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Loss of luteotropic prostaglandin E plays an important role in the regulation of luteolysis in women

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\textit{Running title}: Loss of PGE is important for luteolysis in women

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Abstract

Study question: Do intraluteal prostaglandins contribute to luteal regulation in women?

Summary answer: Prostaglandin (PG) E, which is produced in human granulosa-lutein cells stimulated with luteotropic human chorionic gonadotropin (hCG), plays luteotropic roles in the human corpus luteum (CL). hCG promotes the synthesis of PGE in cultured luteinized granulosa cells (LGCs), and PGE exerts similar luteotropic effects to hCG, namely, promoting genes to produce progesterone, enhancing PGE synthesis, suppressing PGF synthesis, and down-regulating the gene expression of luteolytic activin A and bone morphogenetic proteins (BMPs). The expression of PG synthetic and metabolic enzymes in the human CL is driven towards less PGE, but more PGF during luteolysis.

What is known already: Uterine PGF is a major luteolysin in many non-primate species but not in women. Increases in the PGF synthase, AKR1C3, have been observed in the CL of marmoset monkeys during luteolysis. PGE prevents spontaneous or induced luteolysis in domestic animals.

Study design, size, duration: Human CL tissues staged as the early-luteal (n=6), mid-luteal (n=6), late-luteal (n=5), and menstrual (n=3) phases were obtained at the time of hysterectomy for benign gynecological conditions. LGCs were purified from follicular fluids obtained from patients undergoing assisted conception.

Participants/materials, setting, methods: Quantitative RT-PCR was employed to examine changes in the transcript abundance of PG synthetic and metabolic enzymes, steroidogenic enzymes, luteolytic molecules in the staged human CL, and cultured human LGCs treated with hCG, PGE, and PGF. A PGE withdrawal experiment was also conducted in order to reveal the effects of the loss of PGE in LGCs. Progesterone concentration in the culture medium were measured.
Main results and the role of chance: The key enzyme for PGE synthesis, PTGES was abundant in the functional CL during the mid-luteal phase, while genes involved in PGF synthesis (AKR1B1 and AKR1C1–3) increased in the CL during the late-luteal phase and menstruation. PTGES expression positively correlated with that of HSD3B1 (r=0.7212, P<0.001), while AKR1C3 expression inversely correlated with that of HSD3B1 (r=−0.7167, P<0.001) and PTGES (r=−0.6505, P<0.01). PGE exerted similar effects to hCG-promoting genes to produce progesterone and luteotropic PGE, suppress PGF synthetic enzymes, and down-regulate luteolytic molecules such as activin A and BMPs. PGE withdrawal resulted in reductions in the enzymes that produce progesterone and PGE, and the capacity to produce PGE decreased, while that to produce PGF increased during the culture. The addition of PGF did not recapitulate the luteolytic effects of PGE withdrawal.

Limitations, reasons for cautions: Changes in PG synthetic and metabolic enzymes may not represent actual increases in PGF during luteolysis in the CL. The effects of PGF on luteal cells currently remain unclear. The mechanisms responsible for decreases in the synthesis of PGE during a culture and luteolysis have not yet been elucidated in detail.

Wider implications of the findings: The loss of PGE is more important than the effects of PGF during luteolysis in women. This may be accompanied by decreases in the effects of luteinizing hormone/hCG in luteal cells, particularly a decrease in the activation of cAMP/PKA; however, the underlying mechanisms currently remain unknown.

Study funding and competing interest(s): This study was supported by the Cunningham Trust to WCD, a Postdoctoral Fellowship for Research Abroad from the Japan Society for the Promotion of Science and the Suntory Foundation for Life Sciences to JN-K. WCD is supported by an MRC Centre Grant G1002033 and a Scottish Senior Clinical Fellowship. The authors have nothing to disclose.
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Key words: corpus luteum / hCG / luteinized granulosa cell / luteolysis / prostaglandin
Introduction

Progesterone is essential for the preparation and maintenance of pregnancy, and produced in the corpus luteum (CL) established from an ovulated dominant follicle in the ovary. The luteinization of granulosa cells and theca interna cells in the ovulated follicle is regulated by luteinizing hormone (LH) secreted from the pituitary gland, and granulosa-lutein and theca-lutein cells produce large amounts of progesterone. The CL undergoes degradation during luteolysis in a non-conception cycle, whereas human chorionic gonadotrophin (hCG) secreted from the developing conceptus rescues the CL from luteolysis, and maintains its function during early pregnancy. LH and hCG bind to the luteinizing hormone/choriogonadotropin receptor (LHCGR) on granulosa-lutein and theca-lutein cells in order to stimulate the expression of the genes responsible for progesterone production, such as the steroidogenic acute regulatory protein (STAR) and 3β-hydroxysteroid dehydrogenase (HSD3B1), by activating the cAMP/PKA pathway (Ascoli et al., 2002). The stimulation of progesterone production through LHCGR by LH or hCG is fundamental to the maintenance of luteal cell integrity and functions. We previously demonstrated that paracrine regulators of CL functions, such as vascular endothelial growth factor, SLIT/ROBO, activin A, and bone morphogenetic proteins (BMPs), are secreted from luteal cells under the control of LH/hCG, and play important roles in luteinization and luteolysis (Dickinson et al., 2008; van den Driesche et al., 2008; Nio-Kobayashi et al., 2015). However, the molecular mechanisms underlying luteolysis and its inhibition by increases in hCG during early pregnancy remain unclear.

The involvement of prostaglandins (PGs) in the regulation of CL functions is well-known, particularly in domestic animals in which PGF is the major driver for luteolysis (Fortier et al., 2008). PGs are eicosanoids derived from arachidonic acids, which are released from cell membrane phospholipids through the actions of phospholipases. The synthesis of PGs begins
with the conversion of arachidonic acid to the unstable PGH₂ through the activity of cyclooxygenases (COX1/PTGS1 and COX2/PTGS2). PGH₂ is then converted to bioactive PGs such as PGE₂ (PGE), PGF₂α (PGF), and PGD₂, by specific PG synthases. Three enzymes exhibit PGE synthetic activity, which are involved in the conversion of PGH₂ to PGE: membrane PGES₁ (mPGES1/PTGES), mPGES2 (PTGES2), and cytosolic PGES (cPGES/PTGES3). PGF is produced from either PGH₂ or PGE by the actions of aldo-keto reductase (AKR) family members: AKR1B1 and AKR1C3 are known as PGF synthases and directly convert PGH₂ to PGF (Sugimoto et al., 1994; Suzuki-Yamamoto et al., 1999; Kabututu et al., 2009). On the other hand, two closely related enzymes, AKR1C1 and AKR1C2, exhibit weak PGE 9-ketoreductase activity to convert PGE to PGF (Nishizawa et al., 2000). AKR1C1 is the same enzyme as 20α-HSD, which converts progesterone to its inactive form 20α-hydroxyprogesterone and is involved in the regulation of luteolysis in rodents (Nishizawa et al., 2000). PGE and PGF are both converted into inactive metabolites by 15-hydroxy PG dehydrogenase (PGDH) (Ensor and Tai, 1995).

PGF released from the uterus is the signal that initiates luteolysis in many non-primate species (Michael et al., 1994; Okuda et al., 2002). Although uterine PGs are not involved in luteolysis in women, PGs produced by the CL itself may be effector molecules in luteolysis, because luteolysis may be induced in a small primate, the marmoset monkey by a systemic injection of PGF (Duncan et al., 1998). Bogan et al. (2008) previously reported the significant expression of AKR1B1 and AKR1C1−3 in the monkey during menses, and an increase in AKR1C3 expression in the regressing CL at the very-late stage, suggesting the involvement of intraluteal PGF in luteolysis. On the other hand, PGE is generally considered to be a luteotropic factor. The administration of PGE has been shown to prevent spontaneous or induced luteolysis in ewes, and PGE is considered to be involved in maintaining luteal functions during early

Previous studies indicated that LH and hCG promote PGE synthesis by up-regulating the expression of COX2 and PTGES (Kniss, 1999; Duffy and Stouffer, 2001; Sun et al., 2006). Secreted PGE may stimulate angiogenesis in the CL with the involvement of VEGF (Sakurai et al., 2004; Duncan and Nio-Kobayashi, 2013) and also enhances progesterone secretion from luteal cells through prostaglandin E receptor 2 (PTGER2/EP2) (Harris et al., 2001). On the other hand, AKR1C1−3 expression is also reported to be regulated by hCG in granulosa cells obtained from the periovulatory follicles of monkeys (Dozier et al., 2008). These findings suggest the involvement of intraluteal PGs in the regulation of CL functions and luteolysis; however, the exact functions of intraluteal PGs in the human CL have not yet been elucidated.

This study aimed to investigate 1) the expression profiles of all PG synthetic and metabolic enzymes in the human CL throughout the luteal phase, 2) the roles of PGE, PGF, and PGE withdrawal in the regulation of luteal cell functions, and 3) the regulation of PG synthetic and metabolic enzymes by hCG and PGE.
**Materials & Methods**

*Ethics*

The collection of the CL at the time of hysterectomy was approved by the Lothian Medical Research Ethics Committee. The Lothian Medical Research Ethics Committee and Hokkaido University Research Ethics Committee approved the study using cultured human luteinized granulosa cells (LGC) collected during assisted conception. All women gave informed consent.

*Reagents*

Reagents were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA) unless otherwise stated. Insulin-Transferrin-Selenium (ITS)-X and Matrigel were obtained from Life Technologies Inc. (Carlsbad, CA, USA) and BD Biosciences (San Jose, CA, USA), respectively.

*Collection of the human CL*

Human CLs were collected at the time of surgery from women undergoing hysterectomy for benign conditions such as heavy menstrual bleeding or uterine fibroids. The stages of the CLs were assessed by endometrial morphology and the concentration of LH in daily urine samples, which were obtained prior to surgery as reported previously (Dickinson *et al.*, 2008). The CLs were halved upon collection; one half was snap-frozen and stored at −80°C, and the other half was fixed with formalin and processed in paraffin wax.

*Human LGC culture*

Follicular aspirates were obtained from patients undergoing assisted conception after ovarian stimulation using a standard procedure (Duncan *et al.* 2005). Oocytes were removed, the remaining fluid was centrifuged at 1,500 rpm for 10 min, and cells were re-suspended in culture
medium (DMEM/Ham’s F-12 containing 2 mM L-glutamine, 10 unit/mL penicillin, 0.1 mg/mL streptomycin, 2.5 μg/mL amphotericin B, and 1× concentration of ITS-X). The cell suspension was layered over 45% Percoll in the culture medium mixture, and centrifuged at 1,200 rpm for 30 min to pellet blood cells. LGCs, visible in the interface, were collected by a pipette and washed three times with Dulbecco’s phosphate buffered saline with MgCl₂ and CaCl₂.

A total of 100,000 viable cells were seeded on Matrigel-coated 24 well plates and cultured with 1 mL of culture medium at 37°C in 5% CO₂ in air. The culture medium was changed every two days, and cells were treated on day six of the culture for 24 hours with either 1) 100 ng/mL hCG or 2) 10 ng/mL PGE and PGF dissolved in ethanol. In the PGE withdrawal experiment, after culturing LGCs in medium containing 1 ng/mL PGE for 7 days, medium was changed without PGE or 10 ng/mL PGE or PGF, and collected 24 hours later on day 8. Some cells were collected on culture day 3 or 7 without any treatments. Each experiment was performed in duplicate and repeated at least three times. Other cells were treated with either 100 ng/mL hCG or 10 ng/mL PGE for 48 hours on culture day 6, and, 48 hours later, the concentration of progesterone in the culture medium was outsourced for measurement using Chemiluminescent immunoassay by SRL Inc. (Sapporo, Japan).

**Quantitative RT-PCR (qRT-PCR)**

The CLs used for a quantitative gene expression analysis were classified as the early-luteal (1–5 days after the LH surge, n=4), mid-luteal (6–10 days, n=6), late-luteal (11–14 days, n=5), and menstrual (more than 14 days, n=3) phases. Total RNA was extracted from the frozen human CL or cultured LGCs using a RNeasy Mini Kit (Qiagen Ltd., Crawley, UK) following the manufacturer’s protocol. RNA (200 ng) was used to prepare cDNA using TaqMan Reverse Transcription regents (Applied Biosystems, Foster City, CA, USA).
The sequences of the primer sets used for this study are listed in Table 1 or previously described (Nio-Kobayashi et al., 2015). Primers were pre-validated by standard PCR and by generating standard curves using qRT-PCR. Each reaction buffer contained 5.0 µL 2×PowerSYBR® Green PCR Master Mix (Applied Biosystems), 0.5 µL primer pair (5 µM), 3.5 µL of nuclease-free H2O, and 1.0 µL cDNA, and each reaction was conducted in duplicate. The qRT-PCR cycling program consisted of a denaturing step (95°C for 10 min), annealing and extension step (95°C for 15 sec and 60°C for 1 min, repeated for 40 cycles), and a dissociation step (95°C, 60°C, and 95°C for 15 sec each) using a 7900 Sequence Detection System (Applied Biosystems). The relative expression levels of each target to the housekeeping gene (glucose-6-phosphate dehydrogenase: G6PDH), previously validated using a geNorm analysis (Primerdesign Ltd., Southampton, UK), were quantified using the ∆Ct (for in vivo analysis) or ∆∆Ct (for in vitro analysis) methods.

Statistical analysis
After testing for normality, all statistical analyses were performed using unpaired t-tests, a one-way ANOVA with Sidak’s multiple comparisons test, or a correlation analysis, using GraphPad Prism 6 software (GraphPad Software Inc., San Diego, CA, USA), and P<0.05 was regarded as significant. Values in the graphs represent the mean ± SEM of relative expression to G6PDH.

Immunohistochemistry
Fixed human CLs at the early-luteal (n=6), mid-luteal (n=3), late-luteal (n=3), and menstrual (n=3) phases were used in the immunohistochemical analysis. Tissues were dehydrated through ethanol and embedded into paraffin according to the conventional method. Tissue sections (5
µm thick) were de-waxed and washed twice in distilled water, and antigen retrieval was then performed for 5 minutes in 0.01 M citrate buffer (pH 6.0) using a pressure cooker. After washing twice in phosphate-buffered saline (PBS), sections were incubated with 3% hydrogen peroxide for 20 min followed by Avidin/Biotin blocking solution (Vector Laboratories Inc., Burlingame, CA, USA) for 15 min each. Sections were incubated with normal goat serum at room temperature for 60 min. Thereafter they were incubated with mouse anti-human PTGES (1:200; #160140, Cayman Chemical Company, Ann Arbor, MI, USA) in goat serum at 4°C overnight. Control sections were incubated with non-immune serum and the disappearance of the immunoreaction was confirmed. After washing twice in PBS, sections were incubated with biotinylated anti-mouse IgG (1:500; Vector laboratories Inc.) at room temperature for 60 min. The reaction sites were visualized using a Vectastain ABC Elite kit (Vector Laboratories Inc.) for 60 min followed by an ImmPACT™ DAB Peroxidase Substrate Kit (Vector Laboratories Inc.) for 1 min. Sections were counterstained with hematoxylin and observed under a light microscope (BX51; Olympus corporation, Tokyo, Japan).
Results

PG synthesis and metabolism in the human CL changes across the luteal phase

An analysis of all PG synthases and degradation enzymes in the human CL throughout the luteal phases by qRT-PCR revealed the differential expression patterns of each gene (Table 2). COX1 transcripts increased in the regressing CL collected during menstruation ($P<0.01$) whereas COX2 did not change across the luteal phases. The three PGE synthases were differentially expressed: PTGES peaked in the functional CL during the mid-luteal phase ($P<0.01$), whereas PTGES2 and PTGES3 peaked in the late-luteal phase ($P<0.05$). The transcripts of all genes involved in PGF synthesis (AKR1B1 and AKR1C1–3) increased in the regressing CL during the late-luteal phase, and increases in AKR1C3 were maintained in the CL collected during menstruation ($P<0.001$). Regarding PG degradation enzymes, PGDH expression did not change during the luteal phase. Changes in the enzymes involved in PG synthesis and metabolism suggest a drive towards less PGE and more PGF with the progression of luteolysis (On-line only Supplementary Fig. 1A).

PGE exerts hCG-like luteotropic effects on steroidogenic cells

As expected, hCG enhanced the expression of LHCGR and down-regulated that of follicular stimulating hormone receptor (FSHR) in human LGCs (Fig. 1A). Similarly, PGE increased LHCGR ($P<0.05$) and inhibited FSHR ($P<0.0001$) (Fig. 1B). PGE also exerted similar effects to hCG on the steroidogenic enzyme transcripts in LGCs (Fig. 1C, D). They strongly induced STAR and enhanced the expression of CYP11A1 and HSD3B1, suggesting that PGE as well as hCG supports steroidogenesis in LGCs. On the other hand, the expression of CYP21A2 and HSD17B1 was down-regulated by PGE and hCG (Fig. 1C, D). Unlike hCG, PGE increased transcript abundance for CYP19A1 ($P<0.05$; Fig. 1C, D). These changes suggest that, similar to
hCG, PGE promotes the production of progesterone in luteal steroidogenic cells (On-line only Supplementary Fig. 1B). The progesterone concentrations were significantly increased in the culture medium of hCG- (143.5 ± 7.42; \( P < 0.0001 \)) or PGE-treated cells (106.03 ± 4.44; \( P < 0.0001 \)) when compared to that of non-treated (44.18 ± 1.36) or ethanol-treated cells (44.17 ± 0.99), respectively.

**PGE exerts hCG-like anti-luteolytic effects on steroidogenic cells**

In human LGCs, hCG increased inhibin \( \alpha \)-subunit (INHA) expression, which will reduce activin, and also decreased the \( \beta \)A- and \( \beta \)B-inhibin subunits (INHBA and INHBB), which will also decrease activin synthesis. In addition, hCG reduced BMP expression *in vivo* and *in vitro* (Myers *et al.*, 2007; Myers *et al.*, 2008; Nio-Kobayashi *et al.*, 2015). PGE up-regulated INHA (\( P < 0.001 \), Fig. 2) and decreased INHBA and INHBB in LGCs (\( P < 0.001 \), Fig. 2). Furthermore, PGE significantly reduced BMP2, BMP4, and BMP6 (\( P < 0.001 \), Fig. 2), suggesting that luteolytic molecules are inhibited by PGE in human luteal steroidogenic cells.

**hCG regulates PG synthetic and metabolic enzymes and PGE exerts the same effects**

In order to investigate the effects of hCG and PGE on PG synthesis, we treated LGCs with hCG or PGE and analyzed the expression of synthetic and metabolic enzymes for PG by qRT-PCR. hCG and PGE both had similar effects on the transcript abundance of the investigated genes. COX1 and COX2 were increased in hCG-treated LGCs, with the expression of the latter being 10-fold higher than that in non-treated controls (\( P < 0.001 \), Fig. 3A). PGE also significantly enhanced the expression of COX2 (\( P < 0.01 \), Fig. 3B). The three PGE synthetic enzymes were differentially regulated: hCG and PGE markedly increased PTGES but decreased PTGES2 and PTGES3 (\( P < 0.001 \), Fig. 3A, B). While AKR1B1 was down-regulated by hCG and PGE
AKR1C1 and AKR1C2 were both increased in LGCs by hCG alone (P<0.001 and P<0.05, respectively, Fig. 3A). Neither hCG nor PGE had any effect on the expression of AKR1C3 (Fig. 3A, B). The expression of PGDH was down-regulated by hCG and PGE (P<0.001, Fig. 3A, B). Changes in the enzymes involved in PG synthesis and metabolism suggest a drive towards more PGE and less PGF by luteotropic molecules.

**PGE withdrawal occurs during luteolysis and exerts luteolytic effects**

In the human CL, PTGES immunoreactivity was localized to the cytoplasm of granulosa-lutein cells during the mid-luteal phase, while theca-lutein cells lacked immunoreactivity (Fig. 4A). Non-steroidogenic cells also lacked immunoreactivity (asterisks in Fig. 4A). In the regressing CL collected during menstruation, granulosa-lutein cells were classified into three types as previously reported: intact, apoptotic, and degenerating cells (Nio-Kobayashi et al., 2014). Only intact granulosa-lutein cells were positively immunostained for PTGES (arrow in Fig. 4B), while apoptotic granulosa-lutein cells lacked immunoreactivity (arrowheads in Fig. 4B).

The expression of PTGES and HSD3B1 strongly correlated in vivo in the human CL (r=0.7212, P<0.001; Fig. 4C), consistent with the luteotropic function of PGE. In contrast, AKR1C3 strongly and negatively correlated with the expression of HSD3B1 (r=–0.7167, P<0.001; Fig. 4D). An inverse correlation was observed between PTGES and AKR1C3 in vivo (r=–0.6505, P<0.01; Fig. 4E), suggesting a drive towards less PGE and more PGF synthesis during luteolysis.

In the absence of a luteotropic stimulation, in vitro, the expression of STAR, COX2, and PTGES significantly decreased, whereas that of AKR1C3 increased during the culture (Fig. 4F). When PGE was added during the culture and then acutely withdrawn, a marked decrease was observed in the expression of steroidogenic enzymes (STAR and HSD3B1) and PTGES while the
expression of AKR1C3 was unchanged (Fig. 4G).

**Luteolytic effects of PGE withdrawal are not recapitulated by the addition of PGF**

We assessed the effects of the addition of PGF to human LGCs on the expression of steroidogenic enzymes, PG synthetic and metabolic enzymes, and activin and BMP luteolytic factors. No effects were observed on the transcript abundance of these genes, as shown by STAR and HSD3B1, COX2, PTGES, and AKR1C3, INHA, INHBA, and INHBB, and BMP2, BMP4, and BMP6 (Table 3). In order to investigate whether PGF was biologically active in these cells, we examined C-C motif ligand 2 (CCL2) —a gene known to be induced by PGF— in luteal steroidogenic cells (Luo et al., 2011; Nio-Kobayashi et al., 2015). In the human LGC, CCL2 was increased by PGF ($P<0.05$; Fig. 5A). The addition of PGF did not alter PGE-induced luteotropic STAR or PTGES transcript abundance (Fig. 5B). Furthermore, PGF did not enhance the reductions in STAR or PTGES during PGE withdrawal (Fig. 5C).
Discussion
The results of the present study revealed the expression profiles of the PG synthetic and metabolic enzymes in the human CL and human cultured LGCs. The results obtained strongly support the luteotropic function of PGE in the regulation of the human CL. The production of luteal PGE is enhanced by luteotropic hCG via the up-regulation of key enzymes to produce PGE, namely, COX2 and PTGES. The expression of PTGES was the strongest in the functional CL during the mid-luteal stage and positively correlated with that of HSD3B1, a key enzyme for progesterone production. The enzymes responsible for luteolytic PGF production, AKR1B1 and AKR1C1−3, significantly increased in the regressing CL during the late-luteal phase and menstruation, and the expression of AKR1C3 negatively correlated with that of PTGES and HSD3B1. The capacity to produce PGE decreased, while the expression of AKR1C3 increased during the culture, and the loss of the PGE stimulation resulted in reductions in the potential to produce progesterone and PGE in cultured LGCs. These results suggest that the main PG produced in luteal tissue changes from PGE to PGF during the maturation and regression of the human CL, and PGE withdrawal is more important than the effects of PGF in the regulation of luteolysis in women. We previously demonstrated that PGE enhances the expression of vascular endothelial growth factor to support angiogenesis (Duncan and Nio-Kobayashi, 2013) and inhibits that of CCL2, a chemokine for macrophage infiltration, in the human CL (Nio-Kobayashi et al., 2015). Loss of PGE synthetic activity in luteal cells results in a destabilization of vasculatures, an enhanced macrophage infiltration, and an increased luteolytic factors such as activin A and BMPs in the CL to promote luteolysis in women (Fig. 6).

It is generally accepted that PGF is a luteolysin, whereas PGE is a luteotropin in most mammals. In ruminants, uterine PGF or PGE circulating in the CL are known to initiate or prevent luteolysis, respectively. This system does not function in women because hysterectomy
has no effect on luteal functions; however, similar to other species, luteal cells themselves possess the ability to produce PGs. There is convincing evidence to demonstrate that locally produced luteal PGs contribute to the regulation of CL functions and lifespan in cows (Arosh et al., 2004), sheep (Lee et al., 2012), and pigs (Waclawik et al., 2008). We herein showed the differential expression profiles of the genes responsible for PG biosynthesis in the human CL across the luteal phase. A comprehensive analysis of genes involved in PG biosynthesis has been reported by Bogan et al. (2008) in the CL of rhesus monkeys showing similar expression patterns for PG synthases, particularly for the expression profiles of a PGE synthase (PTGES) and PGF synthase (AKR1C3).

A previous study reported that PGE possesses a luteotropic function: PGE increases progesterone production and cAMP accumulation in cultured bovine luteal cells (Rekawiecki et al., 2005) and cultured human granulosa-lutein cells (Chandras et al., 2007). Increases in luteal PGE biosynthesis during the establishment of pregnancy has also been reported in pigs (Waclawik et al., 2008) and sheep (Lee et al., 2012). Consistent with previous findings, the present study using human LGCs revealed that PGE exerts similar luteotropic effects to hCG on the regulation of the genes responsible for progesterone production as well as luteotropic PGE production itself. The production of activin A and several BMPs increases during luteolysis and these molecules exert luteolytic effects on steroidogenic cell functions. PGE also showed similar effects to hCG on the expression of luteolytic activin A and BMPs expression, further supporting the luteotropic functions of PGE.

We also showed the regulation of PG synthetic and metabolic enzymes by luteotropic hCG and PGE in human LGCs. Luteotropic hCG and PGE both up-regulated the expression of COX2 and PTGES, but suppressed that of AKR1B1, a major PGF synthase in luteal cells. hCG increased the concentration of PGE in the culture medium of certain cells (data not shown).
However, the effects of hCG on actual PGE synthesis were not consistent among samples, although the expression of PTGES was significantly up-regulated by hCG in all samples. We still do not know what influences the effects of hCG on actual PGE synthesis. Further experiments are underway in our laboratory to reveal the mechanisms of differential PGE synthetic ability in LGCs. Luteotropic LH/hCG binds to the common receptor expressed on luteal cells, LHCGR, and promote luteal cell functions mainly via the cAMP/PKA pathway. Human granulosa-lutein cells predominantly express EP2, a receptor for PGE, which acts through the cAMP/PKA pathway, similar to LHCGR (Harris et al., 2001). hCG and PGE may both function mainly through the cAMP/PKA pathway to regulate the expression of PG synthases and degradation enzymes. Luo et al. (2015) recently reported that a decline in luteotropic stimuli leads to the induction of COX2 expression and possibly intraluteal PGF production in women. Przygrodzka et al. (2016) also noted a decline in intraluteal PGE production and increase in PGF concentrations in the regressing porcine CL; however, the increases observed in PGF did appear to be produced in luteal tissue. This study demonstrated that the loss of the luteotropic signal by PGE leads to a decline in the capacity to produce progesterone and PGE in luteal cells. Although the underlying mechanisms remain unknown, the decline observed in the production of PGE, potentially caused by a reduction in cAMP/PKA activation, may be a key event for spontaneous luteolysis in women.

Other paracrine molecules such as activin A and BMPs also possess the ability to regulate the expression of PG synthetic and metabolic enzymes. Liu et al. (2016) recently reported that activin A up-regulates the expression of COX2 in order to promote the synthesis of PGE in immortalized human granulosa lutein cells. Not only luteotropic molecules such as hCG and PGE but also luteolytic molecules including activin A and BMPs are involved in the regulation of PG synthesis in luteal cells. The mechanisms that regulate the production of PG in luteal cells
are complex, and further studies are underway in our laboratory.

Although PGF released from the uterus is a major effector molecule inducing luteolysis in various animals including cows and rodents, it is unlikely that the synthesis of PGF is the major initiator of luteolysis in women. While the luteotropic effects of PGF have been reported in other animals in vitro (Kawaguchi et al., 2013), luteolysis was induced in these animals by an intramuscular or intravenous injection of PGF. PGF produced by luteal cells may mainly act on non-steroidogenic cells such as endothelial cells, fibroblasts, and immune cells in the human CL. It is also likely that the effects of PGF are blocked when luteal cells have sufficient potential to synthesize PGE and/or progesterone. Further studies are needed in order to reveal the effects of PGF and its regulation and sites of action in the CL of women.

In conclusion, the LH/hCG responsive steroidogenic cells of the human CL co-ordinate the processes involved in luteolysis and luteal rescue. Our results suggest that the luteotropic functions of PGE produced by LH/hCG in the human CL are important for the maintenance of the CL. PGE produced from luteal cells promotes luteal functions by up-regulating key enzymes to produce progesterone and luteotropic PGE itself. Luteal PGE promotes luteal functions and blocks the expression of luteolytic molecules. While the expression of PGF synthases is increased in the regressing human CL, suggesting the potent contribution of intraluteal PGF in the ongoing luteolytic process, the withdrawal of luteal PGE may be particularly important. Although detailed mechanistic explanations for how the intraluteal PGE/PGF balance is regulated during luteolysis remain unclear, a reduction in cAMP/PKA activation —as the CL becomes less sensitive to LH— resulting in a reduced capacity for PGE production in luteal cells appears to be a key element underpinning spontaneous luteolysis in women.
Acknowledgments

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Authors’ roles

Study concept and design of the experiments: JN-K and WCD, Acquisition of data: JN-K,

Analysis and interpretation of data: JN-K, Collecting follicular fluids: MK and NS, Drafting of the manuscript: JN-K, WCD, and TI.

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Conflict of interest

None declared.
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Lee J, McCracken JA, Stanley JA, Nithy TK, Banu SK, Arosh JA. Intraluteal prostaglandin biosynthesis and signaling are selectively directed towards PGF2alpha during luteolysis but towards PGE2 during the establishment of pregnancy in sheep. *Biol Reprod* 2012;**87**:97.


Figure legends

**Figure 1. hCG-like luteotropic effects of PGE in human LGCs in vitro.**

hCG (A) and PGE (B) both enhance the expression of *LHCGR*, but suppress the expression of *FSHR*. hCG (C) and PGE (D) exert similar effects on the expression of steroidogenic genes. They stimulate the expression of *STAR, CYP11A1, HSD3B1, CYP17A1*, but inhibit that of *CYP21A2* and *HSD17B1*. PGE significantly increases the expression of *CYP19A1* (D). CYP: cytochrome P450, FSHR: follicular stimulating hormone receptor, hCG: human chorionic gonadotrophin, HSD: hydroxysteroid dehydrogenase, LHCGR: luteinizing hormone/choriogonadotropin receptor, STAR: steroidogenic acute regulatory protein. No change is indicated as 100% of the control while doubling is 200% and halving is 50% in C and D. *P*<0.05, **P*<0.01, ***P*<0.001, ****P*<0.0001.

**Figure 2. Effects of PGE on the expression of luteolytic factors in human LGCs in vitro.**

PGE enhances the expression of inhibin/activin α-subunit (*INHA*), but significantly inhibit that of both inhibin/activin β-subunits (*INHBA* and *INHBB*) as well as three bone morphogenetic proteins (BMPs). ***P*<0.001.

**Figure 3. Effects of hCG and PGE on the expression of PG synthetic and metabolic enzymes in human LGCs in vitro.**

hCG (A) and PGE (B) both enhance the expression of *COX2* and *PTGES*, while only hCG significantly increases that of *COX1*. *PTGES2* and *PTGES3* are both down-regulated by hCG and PGE. Among the four PGF synthases, *AKR1B1* is significantly decreased by the hCG and PGE treatments, whereas hCG stimulates the expression of *AKR1C1* and *AKR1C2*. *AKR1C3* expression is not altered by these two treatments. The expression of *PGDH* is significantly
reduced by the hCG and PGE treatments. AKR: Aldo-keto-reductase, COX: cyclooxygenase, PTGES: PGE synthase, PGDH: 15-hydroxyprostaglandin dehydrogenase. *P<0.05, **P<0.01, ***P<0.001.

Figure 4. Loss of PGE correlates with reduced steroidogenic activity in luteal cells

Immunoreactivity for PTGES is found in the cytoplasm of granulosa-lutein cells, but not in that of theca-lutein cells (t) or non-steroidogenic cells (asterisks) in the human CL during the mid-luteal (ML) stage (A). In the regressing CL collected during menstruation (Men), only intact granulosa-lutein cells show positive immunoreactions for PTGES (arrow in B), while apoptotic cells (arrowheads in B) lack immunoreactivity. The insert in B shows an image of intact and apoptotic granulosa-lutein cells observed at a higher magnification. The mRNA expression of PTGES in the human CL positively correlates with that of HSD3B1 (C). AKR1C3 expression reversely correlates with that of HSD3B1 (D) and PTGES (E). The expression of STAR, COX2, and PTGES decreases, while that of AKR1C3 increases during the culture (F). The expression of STAR, HSD3B1, COX2, and PTGES significantly decreases after PGE withdrawal (G). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Figure 5. PGF cannot recapitulate the luteolytic effect of PGE withdrawal

PGF is biologically active because it enhances the expression of CCL2 (A). The addition of PGF cannot block the luteotropic effects of PGE on the expression of STAR and PTGES (B) and recapitulate those of PGE withdrawal (C). CCL2: C-C motif ligand 2. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.
Figure 6. A schema showing how loss of luteal PGE is involved in luteolysis in women

In the functional CL, PGE promote the synthesis of progesterone, PGE, and vascular endothelial growth factor (VEGF) but inhibit that of PGF, CCL2, and luteolytic factors including Activin A and BMPs to maintain luteal function. Loss of PGE synthesis results in an increased macrophage infiltration and luteolytic factors to promote luteolysis. EC: endothelial cell, GLC: granulosa-lutein cell.

Supplementary Figure 1. Schemas showing the change in the expression of PG synthetic and metabolic enzymes during luteolysis in vivo (A) and effects of PGE on steroidogenesis in vitro (B).

Figure 1 Nio-Kobayashi et al.
Figure 2 Nio-Kobayashi et al.
Figure 3 Nio-Kobayashi et al.
In vivo

A

ML

B

Men

5 μm

20 μm

C

D

E

r = 0.7830
P<0.0001

r = 0.7514
P<0.0012

r = -0.6923
P<0.0002

In vitro

F

G

Figure 4 Nio-Kobayashi et al.
Figure 5 Nio-Kobayashi et al.
Figure 6 Nio-Kobayashi et al.
Supplementary figure 1 Nio-Kobayashi et al.
<table>
<thead>
<tr>
<th>Genes name</th>
<th>Protein name</th>
<th>Accession no.</th>
<th>Forward 5' to 3'</th>
<th>Reverse 5' to 3'</th>
<th>PCR Product size (bp)</th>
</tr>
</thead>
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<td>G6PDH</td>
<td>G6PDH</td>
<td>NM_000402</td>
<td>CGGAAACGCGTCTACCTTC</td>
<td>CCAGACTGATGGAAGCATC</td>
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<tr>
<td>COX1/PTGS1</td>
<td>COX1</td>
<td>NM_000962</td>
<td>GAGTACCAGCAAGAGTTGG</td>
<td>GGAGACCCCTGAGGAAAG</td>
<td>211</td>
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<td>COX2/PTGS2</td>
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<td>NM_000963</td>
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<td>mPGES1/PTGES</td>
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<td>NM_000487</td>
<td>AGTGAGGCTGCGGAAGAAG</td>
<td>AGGGTTAGGACCCAGAAAG</td>
<td>181</td>
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<tr>
<td>mPGES2/PTGES2</td>
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<td>GTGGTGGCATCAGGATAC</td>
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<tr>
<td>cPGES/PTGES3</td>
<td>cPGES</td>
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<td>CAGCTGTCCTGAGGAAATGA</td>
<td>AGGCTTTGCCCTTCTTTTG</td>
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<td>AKR1B1</td>
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<td>NM_001628</td>
<td>AGCCATGGCAAGCCGCTTC</td>
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<td>AKR1C1</td>
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<td>CTGGGATCTCAAGGACAAC</td>
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<td>PGDH</td>
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<td>CAGCGGTTATGTCCTGTTCA</td>
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<td>HSD1B1</td>
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<td>NM_000862</td>
<td>CCAATGAAGAACGCCGCTCTTG</td>
<td>GTTGGTCAGGAGCCTGTTTA</td>
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<tr>
<td>STAR</td>
<td>STAR</td>
<td>NM_000349</td>
<td>GGCTACTGACGCAAGCTGTC</td>
<td>CATCCACACTGACCCAGATG</td>
<td>250</td>
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</table>

Table 2. Changes in the mRNA expression of prostaglandin synthetic and metabolic enzymes in the human corpus luteum across luteal phases

<table>
<thead>
<tr>
<th></th>
<th>EL (n=4)</th>
<th>ML (n=6)</th>
<th>LL (n=5)</th>
<th>Men (n=3)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>COX1</strong></td>
<td>0.02 ± 0.012&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.02 ± 0.004&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.03 ± 0.011&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.09 ± 0.041&lt;sup&gt;b&lt;/sup&gt;</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td><strong>COX2</strong></td>
<td>0.01 ± 0.007</td>
<td>0.02 ± 0.008</td>
<td>0.02 ± 0.007</td>
<td>0.02 ± 0.013</td>
<td>NS</td>
</tr>
<tr>
<td><strong>PTGES</strong></td>
<td>0.97 ± 0.394</td>
<td>1.88 ± 0.378&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.83 ± 0.217</td>
<td>0.09 ± 0.028&lt;sup&gt;b&lt;/sup&gt;</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td><strong>PTGES2</strong></td>
<td>0.49 ± 0.070&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.70 ± 0.077</td>
<td>0.93 ± 0.226&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.59 ± 0.092</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td><strong>PTGES3</strong></td>
<td>3.23 ± 0.408&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.36 ± 0.429&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.11 ± 1.166&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.82 ± 1.821</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td><strong>AKR1B1</strong></td>
<td>1.44 ± 0.392&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.91 ± 0.301</td>
<td>2.66 ± 0.589&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.28 ± 0.612&lt;sup&gt;a&lt;/sup&gt;</td>
<td>P&lt;0.05</td>
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<tr>
<td><strong>AKR1C1</strong></td>
<td>0.04 ± 0.011&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.03 ± 0.008&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.11 ± 0.028&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.07 ± 0.028</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td><strong>AKR1C2</strong></td>
<td>0.33 ± 0.153</td>
<td>0.27 ± 0.088&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.65 ± 0.188&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.46 ± 0.224</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td><strong>AKR1C3</strong></td>
<td>0.02 ± 0.007&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.03 ± 0.012&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.12 ± 0.015&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.16 ± 0.031&lt;sup&gt;b&lt;/sup&gt;</td>
<td>P&lt;0.001</td>
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<tr>
<td><strong>PGDH</strong></td>
<td>0.07 ± 0.027</td>
<td>0.06 ± 0.015</td>
<td>0.08 ± 0.020</td>
<td>0.07 ± 0.038</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values represent the mean ± SEM of relative expression to G6PDH. Statistical analyses were performed using a one-way ANOVA with Sidak's multiple comparisons test, using GraphPad Prism 6 software. Values with different superscript letters indicate significant difference (P<0.05). NS: not significant. EL: early-luteal, LL: late-luteal, Men: menstrual, ML: mid-luteal.
Table 3. Effect of prostaglandin (PG)F on the mRNA expression of steroidogenic enzymes, PG synthases and metabolic enzymes, and luteolytic factors

<table>
<thead>
<tr>
<th>Genes</th>
<th>Control (n=6)</th>
<th>PGF (n=6)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>STAR</td>
<td>2.18 ± 0.669</td>
<td>2.75 ± 0.505</td>
<td>NS</td>
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<tr>
<td>HSD3B1</td>
<td>32.06 ± 2.337</td>
<td>31.42 ± 1.706</td>
<td>NS</td>
</tr>
<tr>
<td>COX2</td>
<td>0.04 ± 0.006</td>
<td>0.04 ± 0.006</td>
<td>NS</td>
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<tr>
<td>PTGES</td>
<td>0.12 ± 0.015</td>
<td>0.11 ± 0.021</td>
<td>NS</td>
</tr>
<tr>
<td>AKR1C3</td>
<td>0.0039 ± 0.0002</td>
<td>0.0035 ± 0.0002</td>
<td>NS</td>
</tr>
<tr>
<td>INHA</td>
<td>0.54 ± 0.121</td>
<td>0.54 ± 0.057</td>
<td>NS</td>
</tr>
<tr>
<td>INHBA</td>
<td>0.74 ± 0.146</td>
<td>0.76 ± 0.103</td>
<td>NS</td>
</tr>
<tr>
<td>INHBB</td>
<td>0.11 ± 0.064</td>
<td>0.09 ± 0.052</td>
<td>NS</td>
</tr>
<tr>
<td>BMP2</td>
<td>0.11 ± 0.064</td>
<td>0.09 ± 0.052</td>
<td>NS</td>
</tr>
<tr>
<td>BMP4</td>
<td>0.07 ± 0.034</td>
<td>0.06 ± 0.025</td>
<td>NS</td>
</tr>
<tr>
<td>BMP6</td>
<td>0.05 ± 0.024</td>
<td>0.05 ± 0.016</td>
<td>NS</td>
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</tbody>
</table>

Values represent the mean ± SEM of relative expression to G6PDH. Statistical analyses were performed by unpaired Student’s t-tests using GraphPad Prism 6 software, and P<0.05 was regarded as significant. NS: not significant.