Two pathways for prostaglandin F2 α synthesis by the primate periovulatory follicle

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Abstract

Prostaglandin E2 (PGE2) has been identified as a PG necessary for ovulation, but the ovulatory gonadotropin surge also increases PGF2 α levels in primate periovulatory follicles. To better understand the role of PGF2 α in ovulation, pathways utilized for PGF2 α synthesis by the primate follicle were examined. Monkeys were treated with gonadotropins to stimulate multiple follicular development; follicular aspirates and whole ovaries were removed before and at specific times after administration of an ovulatory dose of hCG to span the 40 h periovulatory interval. Human granulosa cells were also obtained (typically 34–36 h after hCG) from *in vitro* fertilization patients. PGF2 α can be synthesized from PGH2 via the aldo-keto reductase (AKR) 1C3. AKR1C3 mRNA and protein levels in monkey granulosa cells were low before hCG and peaked 24–36 h after hCG administration. Human granulosa cells converted PGD2 into 11 β -PGF2 α , confirming that these cells possess AKR1C3 activity. PGF2 α can also be synthesized from PGE2 via the enzymes AKR1C1 and AKR1C2. Monkey granulosa cell levels of AKR1C1/AKR1C2 mRNA was low 0–12 h, peaked at 24 h, and returned to low levels by 36 h after hCG administration. Human granulosa cell conversion of [³H]PGE2 into [³H]PGF2 α was reduced by an AKR1C2-selective inhibitor, supporting the concept that granulosa cells preferentially express AKR1C2 over AKR1C1. In summary, the ovulatory gonadotropin surge increases granulosa cell expression of AKR1C1/AKR1C2 and AKR1C3. Both of these enzyme activities are present in periovulatory granulosa cells. These data support the concept that follicular PGF2 α can be synthesized via two pathways during the periovulatory interval.

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Introduction

Prostaglandin (PG) production by the follicle in response to the midcycle luteinizing hormone (LH) surge is necessary for ovulation to occur. Follicular fluid concentrations of both PGE2 and PGF2 α peak just before the expected time of ovulation in primates as in many mammalian species (Sirois 1994, Sirois & Dore 1997, Duffy & Stouffer 2001). While studies with PGF2 α receptor knockout mice suggest that PGF2 α is not required for ovulation (Sugimoto *et al.* 1997), subfertility in mice lacking expression of the PGE2 receptor EP2 focused attention on PGE2 as the key ovulatory PG (Hizaki *et al.* 1999). Blockade of PG production within the follicle prevented cumulus expansion, follicle rupture, and oocyte release; restoration of periovulatory events with coadministration of the PG synthesis inhibitor and PGE2 identified PGE2 as a key mediator of ovulation in mammals (Sogn *et al.* 1987, Peters *et al.* 2004), including primates (Duffy & Stouffer 2002). However, additional studies have demonstrated restoration of ovulation with PGF2 α administration (Wallach *et al.* 1975, Murdoch *et al.* 1986, Sogn *et al.* 1987, Janson *et al.* 1988), suggesting that PGF2 α may also play a role in ovulatory processes.

Synthesis of PGF2 α begins with conversion of arachidonic acid into the unstable intermediate PGH2 through the activity of cyclooxygenase (COX; Vane *et al.* 1998) (Fig. 1). PGH2 can then be converted to bioactive PGs by specific PG synthases. 11 α -PGF2 α (traditionally referred to as PGF2 α) can be produced from either PGH2 or PGE2; a third pathway produces 11 β -PGF2 α from PGH2. All enzymes that produce PGF2 α are members of the aldo-keto reductase (AKR) family (Nishizawa *et al.* 2000). PGH2 can be directly converted to PGF2 α by the action of AKR1C3, also known as PGF synthase (Suzuki-Yamamoto *et al.* 1999); this same enzyme converts PGD2 into 11 β -PGF2 α . 11 β -PGF2 α is equipotent with PGF2 α in the stimulation of PGF2 α receptors (Sugimoto *et al.* 1994). Finally, two closely related enzymes, AKR1C1 and AKR1C2, function as PGE-F isomerases, which interconvert PGE2 and PGF2 α (Nishizawa *et al.* 2000).

To better understand the pathways utilized for PGF2 α synthesis by the primate follicle, follicular expression and activity of enzymes that can produce PGF2 α were assessed. Previous studies by our laboratory demonstrated that the ovulatory gonadotropin surge increases

Figure 1 Three candidate pathways for the synthesis of PGF2 α .

A cyclooxygenase (COX) enzyme produces PGH2 from arachidonic acid. PGF2 α can be synthesized either directly from PGH2 via AKR1C3 or indirectly from PGE2 via AKR1C1 and AKR1C2. 11 β -PGF2 α can be produced from PGH2 via PGD synthase (PGDS) and AKR1C3. PGF2 α is converted to inactive PGF metabolites (PGFM) via the 15-hydroxy PG dehydrogenase (PGDH). Adapted from Watanabe (2002).

granulosa cell expression of key enzymes required for PGE2 synthesis; increased enzyme levels parallel the rising follicular concentration of PGE2 just before ovulation (Duffy & Stouffer 2001, Duffy *et al.* 2005a, 2005b). We hypothesize that the ovulatory gonadotropin surge also stimulates granulosa cell expression of enzymes necessary for the synthesis of PGF2 α . Similarities in periovulatory processes in humans and nonhuman primates are well established (Zelevnik *et al.* 1994). The majority of the studies presented below were conducted using a nonhuman primate, the cynomolgus macaque. Additional experiments used human luteinizing granulosa cells to extend findings to the human periovulatory follicle.

Results

Follicular fluid PGF2 α , PGFM, and 11 β -PGF2 α concentrations

Follicular fluid contained measurable concentrations of PGF2 α throughout the periovulatory interval (Fig. 2). Similar to our previous report of PGF2 α levels in rhesus monkey follicular fluid (Duffy & Stouffer 2001) and PGE2 levels in cynomolgus monkey follicular fluid (Duffy *et al.* 2005c), levels of PGF2 α in follicular fluid of cynomolgus macaques were lowest before (0 h) hCG administration, rose slightly by 12–24 h after hCG, and reached peak levels 36 h after hCG administration, just before the expected time of ovulation ($n=5$ –6/group). Levels of the primary PGF2 α metabolite (PGFM) were also low at 0 h hCG. Mean PGFM levels were higher (though not significantly elevated) 12–24 h after hCG and were highest 36 h after hCG administration ($n=5$ /group). By contrast, 11 β -PGF2 α was not detected in the majority of follicular fluid samples assayed (not shown). No samples obtained 0–12 h after hCG ($n=4$ /time point) and only one out of four samples obtained 24 h after hCG

Figure 2 (A) PGF2 α and (B) PGFM levels in monkey follicular fluid. Follicular fluid was obtained from monkeys experiencing controlled ovarian stimulation before (0 h) and 12, 24, and 36 h after administration of an ovulatory dose of hCG. PG levels were determined by EIA. Within each figure, groups with different superscripts are different by ANOVA and Newman–Keuls test, $P<0.05$. Data are presented as mean $\pm$ s.e.m., $n=5$ –6/group.

administration contained detectable 11 β -PGF2 α . In follicular fluid samples obtained 36 h after hCG, three out of five contained detectable 11 β -PGF2 α , which in these samples was 6.9 ± 3.2 ng/ml.

Expression of PGF2 α synthesis enzymes

AKR1C3 directly converts PGH2 into PGF2 α (Fig. 1). Granulosa cell mRNA levels of AKR1C3 were low at 0 h hCG, intermediate 12 h after hCG, peaked 24 h after hCG, and fell to intermediate levels 36 h after hCG administration (Fig. 3A, $n=4$ –5/group). AKR1C3 protein levels in granulosa cell lysates were assessed by Western blotting. In all monkey granulosa cell lysates examined, AKR1C3 was detected as a single band of 36 kDa (Fig. 3E), consistent with previous detection of a single 36.8 kDa protein in human lung (Suzuki-Yamamoto *et al.* 1999). Granulosa cell levels of AKR1C3 protein were low at 0 h, rose to intermediate levels 12–24 h after hCG, and peaked 36 h after hCG administration (Fig. 3B, $n=4$ /group). AKR1C3 protein was detected in granulosa cells of periovulatory follicles obtained before (0 h) and at all times after hCG administration by immunohistochemistry (Fig. 4A–C). Stromal cells consistent with identification as theca cells of monkey periovulatory follicles were consistently AKR1C3-negative. AKR1C3 detection in luminal epithelial cells of the monkey seminal vesicle served as a positive control (Fig. 4D).

PGE2 and PGF2 α can be interconverted via the activity of AKR1C1 and AKR1C2 (Fig. 1). Levels of AKR1C1/AKR1C2 mRNA were detectable at 0–12 h after hCG, elevated by 24 h after hCG, then returned to 0 h levels by 36 h after hCG administration (Fig. 3D, $n=4$ /group). An antibody recognizing monkey AKR1C1 and/or AKR1C2 protein could not be identified, so *in situ* hybridization with an antisense oligonucleotide probe common to both AKR1C1 and AKR1C2 mRNA was used

Figure 3 Granulosa cell levels of PGF2 α synthesis enzyme mRNA and protein. Granulosa cells obtained from monkeys experiencing controlled ovarian stimulation before (0 h) and 12, 24, and 36 h after hCG were assessed for (A) *AKR1C3* mRNA and (B) protein levels by real-time RT-PCR and Western blotting respectively. (C) Granulosa cell level of mRNA for *AKR1C1/AKR1C2* was determined by real-time RT-PCR. (D) Representative gel shows cDNA amplified by real-time RT-PCR representing detection of mRNA for β -actin (lane 1), *AKR1C1/AKR1C2* (lane 2), and *AKR1C3* (lane 3) in monkey granulosa cells; negative control is also shown (RT omitted, lane 4). (E) A representative Western blot shows detection of AKR1C3 at 36 kDa in lysates of monkey granulosa cells obtained 0, 12, 24, and 36 h after hCG; detection of tubulin in each sample is also shown. All enzyme mRNA levels were normalized to the level of β -actin mRNA in the same sample. Western blotting data were normalized to tubulin content of each sample. Within each figure, groups with no common superscripts are different by ANOVA and Newman-Keuls test, $P < 0.05$. Data are presented as mean $\pm$ S.E.M., $n = 4-5$ /group.

to localize these mRNAs to the cells of the monkey periovulatory follicle (Fig. 4E-G). Dark purple precipitate representing staining for *AKR1C1/AKR1C2* mRNA was observed in granulosa cells of monkey ovarian follicles obtained 0, 12, 24, and 36 h after hCG administration. Staining was occasionally noted in the

stroma immediately surrounding monkey periovulatory follicles, consistent with the location of theca cells (not shown). However, ovarian stroma located at a distance from follicles was generally devoid of staining for *AKR1C1/AKR1C2* mRNA (Fig. 4H). *AKR1C1/AKR1C2* mRNA was also detected in small blood vessels (Eyster

Figure 4 Localization of AKR1C3 protein as well as PGE-F isomerase (*AKR1C1/AKR1C2*) mRNA to cells of monkey follicles obtained (A and E) 0, 12 (not shown), (B and F) 24, and (C and G) 36 h after hCG administration. All follicles are shown with stroma (st) in lower left, granulosa cells (gc) in central, and the follicle antrum in the upper right of the image. AKR1C3 was detected by immunocytochemistry (red/brown) in granulosa cells of monkey follicles (A-C); stromal cells were consistently devoid of staining. No staining was observed when the primary antibody was omitted (inset, A). Monkey seminal vesicle showed intense immunostaining in the luminal epithelium, but not stroma (st), which served as a positive control (D). Sections in (A-D) were not counterstained. *AKR1C1/AKR1C2* mRNA was detected by *in situ* hybridization in granulosa cells of monkey follicles (E-G); staining appears dark purple (arrowhead). Inset in (F) shows absence of dark purple staining in granulosa cells when sense probe was used; only nuclear counterstain (pink) is visible. Small vessels in the monkey ovarian stroma stained for *AKR1C1/AKR1C2* mRNA (H, arrows) and served as a positive control; ovarian stroma located away from follicles did not stain. An enlarged view of image in (H, inset) shows staining in cells near the vessel lumen. For (A-D), bar in (G) is 50 μ m. For (E-H), bar in (G) is 25 μ m. Data shown are representative of $n = 3-4$ animals/group.

et al. 2007) within the ovarian stroma, which served as a positive control (Fig. 4H).

Expression of 11 β -PGF2 α synthesis enzymes

Production of 11 β -PGF2 α requires the activity of PGD synthase (PGDS) to convert PGH2 into PGD2, then AKR1C3 converts PGD2 into 11 β -PGF2 α (Fig. 1). Granulosa cells expressed *H-PGDS*; mRNA levels did not change in response to hCG administration ($n=4-6$ /group, data not shown). Similarly, granulosa cells expressed *L-PGDS* mRNA, but levels were not altered by exposure to an ovulatory dose of hCG ($n=4$ /group, data not shown).

These data support the identification of two pathways that may produce PGF2 α in the primate periovulatory follicle. *AKR1C3* mRNA and protein are expressed in monkey granulosa cells in a manner consistent with the rise in PGF2 α measured in follicular fluid late in the periovulatory interval; previous studies demonstrated that the inducible form of COX2 is expressed by primate granulosa cells to provide PGH2 as substrate for PGF2 α production via *AKR1C3* (Duffy & Stouffer 2001, Duffy *et al.* 2005b). Granulosa cell expression of *AKR1C1/AKR1C2* mRNA also increased late in the periovulatory interval, and high follicular fluid levels of PGE2 produced by the PGE synthase mPGES-1 (Duffy *et al.* 2005a) would provide adequate substrate for PGF2 α production via this enzyme. By contrast, very low levels of 11 β -PGF2 α measured in follicular fluid (above) and granulosa cell cultures (below) argue against a key role for this PG in mediating periovulatory events. Therefore, the following studies focused on PGF2 α production via *AKR1C3* and *AKR1C1/AKR1C2*.

Gonadotropin regulation of PGF2 α synthesis enzyme expression *in vitro*

The ovulatory gonadotropin surge increases granulosa cell levels of mRNA for *AKR1C3* and *AKR1C1/AKR1C2* *in vivo* (Fig. 3). To determine if hCG acts directly at granulosa cells to modulate expression of these mRNAs, granulosa cells obtained from large periovulatory follicles before (0 h) administration of hCG were placed *in vitro* and treated with an ovulatory dose of hCG (Zelinski-Wooten *et al.* 1997). *AKR1C3* mRNA levels were not different between control and hCG-treated granulosa cells after 24 and 48 h *in vitro* (Fig. 5A, $n=4-6$ /group). By contrast, hCG treatment for 24 h increased expression of mRNA for *AKR1C1/AKR1C2* eightfold above mRNA levels measured in control cells (Fig. 5B, $n=5$ /group). After 48 h *in vitro*, *AKR1C1/AKR1C2* mRNA levels in control and hCG-treated granulosa cells were not different. This pattern of gonadotropin-regulated expression of mRNA for

Figure 5 Granulosa cell expression of PGF2 α synthesis enzyme mRNAs *in vitro*. Granulosa cells obtained from monkeys experiencing ovarian stimulation before administration of hCG were cultured in the absence (control) and presence of hCG (100 ng/ml) for 24 (white bars) or 48 (black bars) h. Total RNA was assessed for (A) *AKR1C3* mRNA and (B) *AKR1C1/AKR1C2* mRNA by real-time RT-PCR. All enzyme mRNA levels were normalized to the level of β -actin mRNA in the same sample. Then, mRNA level after hCG treatment was expressed as the fold increase over control cells collected after the same number of hours *in vitro*. Groups with no common superscripts are different by paired *t*-test, $P<0.05$. Data are presented as mean $\pm$ S.E.M., $n=4-6$ /group.

AKR1C1/AKR1C2 was very similar to that seen in granulosa cells exposed to hCG *in vivo* (Fig. 3C).

PGF2 α synthesis enzyme activity in human luteinizing granulosa cells

The PGF2 α precursor PGH2 is highly unstable in solution, so *AKR1C3* activity was examined by assessing the conversion of PGD2 to 11 β -PGF2 α . Human granulosa cells produce very little 11 β -PGF2 α *in vitro* in the absence of added PGD2 (3.4 ± 0.7 pg/ml media, $n=9$); media levels of 11 β -PGF2 α were 60-fold higher in the presence of added PGD2 (Fig. 6A and B). Synthesis of 11 β -PGF2 α from PGD2 was reduced by the *AKR1C3* inhibitor medroxyprogesterone acetate (MPA) in a dose-dependent manner (Fig. 6A), consistent with the reported IC50 of 0.28 μ M (Higaki *et al.* 2003), while the structurally-similar molecule cholesterol had no effect on 11 β -PGF2 α synthesis and served as a negative control (data not shown). To ensure that MPA inhibited *AKR1C3* activity independent of the ability of MPA to regulate transcription via the progesterone receptor, additional cells were cultured with MPA and the progesterone receptor antagonist mifepristone (El-Ashry *et al.* 1989); *AKR1C3* activity in the presence of MPA and mifepristone was similar to *AKR1C3* activity in the presence of MPA alone (data not shown). As an additional approach, the PG analog bimatoprost was also used to inhibit *AKR1C3* activity. Bimatoprost reduced 11 β -PGF2 α production by human luteinizing granulosa cells in a dose-dependent manner (Fig. 6B), consistent with the reported IC50 of 5 μ M (Koda *et al.* 2004).

Figure 6 Activity of PGF2 α synthesis enzymes in human luteinizing granulosa cells. (A) Granulosa cells were incubated with the precursor PGD2 alone (0 μ M) or with the AKR1C3 inhibitor medroxyprogesterone acetate (MPA); media 11 β -PGF2 α concentrations were determined by EIA ($n=4$ /group). (B) Granulosa cells were incubated with the precursor PGD2 alone (0 μ M) or with the AKR1C3 inhibitor bimatoprost; media 11 β -PGF2 α concentrations were determined by EIA ($n=4$ –5/group). (C) To assess AKR1C1 and AKR1C2 activity, human luteinizing granulosa cells were incubated with [3 H]PGE2 in the absence (0 μ M) or presence of the AKR1C2-selective inhibitor taurodeoxycholic acid (Tauro); PGs were separated by thin layer chromatography. Data are expressed as the percentage of total dpms recovered which comigrated with PGF2 α after inhibitor treatment relative to the percentage of total dpms comigrating with PGF2 α in untreated cells ($n=3$ –9/group). Within each figure, groups with no common superscripts are different by ANOVA and Newman–Keuls test, $P<0.05$; data are presented as mean $\pm$ S.E.M..

AKR1C1 and AKR1C2 possess bidirectional PGE-F isomerase activity and are capable of converting PGE2 to PGF2 α as well as PGF2 α to PGE2 (Nishizawa *et al.* 2000). To determine if human luteinizing granulosa cells

convert PGE2 to PGF2 α , cells were incubated with [3 H]PGE2, and production of [3 H]PGF2 α was assessed (Fig. 6C). Granulosa cells converted $9.2 \pm 1.3\%$ of [3 H]PGE2 to [3 H]PGF2 α during the 4 h incubation period. This conversion was inhibited by taurodeoxycholic acid in a dose-sensitive manner, consistent with the reported IC50 for inhibition of AKR1C2 (0.56 μ M) but not AKR1C1 (61 μ M) (Bauman *et al.* 2005). Human luteinizing granulosa cells incubated with [3 H]PGF2 α did not produce [3 H]PGE2 ($n=3$, not shown), so the PGE-F isomerase activity in primate granulosa cells is predominantly or exclusively in the direction of PGE2 to PGF2 α and is catalyzed by AKR1C2.

Discussion

This report is the first to demonstrate that the primate follicle expresses the enzymes and possesses the enzymatic activities required for two pathways of PGF2 α synthesis. Follicular PGF2 α levels peak just before ovulation. PGFM levels in follicular fluid increase in parallel with PGF2 α , supporting the concept that the rate of PGF2 α synthesis peaks late in the periovulatory interval. Granulosa cells of the periovulatory follicle contain enzymatic activities capable of PGF2 α production via AKR1C3 (from PGH2) and via AKR1C2 (from PGE2). By contrast, 11 β -PGF2 α production via AKR1C3 (from PGD2) is unlikely to contribute to bioactive PGF2 α levels within the follicle. AKR1C3 mRNA and protein reach maximal levels in monkey granulosa cells late in the periovulatory interval, and human granulosa cells obtained just before ovulation possess AKR1C3 activity. AKR1C1/AKR1C2 mRNA peaks just before follicular fluid PGF2 α levels reach maximum levels in monkey follicles, and human granulosa cells obtained just before ovulation can convert PGE2 to PGF2 α via AKR1C2. Taken together, these data support the conclusion that PGF2 α can be produced by the primate periovulatory follicle via two pathways.

Primate granulosa cells likely convert PGH2 to PGF2 α via AKR1C3, the traditional PGF synthase (Watanabe 2002). AKR1C3 mRNA and protein were detected in periovulatory monkey granulosa cells, and peak AKR1C3 protein levels coincided with peak follicular fluid PGF2 α concentrations. Interestingly, hCG increased granulosa cell AKR1C3 mRNA level *in vivo*, but not *in vitro*, suggesting that elevated AKR1C3 expression *in vivo* may require participation of a non-granulosa cell type, either as the site of gonadotropin action or for the production of a necessary permissive factor. Human luteinizing granulosa cells possess AKR1C3 activity just before ovulation; these cells converted PGD2 to 11 β -PGF2 α , and both MPA and bimatoprost reduced 11 β -PGF2 α production. The use of two chemically disparate inhibitors supports the conclusion that these agents act directly at the enzyme to reduce AKR1C3 activity as previously reported (Higaki

et al. 2003, Koda *et al.* 2004), not via transcriptional regulation of the *AKR1C3* gene via the progesterone receptor (in the case of MPA) or the PGF2 α receptor (in the case of bimatoprost). These data represent the first demonstration of gonadotropin-regulated *AKR1C3* expression and activity in the mammalian periovulatory follicle and identify granulosa cell *AKR1C3* as a possible enzyme involved in follicular PGF2 α synthesis.

AKR1C1 and *AKR1C2* are closely related enzymes with PGE-F isomerase activity. Monkey granulosa cell *AKR1C1/AKR1C2* mRNA levels increased in response to an ovulatory dose of hCG both *in vivo* and *in vitro*, reaching peak levels 24 h after hCG administration and consistent with the measurement of maximal PGF2 α levels in follicular fluid just before ovulation. While PGE-F isomerase activity can be bidirectional (Nishizawa *et al.* 2000), only conversion of PGE2 to PGF2 α was detected in human granulosa cells in this study. The *AKR1C2*-selective inhibitor taurodeoxycholic acid inhibited PGE-F isomerase activity, supporting the conclusion that *AKR1C2*, but not *AKR1C1*, is the primary PGE-F isomerase in the primate follicle. Taurodeoxycholic acid (0.1–1.0 μ M) most likely reduced PGF2 α synthesis through its ability to inhibit PGE-F isomerase activity (Higaki *et al.* 2003) and not regulation of gene transcription, as similar bile acids regulate transcription with IC50 values of 10–100 μ M (Makishima *et al.* 1999, Parks *et al.* 1999). Expression and activity of PGE-F isomerase has been reported in whole ovaries and periovulatory follicles from rodents and domestic animals (Murdoch & Farris 1988, Iwata *et al.* 1990). In sheep follicles, PGE-F isomerase activity increased in response to an ovulatory gonadotropin surge to peak just before ovulation (Murdoch & Farris 1988). These previous reports, in combination with data from this study, support a role for the *AKR1C2* form of the PGE-F isomerase in the gonadotropin-stimulated conversion of PGE2 to PGF2 α by granulosa cells of the periovulatory follicle.

Despite the presence of two forms of *PGDS* mRNA as well as *AKR1C3* mRNA in monkey granulosa cells, 11 β -PGF2 α levels in follicular fluid were low/nondetectable, suggesting that conversion of PGH2 to PGD2 and then 11 β -PGF2 α within the periovulatory follicle is inefficient. Therefore, it is unlikely that 11 β -PGF2 α acts via the PGF2 α (FP) receptor to play a major role in ovulation. PGD2 produced within the ovulatory follicle may contribute to periovulatory events by stimulating PGD2 (DP) receptors (Saito *et al.* 2002). PGD2 is also a precursor for the production of the peroxisome proliferator activated receptor (PPAR) γ ligand PGJ2. PPAR γ has been implicated in the regulation of periovulatory events such as steroidogenesis (Komar *et al.* 2001) and granulosa cell apoptosis (Lovekamp-Swan & Chaffin 2005). While further study regarding the ovulatory role of PGD2 in the primate follicle is warranted, PGD2 does not represent a likely substrate for PGF2 α synthesis.

Many members of AKR enzyme group are multifunctional, capable of both PG and steroid synthesis (Nishizawa *et al.* 2000). *AKR1C3* possesses primarily PG synthesis activity, as this enzyme clearly prefers PG substrates over steroidogenic substrates (Suzuki-Yamamoto *et al.* 1999, Nishizawa *et al.* 2000). Both *AKR1C1* and *AKR1C2* possess PG reductase activity, but *AKR1C1* possesses 20 α -hydroxysteroid dehydrogenase activity while *AKR1C2* possesses 3 α -hydroxysteroid dehydrogenase activity (Nishizawa *et al.* 2000). Steroid hormones present in the ovarian follicle may reduce the PG reductase activity of *AKR1C1* and *AKR1C2* during the periovulatory interval. Similarly, PGs may reduce steroid hormone synthesis via members of the AKR enzyme family. The primate periovulatory follicle contains micromolar levels of both PGs and steroid hormones just before ovulation (Chaffin *et al.* 1999, Duffy & Stouffer 2001), so determining if the PG synthase activity or the steroid dehydrogenase activity of any individual enzyme predominates in granulosa cells may be exceedingly complex.

While granulosa cells have been established as the primary site of PG synthesis by the periovulatory follicle, a role for theca cells in PG production remains equivocal. *AKR1C3* mRNA was expressed by isolated human theca cells (Nelson *et al.* 2001), but *AKR1C3* protein was not detected in monkey stroma consistent with theca cells (this study). *In situ* hybridization localized a sequence common to *AKR1C1* and *AKR1C2* mRNA to the stroma of monkey periovulatory follicles in this study. *AKR1C1* mRNA and 20 α -hydroxysteroid dehydrogenase activity have been reported in human and bovine theca cells (Nelson *et al.* 2001, Brown *et al.* 2006), so *AKR1C1* is likely the predominant *AKR1C* in monkey theca. Theca cells produce little PGF2 α *in vitro* and theca cell PGF2 α synthesis is relatively insensitive to gonadotropin stimulation (Patwardhan & Lanthier 1981, Wong & Richards 1991, Duffy *et al.* 2005a), so theca *AKR1C1* may have predominantly steroidogenic activity in periovulatory follicles. While a minor contribution to follicular fluid PGF2 α may be made by theca cells, the gonadotropin-stimulated periovulatory rise in follicular fluid PGF2 α is likely due to production primarily, if not exclusively, by granulosa cells.

The specific contribution made by PGF2 α to periovulatory events remains unclear. Administration of PGF2 α to PG-synthesis inhibitor-treated animals can restore ovulation in rodents, domestic animals, and primates (Wallach *et al.* 1975, Murdoch *et al.* 1986, Sogn *et al.* 1987, Janson *et al.* 1988). However, disruption of FP receptor expression did not prevent ovulation in mice (Sugimoto *et al.* 1997), arguing against receptor-mediated action of PGF2 α to initiate ovulatory events in this species. FP receptor mRNA has been detected in follicles of domestic animals and women (Ristimäki *et al.* 1997, Sayasith *et al.* 2006, Bridges & Fortune 2007). FP receptors capable of signal transduction have been

detected in luteal cells of many species (Davis *et al.* 1988, Stocco *et al.* 2003), including monkeys (Houmard *et al.* 1992); functional FP receptors have also been studied in long-term cultures of human luteinized granulosa cells that serve as an *in vitro* model for the corpus luteum (Carrasco *et al.* 1997, Tai *et al.* 2001). However, it is important to note that the presence of functional FP receptors has never been reported for follicular granulosa cells of any mammalian species. Disruption of the PGE2 receptor EP2 reduces the efficiency of ovulation in mice (Hizaki *et al.* 1999), and previous studies support a role for PGE2 to regulate periovulatory events in mammals (Sogn *et al.* 1987, Peters *et al.* 2004), including primates (Duffy & Stouffer 2002). It remains possible that either or both PGE2 and PGF2 α may be able to initiate ovulatory events in the follicles of primates and domestic animals. Treatment of PG synthesis inhibitor-treated animals with non-metabolizable EP and FP receptor-selective agonists will likely be required to determine which PG receptors and, therefore, which PG(s), are needed to restore ovulation in these species.

This study identifies two pathways for the synthesis of PGF2 α by the primate periovulatory follicle. As discussed above, a direct role for PGF2 α in periovulatory events will require demonstration of functional FP receptors in the cells of the follicle. As an alternative, PGF2 α may serve as a substrate for the production of ovulatory PGE2 via the PGE-F isomerase activity present in granulosa cells. Finally, PGF2 α synthesis by the periovulatory follicle may function primarily as a metabolic pathway for removal of follicular PGE2 through conversion into PGF2 α via this PGE-F isomerase activity. In this scenario, PGF2 α synthesis may represent a gonadotropin-stimulated mechanism that acts in concert with PGE2 metabolism via PG dehydrogenase activity (Duffy *et al.* 2005c) to modulate the levels of periovulatory PGE2 within the primate follicle.

Materials and Methods

Animals

Granulosa cells, follicular fluid, and whole ovaries were obtained from adult female cynomolgus macaques (*Macaca fascicularis*) at Eastern Virginia Medical School (EVMS). All animal protocols and experiments were approved by the EVMS Animal Care and Use Committee and were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Adult females with regular menstrual cycles were maintained as previously described and checked daily for menstruation; the first day of menstruation was designated day 1 of the menstrual cycle (Duffy *et al.* 2005c). Blood samples were obtained under ketamine chemical restraint (10 mg/kg body weight) by femoral or saphenous venipuncture, and serum was stored at -20°C . Aseptic surgical procedures (either midline laparotomy or laparoscopy)

were performed under isoflurane anesthesia in a dedicated surgical suite.

A controlled ovarian stimulation model developed for the collection of multiple oocytes for *in vitro* fertilization was used as previously described (Duffy *et al.* 2005c) to obtain monkey granulosa cells and follicular fluid. Recombinant human follicle-stimulating hormone (r-hFSH; Serono Reproductive Biology Institute, Rockland, MA, USA (90 IU/day) or Organon, a part of Schering Plough Corporation, Roseland, NJ, USA (60 IU/day)) was administered for 6–8 days, followed by administration of r-hFSH plus recombinant human LH (Serono, 60 IU/day) for 2–3 days to stimulate the growth of multiple follicles. The gonadotropin-releasing hormone antagonist Antide (0.5 mg/kg body weight; Serono) 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). Aspiration of follicles at least 4 mm in diameter or ovariectomy was performed before (0 h) or 12, 24, and 36 h after administration of 1000 IU r-hCG (Serono) to induce ovulatory events. To obtain undiluted follicular fluid as well as granulosa cells, each follicle was pierced with a 22-gauge needle, and the aspirated contents of all follicles were pooled.

Whole ovaries were bisected to maintain at least two follicles of at least 4 mm in diameter on each piece. One piece of ovarian tissue was fixed in 4% paraformaldehyde and embedded in paraffin; another was coated in OCT compound (Sakura, Torrance, CA, USA), flash frozen in liquid propane, and stored at -80°C until sectioned. Additional monkey tissues such as seminal vesicle were obtained at necropsy and processed as described above.

Monkey granulosa cells

Monkey granulosa cells and follicular fluid were obtained from follicular aspirates as described previously (Chaffin *et al.* 1999). Briefly, aspirates were subjected to centrifugation to pellet the oocytes and granulosa cells; the resulting supernatant (i.e., follicular fluid) was removed and stored at -80°C . 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. The cells were either used immediately for cell culture or were frozen in liquid nitrogen and stored at -80°C for preparation of total RNA or cell lysates.

Human luteinizing granulosa cells

Human luteinizing granulosa cells were obtained after oocyte removal from follicular aspirates from patients experiencing ovarian stimulation at The Jones Institute for Reproductive Medicine, EVMS. This use of discarded human granulosa cells does not constitute human subjects research as determined by the EVMS Institutional Review Board. No information is obtained regarding an individual patient's diagnosis or treatment protocol. However, most patients receive hCG 34–36 h prior to follicle aspiration, so these human cells are very similar to monkey granulosa cells obtained 36 h after hCG

administration. Human granulosa cells were enriched by Percoll gradient centrifugation as described above for monkey granulosa cells.

PG assays

Follicular fluid obtained from aspirates was acidified and extracted with ethyl acetate prior to assay as previously described (Duffy *et al.* 2005c). [^3H]PGE₂ was added to each sample prior to extraction to correct for procedural losses; the mass of [^3H]PGE₂ added was <0.1% of the total PGE₂ content of each follicular fluid sample. Samples were resuspended in assay buffer (see below), and an aliquot was subjected to scintillation counting to calculate PG recovery that averaged 84%. Follicular fluid concentrations of PGF₂ α , 11 β -PGF₂ α , and the primary PGF₂ α metabolite 13,14-dihydroketo PGF₂ α (PGFM) were determined by enzyme immunoassay (EIA; Cayman Chemical, Ann Arbor, MI, USA); the PG content of each sample was corrected based on [^3H]PGE₂ recovery calculated for each sample. Culture media were assayed without extraction. The intra- and inter-assay coefficients of variation were 12.1 and 18.6%, 23.9 and 6.3%, and 23.0 and 7.0% respectively for PGF₂ α EIA, PGFM EIA, and 11 β -PGF₂ α EIA. The lower limit of detection for 11 β -PGF₂ α averaged 1.2 ng/ml after correction for extraction recovery.

Real-time RT-PCR

Multiple mRNAs were analyzed in each granulosa cell sample by real-time RT-PCR using a Roche LightCycler. Total RNA was obtained from granulosa cells using Trizol reagent (Invitrogen) and was stored at -80°C . Total RNA was incubated with DNase and RT was performed as described previously (Chaffin & Stouffer 1999). PCR was performed using the FastStart DNA Master SYBR Green I kit (Roche) with the exception of *L-PGDS*, which was amplified using the QuantiTect SYBR Green PCR kit (Qiagen). PCR primers were designed based on the human or monkey sequences using LightCycler Probe Design software (Roche) and nucleotide sequences of monkey PCR products were determined (Microchemical Core Facility, San Diego

State University, CA, USA) (Table 1). Whenever possible, primers span an intron to prevent undetected amplification of genomic DNA. Due to the significant (98%) nucleic acid identity between human *AKR1C1* and *AKR1C2* cDNA, PCR primers were designed which shared 100% identity with both these human cDNAs as well as reported cynomolgus monkey cDNA sequences for *AKR1C1* (a monkey *AKR1C2* cDNA sequence was not available). At least five log dilutions of the sequenced PCR product was included in each assay and used to generate a standard curve. All data were expressed as the ratio of enzyme mRNA to β -actin mRNA for each sample. Intra- and inter-assay coefficients of variation were less than 10%.

Western blotting for AKR1C3

Granulosa cells were thoroughly lysed on ice in PBS containing 0.5% sodium dodecyl sulfate and 0.1% Triton X-100, mixed with denaturing sample buffer, heated to 95°C for 10 min, and loaded onto 4–20% gradient polyacrylamide Tris–HCl gels (Bio-Rad). Proteins were transferred to PVDF membranes (Immobilon, Millipore, Billerica, MA, USA) and Western blotting proceeded as previously reported (Duffy *et al.* 2005c). The anti-AKR1C3 primary antibody was a rabbit polyclonal generated against the human AKR1C3 protein (Suzuki-Yamamoto *et al.* 1999) and was used at a 1:2000 dilution; an anti-rabbit IgG-horseradish peroxidase conjugate secondary antibody (Amersham) was used at a dilution of 1:20 000. A single band of 36 kDa was consistently detected by ECL (Amersham); no bands were detected when the primary antibody was omitted (not shown). Blots were then stripped of all antibodies following instructions provided by the membrane manufacturer, and Western blotting was performed on the stripped membranes using a mouse anti-tubulin primary antibody (1:1000 dilution, Sigma) and an anti-mouse IgG-horseradish peroxidase conjugate secondary antibody (1:20 000 dilution, Amersham). Molecular size of bands representing AKR1C3 and tubulin proteins were determined by comparison with prestained standards (Bio-Rad). Each experiment included at least four lanes of serial-diluted granulosa cell lysate; detection and densitometric analysis of the protein of interest and tubulin in these samples was used to

Table 1 Reaction conditions for real-time RT-PCR.

Target (primer concentration)	Primer sequences (5' $\rightarrow$ 3')	MgCl ₂ (mM)	Amplified cDNA (bp)	Percentage identity between amplified monkey sequence and sequence used for primer design	Accession number and species of sequence used for primer design	Monkey amplified fragment accession number
<i>AKR1C1/AKR1C2</i> (0.25 μM)	Up: GCAATCCCCATCGACC Down: GCAGAAATCCAGCAGTT	3	359	95.4	NM_001353 NM_001354 Human	DQ124199
<i>AKR1C3</i> (0.25 μM)	Up: AGGAACCTTTCACCAACAG Down: CCACCCATCGTTTGTC	3	309	96.4	AB018580 Human	DQ104393
<i>H-PGDS</i> (0.25 μM)	Up: CTGCTCACGTATAATGCG Down: CTTGAAGGCAACATGG	4	267	95.8	NM_014485 Human	DQ093856
<i>L-PGDS</i> (0.5 μM)	Up: CTAATCATCACACGCTG Down: GGGTCTCAGCTGGTT	4	270	97.7	AB032480 <i>M. fuscata</i>	DQ093857
β -actin (0.5 μM)	Up: ATCCGCAAAGACCTGT Down: GTCCGCCTAGAAGCAT	4	270	97.4	NM_001101 Human	AY765990

generate standard curves for semi-quantitative analysis of granulosa cell lysates. Films were scanned and analyzed densitometrically using SigmaGel software (Jandel Scientific, San Rafael, CA, USA). Tubulin levels in granulosa cell lysates were not different between treatment groups. All data are expressed as a ratio of AKR1C3/tubulin content for each granulosa cell sample.

Immunohistochemical detection of AKR1C3

Immunohistochemical detection of AKR1C3 in ovarian tissues was performed with 5 μ m sections of paraffin-embedded tissues as previously described (Duffy *et al.* 2005c) using the anti-AKR1C3 antibody described above for Western blotting (1:500 dilution), a biotinylated bovine anti-rabbit IgG secondary antibody, and peroxide-conjugated avidin solution (ABC kit, Vector Laboratories, Burlingame, CA, USA); peroxidase activity was visualized with Nova Red chromagen (Vector). Omission of the primary antibody served as a negative control.

In situ hybridization

Primers used for *in situ* detection of a region common to AKR1C1 and AKR1C2 mRNA (antisense 5'–3' GGGATCACTTCCTCACC; sense 5'–3' GGTGAGGAAGTGATCCC) shared 100% sequence identity to human AKR1C1 and AKR1C2. Digoxigenin-labeled oligoprobes were produced using the DIG Oligonucleotide Tailing Kit, 2nd Generation (Roche) according to the manufacturer's protocol. Labeling efficiency was determined by dot blot assay using the DIG Nucleic Acid Detection kit (Roche) according to the manufacturer's protocol. *In situ* hybridization was performed essentially as described by Dijkman *et al.* (1995) with prehybridization in a buffer containing 0.3 M sodium chloride, 0.03 M sodium citrate, pH 7.0 (2 $\times$ SSC), 500 μ g/ml sheared salmon sperm DNA (Eppendorf, Westbury, NY, USA) denatured for 5 min at 95 °C, 10 μ g/ml poly(A) solution (Roche), and 25% deionized formamide (Sigma). Hybridization of probes with slide-mounted frozen ovarian tissue sections was performed using 50 ng/ml oligoprobe for 2 h at 55 °C, followed by washing at 55 °C for 5 min in 2 $\times$ SSC, 1 $\times$ SSC, and twice in 0.25 $\times$ SSC. Sections were then washed twice for 5 min in 2 $\times$ SSC at room temperature. Immunological detection was performed using the DIG Nucleic Acid Detection kit. Sections were then counterstained with Nuclear Fast Red (Vector) and mounted in glycerol.

Granulosa cell culture

Granulosa cells were cultured on tissue culture plates coated with fibronectin and maintained at 37 °C in 5% CO₂ in serum-free DMEM-Ham's F12 medium containing insulin (2 μ g/ml), transferrin (5 μ g/ml), selenium (0.25 nM), aprotinin (25 mg/ml), and human low-density lipoprotein (25 μ g/ml) as previously described (Duffy & Stouffer 2003).

For analysis of RNA, monkey granulosa cells were obtained after ovarian stimulation before (0 h) hCG administration; 100 000 cells/400 μ l media were treated with hCG (100 ng/ml, Serono) or no treatment (control) for up to 48 h. Cells were lysed *in situ* with Trizol reagent, and total RNA was prepared

with the addition of glycogen (10 μ g) to improve recovery; RT-PCR was then performed as described above.

AKR1C3 activity

While PGH2 is unstable in solution, PGD2 is a stable substrate for assay of AKR1C3 activity. Preliminary studies demonstrated that human luteinizing granulosa cells produced very low levels of 11 β -PGF2 α in culture (3.4 $\pm$ 0.7 pg/ml media). Therefore, AKR1C3 activity was assessed by measuring the conversion of PGD2 to 11 β -PGF2 α . Granulosa cells were plated (3 $\times$ 10⁵ cells/ml media) and cultured for 4 h with vehicles (final concentrations 0.03% DMSO+0.04% ethanol), PGD2 (0.5 μ M, Cayman), PGD2+ the AKR1C3 inhibitor MPA (Sigma; Higaki *et al.* 2003), PGD2+ MPA (10 μ M)+ the progesterone receptor antagonist mifepristone (10 μ M, Sigma; El-Ashry *et al.* 1989), or PGD2+ the AKR1C3 inhibitor bimatoprost (Cayman; Koda *et al.* 2004); media were collected and stored at –20 °C until assayed for 11 β -PGF2 α concentration by EIA (Cayman). Preliminary experiments (not shown) confirmed that 11 β -PGF2 α production increased linearly with increased PGD2 concentration (0.01–10 μ M), cell number (1–5.5 $\times$ 10⁵), and incubation time (1–8 h). The concentrations of PGD2, MPA, mifepristone, and bimatoprost used in this study did not alter detection of 11 β -PGF2 α by EIA (not shown).

Activity of AKR1C1/AKR1C2

Human luteinizing granulosa cells were suspended in Hank's buffered salt solution, pH 7.4 (Sigma) +0.1% BSA at a concentration of 4 $\times$ 10⁶ cells/ml. Either [³H]PGE2 or [³H]PGF2 α was added (0.2 μ Ci/ml, Perkin-Elmer, Wellesley, MA, USA), and the cells were incubated for 4 h at 37 °C in a shaking water bath (Murdoch & Farris 1988). In each experiment, media+ [³H]PG was incubated in the absence of cells to determine the dpms of [³H] which comigrated with PGE2 and PGF2 α in the absence of enzymatic conversion. The AKR1C2-selective inhibitor taurodeoxycholic acid (Steraloids, Newport, RI, USA; Bauman *et al.* 2005) was added to some cells before incubation; vehicle (ethanol, 0.04%) did not compromise PG synthesis (not shown). After incubation, HCl was added to achieve a final concentration of 0.1 M, and cells+media were extracted with ethyl acetate (Duffy & Stouffer 2001). The PG-containing organic layer was dried under nitrogen and resuspended in chloroform: methanol (95:5). Radioinert PGE2 and PGF2 α (10 μ g each) were added to each sample before loading onto silica gel 60 plates (E. Merck) and separated in ethyl acetate: isooctane: glacial acetic acid (100:50:20) (Campbell & Ojeda 1987). The locations of PGE2 and PGF2 α in each lane were identified after exposure to iodine vapor. Silica scraped from these portions of the sample lanes was counted in a scintillation counter (LS 6500; Beckman Coulter, Fullerton, CA, USA). To assess conversion of [³H]PGE2 to [³H]PGF2 α , dpms comigrating with PGF2 α were expressed as a percentage of the total dpms recovered minus the percentage of total dpms comigrating with PGF2 α when cells were excluded from the reaction. For each patient, percent of dpms representing PGF2 α in the presence of taurodeoxycholic acid was expressed relative to percent of dpms present as

PGF2 α in absence of the inhibitor. Similar experiments were performed to assess conversion of [³H]PGF2 α to [³H]PGE2.

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 Figs 2, 3, 5B, and 6 were log-transformed before further analysis. Data in Figs 2 and 3 were analyzed by ANOVA, followed by Newman-Keuls test. Data in Fig. 5 were analyzed by two-tailed paired *t*-test. Data in Fig. 6 were analyzed by ANOVA with one repeated measure, followed by Newman-Keuls test. Statistical analyses above were performed using StatPak v4.12 software (North-west Analytical, Portland, OR, USA). Data are presented as mean $\pm$ S.E.M. and significance was assumed at *P*<0.05.

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