Estrogen promotes luteolysis by redistributing prostaglandin F2 α receptors within primate luteal cells

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Abstract

Prostaglandin F2 α (PGF2 α) has been proposed as a functional luteolysin in primates. However, administration of PGF2 α or prostaglandin synthesis inhibitors *in vivo* both initiate luteolysis. These contradictory findings may reflect changes in PGF2 α receptors (PTGFRs) or responsiveness to PGF2 α at a critical point during the life span of the corpus luteum. The current study addressed this question using ovarian cells and tissues from female cynomolgus monkeys and luteinizing granulosa cells from healthy women undergoing follicle aspiration. PTGFRs were present in the cytoplasm of monkey granulosa cells, while PTGFRs were localized in the perinuclear region of large, granulosa-derived monkey luteal cells by mid-late luteal phase. A PTGFR agonist decreased progesterone production in luteal cells obtained at mid-late and late luteal phases, but did not decrease progesterone production by granulosa cells or luteal cells from younger corpora lutea. These findings are consistent with a role for perinuclear PTGFRs in functional luteolysis. This concept was explored using human luteinizing granulosa cells maintained *in vitro* as a model for luteal cell differentiation. In these cells, PTGFRs relocated from the cytoplasm to the perinuclear area in an estrogen- and estrogen receptor-dependent manner. Similar to our findings with monkey luteal cells, human luteinizing granulosa cells with perinuclear PTGFRs responded to a PTGFR agonist with decreased progesterone production. These data support the concept that PTGFR stimulation promotes functional luteolysis only when PTGFRs are located in the perinuclear region. Estrogen receptor-mediated relocation of PTGFRs within luteal cells may be a necessary step in the initiation of luteolysis in primates.

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Introduction

Prostaglandin F2 α (PGF2 α) is widely recognized as a key luteolysin in mammals, including humans and nonhuman primates. In domestic animals, PGF2 α produced by the uterus in the absence of a conceptus is transported to the ovary via a portal or lymphatic circulation, and this PGF2 α initiates luteolysis (reviewed in Niswender & Nett (1994)). A different mechanism appears to control prostaglandin-induced luteolysis in primates as hysterectomy does not alter the length of the luteal phase. For primates, the corpus luteum itself has been proposed as the source of luteolytic PGF2 α (Auletta & Flint 1988). Indeed, treatment of mature granulosa-lutein cells *in vitro* with PGF2 α decreases progesterone production, the classic definition of functional luteolysis (Stouffer *et al.* 1979).

While it has been suggested that PGF2 α is luteolytic, other prostaglandins, most notably PGE2, are possibly luteotropic in primates (reviewed in Stouffer (1991)). Injection of PGF2 α directly into the corpus luteum in women decreased serum progesterone and shortened

luteal phase length (Bennegard *et al.* 1991). Similarly, infusion of PGF2 α directly into the monkey corpus luteum caused a premature decline in progesterone production, while co-infusion of PGF2 α with PGE2 yielded a luteal phase of normal length (Zelinski-Wooten & Stouffer 1990, Auletta *et al.* 1995). These findings are consistent with the concept that actions of PGF2 α are luteolytic, while PGE2 and perhaps other prostaglandins are luteotropic. However, infusion of potentially luteotropic prostaglandins alone did not lengthen luteal life span (Zelinski-Wooten & Stouffer 1990). In these studies, concentrations of luteotropic and luteolytic prostaglandins within luteal tissues did not correlate directly with either maintenance of luteal function or luteolysis. Collectively, these studies do not support the hypothesis that levels of prostaglandins within luteal tissues are primarily responsible for initiation of luteolysis in primates.

Interpretation of these and other studies is complicated by the temporal pattern of PGF2 α receptor (PTGFR) expression in the primate ovary. *PTGFR* mRNA is expressed in both ovulatory follicles and

corpora lutea of monkeys and women (Carrasco *et al.* 1997, Ristimäki *et al.* 1997, Ottander *et al.* 1999, Bogan *et al.* 2008a, Xu *et al.* 2011). PTGFR mRNA and protein are present in the primate corpus luteum throughout its life span, with peak levels measured in late luteal phase (Ottander *et al.* 1999, Bogan *et al.* 2008a,b). PGF2 α levels in follicular fluid and luteal tissue extracts are in the nanomolar to micromolar range (Patwardhan & Lanthier 1981, Lumsden *et al.* 1986, Auletta *et al.* 1995, Ottander *et al.* 1999, Dozier *et al.* 2008), therefore PTGFRs are likely exposed to a receptor-saturating concentration of PGF2 α throughout the ovulatory period and during the entire luteal life span. Importantly, there are no reports of increased luteal levels of PTGFR or PGF2 α specifically at the time that luteolysis is initiated. It has been suggested that changing PTGFR functionality may explain the acquisition of luteolytic responsiveness to PGF2 α (Ottander *et al.* 1999, Tsai *et al.* 2001), but this concept has not been tested.

To test the hypothesis that PTGFR function changes within primate granulosa-lutein cells in order to initiate luteolysis, we examined expression and function of PTGFR in monkey granulosa cells obtained during the ovulatory interval as well as in cells from monkey corpora lutea obtained during the luteal phase. The transition from the granulosa cell phenotype to the granulosa-lutein (luteal) cell phenotype is difficult to assess *in vivo*. For this reason, additional studies were carried out in human luteinizing granulosa cells maintained *in vitro*, using an established cell culture model which promotes the transition from the granulosa cell to the granulosa-lutein (luteal) cell phenotype (Carrasco *et al.* 1997, Ristimäki *et al.* 1997, Chin *et al.* 2004). Using these complementary approaches, we show for the first time that PTGFRs relocate from the cytoplasm/plasma membrane to the perinuclear/nuclear region of granulosa-lutein cells as these cells acquire sensitivity to PGF2 α . Movement of PTGFRs to the perinuclear region is dependent on estrogen, providing a mechanism to explain how the primate corpus luteum may acquire responsiveness to PGF2 α and luteolytic capacity.

Materials and methods

Animals

Granulosa cells, corpora lutea, and whole ovaries were obtained from adult female cynomolgus macaques (*Macaca fascicularis*) at Eastern Virginia Medical School (EVMS). All animal protocols and experiments were approved by the EVMS Animal Care and Use Committee and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animal husbandry and sample collection were performed as described previously (Seachord *et al.* 2005). Briefly, blood samples were obtained under ketamine chemical restraint by femoral venipuncture, and serum was stored at -20°C . Aseptic surgeries were performed in a dedicated surgical suite under isoflurane

anesthesia, and appropriate post-operative pain control was used.

A controlled ovarian stimulation model developed for the collection of multiple oocytes for IVF was used to obtain monkey granulosa cells (Chaffin *et al.* 1999a). Beginning within 3 days of initiation of menstruation, recombinant human (r-h) FSH (90 IU daily, Merck & Co., Inc.) was administered for 6–8 days, followed by daily administration of 90 IU r-hFSH plus 60 IU r-hLH (Serono Reproductive Biology Institute) for 2 days to stimulate the growth of multiple preovulatory follicles. A gonadotropin-releasing hormone (GNRH) antagonist (Antide, 0.5 mg/kg body weight; Serono or Ganirelix, 30 $\mu\text{g/kg}$ body weight; Merck) was also administered daily to prevent an endogenous ovulatory luteinizing hormone (LH) surge. Adequate follicular development was monitored by serum estradiol levels and ultrasonography (Wolf *et al.* 1996). Follicular aspiration was performed during aseptic surgery before (0 h) or up to 36 h after administration of 1000 IU r-hCG (Serono) (Chaffin *et al.* 1999a). At aspiration, each follicle was pierced with a 22-gauge needle, and the contents of all follicles larger than 4 mm in diameter were pooled. Ovulatory follicles in cynomolgus macaques are typically 4–6 mm in diameter as assessed by ultrasonography and confirmed by direct measurement at surgery. Whole ovaries were also obtained from monkeys experiencing ovarian stimulation. These ovaries were bisected, maintaining at least two follicles >4 mm in diameter on each piece. Ovarian pieces were frozen in O.C.T. Compound (Sakura, Tokyo, Japan) and stored at -80°C until sectioned. The sections were fixed in 10% formalin and immunostained.

Corpora lutea were obtained from monkeys experiencing natural menstrual cycles. Serum samples obtained once daily beginning on days 6–9 after menstruation were assayed for estradiol and progesterone. Day 1 of the luteal phase is defined as the 1st day of low serum estradiol following the mid-cycle estradiol peak; serum progesterone is elevated above 1 ng/ml by luteal day 2. The corpus luteum was removed from the ovary during aseptic surgery in the early (days 3–4), mid (days 6–8), mid-late (days 10–11), and late (days 12–15) luteal phases (Duffy & Stouffer 1995). Luteal tissues were either dispersed for culture of luteal cells, frozen without O.C.T. in liquid nitrogen and stored at -80°C until used for preparation of total RNA or tissue lysates, or frozen in O.C.T. as described previously for ovarian tissues, and used for histologic sections.

Monkey granulosa and luteal cells

Monkey granulosa cells and oocytes were pelleted from the follicular aspirates by centrifugation at 300 g. Following oocyte removal, a granulosa cell-enriched population of cells was obtained by Percoll gradient centrifugation (Chaffin *et al.* 1999b); viability was assessed by trypan blue exclusion, and averaged 85%. Granulosa cells were either used for cell culture or frozen in liquid nitrogen and stored at -80°C .

Corpora lutea were dispersed into individual luteal cells as described previously (Sanders *et al.* 1996). Briefly, luteal tissue was minced, and pieces were incubated in Ham's F-10 media containing 1% BSA, 0.16% collagenase, and 0.02% DNase I in an atmosphere of 95% O₂:5% CO₂ at 37 $^{\circ}\text{C}$. Dispersed mixed

luteal cells were held on ice in an atmosphere of 95% O₂:5% CO₂ until placed in culture. Viability of mixed luteal cell preparations was assessed by trypan blue exclusion and averaged 96%.

Granulosa and luteal cells were plated on LabTek glass chamber slides (Nalgene Nunc, Rochester, NY, USA) or culture plates. The cells were cultured on fibronectin-coated surfaces in chemically defined, serum-free DMEM-Ham's F12 medium containing insulin, transferrin, selenium, aprotinin, and human LDL as described previously (Markosyan *et al.* 2006). The PTGFR agonist fluprostenol (1 μ M; Cayman Chemical, Ann Arbor, MI, USA; hCG, 20 IU/ml; Sigma), the general cyclooxygenase inhibitor indomethacin (0.1 μ M; Sigma), the phospholipase C (PLC) inhibitor U73122 (100 μ M; Cayman), the protein kinase C (PKC) inhibitor Ro31-3220 (1 μ M; CalBiochem, Billerica, MA, USA), the mammalian target of rapamycin (mTOR) inhibitor rapamycin (1 μ M; Invitrogen), the 3 β -hydroxysteroid dehydrogenase inhibitor trilostane (250 ng/ml, Stegram Pharmaceuticals, Sussex, UK), progesterone (0.1 μ M; Sigma), the aromatase inhibitor letrozole (4,4-(1,2,3-triazol-1-yl-methylene)-bis-benzonitrile; 0.5 μ M; Novartis Pharm AG), estradiol benzoate (0.1 μ M; Sigma), and the estrogen receptor antagonist ICI 182 780 (1 μ M; Tocris Biosciences, Bristol, UK) were added to cultures as indicated. Spent culture media were stored at -20 °C pending analysis. Preparation of cells for analysis of mRNA or protein is described below. The cells cultured on chamber slides were fixed in 10% formalin for 30 min, and then stored at 4 °C in PBS until used for immunodetection of PTGFR.

Human luteinizing granulosa cells

Luteinizing granulosa cells were obtained from healthy young women undergoing ovarian stimulation for oocyte donation at the Jones Institute for Reproductive Medicine at EVMS. The Institutional Review Board at EVMS determined that this use of discarded human granulosa cells does not constitute human subjects research as defined by 45 CFR 46.102(f). Follicular aspirates were collected 34–36 h after administration of an ovulatory dose of hCG, and granulosa cells were transferred to our laboratory after oocyte removal. A granulosa cell-enriched population of cells was obtained by Percoll gradient centrifugation as described previously for monkey granulosa cells.

Human luteinizing granulosa cells were cultured in a manner similar to that described by others as a model of granulosa-lutein cells (Carrasco *et al.* 1997, Ristimäki *et al.* 1997, Chin *et al.* 2004). Briefly, cells were plated on fibronectin-coated culture ware in DMEM/F12 medium with supplements as described previously for monkey granulosa cells with the addition of 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA, USA) and cultured for up to 10 days. The cells assessed on day 0 (day of plating) were allowed to attach to cell culture ware for 1 h before study. At initiation of *in vitro* treatments, medium was removed and replaced with serum-free DMEM/F12 including all other supplements as described previously for monkey cells. The cells and media were harvested and stored as described previously for monkey granulosa cells.

Quantitative RT-PCR

The levels of mRNA for *PTGFR* were assessed by qPCR using a Roche LightCycler (Roche Diagnostics). Total RNA was obtained from granulosa cells using Trizol reagent, treated with DNase, and reverse transcribed as described previously (Duffy *et al.* 2005). PCR was performed by using the FastStart DNA Master SYBR Green I kit (Roche) following the manufacturer's instructions. The primers were designed using LightCycler Probe Design Software (Roche) based on the human or monkey sequences and span an intron to prevent undetected amplification of genomic DNA. Amplification of monkey cDNA included primers for *PTGFR* (forward: CCTGGTAATCACG-GACT; reverse: GCACACACCACTTAACAT; accession# DQ375448) and *ACTB* (β -actin; forward: ATCCGCAAA-GACCTGT; reverse: GTCCGCTAGAAGCAT; accession# AY765990). Amplification of human cDNA included primers for *PTGFR* (forward: GAGGTTGATGTCGAGCA; reverse: TTGTTTCATACGTGTAGC; accession# NM_000959) and *ACTB* (forward: ATCCGCAAAGACCTGT; reverse: GTCCGCTA-GAAGCAT; accession# NM_001101). The PCR products were sequenced (Microchemical Core Facility, San Diego State University, CA, USA) to confirm amplicon identity. At least five log dilutions of the sequenced PCR product were included in each assay and used to generate a standard curve. For each sample, the content of *PTGFR* and *ACTB* mRNA was determined in independent assays. No amplification was observed when cDNA was omitted. All data were expressed as the ratio of mRNA of interest to *ACTB* mRNA for each sample.

Immunofluorescent detection of PTGFRs

After antigen retrieval with 10 mM sodium citrate (pH 6), slides were blocked with 5% nonimmune goat serum (Vector Laboratories, Burlingame, CA, USA) in PBS containing 0.1% Triton; all antibody solutions were made in this blocking buffer. The slides were incubated for 1 h at room temperature either with primary antibody generated against PTGFR (5 μ g/ml; Cayman) or no primary antibody, followed by incubation with Alexa Fluor 488-conjugated anti-rabbit secondary antibody (4 μ g/ml; Molecular Probes, Eugene, OR, USA). The slides were treated with 1% Sudan Black in 70% methanol to reduce autofluorescence and coverslipped in Vectashield medium containing propidium iodide (Vector). In some experiments, the anti-PTGFR primary antibody was pre-incubated with the blocking peptide at a 1:2 ratio before use in the primary antibody incubation step.

Conventional fluorescence images were obtained using an Olympus BX41 fluorescent microscope fitted with a DP70 digital camera and associated software (Olympus). Confocal laser microscopy was performed by using a Zeiss 510 laser scanning confocal microscope with LSM5 Software for image acquisition (Carl Zeiss, Inc., Thornwood, NY, USA) at 488 nm excitation with a 505/550 band pass filter (green channel) and 543 nm excitation with a 560 nm long-pass filter (red channel).

Western blotting for PTGFR

Whole cells and luteal tissues were lysed on ice in PBS containing 0.5% SDS and 0.1% Triton X-100. Cytoplasmic and

nuclear fractions were prepared using the NE-PER Extraction kit (Thermo Scientific, Waltham, MA, USA). Protein concentration of each cell lysate and cell fraction was determined using the BCA method (Sigma). The lysates and fractions (25 µg protein) were mixed with sample buffer (0.03% bromophenol blue, 5% SDS, 20% glycerol, 0.4 M Tris, pH 6.8), heated to 95 °C for 5 min, and loaded onto 7.5% or 10% precast polyacrylamide Tris-HCl gels (Bio-Rad). Proteins were transferred to polyvinylidene fluoride (PDVF) membranes (Hybond-P, Amersham Biosciences) using the Trans Blot SD Semi-Dry Electrophoresis Transfer Cell (Bio-Rad). The membranes were blocked in 5% nonfat dry milk, 0.1% Tween-20 in PBS. The membranes were incubated for 1 h at room temperature with the PTGFR polyclonal primary antibody (1:500 dilution, 1.0 µg/ml; Cayman) in blocking buffer, followed by exposure to goat anti-rabbit IgG-alkaline phosphatase-conjugated secondary antibody (1:7500 dilution, Applied Biosystems) for 1 h at room temperature. The blots were washed in PBS with 0.3% Tween-20 between antibody incubations and before exposure to chemiluminescence reagents (CDP-Star, Applied Biosystems) for up to 5 min using Fuji medical imaging film (Fuji Photo Film Co., Tokyo, Japan). For each primary antibody, optimal exposure time was determined in preliminary experiments. The blots were then stripped and reprobed using a mouse anti-pan-actin primary MAB (1:1000 dilution, Millipore, Billerica, MA, USA) and an anti-mouse alkaline phosphatase-conjugated secondary antibody (1:10 000 dilution, Applied Biosystems). For examination of nuclear and cytoplasmic fractions, proteins were separated on 10% or 8–16% gradient precast gels (Bio-Rad), blotted and blocked as described previously, and also probed for tubulin (1:5000 dilution, mouse monoclonal, Sigma), sodium/potassium ATPase (Na/K ATPase, 0.1 µg/ml, mouse monoclonal, Millipore), or histone (H3, 1:1000 dilution, mouse monoclonal, Cell Signaling Technology, Danvers, MA, USA). Molecular weights (MW) of bands representing individual proteins were determined by comparison with pre-stained standards (Precision Plus Protein Standard Dual Color, Bio-Rad).

Assay of cAMP and progesterone

Spent culture media was assayed for cAMP by enzyme immunoassay (Cayman) following kit instructions for acetylation of samples; intra-assay and inter-assay coefficients of variation were 11.6 and 11.2% respectively. Additional media samples were assessed for progesterone by enzyme immunoassay following kit instructions (Cayman); intra-assay and inter-assay coefficients of variation were 20.1 and 7.8% respectively. All cAMP and progesterone levels were normalized to media volume of the culture well.

Statistical analysis

All data were assessed for heterogeneity of variance using Bartlett's test; data were log-transformed when Bartlett's test yielded a significance of <0.05. Data were then assessed by either paired *t*-test or ANOVA. When indicated, ANOVA with one repeated measure was used for cell culture experiments to reflect repeated use of cells from an individual woman or monkey in all treatments *in vitro*. For ANOVA, *post hoc* analyses

were performed by Duncan's multiple range test or *t*-test. Statistical analyses were performed by StatPak v4.12 Software (Northwest Analytical, Portland, OR, USA). Data are presented as mean ± S.E.M., and significance was assumed at *P* < 0.05.

Results

PTGFRs in the monkey follicle

PTGFR receptors were expressed in monkey granulosa cells obtained throughout the ovulatory interval. The levels of *PTGFR* mRNA in granulosa cell were low before human chorionic gonadotropin (hCG) (0 h) and increased 12–36 h after administration of an ovulatory dose of hCG (Fig. 1A), with ovulation anticipated 37–40 h after hCG (Weick *et al.* 1973). PTGFR protein was detected by western blot in the lysates of monkey granulosa cells obtained 0 and 36 h after hCG as a single band of 67 MW (Fig. 1B), consistent with previous reports of PTGFR protein in monkey and human tissues (Bogan *et al.* 2008b, Unlugedik *et al.* 2010). Conventional immunofluorescent microscopy showed expression of *PTGFR* observed predominately in the granulosa cells of ovarian tissues obtained throughout the ovulatory interval (Fig. 1C, E, and G) Confocal laser microscopy showed that PTGFR protein was present throughout the granulosa cell and not preferentially in the plasma membrane or the perinuclear area (Fig. 1D, F, and H).

To determine whether PTGFRs in granulosa cells were functional, monkey granulosa cells obtained 0, 24, and 36 h after hCG treatment *in vivo* were treated *in vitro* with the nonmetabolizable PTGFR agonist fluprostenol, the LH-like hormone hCG, or fluprostenol + hCG; media progesterone was assessed. Progesterone levels in culture media were slightly but significantly elevated by exposure to fluprostenol in granulosa cells obtained at 0 h hCG (Fig. 1I). However, fluprostenol did not alter progesterone production by granulosa cells obtained 24 or 36 h after hCG *in vivo*. hCG treatment *in vitro* increased progesterone at all times as expected; the addition of fluprostenol with hCG did not alter progesterone when compared with hCG only. Moreover, fluprostenol did not alter media cAMP (not shown) or intracellular calcium when determined as previously described (Markosyan *et al.* 2006, not shown). Overall, PTGFRs in monkey granulosa cells were not localized to any specific region of the cell, and a selective PTGFR agonist did not alter progesterone production by granulosa cells obtained after the ovulatory stimulus.

PTGFRs in the monkey corpus luteum

PTGFRs were expressed in the monkey corpus luteum throughout the luteal phase. *PTGFR* mRNA levels were lowest early in the luteal phase, higher at mid-luteal phase, and remained elevated thereafter (Fig. 2A). PTGFR protein in luteal tissue lysate was detected as a

Figure 1 PTGFR expression and function in monkey granulosa cells. (A) Levels of *PTGFR* mRNA in monkey granulosa cells obtained before (0) and 12, 24, and 36 h after hCG administration *in vivo* were determined by qPCR and expressed relative to *ACTB* mRNA. Data are expressed as mean ± S.E.M. and were assessed by ANOVA, followed by Duncan's *post hoc* test. Groups with no common superscripts are different, $P < 0.05$; $n = 4$ –5 animals per group. (B) PTGFR protein was detected as a single band of 67 MW in monkey granulosa cells obtained before (0 h) and 36 h after hCG, and monkey corpus luteum (luteal day 10); human luteinizing granulosa cells (lgc) are included for size comparison. Pan-actin detection confirms protein loading; positions of MW size standards are shown on left. (C, D, E, F, G, and H) PTGFR immunodetection (green) in monkey ovarian tissues obtained after controlled ovarian stimulation and before hCG (0 h) (C and D) or 24 h (E and F), and 36 h (G and H) after hCG. In (C), (E), and (G), follicles were imaged by conventional microscopy and are oriented as indicated in (C), with stroma (st) in the lower left, granulosa cells (gc) central, and follicle antrum (an) in upper right. Arrows indicate PTGFR detection throughout granulosa cells at all times examined. (C), (E), and (G) use scale bar in (C) = 25 μ m. (D, F, and H) show PTGFR dispersed throughout granulosa cells as imaged by confocal microscopy and use bar in (D) = 100 μ m. PTGFR detection was reduced when primary antibody was preabsorbed with the peptide used to generate the antibody (insets in (F) and (G)). Nuclei are counterstained red. Images shown are representative of 3–4 monkeys. (I) Granulosa cells obtained at 0, 24, and 36 h were cultured for 16 h with no treatment, fluprostenol (F), hCG, or F + hCG. Progesterone levels (ng/ml media) were determined by EIA. Within each time of hCG exposure, data were assessed by ANOVA with one repeated measure, followed by Duncan's *post hoc* test. Groups with no common superscripts are different, $P < 0.05$; $n = 4$ –5 animals/time point.

single band of 67 MW (Fig. 1B). PTGFR protein was also detected in monkey corpus luteum cells by immunofluorescence and conventional microscopy. Granulosa-derived large luteal cells are easily identified by their characteristic large cytoplasmic area and large, round nuclei (Duffy *et al.* 1994). PTGFR was present throughout the large, granulosa-derived cells of luteal tissues obtained at mid-luteal stage (Fig. 2B, C, and D). PTGFR protein was more concentrated in or near the nuclei of large luteal cells in tissues obtained at mid-late (Fig. 2E, F, and G) and late (Fig. 2H, I, and J) luteal stages. In luteal tissues obtained at the mid-late and late luteal stages, the majority of cells did not colocalize with PTGFR, indicating that other luteal cell types may have little or no PTGFR.

To determine whether PTGFRs regulate luteal progesterone production, dispersed luteal cells from monkey were cultured with fluprostenol or hCG (Fig. 2K). In luteal cells obtained at mid-luteal phase, hCG increased media progesterone, but fluprostenol had no effect on progesterone levels. The luteal cells obtained at mid-late luteal phase showed robust progesterone production in response to hCG. Although fluprostenol alone had no effect on progesterone production, fluprostenol reduced hCG-stimulated progesterone production by luteal cells obtained at mid-late luteal phase. Fluprostenol also decreased hCG-stimulated progesterone production in cells obtained at late luteal phase, although overall levels of progesterone were lower than those measured at mid or mid-late luteal phase. Previous studies suggest that PTGFR agonists may regulate progesterone production by reducing cAMP generation in response to LH receptor stimulation (Channing 1972, Abayasekara *et al.* 1993). However, fluprostenol had no effect on basal or hCG-stimulated cAMP levels (Fig. 2L). Overall, luteal cells responded to PTGFR agonist stimulation to reduce progesterone production when PTGFRs were concentrated in the perinuclear area.

PTGFRs in human luteinizing granulosa cells in vitro

To examine the transition from the granulosa cell to the granulosa-lutein cell (large luteal cell) phenotype, a well-established human cell culture model was used (Carrasco *et al.* 1997, Ristimäki *et al.* 1997, Chin *et al.* 2004). Similar to these previous reports, human luteinizing granulosa cells produced progesterone when cultured in the absence of exogenous gonadotropin support. Media progesterone levels were modest on day 0, peaked on day 2, and declined to low levels on days 6 and 10 of culture (Fig. 3A). Changing progesterone levels *in vitro* parallel the pattern of changes in serum progesterone measured across the luteal phase in monkeys and women (Duffy *et al.* 1994, Groome *et al.* 1996).

PTGFR protein was detected by immunofluorescence and conventional microscopy as being dispersed

Figure 2 PTGFR expression and function in monkey luteal cells. (A) Levels of *PTGFR* mRNA in monkey corpora lutea obtained during the early (days 3–4), mid (days 6–8), mid-late (days 10–11), and late (days 12–15) stages of the luteal phase were determined by qPCR and expressed relative to *ACTB* mRNA. Data are expressed as mean ± S.E.M. and were assessed by ANOVA, followed by Duncan's *post hoc* test. Groups with no common superscripts are different, $P < 0.05$; $n = 3$ –4 animals/group. (B, C, D, E, F, G, H, I, and J) PTGFR immunodetection (green) in monkey luteal tissues obtained at the mid (B, C, and D), mid-late (E, F, and G), and late (H, I, and J) luteal phases. Nuclei are counterstained red. Arrows indicate green fluorescence located in the perinuclear region of large luteal cells. Arrowheads indicate elongated nuclei of nonsteroidogenic cells which do not colocalize with PTGFR. Inset in Panel J shows reduced immunodetection when the PTGFR antibody was preabsorbed with the peptide used to generate the antibody. For (B), (C), (D), (E), (F), (G), (H), (I), and (J), use bar in (G) = 25 μ m; images are representative of 3–4 monkeys/time point. (K and L) Dispersed luteal cells were cultured with no treatment, fluprostenol (F), hCG, or F + hCG for 4 h; media was harvested for assay of progesterone (ng/ml media) and cAMP (pmol/ml media) by EIA. Within each stage of the luteal phase, data were assessed by ANOVA with one repeated measure, followed by Duncan's *post hoc* test. Groups with no common superscripts are different, $P < 0.05$; $n = 4$ –5 animals/luteal stage.

throughout human luteinizing granulosa cells on the day of follicle aspiration (typically 34–36 h after hCG), which is day 0 of culture (Fig. 3B) and comparable with monkey granulosa cells obtained 36 h after hCG (Fig. 1G and H). By day 2 of culture, PTGFR protein was concentrated in

the perinuclear region of the cells (Fig. 3C). On day 6, immunodetection of PTGFR was predominant in the perinuclear region of these human cells (Fig. 3D). Immunodetection of PTGFR was seen throughout the cells on day 10 *in vitro*, with some cells retaining apparent concentration of PTGFR in the perinuclear region (Fig. 3E). PTGFR was detected in a single band in cultured human luteinizing granulosa cells on days 0 and 2 *in vitro* (Fig. 3F). Total cellular levels of PTGFR receptor protein did not vary significantly over the culture period as determined by western blotting, although PTGFR levels were more variable on days 6 and 10 of culture (Fig. 3F and not shown).

Additional studies were performed to determine whether PTGFR was located primarily in cytoplasmic or nuclear fractions of cultured human luteinizing granulosa cells (Fig. 3G). PTGFR was detected in both nuclear and cytoplasmic fractions of cells on day 0 *in vitro*. On day 2, PTGFR was more prominent in the nuclear fraction, with little PTGFR detected in the corresponding cytoplasmic fraction. Successful preparation of nuclear and cytoplasmic fractions was confirmed by detection of tubulin primarily in cytoplasmic fractions and histone H3 primarily in nuclear fractions of cells on days 0 and 2 of culture. The plasma membrane protein Na/K ATPase was detected primarily or exclusively in the cytoplasmic fractions, confirming that nuclear fractions contained little, if any, plasma membrane protein. Overall, these results confirm that PTGFRs were distributed throughout human luteinizing granulosa cells at the time of follicle aspiration, but were located primarily in the perinuclear region after 2 days *in vitro*.

Further experiments focused on human luteinizing granulosa cells cultured for 2 days, when progesterone production peaks in this cell culture model, analogous to monkey luteal cells obtained at mid-late luteal phase (Fig. 2K). To determine whether cultured human luteinizing granulosa cells acquire the ability to respond to PTGFR stimulation as these cells transition from the granulosa cell to the luteal cell phenotype *in vitro*, media progesterone was measured after treatment with fluprostenol or hCG. On day 0 *in vitro*, treatment with fluprostenol did not alter media progesterone levels (Fig. 3H). On day 2 *in vitro*, fluprostenol decreased media progesterone levels (Fig. 3I). hCG increased media progesterone levels at day 2, but not day 0, similar to previous reports (Chin & Abayasekara 2004, Chin *et al.* 2004). Fluprostenol regulation of progesterone could not be attributed to alteration in cAMP levels as fluprostenol did not reduce control or hCG-stimulated cAMP levels after 2 days *in vitro* (Fig. 3J). After 2 days *in vitro*, these cells responded to PTGFR stimulation with reduced progesterone production, consistent with induction of luteolysis.

As members of the seven-transmembrane superfamily of receptors, PTGFR receptors are thought to couple with G proteins to regulate intracellular signaling components

including PLC, PKC, and mTOR (Houmar *et al.* 1992, Abayasekara *et al.* 1993, Carrasco *et al.* 1997, Ristimaki *et al.* 1997, Arvais *et al.* 2006). Human luteinizing granulosa cells were maintained *in vitro* for 2 days, and then treated for 4 h with fluprostenol or fluprostenol in combination with the PLC inhibitor U73122, the PKC inhibitor Ro31-3220, or the mTOR inhibitor rapamycin (Fig. 3K). Fluprostenol treatment reduced progesterone levels when compared with control cultures; this reduction in progesterone was blocked by the PLC inhibitor U73122 and the PKC inhibitor Ro31-3220 but not the mTOR inhibitor rapamycin.

Modulation of PTGFR location in human luteinizing granulosa cells

Additional studies were performed to determine whether the proposed luteolysins estrogen, progesterone, and

PGF2 α modulated PTGFR location within human luteinizing granulosa cells after 2 days *in vitro*. The cells cultured with vehicle only but exposed to endogenously produced estrogen ($\sim 0.1 \mu\text{M}$ (Chang *et al.* 2013); control) showed green immunofluorescence reflecting PTGFR concentrated in the perinuclear region as imaged by confocal microscopy (Fig. 4A, B, and C). To determine whether estrogen receptors were involved in the relocation of PTGFRs, cells were treated with the estrogen receptor antagonist ICI 182 780 to block the action of endogenously produced estrogen. Treatment with ICI 182 780 yielded less apparent PTGFR immunodetection in the perinuclear region (Fig. 4D, E, and F) when compared with control cells. In addition, an ablate-and-replace approach was used to confirm the effect of endogenous estrogen on PTGFR location. Treatment with the aromatase inhibitor letrozole to inhibit estrogen synthesis yielded PTGFR immunodetection throughout the cell, with no apparent concentration in the vicinity of the nucleus (Fig. 4G, H, and I). In contrast, treatment with letrozole+estradiol replacement yielded perinuclear PTGFR localization (Fig. 4J, K, and L).

Figure 3 PTGFR expression and function in human luteinizing granulosa cells differentiating into granulosa-lutein cells *in vitro*. (A) Progesterone ($\mu\text{g/ml}$ media) was assessed in cells after culture for 0, 2, 6, and 10 days. Media were changed on the day of culture indicated, and progesterone accumulation over 4 h was assessed. Data were expressed as mean $\pm$ s.e.m. and assessed by ANOVA with one repeated measure, followed by Duncan's *post hoc* test. Groups with no common superscripts are different, $P < 0.05$; $n = 4-6$ women. (B, C, D, and E) Immunodetection of PTGFR (green) in cells maintained *in vitro* 0 days (A), 2 days (B), 6 days (C), or 10 days (D); cells are not counterstained. Arrowheads indicates cytoplasmic detection of PTGFR; arrows indicate perinuclear PTGFR. Inset in (C) shows reduced immunodetection when the PTGFR antibody was preabsorbed with the peptide used to generate the antibody. For (B, C, D, and E), use bar in (B) = 25 μm . Images shown are representative of 3-4 patients. (F and G) PTGFR (67 MW) detection by western blotting in total human luteinizing granulosa cell lysate (F) and in nuclear (nuc) and cytoplasmic (cyto) fractions of luteinizing granulosa cells (G) on days 0 and 2 of culture (representative of $n = 3-4$ women). Pan-actin detection (37 MW) in (F) was performed on the same blot as PTGFR detection and confirms similar protein loading. Nuclear and cytoplasmic fractions were probed for tubulin (50 MW), Na/K ATPase (112 MW), and H3 (17 MW). (H and I) On day 0 (H) or day 2 (I, J, and K) *in vitro*, fresh media containing fluprostenol (F), hCG, or no treatment (control) were added to each well; media were harvested after 4 h for assay of progesterone by EIA. Progesterone varied widely between women, so control for each woman was set at 1.0, and progesterone levels after hormone treatment are expressed relative to control levels. (J) Media from day 2 cultures were assayed for cAMP by EIA (expressed as pmol cAMP/ml media). (K) On day 2 *in vitro*, fresh media containing fluprostenol (F) alone or in combination with U73122 (F+U7), Ro31-3220 (F+Ro), or rapamycin (F+Rap) were added and collected 4 h later for assay of progesterone. For (H), (I), (J), and (K), data are expressed as mean $\pm$ s.e.m., $n = 4-6$ patients per treatment or time point. Within each panel, data were assessed by ANOVA with one repeated measure, followed by Duncan's *post hoc* test; data with no common superscripts are different, $P < 0.05$.

Figure 4 Estrogen promotes PTGFR localization to the perinuclear region and PTGFR function in cultured human luteinizing granulosa cells. The cells were cultured for 2 days with vehicle (control), ICI 182 780 (ICI), letrozole (Let), or letrozole and estradiol (Let+E₂). (A, B, C, D, E, F, G, H, I, J, K, and L) PTGFR immunodetection (green), nuclear counterstain (red), and merged images are shown. Arrows indicate perinuclear location of PTGFR; arrowheads indicate PTGFR dispersed throughout cells. Images were obtained with confocal microscopy and are representative of $n=3$ women. (M) The cells were cultured for 2 days with vehicle (control), ICI, Let, and Let+E₂ as described previously; media were replaced and cells were treated for 4 h with media containing vehicle or fluprostenol before assay for progesterone by EIA. Progesterone levels after hormone treatment were expressed relative to control levels, which was set at 1.0 for each woman. Fluprostenol reduced media progesterone (asterisks) in control and Let+E₂-treated cells as assessed by paired t -test, $P<0.05$. Data are expressed as mean $\pm$ S.E.M., $n=4$ women.

To determine whether perinuclear location of PTGFR correlated with the ability of PGF2 α to modulate progesterone production, human luteinizing granulosa cells were treated with ICI 182 780, letrozole, or letrozole+estradiol, for 2 days *in vitro*. The media were removed, and cells from each treatment group received fresh media with vehicle or fluprostenol for 4 h before harvest of media for progesterone assay (Fig. 4M). In cells receiving no treatment but exposed to endogenously produced estrogen during 2 days *in vitro* (control), fluprostenol decreased progesterone production, consistent with our findings in Fig. 3I. The cells treated with the estrogen receptor antagonist ICI 182 780 for 2 days *in vitro* did not respond to fluprostenol with altered

progesterone. The cells treated with estrogen synthesis inhibitor letrozole for 2 days *in vitro* also did not respond to fluprostenol with altered progesterone production. In contrast, treatment with letrozole+estradiol replacement for 2 days *in vitro* yielded decreased progesterone in response to fluprostenol treatment. Therefore, only cells exposed to estrogen stimulation during 2 days *in vitro* (i.e., control and letrozole+estradiol-treated cells) responded to fluprostenol with decreased progesterone production.

Progesterone and prostaglandins were not required for the relocation of PTGFRs in human luteinizing granulosa cells. Treatment with either the 3 β -hydroxysteroid dehydrogenase inhibitor trilostane to block progesterone production or trilostane+progesterone replacement for 2 days *in vitro* yielded PTGFRs located in the perinuclear area, similar to control cells ($n=4$; data not shown). PTGFRs were also located in the perinuclear region of cells treated with the prostaglandin synthesis inhibitor indomethacin or indomethacin+fluprostenol for 2 days *in vitro* ($n=3$; data not shown).

Discussion

PGF2 α has been proposed as a key regulator of ovarian function in many species, including monkeys and humans. The observation that follicular PGF2 α increases just before ovulation (Lumsden *et al.* 1986, Murdoch *et al.* 1986, Sirois & Dore 1997, Duffy & Stouffer 2001, Sayasith *et al.* 2006, Dozier *et al.* 2008), coupled with PTGFR expression in follicular granulosa cells (Carrasco *et al.* 1997, Bridges & Fortune 2007, Xu *et al.* 2011), has led to the suggestion that PGF2 α may serve as a local mediator of ovulatory events. While systemic administration of PGF2 α can reverse indomethacin-induced inhibition of ovulation (Wallach *et al.* 1975, Murdoch *et al.* 1986, Sogn *et al.* 1987, Janson *et al.* 1988), disruption of PTGFR expression in mice did not prevent ovulation (Sugimoto *et al.* 1997). To date, functional PTGFRs have not been reported in follicular granulosa cells. Taken together, these findings do not support a role for PGF2 α in ovulatory events.

PGF2 α has also been suggested as a regulator of primate luteolysis. It is well established that PGF2 α of uterine origin acts at the corpus luteum to initiate luteolysis in domestic animal species (Niswender & Nett 1994). A role for PGF2 α to promote luteolysis in primate species is more controversial. Human and monkey luteal tissues synthesize PGF2 α (Auletta *et al.* 1995, Ottander *et al.* 1999) and express PTGFR mRNA and protein (Carrasco *et al.* 1997, Ristimäki *et al.* 1997, Ottander *et al.* 1999, Bogan *et al.* 2008a, Xu *et al.* 2011). Luteal cells express PTGFRs capable of signal transduction in many species (Davis *et al.* 1987, Carrasco *et al.* 1997, Ristimäki *et al.* 1997, Arvisais *et al.* 2006), including monkeys (Houmard *et al.* 1992). Intraluteal or systemic injection of PGF2 α caused a decline in serum

progesterone level and shortened the luteal phase in women and monkeys (Bennegard *et al.* 1991, Auletta *et al.* 1995). Importantly, PGF2 α acts directly at luteal cells to decrease progesterone production. As previously shown (Stouffer *et al.* 1979) and confirmed with more precise timing of tissue collection in this study, PGF2 α decreased hCG-stimulated (but not basal) progesterone production by luteal cells obtained from monkeys at mid-late or late luteal stages, when the corpus luteum is sensitive to luteolytic stimuli *in vivo*. Decreased progesterone production is the hallmark of functional luteolysis, therefore collectively these findings are consistent with the concept that ovarian PGF2 α acts via PTGFRs as a part of the luteolytic process in primates.

Acquisition of luteal cell sensitivity to PTGFR ligands correlates with relocation of the PTGFR from the cytoplasm to the perinuclear area of primate luteal cells. In this study, monkey granulosa cells from ovulatory follicles, monkey luteal cells, and human luteinizing granulosa cells *in vitro* all expressed PTGFR protein. PTGFRs were found distributed throughout monkey granulosa cells. In contrast, PTGFRs were observed primarily in the perinuclear area of mature monkey granulosa-lutein cells. In the human luteinizing granulosa cell model of luteal cell differentiation, PTGFRs were distributed throughout the cells at the start of culture (day 0), with perinuclear PTGFRs observed on day 2 *in vitro*. This relocation of PTGFRs is consistent with observations made in monkey ovarian cells. PTGFRs were distributed throughout human luteinizing granulosa cells on the day of follicle aspiration (about 36 h after the ovulatory gonadotropin stimulus), similar to monkey granulosa cells obtained 36 h after hCG. In contrast, PTGFRs were observed in the perinuclear region of human luteinizing granulosa cells after several days *in vitro*, similar to mature monkey luteal cells. Most importantly, PTGFRs were consistently located in the perinuclear area when cells were sensitive to PTGFR stimulation to reduce progesterone production.

G protein-coupled receptors (GPCRs), including PTGFRs, are often located in the plasma membrane and respond to ligands located outside the cell. Accumulating evidence suggests that functional GPCRs can also be located in the nuclear envelope or perinuclear membranes. While the specific mechanisms which direct nuclear localization of GPCRs remain to be established, structural components of the GPCR referred to as nuclear localization signals or endoplasmic retention sequences have been proposed (Marrache *et al.* 2002, Lee *et al.* 2004). However, such sequences remain to be identified for the majority of GPCRs, including PTGFR. Nuclear membranes do contain components of signal transduction systems for GPCRs, such as G proteins, adenylyl cyclase, PKA, PLC, and PKC; isolated nuclei can respond to ligand binding with generation of intracellular signals (reviewed in Gobell *et al.* (2006)). Our studies in cultured human

luteinizing granulosa cells show that stimulation of perinuclear PTGFRs regulate progesterone production via the PLC/PKC signaling pathway. This is consistent with previous reports in which stimulation of PTGFR by PGF2 α led to increase in the accumulation of inositol trisphosphate (IP3), elevated intracellular calcium, increased PKC activity, and activation of MAP kinases in luteal cells (Davis *et al.* 1987, Abayasekara *et al.* 1993, Chen *et al.* 1998, Tai *et al.* 2001, Hou *et al.* 2008). These observations are consistent with the concept that PTGFRs can activate G protein-coupled signal transduction pathways when located in or near the nucleus.

Despite decades of interest, the key factor which initiates primate luteolysis remains elusive. Several hypotheses have been put forward. Decreasing LH pulse frequency or declining luteal cell sensitivity to LH late in the luteal phase may provide inadequate gonadotropin support for progesterone synthesis (Ellinwood *et al.* 1984, Hutchison *et al.* 1986, Duffy *et al.* 1999a). Alternatively, because the ability of luteal cells to produce progesterone declines as the corpus luteum ages, it has been suggested that reduced substrate availability, decreased activity of steroidogenic enzymes, or declining luteal cell sensitivity to gonadotropin may be the cause of luteolysis (reviewed in Stouffer *et al.* (1996)). Progesterone has also been proposed as a key luteotropin, with declining progesterone itself serving as the initiator of luteolysis (reviewed in Rothchild (1981)). Estrogen has been proposed as a luteolytic signal, but the specific target of estrogen action in experimental models has been disputed (Karsch & Sutton 1976, Hutchison *et al.* 1987). Prostaglandins, and in particular PGF2 α , can modulate progesterone production by luteal cells, but the hypothesis that PGF2 α initiates primate luteolysis *in vivo* remains unproven. This study provides support for a novel hypothesis that unites two of these ideas: estrogen regulates relocation of PTGFRs to the perinuclear area, an event which enhances luteal cell sensitivity to PGF2 α as an early step in luteolysis.

The role of estrogen in luteolysis has been debated for decades. The cells of the corpus luteum express estrogen receptors. ESR1 (ER α), ESR2 (ER β), and the plasma membrane estrogen receptor GPR30 have been reported in luteal tissues of rodents, domestic animals, monkeys, and women, with most studies suggesting that ESR2 predominates (Duffy *et al.* 2000, Hosokawa *et al.* 2001, Diaz & Wiltbank 2004, Shibaya *et al.* 2007, Wang *et al.* 2007, van den Driesche *et al.* 2008, Hazell *et al.* 2009, Maranesi *et al.* 2010). Additional studies have shown that estrogen acts directly at luteal cells to regulate functions as diverse as apoptosis, steroidogenesis, and inhibin production (Diaz & Wiltbank 2004, van den Driesche *et al.* 2008). While expression of enzymes involved in estrogen synthesis increases as luteolysis approaches (Benyo *et al.* 1993, Diaz & Wiltbank 2004), it is widely accepted that tissue estrogen concentrations within the corpus luteum are receptor-saturating

throughout the luteal phase (Duffy *et al.* 1999b). High serum estrogen resulting from the natural preovulatory rise, gonadotropin treatments to stimulate multiple follicles, or systemic administration during the early luteal phase in monkeys and women does not prevent formation of the corpus luteum or promote premature luteolysis (Weick *et al.* 1973, Schoonmaker *et al.* 1981, Hibbert *et al.* 1996, Beckers *et al.* 2006). Exogenous estrogen did elicit premature luteolysis in monkeys when administered at mid/mid-late luteal phase (Schoonmaker *et al.* 1981). In subsequent studies, it was demonstrated that estrogen acts via the hypothalamus to decrease gonadotropin support for the corpus luteum and thereby promote luteolysis (Hutchison *et al.* 1987). However, estrogen implants placed in the corpus luteum itself caused luteolysis, while estrogen delivered systemically or within the contralateral ovary did not, providing strong support for the hypothesis that estrogen can act locally within the corpus luteum to promote luteolysis (Karsch & Sutton 1976). Therefore, while estrogen can shorten luteal life span by inhibiting gonadotropin synthesis/release, well-controlled studies also support a role for estrogen action locally within the corpus luteum to promote luteolysis.

In this study, estrogen promoted movement of PTGFRs to the perinuclear area of luteal cells. In parallel with the estrogen receptor antagonist ICI 182 780 (Howell *et al.* 2000, Thomas *et al.* 2005), use of an ablate-and-replace approach confirmed that PTGFR movement is dependent on both estrogen and estrogen receptors. It is important to note that high estrogen levels within granulosa cells of the follicle *in vivo* did not stimulate PTGFR concentration near the nucleus. Luteal cells may need to mature sufficiently to be receptive to this luteolytic stimulus, and additional experiments would be required to identify the conditions which constitute this receptive environment. One aspect of receptivity may be the ability of estrogen to act via estrogen receptors to stimulate the movement of PTGFRs within granulosa-lutein cells, placing PTGFRs in proximity to the G-protein-coupled apparatus necessary for signal transduction. The findings presented in this report unite two long-standing hypotheses to suggest that estrogen and PGF2 α produced within the corpus luteum are both essential to initiate timely luteolysis during the menstrual cycle in primates.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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