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Prostaglandin dehydrogenase (PGDH) in granulosa cells of primate periovulatory follicles is regulated by the ovulatory gonadotropin surge via multiple G proteins

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Abstract

The ovulatory gonadotropin surge increases granulosa cell prostaglandin synthesis as well as prostaglandin dehydrogenase (PGDH), the key enzyme responsible for prostaglandin metabolism. To investigate gonadotropin regulation of PGDH in the primate follicle, monkey granulosa cells were obtained across the 40-hour periovulatory interval. PGDH activity was low before the ovulatory hCG stimulus, peaked 12-24 hours after hCG, and was low again 36 hours after hCG administration. Granulosa cells maintained in vitro with hCG showed a similar temporal pattern of PGDH. The LH/CG receptor can utilize multiple signaling pathways to regulate intracellular events. Gonadotropin-stimulated cAMP appears to act primarily via the Epacs to increase PGDH mRNA, protein, and activity. In contrast, PLC activation of PKC likely decreases PGDH mRNA, protein, and activity late in the periovulatory interval. Increased, then decreased PGDH activity may delay accumulation of prostaglandins in the follicle until late in the periovulatory interval, contributing to timely ovulation in primates.

Keywords

ovulation; ovary; prostaglandin; granulosa cell; hCG; luteinizing hormone

1.0 INTRODUCTION

The midcycle surge of luteinizing hormone (LH) initiates events within the periovulatory follicle which lead to ovulation. This gonadotropin surge increases prostaglandin production by follicular granulosa cells, and prostaglandins produced within the periovulatory follicle are required for successful ovulation (Murdoch et al. 1993). Numerous studies have demonstrated that gonadotropins stimulate granulosa cell expression of the enzymes required for synthesis of PGE2, the key ovulatory prostaglandin (Diouf et al. 2006; Duffy et al. 2005b; Duffy et al. 2005c; Duffy and Stouffer 2001; Filion et al. 2001; Sirois 1994; Sirois and Dore 1997; Wong et al. 1989).

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While gonadotropin stimulates synthesis of prostaglandins to achieve elevated follicular PGE2 concentration required for ovulation, PGE2 metabolism may also regulate PGE2 levels within the follicle. Prostaglandin dehydrogenase (PGDH), the key enzyme involved in PGE2 metabolism (Tai et al. 2006), is a gonadotropin-regulated gene product in granulosa cells of periovulatory follicles. PGDH mRNA and protein peak midway through the periovulatory interval but decline before ovulation (Duffy et al. 2005a; Sayasith et al. 2007). These data suggest that PGDH activity may contribute to the maintenance of low PGE2 concentrations until just before ovulation, when rising levels of enzymes involved in PGE2 synthesis, coupled with declining levels of PGDH, result in ovulatory levels of PGE2 within the follicle.

The mechanism by which an ovulatory concentration of gonadotropin (LH or hCG) regulates PGDH is unknown. These gonadotropins act through the LH/CG receptor, a member of the G-protein coupled receptor family. The LH/CG receptor is known to couple to multiple effector mechanisms within target cells. Most often, gonadotropin stimulation of the LH/CG receptor on granulosa cells has been shown to activate adenylyl cyclase via Gαs, followed by increased intracellular cAMP to activate protein kinase A (PKA) (Tasken and Aandahl 2004). However, cAMP regulates other pathways, including nucleotide-modulated ion channels (Hofmann et al. 2003) and the guanine nucleotide exchange proteins directly activated by cAMP (Epacs, (de Rooij et al. 1998; Kawasaki et al. 1998)). In addition, coupling of the LH/CG receptor to Gαq and activation of phospholipase C (PLC) has been reported to regulate intracellular calcium, stimulate phosphoinositide turnover, and activate protein kinase C (PKC) (Lee et al. 2002). One or more of these pathways may contribute to gonadotropin-regulation of PGDH mRNA, protein, and activity in granulosa cells of primate periovulatory follicles.

Previous studies by this laboratory demonstrated that administration of an ovulatory dose of hCG increased, then decreased, PGDH mRNA and protein levels in granulosa cells of primate periovulatory follicles (Duffy et al. 2005a); PGDH activity in granulosa cells has never been addressed in vivo or in vitro. The goal of the present study was determine if the ovulatory gonadotropin surge, acting directly via the LH/CG receptor on granulosa cells, is responsible for both increased and decreased PGDH mRNA, protein, and activity during the periovulatory interval. Experiments were designed to 1) determine if PGDH activity parallels the gonadotropin-regulated changes in PGDH mRNA and protein in primate granulosa cells in vivo throughout the periovulatory interval and 2) identify the intracellular signals by which an ovulatory dose of gonadotropin regulates PGDH mRNA, protein, and activity within primate granulosa cells.

2.0 MATERIALS AND METHODS

2.1 Animals

Granulosa cells were obtained from adult female cynomolgus macaques at Eastern Virginia Medical School (EVMS) as previously described (Duffy et al. 2005a). All animal protocols and experiments were approved by the EVMS Animal Care and Use Committee and were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. A controlled ovarian stimulation model developed for the collection of multiple oocytes for in vitro fertilization was used to obtain monkey granulosa cells (VandeVoort et al. 1989). Beginning within 3 days of initiation of menstruation, recombinant human FSH (r-hFSH, 60 IU daily, Serono Reproductive Biology Institute, Rockland, MA or Schering-Plough Corp., now Merck & Co., Inc., Whitehouse Station, NJ) was administered for 6-8 days, followed by twice daily administration of 60 IU of r-hFSH plus 60 IU r-hLH (Serono) for 2 days to stimulate the growth of multiple follicles. A GnRH antagonist, either Antide (0.5 mg/kg body weight; Serono) or Ganirelix (25 μg/kg body weight, Schering-Plough) was also

administered daily to prevent an endogenous ovulatory LH surge. Adequate follicular development was monitored by serum estradiol levels and ultrasonography (Wolf et al. 1996). Follicular aspiration was performed before (0 hour) or 12, 24, and 36 hours after administration of 1000 IU r-hCG (day 9; Serono). In spontaneous menstrual cycles, follicle rupture in monkeys occurs approximately 40 hours after the ovulatory gonadotropin surge (Weick et al. 1973), so these times span the periovulatory interval.

2.2 Granulosa Cell Enrichment and Culture

Monkey granulosa cells were obtained from follicular aspirates as described previously (Chaffin et al. 1999). Briefly, all material aspirated from an individual animal was pooled, and granulosa cells obtained represent n=1 for the experiments described below. Aspirates were subjected to centrifugation to pellet the oocytes and granulosa cells; the resulting supernatant was removed. Oocytes were mechanically removed, and a granulosa cell-enriched population of the remaining cells was obtained by Percoll gradient centrifugation. Viability of granulosa cell-enriched preparations averaged 80% as assessed by trypan blue exclusion.

Granulosa cells were plated on tissue culture plates coated with fibronectin and maintained at 37C in a humidified incubator in 5% CO_2 for up to 36 hours in serum-free DMEM-Ham's F12 medium containing insulin (2 μg/ml), transferrin (5 μg/ml), selenium (0.25 nmol), aprotinin (25 mg/ml), and human low density lipoprotein (25 μg/ml) as previously described (Duffy and Stouffer 2003). Treatments, including ciglitazone (0.3-3.0 μM, Cayman), arachidonic acid (10 μM, Cayman), indomethacin (100 nM, Sigma), hCG (100 ng/ml, Serono), 8-bromo-cAMP (8BR, 0.5 mM, Sigma), PGE2 (0.1 μg/ml, Cayman), 6-benzoyl-cAMP (6BNZ, 0.1 mM, Biolog, Bremen, GE), 8-(4-chlorophenylthio)-2’-O-methyl-cAMP (8CPT, 0.1 mM, Biolog), H89 (0.01 mM, Sigma), diltiazem (0.025 mM, Biolog), phorbol 12-myristate 13-acetate (PMA, 50 nM, Sigma), U-73122 (0.01 mM, Cayman), and vehicle (control, ≤ 0.1% DMSO or EtOH) were added when cell cultures were initiated; media were not changed during the culture interval. A single culture well was established for each treatment except for assay of PGDH activity; replicate cultures for PGDH activity experiments are described in Section 2.3.

2.3 PGDH Activity

PGDH activity converts PGE2 to stable PGE metabolites (PGEM), which can be measured in cell culture media by EIA. PGDH activity was assessed by assay of PGEM using a method similar to one previously published (Lennon et al. 1999). The general COX inhibitor indomethacin was included in all media to prevent PGE2 production. Indomethacin was used at a low concentration (100 nM) to selectively inhibit COX activity (Tegeder et al. 2001) as indomethacin can inhibit PGDH activity at higher concentrations (10-100 μM) (Cho and Tai 2002). Immediately after enrichment, granulosa cells were added to test tubes with culture media containing indomethacin or indomethacin + PGE2 (0.01-1.0 μg/ml, Cayman) as a substrate for PGEM production. After incubation at 37C in a humidified incubator in 5% CO_2 incubator for 10-120 min, cells were pelleted by centrifugation at 350 X g at 4C for 5 min. Media were immediately stored at -20C pending analysis for PGEM by EIA kit (Cayman) as previously described (Duffy et al. 2005a). For each granulosa cell preparation assayed, PGEM concentration in indomethacin-treated cultures was subtracted from the PGEM concentration in indomethacin+PGE2-treated cultures; this is referred to as PGDH Activity in Figures 2-5. Initial studies demonstrated a linear relationship between PGEM in the medium and incubation time, enzyme amount in assay (e.g., cell number), and PGE2 included in the assay as substrate for PGEM production (Figure 1). Additional preliminary studies demonstrated that PGEM concentrations were not different in media from granulosa cells obtained 36 hours after hCG and incubated for 60 min with 0.1 μg/ml

PGE2 when assayed in suspension cultures or plated in vitro (n=4, data not shown). For data presented in Figure 2, 10,000 granulosa cells were incubated with 0.1 μg/ml PGE2 for 60 minutes as a measure of PGDH activity in vivo. For granulosa cells assessed after treatment in vitro, 10,000 cells/well were plated and maintained for 12-36 hours in vitro. Media were then removed and replaced with media containing either indomethacin or indomethacin +PGE2 as described for cell suspension cultures above, then assayed for PGDH activity using 0.1 μg/ml PGE2 as substrate and a reaction time of 60 minutes as described above. Each granulosa cell preparation assessed for PGDH activity was assayed in duplicate (plated cells) or triplicate (suspension cultures). In all experiments, media were harvested and stored at -20C pending PGEM assay. Each media sample was assayed in duplicate for PGEM. The intra- and inter-assay coefficients of variation for the PGEM EIA were 7.0% and 15.8%, respectively.

In some experiments, granulosa cell conditioned media were assayed for PGE2 (in duplicate) by EIA (Cayman). The intra- and inter-assay coefficients of variation for the PGE2 EIA were 14.6% and 13.4%, respectively.

2.4 Real Time Reverse Transcription PCR (RT-PCR)

PGDH mRNA levels were analyzed by real time RT-PCR using a Roche LightCycler (Indianapolis, IN) as previously described (Duffy et al. 2005a). Cultured granulosa cells were lysed in situ with Trizol reagent (Invitrogen, Rockville, MD), and total RNA was prepared according to manufacturer's instructions with the addition of glycogen (10 μg) to improve recovery. Total RNA was incubated with DNase, and reverse transcription was performed as described previously (Chaffin and Stouffer 1999). PCR was performed using the FastStart DNA Master SYBR Green I kit (Roche) following manufacturer's instructions using 0.5 mM of each primer, 4.0 mM $MgCl_2$, and an annealing temperature of 57C. PGDH and β-actin mRNA content of each sample were determined in independent assays. PCR primers for PGDH (up: 5'-TCCAGTGCGATGTGGC; dn; 5'-GCAACGGGCATGAGTC) and β-actin (up: 5'-ATCCGCAAAGACCTGT; dn; 5'-GTCCGCTAGAAGCAT) were previously described (Duffy et al. 2005a). PCR products were 256 base pairs (Accession number AB059653) and 270 base pairs (Accession number AY765990) in length for monkey PGDH and β-actin, respectively. Each cDNA was assayed in duplicate. At least 5 log dilutions of the sequenced PCR product were included in each assay and used to generate a standard curve. Intra- and inter-assay coefficients of variation were less than 10%. All data were expressed as the ratio of PGDH mRNA to β-actin mRNA for each sample. For experiments shown in Figures 3-5, within each time point, control PGDH mRNA levels were set equal to 1.0 for each animal, and treatment PGDH mRNA levels were expressed relative to control.

2.5 Immunohistochemical Detection of PGDH

Granulosa cells were cultured on chamber slides (Nalge Nunc International, Naperville, IL, USA). At the end of the culture period, cells were fixed in 10% formalin for 30 min, and immunohistochemical detection of PGDH proceeded essentially as previously described (Duffy et al. 2005a) using an antibody directed against human PGDH (Cayman Chemical Company, 10 μg/ml) and a biotinylated bovine anti-rabbit IgG secondary antibody and peroxide conjugated avidin solution (Vector Laboratories, Burlingame, CA); peroxidase activity was visualized with nickel diaminobenzidine (Ni DAB, Vector). Slides were not counterstained. In some experiments the primary antibody was preabsorbed with the peptide used to generate the antibody (Cayman, 0.5 μg peptide/1.0 μg primary antibody) for 1 hour at room temperature before incubation with tissue sections; in other experiments the primary antibody was omitted as a negative control. Images were obtained using an Olympus BX41 microscope fitted with a DP70 digital camera and associated software (Melville, NY).

2.6 Data Analysis

All data were assessed for heterogeneity of variance using Bartlett's test and log transformed when Bartlett's test yielded a significance of <0.05; data presented in Figures 2A, 3A, 4G, 4H, 5A, and 5G were log transformed before further analysis. PGEM levels in plated and suspension cultures were compared using unpaired t-test. Data in Figure 2A were compared using one-way analysis of variance (ANOVA), followed by Duncan's Multiple Range test. PGDH mRNA and PGEM levels were compared using one-way ANOVA with one repeated measure (blocked for individual animal) within each time point as well as within each treatment over time, followed by Duncan's Multiple Range test or student's t-test as appropriate. Linear fits were obtained using Origin v7.5 (OriginLab Corp., Northampton, MA). All other analyses were performed using Statpac v4.12 (Northwest Analytical, Portland, OR, USA). Data are presented as mean ± standard error of the mean (SEM), and significance was assumed at $p<0.05$.

3.0 RESULTS

3.1 PGDH activity in monkey granulosa cells in vivo

Granulosa cells obtained from monkeys experiencing controlled ovarian stimulation before (0 hour) and 12, 24, and 36 hours after administration of an ovulatory dose of hCG were assayed for PGDH activity. PGDH activity was present in granulosa cells obtained before hCG, increased more than 6-fold 12-24 hours after hCG treatment, and declined to low levels by 36 hours hCG (Figure 2A). These hCG-induced changes in granulosa cell PGDH activity parallel changes in granulosa cell PGDH mRNA and PGDH protein as previously reported by this laboratory (Duffy et al. 2005a).

As an additional approach to assess PGDH activity, the ability of the PGDH inhibitor ciglitazone to reduce granulosa cell PGDH activity was assessed. Since PGDH converts PGE2 to PGEM, ciglitazone should increase PGE2 accumulation in culture media of granulosa cells which possess PGDH activity. Granulosa cells obtained from monkeys after controlled ovarian stimulation before (0 hour) hCG administration were cultured with ciglitazone for 24 hours (Figure 2B). Granulosa cells cultured in the absence of ciglitazone produced PGE2 in vitro (0.30 ± 0.11 ng/ml). Ciglitazone increased media PGE2 accumulation in a dose-dependent fashion, consistent with the reported IC50=3 μM of ciglitazone for PGDH (Cho and Tai 2002). Granulosa cells obtained from monkeys treated in vivo with hCG for 36 hours also produced PGE2 in the absence of ciglitazone (0.67 ± 0.15 ng/ml), and ciglitazone treatment did not alter PGE2 accumulation (Figure 2C). Taken together, these findings support the concept that granulosa cells obtained midway through the periovulatory interval possess significant PGDH activity, while granulosa cells obtained late in the periovulatory interval possess little or no PGDH activity. The low solubility of ciglitazone in aqueous media prevents testing higher concentrations of this inhibitor. However, these data are consistent with the hypothesis that hCG exposure increases, then decreases PGDH mRNA, protein, and activity within follicular granulosa cells during the periovulatory interval.

3.2 hCG regulation of PGDH mRNA, protein, and activity in vitro

To determine if hCG acts directly at granulosa cells to regulate PGDH, granulosa cells obtained from monkeys before (0 hour) hCG were cultured without hCG (control) or with an ovulatory dose of hCG (100 ng/ml) for 12, 24, or 36 hours to emulate hCG exposure during the periovulatory interval in vivo (Figure 3A). PGDH mRNA was detectable in all granulosa cell cultures examined. hCG increased mean PGDH mRNA levels after 12 hours in vitro, though the increase was not significant (p=0.10). PGDH mRNA levels were significantly

elevated by hCG treatment after 24 hours of culture, but no difference was detected between control and hCG-treated cells after 36 hours in vitro.

Immunocytochemical detection of PGDH in cultured monkey granulosa cells showed modest levels of PGDH protein for 12-36 hours in vitro in the absence of hCG treatment (Figure 3C-E). Treatment of granulosa cells with hCG for 12-24 hours in vitro yielded apparently stronger immunodetection of PGDH when compared to comparable control cultures (Figure 3GH). However, PGDH immunostaining was apparently decreased after 36 hours in vitro with hCG (Figure 3J). PGDH immunostaining was not detected in the absence of primary antibody (not shown) or when primary antibody was preabsorbed with the peptide used to generate the antibody (Figure 3F).

PGDH activity was determined for granulosa cells maintained in vitro in the absence or presence of hCG for 12, 24, and 36 hours (Figure 3B). PGDH activity was detectable in all granulosa cell cultures examined. Control levels of PGDH activity did not change significantly during the culture period, although a trend towards declining PGDH activity with time was noted ($p=0.16$). Treatment with hCG increased PGDH activity in granulosa cells treated with hCG for 24 hours when compared to control cultures, but no difference in PGDH activity was measured between control and hCG-treated granulosa cells by 36 hours after initiation of cultures. Importantly, PGDH activity in hCG-treated cells declined between 24 and 36 hours in vitro.

Overall, granulosa cells treated in vitro with hCG demonstrated changes in PGDH mRNA, protein, and enzyme activity similar to granulosa cells treated with hCG in vivo (Figure 2 and (Duffy et al. 2005a)).

3.3 Regulation of PGDH mRNA, protein, and activity via Gαs

Additional experiments were conducted to determine if cAMP generated via hCG-stimulation of Gαs can regulate PGDH via activation of PKA, activation of Epacs, or regulation of cAMP-gated ion channels.

Initial experiments utilized the general cAMP analog 8-bromo-cAMP (8BR). Granulosa cells obtained before hCG were cultured for 12, 24, or 36 hours with 8BR or vehicle (control) (Figure 4A). 8BR increased PGDH mRNA above control levels after 12, 24, and 36 hours in vitro. Granulosa cells treated with 8BR showed modest PGDH immunoreactivity after 12 hours in vitro (Figure 4D) while immunostaining for PGDH was apparently higher in 8BR-treated cells after 24 and 36 hours in vitro (Figure 4E-F). Treatment with 8BR in vitro also increased PGDH activity after 24 hours, with elevated PGDH activity maintained through 36 hours in vitro (Figure 4B).

Two approaches were used to determine if cAMP acts via PKA. To determine if blockade of PKA activity can prevent hCG-regulated changes in PGDH, granulosa cells were cultured with the PKA inhibitor H89. H89 did not alter control or hCG-stimulated PGDH mRNA levels during the 36 hour culture interval (Figure 4G) Additional granulosa cells were cultured with the PKA-selective cAMP analog 6BNZ (Christensen et al. 2003). PGDH mRNA levels were not different between control and 6BNZ-treated granulosa cells after 12 hours in vitro. 6BNZ did increase PGDH mRNA 1.6-fold above control levels after 24 hours in culture; no difference between control and 6BNZ-treated cells were measured after 36 hours in vitro (Figure 4H). To determine if cAMP acts via the Epacs to regulate PGDH, granulosa cells were treated with the Epac-selective cAMP analog 8CPT (Christensen et al. 2003). 8CPT increased PGDH mRNA 4.8-fold above control levels after 24 hours in vitro; no difference between control and 8CPT-treated granulosa cells was detected after 12 and 36 hours of treatment (Figure 4H).

To determine if blockade of cAMP-gated ion channels could prevent hCG-regulated changes in PGDH, granulosa cells were cultured with the nucleotide-gated channel blocker diltiazem. No differences between control and diltiazem-treated cells were noted at any time examined, and diltiazem did not alter hCG-stimulated increases in PGDH mRNA during culture (not shown).

3.4 Regulation of PGDH mRNA, protein, and activity via Gαq

Treatment with the PKC-activating phorbol ester PMA did not alter PGDH mRNA when compared to controls after 12 or 24 hours of culture. However, PMA decreased PGDH mRNA levels after 36 hours in vitro (Figure 5A). Immunodetection of PGDH protein was low and appeared comparable to or less than control levels at all times examined (Figure 5C-F). PGDH activity did not differ between control and PMA-treated granulosa cells at any time examined (Figure 5B). However, PMA treatment did reduce PGDH activity over the period of culture, with lowest levels of PGDH activity measured after 36 hours of PMA treatment in vitro.

To determine if PLC mediates hCG-regulated changes in granulosa cell PGDH, cells were cultured with hCG in the absence or presence of the PLC inhibitor U-73122 (Figure 5G). Neither hCG or U-73122 altered PGDH mRNA compared with control levels after 12 hours in vitro. However, the combination of hCG+U-73122 did significantly elevate PGDH mRNA above control levels. After 24 hours in vitro, treatment with hCG or U-73122 increased PGDH mRNA above control levels; the combination of hCG+U-73122 also increased PGDH mRNA above control levels. Neither hCG, U-73122, or hCG+U-73122 altered PGDH mRNA levels after 36 hours of treatment.

4.0 DISCUSSION

PGDH activity is regulated by the ovulatory gonadotropin surge in a dynamic fashion in granulosa cells of primate periovulatory follicles in vivo. Previously, we reported that granulosa cell levels of PGDH mRNA and protein were low-to-modest before administration of an ovulatory dose of hCG, high 12-24 hours after hCG, and low 36 hours after hCG (Duffy et al. 2005a). In the present study, a cell-based assay was used to demonstrate that granulosa cell PGDH activity also peaks 12-24 hours after hCG, with very low PGDH activity 36 hours after hCG, just before the expected time of ovulation. These studies show a strong correlation between PGDH mRNA, protein, and activity levels in granulosa cells of primate periovulatory follicles in vivo. Most importantly, these data support a role for the ovulatory gonadotropin surge, acting directly at granulosa cells, to regulate intrafollicular PGE2 levels via modulation of PGDH. Gonadotropin-stimulated expression of COX-2 and PGE2 production begin midway through the primate periovulatory interval (Duffy and Stouffer 2001). Elevated PGDH activity during the middle portion of the periovulatory interval may prevent accumulation of ovulatory PGE2 within the follicle. Falling PGDH activity late in the periovulatory interval may permit accumulation of the high levels of PGE2 required to facilitate ovulatory events and achieve successful ovulation.

To elucidate how the ovulatory gonadotropin surge can increase, then decrease, PGDH mRNA, protein, and activity, granulosa cells were studied in vitro. Treatment of granulosa cells with hCG in vitro led to increased PGDH mRNA, protein, and activity after 24 hours in vitro, with low levels of PGDH mRNA, protein, and activity measured after 36 hours in vitro. This pattern of rising, then falling, PGDH mRNA, protein, and activity parallels gonadotropin-stimulated changes in vivo, suggesting that an ovulatory concentration of hCG (or the midcycle LH surge in a natural menstrual cycle) acting directly at granulosa cells is sufficient to increase, then decrease granulosa cell PGDH mRNA, protein, and activity. For this reason, studies to examine hCG action directly on granulosa cells and the intracellular

signals which regulate PGDH were pursued in vitro. Activators and inhibitors of specific signaling pathways were utilized to identify specific mediators of the gonadotropin signal regulated via the LH/CG receptors (Davis 1994).

Activation of components of the Gαs-coupled signal transduction pathway increased PGDH. The cAMP analog 8BR increased PGDH mRNA, protein and activity. Elevated PGDH mRNA, protein, and activity were maintained throughout the 36 hour culture period by this very potent cAMP analog. The PKA inhibitor H89 did not block hCG-mediated changes in PGDH mRNA, suggesting that cAMP does not act primarily via PKA to regulate PGDH. The cAMP analog 6BNZ (which activates PKA) increased PGDH mRNA less than 2-fold over control levels. In contrast, the Epac-activating cAMP analog 8CPT increased PGDH mRNA more than 4-fold over levels in control cultures, suggesting that cAMP acts primarily via the Epacs to increase PGDH in granulosa cells. 6BNZ and 8CPT are relatively labile in aqueous solution, which may explain the transient nature of activation with these compounds (Holz et al. 2008). The action of cAMP via PKA is well established in granulosa cells (Conti 2002). However, a previous study demonstrated that gonadotropin-stimulated cAMP can act via Epacs in granulosa cells to regulate progesterone production (Chin and Abayasekara 2004). While the cascade of events downstream from Epacs is uncertain, gonadotropin likely acts via the LH/CG receptor, Gαs, cAMP, and Epacs to increase granulosa cell PGDH mRNA, protein, and activity early in the periovulatory interval.

Gonadotropin stimulation of the LH/CG receptor coupled to Gαq may be responsible for declining PGDH late in the periovulatory interval. The phorbol ester PMA, which activates PKC, did not alter PGDH mRNA 12-24 hours after initiation of cultures. PMA did lead to decreased PGDH mRNA and activity after 24-36 hours of treatment in vitro. As a complementary approach, the inhibitor U-73122 was used to block PLC activation in response to LH/CG receptor stimulation. U-73122 increased PGDH mRNA after 24 hours in vitro, with a trend towards additive effects of hCG and U-73122 to further increase PGDH mRNA levels. U-73122 did not maintain increased PGDH mRNA after 36 hours in vitro, perhaps due to the relatively short half-life of this inhibitor in culture media (Wilsher et al. 2007). Overall, hormone binding to the LH/CG receptor coupled to Gαq and mediated via PLC and PKC decreased PGDH mRNA, protein and activity late in the culture period. Previous studies have identified roles for PKC in luteinizing granulosa cells and granulosa-derived cells of the young corpus luteum to regulate angiogenesis and gap junction activity (Grazul-Bilska et al. 2001; Witt et al. 2004). Better understood is the role of PKC in mature luteal cells, where this important kinase mediates hormonal signals to initiate luteolysis (Wiltbank et al. 1991). Granulosa cells begin the process of luteinization prior to ovulation (Stouffer et al. 2007), so coupling of the LH/CG receptor to the Gαq pathway may occur late in the periovulatory interval as granulosa cells luteinize. Gonadotropin action via the LH/CG receptor coupled to Gαq may be responsible for the decline in PGDH mRNA, protein, and activity late in the periovulatory interval, which permits PGs to accumulate to levels which will trigger ovulatory events.

This report is the first detailed examination of the regulation of PGDH mRNA, protein, and activity in ovarian granulosa cells. However, PGDH has been examined in numerous nonovarian cells and tissues. The 5’-flanking region of the human PGDH gene contains binding sites for the cAMP-response element binding protein (CREB), AP-1, and Ets (Nandy et al. 2003). While the PKA-CREB pathway is active in granulosa cells (Mukherjee et al. 1996), cyclic AMP appears to increase PGDH mRNA levels in primate granulosa cells via Epacs. Epacs can activate Rap and MAPK family members (Holz et al. 2008), but how these signals may increase PGDH mRNA levels is currently unknown. In contrast, PKC activation decreased PGDH mRNA levels in primate granulosa cells. PKC may regulate transcription of PGDH via AP-1 and Ets as previously described for other genes (Nandy et

al. 2003). The ability of cAMP or PKC to modulate PGDH promotor activity differs between cell lines, so cell-specific factors likely modulate expression of PGDH in each type of cell (Greenland et al. 2000). For example, cAMP generally stimulates PGDH expression, but cAMP decreased PGDH expression and activity in human trophoblast cells (Lennon et al. 1999). Though PMA generally increases PGDH expression (Tai et al. 2006), the ability of PMA to decrease PGDH expression has been reported (Casciani et al. 2008). These examples provide precedent for the regulation of PGDH observed in the present study, where cAMP/Epac activation increased, and PLC/PKC activity decreased, PGDH mRNA, protein, and activity.

Elevated PGs in the primate follicle are a late signal in the cascade of events which lead to successful ovulation. The ovulatory surge of gonadotropin increases granulosa cell levels of COX-2 mRNA and protein, thought to catalyze the rate-limiting step in PG production (Duffy and Stouffer 2001; Sirois and Dore 1997; Wong et al. 1989). There is a strong correlation between this increased expression of PG synthesis enzymes, follicular PG levels, and follicle rupture in primates (Duffy et al. 2005a; Duffy et al. 2005b; Duffy et al. 2005c; Duffy and Stouffer 2002). While levels of PG synthesis enzymes increase rapidly after the ovulatory surge of gonadotropin, follicular PG levels increase slightly from 0 to 24 hours and do not reach peak levels in primate follicles until 36 hours after hCG, or just before ovulation, leading to the hypothesis that PG metabolism may play a role in the regulation of intrafollicular PG levels. The present study, coupled with our previous work (Duffy et al. 2005a), demonstrates that PGDH mRNA, protein, and activity increase, then decrease after the ovulatory gonadotropin surge. Elevated PGDH activity may maintain low intrafollicular PG levels until midway through the periovulatory interval, when continued PG synthesis and falling PGDH activity lead to rising PG levels, which trigger events which lead to successful ovulation. Direct testing of this hypothesis in vivo will require development of highly-soluble, specific inhibitors of PGDH. Inhibition of follicular PGDH activity may lead to premature accumulation of PGs and premature or dysfunctional ovulation. In converse, maintenance of elevated PGDH activity may prolong the periovulatory interval and delay or prevent ovulation. Pharmacological control of follicular PGDH activity may be helpful in the treatment of some types of infertility or in the development of novel contraceptives.

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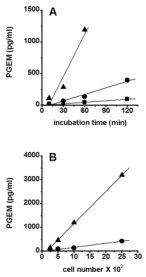

Figure 1.

PGDH activity as assessed by conversion of PGE2 to PGEM. Panel A. Granulosa cells (10,000 cells/reaction) were incubated in the presence of PGE2 (▲, 1.0 μg/ml; •, 0.1 μg/ml; ■, 0.01 μg/ml) for 10-120 minutes. Panel B. Granulosa cells (2,500-25,000 cells/reaction) were incubated in the presence of PGE2 (▲, 1.0 μg/ml; •, 0.1 μg/ml) for 60 minutes. Media PGEM was determined by EIA.

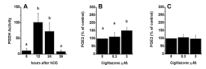

Figure 2.

PGDH activity in monkey granulosa cells obtained throughout the periovulatory interval. Panel A. Granulosa cells from monkeys experiencing controlled ovarian stimulation were obtained before (0 hour) and 12, 24, and 36 hours after administration of an ovulatory dose of hCG. PGDH activity was assessed by conversion of PGE2 to PGEM (see Figure 1). Panels B-C. Ciglitazone inhibition of PGE2 accumulation in media of granulosa cell cultures. Granulosa cells obtained before (B) or 36 hours after (C) hCG administration were incubated for 24 hours with vehicle (0.1% DMSO; control) or with the PGDH inhibitor ciglitazone (0.3-3.0 μM); all cultures contained arachidonic acid (10 μM) as substrate for PGE2 synthesis. Media PGE2 levels were determined by EIA. For each animal, PGE2 levels in ciglitazone-treated cultures are expressed as a percentage of control PGE2 levels. Within each panel, treatment groups with no common superscripts are different, $p<0.05$. Data are expressed as mean ± SEM; n=3-4 animals/treatment group.

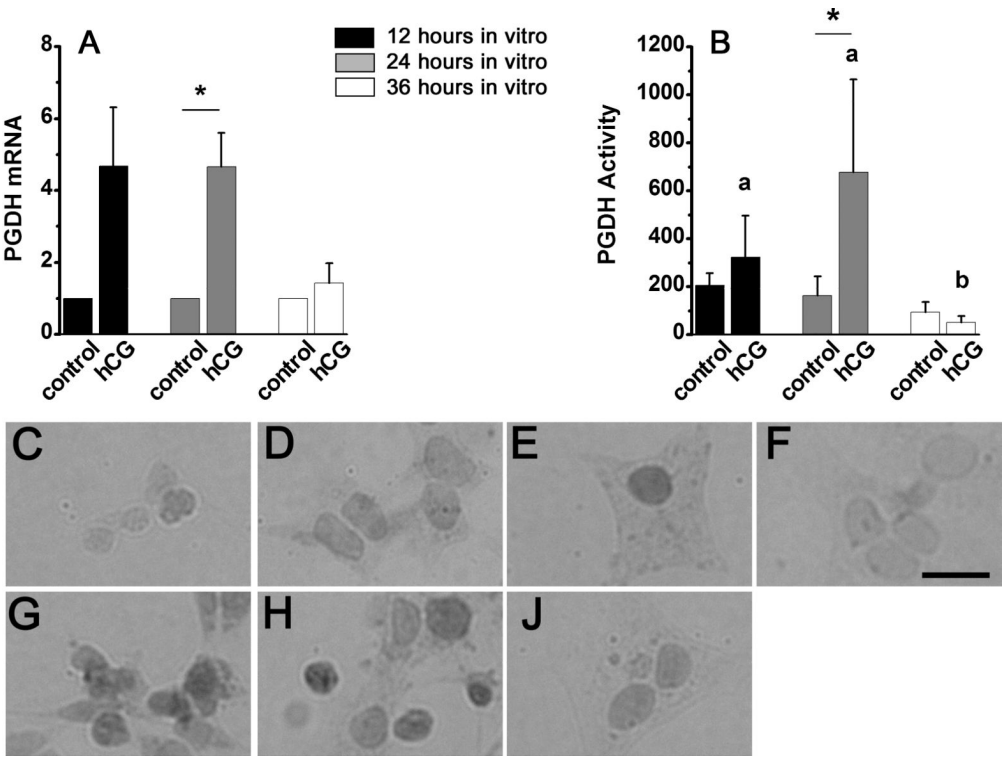

Figure 3.

hCG regulates PGDH mRNA, protein, and activity in monkey granulosa cells in vitro. Granulosa cells obtained from large follicles before administration of hCG were treated without (control) or with hCG (100 ng/ml) for 12, 24, or 36 hours in vitro. A. RNA harvested from cultured cells was assessed for PGDH mRNA by real time RT-PCR and expressed relative to the β-actin mRNA level in each granulosa cell sample (n=4-5 animals/ treatment group). B. PGDH activity was assessed by conversion of PGE2 to PGEM (n=4 animals/treatment group). For Panels A and B, within each time point, control and hCG are different as indicated by the asterisk (*), $p<0.05$. Within hCG-treated cells, groups with no common superscripts are different, $p<0.05$. Data are expressed as mean ± SEM. Panels C-J. Modest levels of PGDH protein were detected by immunostaining in granulosa cells cultured without hCG for 12 hours (C), 24 hours (D), and 36 hours (E). Granulosa cells cultured with hCG for 12 hours (G) and 24 hours (H) showed stronger immunodetection of PGDH; little PGDH was detected after 36 hours with hCG in vitro (J). Dark precipitate represents immunodetection of PGDH; cells were not counterstained. Reduced immunostaining was observed when the primary antibody was omitted (not shown) or preabsorbed with the peptide used to generate the primary antibody (F). PGDH protein detection is representative of granulosa cells from n=3 animals.

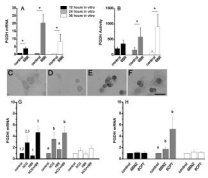

Figure 4.

cAMP regulates PGDH mRNA, protein, and activity. Granulosa cells obtained from large follicles before administration of hCG were maintained in vitro without treatment (control) or with treatments as described below for 12, 24, or 36 hours. A. RNA harvested from untreated cells (control) and cells treated with the cAMP analog 8BR (0.5 mM) was assessed for PGDH mRNA by real time RT-PCR and expressed relative to the β-actin mRNA level in each sample (n=4-5 animals/treatment group). B. PGDH activity was assessed by conversion of PGE2 to PGEM (n=4 animals/treatment group). Within each time point, control and 8BR are different as indicated by the asterisk (*), $p<0.05$. Modest levels of PGDH protein were detected by immunostaining in granulosa cells cultured with 8BR for 12 hours (D); strong PGDH immunodetection was observed after treatment with 8BR for 24 hours (E) and 36 hours (F). PGDH immunostaining in control cells after 24 hours in vitro is also shown (C). PGDH protein detection is representative of granulosa cells from n=3 animals. G. Additional granulosa cells were treated for 12-36 hours in vitro with hCG (100 ng/ml), the PKA inhibitor H89 (0.01 mM), hCG+H89, or no treatment (control); PGDH mRNA was assessed as described above (n=4 animals/treatment group). H. Granulosa cell treated for 12-36 hours in vitro with the cAMP analogs 6BNZ (0.1 mM) or 8CPT (0.1 mM); PGDH mRNA was assessed as described above (n=5-8 animals/treatment group). For Panels G-H, within each time point, groups with no common superscripts are different, $p<0.05$. Data are expressed as mean ± SEM.

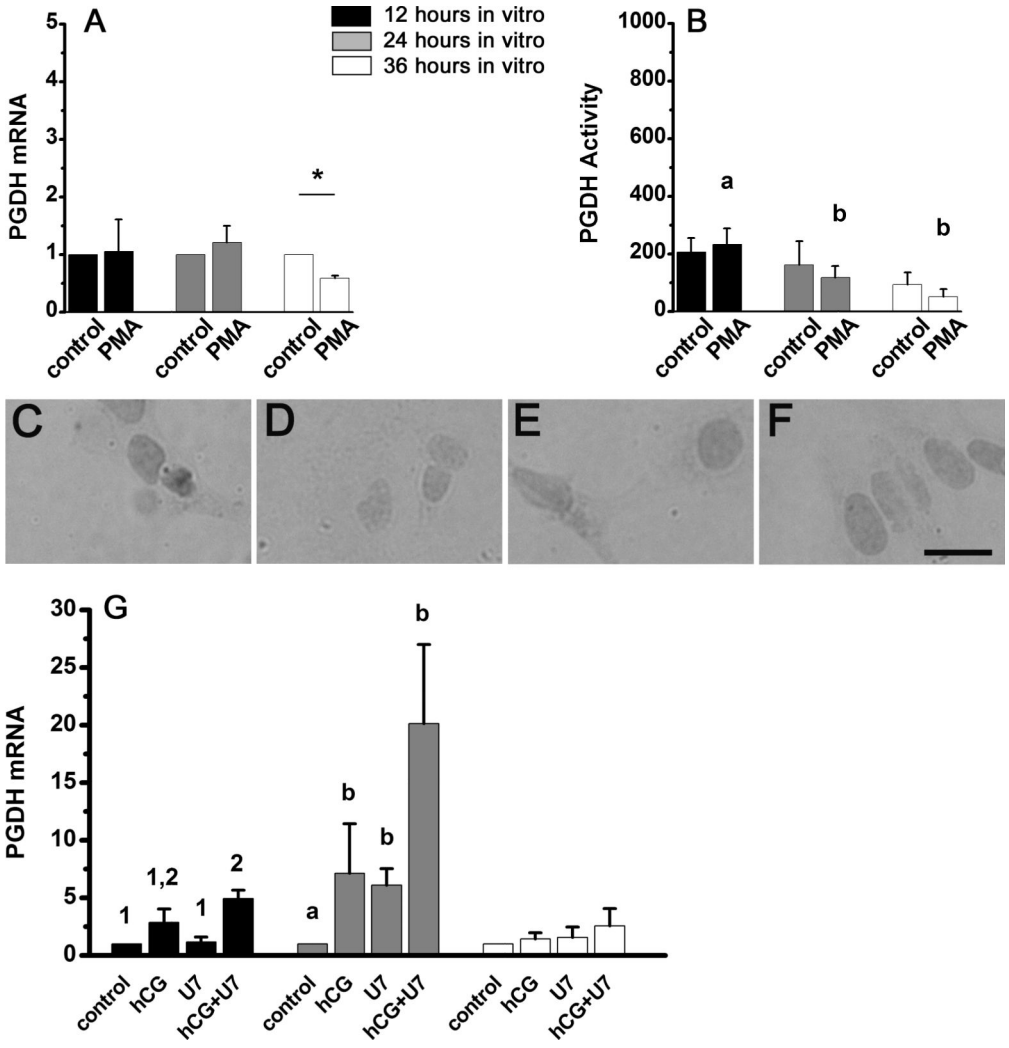

Figure 5.

PLC and PKC regulate PGDH mRNA, protein, and activity. Granulosa cells obtained from large periovulatory follicles before administration of hCG were maintained in vitro without treatment (control) or with treatments as described below for 12, 24, or 36 hours. A. RNA harvested from untreated cells (control) and cells treated with the PKC activator PMA (50 nM) was assessed for PGDH mRNA by real time RT-PCR and expressed relative to the β-actin mRNA level in each sample (n=4 animals/treatment group). Within each time point, control and PMA are different as indicated by the asterisk (*), $p<0.05$. B. PGDH activity was assessed by conversion of PGE2 to PGEM (n=4/group). Within PMA-treated cells, groups with no common superscripts are different, $p<0.05$. Immunodetection of PGDH protein in granulosa cells was similar to or less than that observed in control cells after treatment with PMA for 12 hours (D), 24 hours (E), and 36 hours (F) in vitro. PGDH immunostaining in control cells after 24 hours in vitro is also shown (C). PGDH protein detection is representative of granulosa cells from n=3 animals. G. Additional granulosa cells were treated for 12-36 hours in vitro with hCG (100 ng/ml), the PLC inhibitor U-71322 (U7, 0.01 mM), hCG+U7, or no treatment (control); PGDH mRNA was assessed as described above (n=4-6 animals/treatment group). Within each time point, groups with no common superscripts are different, $p<0.05$. Data are expressed as mean ± SEM.