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Research

Cloning of pig prostaglandin $F_{2\alpha}$(FP) receptor cDNA and expression of its mRNA in the corpora lutea

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Changes in the expression and localization of luteal mRNA for $PGF_{2\alpha}$ (FP) receptors may be critical in determining the luteolytic action of $PGF_{2\alpha}$ in pig corpora lutea. In this study, a full-length FP receptor (FPr) cDNA was isolated and cloned from pig corpora lutea. This isolate (GenBank accession no. U91520) contains an open reading frame of 1086 bases coding for a protein of 362 amino acids with seven potential transmembrane domains. The predicted amino acid sequence of this isolate was 83% identical to the FPr amino acid sequence of other species including sheep, cattle and humans. Northern blot analysis showed the presence of an FPr message of about 5 kb in mRNA from pig corpora lutea. Relatively weak FPr mRNA expression was detected on day 4 and day 7 of the oestrous cycle. The expression was greater ($P < 0.05$) on days 10, 13 and 15 than on days 4 and 7. *In situ* hybridization analysis revealed that mRNA for FPr was expressed predominantly in the steroidogenic large luteal subtype of cell, although there was some expression in small luteal cells, with histological appearance of steroidogenic small cells. Localization of hybridization signals of FPr was observed in luteal tissue at all stages examined. These data demonstrate that FPr is expressed in pig corpora lutea throughout the oestrous cycle and that upregulation of the FPr mRNA occurs when the corpora lutea becomes sensitive to $PGF_{2\alpha}$. Direct luteal targets of $PGF_{2\alpha}$ appear to be primarily large steroidogenic cells in this species.

Introduction

In pigs, the lifespan of the corpus luteum is naturally terminated by endometrial production of $PGF_{2\alpha}$ or may be artificially shortened by exogenous $PGF_{2\alpha}$ and its analogues (Hallford *et al*., 1975; Guthrie and Polge, 1976; Moeljono *et al*., 1976). Endogenous $PGF_{2\alpha}$ and commercial $PGF_{2\alpha}$ analogues mediate regression (luteolysis) of the corpora lutea by means of specific plasma membrane-associated $PGF_{2\alpha}$ FP receptors (FPr) (Niswender *et al*., 2000). FPr is a G-protein-coupled receptor containing seven transmembrane domains (Anderson *et al*., 2001).

In contrast to ruminant corpora lutea, which become sensitive to $PGF_{2\alpha}$ by day 5 or 6 of the oestrous cycle (Acritopoulou and Haresign, 1980; Beal *et al*., 1980), pig corpora lutea do not undergo regression in response to a single dose of $PGF_{2\alpha}$ until after day 12 of the oestrous cycle (Hallford *et al*., 1975; Guthrie and Polge, 1976; Moeljono *et al*., 1976). In ruminants, it has been shown that only corpora lutea with luteolytic capacity (that is, sensitive to $PGF_{2\alpha}$) are capable of responding to $PGF_{2\alpha}$ with an induction of prostaglandin G/H synthase (PGHS)-2, which in turn enables corpora lutea to produce $PGF_{2\alpha}$ locally, and thus amplify the luteolytic signal (Tsai and Wiltbank, 1997, 1998). However, induction of PGHS-2 by cloprostenol, a $PGF_{2\alpha}$ analogue, was similar in pig corpora lutea with and without luteolytic capacity (Diaz *et al*., 2000), thereby indicating that there is a different explanation for corpora lutea refractoriness in this species.

FPr plays a critical role in determining luteal sensitivity to the luteolytic actions of $PGF_{2\alpha}$. In pigs, concentrations of luteal FPr ($PGF_{2\alpha}$ binding sites) increase significantly on day 13 of the oestrous cycle, concurrently with the onset of luteal sensitivity to $PGF_{2\alpha}$ (Gadsby *et al*., 1990, 1993). Furthermore, as circulating $PGF_{2\alpha}$ is rapidly metabolized in the lung (Bonnin *et al*., 1999), and $PGF_{2\alpha}$ decreases the concentration of luteal $PGF_{2\alpha}$ binding sites (Estill *et al*., 1995) and mRNA expression (Diaz *et al*., 2000) of its own receptor, there must be sufficient FPr in pig corpora lutea to enable $PGF_{2\alpha}$ to bind and induce an optimal luteolytic signal. Currently, there are limited data to confirm that an increase in $PGF_{2\alpha}$ binding sites and receptors is related to an increased expression of the FPr gene in pigs.

Luteal FPr is localized only in large luteal cells in sheep (Juengel *et al*., 1996), and in large and small luteal cells in cattle (Mamluk *et al*., 1998). In pigs, large

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and small steroidogenic cells bind to $PGF_{2\alpha}$, although most high-affinity binding sites are detected on large cells (Gadsby *et al*., 1990). However, the precise cellular localization of FPr mRNA expression has not been described for the corpora lutea of this species. Finally, although a partial nucleotide sequence for this receptor has been reported (GenBank accession no. U91520), a full-length cDNA clone coding for pig FPr is not available. Thus, the present study was designed to clone and characterize a complete coding sequence for pig FPr, and to investigate the expression and cellular localization of FPr mRNA in pig corpora lutea throughout the oestrous cycle.

Materials and Methods

Materials

Pig cDNA for 18s rRNA was generously provided by R. MacNeel (Baylor College of Medicine, Houston, TX) (Ding *et al*., 1999). PG600® was donated by Intervet Inc. (Millsboro, DE). Other materials and reagents included: [^{35}S]UTP (NEN® Life Science Products Inc., Boston, MA), [α-^{32}P]dCTP (ICN, Costa Mesa, CA), Denhart solution (USB Scientific, Cleveland, OH), MAXIscript SP6/T7 Kit (Ambion Inc., Austin, TX), Qiagen® Plasmid MidiKit (Qiagen Inc., Valencia, CA), Zeta-Probe® GT blotting membrane (Bio-Rad, Richmond, CA), primers (Genosys Biotechnologies Inc., The Woodlands, TX), collagenase type IV (Worthington Biochemical Corp., Freehold, NJ), SuperScript™II RNase H-Reverse Transcriptase, Random Primer DNA labelling system (Gibco–BRL Life Technologies, Grand Island, NY), autoradiography emulsion (Eastman Kodak, Rochester, NY), pGEM®-T Easy Vector System II, oligodeoxythymidine (oligo $[dT]_{12-18}$) (0.5 $\mu g\,\mu l^{-1}$), JM109 competent cells, RNase inhibitor, RNA molecular weight marker, dithiothreitol (Promega, Madison, WI), Taq DNA polymerase, Sephadex G-50 Quick Spin columns, sperm DNA, yeast total RNA, tRNA, deionized formamide, ribonuclease A (Roche, Indianapolis, IN), paraformaldehyde, TRI reagent, triethanolamine, acetic anhydride, dextran sulphate, Hank's balanced salt solution (HBSS; without calcium and magnesium), BSA (fraction V), Hepes, hyaluronidase (type V from ovine testis), deoxyribonuclease (DNase type I from bovine pancreas), penicillin–streptomycin, gentamycin and 0.2–10 kb RNA marker (Sigma Chemical Co., St Louis, MO). All other materials and chemicals were purchased from Fisher Scientific, Pittsburgh, PA, unless otherwise specified.

Animals and tissues

All procedures involving animals were performed with the approval of the North Carolina State University Institutional Animal Care and Use Committee. Twenty crossbred prepubertal gilts (Yorkshire × Landrace × Chester White; 170–180 days of age) were obtained from the NCSU Swine Education and Research facility. Puberty was synchronized by treating each pig with PG600®. Commencing 2 days after administration of PG600®, pigs were checked twice a day for oestrus with a mature boar. The first day of oestrus was designated day 0. Of the initial 20 animals, two did not show oestrus and three failed to ovulate. These five animals were discarded from the study. The remaining 15 pigs ($n = 3$ per day) were killed at days 4, 7, 10, 13 and 15 after oestrus. After removal from the ovaries, two corpora lutea were embedded in OCT media on dry ice, three or four corpora lutea were snap-frozen in liquid nitrogen and stored at –80°C until RNA extraction, and the remaining corpora lutea were dissected into small pieces and dissociated enzymatically as described by Gadsby and Earnest (1994). After dissociation and filtration through nylon mesh (120 μm), dispersed cells were suspended in Hank's media with 0.1% (w/v) BSA, 25 mmol Hepes l^{-1} and 25 mmol $NaHCO_3\,l^{-1}$. On the basis of Trypan blue exclusion, dispersed cells had greater than 80% viability. Mixed luteal cells were spun (cytospin) onto slides (20 000 large luteal cells per slide), fixed in 4% (w/v) paraformaldehyde–PBS, dehydrated through a graded ethanol series and stored at –80°C. For *in situ* hybridization studies, frozen corpora lutea in OCT were sectioned (8 μm) using a cryostat, mounted on Superfrost Plus slides and stored at –80°C.

Molecular cloning and sequence characterization of pig FP receptor cDNA

The cloning of a full-length cDNA sequence encoding pig FPr was based on sequence identity of previously cloned FPr cDNA in other species. Briefly, a sense and antisense primer pair was designed by comparing the highly conserved regions of the cDNA for FPr of human (accession no. AF004021) with sheep (Graves *et al*., 1995) and mouse (Sugimoto *et al*., 1994) sequences, and human with cattle (Sakamoto *et al*., 1994) sequences, respectively. The primers were 5′-GGA GAT GAC TTG AGT GGT CGG C-3′ (sense) and 5′-TCC CAT GGC ATT ATT TTA TTG-3′ (antisense), corresponding to nucleotides 114–135 and 1465–1445 of the human FPr cDNA. A reverse transcriptase PCR was conducted to amplify a product (about 1364 bp) of the pig FPr gene from day 13 corpora lutea. Initially, oligo d(T)15 primers were used to reverse transcribe pig corpora lutea total RNA (Superscript™II) before the PCR reaction. The PCR products were purified by gel extraction, cloned into the pGEM®-T Easy Vector, and three individual clones from independent PCR amplifications were sequenced at the DNA Sequencing and Synthesis Facility (Iowa State University, Ames, IA). The plasmids were sequenced in both directions with the dideoxy chain termination method (Sanger *et al*., 1977) using the Applied Biosystems (Foster City, CA) Prism BigDye

terminator cycle sequencing kit and electrophoresis was carried out on an Applied Biosystems Prism 377 DNA sequencer. The middle region of the entire coding insert was confirmed by using an additional primer pair within the interior of the entire insert for a new PCR reaction. The new primers were 5′-GGC ATC GTG ACT ACA AAA TTC AAG CA-3′ (sense) and 5′-AGA GAT TCT TAA GGA CTG CCT TCC G-3′ (antisense). The new PCR product (429 bp) was cloned and sequenced, showing that this internal fragment was identical to that obtained within the entire coding region. The pig FPr clone was then submitted to GenBank and obtained accession numbers AY043485 (09–02–01) and AAK95379 for its predicted peptide.

After the entire coding sequence for the pig FPr was determined, comparisons with other FPr and prostanoid receptor family members were performed using sequence alignments and phylogenetic trees constructed by the Clustal method in MegAlign (DNASTAR Inc., Madison, WI) with per cent accepted mutations 250 residue weighting. Highly diverged regions were excluded from the alignment and the remaining regions (mainly the transmembrane domains) were based on the regions suggested by Toh *et al*. (1995). Positions of the putative transmembrane domains were determined on the basis of a hydropathicity profile (Kyte and Doolittle, 1982) and comparison with other FPr peptides. GenBank accession numbers for sequences used in the analysis were as follows: FP receptor (pig, AAK95379; sheep, Q28905; cattle, P37289; human, P43088; mouse, P43117; rat, P43118), EP_1 receptor (human, P34995), EP_2 receptor (human, P43116), EP_3 receptor (human, P43115; pig, P50131), EP_4 receptor (human, P35408), thromboxane A_2 (TP) receptor (human, P21731), DP receptor (human, Q13258), prostacyclin (IP) receptor (human, P43119) and platelet-activating factor receptor (human, AAA60001).

A primer pair for LHr was designed based on a pig cDNA sequence reported by Loosfelt *et al*. (1989) to localize mRNA for luteinizing hormone receptor (LHr) as a positive control for small steroidogenic luteal cells. The primers were 5′-AAT GCC TTT GTG AAA ATT GCG TAT AG-3′ (sense) and 5′-TAT TCA GCT GGA CCA AAA GCT ACG-3′ (antisense), corresponding to nucleotides 1477–1499 and 1953–1928 of pig LHr cDNA, respectively. The PCR product (477 bp) was subcloned, sequenced and excised for generating a cRNA probe as described below.

Preparation of pig FPr, LHr and 18s RNA probes

The full-length (1364 bp) and shorter (429 bp) sequence for the pig FPr cDNA were used to generate a cDNA probe for northern blot analysis and a cRNA probe for *in situ* hybridization, respectively. For northern blot hybridization, the sequences of cloned FPr and 18s cDNA were excised from the plasmids using restriction enzymes (EcoRI for FPr cDNAs; NotI and EcoRI for 18s cDNA). After linearization, gel electrophoresis was performed and a DNA extraction column (Ultrafree-DA, Millipore Corp., Bedford, MA) was used to isolate and recover the purified template for transcription of cDNA probes. The Random Primer DNA labelling system and [^{32}P]dCTP (3000 Ci mmol^{-1}) were used to synthesize radiolabelled FPr and 18s cDNA probes. For *in situ* hybridization, the cloned FPr cDNA and LHr cDNA were linearized by restriction enzymes (NcoI for antisense and PstI for sense probe preparation) and gel-purified as mentioned above. Preparation of ^{35}S-labelled complementary RNA (cRNA) probes was conducted by RNA polymerase-mediated transcription of linearized plasmids using a transcription reaction kit (MAXIscript SP6/T7 Kit). An FPr and LHr cRNA sense probe was used as a negative control. Unincorporated nucleotides were removed by filtration on Sephadex G-50 Quick Spin columns. All procedures were performed according to the manufacturer's recommendations.

RNA preparation and northern blot analysis

Total cellular RNA was prepared by the method of Chomczynski and Sacchi (1987). Briefly, frozen corpora lutea were weighed, crushed in liquid nitrogen and homogenized in 10% (w/v) TRI reagent. Extraction of the RNA was performed according to the manufacturer's protocol. The purified RNA was re-suspended in RNase-free water and concentration was measured in a spectrophotometer at 260 nm.

The northern blot procedures used in this study were adapted from that described by Nicholson *et al*. (1999). Briefly, total cellular RNA (20 μg of each sample) and a molecular size marker (0.2–10 kb RNA ladder) were denatured and separated by electrophoresis in 1% (w/v) agarose–formaldehyde gels. After electrophoresis, the gels were stained with ethidium bromide and the ribosomal RNA bands were visualized under ultraviolet illumination to ensure the integrity of the RNA samples. Size of the mRNA transcripts was calculated on the basis of molecular size markers run on the same gel. Gels were transferred to nylon membranes overnight using the Turboblotter™ Rapid Downward Transfer system (Schleicher & Schuell, Keene, NH). The blotted membrane was rinsed, and dried at 80°C for 60 min. The membranes were prehybridized at 65°C for 30 min and then placed in fresh prehybridization solution with denatured DNA probe (2×10^6 c.p.m. ml^{-1}; total volume 5 ml) and hybridization was continued overnight. The membrane was washed twice with 0.1 × SSC containing 0.1% (w/v) SDS and 1 mmol EDTA l^{-1}, pH 8.0 at room temperature for 30 min, and twice with 20 mmol $NaHPO_4$ l^{-1}, 1% (w/v) SDS, 1 mmol EDTA l^{-1}, pH 8.0 at 65°C for 5–15 min. The membrane was exposed to a phosphorimager screen (Molecular Dynamics, Sunnyvale, CA) at room temperature for

5 h. Subsequently, the blots were stripped in a shaking chamber containing a boiling solution of 0.1 × SSC and 0.5% (w/v) SDS for 15 min and then boiling water for 15 min. The membrane was prehybridized as before and then hybridized with the 18s probe. The blot was washed and exposed (15 min) to a phosphorimager screen. Quantification of hybridization signals was obtained by phosphorimage analysis using ImageQuant software (Molecular Dynamics). The values were reported as arbitrary units above background. All time points (days 4–15) were represented in a single blot. A pool of RNA was made by combining purified RNA extracted from days 4–15 corpora lutea. The pool RNA was used as an internal control between each blot. The intensities for FPr signals were adjusted (normalized) according to the difference between the signals of the pooled RNA samples among blots. The signal values for 18s were adjusted by calculating the difference of intensities between blots for the pooled sample, and then used to normalize the signals between blots. Therefore, the FPr and 18s signals were adjusted before determining the FPr:18s ratios.

In situ *hybridization*

In situ hybridization was performed as described by Wandji *et al.* (1998), with minor modifications. Briefly, the tissue slides were retrieved from –80°C, fixed in 4% (w/v) paraformaldehyde–PBS, rinsed in PBS, dehydrated gradually and allowed to air-dry at room temperature. The slides were then rehydrated in DEPC water (cytospin slides were added at this step), treated with 0.5% (v/v) acetic anhydride, 0.05 mol triethanolamine l^{-1}, 0.3% (v/v) acetic acid for 10 min and washed in 0.2 × SSC, then dehydrated. Sections were incubated for 2 h in a moist chamber at room temperature with prehybridization buffer (50% (v/v) deionized formamide, 0.6 mol NaCl l^{-1}, 10 mmol Tris HCl l^{-1}; pH 7.5, 0.02% (w/v) Ficoll, 0.02% (w/v) BSA, 0.02% (w/v) polyvinylpyrrolidone, 1 mmol EDTA l^{-1}, 0.4 mg sheared herring sperm DNA ml^{-1}, 0.5 mg yeast total RNA ml^{-1} and 0.05 mg transfer RNA ml^{-1}). The sections were incubated overnight (18–20 h) in a moist chamber at 55°C with the radiolabelled cRNA probe (4×107 c.p.m. ml^{-1}) in hybridization buffer (prehybridization buffer plus 10% (w/v) dextran sulphate, 0.1% (w/v) SDS and 10 mmol dithiothreitol l^{-1}). After hybridization, slides were briefly dipped in 2 × SSC, soaked in solution containing 50% (v/v) formamide, 1 × SSC, and 10 mmol dithiothreitol l^{-1} at 45°C for 30 min and washed in 0.5 × SSC at room temperature for 30 min. Unhybridized single-strand probes were degraded with ribonuclease A (100 mg ml^{-1}) in digestion buffer for 30 min at room temperature, washed twice with digestion buffer (no ribonuclease), and washed in 0.2 × SSC at 55–60°C for 2 h. After washing, slides were dehydrated through graded ethanols, dried and exposed to autoradiographic film (Kodak BiomaxMR) to evaluate the intensity of hybridization signals. Slides were then dipped in Kodak NTB2 liquid emulsion and exposed for 3 weeks at 4°C. The emulsion-coated slides were developed in Kodak-D19 developer and fixed according to the manufacturer's recommendations. The slides were counterstained with haematoxylin and eosin, and examined with light microscopy.

Blood samples and hormone assays

Blood samples, for determination of serum progesterone concentration, were collected from each animal before administration of PG600® and before they were killed. Progesterone was quantified using commercial radioimmunoassay kits as described by Feng and Almond (1996). All samples were assayed in duplicate and 100 μl serum was used in each replicate. The intra-assay coefficient of variation for both high (18 ng ml^{-1}) and low (0.3 ng ml^{-1}) progesterone reference sera was less than 5%.

Statistical analysis

All northern blot data were expressed as the ratio of the signal intensity for FPr to 18s rRNA (internal control) and shown as the mean arbitrary units ± SEM. The mean for a specific day represents luteal mRNA expression from three animals. Data were initially analysed for heterogeneity of variance (Steel *et al.*, 1997) using the univariate procedure (SAS, 1988). As the variance was not heterogeneous, data were analysed by ANOVA using the General Linear Model procedure (SAS, 1988). The classes in the ANOVA were day and replicate. Differences in means were compared by Duncan's multiple-range test when a significant F statistic ($P < 0.01$) was obtained.

Results

Cloning of pig FPr cDNA

The 1364 bp PCR cloning cDNA (GenBank accession no. AY043485) contains the entire coding region for pig FPr and includes 37 bp of additional upstream (5′) sequence and 238 bases in the 3′ non-translated region. The open reading frame of 1086 bases translates to a protein of 362 amino acids with seven potential transmembrane domains (Fig. 1). The nucleotide and its deduced amino acid sequences of the pig FPr were used for a BLAST homology search against the GenBank database. Identities for nucleotide and amino acid sequences were 91 and 83% for ovine (Graves *et al.*, 1995); 90 and 83% for bovine (Sakamoto *et al.*, 1994);

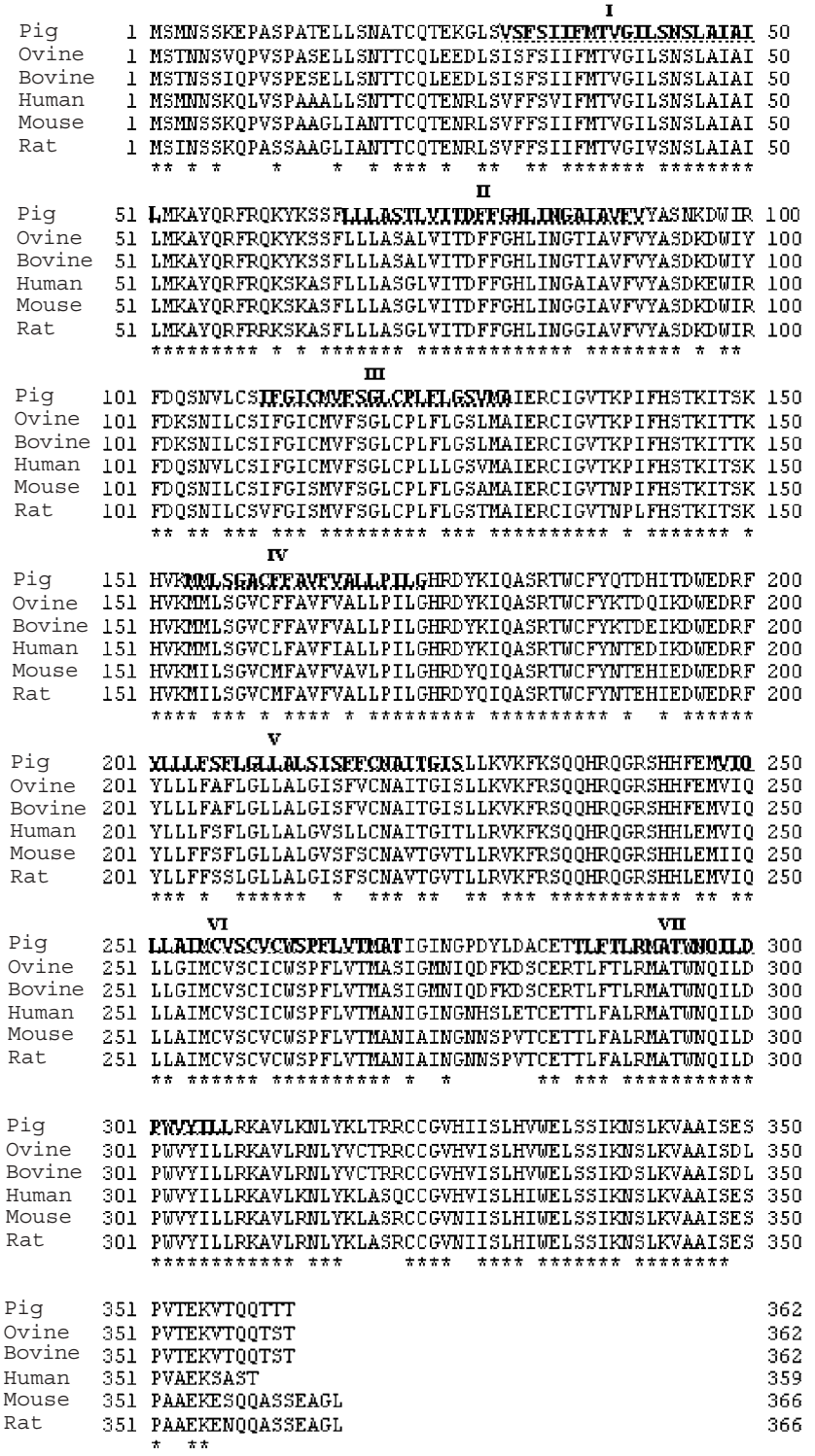

Fig. 1. Comparison of the predicted amino acid sequences for pig $PGF_{2\alpha}$ receptor (FPr) with peptide sequences for known FPr. The sequences were obtained from the GenBank database via www.ncbi.nlm.nih.gov. The accession number for each species was selected as follows: pig AAK95379, ovine Q28905, bovine P37289, human P43088, mouse P43117 and rat P43118. The peptide sequences are aligned to match identical amino acids, which are denoted by asterisks (*). The positions of the putative transmembrane domains I–VII (based on hydropathicity profile) are indicated by bold letters and underlined with dots.

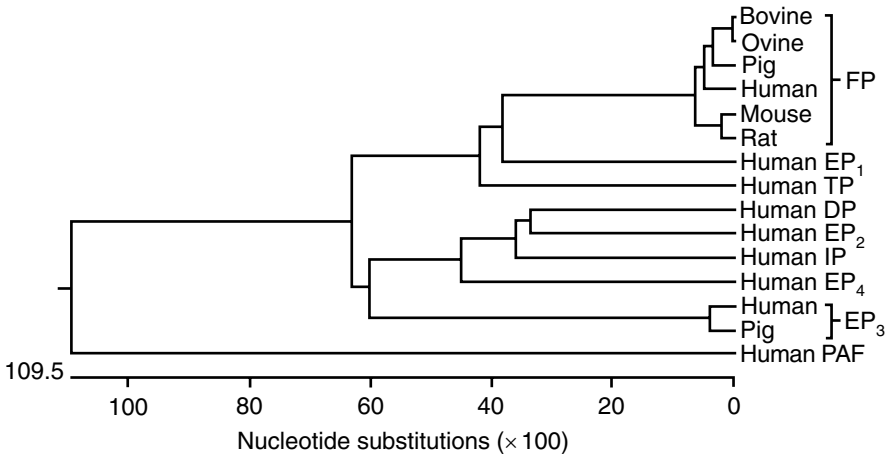

Fig. 2. Phylogeny of selected prostanoid receptor family members using Clustal method in MegAlign (DNASTAR) with per cent accepted mutations 250 residue weighting. The scale denotes substitution distance. The human platelet-activating factor receptors are another rhodopsin family member used as the outgroup for the prostanoid receptors. FP, prostaglandin F receptor; EP, prostaglandin E receptor; TP, thromboxane receptor; DP, prostaglandin D receptor; IP, prostaglandin I receptor; PAF, platelet-activating factor receptor.

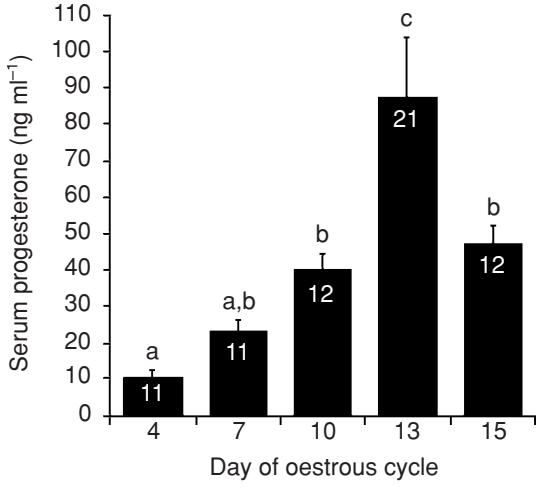

Fig. 3. Serum progesterone concentration (mean ± SEM; $n = 3$ animals day^{-1}) differed between days of the oestrous cycle (values with different letters are significantly different; $^{a,b,c}P < 0.05$). The number in each bar represents the mean number of corpora lutea per animal.

89 and 83% for human (accession no. AF004021); 82 and 79% for mouse (Sugimoto *et al.*, 1994); and 80 and 78% for rat (Olofsson *et al.*, 1996) FP receptor, respectively. A phylogenetic tree was generated by comparison of the deduced amino acid sequence for pig FPr with that for other prostanoid receptors in the GenBank database (Fig. 2). The phylogenetic analysis of the prostanoid receptors assigned the pig FPr in the same cluster with FPr peptides of other species. Pig FPr is most closely related to ruminant FPr protein.

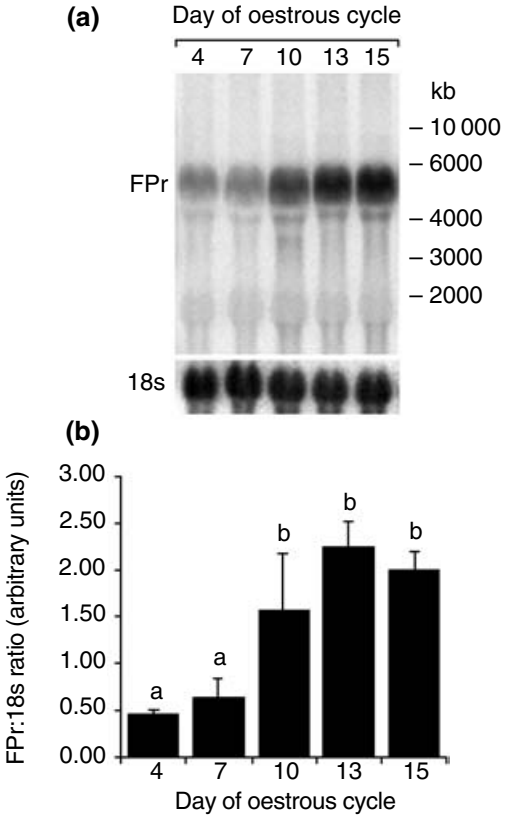

Fig. 4. (a) Northern blot analysis for luteal FP receptor (FPr). Only one positive band was detected, which was approximately 5 kb. (b) The blots were re-probed for 18s rRNA as a control and the relative amount of FPr mRNA to 18s rRNA (arbitrary units ± SEM; $n = 3$ animals day^{-1}) in pig corpora lutea differed between days of the oestrous cycle (values with different letters are significantly different; $^{a,b}P < 0.05$).

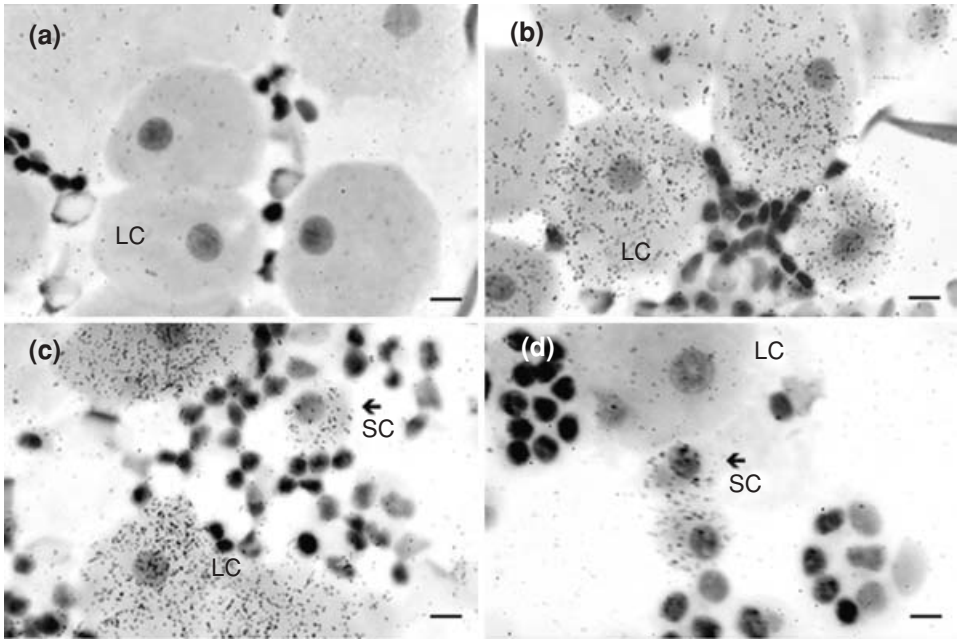

Fig. 5. *In situ* hybridization of FP receptor (FPr) mRNA in pig luteal cells (cytospin slides) obtained from day 13 corpora lutea. *In situ* hybridization for mRNA encoding FPr with the (a) sense probe and (b,c) antisense probe. (d) *In situ* hybridization for mRNA encoding luteinizing hormone receptor (LHr) with antisense probe. Hybridization signals for mRNA encoding FPr seen as silver grains (black dots) are intense in (b,c) large cells and in (c) some small cells (arrow). Hybridization signals for mRNA encoding LHr (d) are present in small cells (arrows) that are morphologically similar to cells observed for mRNA encoding FPr (c). Slides were stained with haematoxylin and eosin. LC, large cells; SC, small cells. Scale bars represent 10 μm.

Progesterone radioimmunoassay

Serum progesterone concentrations obtained from all animals before the administration of PG600® were below 0.2 ng ml^{-1} (data not shown). Concentrations of serum progesterone during the oestrous cycle (Fig. 3) were used to assess the functional status of the corpora lutea and to monitor the stage of oestrous cycle in animals that ovulated. Serum progesterone concentration increased ($P < 0.05$) with age of the corpora lutea and reached a maximum on day 13. Progesterone concentrations were similar on days 7, 10 and 15.

Northern blot analysis

Northern blot analysis of mRNA extracted from pig luteal tissues collected throughout the cycle showed a strong positive band of about 5.0 kb (Fig. 4a). Expression of pig FPr transcript in the corpora lutea was found throughout the cycle, but expression was greater ($P < 0.05$) at days 10, 13 and 15 than at days 4 and 7 (Fig. 4b).

In situ *hybridization*

High-resolution examination of the cytospin slides by silver emulsion autoradiography indicated that the FPr mRNA localized primarily to large luteal cells (Fig. 5b,c). The hybridization signals were also observed in small luteal cells (Fig. 5c). When the same technique was used for LHr, the small luteal cells that showed the hybridization signals (Fig. 5d) were morphologically similar to those for FPr.

Hybridization signals of FPr were localized primarily in the luteal tissue at all stages examined, that is day 4 (Fig. 6d) and days 4–15 (Fig. 7a–j). When LHr was used as a positive control, the expression of LHr mRNA was observed primarily in the follicular tissues (Fig. 6f).

Discussion

A high nucleotide similarity among previously cloned FPr from other species facilitated the identification and cloning of a full-length cDNA of pig FPr from luteal tissues. Neither the binding of this receptor to its ligand ($PGF_{2\alpha}$) nor its functional activity were tested in the present study; however, the following criteria, established during the molecular characterization, provide strong evidence that the cloned receptor was FPr. First, the pig FPr cDNA coded for a predicted 362 amino acid protein, which has the characteristics of a G-protein-coupled receptor, including the seven potential transmembrane domains as determined by Kyte and Doolittle (1982). The FPr cDNA also shared a high amino acid sequence

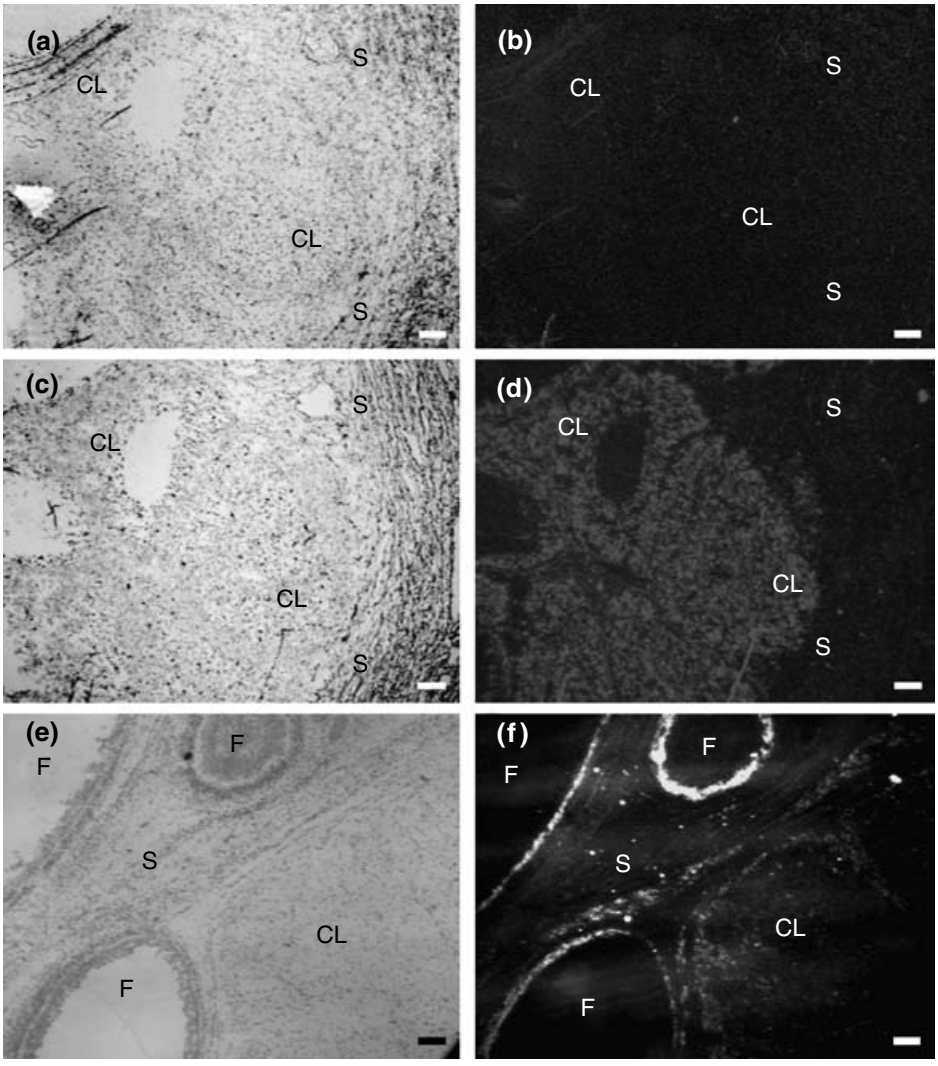

Fig. 6. Sections of pig ovary at day 4 of the oestrous cycle. (a) and (b) are brightfield and darkfield images, respectively, of hybridization in the ovary using a sense FP receptor (FPr) RNA probe (negative control). (c) and (d) are brightfield and darkfield views, respectively, of adjacent sections hybridized with an antisense FPr RNA probe. (e) and (f) are brightfield and darkfield photos, respectively, of sections hybridized with an antisense luteinizing hormone receptor (LHr) RNA probe. High amounts of hybridization signals for FPr mRNA are seen exclusively in the corpora lutea, whereas hybridization signals for LHr mRNA are also seen in the follicular tissues. CL, corpus luteum; F, follicle; S, luteal stroma. Scale bars represent 100 μm.

identity with previously cloned FP receptors. Second, the phylogenetic analysis included the cloned pig FPr in the same FPr cluster as those from other species. Third, northern hybridization and *in situ* hybridization analysis showed the expression and localization of the mRNA for FPr in the corpora lutea, and largely confirm data obtained from radioactive ligand-binding analysis (Gadsby *et al.*, 1990, 1993).

Northern blot analysis in the present study identified the major mRNA transcript for FPr at about 5 kb in pig luteal tissue. A smaller, minor transcript (range from 2 kb to 4 kb) reported in other species (Sugimoto *et al.*, 1994;

Fig. 7. Sections of pig ovary at days (a,b) 4, (c,d) 7, (e,f) 10, (g,h) 13 and (i,j) 15 of the oestrous cycle. Left and right panels are brightfield and darkfield images, respectively, of hybridization in the ovary using an antisense FP receptor (FPr) RNA probe. CL, corpus luteum; F, follicle; S, luteal stroma. Scale bars represent 100 μm.

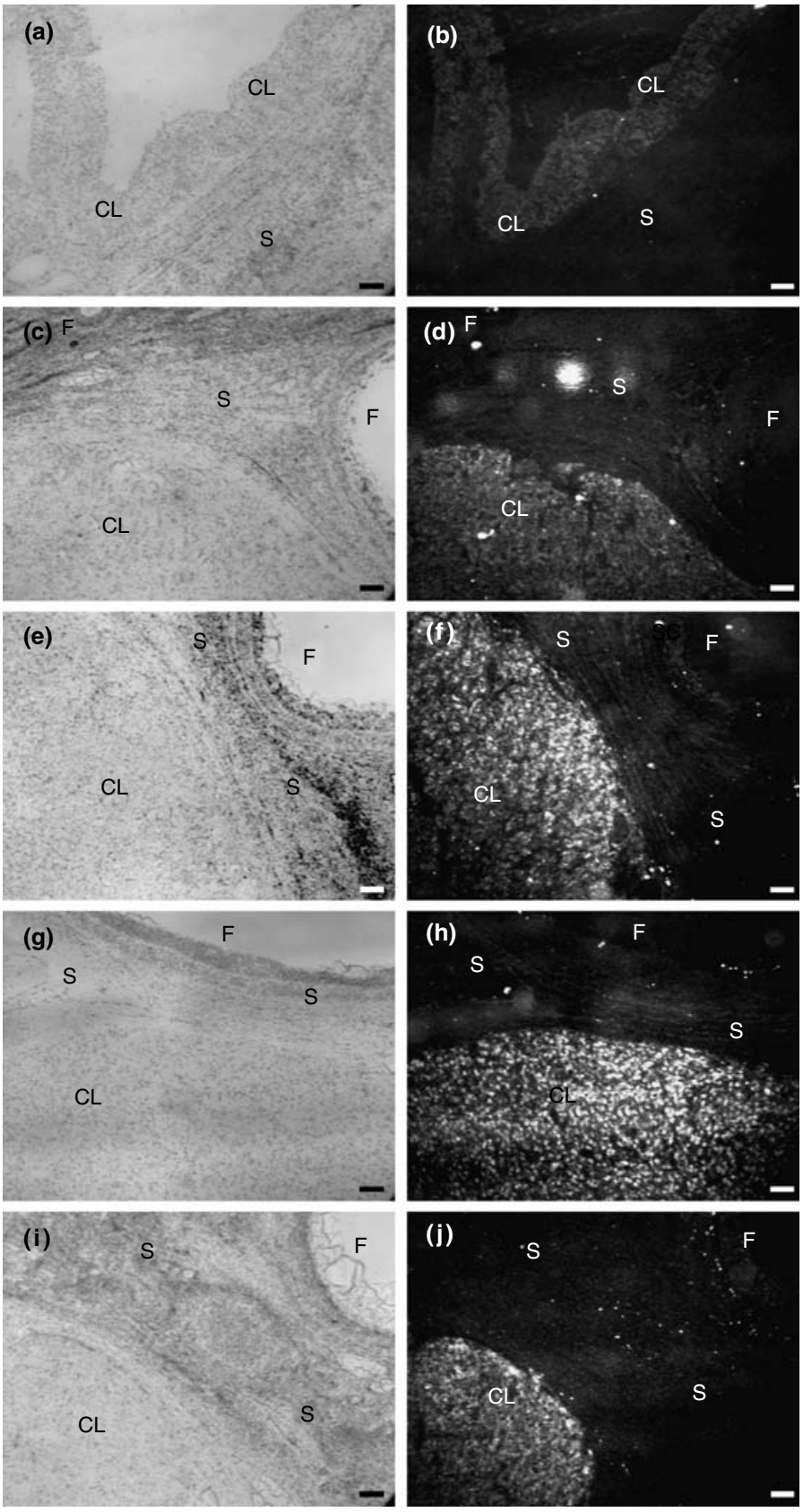

Fig. 7. For legend see facing page.

Graves *et al*., 1995; Juengel *et al*., 1996; Pierce *et al*., 1997) was not found in pig corpora lutea. This additional transcript was observed in enriched (that is, poly A+) mRNA preparations, and it may not be present in high enough concentrations in the total corpora lutea RNA preparations used in the present study for detection on northern blots.

Although the concentrations and localization of FPr on pig luteal cells in cyclic pigs have been reported (Gadsby *et al*., 1990, 1993), the present study illustrates a marked increase in expression of the FPr gene at the transcriptional level on day 10 of the oestrous cycle. Gadsby *et al*. (1990, 1993) detected increased functional FPr protein only by days 13–14. The current observations indicate that pig corpora lutea begin to develop $PGF_{2\alpha}$-responsive pathway components earlier than was previously believed. Despite relatively low amounts of $PGF_{2\alpha}$ receptors (proteins) on day 10 (Gadsby *et al*., 1990), these receptors are capable of mediating a steroidogenic response to physiological doses of $PGF_{2\alpha}$ (ED_{50} value 6.9 ng ml) on this day (Gadsby and Earnest, 1994). In fact, the steroidogenic response noted by Gadsby and Earnest (1994) showed no significant variation in either maximal response or ED_{50} values between day 6 and day 14 of the oestrous cycle, thereby indicating that some FPr mediated pathways are present and functional, irrespective of number of FPr. There is possibly more than one second messenger system, namely a protein kinase C system involved in luteolysis (Diaz *et al*., 2000) and another system, which facilitates a steroidogenic response to $PGF_{2\alpha}$.

In ruminants, high-affinity binding sites for $PGF_{2\alpha}$ and mRNA encoding FPr are localized to large luteal cells (Fitz *et al*., 1982; Balapure *et al*., 1989; Sakamoto *et al*., 1994; Graves *et al*., 1995; Juengel *et al*., 1996). Similarly, the present study shows that in pigs, only large luteal cells display a binding component showing the characteristics of FPr (high affinity, low capacity). In contrast, small cells bind $PGF_{2\alpha}$ with low affinity and high capacity (Gadsby *et al*., 1990). In the current study, FPr mRNA was localized in both large and small luteal cells, indicating that small cells express FPr mRNA, although as described, they do not appear to express the high-affinity binding form of the protein. This finding indicates that small cells express a variant form of FPr that has reduced $PGF_{2\alpha}$ binding affinity. Although the technique used in the present study cannot determine the precise type of cell in the small luteal cell subpopulation that expresses FPr, the mRNA encoding LHr was localized to a morphologically similar small subtype of luteal cell, which is presumably steroidogenic. The observation that FPr mRNA was expressed in small steroidogenic cells in pig corpora lutea indicates that $PGF_{2\alpha}$ has direct luteolytic effects on this cell subpopulation, as the $PGF_{2\alpha}$ binding site on these cells has lower affinity, as mentioned above. The hybridization signals for FPr in other small non-steroidogenic luteal cell subpopulations (for example, endothelial cells) were similar to the background, indicating that there is little or no FPr mRNA expression in these small types of cell. In contrast to FPr, luteal endothelial cells have been identified as sites of mRNA expression of endothelial cell-specific genes such as *VEGFR-1* and *VEGFR-2*, the receptors for vascular endothelial growth factor (VEGFR) using *in situ* hybridization (Boonyaprakob *et al*., 2000). The finding that mRNA encoding FPr does not localize in small non-steroidogenic luteal cell subpopulations, such as the endothelial cells, indicates that the action of $PGF_{2\alpha}$ on these cells is indirect during luteolysis in pigs.

The expression of mRNA encoding FPr was found throughout the oestrous cycle. Expression of FPr mRNA increased ($P < 0.05$) more than fourfold from day 4 to days 13–15, with the highest expression seen on day 13 corpora lutea. The pattern of mRNA expression was similar to that reported previously for $PGF_{2\alpha}$ binding sites, but the present study found that mRNA expression increased on day 10, whereas at the protein level ($PGF_{2\alpha}$ binding sites), the earliest significant increase was seen on day 13 (Gadsby *et al*., 1990). The results of the present study in pigs are consistent with that reported in cattle (Sakamoto *et al*., 1995) and rabbits (Boiti *et al*., 2001) regarding the increased number of FPr as corpora lutea development progresses during the luteal phase. However, in sheep (Juengel *et al*., 1996) and cattle (Tsai *et al*., 1996) there are few if any significant changes in FPr expression, indicating major species differences in the expression pattern of luteal FPr during the oestrous cycle.

It has been suggested that low concentrations of FPr, *per se*, do not explain the refractoriness of pig corpora lutea to $PGF_{2\alpha}$ during the early phase of the oestrous cycle, as FPr are present at this stage (albeit at low amounts), and appear to be capable of mediating some responses to $PGF_{2\alpha}$, if not complete luteolysis (Diaz *et al*., 2000). Nevertheless, a marked increase in FPr mRNA expression during the mid–late luteal phase (days 10–15) in the present study agrees with the observation of increased binding sites, presumably receptors for $PGF_{2\alpha}$, during this period (Gadsby *et al*., 1990). These events correspond to the increased sensitivity of pig corpora lutea to the luteolytic actions of $PGF_{2\alpha}$. At this time, a single treatment with exogenous $PGF_{2\alpha}$ initiates complete luteolysis (Hallford *et al*., 1975; Guthrie and Polge, 1976; Moeljono *et al*., 1976) and natural luteolysis is initiated with increased pulsatile uterine $PGF_{2\alpha}$ secretion (Bazer *et al*., 1984). On the basis of the ability of peripheral $PGF_{2\alpha}$ to stimulate intraluteal production of $PGF_{2\alpha}$ (Diaz *et al*., 2000), it is plausible that profound upregulation of FPr is critical for the rapid autoamplification of luteal $PGF_{2\alpha}$ and for the acquisition of luteolytic capacity.

In rabbit corpora lutea, it is believed that during the early luteal phase a 'luteotrophic pathway' involving PGE_2 and stimulation of adenylate cyclase and protein kinase-A is favoured, which in turn acts to reduce the

sensitivity of the corpora lutea to the luteolytic effects of $PGF_{2\alpha}$ (Boiti *et al*., 2001). Only during the late luteal phase when the $PGF_{2\alpha}$ pathway predominates do corpora lutea become sensitive to $PGF_{2\alpha}$ (Boiti *et al*., 2001). Similarly, in pigs, PGE_2 receptors in luteal tissue decrease between day 10 and day 14 (Feng and Almond, 1999) while during the same time frame, $PGF_{2\alpha}$ receptors increase significantly (Gadsby *et al*., 1990). In addition, $PGF_{2\alpha}$ and PGE_2 receptors are co-localized in large luteal cells (Gadsby *et al*., 1990; Feng and Almond, 1999), indicating that as for rabbits, these two different receptor-mediated signalling pathways counterbalance each other and the expression of luteolytic capacity may depend on the relative amounts of ligand, receptor and transduction pathways for the two prostanoids.

In summary, an FPr from pig corpora lutea was cloned and sequenced. The FPr was highly expressed in large luteal cells and small subtypes of luteal cell expressed LHr, indicating that the large luteal cells are the primary targets for $PGF_{2\alpha}$ action. The mRNA for FPr in corpora lutea is transcriptionally upregulated on days 10–15 of the oestrous cycle. These results indicate a role for increased FPr in the acquisition of luteolytic capacity in pigs.

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References

Acritopoulou S and Haresign W (1980) Response of ewes to a single injection of an analogue of $PGF_{2\alpha}$ given at different stages of the oestrous cycle *Journal of Reproduction and Fertility* **58** 219–221

Anderson LE, Wu YL, Tsai SJ and Wiltbank MC (2001) Prostaglandin $F_{2\alpha}$ receptor in the corpus luteum: recent information on the gene, messenger ribonucleic acid and protein *Biology of Reproduction* **64** 1041–1047

Balapure AK, Caicedo IC, Kawada K, Watt DS, Rexroad CE, Jr and Fitz TA (1989) Multiple classes of prostaglandin F_2 alpha binding sites in subpopulations of ovine luteal cells *Biology of Reproduction* **41** 385–392

Bazer FW, Marengo SR, Geisert RD and Thatcher WW (1984) Exocrine versus endocrine secretion of prostaglandin $F_{2\alpha}$ in the control of pregnancy in swine *Animal Reproduction Science* **7** 115–132

Beal WE, Milvae RA and Hansel W (1980) Oestrous cycle length and plasma progesterone concentrations following administration of prostaglandin $F_{2\alpha}$ early in the bovine oestrous cycle *Journal of Reproduction and Fertility* **59** 393–396

Boiti C, Zampini D, Zerani M, Guelfi G and Gobbetti A (2001) Prostaglandin receptors and role of G protein-activated pathways on corpora lutea of pseudopregnant rabbit *in vitro*. *Journal of Endocrinology* **168** 141–151

Bonnin P, Huynh L, L'Haridon R, Chene N and Martal J (1999) Transport of uterine $PGF_{2\alpha}$ to the ovaries by systemic circulation and local lymphovenous-arterial diffusion during luteolysis in sheep *Journal of Reproduction and Fertility* **116** 199–210

Boonyaprakob U, Ge Z, Hedgpeth V, Routh P, Gadsby JE and Almond GW (2000) *In situ* localization of VEGF mRNA during luteal formation and regression in the pig *Biology of Reproduction* **62** *Supplement* **1** 270

Chomczynski P and Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction *Analytical Biochemistry* **162** 156–159

Diaz FJ, Crenshaw TD and Wiltbank MC (2000) Prostaglandin $F_{2\alpha}$ induces distinct physiological responses in porcine corpora lutea after acquisition of luteolytic capacity *Biology of Reproduction* **63** 1504–1512

Ding ST, MacNeel RL and Mersmann HJ (1999) Expression of porcine adipocyte transcripts: tissue distribution and differentiation *in vitro* and *in vivo*. *Comparative Biochemistry and Physiology* **123B** 307–318

Estill CT, Britt JH and Gadsby JE (1995) Does increased $PGF_{2\alpha}$ receptor concentration mediate $PGF_{2\alpha}$-induced luteolysis during early diestrus in the pig? *Prostaglandins* **49** 255–267

Feng SM and Almond GW (1996) Identification and distribution of prostaglandin E receptors on porcine luteal cells *Biology of Reproduction* **54** 1366–1374

Feng SM and Almond GW (1999) PGE receptor characteristics on porcine luteal cells during the estrous cycle and early pregnancy *Prostaglandins and Other Lipid Mediators* **57** 13–22

Fitz TA, Mayan MH, Sawyer HR and Niswender GD (1982) Characterization of two steroidogenic cell types in the ovine corpus luteum *Biology of Reproduction* **27** 703–711

Gadsby JE and Earnest KL (1994) Prostaglandin $F_{2\alpha}$ stimulates progesterone secretion by porcine luteal cells *in vitro* throughout the oestrous cycle *Prostaglandins* **48** 109–125

Gadsby JE, Balapure AK, Britt JH and Fitz TA (1990) Prostaglandin $F_{2\alpha}$ receptors on enzyme-dissociated pig luteal cells throughout the estrous cycle *Endocrinology* **126** 787–795

Gadsby JE, Lovdal JA, Britt JH and Fitz TA (1993) Prostaglandin $F_{2\alpha}$ receptor concentrations in corpora lutea of cycling, pregnant, and pseudopregnant pigs *Biology of Reproduction* **49** 604–608

Graves PE, Pierce KL, Bailey TJ, Rueda BR, Gil DW, Woodward DF, Yool AJ, Hoyer PB and Regan JW (1995) Cloning of a receptor for prostaglandin $F_{2\alpha}$ from the ovine corpus luteum *Endocrinology* **136** 3430–3436

Guthrie HD and Polge C (1976) Luteal function and oestrus in gilts treated with a synthetic analogue of prostaglandin $F_{2\alpha}$ (ICI 79,939) at various times during the oestrous cycle *Journal of Reproduction and Fertility* **48** 423–425

Hallford DM, Wettemann RP, Turman EJ and Omtvedt IT (1975) Luteal function in gilts after prostaglandin $F_{2\alpha}$ *Journal of Animal Science* **41** 1706–1710

Juengel JL, Wiltbank MC, Meberg BM and Niswender GD (1996) Regulation of steady-state concentrations of messenger ribonucleic acid encoding prostaglandin $F_{2\alpha}$ receptor in ovine corpus luteum *Biology of Reproduction* **54** 1096–1102

Kyte J and Doolittle RF (1982) A simple method for displaying the hydropathic character of a protein *Journal of Molecular Biology* **157** 105–132

Loosfelt H, Misrahi M, Atger M *et al.* (1998) Characterization of messenger ribonucleic acid expression for prostaglandin $F_{2\alpha}$ and luteinizing hormone receptors in various bovine luteal cell types *Biology of Reproduction* **58** 849–856

Mantel A, Sar S, Jallal B, Garnier J and Milgrom E (1989) Cloning and sequencing of porcine LH-hCG receptor cDNA: variants lacking transmembrane domain *Science* **245** 525–528

Moeljono MP, Bazer FW and Thatcher WW (1976) A study of prostaglandin $F_{2\alpha}$ as the luteolysin in swine. I. Effect of prostaglandin $F_{2\alpha}$ in hysterectomized gilts *Prostaglandins* **11** 737–743

Nicholson WC, Ge Z, Plotner DM, Farin CE and Gadsby JE (1999) Insulin-like growth factor (IGF)-I, IGF-I receptor, and IGF binding protein-3 messenger ribonucleic acids and protein in corpora lutea from prostaglandin $F_{2\alpha}$-treated gilts *Biology of Reproduction* **61** 1527–1534

Niswender GD, Juengel JL, Silva PJ, Rollyson MK and MacIntush EW (2000) Mechanisms controlling the function and life span of the corpus luteum *Physiological Review* **80** 1–29

Olofsson JI, Leung CH, Bjurulf E, Ohno T, Selstam G, Peng C and Leung PC (1996) Characterization and regulation of a mRNA encoding the prostaglandin $F_{2\alpha}$ receptor in the rat ovary *Molecular Cellular Endocrinology* **123** 45–52

Pierce KL, Bailey TJ, Hoyer PB, Gil DW, Woodward DF and Regan JW (1997) Cloning of a carboxyl-terminal isoform of the prostanoid FP receptor *Journal of Biological Chemistry* **272** 883–887

Sakamoto K, Ezashi T, Miwa K, Okuda-Ashitaka E, Houtani T, Sugimoto T, Ito S and Hayaishi O (1994) Molecular cloning and expression of a cDNA of the bovine prostaglandin $F_{2\alpha}$ receptor *Journal of Biological Chemistry* **269** 3881–3886

Sakamoto K, Miwa K, Ezashi T, Okuda-Ashitaka E, Okuda K, Houtani T, Sugimoto T, Ito S and Hayaishi O (1995) Expression of mRNA encoding the prostaglandin $F_{2\alpha}$ receptor in bovine corpora lutea throughout the oestrous cycle and pregnancy *Journal of Reproduction and Fertility* **103** 99–105

Sanger F, Nicklen S and Coulson AR (1977) DNA sequencing with chain-terminating inhibitors *Proceedings National Academy of Sciences USA* **74** 5463–5467

SAS (1988) *SAS User's Guide: Statistics* Statistical Analysis System Institute Inc., Cary, NC

Steel RGD, Torrie JH and Dickey DA (1997) *Principles and Procedures of Statistics: A Biometrical Approach* 3rd Edn. McGraw-Hill Companies Inc., New York, NY

Sugimoto Y, Hasumoto K, Namba T, Irie A, Katsuyama M, Negishi M, Kakizuka A, Narumiya S and Ichikawa A (1994) Cloning and expression of a cDNA for mouse prostaglandin $F_{2\alpha}$ receptor *Journal of Biological Chemistry* **269** 1356–1360

Toh H, Ichikawa A and Narumiya S (1995) Molecular evolution of receptors for eicosanoids *FEBS Letters* **361** 17–21

Tsai SJ and Wiltbank MC (1997) Prostaglandin $F_{2\alpha}$ induces expression of prostaglandin G/H synthase-2 in the ovine corpus luteum: a potential positive feedback loop during luteolysis *Biology of Reproduction* **57** 1016–1022

Tsai SJ and Wiltbank MC (1998) Prostaglandin $F_{2\alpha}$ regulates distinct physiological changes in early- and mid-cycle bovine corpora lutea *Biology of Reproduction* **58** 346–352

Tsai SJ, Wiltbank MC and Bodensteiner KJ (1996) Distinct mechanisms regulate induction of messenger ribonucleic acid for prostaglandin (PG) G/H synthase-2, PGE (EP3) receptor, and PGF_2 alpha receptor in bovine preovulatory follicles *Endocrinology* **137** 3348–3355

Wandji SA, Wood TL, Crawford J, Levison SW and Hammond JM (1998) Expression of mouse ovarian insulin-like growth factor system components during follicular development and atresia *Endocrinology* **139** 5205–5214

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