Temporal gene expression in bovine corpora lutea after treatment with PGF_{2α} based on serial biopsies *in vivo*

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There is growing evidence to indicate that PGF_{2α}-induced luteolysis involves altered gene expression in the corpus luteum. Concentrations of mRNA encoding nine different gene products were quantified at three time points from corpora lutea *in situ*. Serial luteal biopsies (2.1–5.5 mg per biopsy) were collected using an ultrasound-guided transvaginal method and mRNA concentrations were quantified with standard curve quantitative competitive RT-PCR. In the first experiment, three luteal biopsies were collected from three heifers and analysed in multiple assays to evaluate the repeatability of the methods. Concentrations of mRNA for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), PGF_{2α} receptor (FP receptor) and LH receptor were found to be highly repeatable between assays, between multiple biopsies and between animals (coefficients of variation 1.3–17.3%). In the second experiment, heifers on days 9–11 after ovulation were assigned randomly to receive saline only ($n = 6$), saline with biopsies taken at $t = 0, 0.5$ and 4.0 h after injection

($n = 6$), PGF_{2α} only ($n = 6$) or PGF_{2α} with biopsies taken at $t = 0, 0.5$ and 4.0 h after treatment ($n = 7$). Biopsy alone did not change corpus luteum diameter, serum progesterone concentrations or days to next ovulation within the saline- or PGF_{2α}-treated groups. Concentrations of mRNA for steroidogenic acute regulatory protein, FP receptor, 3β-hydroxysteroid dehydrogenase, cytosolic phospholipase A₂ and LH receptor were decreased at 4.0 h after PGF_{2α} injection. In contrast, PGF_{2α} increased mRNA concentrations for prostaglandin G/H synthase-2, monocyte chemoattractant protein-1 and c-fos but the time course differed for induction of these mRNAs. Concentrations of mRNA for GAPDH did not change after PGF_{2α} treatment. In conclusion, the techniques allowed analysis of multiple, specific mRNAs in an individual corpus luteum at multiple time points without altering subsequent luteal function. Use of these techniques confirmed that luteolysis involves both up- and down-regulation of specific mRNA by PGF_{2α}.

Introduction

The action of PGF_{2α} on luteal regression has been studied intensively for almost three decades. Morphologically, there is an increase in accumulation of lipid droplets and dense bodies within steroidogenic luteal cells after exposure to PGF_{2α}, and luteal cells undergo DNA fragmentation (Juengel *et al.*, 1993). In addition, PGF_{2α} induces rigidification of the plasma membrane of luteal cells, potentially causing decreased receptor binding or activation (Sawada and Carlson, 1991). Functionally, luteal production of progesterone decreases by 4 h after PGF_{2α} treatment and reaches basal concentrations by 48 h after PGF_{2α} treatment (for example see Rodgers *et al.* (1995)). Recently, researchers using molecular biology techniques have provided substantial information on intracellular effector systems (Chen *et al.*, 1998), gene regulation

(Hawkins *et al.*, 1993; Sakamoto *et al.*, 1994; Juengel *et al.*, 1996; Tsai and Wiltbank, 1997), and involvement of the immune system (Pate, 1995; Townson *et al.*, 1996a; Tsai *et al.*, 1997; Haworth *et al.*, 1998) in the mechanisms of PGF_{2α} action. These studies involved either cell culture techniques or removal of corpora lutea at specific times after treatment to analyse PGF_{2α} action. In the present study, a methodology that allows multiple analyses of gene expression *in vivo* from a single corpus luteum with no detectable alteration in subsequent function of the corpus luteum is described. Transvaginal ultrasonography can be used to collect a small amount (20 µl) of follicular fluid (Ginther *et al.*, 1997) or luteal tissue (Kot *et al.*, 1999) without altering subsequent ovarian function. These techniques were modified to allow quantification of steady-state concentrations of multiple mRNAs from multiple, sequential luteal biopsies using standard curve quantitative competitive RT-PCR introduced by Tsai and Wiltbank (1996). The use of these procedures to study temporal changes in nine different mRNAs after treatment of heifers with PGF_{2α} is described.

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Materials and Methods

Chemicals and reagents

All chemicals used in this study, unless otherwise specified, were purchased from Sigma Chemical Company (St Louis, MO). T7 RNA polymerase, pGEM™-T cloning system, SuperScript™ II RNase H minus M-MLV reverse transcriptase and restriction enzymes were from Promega (Madison, WI). PCR2.1™ cloning system was from Invitrogen (Carlsbad, CA). Taq DNA polymerase and 1 kb DNA ladders were from GIBCO/BRL (Gaithersburg, MD). Magnetight™ Oligo(dT) particles were from Novagen (Madison, WI). Pharmacia-Upjohn Co (Kalamazoo, MI) donated PGF_{2α} (Lutalyse®).

Animals

Nulliparous Holstein heifers (age 18–30 months, body weight 400–580 kg) with normal oestrous cycles were used in this study. Heifers were housed in the University of Wisconsin-Madison Charmany facility and kept in outdoor paddocks with free access to shelter. Oestrus was synchronized by giving 25 mg PGF_{2α} (Lutalyse®) i.m. to heifers with a mature corpus luteum. Daily transrectal ultrasonography was performed to monitor time of ovulation and cross-sectional diameter of each corpus luteum.

Experiment 1

Three biopsies were taken during the same examination from the corpus luteum of three heifers on days 9 or 10 after ovulation by the transvaginal ultrasound-guided technique described below to evaluate the repeatability of the biopsy technique. All three biopsies were removed as quickly as possible with no more than 15 min elapsing between all three biopsies. The biopsies were frozen immediately in liquid nitrogen and stored at –80°C for subsequent quantification of specific RNA transcripts. Steady-state concentrations of mRNA encoding for PGF_{2α} receptor (FP receptor), LH receptor or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were quantified by standard curve quantitative competitive RT-PCR. The concentration of each mRNA was quantified from each biopsy in three separate assays. The coefficients of variation ((standard deviation/mean) × 100) between assays, biopsies and heifers were calculated.

Experiment 2

Twenty-five heifers on days 9–11 after ovulation were assigned randomly into one of four treatment groups. Heifers in group 1 (saline only; *n* = 6) received an i.v. injection of 5 ml saline solution. Heifers in group 2 (saline plus biopsy; *n* = 6) received an i.v. saline injection and had biopsies taken immediately before injection (0 h) as well as at 0.5 and 4.0 h after injection. Heifers in group 3 (PGF_{2α} only; *n* = 6) received an i.v. injection of 25 mg PGF_{2α}. Heifers in group 4 (PGF_{2α} plus biopsy; *n* = 7) received an i.v. PGF_{2α} injection and biopsies were taken immediately

before injection (0 h) as well as at 0.5 and 4.0 h after PGF_{2α} injection. Daily transrectal ultrasonography was performed from the day before treatment until the next ovulation using an Aloka SSD-500V Micrus machine equipped with a 7.5 MHz linear array probe (Aloka Co, Wallingford, CT). Blood samples were taken from the coccygeal vessels at 0, 0.5 and 4.0 h after treatment and once a day thereafter until the next ovulation. Blood samples were allowed to clot and were centrifuged at 500 *g* for 20 min. The serum was stored at –20°C until assayed for progesterone. Progesterone was extracted from serum and concentrations were quantified by competitive ELISA as described by Tsai and Wiltbank (1998). The assay had 80% binding at 0.13 ng ml^{–1} with mean intra- and interassay coefficients of variation of 8 and 17%, respectively. Biopsies were frozen in liquid nitrogen and stored at –80°C for subsequent isolation and quantification of specific mRNA.

Transvaginal ultrasound-guided biopsy of the corpus luteum

Heifers were confined in a chute and caudal epidural anaesthesia was induced with 6 ml lidocaine hydrochloride (2% (w/v); Phoenix Pharmaceutical Inc, St Joseph, MO). A plastic handle containing a needle guide 45 cm in length was mounted to a 5.0 MHz convex array ultrasound transducer (Aloka UST-9111) for biopsy. The transducer face was applied to the wall of the vaginal fornix and a 48 cm, 18-gauge biopsy needle (US Biopsy, Division of Promex Inc, Indianapolis, IN) was inserted into the needle guide. The needle spring was locked into a ready position (the specimen notch of the inner stylet was surrounded by a cutting needle) before insertion of the needle into the needle guide. The ovary containing the corpus luteum was positioned transrectally against the vaginal wall and over the transducer face so that the targeted corpus luteum was clearly visible on the monitor. The needle was then advanced through the vaginal wall and into the corpus luteum (as determined on the ultrasound image). The outer cutting needle was sprung, cutting off trapped tissue within the specimen notch (Fig. 1). The needle was then withdrawn from the needle guide and the tissue biopsy was removed from the specimen notch, placed into a 1.5 ml microcentrifuge tube and frozen immediately in liquid nitrogen.

Isolation of mRNA from biopsy sample

The tissue biopsy was removed from –80°C, placed in a 1 ml glass tissue grinder (Duell® 20; Kontes Glass Co, distributed by PGC Scientifics, Gaithersburg, MA) and weighed. Lysis buffer (50 µl; 4 mol guanidinium isothiocyanate l^{–1}, 0.1 mol Tris–HCl l^{–1}, pH 8.0, 1% (w/v) dithiothreitol and 0.5% (w/v) *N*-lauroylsarcosine) was added and the tissue was homogenized with a pestle. Binding buffer (100 µl; 100 mmol Tris–HCl l^{–1}, pH 8.0, 400 mmol NaCl l^{–1} and 20 mmol EDTA l^{–1}) was added to the glass tube and the solution was transferred to a 1.5 ml

Fig. 1. (a) Schematic drawing of the biopsy needle with the outer cutting needle locked in the ready position. The inner needle is advanced into the tissue and a portion of the tissue protrudes into the specimen notch. (b) Schematic drawing of the needle after the outer needle is sprung showing the tissue trapped in the specimen notch. (c) Needle and tissue after luteal biopsy has been removed. Black arrowhead indicates the luteal biopsy within the biopsy needle. Black arrow is at the end of the luteal biopsy. White arrow points to the edge of the outer cutting needle. (d) Typical luteal biopsy collected in this study. Scale bar represents 5 mm.

microcentrifuge tube. Poly (A+) RNA was isolated using Magnetight™ oligo(dT) beads as described by Tsai and Wiltbank (1998). In brief, cell debris was pelleted by centrifuging the whole lysate at 16 000 *g* for 5 min at 4°C and the supernatant was transferred to a new microcentrifuge tube. Magnetight™ oligo (dT) solution (50 µl; 10 mg oligo(dT) beads ml⁻¹ solution) was added and allowed to hybridize with mRNA at room temperature for 5 min. Magnetight™ beads were captured with a magnetic stand. The supernatant was removed for determination of DNA content by Hoechst 33258 fluorescent dye and a fluorometer (DyNA Quant 200; Pharmacia, Piscataway, NJ). The beads were washed five times with 400 µl washing buffer (0.15 mol NaCl l⁻¹, 10 mmol Tris-HCl l⁻¹, pH 8.0, and 1 mmol EDTA l⁻¹). After the final wash, 15 µl elution buffer (2 mmol EDTA l⁻¹) was added to elute mRNA from Magnetight™ beads at 65°C for 3–5 min. The mRNA was divided into aliquots and stored at –80°C until used.

Preparation of native and competitor RNAs

The construction of plasmids containing native and competitor RNA for PGF_{2α} receptor, prostaglandin G/H synthase-2 (PGHS-2), GAPDH and 3β-hydroxysteroid dehydrogenase (3β-HSD) has been described previously (Tsai *et al.*, 1996, 1998; Tsai and Wiltbank, 1998). Plasmids were also constructed containing native or competitor RNA for monocyte chemoattractant protein 1 (MCP-1), steroidogenic acute regulatory protein (StAR) and LH receptor (Fig. 2) using the techniques of Tsai and Wiltbank (1996). In brief, specific primer pairs (Table 1) were designed to amplify a fragment of DNA from mRNA transcripts. These DNA fragments were cloned into PCR cloning vectors

Fig. 2. (a) Schematic drawing of strategy for generating native and competitor DNA standards for LH receptor and (b,c) production of standard curve for quantification of LH receptor mRNA. The native mRNA was amplified using a forward (P1) and reverse (P2) primer. To produce the competitor, a combination internal primer (IP) was designed that contained the P2 sequence of the reverse primer on the 5' end of the internal primer and also contained an internal sequence that was 109 bp upstream from the P2 sequence on the 3' end of the internal primer. This allowed amplification of a competitor that was only 307 bp using the same P1 and P2 primers that amplify a 395 bp sequence from the native molecule. The competitor was added at a constant amount (1 amol in each final PCR tube) into the PCR master mix. Increasing amounts of native RNA (0.1–12.8 amol) were added to the standard curve tubes and amplified by 30 rounds of PCR. The products were separated by PAGE and stained with ethidium bromide. (b) The ethidium bromide-stained PCR products were visualized on a UV box and the intensity of each band was quantified. (c) The ratios of native to competitor RNA were plotted against initial amounts of native added to construct the standard curve. $r^2 = 0.998$.

(PCR2.1™ for LH receptor and pGEM™-T for MCP-1 and StAR), amplified and sequenced. For LH receptor and MCP-1, internal primers were designed consisting of a portion of complementary sequence to the mRNA at the 3' end of the internal primer and sequences identical to the downstream primer at the 5' end of the internal primer (Fig. 2). The internal primer was paired with the upstream primer to amplify a shorter fragment that also contained the downstream primer sequences. The amplified fragment was again cloned into a PCR cloning vector and a positive clone was identified. This clone served as the competitor plasmid for LH receptor or MCP-1. The competitor for StAR was

Table 1. Details of primer sets used

Gene	Primer name	Sequence	Species	Reference
FP receptor	A0035	5' GTAAAAAGGGTTTCACAGG 3'	Cow	Sakamoto <i>et al.</i> (1994)
	A0034	5' CAAAGACTGGGAAGATAGGTT 3'		
LH receptor	LHR-5	5' CTTCTGCATGGGGCTCTA 3'	Cow	Lussier <i>et al.</i> (1995)
	LHR-3	5' TCACGTTGAGAATCAGGATGG 3'		
PGHS-2	bPGS2A	5' AGGTGTATGTATGAGTG-TAGGA 3'	Cow	Tsai <i>et al.</i> (1996)
	bPGS2B	5' GTGCTGGGCAAAGAATGCAA 3'		
cPLA ₂	bcPLA-F	5' GGATGCCAATTATGTTATGGATGA 3'	Cow	S.-J. Tsai and M. C. Wiltbank, unpublished
	bcPLA-R	5' TGTGAGAAGTAAAAGAGGGTTGTG 3'		
StAR	bSTAR-1	5' CCTCTCTACAGCGACCAA 3'	Cow	Hartung <i>et al.</i> (1995)
	bSTAR-2	5' TCGTGAGTGATGACCGTG 3'		
3 β -HSD	3bHSD-1	5' TGTGGTGGAGGAGAAGG 3'	Cow	Zhao <i>et al.</i> (1989)
	3bHSD-2	5' GGCCGTCTTGGATGATCT 3'		
MCP-1	MCP1-F	5' TCCTGTGCCTGCTACTCA 3'	Cow	Wempe <i>et al.</i> (1991)
	MCP1-R	5' TCAAGGCTTTGGAGTTTGGT 3'		
GAPDH	GP1	5' TGTCCAGTATGATTCCACCC 3'	Cow	Tsai <i>et al.</i> (1996)
	GP2	5' TCCACCACCCTGTTGCTGTA 3'		
c-Fos	hFOS-F	5' GGAAAGGAATAAGTGGCTG 3'	Human	van Straaten <i>et al.</i> (1983)
	hFOS-R	5' AGTCTGCTGCATAGAAGG 3'		

FP receptor: PGF_{2 α} receptor; PGHS-2: prostaglandin G/H synthase 2; cPLA₂: cytosolic phospholipase A₂; StAR: steroidogenic acute regulatory protein; 3 β -HSD: 3 β -hydroxysteroid dehydrogenase; MCP-1: monocyte chemoattractant protein 1; GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

produced by digestion of the native cDNA with Styl to remove 32 bp from the internal sequence, similar to the method used for production of a competitor for PGF_{2 α} receptor (Tsai and Wiltbank, 1996). For cytosolic phospholipase A₂ (cPLA₂), primers were designed according to the published human sequence (Sharp *et al.*, 1991) and used to amplify a fragment of DNA from mRNA isolated from bovine granulosa cells. This DNA fragment was cloned into pGEMTM-T vector and sequenced by ABI PrismTM terminator cycle sequencing kit (Perkin Elmer, Foster City, CA) according to the manufacturer's protocol. Bovine specific primers were designed from the partial cDNA sequence and used to construct native and competitor plasmids as described above.

Plasmids containing native or competitor DNA were linearized by proper restriction enzymes and transcribed *in vitro* using T7 RNA polymerase. The transcribed RNAs were precipitated twice using 0.3 mol sodium acetate l⁻¹ (pH 4.2) and 2.5 volumes of 100% ethanol after removal of DNA and protein from the solution. The concentrations of RNAs were quantified by absorbance at 260 nm, divided into aliquots and stored at -80°C. Each RNA aliquot was used only once to reduce variation due to potential degradation of RNA after freezing and thawing.

Standard curve of the quantitative competitive RT-PCR

The detailed procedure of standard curve quantitative competitive RT-PCR has been described by Tsai and Wiltbank (1996) and Tsai *et al.* (1996). In brief, a constant

amount of competitor RNA was added into reverse transcription master mix (50 mmol Tris-HCl l⁻¹, 75 mmol KCl l⁻¹, 3 mmol MgCl₂ l⁻¹, pH 8.3, 10 mmol dithiothreitol, 100 pmol random primer, 4 mmol dNTPs l⁻¹ and 50 U SuperScriptTMII RNase H-reverse transcriptase). The competitor RNA was at a constant amount in all reactions because final results were all expressed as a ratio of the native band to the band from this competitor. This mix was then dispensed into 0.2 ml thin wall PCR tubes (USA/Scientific Plastics®, Ocala, FL) and known amounts of native RNA in 2 μ l diethyl pyrocarbonate-treated water or 2 μ l unknown mRNA samples were added individually to each tube. The final volume of reverse transcription mix was 20 μ l and reverse transcription was performed at 42°C for 60 min followed by heating to 95°C for 10 min and quick chilling to 4°C in a programmable thermocycler (PTC-100; MJ Research, Watertown, MA). Five microlitres of reverse transcription products were added to 15 μ l PCR mix (final concentration: 10 mmol Tris-HCl l⁻¹ (pH 9.0 at 25°C), 1.5 mmol MgCl₂ l⁻¹, 50 mmol KCl l⁻¹, 0.1% (v/v) Triton-X100®, 0.2 mmol dNTPs l⁻¹, 0.5 U Taq polymerase and 0.4 μ mol primers l⁻¹). This mixture was subjected to 30 cycles of amplification (each cycle consisting of 30 s denaturation at 95°C, 30 s annealing at 57°C and 30 s elongation at 72°C) followed by final elongation at 72°C for 5 min. A sample (10 μ l) of PCR products was separated directly on a 5% (w/v) acrylamide gel with 1 $\times$ TBE (0.09 mol Tris l⁻¹, 0.09 mol boric acid l⁻¹, 0.001 mol EDTA l⁻¹, pH 8.0) buffer at 110 V for 40 min using Mini-protein® II electrophoresis system (BioRad, Richmond, CA). The gel

Table 2. Results of Expt 1 showing concentrations of mRNAs for FP receptor, LH receptor and GAPDH in luteal biopsies from three heifers

Animal	Biopsy identity	Mass of biopsy (mg)	mRNA copies per cell (mean $\pm$ SD)		
			FP receptor ($\times 10^3$)	LH receptor ($\times 10^4$)	GAPDH ($\times 10^4$)
R18	1	5.0	1.6 $\pm$ 0.1	1.2 $\pm$ 0.3	8.6 $\pm$ 1.1
	2	2.5	2.0 $\pm$ 0.1	1.4 $\pm$ 0.1	7.3 $\pm$ 1.6
	3	2.1	2.0 $\pm$ 0.3	1.4 $\pm$ 0.2	7.1 $\pm$ 0.5
	Mean	3.2 $\pm$ 1.3	1.9 $\pm$ 0.1	1.4 $\pm$ 0.2	7.6 $\pm$ 0.7
Y4 ^a	1	5.5	1.8 $\pm$ 0.1	1.5 $\pm$ 0.2	9.2 $\pm$ 1.3
	3	2.1	1.6 $\pm$ 0.1	1.2 $\pm$ 0.2	9.5 $\pm$ 0.7
	Mean	3.8 $\pm$ 1.7	1.7 $\pm$ 0.1	1.4 $\pm$ 0.1	9.3 $\pm$ 0.7
R25	1	4.9	1.8 $\pm$ 0.1	1.3 $\pm$ 0.1	7.3 $\pm$ 0.6
	2	3.0	1.5 $\pm$ 0.3	1.5 $\pm$ 0.3	8.3 $\pm$ 0.8
	3	5.3	1.8 $\pm$ 0.1	1.3 $\pm$ 0.4	6.0 $\pm$ 0.4
	Mean	4.4 $\pm$ 1.0	1.7 $\pm$ 0.1	1.4 $\pm$ 0.2	7.2 $\pm$ 0.5
Coefficient of variation	Between assays		8.7%	17.3%	13.5%
	Between biopsies		8.1%	7.5%	7.9%
	Between animals		4.9%	1.3%	11.3%

FP receptor: PGF_{2α} receptor; GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

^aBiopsy 2 contained too little tissue owing to a large cavity in the corpus luteum and was therefore excluded from the study.

Three biopsies were taken from each of three heifers. Each biopsy was analysed in three separate assays (SD between assays shown for each biopsy) and the mean and SD for each heifer were calculated using the mean for each biopsy. The mean coefficients of variation ((SD/mean) $\times$ 100) were calculated between assays, between biopsies and between animals for each individual mRNA. There were no significant differences between biopsies within or among heifers for any end point.

was then stained with ethidium bromide and placed on a UV illuminator equipped with a camera connected to a Macintosh computer. The gel image was analysed using Collage™ software (Fotodyne, Hartland, WI). In each lane of the gel, the intensities of the native and competitor bands were quantified and a ratio of these intensities was calculated. The logarithmic ratio of native to competitor RNA was plotted against the logarithmic initial amounts of native RNA to produce the standard curve (Fig. 2). Concentrations of specific mRNA transcripts in samples were calculated by comparison to the standard curve as described by Tsai and Wiltbank (1996). The DNA concentration that was determined during mRNA isolation was used to determine the cell number in the luteal biopsies and this number was used to express results as number of copies of specific mRNA per cell.

Semi-quantitative RT-PCR

Primers were designed according to the published human cDNA sequence to quantify steady-state concentration of mRNA encoding c-fos (van Straaten *et al.*, 1983). This primer pair amplified a fragment of 476 bp from bovine mRNA. Semi-quantitative RT-PCR using GAPDH as internal control was performed to quantify steady-state concentrations of mRNA for c-fos in a similar way to that described by Tsai *et al.* (1996, 1997). The combination of GAPDH primers and c-fos primers produced two products

corresponding to GAPDH (841 bp) and c-fos (476 bp). The products were separated on a 5% (w/v) acrylamide gel and the intensities of the two bands were analysed as described above.

Statistical analyses

In Expt 1, mRNA data were analysed through use of general linear model (GLM) of the Statistical Analysis System (SAS). In Expt 2, corpus luteum diameters and serum progesterone concentrations were analysed by repeated measures ANOVA using the GLM procedure of SAS. There were no differences between saline only and saline plus biopsy groups or between PGF_{2α} only and PGF_{2α} plus biopsy groups. Therefore, the data in saline- and PGF_{2α}-treated groups were combined into respective groups for the test of treatment effect. Serum progesterone concentrations at 0, 0.5 or 4.0 h and number of days to next ovulation were evaluated by one-way ANOVA with differences between groups tested by Duncan's multiple range test. The concentrations of each specific mRNA were analysed by one-way ANOVA followed by Duncan's multiple range test if significant differences were found. There were no significant changes in any of the nine individual mRNAs between any of the times after saline or between any saline group and 0 h of PGF_{2α} treated group; therefore, data from these four groups were combined as a single control group (0 h).

Table 3. Biopsy mass, serum progesterone concentration and days to next ovulation after saline or PGF_{2α} treatment of cows with or without collection of a biopsy sample

Group (n)	Serum progesterone (ng ml ⁻¹)			Days to next ovulation (range)	Days between ovulations
	0 h	0.5 h	4.0 h		
Saline only (6)	2.6 ± 0.6 ^a	2.3 ± 0.4 ^a	2.8 ± 0.7 ^a	10.3 ± 1.0 ^a (6–13)	20.2 ± 0.9 ^a
Saline plus biopsy (6)	2.6 ± 0.4 ^a	2.7 ± 0.3 ^a	2.4 ± 0.2 ^a	11.0 ± 1.3 ^a (8–16)	20.8 ± 1.1 ^a
PGF _{2α} only (6)	2.7 ± 0.4 ^a	2.3 ± 0.4 ^a	1.6 ± 0.2 ^b	4.8 ± 0.9 ^b (3–9)	15.0 ± 1.2 ^b
PGF _{2α} plus biopsy (7)	2.7 ± 0.2 ^a	2.3 ± 0.2 ^a	1.6 ± 0.1 ^b	5.1 ± 0.5 ^b (4–6)	15.0 ± 0.7 ^b

^{ab}Values within a column with different superscripts are significantly different ($P < 0.05$).

Results

Effects of biopsy and PGF_{2α} on luteal function and oestrous cycle

Each ultrasound-guided transvaginal biopsy removed a mean 4.4 mg of luteal tissue (Table 2). Removal of three luteal biopsies did not change serum progesterone concentrations or corpus luteum diameter in either the saline- or PGF_{2α}-treated groups (Table 3 and Fig. 3). In contrast, PGF_{2α} significantly decreased serum progesterone concentration by 4.0 h (Table 3) with basal concentrations observed by day 2 after treatment (Fig. 3). The cross-sectional diameter of corpora lutea was not affected by biopsy but was smaller by day 2 after PGF_{2α} treatment (Fig. 3). The decrease in progesterone concentration occurred more rapidly than did the decrease in luteal diameter. The duration of the oestrous cycle was not changed by biopsy but was shortened by PGF_{2α} treatment (Table 3).

Repeatability of biopsy technique

Concentrations of mRNAs encoding for FP receptor, LH receptor or GAPDH were not different among three different biopsies taken from the same heifer or among biopsies from three different heifers (Table 2).

Effect of biopsy and PGF_{2α} on luteal mRNA

There was no difference in any mRNA concentrations among saline groups (0, 0.5 and 4.0 h) and the 0 h PGF_{2α} group; therefore, these data were combined for analysis. Treatment with PGF_{2α} decreased steady-state concentrations of mRNA encoding for LH receptor, FP receptor, cPLA₂, StAR and 3β-HSD at 4 h after treatment ($P < 0.05$, Table 4). In contrast, steady-state concentrations of mRNA encoding for PGHS-2 were greatest at 0.5 h after PGF_{2α} treatment and decreased at 4 h after treatment although they continued to be greater than the control values ($P < 0.05$). The mRNA for MCP-1 increased by 0.5 h with a further increase by 4 h after PGF_{2α} treatment ($P < 0.05$). Steady-state concentration of mRNA for the nuclear transcription factor c-fos increased after PGF_{2α} administration at 0.5 h but returned to basal concentration by 4 h after treatment. Steady-state concentration of mRNA for the housekeeping

Fig. 3. (a) Serum progesterone concentrations and (b) corpus luteum diameter after treatment with PGF_{2α}. ○: Saline injection only; ●: saline injection and biopsies at 0, 0.5 and 4.0 h; □: PGF_{2α} injection only; and ■: PGF_{2α} injection and biopsies at 0, 0.5 and 4.0 h. *Indicate differences between saline and PGF_{2α} treated groups ($P < 0.05$). There were no differences between saline and saline biopsy groups or between PGF_{2α} and PGF_{2α} biopsy groups.

Table 4. Effect of treatment with PGF_{2α} on concentrations of mRNAs in bovine luteal biopsies

mRNA*	Treatment time		
	Control	0.5 h	4.0 h
PGHS-2 ($\times 10^2$)	0.7 $\pm$ 0.2 ^a	3.5 $\pm$ 1.2 ^c	1.4 $\pm$ 0.4 ^b
MCP-1 ($\times 10^3$)	0.6 $\pm$ 0.1 ^a	1.3 $\pm$ 0.6 ^b	2.8 $\pm$ 0.6 ^c
FP receptor ($\times 10^3$)	1.5 $\pm$ 0.2 ^a	1.2 $\pm$ 0.3 ^{ab}	0.6 $\pm$ 0.1 ^b
cPLA ₂ ($\times 10^2$)	2.1 $\pm$ 0.3 ^a	1.6 $\pm$ 0.4 ^{ab}	1.2 $\pm$ 0.4 ^b
LH receptor ($\times 10^4$)	1.8 $\pm$ 0.3 ^a	1.6 $\pm$ 0.5 ^a	0.7 $\pm$ 0.2 ^b
3 β -HSD ($\times 10^5$)	1.4 $\pm$ 0.2 ^a	1.1 $\pm$ 0.4 ^{ab}	7.5 $\pm$ 0.3 ^b
StAR ($\times 10^4$)	8.9 $\pm$ 1.4 ^a	7.1 $\pm$ 2.3 ^{ab}	2.7 $\pm$ 0.9 ^b
GAPDH ($\times 10^4$)	8.9 $\pm$ 1.0	7.8 $\pm$ 1.8	7.3 $\pm$ 1.7
c-fos ($\times 10^{-1}$)	1.4 $\pm$ 0.2 ^a	8.2 $\pm$ 1.4 ^b	2.8 $\pm$ 0.8 ^a

PGHS-2: prostaglandin G/H synthase 2; MCP-1: monocyte chemoattractant protein 1; FP receptor: PGF_{2α} receptor; cPLA₂: cytosolic phospholipase A₂; 3 β -HSD: 3 β -hydroxysteroid dehydrogenase; StAR: steroidogenic acute regulatory protein; GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

*Units for mRNA (mean $\pm$ SEM) are copies per cell except for c-fos, which is ratio to GAPDH.

^{ab}Values within a row with different superscripts are significantly different ($P < 0.05$).

gene, GAPDH, was not changed at 0.5 or 4.0 h after PGF_{2α} treatment.

Discussion

These studies combined two techniques, ultrasound-guided luteal biopsy and standard curve quantitative competitive RT-PCR, to analyse changes in luteal mRNA serially after treatment with PGF_{2α}. There was no detectable effect of luteal biopsy on any measure of luteal function that was evaluated. Normal serum concentrations of progesterone and luteal diameter were maintained both acutely and chronically after luteal biopsy. In addition, luteal biopsy alone (saline plus biopsy) did not alter acute expression of any of the nine mRNAs evaluated. Thus, three biopsies from a corpus luteum had no detectable impact on luteal function. Three biopsies would have removed only about 0.25% of the luteal tissue (15 mg of 6 g corpus luteum). It seems likely that more than three biopsies can be performed without alteration of luteal function, but this possibility was not studied.

Expt 1 was designed to analyse the repeatability of various aspects of the techniques. Three different mRNAs were chosen for analysis due to distinct luteal expression patterns. The FP receptor mRNA is present primarily in large luteal cells, whereas LH receptor is expressed mainly in small luteal cells (Sakamoto *et al.*, 1994; Guy *et al.*, 1995; Juengel *et al.*, 1996). The mRNA for GAPDH appears to be ubiquitous and has been referred to as a housekeeping gene. It was found that there was substantial variation in the mass of luteal biopsies (from 2.1 mg to 5.5 mg). However, the mRNA concentrations expressed as copies per cell were very consistent (inter-biopsy coefficient of variation was

< 10%) despite differences in biopsy mass. The coefficient of variation appeared to be greatest for between assays indicating that most of the variation in this procedure may result from the standard curve quantitative competitive RT-PCR, and even this variation was clearly within acceptable limits (coefficients of variation < 20%). There was great variation among concentrations of the three different mRNAs (coefficient of variation among different genes was 132%); however, there was minimal variation among different heifers in expression of any specific mRNA (coefficients of variation 1.3–11.3%). Thus, the described luteal biopsy technique provided biopsies with representative amounts of different luteal cell types. In addition, isolation of mRNA from these biopsies followed by standard curve quantitative competitive RT-PCR provided repeatable measurements of the concentrations of different specific mRNAs in the corpus luteum.

In addition to providing novel methodology for analysis of temporal gene expression, the present study provides information on the mechanism of PGF_{2α} action during luteolysis. The earliest responses to PGF_{2α} were an increase in mRNAs encoding for c-fos, MCP-1 and PGHS-2. MCP-1 is a potent chemotactic factor that recruits monocytes and macrophages from the systemic circulation to the regressing corpus luteum and may be involved in activation of macrophages (Pate, 1995; Townson *et al.*, 1996a). PGF_{2α} has been shown to upregulate MCP-1 mRNA expression in bovine and ovine mid-cycle corpora lutea (Tsai *et al.*, 1997; Haworth *et al.*, 1998). The induction of MCP-1 was detectable by 0.5 h after PGF_{2α} treatment and there was a further increase by 4 h after treatment. This increase in MCP-1 mRNA occurred only in corpora lutea that subsequently underwent luteolysis in response to PGF_{2α} (Tsai *et al.*, 1997).

The rapid induction of c-fos may be important for regulation of luteal gene transcription after PGF_{2α} treatment. c-fos is a nuclear transcription factor that can interact with c-jun (another transcription factor) and bind to AP-1 cis-regulatory regions to regulate gene transcription. Expression of c-fos is tightly regulated and alters gene transcription in many different cell types (Karin, 1995). It seems likely that the intracellular effector systems regulated by PGF_{2α} such as increased free intracellular calcium, directly activate transcription of the c-fos gene as described in other systems (Shimokawa *et al.*, 1998; Templeton *et al.*, 1998). Both c-fos and PGHS-2 have been termed immediate early genes because of their direct induction by intracellular second messenger pathways. Induction of the c-fos nuclear transcription factor could subsequently be involved in later gene transcriptional events such as up- or downregulation of specific genes involved in luteal steroidogenesis or cell death. PGF_{2α} has been reported to increase both c-fos and c-jun mRNA in cultured bovine luteal cells (Fong and Davis, 1996) and the results of the present study demonstrate that induction of c-fos also occurs after *in vivo* treatment with PGF_{2α}.

The finding that PGF_{2α} rapidly induced expression of

PGHS-2 mRNA is consistent with previous reports using mid-cycle ovine (Tsai and Wiltbank, 1997) and bovine (Tsai and Wiltbank, 1998) corpora lutea and cultured ovine large luteal cells (Tsai and Wiltbank, 1997). The autoamplification of $\text{PGF}_{2\alpha}$ by luteal cells may play an important role in luteolysis. The cPLA_2 (the enzyme that catalyses the release of arachidonic acid from phospholipid) mRNA content was decreased about 40% at 4 h after $\text{PGF}_{2\alpha}$ treatment. Similar results were observed in mid-cycle ovine corpora lutea treated with $\text{PGF}_{2\alpha}$ (S.-J. Tsai and M. C. Wiltbank, unpublished). Sawada and Carlson (1991) showed that there was increased PLA_2 activity in rat corpora lutea within 1 h after $\text{PGF}_{2\alpha}$ treatment. Primary regulation of PLA_2 activity appears to be due to phosphorylation of the PLA_2 protein and, therefore, changes in mRNA may not be indicative of PLA_2 activity. Thus, increased production of luteal $\text{PGF}_{2\alpha}$ (Tsai and Wiltbank, 1997) is probably due to increased PGHS-2 and increased PLA_2 activity but is not due to increased cPLA_2 mRNA. There was also a $\text{PGF}_{2\alpha}$ -induced decrease in FP receptor mRNA as has been shown previously (Sakamoto *et al.*, 1994; Juengel *et al.*, 1996; Tsai and Wiltbank, 1998).

The decrease in serum progesterone concentrations caused by $\text{PGF}_{2\alpha}$ was reflected in corresponding decreases in mRNA for StAR, 3β -HSD and LH receptor. The decrease in 3β -HSD mRNA has been documented clearly in previous studies (Hawkins *et al.*, 1993; Juengel *et al.*, 1998; Tsai and Wiltbank, 1998); nevertheless, there appears to be no change in protein or enzyme activity for 3β -HSD even at 24 h after $\text{PGF}_{2\alpha}$ treatment (Rodgers *et al.*, 1995; Juengel *et al.*, 1998). Thus, the decrease in 3β -HSD mRNA is unlikely to be the cause of decreased luteal steroidogenesis. Indeed, early corpora lutea that do not regress after $\text{PGF}_{2\alpha}$ also demonstrate a significant decrease in 3β -HSD mRNA after $\text{PGF}_{2\alpha}$ treatment despite continued luteal progesterone production (Tsai and Wiltbank, 1998). In contrast, the StAR protein is fairly labile and the observed 70% decrease in StAR mRNA is likely to have important implications in acute regulation of luteal steroidogenesis (Stocco and Clark, 1996). A decrease in StAR mRNA has been demonstrated in a number of different species during natural or exogenous $\text{PGF}_{2\alpha}$ -induced luteolysis (Juengel *et al.*, 1995; Stocco and Clark, 1996; Townson *et al.*, 1996b). A decrease in LH receptor mRNA in response to $\text{PGF}_{2\alpha}$ has also been reported by Guy *et al.* (1995) and this decrease may disrupt LH-stimulated steroidogenesis during luteolysis.

In conclusion, use of the serial ultrasound-guided transvaginal biopsy technique in conjunction with standard curve quantitative competitive RT-PCR provided a powerful method to analyse temporal changes in mRNA concentrations associated with $\text{PGF}_{2\alpha}$ -induced luteolysis. These methods allowed characterization and correlation of changes in luteal concentrations of mRNA with subsequent functional changes in the corpus luteum. It was found that treatment with $\text{PGF}_{2\alpha}$ produced patterns of mRNA that were distinct for different mRNA. Genes that are important in maintaining luteal function, such as StAR, 3β -HSD and LH

receptor, were inhibited by $\text{PGF}_{2\alpha}$. Other genes such as *c-fos*, *MCP-1* and *PGHS-2*, were induced after $\text{PGF}_{2\alpha}$ treatment. Two of these genes, *c-fos* and *PGHS-2*, which are considered immediate early genes, reached high concentrations by only 0.5 h after treatment.

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