionic gonadotrophin in primates during pregnancy (Hearn, 1986; Webley and Hearn, 1994). In cattle, it is generally agreed that the corpus luteum of pregnancy is largely dependent on pituitary LH and, between day 16 and days 24–27 of pregnancy (Helmer *et al.*, 1987), the conceptus synthesizes interferon τ to abrogate the luteolytic effects of PGF_{2 α} from the uterus (Bazer *et al.*, 1986; Godkin *et al.*, 1988). As cows require a functional corpus luteum for the greater portion of gestation (Estergreen *et al.*, 1967), and interferon τ is produced transiently (Helmer *et al.*, 1987), the possibility of additional placental luteotrophic support in this species should not be ruled out. Other studies have provided preliminary evidence for bovine conceptus luteotrophins, in particular molecules with bovine LH (bLH)-like activity with molecular mass greater than that of pituitary bLH (Ailenberg and Shemesh, 1983; Hickey *et al.*, 1989).

The present study investigated the transcription of bLH- α and - β subunit mRNA using RT-PCR to determine whether bovine conceptuses synthesize bLH-like molecules during early development. Analysis of PCR products was

performed using Southern blot analysis, restriction endonuclease digestion, ribonuclease protection analysis and sequencing. The potential physiological implications of bLH synthesis within the uterus were investigated further by examining the effect of administering small amounts of hCG either into the uterine lumen or into the circulation on circulating progesterone concentrations in an attempt to determine the likelihood of luteotrophic or luteolytic effects that could result from LH synthesis by the conceptus.

Materials and Methods

Tissue collection and RNA isolation

The tissue samples for RNA isolation were obtained from conceptuses recovered from beef cows immediately after they had been killed at one of four stages of gestation after artificial insemination: day 16 (eight sets of pooled samples), day 23 (six single whole samples), day 30 (16 conceptuses) and day 35 (12 conceptuses). The cows that were used to obtain conceptuses at day 16 were synchronized for oestrus and superovulated, whereas the cows that provided conceptuses at days 23, 30 and 35 were not superovulated. Adult tissues were also obtained from cattle immediately after they were killed, namely the anterior pituitary gland ($n = 7$), liver ($n = 4$), kidney ($n = 4$) and brain ($n = 4$). Conceptuses at day 16 from superovulated cows were flushed from the uterus and pooled for each analysis. Conceptuses at days 23 and 30 were obtained by dissection of the uterus. Conceptuses at day 23 were used as single whole samples for each analysis. Conceptuses at days 30 and 35 were dissected into allantois, embryo and chorion, each of which was analysed separately.

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All animal procedures were approved by the University Animal Care Committee in accordance with the regulations of the Canadian Committee for Animal Care. Total RNA was isolated from each sample immediately after recovery essentially as described by Maniatis *et al.* (1982).

RT-PCR using specific primers for bLH- α and bLH- β subunits

Total RNA (100 ng–2 μ g) from conceptuses and adult tissues was mixed with random primers (Gibco BRL, Burlington, ON) in a proportion of 40 ng primer per μ g RNA and the volume was made up to 10 μ l with diethylpyrocarbonate (DEPC)-treated water. The sample was heated for 10 min at 70°C, cooled immediately on ice and spun briefly to ensure the sample was at the bottom of the tube before storage. The mixture was stored on ice and combined with 5 μ l of 5 $\times$ reverse transcription buffer (Gibco BRL), 2 μ l of 0.1 mol dithiothreitol (DTT) l⁻¹ (Gibco BRL), 2 μ l of 10 mmol dNTP l⁻¹ (Pharmacia Biotech, Baie d'Urfe, Quebec), 40 U RNAGuard RNase inhibitor (Pharmacia), 300 U Moloney murine leukaemia virus RNase H⁻ reverse transcriptase (M-MLV H⁻ RT (Superscript)TM; Gibco BRL) and DEPC-treated water to a volume of 25 μ l. The sample was left for 10 min at room temperature followed by incubation for 1 h at 38°C. The reaction products were heated at 95°C for 5 min. A 3 μ l volume of the final reaction mixture was used for PCR analysis with primer pairs designed according to the published bovine DNA sequences for LH- β (P1, P2) (Maurer, 1985; Virgin *et al.*, 1985) and the glycoprotein hormone α subunit (P3, P4) (Goodwin *et al.*, 1983; Nilson *et al.*, 1983). The sequences of the primers are as follows: P1: 5' TGCCCTGTCTGTATC-ACTTT 3'; P2: 5' CCACGGGGAAGGAGACCATT 3'; P3: 5' GCAGCTGTCATTCTGGCCAT 3'; and P4: 5' GCACTGAT-AGATTGGAGCAT 3'. PCR analysis was performed using a carryover contamination prevention kit (Gibco BRL) that substituted deoxyuridine triphosphate (dUTP) for deoxythymidine triphosphate (dTTP) and used uracil DNA glycosylase (UDG) to destroy uracil-containing DNA from previous reactions (Erlich *et al.*, 1991; Bebee *et al.*, 1992). The reaction components were 10 μ l of 10 $\times$ reaction buffer (100 mmol Tris-HCl l⁻¹, pH 8.3, and 500 mmol KCl l⁻¹; Gibco BRL), 2 μ l dU-dNTP mix (10 mmol l⁻¹ each of dATP, dCTP, dGTP and 20 mmol dUTP l⁻¹; Gibco BRL), 2 μ l 5' primer (20 pmol), 2 μ l 3' primer (20 pmol), 1 ng template DNA, 1 μ l UDG (1 U), 15 μ l MgCl₂ (10 mmol l⁻¹) and water to bring the final reaction volume to 100 μ l. For each PCR, a 'no template' and a 'no reverse transcriptase' control was included. The solution was incubated at 37°C for 10 min followed by incubation at 94°C for 10 min. Taq DNA polymerase (2.5 U; Promega Inc., Madison, WI) was added and the mixture was subjected to 40 cycles of amplification, each consisting of 94°C for 30 s (denaturation), 55°C for 60 s (annealing) and 72°C for 30 s (primer extension). At the end of the temperature cycles the solution was incubated at 72°C for 15 min. The PCR

products (20 μ l samples) were subjected to electrophoresis on 2% (w/v) agarose gels containing 1 μ g ethidium bromide ml⁻¹ with a DNA molecular size marker (Gibco BRL), and the amplified fragments were viewed under ultraviolet light and photographed.

Restriction endonuclease digestion of PCR products

Restriction endonuclease digestion was performed to verify the identity of the amplified cDNA fragments. The reaction mixture consisted of 15 μ l DNA, 2 μ l of 10 $\times$ restriction endonuclease buffer (buffer A for *Alu* I or buffer L for *Hpa* II; Boehringer Mannheim, Indianapolis, IN), 2 μ l restriction endonuclease (*Hpa* II for LH- β products or *Alu* I for glycoprotein hormone α subunit products), 0.8 μ l spermidine (100 mmol l⁻¹) and water to a final volume of 20 μ l. The samples were incubated at 37°C for 5–24 h. At the end of the reaction the digests were resolved on 2% (w/v) agarose gels.

Southern blot analysis of RT-PCR products using digoxigenin-labelled cDNA probes

After electrophoresis of the amplified fragments, the agarose gel was immersed twice (2 $\times$ 15 min) in denaturation solution (0.5 mol NaOH l⁻¹, 1.5 mol NaCl l⁻¹) at room temperature with gentle shaking. The gel was rinsed with water and submerged in neutralization solution (0.5 mol Tris-Cl l⁻¹, pH 7.5, 3 mol NaCl l⁻¹) for 2 $\times$ 15 min at room temperature. The DNA was transferred from the gel to positively charged nylon membranes (Boehringer Mannheim) by capillary transfer in 20 $\times$ saline-sodium citrate buffer (SSC) overnight, as described by Sambrook *et al.* (1989). After transfer the DNA was fixed on the membrane by heating in a vacuum oven at 80°C for 2 h. Hybridization was performed as described for the northern blotting (see below) except that for the Southern blot incubations with both the prehybridization solution (5 $\times$ SSC, 1% (w/v) blocking reagent, 0.1% (w/v) *N*-lauroylsarcosine, 0.02% (w/v) SDS) and the hybridization solution were performed at 68°C. Chemiluminescent detection was performed as for the northern blot except that the Southern blot was blocked using buffer 2 (1% (w/v) blocking reagent in buffer 1; 100 mmol maleic acid l⁻¹, 150 mmol NaCl l⁻¹, pH 7.5) for 1 h and was washed for 2 $\times$ 15 min in buffer 1 after the antibody incubation step.

Northern blot analysis

Eight microlitres of deionized formamide, 2 μ l of 20 $\times$ running buffer (800 mmol MOPS l⁻¹, 200 mmol sodium acetate l⁻¹, 20 mmol EDTA l⁻¹, pH 7), 4 μ l formaldehyde (12.3 mol l⁻¹, 37% (v/v) solution in water; Fisher Scientific, Unionville, ON) and 1 μ l ethidium bromide solution (1 μ g μ l⁻¹ water) were added to each total RNA sample (20 μ g) from the conceptuses at day 30 and the adult tissue and to a 5 μ l sample of digoxigenin-labelled RNA molecular mass marker III (Boehringer Mannheim). The

samples were incubated for 15 min at 65°C followed by the addition of 1.5 µl loading buffer (50% (v/v) glycerol, 0.2% (w/v) bromophenol blue and 0.2% (w/v) xylene cyanol). The samples were loaded into the wells of a 3 mm thick 1% (w/v) agarose-formaldehyde gel covered with 1 × running buffer and subjected to electrophoresis at 60 V for 2 h. The RNA bands were viewed over ultraviolet light and the integrity of the ribosomal RNA was confirmed.

After electrophoresis, the agarose-formaldehyde gel was equilibrated by soaking in 20 × SSC buffer for 2 × 15 min. RNA was blotted to a positively charged nylon membrane (Boehringer Mannheim) by capillary transfer in 20 × SSC buffer for 15–24 h at room temperature, as described by Sambrook *et al.* (1989). After transfer the membrane was heated in a vacuum oven at 80°C for 2 h to fix the RNA and was stored in a vacuum-sealed plastic bag.

Hybridization was performed using the procedure and reagents provided with the digoxigenin DNA labelling and detection kit (Boehringer Mannheim). The blot was placed in a hybridization bag containing 20 ml northern prehybridization solution (5 × SSC, 50% (v/v) formamide, 7% (w/v) SDS, 0.1% (w/v) *N*-lauroylsarcosine and 2% (w/v) blocking reagent) per 100 cm² of membrane surface area and was incubated at 42°C for 2 h with gentle shaking. The probes used were: pSP65-βbLH containing a bovine LH-β cDNA insert cloned into the *Eco* RI site of pSP65 (Maurer, 1985; provided by R. A. Maurer, Department of Physiology and Biophysics, University of Iowa, Iowa City, IA); pBα consisting of a bovine pituitary glycoprotein hormone α subunit cDNA insert cloned into the *Pst* I site of pBR 322 (Nilson *et al.*, 1983; provided by J. H. Nilson, Department of Pharmacology and Medicine, Case Western Reserve University, Cleveland, OH); and pBS SK(+) actin, which was constructed by subcloning a bovine γ actin cDNA insert from an original pBR 322-based clone (Degan *et al.*, 1983) into the *Pst* I site of pBluescript SK(+)TM (Stratagene Cloning Systems, La Jolla, CA), and was provided by D. Morris (Department of Biochemistry, University of Washington, Seattle, WA). Digoxigenin-labelled cDNA probe (LH-β, glycoprotein hormone α subunit or γ actin) was denatured by heating in a boiling waterbath for 10 min and chilled immediately on ice, after which it was added to 10 ml prehybridization solution at a concentration of 5–25 ng ml⁻¹. The northern prehybridization solution was discarded from the bag and was replaced with the hybridization solution containing the probe. The bag was incubated at 42°C overnight with gentle shaking. The membrane was placed in a Pyrex dish and was washed (2 × 5 min) in 2 × wash solution (2 × SSC, 0.1% (w/v) SDS) at room temperature, after which it was washed (2 × 15 min) in 0.5 × wash solution (0.5 × SSC, 0.1% SDS) at 68°C.

Chemiluminescent detection

Chemiluminescent detection was performed using a modified version of the method of Lanzillo (1991) according to the instructions provided with the digoxigenin

DNA labelling and detection kit (Boehringer Mannheim). All incubations were performed at room temperature with gentle agitation. After post-hybridization washes the membrane was equilibrated in 50 ml buffer 1 per 100 cm² for 1 min. The membrane was then incubated in 50 ml northern blocking solution (7% (w/v) blocking reagent in buffer 1) for 2 h. Before completion of the blocking step, the anti-digoxigenin alkaline phosphatase (Boehringer Mannheim) was diluted to 150 mU ml⁻¹ (1:5000) in 30 ml blocking solution per 100 cm² membrane surface area. The blot was incubated for 30 min in the antibody solution, followed by 3 × 20 min washes in 200 ml buffer 1, after which the membrane was equilibrated for 5 min in buffer 3 (100 mmol Tris-HCl l⁻¹, pH 9.5, 100 mmol NaCl l⁻¹, 50 mmol MgCl₂ l⁻¹). The membrane was placed between two sheets of acetate and 0.5 ml per 100 cm² of LumigenTM PPD (Boehringer Mannheim) diluted 1:100 in buffer 3 was added and distributed evenly between the upper sheet of acetate and the upper surface of the membrane. For detection of chemiluminescence, the membrane was placed in a cassette for exposure of X-ray film (Kodak X-OMAT AR-5; Medtech, Mississauga, Ontario) for 1–12 h in the dark at room temperature.

Ribonuclease protection analysis

Ribonuclease protection analysis was performed essentially according to Lee and Costlow (1987) to determine the presence of full-length LH-β transcripts. The plasmid pSP65-βbLH contained a 600 bp bovine LH-β cDNA insert oriented such that an antisense RNA transcript was generated with SP6 RNA polymerase (Maurer, 1985). The second plasmid, pBS SK(+) actin, included a 1.3 kbp bovine γ actin cDNA insert (Degen *et al.*, 1983) with an orientation, as determined by *Hind* III digestion, allowing transcription of an RNA probe using T7 RNA polymerase. The 564 bp glycoprotein α subunit cDNA insert from a pBα clone (Nilson *et al.*, 1983) was subcloned into the *Sma* I site of pBluescript SK(+)TM, which has transcription promoters, to enable synthesis of an RNA probe. The three plasmids were digested at the 5' ends of the inserts, using *Xba* I for pSP65-βbLH, *Bst* EII for pBS SK(+) actin and *Pvu* II for pBS SK(+)-α, to enable transcription of antisense RNA probes. A digoxigenin RNA labelling kit (Boehringer Mannheim) was used to transcribe and label the RNA simultaneously.

Twenty micrograms of total RNA sample from day 30 conceptuses and adult tissues was dissolved in 20 µl hybridization buffer (80% (v/v) deionized formamide, 100 mmol sodium citrate l⁻¹, pH 6.4, 300 mmol sodium acetate l⁻¹, pH 6.4, 1 mmol EDTA l⁻¹; Soln A, Ambion kit, Ambion, Austin, TX). The samples were incubated with DNase I (20 U; Gibco BRL) for 15 min at 37°C to remove possible contaminating genomic DNA. The samples were then mixed with 600 pg digoxigenin-labelled RNA probe (bLH-β, α subunit or γ actin) before ribonuclease protection analysis using the ribonuclease protection kit (RPA IITM; Ambion, Austin, TX).

Fig. 1. (a) Detection of LH- β transcripts in bovine conceptuses by RT-PCR. Sizes of the products expected from spliced (187 bp) and unspliced (435 bp) LH- β transcripts are indicated on the right-hand side of the gel. The total RNA samples used for RT-PCR in the upper (conceptuses at day 30, $n=3$) and lower (conceptuses at days 16 and 23, $n=4$) parts of the gel are as indicated. (b) Hybridization of a bLH- β cDNA probe to bovine conceptus and adult tissue RT-PCR products. The RT-PCR products shown in (a) were transferred to positively charged nylon membrane and hybridized with digoxigenin-labelled bLH- β cDNA probe. (c) Verification of the identity of RT-PCR products from bovine

Sequencing of amplified LH- β DNA fragments

LH- β DNA fragments amplified using primers P1 and P2 from chorion and allantois of conceptuses from days 33 and 35 were sequenced using the ABI-377 prism sequencer (Perkin Elmer Corporation, Alameda, CA) using primers P1 and P2.

Intrauterine administration of hCG

Holstein heifers ($n=22$, age 12–15 months) were synchronized into oestrus using two injections (500 μg i.m.) of cloprostenol (Estrumate, Coopers Agropharm Inc., Willowdale, Ontario) 11 days apart and were assigned randomly to four treatment groups. On days 14–16 of the synchronized oestrous cycle, the animals received intravenous (hCG-IV, $n=7$) or intrauterine (hCG-IU, $n=7$) administration of 200 iu highly purified hCG (CR-127; National Hormone and Pituitary Program, Torrance, CA) once a day. Control groups received equivalent intravenous (Sal-IV, $n=4$) or intrauterine (Sal-IU, $n=4$) volumes of saline. The intrauterine administration of hCG or saline was performed using a 0.5 ml Cassou pipette inserted into the uterine body by cervical manipulation per rectum. Blood samples were collected once a day by coccygeal venepuncture into 10 ml heparinized vacutainer tubes (Beckton-Dickinson, Mississauga, Ontario) for determination of plasma progesterone concentrations. All animals were equipped with indwelling jugular catheters on day 13 to facilitate frequent blood sampling on days 14–16. Blood samples (10 ml) were also collected every 2 h starting before hCG treatment to 10 h after treatment. All the blood samples were centrifuged at 1500 g for 20 min and plasma was stored at -20°C until progesterone concentrations in the plasma were determined using a validated radioimmunoassay (Robinson *et al.*, 1989). Ovarian activity was monitored each day from day 12 to day 26 using transrectal ultrasonography (Aloka SSD-500 real-time B-mode linear array ultrasound scanner equipped with a 5.0 MHz transducer; Aloka Co. Ltd, Tokyo).

Plasma progesterone concentrations from each day were analysed by repeated measurement ANOVA using the general linear model procedure of SAS (SAS, 1990). Variables containing single observations in time were examined for treatment effects by one-way ANOVA.

conceptuses at day 30 in (a) using restriction endonuclease digestion analysis. Examples of the typical pattern of *Hpa* II-digested bLH- β -like RT-PCR products from conceptuses at day 30 displaying the expected sizes for unspliced (212, 91, 50, 44 and 38 bp) and spliced (105, 44 and 38 bp) transcripts are shown. P: pituitary; A: allantois; B: brain; C: chorion; d: digested; E: embryo; K: kidney; L: liver; M: (a,c) 123 bp DNA size marker, (b) digoxigenin-labelled DNA size marker; u: undigested; 1: conceptus at day 16; 2: conceptus at day 23.

Results

RT-PCR using bLH- β -specific primers

RT-PCR results obtained using the LH- β primers are shown (Fig. 1a). The expected 187 bp band was present in the tissues from bovine conceptuses at day 30 (allantois, embryo and chorion) and in the adult pituitary. The lower band appearing in all the samples represents unused primers. The adult kidney, liver and brain tissues yielded predominantly a fragment of larger size (435 bp), with comparatively fewer, if any, 187 bp fragments and one additional faint band just below the 435 bp fragment. Likewise, the expected 187 bp fragment was present in conceptus samples from day 16 (four pooled sets) and day 23 (four whole singles) (Fig. 1a). Southern blot analysis displayed strong hybridization of the 187 bp fragment from conceptuses at days 16, 23 and 30 with the bLH- β cDNA probe (Fig. 1b). The RT-PCR products from the adult kidney, liver and brain tissues showed hybridization predominantly at the 435 bp fragment. Restriction endonuclease digestion with *Hpa* II of the RT-PCR products resulted in fragments of expected sizes for pituitary and conceptus (105, 44 and 38 bp) and for kidney, liver and brain (212, 91, 50, 44 and 38 bp) products (Maurer, 1985; Virgin *et al.*, 1985) (Fig. 1c). The amplified fragments of LH- β DNA were sequenced using the 5' and 3' LH- β primers (P1 and P2). The forward and reverse sequences were compared with the bovine LH- β nucleotide sequence published in GenBank, using PC GENE® (IntelliGenetics, Inc., Mountain View, CA). Combining the forward and reverse nucleotide information into one sequence allowed complete (100%) alignment of pituitary, allantois- and chorion-amplified sequences with the published 187 bp LH- β nucleotide sequence between primers P1 and P2.

RT-PCR using bovine glycoprotein hormone α -specific primers

The PCR products generated using the glycoprotein hormone α subunit primers for the same reverse-transcribed samples as used for the LH- β products are shown (Fig. 2). The expected 147 bp band was observed in the different tissues of conceptuses at day 30 (allantois, embryo and chorion), similar to that for the adult pituitary. The adult kidney, liver and brain yielded predominantly amplified products of 1.2 kbp. In addition, other intermediate bands were observed. Conceptuses at days 16 and 23 displayed a similar pattern of expression of the α -like transcripts to that observed for the conceptuses at day 30, yielding predominantly the expected 147 bp band. Southern blot analysis and restriction endonuclease digestion with *Alu* I were used to confirm the identity of the amplified products (data not shown).

Northern blot analysis

Bovine pituitary RNA of approximately 700 bases elicited a strong hybridization signal with the bLH- β cDNA

Fig. 2. Detection of bovine glycoprotein hormone α subunit transcripts in bovine conceptuses by RT-PCR. Sizes of the products expected from completely spliced (147 bp) and unspliced (1.2 kbp) bovine glycoprotein hormone α subunit transcripts are indicated on the right-hand side of the gel. The total RNA samples used for RT-PCR in the upper (conceptuses at day 30) and lower (conceptuses at days 16 and 23) parts of the gel are as indicated. A: allantois; B: brain; C: chorion; E: embryo; K: kidney; L: liver; M: 123 bp DNA size marker; P: pituitary; 1: conceptus at day 16; 2: conceptus at day 23.

probe in northern blot analysis, whereas the RNA from conceptuses at day 30 and from adult kidney, liver and brain tissue did not hybridize. Re-probing of the same blot with the bovine γ actin cDNA probe revealed transcripts of approximately 2 kb in all the samples examined. Similar results were obtained when parallel samples were hybridized with the glycoprotein hormone α subunit cDNA probe: pituitary RNA of approximately 730 bases yielded a signal, whereas there was no hybridization to the conceptus or other tissue samples.

Ribonuclease protection analysis

Like the adult pituitary, but unlike the kidney, all the RNA samples from the conceptuses at day 30 appeared to protect a fragment of the size expected from full-length bLH- β transcripts (approximately 600 bases) (Fig. 3). However, the conceptus product was of very low intensity. Hybridization of parallel samples with a bovine γ actin RNA probe as a control revealed the presence of a protected fragment of the size (1.1 kb) expected for full-length γ actin transcripts.

Intrauterine administration of hCG

Intravenous administration of 200 iu hCG from day 14 to day 16 increased circulating concentrations of progesterone on days 16, 17 and 18 ($P < 0.05$; Fig. 4). However, infusion of the same dose of hCG into the uterus or the administration of saline (pooled by route of administration)

Fig. 3. Ribonuclease protection analysis of bLH- β RNA probe with total RNA from bovine conceptuses at day 30 and adult tissue. Total RNA (20 μ g) from bovine conceptuses at day 30 ($n = 2$) and adult tissues was hybridized with 600 pg digoxigenin-labelled bLH- β RNA, followed by ribonuclease digestion, polyacrylamide gel electrophoresis, electroblotting to positively charged nylon membrane and chemiluminescent detection. A: allantois; C: chorion; E: embryo; K: kidney; M: digoxigenin-labelled RNA size marker; P: pituitary. M and P on the right-hand side are M and P of the left-hand side after shorter exposure of the film.

was without effect. There were also no differences among treatments in the size or progression of either the existing corpus luteum or the ovulatory dominant follicle during or after administration of hCG or saline.

Discussion

RT-PCR analysis of total RNA from bovine conceptuses at days 16, 23 and 30 using primers for bLH- β resulted in amplified fragments of 187 bp, similar to those from the adult pituitary gland. On the basis of the published bovine LH- β DNA sequences (Maurer, 1985; Virgin *et al.*, 1985), the primers should amplify fragments of 187 bp for cDNA from processed bLH- β transcripts. Detection of bLH-like transcripts at day 30 in the chorion might be expected, as primate conceptuses synthesize hCG in the chorion (Hearn, 1986). The expression of LH-like transcripts in the allantois is consistent with studies that demonstrated the presence of a luteotrophic glycoprotein in allantoic fluid from day 24 to day 37 in bovine conceptuses (Hickey *et al.*, 1989). Although the embryonic pituitary gland is not visible before week 12 of gestation in humans, appearance of the pituitary primordium has been demonstrated using ultrastructural studies in human embryos at week 4 of pregnancy (Stefanovic *et al.*, 1993), possibly explaining the presence of bLH-like transcripts in the embryo tissue at day 30, given the similarities in the duration of gestation between humans and cows.

The expected size for the RT-PCR products of unprocessed bLH- β transcripts obtained using primers P1 and P2 is 435 bp (Maurer, 1985; Virgin *et al.*, 1985). The adult kidney, liver and brain tissues generated mainly fragments of larger size (435 bp), which represent products

Fig. 4. Plasma concentrations of progesterone (mean $\pm$ SEM) of heifers that received daily intravenous injection of 200 iu hCG ($\bullet$, $n = 7$), intrauterine infusion of 200 iu hCG ($\blacklozenge$, $n = 7$) or equivalent volume of saline ($\square$, $n = 8$); data pooled from intravenous saline ($n = 4$) and intrauterine saline ($n = 4$) on days 14–16 (indicated by arrows). Progesterone concentrations before treatment on day 13 were used as a covariate for statistical analysis. Asterisks indicate means from the intravenous hCG group that are significantly different from the other groups ($P < 0.05$).

from unprocessed transcripts, with fewer, if any, products from processed transcripts. Southern blot analysis, using a bLH- β cDNA probe (Maurer, 1985) and performed under stringent conditions that allow hybridization of only homologous sequences (Sambrook *et al.*, 1989), confirmed the specificity of the RT-PCR products. Further verification using restriction endonucleases that have sequence-specific sites for bLH- β (Virgin *et al.*, 1985) and sequencing confirmed that the amplified fragments are identical to LH- β cDNA in this region of the gene.

Examination of total RNA from bovine conceptuses at days 16, 23 and 30 by RT-PCR using the glycoprotein hormone α primers resulted in amplified fragments of 147 bp, similar to those produced using the adult pituitary gland. On the basis of published DNA sequences for the bovine glycoprotein hormone α subunit (Goodwin *et al.*, 1983; Nilson *et al.*, 1983), these primers should amplify fragments of 147 bp for cDNA from processed bovine glycoprotein hormone α subunit transcripts. Detection of conceptus α subunit-like transcripts lends support to the contention that the conceptus bLH- α - and - β -like transcripts could result in functional LH synthesis, as both subunits are required for the assembly of the glycoprotein (Pierce and Parsons, 1981).

Therefore, the experiments using RT-PCR demonstrate that bovine conceptuses at days 16, 23 and 30 express both LH- β -like and glycoprotein hormone α -like transcripts that appear to be processed. However, amplification by PCR of internal sequences does not prove that full-length transcripts are present; therefore, northern blot analysis was performed. Northern blot analysis of total RNA from bovine

conceptuses at day 30 did not demonstrate the presence of bLH- β or bovine glycoprotein α subunit transcripts, whereas both transcripts were readily detected in the adult pituitary. These results imply that if there are LH- β and glycoprotein α subunit transcripts in the bovine conceptus, they are considerably less abundant than in the pituitary. Therefore, evidence for the presence of full-length LH- β transcripts in bovine conceptuses was sought using the more sensitive ribonuclease protection analysis.

Solution hybridization analysis of total RNA from bovine conceptuses at day 30 with an RNA probe transcribed from bLH- β cDNA (Maurer, 1985) revealed a 600 base protected fragment, the approximate size expected for full-length mRNA, but in very low amounts. Similar results were observed for the glycoprotein α subunit, indicating that bovine conceptuses at day 30 express full-length processed LH- α - and - β -like transcripts.

Therefore, the present study provides evidence for the presence of bLH- β -like and glycoprotein hormone α -like transcripts in bovine conceptuses. If the conceptus synthesizes glycoprotein from the LH-like transcripts, albeit in small amounts, the potential role of this LH is worthy of further consideration. Conceptus LH-like glycoproteins could have a paracrine role within the conceptus or on the uterus, or an endocrine role on the corpus luteum. These functions would require adequate concentrations and transport of the glycoproteins and the presence of LH-hCG receptors on the respective target cells (Pierce and Parsons, 1981).

The presence of uterine LH-hCG receptors has been demonstrated in pigs (Ziecik *et al.*, 1986), rabbits (Jensen and Odell, 1988; Sawitzke and Odell, 1991), humans (Reshef *et al.*, 1990), rats (Bonnamy *et al.*, 1990, 1993) and cows (Friedman *et al.*, 1995). The presence of LH-hCG receptors in pregnant pig uterus indicates that these receptors could mediate the direct effects of LH, or a conceptus LH-like substance, for instance in the relaxation of myometrial smooth muscles (Ziecik *et al.*, 1986) or in the modulation of progesterone concentrations in the endometrium (Bonnamy *et al.*, 1990). Moreover, the observation that hCG administration increases uterine blood flow in gilts (Ziecik *et al.*, 1992) strengthens the concept that a paracrine effect of conceptus LH on uterine blood flow in cows could be mediated through the uterine LH-hCG receptors described by Friedman *et al.* (1995).

In addition to a local effect on the uterus, the possibility that potential glycoprotein products of the conceptus bLH-like transcripts have endocrine effects on the corpus luteum was considered. In general, an endocrine role would require synthesis of sufficient amounts of the bLH-like glycoprotein to overcome the dilution in systemic circulation and stimulate progesterone synthesis by the corpus luteum. Nevertheless, evidence is available for luteotrophic effects of conceptus products, as studies have demonstrated that both plasma and milk progesterone concentration are higher in pregnant compared with non-pregnant cows during the early luteal phase after ovulation, which is before the onset of luteolysis (days 16–17) and the

secretion of interferon τ (Henricks *et al.*, 1970; Bulman and Lamming, 1978; Lukaszewska and Hansel, 1980). Evidence for direct LH-like effects of bovine conceptus products on the corpus luteum also stems from studies that demonstrated the ability of bovine conceptus extracts or secretions to stimulate progesterone secretion by bovine luteal cells (Beal *et al.*, 1981; Ailenberg and Shemesh, 1983; Hickey *et al.*, 1989; Izhar and Shemesh, 1989; Thibodeaux *et al.*, 1994).

In an attempt to determine whether a small amount of bLH from the uterus was capable of exerting a luteotrophic response, hCG was administered directly into the uterus. As luteal and endometrial LH receptors have similar affinity for, and responsiveness to, hCG, this was used as a longer-acting stimulus. At a dose of hCG that stimulated progesterone synthesis by the existing corpus luteum when infused intravenously, there was no effect of the intrauterine administration of hCG. As the uterus at this time contains a significant population of LH-hCG receptors, the uterus could act to prevent escape of hCG to the vasculature and thus prevent action at the corpus luteum. As the endometrial LH-hCG receptors are coupled to cyclooxygenase activity and prostaglandin synthesis (Friedman *et al.*, 1995; Stepien *et al.*, 1999), the absence of a luteolytic effect is also of interest.

Therefore, these data support the conclusion that, at the level of transcription observed using RT-PCR and ribonuclease protection analysis, synthesis of active LH by the conceptus can have only physiological significance as a local mediator either within the developing conceptus or, possibly more likely, through interaction with the endometrial receptors for LH-hCG to influence uterine contractility or blood flow.

In summary, evidence is presented to indicate that there is expression of LH- α and - β subunit genes by bovine conceptuses between day 16 and day 35 of gestation, albeit at very low levels. At this level of detection, expression may be either non-specific 'leaky' transcription or low level extra-pituitary gene expression as reported in human leucocytes (Hotakainen *et al.*, 2000). As intrauterine administration of hCG had no effect on luteal function, it appears that any local synthesis of small amounts of LH would have only local physiological significance as a paracrine influence either on the developing conceptus or on the epithelium of the endometrium.

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