Stimulation of progesterone production in bovine luteal cells by co-incubation with bovine blastocyst-stage embryos or trophoblastic vesicles

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A study was conducted to determine whether bovine blastocyst-stage embryos and trophoblastic vesicles stimulate the production of progesterone in bovine luteal cells during incubation in vitro. The effects of co-incubation of these embryos and vesicles with uterine endometrial tissue on progesterone production was also investigated. Bovine small and large luteal cells were obtained on day 12 of the oestrous cycle, dispersed by unit gravity sedimentation and recombined to provide preparations free of accessory cells. Blastocyst-stage embryos were obtained on day 7 and trophoblastic vesicles were obtained from bovine embryos on day 12. A uterine endometrial tissue sample was obtained from the same cow from which the corpus luteum was taken. Treatment groups were arranged in 24-well plates as follows: luteal cells alone; luteal cells and one trophoblastic vesicle; luteal cells and one blastocyst embryo; luteal cells and a 10 mg uterine endometrial sample; luteal cells, one trophoblastic vesicle and a uterine endometrial sample; and luteal cells, one blastocyst embryo and a uterine endometrial sample. All treatment groups were incubated (at 37°C under 5% CO2) in Ham’s F-12 medium supplemented with antibiotics (100 μg penicillin ml–1 and 100 U streptomycin ml–1), L-glutamine (0.29 mg ml–1), insulin (5 μg ml–1), transferrin (5 μg ml–1) and selenium (5 ng ml–1) for 12 h. Samples of the medium were harvested 10 min (basal concentration) and 2, 6 and 12 h after incubation to determine the concentrations of progesterone and prostaglandin. The major findings of this study were that both trophoblastic vesicles and blastocyst-stage embryos stimulated progesterone production during the 12 h incubation. In addition, the uterine endometrial sample partially inhibited the stimulatory actions of trophoblastic vesicles and blastocyst embryos after 12 h of incubation.

Introduction

Significant increases in progesterone concentrations in plasma (Łukaszewska and Hansel, 1980) and milk (Lamming et al., 1989) in pregnant compared with nonpregnant cattle have been found as early as day 8 after oestrus. This finding indicates that some form of luteotropic activity is produced by the bovine conceptus before day 16, the day most often cited as the day of maternal recognition of pregnancy (Betteridge et al., 1980; Northeby and French, 1980). Homogenates and aqueous extracts of homogenates of bovine conceptuses at day 13–18 stimulate progesterone synthesis when added to dispersed bovine luteal cells (Beal et al., 1981). The active compound(s) is a lipido-soluble, heat-labile luteotrophin(s) of < 10 kDa (Hickey and Hansel, 1987). In addition, intra-

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uterine infusion of 'conceptus secretory proteins' from cultures of day 17–18 conceptuses between day 15 and day 17 after oestrus extends the duration of the oestrous cycle (Thatcher et al., 1985).

Specific genes encoding products that exhibit luteotropic or antiluteolytic properties have been detected in early-stage bovine embryos. Platelet-derived growth factor stimulates progesterone production in luteal cells (Battista et al., 1989) and the gene and receptor for this ligand are present in all developmental stages of bovine embryos before they hatch from the zona pellucida (Watson et al., 1992). In addition, mRNA for bovine trophoblast protein-1 (bTP-1) was detected in blastocyst-stage bovine embryos (Hernandez-Ledesma et al., 1992); intrauterine infusion of this protein into cyclic cows between day 14 and day 17 extends the lifespan of the corpus luteum and decreases the concentration of PGF2α (Helmer et al., 1989a). Endometrial explants exhibit a decrease in PGF2α concentration and an increase in the secretion of PGE in response to bTP-1 (Helmer et al., 1989b).
Several factors secreted by bovine embryos, such as platelet-activating factor (Stock and Hansel, 1992), steroids and prostaglandins (Shemesh et al., 1979; Shemesh et al., 1984; Hwang et al., 1988; Lewis, 1989; Wilson et al., 1992) and bTP-1 (Thatcher et al., 1989; Hernandez-Ledezma et al., 1992) before day 16 may be involved in the maternal recognition of pregnancy. However, many of these products may exert their effects through localized interactions with the uterine endometrium.

This experiment was designed to determine whether blastocyst-stage embryos before they hatch from the zona pellucida, or trophoblastic vesicles obtained from bovine conceptuses at day 12 exhibit luteotrophic activity alone or in combination with uterine endometrial tissue when added to mixed populations of dispersed small and large bovine luteal cells.

Materials and Methods

Superoxidation

A group of normally cyclic mixed breed beef cows was injected i.m. once a day with FSH (FSH-P; Schering, Kenilworth, NJ), beginning on day 8–12 of the oestrous cycle (oestrus = day 0) for 5 consecutive days. A single injection of PGF2α (Upjohn, Midland, MI) was administered on the evening of the fourth FSH-P treatment. Animals were artificially inseminated and mated with a fertile bull 12 h and 24 h after the onset of oestrus.

Embryo collection and culture

Twelve days after the induction of the superovulatory oestrus, the uterus of each cow was flushed nonsurgically with Dulbecco’s PBS (D-PBS; Gibco Laboratories, Grand Island, NY), containing 1% calf serum (Gibco) and antibiotics (100 µg penicillin ml⁻¹ and 100 µU streptomycin ml⁻¹; Gibco). The conceptuses were removed from the flushing medium and washed in fresh D-PBS plus 10% fetal bovine serum (FBS: Gibco) and antibiotics in sterile Petri dishes.

Conceptuses were washed through several drops of fresh medium and dissected to prepare trophoblastic vesicles according to procedures described by Pool et al. (1988). After dissection, tissue segments were washed several times in Ham’s F-12 medium (Sigma Chemical Co., St Louis, MO) containing 10% FBS and antibiotics, and incubated for approximately 24 h at 39°C under 5% CO₂ in humidified air to allow the conceptus tissue to re-expand.

Embryos at the morula and blastocyst stages were nonsurgically recovered from donor animals at a commercial embryo transplant unit, and frozen in liquid nitrogen. Embryos were thawed and co-cultured for approximately 24–48 h in vitro on oviductal epithelial cells as described by Thibodeaux et al. (1992, 1993) to obtain embryos at the blastocyst or expanded blastocyst stage for the experimental protocol. Embryos were initially cultured in tissue culture medium-199 (TCM-199; Gibco) containing 10% FBS and antibiotics at 39°C under 5% CO₂ in humidified air. Immediately before allocation to treatment groups, blastocysts were washed several times in Ham’s F-12 medium without serum.

Luteal cell preparations

Corpora lutea (n = 6) were enucleated from normal cyclic mixed breed beef and Holstein cows under epidural anaesthesia on day 10 of the oestrous cycle (oestrus = day 0) through a small incision in the anterior vagina, and placed in chilled (4°C) TCM-199 containing antibiotics. Luteal tissue was dissociated using 2000 U of collagenase g⁻¹ of tissue ( Worthington Biochemicals, Freehold, NJ) in TCM-199 at 34°C by the procedure described by Alila et al. (1988a). The cells were filtered through a silkscreen (pore diameter of 150 µm) and monofilament mesh (44 µm in diameter) and separated by unit gravity sedimentation (Koos and Hansel, 1981). After unit gravity sedimentation, small and large luteal cell fractions were pooled to obtain mixed luteal cell preparations nearly free of accessory cells. The mean (±SEM) viability of luteal cells after unit gravity sedimentation, as estimated by trypan blue dye exclusion, was 87.2 ± 1.5% (n = 6 corpora lutea).

Endometrial tissue

A portion of uterine endometrial tissue was harvested from the same experimental animal that served as the corpus luteum donor. The tissue sample was harvested from the uterine horn ipsilateral to the corpus luteum using a uterine biopsy tool (Pilling Corporation, Fort Washington, PA). The recovered tissue was immediately placed on ice in TCM-199 containing antibiotics and transported to the laboratory.

After several washings in fresh medium, the uterine tissue sample was blotted with sterile gauze and cut into small segments that weighed approximately 10 mg each. Each 10 mg tissue segment was washed and placed into individual wells of a 24-well tissue culture plate (Corning Glass Works, Corning, NY) containing 500 µl of cell culture medium, and incubated for 2–3 h at 37°C under 5% CO₂ in humidified air before the experiment began. The cell culture medium consisted of Ham’s F-12 medium supplemented with antibiotics, L-glutamine (0.29 mg ml⁻¹), insulin (5 µg ml⁻¹), transferrin (5 µg ml⁻¹) and selenium (5 ng ml⁻¹). Immediately before the experimental protocol began, the culture medium was removed from each well and the endometrial tissue was washed twice with fresh cell culture medium.

Incubation conditions

Treatment groups were arranged in individual wells of a 24-well tissue culture plate as follows: luteal cells alone, one trophoblastic vesicle alone; one blastocyst-stage embryo alone; one 10 mg sample of uterine endometrial tissue alone; luteal cells and one trophoblastic vesicle; luteal cells and one blastocyst-stage embryo; luteal cells and a 10 mg sample of uterine endometrial tissue; luteal cells, one trophoblastic vesicle and a 10 mg uterine endometrial sample; and luteal cells, one blastocyst-stage embryo and a 10 mg uterine endometrial sample. Luteal cells were added to each well at a concentration of 250 000 viable cells per well and the total volume of medium in each well was 500 µl. There were two to four replicates for each treatment for each corpus luteum.

All treatment wells were incubated at 37°C in an atmosphere of 5% CO₂ in humidified air for 12 h. A sample of medium

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(100 µl) from each treatment was withdrawn 10 min (basal concentration), and 2 and 6 h after the treatment began, and was stored at −20°C for progesterone assay. Equal volumes of fresh medium were replaced following sample collection. After 12 h of treatment, the culture medium (approximately 500 µl) for each treatment group was removed and stored at −20°C for progesterone assay. In addition, media samples harvested from the uterine endometrium culture treatment were assayed for PGF₂α, PGE₂ and 6-keto PGF₁α.

**Progesterone determination**

The concentration of progesterone in the culture medium was measured by enzymeimmunoassay procedures that have been reported and validated by Del Vecchio et al. (1993). The sensitivity of the assay was 1.56 pg per well and allowed for the measurement of 0.03125 ng ml⁻¹; the intra-assay and interassay coefficients of variation were 0.3 and 13.2%, respectively.

**Prostaglandin determination**

Culture media from wells that contained only a 10 mg endometrial tissue section were harvested parallel to other treatment groups and assayed for prostaglandins. The concentrations of prostaglandins were determined using commercial PGF₂α, PGE₂ and 6-keto PGF₁α enzymeimmunoassay kits (Cayman Chemical Co., Ann Arbor, MI). The kits were used in accordance with the supplier’s instructions. All samples were run in single assays and the intra-assay coefficient of variation of all assays ≤10%.

According to the manufacturer, the PGF₂α antibody has a crossreactivity of 100% with PGF₂α, PGF₁α and PGF₃α, 7% with PGD₂, 2% with 6-keto PGF₁α < 0.5% with PGA₂, 0.3% with 2,3-dinor-6-keto PGF₁α, and < 0.1% with PGE₂, 6.15-diketo-13,14-dihydro PGF₁α, thromboxane B₂ and PGE₃. The 6-keto PGF₁α antibody has a crossreactivity of 100% with 6-keto PGF₁α, 8.7% with 2,3-dinor-6-keto PGF₁α, 2.1% with PGF₂α, 0.8% with PGF₁α, 0.1% with 6,15-diketo-13,14-dihydro PGF₁α and < 0.01% with PGD₂, 13,14-dihydro PGF₁α and thromboxane B₂. The PGE₂ antibody has a crossreactivity of 100% with PGE₂ and PGE₃, 9.2% with 15-keto PGE₂, 0.02% with PGF₃α and < 0.01% with PGA₁, PGA₂, PGF₁α, 6-keto PGF₁α, PGF₂α, thromboxane B₂ and 13,14-dihydro-15-keto PGF₂α.

**Statistical analysis**

To reduce the variation arising from individual corpora lutea, embryos and uterine endometrium, data concerning progesterone concentrations were converted to units of stimulation index (progesterone treated/progesterone control), as described by Hickey and Hansel (1987): the progesterone control was the sample harvested 10 min after the treatment began (basal concentration). Progesterone stimulation index values were analysed by least squares analysis of variance using the General Linear Models procedure of SAS (SAS Institute Inc., Cary, NC). Data were analysed using the model components of corpus luteum, treatment, corpus luteum × treatment, replicate (corpus luteum × treatment), time, corpus luteum × time, treatment × time, corpus luteum × treatment × time, and residual. The effect of corpus luteum and corpus luteum × treatment was tested using replicate (corpus luteum × treatment). The effect of treatment was tested using corpus luteum × treatment and the effect of time was tested using corpus luteum × time. The effect of treatment × time was tested using corpus luteum × treatment × time, and the remaining terms were tested with the residual. Data for prostaglandin concentrations were analysed by a one-way analysis of variance and the differences between treatment means were analysed using the Bonferroni multiple comparison test.

**Results**

Only very small amounts of progesterone were produced by trophoblastic vesicles, blastocyst embryos and uterine endometrial tissue alone during the 12 h incubation period. The concentrations (ng ml⁻¹) of progesterone (mean ± SE) in the culture medium after 2, 6 and 12 h of incubation were 1.1 ± 0.4, 3.6 ± 0.7, and 3.2 ± 1.0, respectively, for trophoblastic vesicles, 0 ± 0, 0.2 ± 0.2, and 0.2 ± 0.2, respectively, for blastocyst-stage embryos, and 0.2 ± 0.1, 1.1 ± 0.4, and 1.1 ± 0.6, respectively, for uterine endometrial tissue. The mean concentrations (ng ml⁻¹) of progesterone in the culture medium after 2, 6 and 12 h of incubation from luteal cells alone were 51.0, 234.2 and 863.4, respectively.

After 2 h, progesterone synthesis was stimulated by a combination of luteal cells, blastocyst-stage embryos and uterine endometrium (P < 0.10) (Fig. 1a). Progesterone was not stimulated by trophoblastic vesicles, blastocyst-stage embryos or endometrial tissue alone (P > 0.10). After 6 h, progesterone was stimulated by trophoblastic vesicles (P < 0.01), blastocyst-stage embryos (P < 0.01), trophoblastic vesicles and uterine endometrium (P < 0.01), and blastocyst embryos and uterine endometrium (P < 0.001) (Fig. 1b). In addition, there was a trend for progesterone to be stimulated by treatment with a uterine endometrium sample (P < 0.10).

After incubation for 12 h, trophoblastic vesicles and blastocyst-stage embryos stimulated progesterone synthesis (P < 0.001) (Fig. 1c). In addition, the uterine endometrium lost its stimulatory effect on progesterone synthesis and appeared to inhibit synthesis alone or in combination with trophoblastic vesicles (P < 0.10).

Concentrations of prostaglandins secreted into the culture medium from a uterine endometrial sample during the 12 h incubation period were determined and assessed for a possible correlation with progesterone data. Concentrations of PGE₂, 6-keto PGF₁α and PGF₂α were not different (P > 0.05) in samples taken either after 2 h or 6 h of incubation. However, concentrations of all three compounds were significantly higher (P < 0.05) in the samples taken after 12 h, compared with those taken after 2 and 6 h (Fig. 2).

**Discussion**

This study demonstrates that bovine blastocyst-stage embryos at day 7 and trophoblastic vesicles from embryos at day 12
produce a luteotrophic substance(s) during incubation in vitro. In addition, uterine endometrial tissue exhibits a trend to stimulate progesterone production in luteal cells after 6 h of incubation, although after 12 h it has not only lost its stimulatory capability but also inhibits the luteotrophic effects of trophoblastic vesicles. To our knowledge, this is the first report of the production of a luteotrophic substance by a single blastocyst at day 7. However, Beal et al. (1981) reported that both homogenates and aqueous extracts of homogenates of embryos at days 13–18 stimulate progesterone production by dispersed bovine luteal cells. In addition, Hickey and Hansel (1987) reported that culture medium harvested from cultured bovine conceptus tissue at days 13–18 exhibits luteotrophic activity in dispersed bovine luteal cells and showed that the luteotrophic substance has a low molecular weight, is lipid soluble, and heat labile. This same substance may have been responsible for the increased progesterone production observed in these experiments.

Fig. 1. Progesterone synthesis (least squares means) by bovine luteal cells (C), luteal cells with a trophoblastic vesicle (C + TV), luteal cells with a blastocyst-stage embryo (C + BL), luteal cells with a uterine endometrial sample (C + UE), luteal cells with a trophoblastic vesicle and uterine endometrial sample (C + TV + UE), and luteal cells with a blastocyst-stage embryo and uterine endometrial sample (C + BL + UE). Results from six corpora lutea are expressed as units of stimulation index (progesterone treated/progesterone control) from samples harvested (a) 2 h, (b) 6 h and (c) 12 h after the treatment protocol began. The pooled SEMs of the mean is 8.0 and statistical notations are comparisons with luteal cells alone: *P < 0.10; **P < 0.05; ***P < 0.01; ****P < 0.001.

Fig. 2. Concentrations (ng ml⁻¹) of the prostaglandins (a) PGE₂, (b) PGF₂α, and (c) 6-keto-PGF₁α secreted by bovine uterine endometrial tissue during a 12 h incubation period. Data are means ± SEM from five cows using approximately 10 mg of tissue; the experiment was replicated twice for each sampling period. Values with different letters above error bars within assays differ significantly (P < 0.05).

The amounts of progesterone produced directly by blastocyst-stage embryos, trophoblastic vesicles and uterine endometrium were insignificant, and the large amounts of progesterone produced in this experiment were clearly due to synthesis by the dispersed luteal cells. Wilson et al. (1992) reported that progesterone production is highly variable in bovine embryos at days 8–18 in a perfusion culture system. Bovine embryos also produce several prostanoids (Lewis et al., 1982; Shemesh et al., 1984; Hwang et al., 1988; Wilson et al., 1992) that are luteotrophic and that may be possible signals for
the maternal recognition of pregnancy (Pratt et al., 1977; Silvia et al., 1984). In our experience, luteotropic effects of the magnitude produced by the addition of a single blastocyst or trophoblastic vesicle to luteal cells uncontaminated with accessory cells have been produced only by LH, or by agents that increase the concentration of cyclic AMP or that activate protein kinase C (in small cells only).

Alila et al. (1988b) reported that PGE₂, PGF₂α, and PGI₂ all stimulate progesterone synthesis in small bovine luteal cells, but that only PGE₂ and PGI₂ stimulate progesterone in large cells. Alila et al. (1988b) also reported that PGF₂α does not affect the basal concentration of progesterone in large luteal cells. In addition, the stimulation of progesterone production by PGF₂α and PGI₂ in the large cells is inhibited by PGF₂α (Alila et al., 1988b). However, in our experiment, the apparent switch in the effect of endometrial tissue on progesterone production in luteal cells from stimulatory to inhibitory between 6 and 12 h of incubation does not appear to be due to an alteration in the relative amounts of luteotropic and luteolytic cyclooxygenase products. The concentration of all the cyclooxygenase products (PGF₂α, PGE₂, and PGI₂) increased over time. The ratios of PGE₂:PGF₂α and of 6-keto-PGF₁α:PGF₂α after 2, 6 and 12 h of incubation were 0.51:1 and 2.45:1, 0.44:1 and 2.36:1, and 0.39:1 and 1.94:1, respectively. It seems more likely that these results could be attributed to an increased production of lipoxigenase products over time; these products are known to have luteolytic properties (Milvae et al., 1986). However, the attenuation of the inhibitory effects of uterine endometrium on progesterone production when co-incubated with a trophoblastic vesicle or blastocyst-stage embryo could be due to a shift in the metabolism of arachidonic acid toward PGE₂ synthesis rather than PGF₂α, as described by Lewis and Waterman (1983). The inhibitory effects of the uterine endometrium could also be due to the metabolism of progesterone in the culture medium (Eley et al., 1983).

These data indicate that early-stage bovine embryos produce a factor(s) that can stimulate progesterone production in luteal cells several days before the generally accepted period of maternal recognition of pregnancy. This factor can act directly on luteal cells to stimulate progesterone production. The endometrium also produces both luteotropic and luteolytic arachidonic acid metabolites, but these do not appear to enhance the luteotropic effects of the early embryo and may even inhibit them under certain circumstances.

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