Bovine luteal cell production *in vitro* of prostaglandin E₂, oxytocin and progesterone in response to pregnancy-specific protein B and prostaglandin F_{2α}

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A study was conducted to determine the effects of pregnancy-specific protein B (PSPB) and prostaglandin F_{2α} (PGF_{2α}) on bovine luteal cell progesterone, prostaglandin E₂ (PGE₂) and oxytocin production *in vitro*. Corpora lutea were enucleated from multiparous cows with normal oestrous cycles during the mid-luteal (days 10–12; *n* = 5) or late-luteal (days 17–18; *n* = 5) stage. Mixed large and small cells (1.5×10^5 cells per well) were incubated in 500 µl modified Ham's F-12 medium. Cells were incubated for 18 h before treatments were added. Cells were treated with PSPB (0, 2.5, 5.0 µg) and PGF_{2α} (0, 100, 200 ng) in a 3 × 3 factorial arrangement. After treatments were added, media samples were collected at 6 and 12 h. During the 18 h pretreatment incubation, progesterone, PGE₂ and oxytocin production was similar between the prospective treatment groups. Progesterone production was greater (*P* < 0.001) by mid-stage than by late-stage cells. In addition, progesterone decreased (*P* < 0.001) as incubation time increased. Progesterone production was not affected by PGF_{2α}, but PSPB increased (*P* < 0.02) progesterone at the 5.0 µg dose. Late-stage luteal cells produced more (*P* < 0.001) PGE₂ than did mid-stage cells; PGE₂ production decreased (*P* < 0.001) with increased incubation time. Luteal PGE₂ production increased in response to PSPB treatment (*P* < 0.01) and PGF_{2α} treatment (*P* < 0.001). Luteal oxytocin production was greater (*P* < 0.01) by mid-stage compared with late-stage cells. Oxytocin production decreased (*P* < 0.001) with incubation time in mid-stage cells, but in late-stage cells oxytocin production was similar over time. Neither PSPB nor PGF_{2α} had an effect on oxytocin. These results indicate that PSPB does not affect luteal oxytocin, but does increase progesterone and PGE₂ production. In addition, PGF_{2α} increases luteal PGE₂, but does not affect progesterone or oxytocin production. These data do not show an interaction between PSPB and PGF_{2α} in regulating bovine luteal cell endocrine function.

Introduction

Pregnancy-specific protein B (PSPB) has been used successfully as the target antigen in the development of a radioimmunoassay for pregnancy determination in many domestic and wildlife species, including cattle (Sasser *et al.*, 1986, 1989; Haigh *et al.*, 1993; Willard *et al.*, 1994). Despite knowledge of its presence and specificity to pregnancy in these species, studies designed to test the physiological function(s) of this protein are only now being carried out (Del Vecchio *et al.*, 1990, 1995a, b; Kiracofe *et al.*, 1993; Weems *et al.*, 1994).

Kiracofe *et al.* (1993) suggested that PSPB is involved in regulation of postpartum ovarian follicular development. Del Vecchio *et al.* (1990) reported that PSPB increases prostaglandin E₂ (PGE₂) and, to a lesser extent, prostaglandin F_{2α} (PGF_{2α}) secretion rates in bovine endometrial tissue.

Weems *et al.* (1994) reported data indicating a positive relationship between placental PSPB, PGE₂ and progesterone production in pregnant ewes. Other work which studied the effect of PSPB on luteal cell endocrine function indicated that PSPB does not affect progesterone or oxytocin production, but does stimulate PGF_{2α} and PGE₂ production (Del Vecchio *et al.*, 1995a, b). Furthermore, the data suggest that PSPB does not interact with LH or PGE₂ to enhance their luteotrophic actions. Although limited, these studies suggest a relationship between PSPB and prostaglandins; however, they are the only available data on the physiological functions of PSPB.

In contrast, the luteolytic effects of PGF_{2α} have been well documented and, as a result, PGF_{2α} is commonly referred to as the endogenous luteolysin in cattle (Inskeep and Murdoch, 1980). Cyclic luteal regression is generally thought to be accomplished by a positive feedback loop, which occurs between luteal oxytocin and endometrial PGF_{2α} (Silvia *et al.*, 1991). Furthermore, for pregnancy to be maintained, this

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positive feedback loop needs to be interrupted. PSPB may help to mediate this process by modifying luteal cell oxytocin secretion and hence compromising the positive feedback loop resulting in extension of the lifespan of the corpus luteum. In addition, PSPB may also act as a luteotrophic and luteo-protective agent, again resulting in maintenance of the corpus luteum of pregnancy.

This study was designed to complement previous work (Del Vecchio *et al.*, 1995a, b) by: (1) testing the effects of PSPB alone on luteal cell progesterone, PGE₂ and oxytocin production; and (2) determining whether PSPB interacts with PGF_{2α} to alter luteal cell progesterone, PGE₂ and oxytocin production, during both the mid- and late-luteal stages of the bovine oestrous cycle.

Materials and Methods

Materials

Pregnancy-specific protein B was supplied by R. G. Sasser (University of Idaho, Moscow, ID). Isolation of PSPB was as described by Del Vecchio *et al.* (1995b). Prostaglandin F_{2α} was purchased from Cayman Chemical Company (Ann Arbor, MI). Medium 199 and antibiotics (penicillin and streptomycin) were purchased from Gibco Laboratories (Grand Island, NY). Collagenase was purchased from Worthington Biomedical (Freehold, NJ). Ham's F-12, L-glutamine, insulin-transferrin-sodium selenite medium supplement, and BSA were purchased from Sigma Chemical Company (St Louis, MO).

Tissue dissociation and cell separation

Corpora lutea were enucleated from an ovary through an incision in the anterior vagina from normal cyclic multiparous cows during either the mid-luteal (days 10–12; *n* = 5) or late-luteal (days 17–18; *n* = 5) stage of the oestrous cycle (day 0 = oestrus) under epidural anaesthesia. The corpora lutea were immediately placed in approximately 30 ml of ice-cold Medium 199 containing penicillin and streptomycin (100 units penicillin ml⁻¹ and 100 µg streptomycin ml⁻¹ and transported to the laboratory. Once in the laboratory, the corpora lutea were weighed and thinly sliced, and the slices placed into a spinner flask for cell dispersion. The luteal cells were dispersed with collagenase (2500 units g⁻¹ tissue) in Medium 199 (10 ml g⁻¹ tissue) as described by Alila *et al.* (1988a). The luteal cells were then separated from accessory cells by unit gravity sedimentation (Koos and Hansel, 1981). After the unit gravity sedimentation separation step all the luteal cells, both large and small, were pooled together. Viability of the large and small luteal cells after separation averaged 89.6 ± 3.6 and 93.9 ± 2.0% for the mid-luteal, and 94.1 ± 1.7 and 95.9 ± 0.8% for the late-luteal stage of the oestrous cycle, respectively, as determined by Trypan blue dye (0.4%) exclusion under the light microscope.

Incubation procedure

Mixed large and small luteal cells (1.5 × 10⁵ viable cells per well) were incubated in 500 µl Ham's F-12 medium

supplemented with insulin (5 µg ml⁻¹), transferrin (5 µg ml⁻¹) and selenium (5 ng ml⁻¹), L-glutamine (0.29 mg ml⁻¹), penicillin (100 iu ml⁻¹) and streptomycin (100 µg ml⁻¹). All accessory cells were absent in the cultures as determined by examination under a light microscope of the luteal cell preparations after the unit gravity sedimentation separation. Culture wells were coated with 200 µl 0.5% (w/v) BSA in Ham's F-12 medium for 1 h to facilitate cell adhesion to the plates; the medium was then aspirated and discarded. Luteal cells were incubated for 18 h before starting the treatments. Incubation temperature was maintained at 37°C in 5% CO₂ in humidified air. Luteal cells were treated with PSPB (0, 2.5 or 5.0 µg) and PGF_{2α} (0, 100 or 200 ng) in a 3 × 3 factorial arrangement. After the 18 h pretreatment incubation period, media samples were collected and replaced with media containing treatments. The cells were incubated for an additional 12 h and media samples were collected and replaced with treatment medium at intervals of 6 h. All culture treatments were conducted in duplicate.

Hormone determinations

The concentration of progesterone in the culture medium was measured directly by enzymeimmunoassay as described by Del Vecchio *et al.* (1994). Intra-assay and interassay coefficients of variation were 6.6% and 15.1%, respectively.

Prostaglandin E₂ was measured by enzymeimmunoassay as described by Del Vecchio *et al.* (1992) with the following variations. Antisera to PGE₂ purchased from Cayman Chemical was used according to supplier's recommendation. Cross-reactivity of the PGE₂ antisera has been reported by Gobetti *et al.* (1994) and Thibodeaux *et al.* (1994). Enzyme-labelled antigen (tracer) for PGE₂ was purchased from Cayman Chemical Company. Prostaglandin E₂ tracer was used at a 1:2 dilution in relation to the suppliers' recommendations. Prostaglandin E₂ for the standard curves was purchased from Sigma Chemical Company. Intra-assay and interassay coefficients of variation were 5.9 and 14.3%, respectively.

The concentration of oxytocin in the culture medium was measured directly by radioimmunoassay (Sheldrick and Flint, 1981). Anti-oxytocin antibody was generously provided by D. Schams (Freising) and used at a dilution of 1:37 500. Crossreactivity of the oxytocin antisera was reported by Schams (1983). Synthetic oxytocin for the standard curves was purchased from Sigma Chemical Company. Oxytocin ¹²⁵I-labelled Tyr² was purchased from Dupont Chemical Company (Mississauga, ON). Intra-assay and inter-assay coefficients of variation were 6.5 and 12.6%, respectively.

Statistical analyses

All data were analysed using general linear models procedure of SAS (1985). The data were transformed to square-roots to stabilize the variance before statistical analysis (Kleinbaum and Kupper, 1978). Analyses of covariance was used to determine differences in overall progesterone, PGE₂ and oxytocin production between the main effects (replicate, stage of the oestrous cycle (stage), sample collection time (time), PSPB treatment, PGF_{2α} treatment and the interactions.

Cow was used as the covariate within each hormone analysis to minimize bias due to variability in production of the respective hormone among the different corpora lutea collected. When statistically significant differences were detected, the predicted difference option of SAS, which tests the equality of all pairs of class means, was used as the means separation procedure. All data are reported on a square-root transformation basis $\pm$ SEM.

Results

Before adding treatments (that is, during the 18 h pretreatment incubation period) luteal cell production of progesterone, PGE₂ and oxytocin was similar between the prospective PSPB and PGF_{2α} treatment groups, regardless of whether the cells were collected during the mid- or late-luteal stage of the oestrous cycle.

Progesterone production was greater ($P < 0.001$) by mid-stage cells than by late-stage cells (Fig. 1a). Production of progesterone also decreased ($P < 0.001$) between 6 and 12 h of incubation (Fig. 1a). Luteal cell progesterone production was not affected by PGF_{2α} treatment (Fig. 1b). However, treatment with PSPB increased ($P < 0.02$) luteal cell progesterone production at the 5.0 μ g dose as compared with the controls; the effect of the 2.5 μ g dose was intermediate (Fig. 1c). The PSPB $\times$ PGF_{2α} interaction, as well as all other interactions not mentioned, did not affect progesterone production.

Late-stage luteal cells produced greater ($P < 0.001$) amounts of PGE₂ than did mid-stage cells (Fig. 2a). Production of PGE₂ decreased ($P < 0.001$) with increased incubation time, that is, between the 6 and 12 h incubation times (Fig. 2a). Luteal cell PGE₂ production increased similarly in response to the 2.5 and 5.0 μ g treatment doses of PSPB treatment ($P < 0.01$; Fig. 2b) compared with the controls. In addition, both the 100 and 200 ng treatment doses of PGF_{2α} increased ($P < 0.001$) PGE₂ production (Fig. 2c) more than in controls. The PSPB $\times$ PGF_{2α} interaction, as well as all other interactions not mentioned, did not affect PGE₂ production.

Both the PGF_{2α} (Fig. 3a) and PSPB (Fig. 3b) treatments had no effect on luteal cell oxytocin production. The stage of the oestrous cycle $\times$ time interaction indicated that luteal oxytocin production was greater ($P < 0.01$) by mid-stage cells compared with late-stage cells. In addition, oxytocin decreased ($P < 0.001$) as incubation time increased in mid-stage cells; however, oxytocin production by late-stage cells remained unchanged over time (Fig. 3c). The PSPB $\times$ PGF_{2α} interaction, as well as all other interactions not mentioned, did not affect oxytocin production.

Discussion

The data reported here are in agreement with earlier reports that PSPB increases PGE₂ production in cultured bovine luteal cells (Del Vecchio *et al.*, 1995a). In addition, PSPB has been shown to increase PGE₂ secretion by endometrial tissue collected on day 16 of the oestrous cycle (Del Vecchio *et al.*, 1990). The consistency of these findings suggests a role for PSPB in recognition and maintenance of pregnancy through

Fig. 1. Progesterone production by cultured bovine luteal cells: (a) during the mid- and late-stage of the oestrous cycle and at 6 and 12 h of culture. ^{a,b}Values with different superscripts are significantly different ($P < 0.001$); (b) treated with 0, 100 or 200 ng prostaglandin F_{2α} (PGF_{2α}). No differences were detected among treatment groups; (c) treated with 0, 2.5 or 5.0 μ g pregnancy-specific protein B (PSPB). ^{a,b}Values with different superscripts are significantly different ($P < 0.02$). Five corpora lutea were used per stage of the oestrous cycle.

regulation of prostaglandins. Prostaglandin E₂ possesses luteotrophic/luteoprotective properties in pigs (Akinlosotu *et al.*, 1988; Christenson *et al.*, 1994; Richards *et al.*, 1994), sheep (Pratt *et al.*, 1977, 1979; Fitz *et al.*, 1984), horses (Vanderwall *et al.*, 1994), and cattle (Reynolds *et al.*, 1983; Shelton *et al.*, 1990; Thibodeaux *et al.*, 1992). Prostaglandin E₂ is also produced in increasing quantities by bovine embryos immediately after fertilization (Gurevich *et al.*, 1993) up to day 18 (Wilson *et al.*, 1992), which indicates that this prostaglandin may be part of the embryonic signal to the maternal unit responsible for luteostasis, thereby permitting embryo survival. Together these data certainly support the implication that PGE₂ is a key component in the establishment and maintenance of pregnancy in many species. Furthermore, the fact that PSPB regulates production of PGE₂ suggests PSPB has an indirect role in pregnancy maintenance.

Prostaglandin F_{2α} increased luteal cell PGE₂ production. To our knowledge, this is the first report that PGF_{2α} stimulates the

Fig. 2. Prostaglandin E₂ (PGE₂) production by cultured bovine luteal cells: (a) during the mid- and late-stage of the oestrous cycle and at 6 and 12 h of culture. ^{a,b}Values with different superscripts are significantly different ($P < 0.001$); (b) treated with 0, 2.5 or 5.0 μg pregnancy-specific protein B (PSPB). ^{a,b}Values with different superscripts are significantly different ($P < 0.01$); (c) treated with 0, 100 or 200 ng prostaglandin F_{2α} (PGF_{2α}). ^{a,b}Values with different superscripts are significantly different ($P < 0.001$). Five corpora lutea were used per stage of the oestrous cycle.

release of PGE₂ by bovine luteal cells. Other researchers have, however, shown an increase in uterine PGE₂ production in response to PGF_{2α} treatment (Weems *et al.*, 1993). Weems *et al.* (1993) reported increased concentrations of PGE₂ in uterine venous plasma by 64 h after the start of PGF_{2α} treatment in intact ewes at day 90–100 of pregnancy. In an earlier study, we reported that the inverse is also true, that treatment of bovine luteal cells with PGE₂ results in increased PGF_{2α} secretion (Del Vecchio *et al.*, 1995b). Chegini *et al.* (1991) showed that both small and large bovine luteal cells contain binding sites for PGE₂, PGF_{2α}, PGI₂, leukotriene C₄ and hCG/LH. In addition, Milvae and Hansel (1983), Hu *et al.* (1990) and Townson and Pate (1994) and our data show that bovine luteal cells actively secrete a variety of prostaglandins. Milvae and Hansel (1985) showed that by blocking uterine and ovarian prostaglandin production with indomethacin, the development and function

Fig. 3. Oxytocin production by cultured bovine luteal cells: (a) treated with 0, 100 or 200 ng prostaglandin F_{2α} (PGF_{2α}). No differences were detected among treatment groups; (b) treated with 0, 2.5 or 5.0 μg pregnancy-specific protein B (PSPB). No differences were detected among treatment groups; (c) during the mid- (■) and late- (□) stage of the oestrous cycle and at 6 and 12 h of culture. ^{a,b,x,y}Values with different superscripts are significantly different (^{a,b} $P < 0.01$; ^{x,y} $P < 0.001$). Five corpora lutea were used per stage of the oestrous cycle.

of the bovine corpus luteum could be inhibited. Peters *et al.* (1994) reported that LH is not required for maintenance of the bovine corpus luteum after day 12 of the oestrous cycle, which suggests that during the latter stages of the cycle, PGE₂ and PGI₂ may be the principal luteotrophins. This is in agreement with our data showing that PGF_{2α} and PGE₂ production by bovine luteal cells is greater in the late stages of the cycle than at mid-stage and that an autocrine regulatory mechanism may be involved (Del Vecchio *et al.*, 1995a). Taken together, these data indicate that prostaglandins of uterine (paracrine) or ovarian (autocrine) origin are required for normal development and function of the bovine corpus luteum.

Treatment with 5.0 μg PSPB did increase progesterone production by luteal cells. This finding is not consistent with earlier reports that concluded that PSPB does not influence luteal cell progesterone (Del Vecchio *et al.*, 1995a, b). However,

given the data reported thus far concerning the effect of PSPB on luteal prostaglandin production, both in this study and an earlier study (Del Vecchio *et al.*, 1995a), it may be possible that PSPB increased luteal PGE₂ production, and the increased PGE₂ acted in an autocrine fashion to increase progesterone. PGE₂ is luteotrophic and luteoprotective. Other researchers have reported similar findings in pregnant ewes (Weems *et al.*, 1994). On the basis of their findings, these researchers proposed that PSPB indirectly regulates ovine placental secretion of progesterone through regulation of placental secretion of PGE₂. In both the present study and our earlier work (Del Vecchio *et al.*, 1995a) the luteal cells were incubated for a total of 12 h with PSPB treatment. Perhaps the limited time in culture did not consistently allow for the PSPB-induced rise in PGE₂ to increase progesterone production.

It was expected that PGF_{2α} would decrease luteal progesterone production; however, no effect was detected in this study. Although PGF_{2α} is considered the endogenous luteolysin, other work *in vitro* has shown that PGF_{2α} has little or no effect on progesterone production (Pate and Condon, 1984). In fact some researchers have reported a stimulatory effect of PGF_{2α} on bovine luteal cell progesterone production (Alila *et al.*, 1988b; Hansel *et al.*, 1991); however, this effect was limited to the small luteal cells only. Gadsby and Earnest (1994) reported that PGF_{2α} stimulates progesterone production by pig luteal cells *in vitro* throughout the oestrous cycle and that progesterone production by large, but not small, luteal cells was increased in a dose-dependent fashion by PGF_{2α}. Furthermore, Miyamoto *et al.* (1993) reported that PGF_{2α} has a stimulatory role when intact corpora lutea were infused with 10⁻¹⁰–10⁻⁵ mol PGF_{2α} l⁻¹ through an *in vitro* microdialysis system. In contrast, other researchers have reported luteolytic actions of PGF_{2α} on luteal tissue and cells in culture (Pate, 1994), and the luteolytic effect/action of PGF_{2α} seems to be restricted to the large luteal cells. However, when PGF_{2α} treatment is administered alone it appears to have little effect on progesterone production, but PGF_{2α} does seem to inhibit the LH-, PGE₂- and PGI₂-stimulated production of progesterone (Alila *et al.*, 1988b; Pate, 1994). This discrepancy between *in vitro* and *in vivo* studies as well as among *in vitro* studies suggests that there may be other factors working in concert with PGF_{2α} to cause luteolysis *in vivo* which may be absent in tissue and dispersed cells maintained *in vitro*.

These data agree with previous data (Del Vecchio *et al.*, 1995a, b) that PSPB does not affect oxytocin production by cultured bovine luteal cells. Therefore, the likelihood that PSPB alters luteal cell release of oxytocin, thereby compromising the integrity of the proposed positive feedback loop mechanism of cyclic luteal regression and favouring maintenance of the corpus luteum, is not supported. In addition, PGF_{2α} had no effect on luteal cell oxytocin production in this study. Prostaglandin F_{2α} has been shown to cause an increase in luteal oxytocin production when administered to intact tissue, that is, intact corpora lutea or luteal slices *in vitro* (Abdelgadir *et al.*, 1987, 1994) and dispersed luteal cells (Lafrance and Hansel, 1992). However, these researchers were able to stimulate oxytocin release only from early, day 8, corpora lutea. Data for corpora lutea collected during the later stages of the oestrous cycle, days 12–16, indicate that PGF_{2α} is not effective in inducing luteal oxytocin release (Abdelgadir *et al.*, 1987).

Similar data have been reported for ewes (Cooke and Ahmad, 1994). The time within the oestrous cycle during which PGF_{2α} stimulates oxytocin release is not consistent with the positive feedback loop theory. More specifically, early stage corpora lutea respond to PGF_{2α} by releasing oxytocin while late stage corpora lutea are unresponsive, and it is during the late-stage that the PGF_{2α}–oxytocin positive feedback loop is supposed to occur. Perhaps by removing the corpus luteum from its *in vivo* environment into an *in vitro* environment we are eliminating factors that are necessary for the normal physiological processes associated with cyclic luteal regression to take place. Additional research is needed to understand this relationship more clearly.

In conclusion, these data suggest that PSPB is a regulator of luteal prostaglandin secretion, and this in turn may help regulate progesterone production. However, these data did not provide evidence that PSPB alters luteal oxytocin secretion, nor was there any indication that PSPB and PGF_{2α} interact directly to modify luteal endocrine function.

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