Endocrine, *vol. 22, no. 3, 249–256, December 2003* 0969–711X/03/22:249–256/$25.00

Luteinizing Hormone Acts Directly at Granulosa Cells to Stimulate Periovulatory Processes

Modulation of Luteinizing Hormone Effects by Prostaglandins

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The midcycle surge of luteinizing hormone (LH) triggers events within the primate periovulatory follicle that culminate in follicle rupture and luteinization of the follicle wall; these events include the shift from primarily estrogen to primarily progesterone production, vascularization of the granulosa cell layer, and expression of matrix metalloproteinases and their inhibitors (MMPs and TIMPs) thought to be necessary for follicle rupture. However, it is unknown if LH acts directly at granulosa cells to regulate these important periovulatory processes. The ovulatory LH surge also stimulates the production of prostaglandins (PGs) by the follicle just before follicle rupture, suggesting that LH may have both PG-dependent and PG-independent actions. To address these questions, gonadotropins were administered to adult female rhesus monkeys to stimulate the development of multiple, large preovulatory follicles. Granulosa cells were aspirated and maintained in vitro for up to 48 h in serum-free, chemically defined medium. Granulosa cells were cultured with LH alone or in combination with PGs to determine if these hormones act directly at granulosa cells to induce the production of factors implicated in periovulatory processes. LH treatment increased media progesterone ($p < 0.05$) and vascular endothelial growth factor (VEGF; $p < 0.05$) levels as well as stimulating expression of mRNAs for MMP-1 ($p = 0.05$), MMP-9 ($p < 0.05$), and TIMP-1 ($p < 0.05$), similar to the effects of an ovulatory dose of gonadotropin in vivo. PGE2 alone elevated media progesterone levels but decreased LH stimulation of MMP-1 mRNA ($p < 0.05$). PGF2α reduced LH-stimulated TIMP-1 mRNA ($p < 0.05$) levels. These studies suggest a direct action of LH on granulosa cells to stimulate the processes involved in tissue remodeling and neovascularization, i.e., MMPs/TIMPs and angiogenic factors, as well as steroidogenesis. LH-stimulated PGs may have a regulatory role to modulate some effects of the LH surge, such as MMP/TIMP expression.

Key Words: Ovulation; luteinizing hormone; prostaglandin; granulosa cell; follicle.

Introduction

A critical role for the midcycle LH surge in initiating periovulatory events, such as changes that lead to oocyte maturation, follicle rupture, and luteinization of the follicle wall, in mammalian ovaries is well established. Acting through LH/CG receptors located on granulosa and theca cells *(1)*, LH stimulates processes culminating in follicle rupture, oocyte release, and luteinization of the follicle wall. Several specific biochemical processes regulated by the ovulatory gonadotropin surge within the follicle have been identified. The LH surge initiates a shift in steroidogenesis from primarily estrogen to primarily progesterone production in primate follicles. Elevated follicular fluid concentrations of progesterone *(4)* correlate with increased granulosa cell expression of steroidogenic acute regulatory (StAR) protein and 3β-hydroxysteroid dehydrogenase (3β-HSD) as well as decreased expression of aromatase *(5)*. Changes in follicular blood flow in response to gonadotropin appear to be essential for ovulation to occur *(6)*, and the ovulatory gonadotropin surge stimulates granulosa cell production of the angiogenic factor VEGF *(7,8)*. In addition, granulosa cell expression of several MMPs and TIMPs is elevated following the ovulatory gonadotropin surge in monkeys *(2)* and rodents *(3)*; these enzymes have been implicated in follicle rupture and structural remodeling of the follicle into the corpus luteum. While a role for LH to stimulate granulosa cell production of progesterone and VEGF has been demonstrated, it is unclear if LH acts directly at granulosa cells to stimulate MMP/TIMP expression in the primate follicle.

Received July 8, 2003; Revised August 21, 2003; Accepted September 30, 2003.

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In many species *(9,10)*, including monkeys *(11)*, the gonadotropin surge induces granulosa cells of the periovulatory follicle to express the PG synthetic enzyme cyclooxygenase-2 (COX-2). Prostaglandins produced following LH induction of COX-2 expression are essential for follicle rupture to occur. Follicular fluid concentrations of PGE2 and PGF2α peak just before the expected time of ovulation *(9–11)*. Blockade of PG synthesis during the periovulatory interval can delay or prevent follicle rupture in monkeys *(12, 13)*, women *(14,15)*, and other mammals *(16,17)*; coadministration of PGE2 or PGF2α can restore ovulation in the presence of COX inhibitors *(13,16–18)*. Granulosa cells obtained from several mammalian species *(19–21)* including humans *(22–24)* express receptors for PGE2 and PGF2α, so these cells are likely targets for PG action. Therefore, PGs may mediate at least some of the periovulatory effects of the LH surge, although specific processes regulated by LH-stimulated PGs within the periovulatory follicle remain poorly understood.

These experiments were designed to determine if the ovulatory surge of LH acts directly at the granulosa cells obtained from large preovulatory follicles to regulate specific LH-stimulated periovulatory processes in the rhesus monkey. Because the ovulatory LH surge stimulates follicular PG production in vivo, the ability of intrafollicular concentrations of PGE2 and PGF2α to mediate or modulate the periovulatory effects of LH through direct action at granulosa cells was also examined.

Results

Maintenance of monkey preovulatory granulosa cells in chemically defined (serum-free) conditions in vitro altered PG production. Media levels of PGE2, but not PGF2α, increased during the 36 h after initiation of cultures in the absence of gonadotropin treatment (Fig. 1A, $p < 0.05$). Progesterone and VEGF concentrations were not different between 24 and 48 h of culture in the absence of gonadotropin and PGs (not shown). In addition, granulosa cell mRNA levels of MMP-1, MMP-9, and TIMP-1 did not change during time in culture in the absence of treatment (not shown).

To determine if LH stimulated PG production in vitro, granulosa cells were cultured without or with an ovulatory concentration of LH (100 ng/mL). Media levels of PGE2 and PGF2α were low when cells were cultured without LH (Fig. 1B). PGE2 levels did not change in response to LH treatment. However, a threefold increase in PGF2α concentrations was measured in response to LH stimulation when compared to control cultures ($p < 0.05$). Media PG levels in control and LH-stimulated cultures (less than 1 ng/mL) were 1000-fold lower than the levels of PGE2 and PGF2α measured in monkey follicular fluid just before ovulation [approx 1 μg/mL *(11)*]. In order to expose all granulosa cells to the same concentrations of PGs in subsequent experiments, endogenous PG production was inhibited by

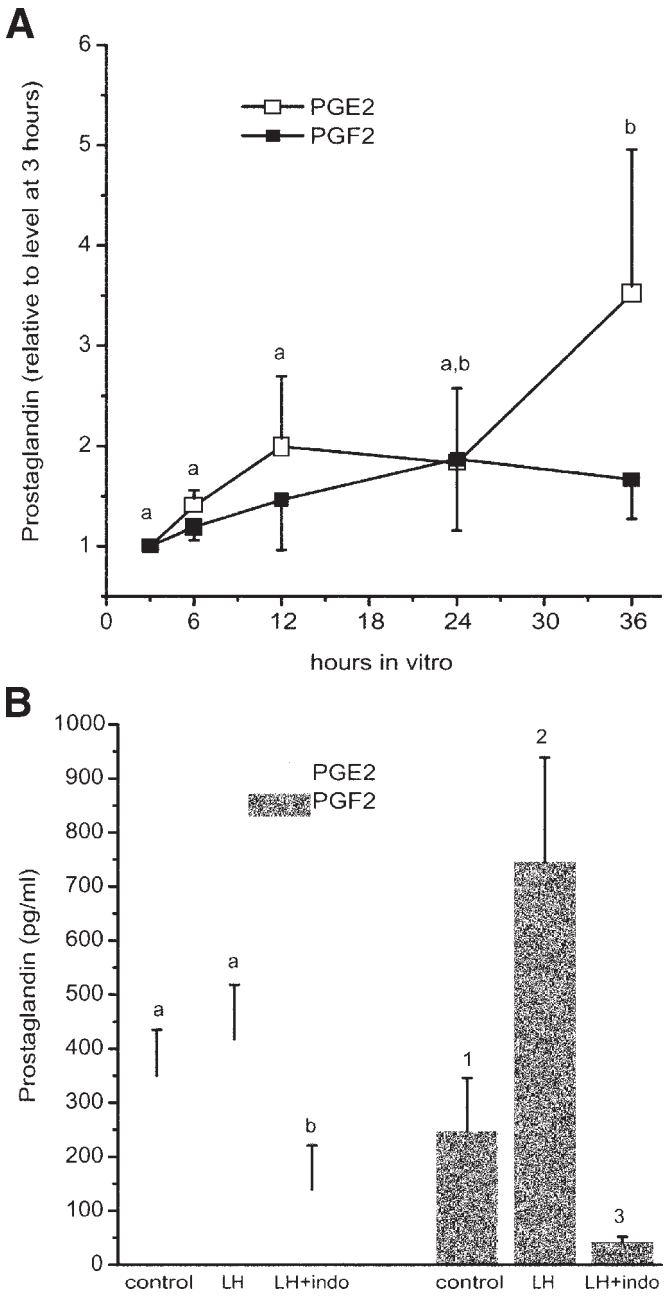

Fig. 1. Prostaglandin production by preovulatory granulosa cells in vitro. (**A**) Granulosa cells from large, preovulatory follicles were maintained in vitro for up to 36 h in chemically defined media in the presence of vehicle (<0.1% ethanol) only. Media concentrations for each PG were expressed in relation to the levels measured after 3 h of culture. (**B**) Granulosa cells were cultured for 36 h in the presence of LH (100 ng/mL) or LH with the addition of indomethacin (0.1 μ*M*, LH+indo). Control cells were cultured with vehicle only. Media concentrations of PGE2 (open symbols and bars) and PGF2α (shaded symbols and bars) were determined by EIA. For PGE2 (a,b) and PGF2α (1,2,3), groups with different superscripts are different, $p < 0.05$. Data are expressed as mean ± SEM; $n = 4$/group.

treatment with the general COX inhibitor indomethacin (Fig. 1B). Dose–response studies (not shown) demonstrated that 0.1 μ*M* was the lowest concentration of indomethacin

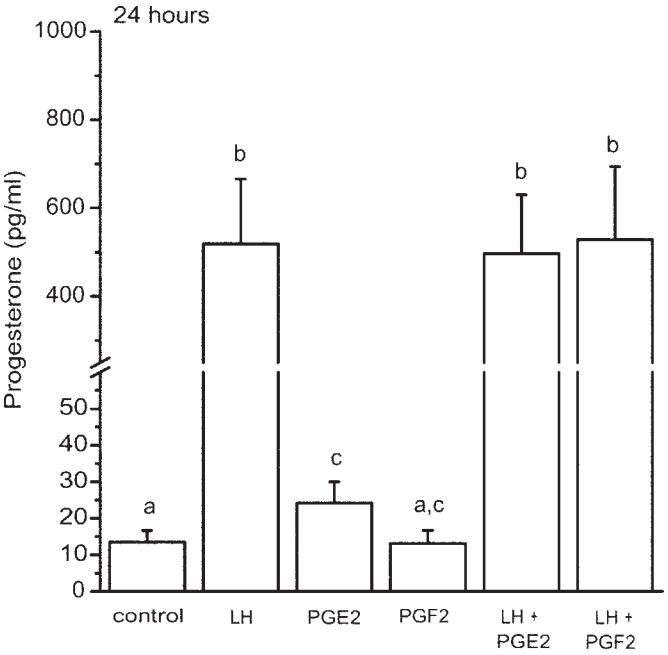

Fig. 2. Progesterone production in response to LH and PGs. Granulosa cells from large, preovulatory follicles were maintained in vitro for 24 h with LH (100 ng/mL), PGE2 (1 μg/mL), PGF2α (1 μg/mL), or a combination of LH+PG. All cells were cultured in the presence of 0.1 μ*M* indomethacin to reduce endogenous PG production; control cells received indomethacin only. Media were assayed for progesterone by RIA. Note the position of the axis break. Groups with different superscripts are different, $p < 0.05$. Data are expressed as mean ± SEM; $n = 5$/group.

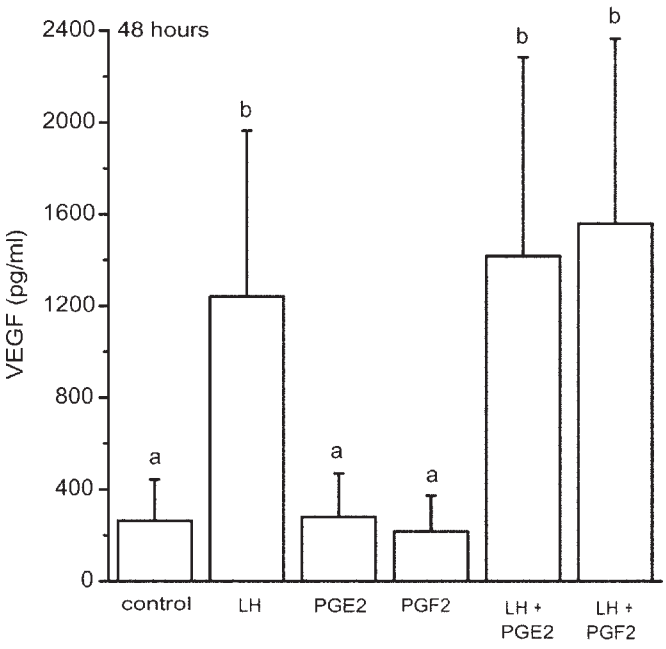

Fig. 3. VEGF production in response to LH and PGs. Granulosa cells were obtained and cultured for 48 h as described for Fig. 2. Media were assayed for VEGF by EIA. Groups with different superscripts are different, $p < 0.05$. Data are expressed as mean ± SEM; $n = 5$/group.

that consistently reduced LH-stimulated PGE2 and PGF2α concentrations to levels at or below those measured in control cultures. Indomethacin at this concentration did not alter LH-stimulated progesterone production (not shown), suggesting that this concentration of indomethacin was not detrimental to granulosa cell function. Indomethacin (0.1 μ*M*) was added to all subsequent cultures to minimize control and LH-stimulated PG production.

Treatment with an ovulatory dose of LH stimulated progesterone production by preovulatory granulosa cells at least 30-fold over control after 24 and 48 h of culture (Fig. 2 and data not shown, $p < 0.05$). To determine if PGs stimulated progesterone production by granulosa cells, cells were cultured with PGE2 and PGF2α at concentrations similar to those measured in monkey follicular fluid near the expected time of ovulation *(11)*. After 24 h of culture, media progesterone concentrations in cultures treated with PGE2 were greater than in control cultures ($p < 0.05$); PGF2α treatment did not alter progesterone concentrations. Exposure to PGE2 or PGF2α in addition to LH for 24 h did not alter progesterone concentrations compared with those measured after treatment with LH only. After 48 h of culture, PGs did not alter control- or LH-stimulated progesterone levels.

LH exposure increased granulosa cell VEGF production when compared to control cultures after 24 and 48 h in vitro (Fig. 3 and data not shown, $p < 0.05$). Treatment with PGs did not alter media VEGF concentrations when compared to control cultures; PGs also did not affect LH-stimulated VEGF levels after 24 or 48 h of culture.

After 24 h of culture, no effect of treatment on MMP-1 mRNA levels was observed (data not shown). LH exposure tended to increase MMP-1 mRNA levels after 48 h of treatment (Fig. 4, $p = 0.05$). Exposure to PGE2 or PGF2α for 48 h did not alter MMP-1 mRNA content when compared to control cultures, and PGF2α did not affect LH-stimulated MMP-1 mRNA levels. However, PGE2 reduced LH-stimulated MMP-1 mRNA after 48 h of culture ($p < 0.05$).

MMP-9 mRNA levels were increased in response to LH stimulation after 24 h of culture (Fig. 5, $p < 0.05$). LH did not alter MMP-9 mRNA levels after 48 h in vitro (data not shown). Prostaglandin treatment did not alter MMP-9 mRNA alone or in combination with LH after 24 or 48 h of culture.

Levels of TIMP-1 mRNA increased in response to LH after 24 and 48 h of treatment (Fig. 6 and data not shown, $p < 0.05$). PGE2 or PGF2α alone had no effect on TIMP-1

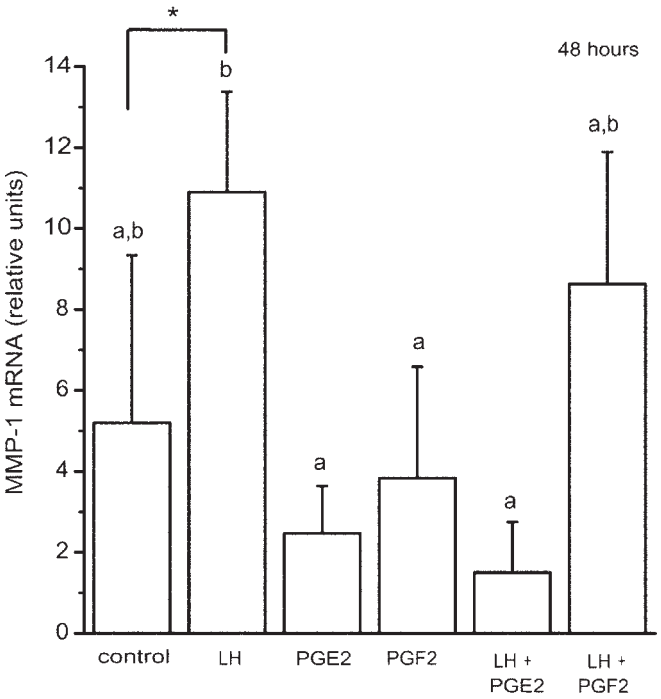

Fig. 4. Expression of MMP-1 mRNA by granulosa cells in response to LH and PGs. Granulosa cells were obtained and cultured for 48 h as described for Fig. 2. MMP-1 mRNA levels were determine by RT-PCR and are expressed relative to the expression of the housekeeping gene cyclophilin. Groups with different superscripts are different, $p < 0.05$. Control tended to be less than LH by paired *t*-test *, $p = 0.05$. Data are expressed as mean ± SEM; $n = 3$/group.

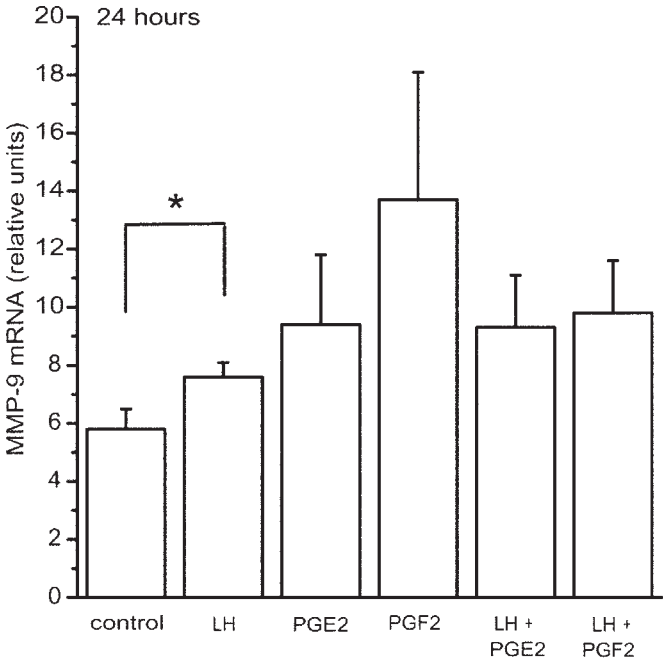

Fig. 5. Expression of MMP-9 mRNA by granulosa cells in response to LH and PGs. Granulosa cells were obtained and cultured for 24 h as described for Fig. 2. MMP-9 mRNA levels were determine by RT-PCR and are expressed relative to the expression of the housekeeping gene cyclophilin. Data were log-transformed and analyzed by one-way ANOVA; no differences were identified. Control was less than LH by paired *t*-test *, $p < 0.05$. Data are expressed as mean ± SEM; $n = 4$/group.

levels when compared with control cultures, and PGE2 failed to alter LH-stimulated TIMP-1 expression after 24 or 48 h in vitro. PGF2α decreased LH-stimulated TIMP-1 levels after 24 h of culture ($p < 0.05$), but this treatment did not alter TIMP-1 levels after 48 h in vitro.

Discussion

This report demonstrates that gonadotropin acts directly at monkey granulosa cells to enhance production of several important molecules implicated in periovulatory events. Previous studies have shown that LH stimulates preovulatory granulosa cells to increase production of progesterone *(25)* and VEGF *(26)*, and these results were confirmed in the present study. We show for the first time that monkey granulosa cell expression of MMP-1, MMP-9, and TIMP-1 mRNA was elevated in response to LH exposure in vitro, suggesting that the ability of ovulatory gonadotropin surge to increase granulosa cell content of these mRNAs in vivo results, at least in part, from the direct action of gonadotropin on granulosa cells. Notably, PGs blunted LH-induced expression of MMP-1 and TIMP-1, suggesting a mechanism by which PGs, acting directly at granulosa cells, can modulate the effects of the LH surge to regulate ovarian tissue remodeling, which is essential for periovulatory processes including follicle rupture, oocyte release, and luteinization of the follicle.

LH and, to a lesser extent, PGE2, but not PGF2α, increased media progesterone levels in cultures of monkey preovulatory granulosa cells. LH action directly at monkey granulosa cells stimulated progesterone production, confirming previous reports of gonadotropin stimulated progesterone production in vitro *(25)* and in vivo *(4)*. In contrast, the ability of PGs to regulate progesterone production by monkey preovulatory granulosa cells has received little attention. Studies published by Channing over 30 years ago showed a modest effect of PGE2 to stimulate progesterone production by preovulatory monkey granulosa cells in vitro while PGF2α did not alter media progesterone levels *(27)*. However, the data presented in these studies were not subjected to statistical analysis. Studies using sheep *(28)* and mouse *(29)* preovulatory granulosa cells showed that PGE2 tended to raise progesterone levels, comparable to the twofold increase measured in the present study. In studies using cultured bovine *(30)* and sheep *(28)* preovulatory granulosa cells, 1 μ*M* PGF2α also increased progesterone production, although the effect was modest; a similar effect of PGF2α on monkey granulosa cells was not observed in the present

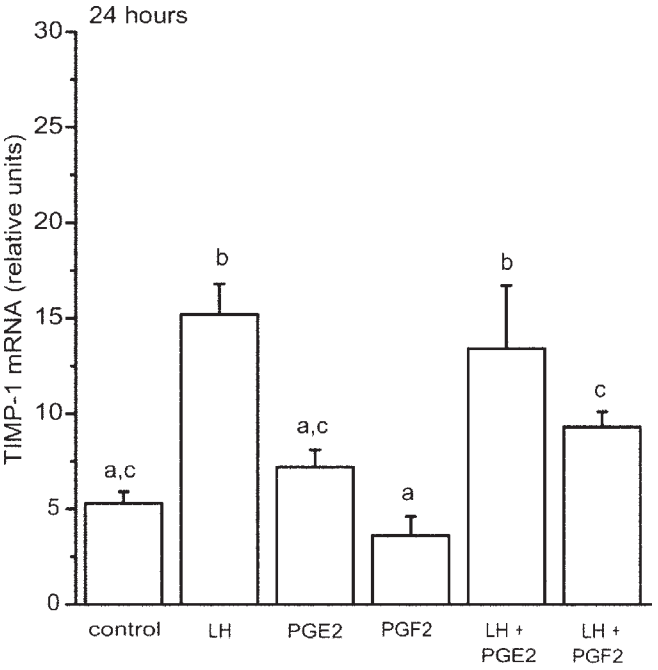

Fig. 6. Expression of TIMP-1 mRNA by granulosa cells in response to LH and PGs. Granulosa cells were obtained and cultured for 24 h as described for Fig. 2. TIMP-1 mRNA levels were determine by RT-PCR and are expressed relative to the expression of the housekeeping gene β2-microglobulin. Groups with different superscripts are different, $p < 0.05$. Data are expressed as mean ± SEM; $n = 4$/group.

study. Moon et al. demonstrated that PGE2, but not PGF2α, stimulated progesterone levels in cultures of rat granulosa cells *(31)* and concluded that this effect likely results from inhibition of 20α-hydroxysteroid dehydrogenase, which converts progesterone to an inactive metabolite and not an increase in progesterone synthesis *(31)*. In all of these studies, media progesterone levels achieved in response to PG treatment in vitro were lower than gonadotropin-stimulated progesterone production. However, PG regulation of progesterone production may be physiologically relevant in cumulus cells, which are thought to express few or no LH receptors *(32)*. Cumulus cells are a likely target for PGE2 action as mice lacking expression of the PGE2 receptor EP2 are infertile secondary to a failure of cumulus expansion *(33)*. Functional EP2 receptors are present on human luteinizing granulosa cells *(23)*, and stimulation of these cells with an EP2 receptor-specific agonist increased intracellular cAMP *(23)*, presumably through coupling with the G_s protein *(34)*. The LH receptor also utilizes G_s *(35)*, and use of this common intracellular signaling pathway may prevent LH+PGE2 stimulation of progesterone production above the levels observed in response to LH alone. However, LH may utilize intracellular signals in addition to cAMP *(35)* or may couple to G_s more efficiently than the EP2 receptor in granulosa cells; these hypotheses may explain the higher levels of progesterone production resulting from LH exposure when compared with PGE2 stimulation.

While LH stimulated VEGF production, PG exposure did not alter control or LH-stimulated VEGF production by monkey preovulatory granulosa cells in vitro. Previous studies demonstrated that the ovulatory gonadotropin surge in vivo stimulated VEGF production by monkey follicles *(8)*, and here we confirm the results of a previous study *(26)* that LH acts directly at granulosa cells to increase VEGF levels in vitro. Bovine preovulatory granulosa cells also produced VEGF in response to gonadotropin stimulation *(36)*. Our findings that PGE2 and PGF2α do not alter VEGF accumulation in culture media extend results of a previous study using bovine granulosa cells which indicate that VEGF production by granulosa cells is not PG-regulated *(36)*. In non-granulosa cell types, VEGF expression is regulated by factors induced in response to hypoxia *(37)* as well as oncogenes *(38)*; the roles of these factors on granulosa cell VEGF production remain to be examined. Taken together, these data suggest that LH may act through intracellular signaling pathways not utilized by PGs to increase VEGF production by granulosa cells in vitro. Additional studies targeting protein kinase A, protein kinase C, and other signaling intermediaries potentially utilized by LH and PGE2 would be required to address this hypothesis.

Exposure of monkey preovulatory granulosa cells to an ovulatory dose of LH increased MMP-9, increased TIMP-1, and tended to increase MMP-1 mRNA expression. The present study extends the findings of Mann et al. *(39)*, who demonstrated that gonadotropin stimulated rat preovulatory granulosa cells to increase both matrix metalloproteinase inhibitor activity and TIMP-1 mRNA expression in vitro. These findings are consistent with previous studies which showed that gonadotropin administered in vivo to monkeys *(2)* and other mammalian species *(40,41)* increased granulosa cell expression of MMP-1, MMP-9, and TIMP-1. Coordinated regulation of MMP and TIMP expression by cells of the periovulatory follicle may be essential for follicle rupture, oocyte release, and formation of the corpus luteum. MMP-1 degrades collagen types I and III, which are found in the theca externa and tunica albuginea of the follicle, while MMP-9 degrades collagen type IV, a major component of the granulosa cell basement membrane *(42)*. Less well understood is the regulation of MMP and TIMP activities, which involves changes in transcription, translation, and substrate availability as well as locally regulated activation and inactivation of these enzymes. For example, TIMP-1 inhibits the activity of MMPs such as MMP-1 *(43)*. Further studies will be required to determine if MMP and TIMP activity are gonadotropin-regulated in the periovulatory follicle.

The ability of PG synthesis inhibitors to prevent follicle rupture in response to an ovulatory gonadotropin stimulus is well established for many mammalian species *(12–14,*

16,17), but the specific functions of PGs in this process are unclear. In the present study, LH-stimulated MMP-1 expression by monkey preovulatory granulosa cells was markedly reduced by PGE2 exposure; LH-stimulated TIMP-1 expression was modestly decreased by PGF2α treatment. Expression of MMP-1 and TIMP-1 was not altered by PG alone, suggesting that PGs may act in vivo to prevent overexpression of these important molecules in response to the ovulatory LH surge. Systemic administration of PG synthesis inhibitors to rats increased ovarian MMP-9 and decreased MMP-1 expression *(41)* but did not alter the LH-stimulated increase in TIMP-1 expression *(39,41)*. These limited data suggest that differences may exist between species and follicular compartments regarding the role of PGs in the regulation of MMP and TIMP expression in of the periovulatory follicle.

Systemic administration of PG synthesis inhibitors blocks periovulatory events in various mammalian species, including primates. Inhibition of COX activity during the periovulatory period led to ovulatory failure and the development of luteinized, unruptured follicles (LUF) in monkeys *(12)* that was reversed with systemic PGF2α treatment *(18)*. Oral administration of COX inhibitors also led to the formation of LUF *(14)* and delayed follicle rupture *(15)* in women. Intrafollicular administration of indomethacin prevented oocyte release and caused the formation of abnormal ovulatory stigmata in monkeys *(13)*; these effects of indomethacin administration were reversed with coadministration of PGE2 *(13)*. Cumulus expansion and detachment of the cumulus–oocyte complex from the follicle wall fails in mice that do not express the PGE2 receptor EP2 *(33)*; failure of oocyte release following COX inhibitor administration in monkeys *(13)* may occur through a similar mechanism. While mice lacking the PGF2α (FP) receptor have normal ovulatory function *(44)*, PGF2α can restore ovulation in COX-inhibited monkeys *(18)*, supporting an important role for PGs of both the E and F series in primate periovulatory process. Taken together, these data support an essential role for COX products, most likely PGE2 and/or PGF2α, in timely follicle rupture and oocyte release in primates. In most studies, little or no disruption of follicle luteinization or subsequent luteal function occurred following inhibition of PG synthesis *(13,15)*, suggesting that the essential role of PGs in periovulatory events may be primarily related to the regulation of oocyte release and the structural changes associated with follicle rupture.

In summary, the ovulatory surge of gonadotropin likely acts directly at granulosa cells of the monkey preovulatory follicle to stimulate expression of MMP-1, MMP-9, and TIMP-1 as well as increasing production of progesterone and VEGF. LH-stimulated PGs may also have a regulatory role within the follicle to modulate some effects of the LH surge in vivo, such as regulation of MMP/TIMP expression, supporting a role for ovulatory PGs in the process of tissue remodeling within the periovulatory follicle.

Materials and Methods

Animals

The general care and housing of rhesus monkeys (*Macaca mulatta*) at the Oregon National Primate Research Center (ONPRC) were described previously *(45)*. Animal protocols and experiments were approved by the ONPRC Animal Care and Use Committee, and studies 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 *(46)* was used to obtain granulosa cells for these studies. Adult females with regular menstrual cycles were checked daily for menstruation, and blood samples were obtained daily from unanesthetized monkeys by saphenous venipuncture beginning on the first day of treatment. Multiple follicular development was stimulated by administration of recombinant human gonadotropins (r-hFSH, 30 IU twice daily for 8 d; r-hLH 30 IU twice daily on d 7 and 8; Serono Reproductive Biology Institute, Rockland, MA) beginning 1–3 d after the onset of menstruation. Monkeys also received a daily subcutaneous injection of the GnRH antagonist Antide [0800 h, 0.5 mg/kg body weight in propylene glycol: water (1:1); Serono] throughout the stimulation protocol to prevent an endogenous LH surge. Adequate follicular development was monitored by serum estradiol levels and by ultrasonography *(47)*, and follicular aspiration was performed by laparoscopy on anesthetized animals during aseptic surgery *(48)*.

Cell Culture

Granulosa cells were obtained from follicular aspirates as described previously *(4)*. Briefly, after oocyte removal, aspirates were subjected to Percoll gradient centrifugation to obtain a granulosa cell-enriched population. Granulosa cells (100,000 cells/well) were cultured on fibronectin-coated 48-well plates in DMEM–Ham's F-12 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). Granulosa cells were also cultured with indomethacin, LH (100 ng/mL), PGE2 (1 μg/mL), and PGF2α (1 μg/mL). These concentrations of LH and PGs were selected to reflect the concentrations of these hormones to which granulosa cells are exposed in the monkey preovulatory follicle and which are thought to stimulate periovulatory processes *(11,26)*. Ethanol (0.1% final concentration) was added to control cultures for treatment vehicle when appropriate. Cells were maintained without change of medium at 37°C in a humidified atmosphere of 95% O_2/5% CO_2. After 24–48 h of culture, media was collected and stored at –20°C. Cells were lysed *in situ* with Trizol reagent (Invitrogen, Carlsbad, CA), and RNA was prepared according to manufacturer's instructions with the addition of glycogen (10 μg/sample) to aid in RNA recovery. PGs were obtained from Cayman Chemical (Ann Arbor,

MI); all other chemicals were obtained from Sigma-Aldrich (St. Louis, MO) except where noted.

Hormone Assays

Serum and culture media levels of progesterone *(49)* and serum levels of estradiol *(50)* were determined by RIA; intra- and interassay coefficients of variation did not exceed 15%. Media PGE2 and PGF2α levels were determined by enzyme immunoassay (EIA; Cayman Chemical) as previously described *(11)*; intra- and interassay coefficients of variation for these assays were <20% and <10%, respectively. VEGF concentrations in culture media were determined by enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN) as previously described *(26)*.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA from each granulosa cell sample (approx 200 ng) was treated with DNase I (Invitrogen) prior to reverse transcription, which was performed using Molony murine leukemia virus reverse transcriptase (Invitrogen) as previously reported *(2)*. Sequences for the oligonucleotide primers (Invitrogen) and methods for sensitive semiquantitative RT-PCR based assays used to assess MMP-1, MMP-9, and TIMP-1 mRNA levels were previously described *(2)*. The $MgCl_2$ concentration, amount of cDNA included in each PCR reaction, number of PCR cycles, and primer concentrations (for which the amount of co-amplified products for experimental and the internal standard were linear and parallel with increasing amount of cDNA) were determined empirically for each primer set. The products of each primer set were also in the exponentially increasing phase relative to the number of PCR cycles. PCR products were separated on 2% agarose gels, stained with ethidium bromide, and photographed using Polaroid 667 film (Polaroid Corp, Cambridge, MA). Each mRNA sample was assayed in duplicate. A pool containing cDNA from RT preparations of several different groups of granulosa cells was included in triplicate in each PCR reaction. Intraassay and interassay variability calculated using this pool were less than 15% for each assay. Because data for each set of primers was collected in two or three separate PCR reactions, pool triplicates were also used to normalize data between reactions.

Statistical Analysis

Bartlett's test was used to assess heterogeneity of variance, and data were log transformed when necessary. Within each time point, data were analyzed by one-way ANOVA with one repeated measure (blocked for individual animal), followed by Duncan's or Newman–Keuls' tests when indicated. A paired t-test was used to test the hypothesis that LH increased granulosa cell expression of MMP-1 and MMP-9 mRNA levels when compared to control cultures. All data were expressed as mean ± standard error of the mean (SEM), and significance was assumed at $p < 0.05$.

Acknowledgments

Supported by The Medical Research Foundation of Oregon (DMD), the Andrew W. Mellon Foundation, and NIH Grants HD39872 (DMD), HD20869 (RLS), HD18185, and RR00163. The authors thank the Animal Care and Surgical Staffs at ONPRC for their outstanding contribution to these studies. Also appreciated is the work of the Morphology and Hormone Assay Core Laboratories at ONPRC. Serono Reproductive Biology Institute (Rockland, MA) provided the Antide as well as the r-h FSH and r-hLH used for these studies.

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