Endocrine, *vol. 8, no. 3, 261–267, June 1998* 0969–711X/98/8:*261–267*/$9.75

Regulation of Prostaglandin $F_{2\alpha}$-Receptor mRNA in Human Granulosa-Luteal Cells by Human Chorionic Gonadotrophin and Prostaglandin $F_{2\alpha}$

Jeffrey E. Väänänen, Céline C. M. Väänänen, Suzie Lee, Basil Ho Yuen, and Peter C. K. Leung

Department of Obstetrics and Gynaecology, University of British Columbia, Vancouver, British Columbia, Canada

This study examined the effects of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and human chorionic gonadotropin (hCG) on the levels of $PGF_{2\alpha}$-receptor ($PGF_{2\alpha}$-R) mRNA and steroidogenesis, in the human granulosa luteal cell (hGLC). Human GLCs collected from patients undergoing in vitro fertilization, were cultured for 24 h, after which cells were exposed to culture media containing either vehicle, hCG (1 IU/mL), or hCG plus $PGF_{2\alpha}$ (10^{-11}–10^{-6} *M*), for a further 24 h. Following the treatment period, media were collected and stored (–20°C) until assayed for progesterone and 17β-estradiol (estradiol). Immediately following the treatment period, cells were extracted for total RNA. Transcripts for $PGF_{2\alpha}$-R were detected by PCR with two different sets of oligonucleotide primers based on the published human and rat $PGF_{2\alpha}$-R sequences. PCR products were confirmed to be those of $PGF_{2\alpha}$-R by size and by Southern blot hybridization with an internal oligo nucleotide probe. All experiments were performed a minimum of three times, on cells from a minimum of three separate patients. Prostaglandin $F_{2\alpha}$-R mRNA was significantly downregulated, whereas progesterone and estradiol production were significantly stimulated by hCG. Conversely, both low (10^{-11} *M*) and high concentrations (10^{-6} *M*) of $PGF_{2\alpha}$ restored $PGF_{2\alpha}$-R mRNA levels to those of the controls, whereas steroidogenesis was significantly inhibited by these conditions. At a concentration of 10^{-9} *M* $PGF_{2\alpha}$-R mRNA was barely detectable. Progesterone and estradiol production were inversely related to $PGF_{2\alpha}$-R levels, since hCG-stimulated progesterone and estradiol production were completely restored in the presence of 10^{-9} *M* $PGF_{2\alpha}$. Messenger RNA levels for the housekeeping gene β-actin were unaltered by the above treatments. In conclusion, in the human granulosa luteal cell, $PGF_{2\alpha}$-R mRNA levels are inversely related to hCG-stimulated steroidogenesis (which was biphasic in nature). Moreover, in the presence of hCG, $PGF_{2\alpha}$ downregulates its receptor mRNA, thus providing a potential form of negative feedback on its own actions, which may be important in rescuing the corpus luteum from $PGF_{2\alpha}$-mediated luteolysis should pregnancy occur.

Key Words: Prostaglandin $F_{2\alpha}$; human; granulosa; luteal; FP receptor.

Received December 4, 1997; Revised March 2, 1998; Accepted March 2, 1998.

Author to whom all correspondence and reprint requests should be addressed: Dr. P. C. K. Leung, Department of Obstetrics and Gynaecology, University of British Columbia, 2H30-4490 Oak St., Vancouver, BC, Canada, V6H-3V5.

Introduction

Prostaglandin $F_{2\alpha}$ lowers both gonadotrophin- and prostaglandin E_2-stimulated rises in cAMP, as well as increases intracellular calcium and inositol phosphates in reproductive tissues *(1–4)*. Numerous prostanoid receptors have been cloned from mammalian tissues. These receptors include the PGD_2 receptor (DP), the PGE_2 receptors (EP_1, EP_2, and EP_3 family), the $PGF_{2\alpha}$ receptor (FP), and the prostacyclin or PGI_2 receptor (IP) *(5–9)*. Recently, $PGF_{2\alpha}$-receptor ($PGF_{2\alpha}$-R) mRNA has been detected in the human granulosa luteal cell (hGLC) *(10)*. Ligand binding studies have demonstrated that the human $PGF_{2\alpha}$-R binds $PGF_{2\alpha}$ with an equilibrium dissociation constant (K_d) of approx 1–1.63 n*M (6,7)*. The binding characteristics of the rat $PGF_{2\alpha}$-R suggest a two-site model, with a high-affinity (K_d = 3.9 n*M*) and a lower affinity site (K_d = 34 n*M*) *(6)*.

Temporal and confounding relationships of ovarian hormones may be important in preventing corpus luteum (CL) regression should pregnancy occur *(11–13)*. For example, $PGF_{2\alpha}$ is well accepted as being able to inhibit human chorionic gonadotropin (hCG) stimulated progesterone production in studies where these two hormones are administered together. However, when hCG treatment precedes $PGF_{2\alpha}$, this luteolytic effect is not seen *(11)*. The blockade of luteolysis by pretreatment with hCG is suggested as being a means by which the placenta rescues the corpus luteum from $PGF_{2\alpha}$-mediated regression *(12)*, thus allowing pregnancy to proceed.

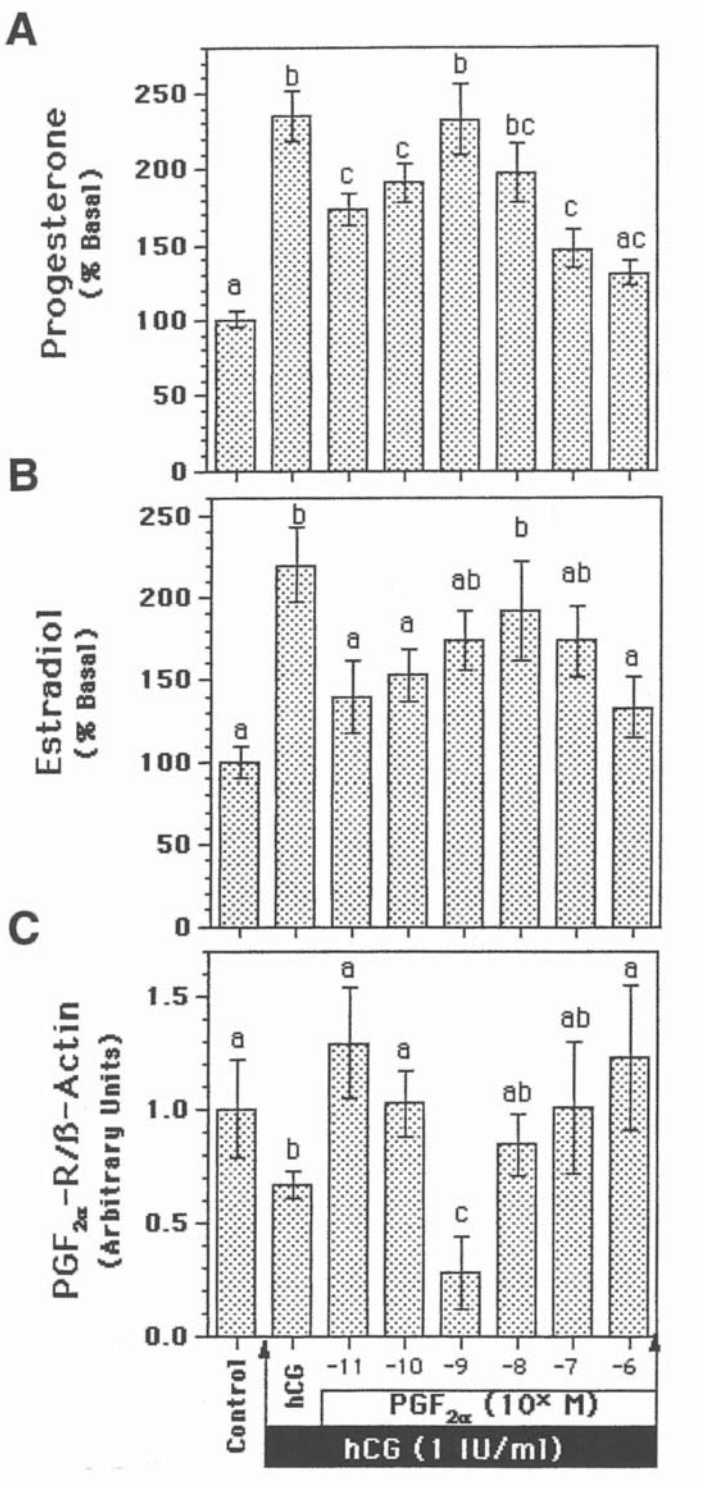

Fig. 1. (A) The effects of $PGF_{2\alpha}$ on hCG-stimulated progesterone production (over 24 h) from 1-d precultured hGLCs ($n = 11$; a ≠ b ≠ c, $p < 0.001$ by ANOVA). **(B)** The effects of $PGF_{2\alpha}$ on hCG-stimulated estradiol production from 1-d precultured hGLCs ($n = 8$; a ≠ b, $p < 0.01$ by ANOVA). **(C)** The effects of $PGF_{2\alpha}$ on $PGF_{2\alpha}$-R mRNA levels in the presence of hCG, in 1 d precultured human granulosa-luteal cells from three separate patients. Following the treatment period, cells were extracted for RNA, which was reverse-transcribed (RT) to cDNA and subjected to semiquantitative polymerase chain reaction (PCR). The housekeeping gene β-actin was unaffected by any of the above *(Figure 1 caption continued)* treatments (not shown). Photographs were subjected to densitometric analysis, and normalized to β-actin responses and averaged. Significant inhibition of $PGF_{2\alpha}$-R mRNA levels was seen in cells treated with hCG and hCG plus 10^{-9} *M* $PGF_{2\alpha}$ (a ≠ b ≠ c, $p < 0.05$ by ANOVA).

This study sought to confirm or disaffirm the presence of $PGF_{2\alpha}$-R mRNA in the hGLC, and to examine the effects of $PGF_{2\alpha}$ on these mRNA levels in the presence and absence of hCG. Furthermore, receptor mRNA levels were correlated with progesterone and estradiol levels. The results of this study provide a potential mechanism for the reported biphasic actions of $PGF_{2\alpha}$ *(13)*, in addition to suggesting a potential mechanism by which $PGF_{2\alpha}$ may downregulate its luteolytic actions rescuing the CL from luteolysis should pregnancy occur.

Results

The Effects of $PGF_{2\alpha}$ on Basal Steroid Levels

In the absence of hCG, progesterone and estradiol levels were not significantly affected in response to $PGF_{2\alpha}$ (10^{-11}–10^{-6} *M*), in hGLCs in these sets of experiments and under these culture conditions (not shown; $n = 5$).

The Effects of $PGF_{2\alpha}$ on hCG-Stimulated Steroidogenesis

Prostaglandin $F_{2\alpha}$ (10^{-11}, 10^{-10}, 10^{-7}, and 10^{-6} *M*) inhibited hCG-stimulated progesterone production in GLCs (Fig. 1A; $p < 0.001$; $n = 11$). Conversely, $PGF_{2\alpha}$ (10^{-9} and 10^{-8} *M*) did not effect hCG-stimulated progesterone production in cultured GLCs. A similar trend was seen with estradiol production. Prostaglandin $F_{2\alpha}$ (10^{-11}, 10^{-10}, and 10^{-6} *M*) inhibited hCG-stimulated estradiol production from cultured GLCs (Fig. 1B; $p < 0.01$; $n = 8$). However, $PGF_{2\alpha}$ (10^{-9}, 10^{-8}, and 10^{-7} *M*) did not inhibit hCG-stimulated estradiol production from cultured GLCs ($p > 0.05$).

Confirmation of $PGF_{2\alpha}$-R cDNA Derived from hGLCs

Amplification of $PGF_{2\alpha}$-R cDNA using PCR incorporating ^{32}P-dCTP revealed the presence of products in samples obtained from hGLCs from three separate patients and in placental tissues from two separate patients (Fig. 2A). However, PCR failed to detect $PGF_{2\alpha}$-R cDNA in human leukocyte cDNA samples from two patients. The autoradiogram of this gel was further validated when lanes from this experiment were cut and counted with a β-counter (Fig. 2B).

Amplification of $PGF_{2\alpha}$-R and β-Actin cDNAs Derived from hGLCs

Prostaglandin $F_{2\alpha}$-R cDNA was amplified from human GLCs (obtained from two different patients) with two different sets of oligonucleotide primers (hPGF± and rPGF±). Products of the expected size (802 and 720 bp) were

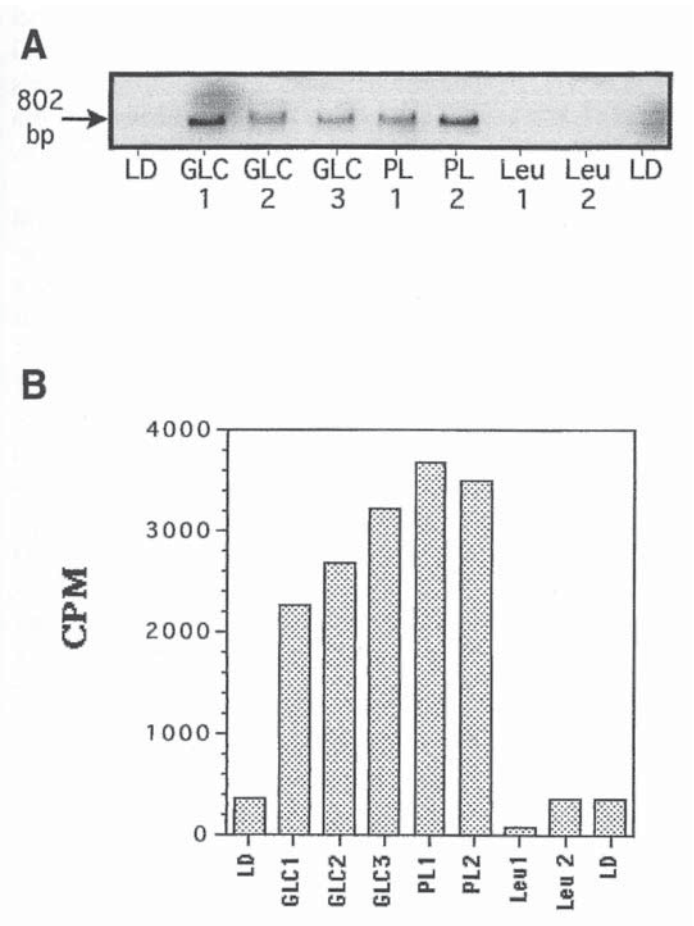

Fig. 2. PCR amplification of $PGF_{2\alpha}$-R cDNA from hGLCs, placenta, and leukocytes. **(A)** An autoradiograph of amplified $PGF_{2\alpha}$-R cDNA with the oligonucleotides hPGF+ and hPGF– in the presence of ^{32}P-dCTP. PCR products of the predicted size were amplified from three different hGLC (GLC1, 2 and 3) and two different human placenta (PL1 and 2; uncultured samples) cDNA samples. Conversely, gel lanes loaded with mol-wt ladder (LD) or two different leukocyte (Leu) cDNA samples did not show specific labeling of product. **(B)** Gel bands (from A) were also separated and counted with a β-counter.

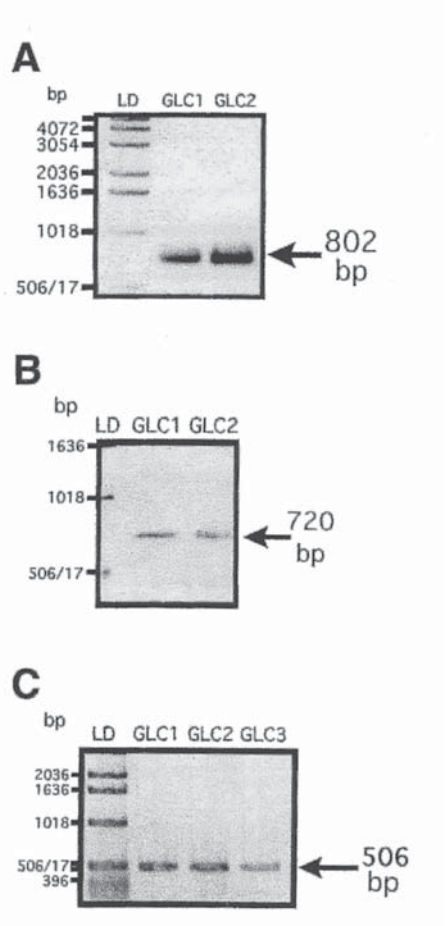

Fig. 3. Ethidium bromide-stained gels of PCR amplification of $PGF_{2\alpha}$-R and β-actin cDNA. Two different sets of oligonucleotide primers were utilized to amplify P $F_{2\alpha}$-R cDNA, and one set was utilized to amplify β-actin cDNA, from 1 d precultured hGLCs. These primers were hPGF+ and hPGF– **(A)**, rPGF+ and rPGF+ **(B)** and Act+ and Act– **(C)**. All three sets of oligonucleotides were able to amplify products of the predicted size from hGLCs from up to three different patients.

amplified by both primers (hPGF± and rPGF±; Fig. 3A,B). Additionally, oligonucleotide primers for β-actin cDNA (Act±) successfully amplified a product of the expected size (506 bp) from human GLCs obtained from three different patients (Fig. 3C).

The Effects of $PGF_{2\alpha}$ on $PGF_{2\alpha}$-R mRNA Levels

Prostaglandin $F_{2\alpha}$ (10^{-11}–10^{-6} *M*) had no detectable effect on basal $PGF_{2\alpha}$-R mRNA levels in 1-d precultured human GLCs (not shown).

The Effects of $PGF_{2\alpha}$ on $PGF_{2\alpha}$-R mRNA Levels in the Presence of hCG

Human GLCs were incubated with vehicle, hCG (1 IU/mL), or hCG plus $PGF_{2\alpha}$ (10^{-11}–10^{-6} *M*). The effects of these treatments on $PGF_{2\alpha}$-R and β-actin cDNA levels were examined by RT-PCR followed by densitometry (Fig. 1C) and Southern blot hybridization (Fig. 4A,B).

Briefly, $PGF_{2\alpha}$-R message was downregulated by hCG. Prostaglandin $F_{2\alpha}$ at low (10^{-11} *M*) and high (10^{-6} *M*) concentrations prevented this downregulation. However, densitometric analysis revealed significant inhibition of $PGF_{2\alpha}$-R mRNA levels in cells treated with hCG and hCG plus 10^{-9} *M* $PGF_{2\alpha}$ ($n = 3$; $p < 0.05$ by ANOVA; Fig. 1C). The housekeeping gene β-actin was unaffected by any of the above treatments (not shown).

Southern blot hybridization of a semiquantitative PCR experiment (presented in Fig. 1C), with an oligonucleotide probe confirmed the identity of the PCR products (Fig. 4A). Moreover, densitometric analysis of the autoradiogram revealed a pattern of mRNA regulation similar to that found in Fig. 1C (small differences may be attributed to the fact that Southern blot data were not ratioed over β-actin levels). In short, hCG attenuated $PGF_{2\alpha}$-R mRNA levels in this experiment. Additionally, in presence of hCG, $PGF_{2\alpha}$ (10^{-10}–10^{-8} *M*) further reduced $PGF_{2\alpha}$-R mRNA levels.

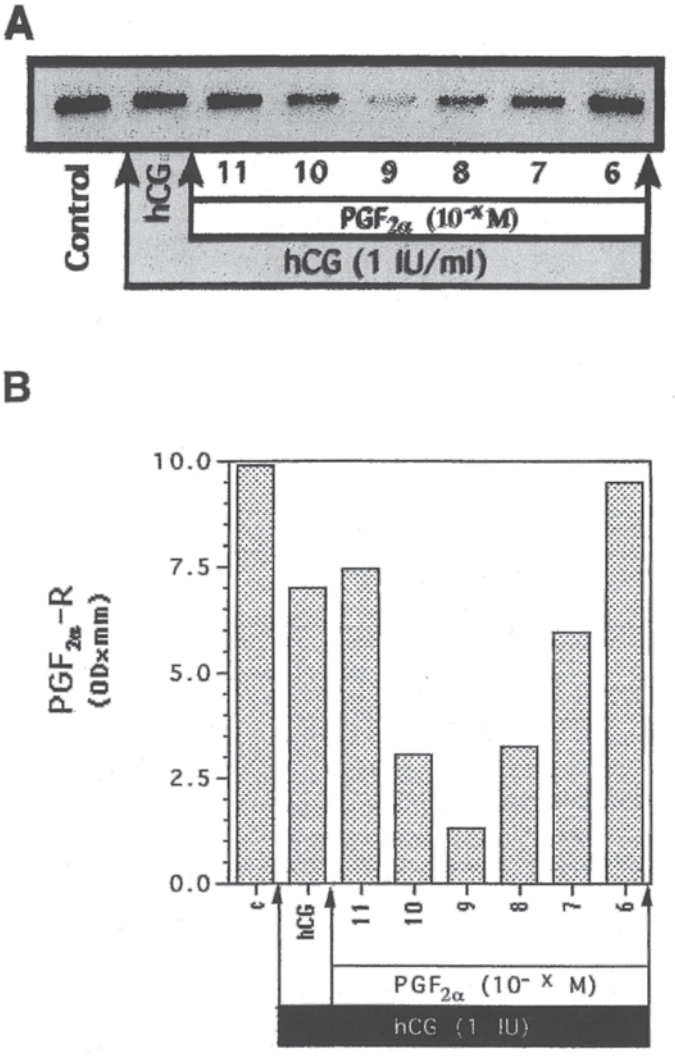

Fig. 4. (A) Southern blot hybridization of a semiquantitative PCR experiment, with an oligonucleotide probe for $PGF_{2\alpha}$-R, in cells from 1 d precultured HGLCs. These data confirm the identity of the PCR products presented in (Fig. 4A). **(B)** Densitometric analysis of the autoradiogram presented in A. Human chorionic gonadotrophin (hCG) inhibited $PGF_{2\alpha}$-R mRNA message in this experiment. Moreover, in the presence of hCG, $PGF_{2\alpha}$ (10^{-11}–10^{-7} *M*) inhibited $PGF_{2\alpha}$-R mRNA message, potentiating hCG-mediated inhibition at concentrations of (10^{-10}–10^{-8} *M*, $PGF_{2\alpha}$).

Discussion

Wide-ranging concentration–response studies (1 p*M* to 1 μ*M* $PGF_{2\alpha}$) were performed in both the presence and absence of hCG, in short-term cultures of human GLCs. The concentration range of $PGF_{2\alpha}$ used in these studies resulted in bell curve-like inhibition of hCG-stimulated progesterone and estradiol production. As previously discussed *(13)*, this biphasic nature is somewhat usual for prostaglandin actions *(14–17)*.

Inhibition of $PGF_{2\alpha}$-R mRNA and presumably $PGF_{2\alpha}$-R would reduce the effectiveness of $PGF_{2\alpha}$-mediated luteolytic effects. Thus, the inverse bell curve-like autoregulation of $PGF_{2\alpha}$-R mRNA by $PGF_{2\alpha}$ may explain its bell curve-like effects on progesterone production *(13)*. Notably, maximal stimulation of progesterone production in the presence of hCG and $PGF_{2\alpha}$ (10^{-9} *M*) occurred when $PGF_{2\alpha}$-R mRNA levels were at their lowest. Thus, rather than potentiating hCG in a true sense, $PGF_{2\alpha}$ is inhibiting its own luteolytic effects, allowing more effective stimulation by gonadotrophins.

It is unknown what mechanism mediates the inverse bell curve-like response of $PGF_{2\alpha}$-R mRNA to $PGF_{2\alpha}$ treatment. However, the bell curve-like progesterone response to $PGF_{2\alpha}$ treatment does not appear to mediate this effect as suggested by the fact that treatment with $PGF_{2\alpha}$ alone (10^{-11}–10^{-6} *M*; in the absence of hCG) did not alter $PGF_{2\alpha}$-R mRNA levels, but a bell curve-like progesterone response could still be observed in cells of this culture time (not shown). Thus, the mechanism by which $PGF_{2\alpha}$ autoregulates its receptor mRNA needs to be studied further.

Prostaglandin $F_{2\alpha}$ has been shown to mediate functional luteolysis and luteal regression, in the mammalian ovary *(11,18–23)*. However, the presence of $PGF_{2\alpha}$ in the ovary only roughly correlates with this action, since $PGF_{2\alpha}$ levels are highest in mid- rather than late-luteal phase in the human. This discrepancy has been accounted for with the examination of PGE_2, which is suggested to counteract $PGF_{2\alpha}$-induced luteolysis. Prostaglandin E_2 levels in midluteal phase are high, but they are not in late-luteal phase. Thus, it is postulated that during the midluteal phase, the ratio of $PGF_{2\alpha}$:PGE_2 is low and not suitable for luteolysis, although in the late-luteal phase this ratio is high, allowing for luteolysis in the human *(24)*. The present results suggest that $PGF_{2\alpha}$-mediated $PGF_{2\alpha}$-R down-regulation may be another mechanism by which the CL is rescued from luteolysis in the case of pregnancy.

Additionally, studies have demonstrated that temporal and confounding relationships of ovarian hormones may be important in preventing CL regression, should pregnancy occur *(11)*. For example, $PGF_{2\alpha}$ is well accepted as being able to inhibit hCG-stimulated progesterone production in studies where these two hormones are administered together. However, when hCG treatment precedes $PGF_{2\alpha}$, this luteolytic effect is not seen *(11)*. Similarly, prolactin, luteinizing hormone (LH), and follicle-stimulating hormone (FSH), alone and in combination, were not capable of blocking $PGF_{2\alpha}$-induced luteolysis. Other confounding interactions have been described in the hamster *(25)*. The blockade of luteolysis by pretreatment with hCG is suggested as being a means by which the placenta rescues the CL from $PGF_{2\alpha}$-mediated regression *(12)*, thus allowing pregnancy to proceed. Since the $PGF_{2\alpha}$ receptor has only been recently cloned in the human and rat *(6,7)*, there exists only one other report of $PGF_{2\alpha}$-R mRNA regulation by hCG in the literature *(10)*. The ability of hCG alone to inhibit $PGF_{2\alpha}$-R mRNA supports the findings of Michael and Webley *(11)*, in that it provides another potential mechanism by which the pre-exposure of GLCs to hCG may prevent the luteolytic effects of $PGF_{2\alpha}$.

Contrary to these results, Ristimaki et al. demonstrated an hCG-mediated upregulation of $PGF_{2\alpha}$-R mRNA levels in midterm cultured cells, whereas in 1-d cultured cells, they showed no effect *(10)*. The inability of Ristimaki and associates to show regulation by hCG in earlier cultures may be owing to differing culture conditions, cell densities, differential exposure of the cells to pharmacological agents (such as gonadotropins) prior to collection, or even rank order of treatment application.

In brief, $PGF_{2\alpha}$ inhibits hCG-stimulated steroidogenesis in a bell curve-like manner. Prostaglandin $F_{2\alpha}$ negatively autoregulates its receptor mRNA, thus providing a form of negative feedback on its own actions, which may be important in rescuing the CL from $PGF_{2\alpha}$-mediated luteolysis should pregnancy occur. Interestingly, this negative feedback is exerted at one specific concentration (10^{-9} *M* $PGF_{2\alpha}$), which corresponds to the reported K_d of the $PGF_{2\alpha}$-receptor *(6,7)*. This report further supports previous reports that $PGF_{2\alpha}$ may exert both luteolytic and luteotrophic effects *(13)*. In conclusion, these results provide a potential mechanism by which the biphasic response to $PGF_{2\alpha}$ may occur. In addition, they strongly support the idea that $PGF_{2\alpha}$ is a complex local regulator of the ovarian GLC capable of exerting multiple actions that are dependent on concentration and confounding interactions.

Materials and Methods

GLC Collection and Culture

The use of human GLC was approved by the Clinical Screening Committee for Research and Other Studies Involving Human Subjects of the University of British Columbia. GLCs were harvested in conjunction with oocyte collection in the University of British Columbia's in vitro fertilization program, as previously described *(13)*, with the exception that owing to the low level of $PGF_{2\alpha}$-R mRNA expressed in these cells, all available cells (from all follicles >12 mm in diameter) from each patient were plated on a single plate in order to optimize the chances of obtaining a detectable signal. Cell viability (>95% viable) was checked with a tryphan blue dye exclusion test prior to and following treatment. It should be noted that these cells were exposed in vivo to GnRH analog (Synarel, Syntex; Montreal, Quebec, Canada), hMG (Humegon 75 IU FSH and 75 IU LH, Organon, Scarborough, Ontario, Canada; or Fertinorm 75 IU FSH, Serono, Oakville, Ontario, Canada) and hCG (10,000 IU; Serono) as previously described *(13)*.

Prostaglandin $F_{2\alpha}$ concentration–response curves (10^{-11}–10^{-6} *M*) were performed in the absence and presence of hCG (1 IU/mL), in FBS-free media supplemented with androstenedione (5×10^{-7} *M*; as substrate for estradiol production). A prewash with FBS-free media was performed prior to this treatment period. Following the treatment period (24 h), media were collected and stored (–20°C) until assayed for progesterone or estradiol with radioimmunoassay (RIA). Cells were extracted for total RNA, which was reverse-transcribed and examined with semiquantitative PCR (as described below). All hormones utilized in these experiments were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated.

Fresh human placental tissues collected immediately postpartem, and human leukocytes collected from blood samples were utilized as positive and negative controls, respectively, for $PGF_{2\alpha}$-R gene expression *(15)*.

RIA of Progesterone and Estradiol

The progesterone and estradiol concentrations in culture media were determined by specific RIAs, as previously described *(13,26,27)*.

Total RNA Preparation

Following experiments, human GLCs, placental cells, and leukocytes were extracted for total RNA with an RNaid™ kit (Bio 101, La Jolla, CA), as outlined in the provided instructions. In order to check the relative efficacy of the RNA extraction procedure and the integrity of the RNA, the extraction products were run on an RNA gel. The RNA gel was composed of agarose (1.0%) dissolved in dH_2O (21.6 mL). Additionally, RNA gel-running buffer (GRB-R, 3 mL, as defined below) and formaldehyde (5.34 mL) were added, and the solution was allowed to cool (5 min) before pouring into a gel tray. RNA samples were loaded (0.5–2 µg in 10 µL) along with GLB-R (3 µL), and the gel was run (100 V, 50 min). Total RNA was found to be intact as determined by the presence of 28 and 18 S bands. Moreover, the relative intensity of the bands were similar under different treatment regimens as determined by densitometry (not shown).

Lysis buffer was composed of guanidine thyocyanate (4.0 *M*), sodium citrate (pH 7.0; 5 m*M*), sarcosyl (0.5 % w/v), and β-mercaptoethanol (0.7% v/v) in diethylpyrocarbonate-(DEPC) treated water. The GRB (10X) consisted of MOPS (0.2 *M*), NaOAc (80 m*M*), and EDTA (10 m*M*) in dH_2O. GLB-R was composed of glycerol (50%), EDTA (1 m*M*), bromophenol blue (0.4%), xylene cyanol (0.4%), and ethidium bromide.

RT-PCR Amplification

Total RNA levels were determined by spectrophotometric estimation, which was validated by repeated estimation ($n = 6$) of a known concentration of DNA (salmon sperm DNA serially diluted in 2× increments from 5000 to 5 ng/mL). The difference between the known (by weight) and estimated (by spectrophotometry) concentrations of DNA was within 12.7 and 1.0% for DNA concentrations between 80–625 and 1250–5000 ng/mL, respectively. Spectrophotometric estimation of DNA concentrations lower than 40 ng/mL resulted in an overestimation of DNA levels by up to 90%. Thus, only samples estimated within the higher concentrations (500–5000 ng/mL) were utilized for reverse transcription to complementary DNA (cDNA)

Table 1
Oligonucleotide Sequences Utilized for PCR and Southern Blot Hybridization[a]

Gene	±	Primer sequence, 5' to 3'	Name	Mol wt	Ref.
Human $PGF_{2\alpha}$	+	CTC ATG AAG GCA TAT CAG AG	hPGF+	6127	*7*
Receptor	–	GTT GCC ATT CGG AGA GCA A	hPGF–	5831	
	+*	GCT TCT GAT AAA GAA TGG ATC CGC TT	hPGFP+	7955	
Rat $PGF_{2\alpha}$	+	CCA TTG CCA TCC TCA TGA AGG	rPGF+	6407	*6*
Receptor	–	AGC GTC GTC TCA CAG GTC AC	rPGF–	6120	
	+*	CAG TAC GAT GGC CAT TGA GAG GTG CAT	rPGFP+	8399	

[a]+, sense; –, antisense. *, utilized as an internal probe for blots. Mol wt, Molecular weight.

in this study. Reverse transcription was performed as follows: A fixed quantity of total RNA (between 0.5 and 2 µg in 10 µL DEPC-treated water) was heated (70°C, 10 min), and then spun down (5 min, 10,000*g*). DTT (1 µL), oligo-dT (1 µL), and bulk mixture (5 µL) were added, followed by a 1-h incubation (37°C; Pharmacia First Strand cDNA Kit™, Uppsala, Sweden). Following this incubation the preparation was boiled for 10 min, spun down, and frozen (–20°C) until used.

cDNA obtained from reverse transcription reactions were amplified by PCR such that relative changes in $PGF_{2\alpha}$-R expression could be examined. Briefly, a fixed quantity of cDNA between 1 and 5 µL depending on availability for each experiment was added to a master mix (22 µL, as defined below), which was premixed with a sense and antisense primer (0.5 µ*M* in 1 µL each; Table 1) and *Taq* polymerase (1 U, 0.2 µL) in a microcentrifuge tube (0.5 mL, Canlab). Two different pairs of primers were used to amplify cDNA for the $PGF_{2\alpha}$-R (Table 1). One pair of primers (hPGF+ and hPGF–) were based on the published human sequence *(7)*, whereas the second pair (rPGF+ and rPGF–) were based on the published rat sequence *(6)*. Expected product sizes were 802 and 720 bp for the hPGF and rPGF products, respectively. The housekeeping gene β-actin was also amplified (as a control) using primers (Act+ and Act–) based on the published human sequence *(28)*.

Master mix was composed of 10X PCR buffer (1/10 vol) plus deoxynucleotide-triphosphates (dNTPs; 0.2 m*M*). Ten times PCR buffer consisted of Tris-HCl (100 m*M*, pH 8.3), KCl (500 n*M*), $MgCl_2$ (1.5 m*M*), and gelatin (0.1%) in ddH_2O. Radiolabeled PCR contained 4.0 nCi of ^{32}P-dCTP. TBE (5X) was composed of Tris-base (10.8 g), boric acid (5.5 g), and EDTA (0.5 *M*, pH 8.0) dissolved in dH_2O (final volume 1 L). GLB-D consists of glycerol (50 mL), EDTA (0.5 *M*, 20 mL), bromophenol blue (0.1 g), xylene cyanol (0.1 g), and H_2O (20 mL).

Prior to thermocycling, PCR reactions were denatured at 96°C (3 min). Reactions incorporating hPGF± primers were denatured at 96°C (30 s), annealed at 57°C (30 s) and polymerized at 72°C (90 s) for 40 cycles. Reactions incorporating rPGF± primers were denatured at 96°C (30 s), annealed at 50°C (30 s), and polymerized at 72°C (90 s) for 40 cycles. Reactions incorporating Act± primers were denatured at 96°C (30 s), annealed at 55°C (30 s), and polymerized at 72°C (90 s) for 30 cycles. For all reactions, a 7-min extension period followed the last PCR thermocycle (72°C). The number of cycles for each primer pair were determined by PCR cycle experiments, which revealed a linear amplification between 35–48 and 20–44 cycles for hPGF± and Act±, respectively. PCR products were run on an agarose gel (1.5%) in a Tris-borate-EDTA buffer (TBE) as defined above (120–140 V). The gel was poststained with ethidium bromide and photographed with a Polaroid MP5 camera under UV light.

Several controls were performed to determine the accuracy of the PCRs. First, reverse transcriptions without the addition of reverse transcriptase were carried out, and aliquots were subjected to PCR amplification to test the presence of contaminating genomic DNA in the RNA samples. Second, reverse transcriptions were performed in the absence of RNA in order to rule out RNA contamination of stock solutions. Third, as mentioned above, the integrity of RNA samples was confirmed by gel electrophoresis and PCR for β-actin, which was run in parallel. Fourth, PCRs were performed in the absence of cDNA to examine the crosscontamination of samples. Finally, PCR reactions were all made up in a bulk stock and aliquoted into tubes prior to the addition of template cDNA in order to ensure the homogeneity of reactions, and to preserve the ability to infer quantitative changes owing to differences in template, rather than buffer heterogeneity.

Southern Blot Hybridization

The identity of PCR products was verified with hybridization of an oligonucleotide to the internal sequence of the predicted product (Southern blot). In short, an agarose gel containing the expected PCR product was denatured by immersion and agitation (15 min, room temperature) in a solution containing NaCl (1.5 *M*) and NaOH (0.5 *M*). Sodium hydroxide was then neutralized with a solution of NaCl (3 *M*) and Tris (0.5 *M*) at pH 8.0. Three washes with a sodium chloride/sodium citrate buffer (SSC) followed (5 min each), after which an overnight transfer to a nylon membrane was performed. The SSC buffer was composed of sodium chloride (26.3 g/L) and sodium citrate (13.2 g/L), in dH_2O (pH 7.0).

Following transfer of the gel to a nylon membrane, the membrane was washed (in SSC), dried wrapped in Saran Wrap™, and exposed to UV light (2 min). The membrane was stored (4°C) until hybridization, which was performed with a radiolabeled oligonucleotide, specific to the inner sequence of the predicted PCR product (hPGFP+ and rPGFP+; Table 1). Radiolabeling of the oligonucleotide was performed by a kination reaction as follows. Primer (10 pmol, 1 µL), T_4 kinase buffer (1 µL, 10X), dH_2O (2 µL), γ^{32}P-ATP (5 µL), and T_4 kinase (10 U, 1 µL) were mixed and incubated (1 h, 37°C). The probe was then boiled (2 min), and spun (1 min, 10,000 g). Just prior to hybridization the nylon membrane was removed from the refrigerator and preincubated in a prehybridization solution. The probe was then diluted in a hybridization solution and hybridized (40°C, overnight). The following day the membrane was washed three times in SSC (15 min each at 40–50°C). The washed membrane was blotted, rewrapped in Saran Wrap, and then autoradiographed for 20 min to several days (–70°C) depending on signal strength.

Densitometry of Photographed Gels and Autoradiographs

RNA and DNA gels stained with ethidium bromide (200 µg/100 mL gel; Sigma) were visualized with UV illumination (Photoprep, Bio/Can Scientific, Mississauga, ON). Quantification of products required gels to be photographed with a negative film (Polaroid, 665, Toronto, Ontario). Negatives and autoradiographs from Southern blotting were scanned with a transluminescence video densitometer (Model 620, Bio-Rad, Mississauga, Ontario). Scanning software was utilized to calculate the relative optical density of each product band. In order to reduce variability, three scans of each film were performed, and the means of the three scans were plotted.

Data Analysis

Prostaglandin $F_{2\alpha}$-R mRNA levels estimated by quantitative PCR were expressed as the ratio of $PGF_{2\alpha}$ to β-actin. Data obtained from one patient were normalized as a percentage of the basal levels. Mean progesterone and estradiol levels of six to eight wells that received the same treatment were also expressed as a percentage of the basal value before complete experiments from different patients were pooled and presented as mean ± SEM. Cells from each patient were exposed to all of the treatments. Statistical analysis was carried out using one-way analysis of variance (ANOVA), followed by Fisher's protected least-significant difference test (Statview for Macintosh) when multiple groups were compared.

Acknowledgments

The authors wish to acknowledge the staff of the UBC Human IVF Program for their technical assistance in the collection of human granulosa-luteal cells. This research was supported by the MRC of Canada. This work was supported by a grant from the Medical Research Council of Canada (to P. C. K. L.). P. K. Leung is a career investigator of the British Columbia Research Institute for Child and Family Health.

References

1. Davis, J. S., Tedesco, T. A., West, L. A., Maroulis, G. B., and Weakland, L. L. (1989). *Mol. Cell. Endocrinol.* **65,** 187–193.
2. Currie, W. D., Li, W., Baimbridge, K. G., Ho Yuen, B., and Leung, P. C. K. (1992). *Endocrinology* **130,** 1837–1843.
3. Pepperell, J. R., Preston, S. L., and Behrman, H. R. (1989). *Endocrinology* **125,** 144–151.
4. Lahav, M., Rennert, H., Sabag, K., and Barzilai, D. (1987). *J. Endocrinol.* **113,** 205–212.
5. Adam, M., Boie, Y., Rushmore, T. H., Muller, G., Bastien, L., McKee, K. T., et al. (1995). *Biochemistry* **34,** 15,407–15,414.
6. Lake, S., Gullberg, H., Wahlqvist, J., Sjogren, A. M., Kinhult, A., Lind, P., et al. (1994). *FEBS Lett.* **355,** 317–325.
7. Abramovitz, M., Boie, Y., Nguyen, T., Rushmore, T. H., Bayne, M. A., Metters, K. M., et al. (1994). *J. Biol. Chem.* **269,** 2632–2636.
8. Boie, Y., Rushmore, T. H., Darmon, G. A., Grygorczyk, R., Slipetz, D. M., Metters, K. M., et al. (1994). *J. Biol. Chem.* **269,** 12,173–12,178.
9. Funk, C. D., Furci, L., FitzGerald, G. A., Grygorczyk, R., Rochette, C., Bayne, M. A., et al. (1993). *J. Biol. Chem.* **268,** 26,767–26,772.
10. Ristimaki, A., Jaatinen, R., and Ritvos, O. (1997). *Endocrinology* **138,** 191–195.
11. Michael, A. E. and Webley, G. E. (1991). *J. Endocrinol.* **131,** 319–325.
12. Webley, G. E., Richardson, M. C., Given, A., and Harper, J. (1991). *Hum. Reprod.* **6(6),** 779–782.
13. Väänänen, J. E., Li-Pak-Tong, B., Väänänen, C. C. M., Hok-Yue-Chan, I., Ho-Yuen, B., and Leung, P. C. K. (1997). *Biol. Reprod.* **57,** 1346–1353.
14. Cohen, L. R. and Rimon, G. (1992). *Cell Signal* **4,** 331–335.
15. Sano, N. and Shichi, H. (1993). *Prostaglandins Leukotrienes Essent. Fatty Acids* **49,** 765–769.
16. Puschel, G. P., Kirchner, C., Schroder, A., and Jungermann, K. (1993). *Eur. J. Biochem.* **218,** 1083–1089.
17. Hargrove, J. L., Seeley, R. R., and Ellis, L. C. (1975). *Am. J. Physiol.* **228,** 810–814.
18. Jalkanen, J., Ritvos, O., Huhtaniemi, I., Stenman, U. H., Laatikainen, T., and Ranta, T. (1987). *Mol. Cell Endocrinol.* **51,** 273–276.
19. Korda, A. R., Shutt, D. A., Smith, I. D., Shearman, R. P., and Lyneham, R. C. (1975). *Prostaglandins* **9,** 443–449.
20. Grinwich, D. L., Ham, E. A., Hichens, M. , and Behrman, H. R. (1976). *Endocrinology* **98,** 146–150.
21. Moon, Y. S., Duleba, A. J., Kim, K. S., and Ho Yuen, B. (1986). *Biochem. Biophys. Res. Commun.* **135,** 764–769.
22. Hanzen, C. (1984). *J. Gynecol. Obstet. Biol. Reprod. (Paris)* **13,** 351–361.
23. Richardson, M. C. and Masson, G. M. (1980). *J. Endocrinol.* **87,** 247–254.
24. Patwardhan, V. V. and Lanthier, A. (1985). *Prostaglandins* **30(1),** 91–98.
25. Harris, K. H. and Murphy, B. D. (1981). *Prostaglandins* **21(2),** 177–187.
26. Li, W., Khorasheh, S., Ho Yuen, B., Ling, N., and Leung, P. C. K. (1993). *Endocrinology* **132,** 1750–1756.
27. Rodway, M. R., Ho Yuen, B., and Leung, P. C. K. (1990). *Am. J. Obstet. Gynecol.* **163,** 1546–1551.
28. Ng, S. Y., Gunning, P., Eddy, R., Ponte, P., Leavitt, J., Shows, T., et al. (1985). *Mol. Cell. Biol.* **5,** 2720–2732.