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Comparison of mRNA levels for the $PGF_{2\alpha}$ receptor (FP) during luteolysis and early pregnancy in the ovine corpus luteum[1]

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Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is the physiological signal that triggers luteolysis in the non-pregnant ewe. This is associated with a decline in circulating levels of progesterone, beginning around day 14 of a 16–17 day estrous cycle. Recently, the receptor for $PGF_{2\alpha}$ (FP) was cloned from an ovine day 10 large luteal cell cDNA library. The purpose of this study was to measure relative abundance of FP mRNA as it may change with luteolysis during the luteal phase. Corpora lutea (CL) were collected from ewes on day 10, 12, 14 or 16 ($n \geq 4$/day; day 0 = synchronized estrus); 12 h after $PGE_{2\alpha}$-treatment on day 10, 12 or 14 ($n \geq 3$/day) of the estrous cycle; or on day 16 of pregnancy ($n = 6$). Pregnancy was confirmed by visualization of the conceptus. Blood samples were collected 12 h prior to and at the time of tissue collection to determine levels of progesterone. Serum concentrations of progesterone declined with the onset of luteolysis in control animals (day 14, day 16; $P < 0.05$) as well as 12 h following $PGF_{2\alpha}$-treatment (day 10, day 12; $P < 0.05$). Genomic DNA from these tissues was prepared and visualized by agarose gel electrophoresis. Internucleosomal fragmentation (indicative of apoptosis) was seen in CL from animals in which luteolysis had been initiated (all $PGE_{2\alpha}$-treated and day 16 control ewes), but not in ewes with functional CL. Total RNA isolated from each CL was separated through a denaturing 1% agarose gel, transferred to nylon membranes and hybridized to a radioactive ovine FP cDNA probe. Hybridization to a radiolabeled 18S ribosomal cRNA probe was used to confirm equal loading of RNA in each lane. By northern analysis, a major transcript was seen at ~6.1 kb. A relatively high level of FP mRNA was measured in CL collected from control non-pregnant ewes during the mid luteal phase (day 10, 2.73 ± 0.17; day 12, 2.47 ± 0.91; FP/18S ratio), but varied among animals (3.09 ± 1.59) on day 14. Administration of $PGF_{2\alpha}$ resulted in the lowest amounts of FP mRNA on days 10, 12, and 14 ($P < 0.05$). Amounts of FP mRNA were higher ($P < 0.05$) on day 16 of pregnancy as compared to day 10 (by 1.9-fold) or to day 16 (by 5.9-fold) of the estrous cycle. From these observations we conclude that $PGF_{2\alpha}$ or some event associated with luteolysis appears to down regulate amounts of the FP mRNA. Furthermore, pregnancy, and/or the antiluteolytic signals associated with maternal recognition of pregnancy may prevent the decline in the amount of FP mRNA.

Keywords: ovine, corpus luteum, $PGF_{2\alpha}$ receptor, mRNA, FP

Introduction

The ovine corpus luteum is composed of a variety of cell types, including two morphologically and functionally distinct steroidogenic types, designated large and small (Fitz *et al.*, 1982; Rodger & O'Shea, 1982). Although large and small steroidogenic cells synthesize progesterone, hormone production in the two cell types is differentially regulated. Small luteal cells have receptors for luteinizing hormone (LH), the primary luteotropin (Kaltenbach *et al.*, 1968) which are functionally coupled to progesterone secretion via a cAMP-dependent second messenger pathway (Fitz *et al.*, 1982; Hoyer *et al.*, 1984; Hoyer & Niswender, 1985) involving protein kinase A (Hoyer & Niswender, 1986). However, basal progesterone synthesis on a per cell basis is greater in large cells than in small and is independent of luteinizing hormone (LH) stimulation (Hoyer *et al.*, 1984).

Prostaglandin $F_{2\alpha}$ produced in the uterus, has been identified as the physiological signal for luteal regression in the ewe (McCracken *et al.*, 1972). Prostaglandin $F_{2\alpha}$ binds to high affinity receptors on ovine large, but not small steroidogenic luteal cells (Balapure *et al.*, 1989; Wiepz *et al.*, 1992). Receptors for $PGF_{2\alpha}$ are pharmacologically designated FP (Kennedy *et al.*, 1982). Upon binding to receptor, $PGF_{2\alpha}$ activates the phosphatidyl inositol pathway (Jacobs *et al.*, 1991) and, in large cells, stimulates an increase in cytosolic-free calcium (Wiltbank *et al.*, 1989a) from intracellular stores (Wegner *et al.*, 1990, 1994). Activation of the $PGF_{2\alpha}$ response is believed to also activate protein kinase C (Wiltbank *et al.*, 1989b), and inhibits steroid synthesis (functional regression, Wiltbank *et al.*, 1990; Wegner *et al.*, 1991). Inhibition of steroid synthesis precedes cellular disruption associated with regression (structural luteolysis) and appears to be reversible (McGuire *et al.*, 1994). Once the integrity of the CL has been challenged, cell death results. Apoptotis, a form of physiological cell death, has been demonstrated by morphological (Sawyer *et al.*, 1990) and biochemical (Juengel *et al.*, 1993; Rueda *et al.*, 1995a) analyses in ovine and bovine CL undergoing luteolysis. Therefore, in addition to mediating functional regression, $PGF_{2\alpha}$ binding may directly or indirectly initiate specific intracellular mechanisms for induction of cell death (structural luteolysis). Specific information about the $PGF_{2\alpha}$ receptor (FP) has been limited to binding studies (Powell *et al.*, 1974; Rao *et al.*, 1979; Balapure *et al.*, 1989; Wiepz *et al.*, 1992; Wiltbank *et al.*, 1995). Fluctuations reported in the number of receptors for $PGF_{2\alpha}$ may reflect alterations in receptor expression at the transcriptional or translational level. Recently, cDNA encoding the receptor for $PGF_{2\alpha}$ was isolated and cloned from mouse, bovine, human and ovine tissues (Abramovitz *et al.*, 1994; Sakamoto *et al.*, 1994; Sugimoto *et al.*, 1994; Graves *et al.*, 1995). We hypothesized that the amount of FP mRNA would be regulated in the ovine CL and that this amount would vary in animals at the time of luteolysis. We further speculated that availability of FP mRNA would be different in pregnant *vs* non-pregnant ewes. Therefore, the purpose of this study was to compare relative amounts of mRNA for FP during the luteal phase of the ovine estrous cycle and early in pregnancy.

Results

Circulating concentrations of pregosterone

Circulating levels of progesterone were similar in ewes on days 10 and 12 of the luteal phase (Figure 1). Conversely,

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circulating levels of progesterone had decreased ($P<0.05$) 12 h following the administration of $PGF_{2\alpha}$ on days 10 and 12 and was already significantly reduced on days 14 and 16 in control animals as compared to day 10 control ewes. Therefore, luteal tissue was classified as functional when it was collected from ewes in which the concentration of progesterone had not declined at both measurements (12 h prior to and at the time of tissue collection). Alternatively, luteal tissue was classified as luteolytic, if circulating levels were $<0.5\ ng/ml^{-1}/CL^{-1}$ at both time points or if there was a significant reduction in circulating levels of progesterone at the time of tissue collection, when compared with 12 h before.

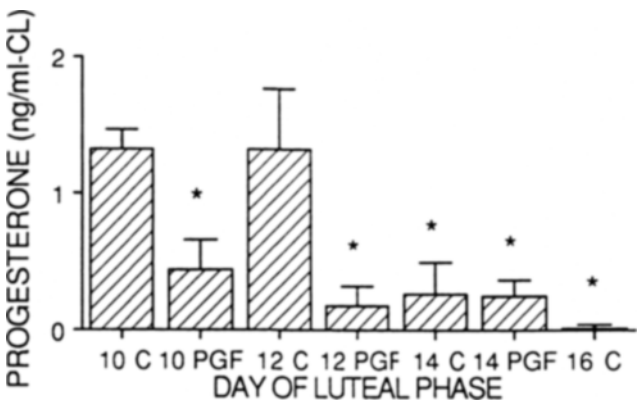

Figure 1 Circulating levels of progesterone in control or $PGF_{2\alpha}$-treated ewes just prior to CL collection on days 10, 12, 14 or 16 of the luteal phase. Progesterone was measured by RIA as described in methods (mean ± SEM). *different from day 10 control ($P<0.05$)

Identification of apoptosis in luteolytic CL

Additional evidence of regression in individual CL was obtained by demonstration of increased internucleosomal fragmentation of genomic DNA (laddering) which has been associated with structural luteolysis. Apoptosis appears as multiples of 185 bp fragments as radioactively labeled genomic DNA is electrophoresed through an agarose gel and visualized by autoradiography (Wyllie *et al.*, 1980). CL collected from day 10 and 12 of the estrous cycle or from pregnant ewes (day 16) contained intact, high molecular weight genomic DNA with no evidence of internucleosomal fragmentation (Figure 2). This demonstrated that tissue was collected prior to the onset of luteolysis. Internucleosomal DNA fragmentation was observed in some of the CL collected on day 14 of the estrous cycle. In contrast, internucleosomal DNA fragmentation was present in all CL collected from ewes on day 16, or 12 h following administration of $PGF_{2\alpha}$ on days 10, 12, or 14 of the estrous cycle.

FP mRNA levels in the CL

Northern and slot blot analysis of total RNA extracted from ovine CL demonstrated that levels of 18S ribosomal RNA were not different between days (10, 12, 14, or 16; $P<0.05$). Therefore, 18S ribosomal levels were used to normalize amounts of FP mRNA between tissues. Data are expressed as an FP/18S ratio. Multiple transcripts of FP mRNA were detected by northern analysis. Two major transcripts were observed, one at ~6.1 kb with a second at ~4.1 kb (Figures 3, 4, 5, 6). A third transcript was occasionally seen at ~3.0 kb. The relative amount of FP mRNA was similar in CL from day 10 and 12 ewes; however, the concentration of FP mRNA on day 14 varied greatly among ewes (3.09 ± 1.59; $n=4$; Figure 3). In contrast, the relative abun-

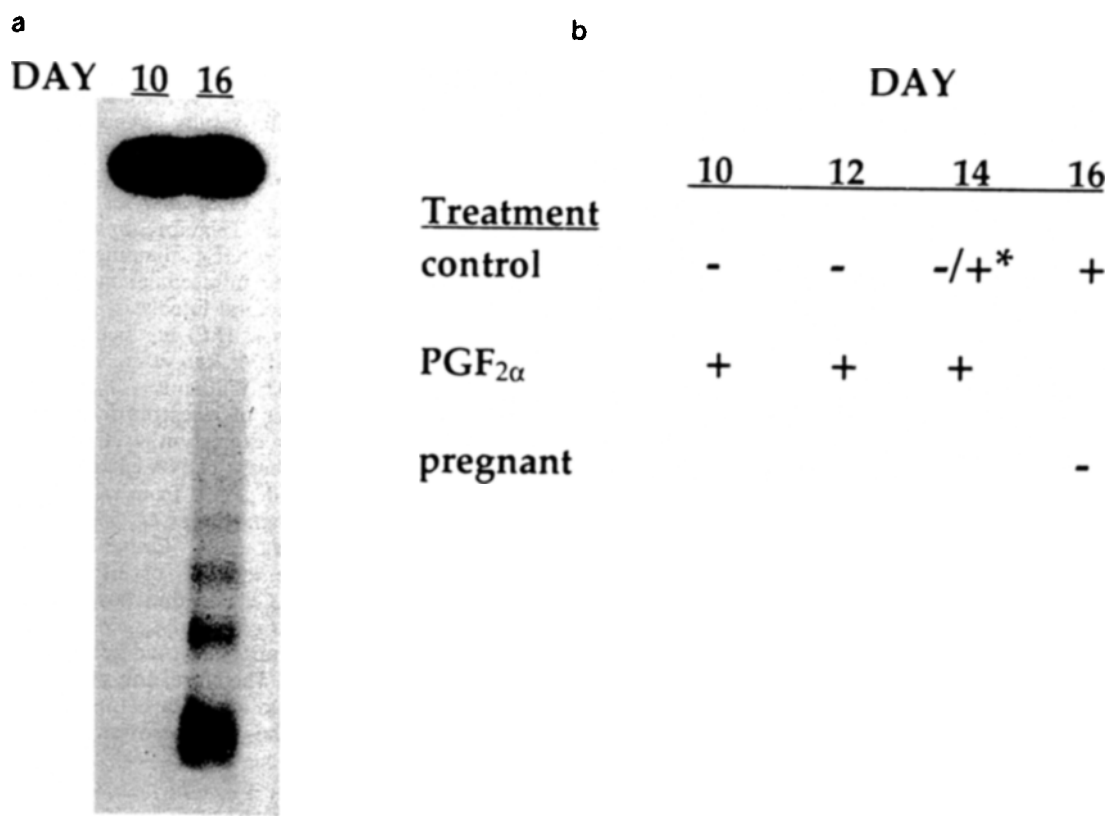

Figure 2 (**a**) Autoradiographic visualization of genomic DNA isolated from luteal tissue collected on day 10 and 16 of the luteal phase from non-pregnant ewes. Genomic DNA was analysed by agarose gel electrophoresis for oligonucleosomal DNA fragmentation as described in methods. (**b**) Qualitative assessment of internucleosomal DNA fragmentation (laddering) in non-pregnant control (d10, 12, 14, 16; $n \geq 4$/day), $PGF_{2\alpha}$-treated (d10, 12, 14; $n \geq 3$/day) or pregnant ewes (d16; $n=6$). (−) absence of DNA laddering; (+) presence of DNA laddering. *reflects the variability of DNA laddering on day 14 of the luteal phase in non-pregnant control ewes ($n=4$)

dance of FP mRNA was reduced ($P<0.05$) in tissue collected from all $PGF_{2\alpha}$-treated ewes. The quantity of FP mRNA was not different between days following $PGF_{2\alpha}$-treatment ($P>0.05$). To determine if changes in amounts of FP message were associated with the decline in circulating concentrations of progesterone and the onset of internucleosomal DNA laddering, results from individual day 14 ewes were compared (Figure 4a,b,c). The relative amount of FP message declined in ewes with decreased circulating concentrations of progesterone. This decrease inversely corresponded to the onset of internucleosomal DNA laddering. FP mRNA levels in CL collected from pregnant ewes (day 16) were 5.9-fold higher than that from day 16 non-pregnant ewes and 1.9-fold higher than that from ewes on day 10 of the estrous cycle (Figure 5).

The relative distribution of mRNA for FP in other ovine tissues was also determined (Figure 6). The greatest amount was observed in luteal tissue (day 10; 6.1 kb). Whereas, all 3 transcripts (6.1, 4.1, 3.0 kb) for FP mRNA were observed in luteal tissue, the 6.1 transcript was not evident in any of the other tissues evaluated (adrenal, brain, heart, kidney, liver, lung and uterus). The major transcript in lung and uterine tissue was at ~4.1 kb, which was also observed in heart and kidney. All tissues displayed some evidence of the 3 kb transcript.

Discussion

Prostaglandin $F_{2\alpha}$, the physiological luteolysin in many species including the ewe, is associated with a decline in circulating levels of progesterone (McCracken *et al.*, 1972). Uterine derived, as well as exogenous $PGF_{2\alpha}$ binds high affinity receptors located on large steroidogenic cells of the ovine CL (Fitz *et al.*, 1982; Balapure *et al.*, 1989). Wiepz *et al.* (1992) determined by ligand binding studies that concentrations of luteal $PGF_{2\alpha}$ receptors are similar in pregnant and non-pregnant ewes on days 10 and 13 post-estrus with the highest concentration of FP receptors observed in luteal tissue collected from pregnant ewes on days 13 and 15. On day 15 of the estrous cycle, however, the number of FP receptors were lower compared to that on day 15 of pregnancy. Furthermore, the concentration of luteal FP receptor was lower on day 13 than on day 10 of pregnancy. In the present study, a relatively high amount of FP mRNA was observed in CL collected from non-pregnant ewes during the mid luteal phase (day 10, 12) as well as in CL from pregnant ewes (day 16; $P<0.05$). Relative amounts of mRNA in CL on day 14 varied among animals, and were reduced ($P<0.05$) in tissue collected from control day 16 and all $PGF_{2\alpha}$-treated ewes. Therefore, alterations in the concentration of receptors for $PGF_{2\alpha}$ during the estrous cycle and early pregnancy reported by Wiepz *et al.* (1992) are in agreement with the changes in the amount of mRNA for the receptor presented here.

Multiple transcripts for FP were observed by northern analysis. This is similar to reports in mouse and bovine tissues (Sakamoto *et al.*, 1994; Sugimoto *et al.*, 1994; respectively). The amount of mRNA for FP was greatest in the CL, with the major transcript migrating at ~6.1 kb, and a minor transcript at ~4.1 kb. Interestingly, the 6.1 FP transcript observed in the luteal tissue was not abundant in other tissues, where mainly the 4.1 transcript was observed.

To associate mRNA FP levels with the functional status of the CL, circulating levels of progesterone and integrity of genomic DNA were determined in each animal. Circulating concentrations of progesterone above 0.5 ng/ml, measured at

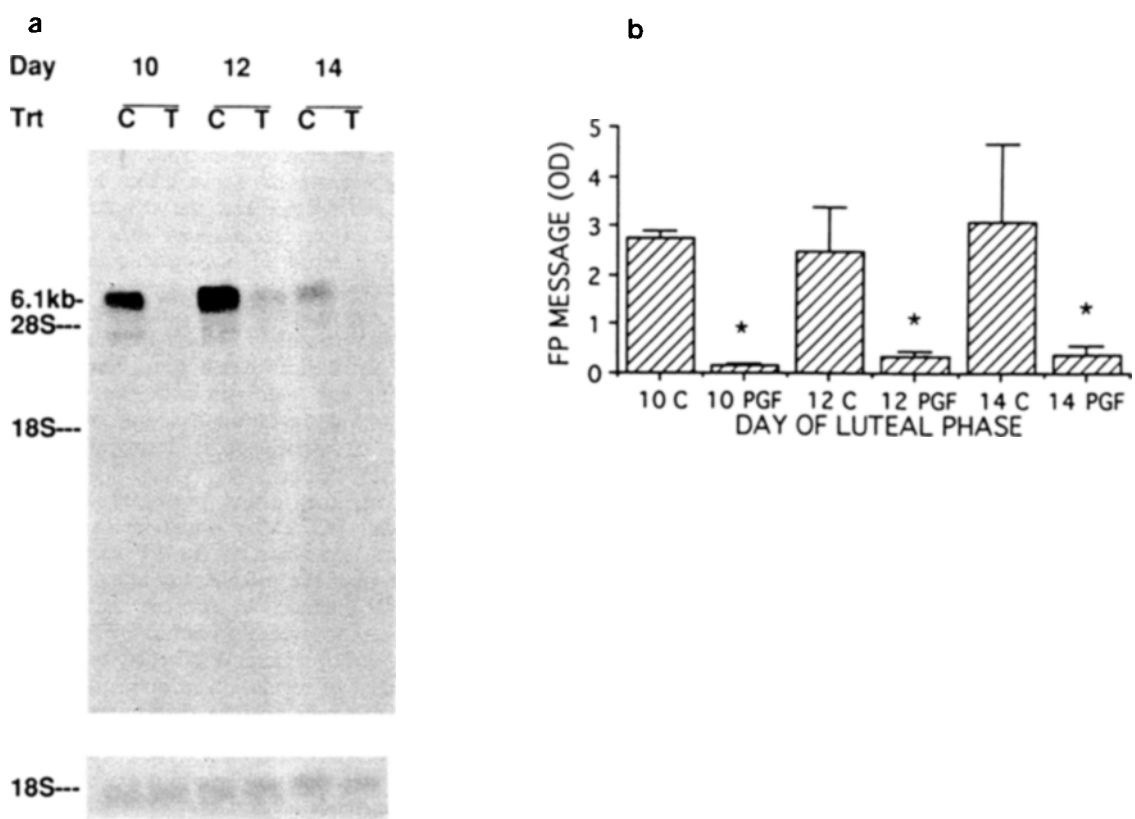

Figure 3 Representative northern analysis of FP mRNA at different times during the estrous cycle. (**a**) Total RNA was isolated from ovine luteal tissue collected on day 10, 12, or 14 of the luteal phase in control (C) or $PGF_{2\alpha}$-treated (T) ewes. A ^{32}P-labeled cDNA FP probe was used for hybridization as described in methods. Membranes were rehybridized with a 18S ribosomal cRNA probe for normalization (lower panel). (**b**) Quantification of data obtained by northern and slot blot analysis following normalization against 18S RNA levels in each sample (C = control, PGF = $PGF_{2\alpha}$-treated ewes, mean ± SEM, $n \geq 3$/day). *$P<0.05$ different from day 10 non-treated

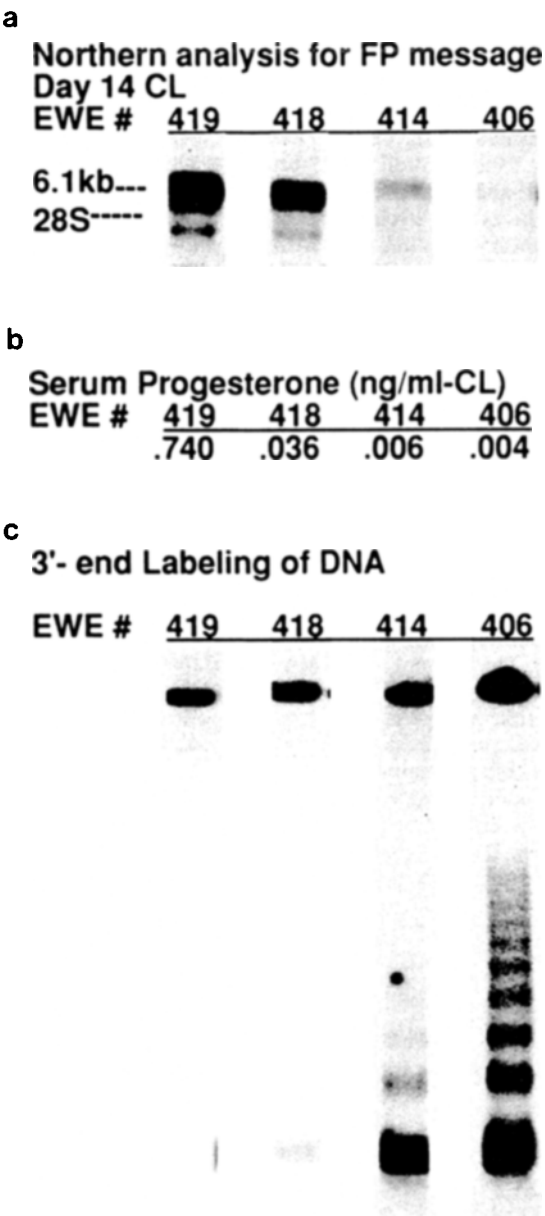

Figure 4 Comparison of (**a**) the relative amounts of FP message with (**b**) circulating concentrations of progesterone and (**c**) internucleosomal genomic DNA fragmentation in individual ewes on day 14. In addition to total RNA and nuclear DNA isolated from CL, blood samples were collected on day 14 from the same 4 non-pregnant ewes (ewe 419, 418, 414, 406). A ^{32}P-labeled cDNA FP probe was used to hybridize to northern blot (estimated transcript size in kb, and migration distance of 28S ribosomal RNA, is indicated) as described in methods. Genomic DNA was analyzed by agarose gel electrophoresis for oligonucleosomal DNA fragmentation as described in methods. Progesterone was measured by RIA as described in methods

the time of tissue collection, were assumed to reflect a functional CL. Alternatively, CL were classified as undergoing luteolysis, by either: (1) a marked reduction in progesterone during the 12 h before tissue collection or by (2) very low (<0.5 ng) circulating levels of progesterone at both time points. Circulating levels of progesterone indicated adequate luteal function on days 10 and 12; however, by day 14 serum concentrations of progesterone indicated that luteal regression had begun. Day 14 is considered to be the initial period of luteolysis and differences in serum progesterone concentrations can be attributed to variability in the initiation of luteolysis between animals (Rueda *et al.*, 1995a). Circulating levels of progesterone in $PGF_{2\alpha}$-treated ewes declined on all days within 12 h following injection and were similar to the levels observed in day 16 non-pregnant ewes. Therefore, CL collected from all day 16 non-pregnant as well as all $PGF_{2\alpha}$-treated ewes were functionally inhibited or regressed.

Since the functional aspect of luteal regression is considered reversible (McGuire *et al.*, 1994; Pate, 1994), we also wanted to associate the changes in the relative abundance of FP mRNA with the onset of cell death. Apoptosis, a form of physiological cell death, has been identified in the disruption of cellular integrity during structural luteolysis in cyclic ewes as well as following the administration of $PGF_{2\alpha}$ (McGuire *et al.*, 1994; Rueda *et al.*, 1995a). No internucleosomal DNA fragmentation (laddering) was observed in CL collected on day 10 and 12 of the estrous cycle or on day 16 of pregnancy. Some CL collected on day 14 and all CL from day 16 control ewes displayed DNA laddering. Furthermore, CL collected from ewes which had been classified by circulating progesterone levels as luteolytic (day 10, 12, 14 $PGF_{2\alpha}$-treated and day 16 control) had evidence of internucleosomal DNA fragmentation.

Low abundance of mRNA for FP was associated with functional regression (as determined by progesterone levels) in all ewes except day 14 control. At this time, circulating progesterone was significantly lower than day 10; however levels of mRNA for FP were not different from day 10. This suggests that functional regression (decreased progesterone secretion) precedes down regulation of FP message. However, this finding must be viewed with caution due to the large animal variability observed in these experiments on day 14 (onset of luteolysis). A study must be specifically designed to address this issue. On the other hand, internucleosomal fragmentation of DNA (indication of structural luteolysis), was in all cases associated with reduced levels of FP mRNA.

To determine if the reduction in relative amounts of FP message was associated with the decline in circulating concentrations of progesterone and/or the onset of internucleosomal DNA laddering, results from day 14 were compared (Figure 4). These data demonstrate that in individual ewes, the decreased amount of FP message is associated with a decline in concentrations of progesterone and is inversely associated with the onset of internucleosomal DNA laddering. This provides support for a direct association between regulation of FP mRNA and the structural component of luteolysis. It does not seem likely that the decline in FP message was the result of non-specific degradation during cellular destruction rather than a specific down regulation of expression of the receptor itself since 18S ribosomal RNA levels were constant among tissues. Furthermore, in another study mRNA for the cell death gene, *bax*, was significantly increased during late luteolysis in bovine CL (Rueda *et al.*, 1995b). This provides evidence that random degeneration of RNA does not necessarily occur during the initial stages of physiological cell death.

In conclusion, the results presented here support our hypothesis that $PGF_{2\alpha}$ or some event associated with luteolysis alters expression of the FP receptor. Specifically, down regulation of the mRNA for luteal FP may occur in CL of luteolytic animals. We propose that this event is also reflected in receptor levels. In contrast, FP mRNA levels are maintained in early pregnancy. Whether this reflects an additional component of regulation in maintenance of the CL in early pregnancy remains to be determined.

Materials and methods

Animals and tissue collection

The estrous cycles of Western range ewes (2–5 years of age) were synchronized by injection of $PGF_{2\alpha}$ (Lutalyse; Upjohn Company, Kalamazoo, MI; 10 mg i.m.) on days -12 and -3 (day 0 being synchronous estrus). Follicular development was

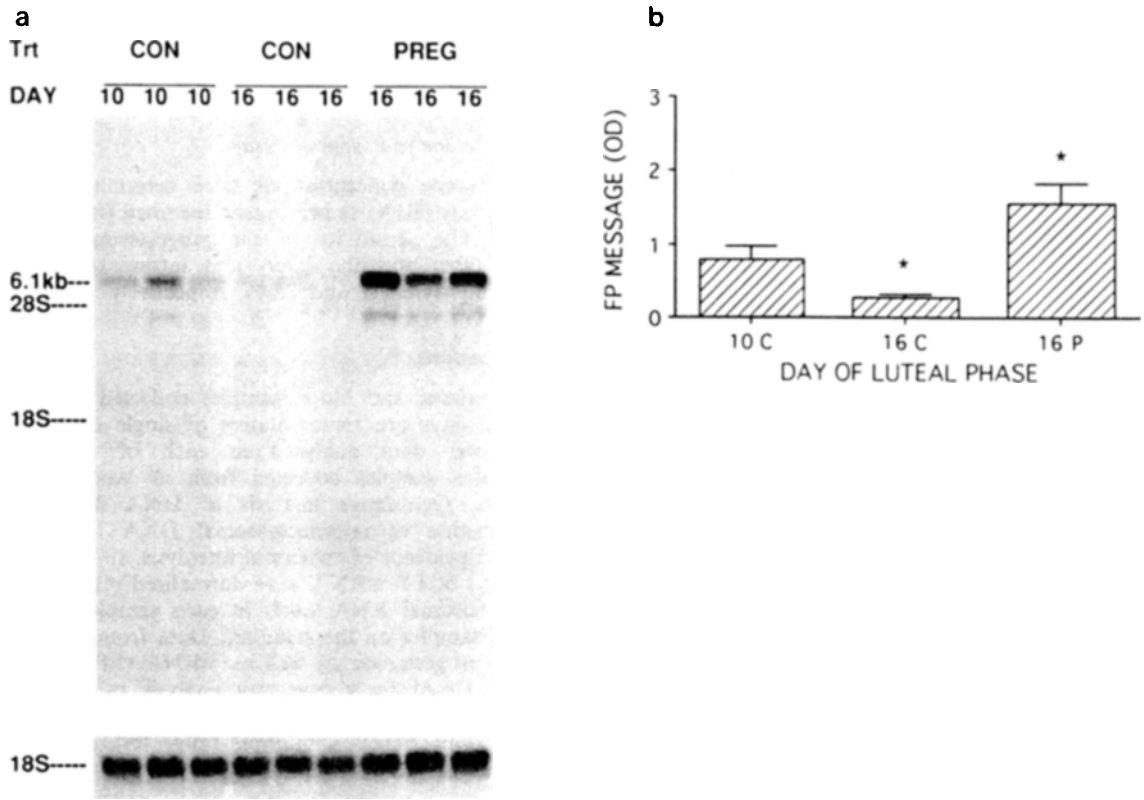

Figure 5 Northern analysis of FP mRNA in pregnant and non-pregnant ewes. (**a**) Total RNA isolated from ovine luteal tissue collected on day 10 or 16 from non-pregnant (CON) and on day 16 from pregnant ewes (PREG). A ^{32}P-labeled cDNA FP probe was used to hybridize to both northern and slot blots (estimated transcript size in kb, and migration distances of 18S and 28S ribosomal RNAs, are indicated) as described in methods. Membranes were rehybridized with 18S ribosomal RNA probe for normalization (lower panel). (**b**) Quantification of data was obtained by northern and slot blot analysis following normalization (FP/18S mRNA levels) in each sample (mean ± SEM, $n \geq 3$/day). $^{*}P < 0.05$ different from day 10

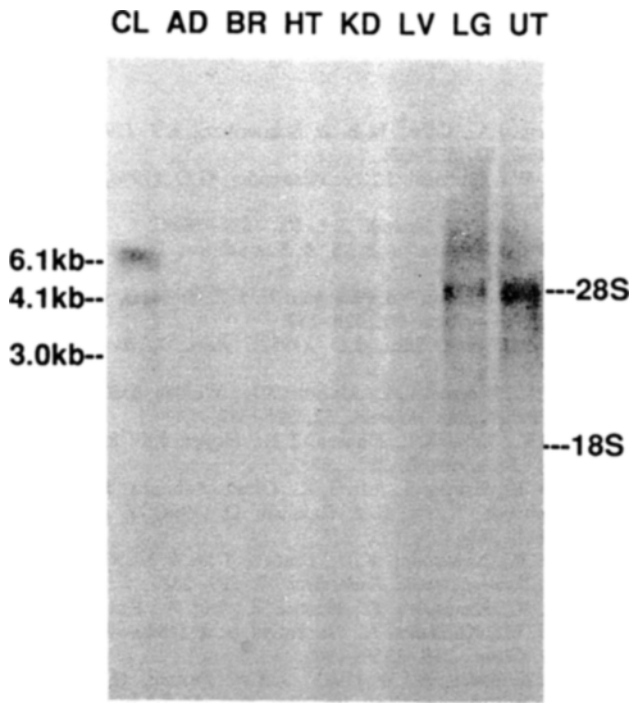

Figure 6 Autoradiography of northern blot analysis of FP mRNA in ovine tissue. Total RNA was isolated from ovine CL, adrenal (AD), brain (BR), heart (HT), kidney (KD), liver (LV), lung (LG), and uterine (UT) tissue collected on day 10 from a non-pregnant ewe. A ^{32}P-labeled cDNA FP probe was used to hybridize the northern blot (estimated transcript size in kb, and migration distances of 18S and 28S ribosomal RNAs, are indicated) as described in methods. Following hybridization with FP probe the membranes were rehybridized with 18S ribosomal RNA probe (lower panel)

stimulated with a subcutaneous injection of pregnant mare's serum gonadotropin (750 IU) on day -3 followed on day 0 with an injection of human chorionic gonadotropin (Sigma Chemical Co., St Louis, MO; 750 IU). CL were collected on day 10, 12, 14, and 16 of the estrous cycle and day 16 of pregnancy. To obtain CL from pregnant animals, ewes were exposed to fertile rams and breeding dates were recorded. Pregnancy was confirmed in day 16 ewes by retrieval of the conceptus(es) following uterine flushing. In addition, CL were collected 12 h after administration of $PGF_{2\alpha}$ to initiate luteolysis on day 10, 12, and 14. The CL were surgically collected by mid-ventral laparotomy and immediately frozen in liquid nitrogen. Blood samples were collected via jugular venipuncture 12 h prior to tissue collection and again at the time of surgery for determination of circulating levels of progesterone. Additionally, in one experiment, adrenal, brain, heart, kidney, liver, lung and uterine tissues were collected from a day 10 ewe and quick frozen and stored at −70°C for future isolation and analysis of RNA.

Extraction of DNA and analysis

Genomic DNA was prepared as previously described (Tilly & Hsueh, 1993) and evaluated for its integrity (Rueda *et al.*, 1995a). The quantity and purity of nucleic acid preparations were estimated by measuring the optical density of each sample (A260/A280). An equivalent amount of genomic DNA from each treatment group was radiolabeled on 3′-ends with [α^{32}P] dideoxy-ATP, (3000 Ci/mmol; Amersham, Arlington Heights, IL) using 25 U terminal transferase (Boehringer-Mannheim, Indianapolis, IN). DNA samples were separated by electrophoresis through 2% agarose gels (500 ng of labeled DNA per well) for approximately 3.5 h at 65 V. The gels were dried without heat in a slab dryer and exposed to

film (Kodak X-Omat films at −70°C) for autoradiographic analysis.

Northern blot analysis

Total RNA was extracted by the guanidinium thiocyanate-phenol-chloroform extraction procedure (Chomczynski & Sacchi, 1987). The purity and quantity of RNA was estimated by measuring the optical density of each sample (A260/A280). Isolated RNA was analysed by northern or slot blot analysis using either cDNA or cRNA probes. For northern blot analysis, 5 μg of total RNA from each sample was separated through a 1% denaturing gel and transferred to nylon membranes (Stratagene, LaJolla, CA) as previously described (Graves *et al.*, 1995). An FP cDNA, 752 bp in length (639 through 1390 bp) was labeled by random priming (Feinberg & Vogelstein 1983) using [α^{32}P] deoxy-CTP (3000 Ci/mmol; Amersham) and purified from unincorporated radionucleotide by column chromatography (Nuc Trap Push column, Strategene) as previously described (Rueda *et al.*, 1995b). Following hybridization analysis, the blots were re-hybridized with the radiolabeled 18S ribosomal cRNA probe as previously described (Graves *et al.*, 1995).

The quantity of total RNA was normalized using a radiolabeled 18S ribosomal cRNA probe as previously described (Graves *et al.*, 1995). The antisense RNA probe complimentary to 18S ribosomal RNA was synthesized by *in vitro* transcription from linearized plasmid templates using RNA polymerase, [α^{32}P]-CTP (3000 Ci/mmol; Amersham) and the Gemini II Riboprobe Core System (Promega, Madison, WI) as described (Melton *et al.*, 1984).

Northern blots were prehybridized 2 to 4 h (42° or 65°C), followed by overnight hybridization with either the radiolabeled cDNA or cRNA probes. Membranes were washed at room temperature with 2 × SSC-1% SDS, followed by 1–2 consecutive 15 min washes with 0.5 × SSC-0.1% SDS. Membranes were then exposed to Kodak X-Omat films as previously described (Rueda *et al.*, 1995b). Changes in FP mRNA levels were normalized against 18S ribosomal RNA levels in each sample following scanning densitometry of hybridization signal intensities.

Progesterone radioimmunoassay

Progesterone concentrations were determined by radioimmunoassay (RIA) as previously described (Hild-Petitio *et al.*, 1987). The sensitivity of the progesterone antibody was .56 pg/tube, and the inter- and intraassay coefficients of variation were 9.1 and 7.0%, respectively.

Data analysis

Luteal tissue and blood samples collected on each of the specific days are representative of single ewes ($n \geq 3$/day). Therefore, data analysed in each of the experiments represents samples collected from at least three different animals. Qualitative analysis of DNA integrity was by visualization of internucleosomal DNA fragmentation to confirm evidence of structural luteolysis. In each experiment, amounts of FP mRNA were normalized relative to levels of 18S ribosomal RNA levels in each sample and compared within samples on the same gel. Data from measurement of serum progesterone as well as mRNA (FP/18S ratio) were first analysed by a one way analysis of variance. When significant differences were identified, the data were further analysed by Scheffe's multiple range test. Significance was assigned at the 0.05 level.

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