Induction of Ectonucleotide Pyrophosphatase/Phosphodiesterase 3 During the Periovalutary Period in the Rat Ovary

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Abstract

Ectonucleotide pyrophosphatase/phosphodiesterase 3 (*Enpp3*) is involved in multiple physiological processes, such as morphological changes and inflammatory processes. The present study investigated the spatiotemporal expression pattern and regulatory mechanisms controlling expression of *Enpp3* in the rat ovary during the periovalutary period. Immature female rats were injected with pregnant mare serum gonadotropin to stimulate follicular development. Ovaries, granulosa cells, or theca-interstitial cells were collected at various times after human chorionic gonadotropin (hCG) administration. Real-time polymerase chain reaction analysis revealed that messenger RNA (mRNA) for *Enpp3* was highly induced in both granulosa cells and theca-interstitial cells by hCG. In situ hybridization analysis demonstrated that *Enpp3* mRNA expression was induced in theca cells at 4 hours after hCG, and the expression remained elevated until 12 hours after hCG. The expression of *Enpp3* mRNA was stimulated in granulosa cells at 8 hours and reached the highest expression at 12 hours. Localization of *Enpp3* mRNA was observed in newly forming corpora lutea by in situ hybridization. The hCG-stimulated expression of *Enpp3* mRNA was blocked by a protein kinase C inhibitor (GF109203) instead of the protein kinase A inhibitor (H89). Furthermore, *Enpp3* induction is dependent on new protein synthesis. Inhibition of progesterone action did not alter *Enpp3* mRNA expression, whereas inhibition of prostaglandin synthesis or the epidermal growth factor pathway diminished *Enpp3* mRNA levels. In conclusion, our findings suggest that the induction of the *Enpp3* mRNA may be important for the morphological changes and inflammatory response during ovulation and luteinization.

Keywords

Enpp3, ovulation, luteinization, inflammation, ovary

Introduction

Ectonucleotide pyrophosphatase/phosphodiesterase family member 3 (ENPP3/CD203c/PD-1 β /B10/gp130^{RB13-6}) is type II transmembrane metalloenzyme. It can catalyze the hydrolysis of pyrophosphate and phosphodiester bonds. This hydrolysis of 5'-phosphodiester bonds in nucleotides results in the release of 5'-nucleotide monophosphates. *Enpp3* is involved in multiple physiological processes including nucleotide recycling, stimulation of cell motility, and tumoral transformation.¹⁻⁶ *Enpp3* expression can induce synthesis of differentiation factors, morphological changes, and enhanced invasive properties in fibroblasts and glioma cells.⁷ It was also reported that *Enpp3* is highly expressed in activated basophils and was considered as a good activation marker of basophils and mast cells.^{8,9} *Enpp3* has also been shown to be rapidly induced by immunoglobulin receptor Fc ϵ RI crosslinking and negatively regulated by chronic allergic inflammation.¹⁰ Basophil and mast cell numbers increased in *Enpp3* mutant mice with augmented serum adenosine triphosphate (ATP) concentrations. ENPP3 can decrease ATP concentration and suppress basophil

and mast cell activity. Together, these findings demonstrate that *Enpp3* regulates inflammatory responses in various tissues.

The ovary undergoes dynamic morphological changes during the reproductive cycle. The maturing follicles undergo rupture and complete reorganization after ovulation. Then an integration of cells, including both theca-interstitial and granulosa cells, is stimulated to develop into the corpus luteum (CL). Theca cells differentiate into small luteal cells, whereas

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granulosa cells become large luteal cells. Ovulation has similar features of inflammatory response: vasodilatation and cellular infiltration. This ovulatory inflammatory response is the result of a cascade of biochemical changes in follicular cells and macrophages initiated by luteinizing hormone (LH) surge. These changes include progesterone production, increased proinflammatory cytokine production, and increased proteolysis.^{11,12} Basophils and possibly other types of leukocytes accumulate in mature follicles near the time of ovulation. The maximum accumulation is reached several hours after ovulation.^{13,14}

Based on the potential role of *Enpp3* in the regulation of inflammatory responses and morphological changes, we hypothesized that *Enpp3* may play a role in the ovulatory process and subsequent changes during luteinization. To test our hypothesis, the expression and regulation of *Enpp3* were characterized during the periovulatory period in the rat ovary.

Materials and Methods

Materials and Reagents

Unless otherwise noted, all chemicals and reagents were purchased from Sigma-Aldrich Chemical Co (St. Louis, Missouri). Molecular biological enzymes, molecular size markers, oligonucleotide primers, culture media, and TRIzol were purchased from Invitrogen Life Technologies, Inc (Carlsbad, California). Chemicals and reagents (pregnant mare serum gonadotropin [PMSG], human chorionic gonadotropin [hCG], forskolin [FSK], phorbol 12 myristate 13-acetate [PMA], RU486, NS398, and AG1478) were all purchased from Sigma Chemical Co.

Isolation of Granulosa Cells and Theca-Interstitial Cells

All animal procedures for these experiments were approved by the Committee of Laboratory Animals at Hangzhou Normal University, China, and the University of Kentucky Institutional Animal Care and Use Committee. Sprague Dawley rats were obtained from Hangzhou Normal University Animal Center and Harlan Sprague Dawley Inc (Indianapolis, Indiana) and maintained on a 12:12 light/dark cycle. Immature female rats (22–23 days old) were injected with PMSG (10 IU) subcutaneously (SC) to stimulate follicular development. Forty-eight hours after PMSG administration, rats were then injected with hCG (5 IU) SC to induce ovulation and subsequent formation of corpora lutea. Ovulation in this model occurs at 12 to 16 hours after hCG (personal observation).

The ovaries collected from gonadotropin-primed immature rats (48 hours after PMSG) at defined time points ($n = 3$ –4 animals/time point) were dissected and punctured to release granulosa cells.^{15,16} Then, the residual ovarian tissues were minced and incubated for 90 minutes at 37°C in a medium containing 2.5 mg/mL collagenase plus 10 µg/mL deoxyribonuclease. The dispersed theca-interstitial cells were collected after washing 3 times in Opti-MEM medium. The collected

granulosa and theca-interstitial cells were processed for later isolation of total RNA or protein.

In Situ Hybridization of *Enpp3* Messenger RNA

Ovaries were collected at defined time points after hCG administration ($n = 3$ –4 animals/time point). In situ hybridization was performed as previously described.¹⁷ Oligonucleotide primers corresponding to complementary DNA (cDNA) for rat *Enpp3* (forward 5'-GAAAACATGTGGACTCCATTCC-3', reverse 5'-TCAAACCCTCCATCAGCATCC-3') were designed using PRIMER3 software (version 0.4.0).¹⁸ The polymerase chain reaction (PCR) products were cloned into a pCRII-TOPO vector (Invitrogen Life Technologies, Inc., Carlsbad, CA). Probes were labeled with [α -³⁵S] uridine 5'-triphosphate (10 mCi/mL; MP Biomedicals, Inc, Costa Mesa, California). A sense complementary RNA probe was used as a control for nonspecific binding. One ovary each from 3 animals was used for in situ hybridization ($n = 3$ –4 ovaries/time point). A minimum of 4 sections were examined from each ovary for a minimum of 12 sections per time point.

Rat Granulosa Cell and Theca-Interstitial Cell Culture

For the in vitro studies, ovaries were collected 48 hours after PMSG administration and processed as described previously.^{16,19} Briefly, granulosa cells or theca-interstitial cells isolated from ovaries were resuspended in Opti-MEM medium supplemented with 0.05 mg/mL of gentamycin and $1 \times$ insulin, transferrin, and selenium. The cells were cultured in the absence or presence of various reagents discussed below for different time points at 37°C in a humidified atmosphere of 5% CO₂. Cells were collected at the end of each culture period for later isolation of total RNA or protein.

Quantification of Messenger RNA for *Enpp3* Gene

Real-time PCR reactions were performed on a Mx3000P QPCR System (Stratagene, La Jolla, California). Oligonucleotide primers corresponding to cDNA for rat *Rpl32* (forward 5'-GAAGCCCAAGATCGTCAAAA-3', reverse 5'-AGGATCTGGCCCTTGAATCT-3') and rat *Enpp3* (forward 5'-TGCCCAAAGCTGAACGACC-3', reverse 5'-CATCATCAACTAAGTGAAGG-3') were designed using PRIMER3 software.¹⁸ The specificity for each primer set was confirmed by both PCR products sequencing and analyzing the melting (dissociation) curve in the MxPro real-time PCR analysis program (Stratagene) after each real-time PCR reaction. The real-time PCRs were carried out as previously described.^{20,21} The thermal cycling steps included 2 minutes at 50°C to permit optimal AmpErase uracil-N-glycosylase activity, 10 minutes at 95°C for initial denaturation, and then 40 cycles of 30 seconds at 95°C, 30 seconds at 55°C, and 30 seconds at 72°C, followed by 1 minute at 95°C, 30 seconds at 58°C, and then 30 seconds at 95°C for ramp dissociation. The relative amount of the transcripts for

the gene was calculated using the $2^{-\Delta\Delta CT}$ method²² and normalized to the endogenous *Rpl32* reference gene.

Western Blot Analysis

Western blotting was performed as described previously.^{23,24} Intact ovary lysates were prepared using radioimmunoprecipitation assay buffer/protease inhibitor cocktail. The protein concentration was measured by the Lowry method.²⁵ Thirty micrograms of protein was separated on a 15% polyacrylamide gel with sodium dodecyl sulfate and transferred to a nitrocellulose membrane (Whatman, Sanford, Maine). Blots were incubated with the primary antibody for ENPP3 (Abcam, Cambridge, Massachusetts) or β -actin (Cell Signaling Technology, Danvers, Massachusetts), overnight at 4°C. Then blots were washed 3 times with phosphate-buffered saline + 0.1% Tween and incubated with respective secondary antibodies for 1 hour. Western blots were analyzed using an enhanced chemiluminescence detection system (GE Healthcare, Piscataway, New Jersey) and exposed to X-ray film.

Statistical Analyses

All data are presented as means $\pm$ standard error of the mean (SEM). One-way analysis of variance (ANOVA) was used to test differences in *Enpp3* messenger RNA (mRNA) expression across time of culture or among treatments. If ANOVA revealed significant effects of treatments, the means were compared by Duncan test, with $P < .05$ considered significant.

Results

Human Chorionic Gonadotropin-Stimulated *Enpp3* Expression in Periovarian Rat Ovaries, Granulosa Cells, and Theca-Interstitial Cells In Vivo

Human chorionic gonadotropin has been widely used as an effective substitute for LH to stimulate periovarian processes.^{23,26,27} Therefore, we used hCG to mimic the LH surge in vivo. The expression patterns of *Enpp3* mRNA and protein were analyzed in the ovary at different times after hCG treatment. *Enpp3* mRNA expression was highly stimulated at 8 and 12 hours after hCG administration in intact ovaries. *Enpp3* mRNA expression reached its highest level at 12 hours, approximately 22-fold higher than that of the 0 hour (ie, control; Figure 1A). Western blot results showed that ENPP3 protein levels increased at 8 hours after hCG treatment and expression remained high until 24 hours after hCG (Figure 1B).

The relative expression patterns of *Enpp3* mRNA in the granulosa cell and theca-interstitial cell compartments were similar to that in intact ovaries. The expression of *Enpp3* mRNA in granulosa cells reached the highest levels at 12 hours, approximately 107-fold higher than that of the 0 hour (ie, control; Figure 1C). In the theca-interstitial cell compartment, the highest expression level of *Enpp3* mRNA at 12 hours was approximately 9-fold higher than that of the 0 hour (ie, control;

Figure 1D). These results suggest that the hCG-induced stimulation of *Enpp3* mRNA in the granulosa cells was much more significant than that in the theca-interstitial cell compartment.

Localization of *Enpp3* mRNA in the Granulosa and Theca Cells of the Ovary

In situ hybridization was used to analyze the localization of *Enpp3* mRNA in intact ovaries after hCG. *Enpp3* mRNA was rarely detected prior to hCG treatment (0 hour; Figure 2). *Enpp3* mRNA began to be expressed in the theca-interstitial cells at 4 hours after hCG and remained high until 12 hours (Figure 2). *Enpp3* mRNA was strongly localized in the granulosa cells at 12 hours after hCG (12 hours; Figure 2). The signal of *Enpp3* mRNA was still present in the forming CL at 24 hours after hCG treatment (24 hours; Figure 2).

Stimulation of *Enpp3* mRNA by hCG in Granulosa Cells In Vitro

To determine whether the increase in *Enpp3* mRNA in vivo can be mimicked in vitro, granulosa cells were isolated from the ovaries collected 48 hours after PMSG treatment. Treatment with hCG increased the expression of *Enpp3* mRNA in granulosa cells in vitro (Figure 3A), with the highest levels observed at 12 to 24 hours after hCG. Forskolin, which is an activator of adenylate cyclase, and PMA, an activator of protein kinase C (PKC), were used to mimic the action of hCG. The FSK + PMA treatment also stimulated the expression of *Enpp3* mRNA in cultured granulosa cells (Figure 3B). *Enpp3* mRNA reached the highest expression at 24 hours after FSK + PMA administration.

Intracellular Signaling Pathways Regulating Granulosa Cell *Enpp3* mRNA Expression

To investigate which signaling pathway(s) is involved in *Enpp3* mRNA expression, both FSK and PMA were used to activate the protein kinase A (PKA) and PKC signaling pathways, respectively. Granulosa cells from rat preovulatory ovaries (48 hours post-PMSG) were cultured with FSK or PMA for 12 hours. Interestingly, only PMA treatment induced the expression of *Enpp3* mRNA (Figure 4). These results suggest that only the PKC signaling pathway instead of the PKA pathway is involved in the hCG-induced *Enpp3* mRNA expression.

To further investigate the mechanisms regulating *Enpp3* mRNA expression, granulosa cells were cultured with medium alone (Ctrl), hCG, inhibitors of various hCG-stimulated signaling molecules (H89, an inhibitor of PKA [10 μ M]; LY294002, an inhibitor of phosphatidylinositol 3 kinase [PI3 kinase; 25 μ M]; PD98059, an inhibitor of MAPK kinase [MEK; 20 μ M]; GF109203X, an inhibitor of PKC [1 μ M]), or hCG in combination with the above inhibitors for 12 hours. Real-time PCR results showed that LY294002 significantly inhibited the hCG stimulation of *Enpp3* mRNA and GF109203X completely blocked *Enpp3* mRNA to control levels (Figure 5). These

Figure 1. Stimulation of ectonucleotide pyrophosphatase/phosphodiesterase (*Enpp3*) expression by human chorionic gonadotropin (hCG) in vivo. Real-time polymerase chain reaction (PCR) analysis shows the expression of *Enpp3* messenger RNA (mRNA) in whole ovaries (A), rat granulosa cells in vivo (C), and theca-interstitial cells in vivo (D). Rat ovaries, granulosa cells, and theca-interstitial cells were collected at 0, 4, 8, 12, and 24 hours after hCG treatment. Relative levels of mRNA for *Enpp3* were normalized to the *Rpl32* in each sample (mean $\pm$ standard error of the mean [SEM]; $n = 3$ independent culture experiments). Western blot analysis (B) shows ENPP3 protein expression at different time points after hCG treatment in intact rat ovaries. All the 0 hour time points were standardized to "1." Bars with no common superscripts are significantly different ($P < .05$).

H89 and PD98059 had no effect on the hCG-induced expression of *Enpp3* mRNA.

AG1478 (Figure 6), whereas RU486 had no effect on the stimulation of *Enpp3* mRNA (Figure 6).

Hormonal Regulation of *Enpp3* mRNA Expression in Granulosa Cells

To investigate whether the hCG-induced increase in *Enpp3* mRNA level requires de novo protein synthesis, granulosa cells were incubated for 12 hours with or without hCG treatment in the absence or presence of cycloheximide, an inhibitor of new protein synthesis. Cycloheximide completely blocked hCG-induced *Enpp3* mRNA expression (Figure 6). Stimulation of prostaglandin-endoperoxide synthase 2 (PTGS2), induction of progesterone receptors (PGR), and activation of epidermal growth factor (EGF) signaling by hCG are also crucial for ovulation and luteinization.²⁸ Signaling inhibitors NS398 (PTGS2 inhibitor), RU486 (PGR inhibitor), and AG1478 (EGF-signaling inhibitor) were used to test whether the induction of *Enpp3* mRNA is mediated by the hCG-induced activation of these signaling pathways. The induction of *Enpp3* mRNA expression was partly blocked by NS398 and

Discussion

Seven members of the ENPP family have been identified and numbered according to their order of cloning (ENPP1-ENPP7).²⁹ This family of ectonucleotidases acts to hydrolyze pyrophosphate and phosphodiester bonds but also has been reported to be multifunctional proteins that can interact with the extracellular matrix or even signal into the cell.²⁹ This led us to explore the expression of *Enpp3* in the ovary around the time of ovulation.

ENPP3 has been shown to regulate immune cell function, induce morphological changes in fibroblasts, and regulate synthesis of differentiation specific proteins.⁷ During ovulation, the ovary undergoes dynamic changes in differentiation, inflammation, and morphology. The LH surge initiates an acute inflammatory reaction in mature follicles, leading to dissolution of the follicle wall, and differentiation of the granulosa and theca cells into the CL. In other systems, many of these changes

Figure 2. Cellular localization of *Enpp3* mRNA in periovulatory rat ovaries. Sections of rat ovaries obtained at 0, 4, 8, 12, and 24 hours after hCG injection were hybridized with the appropriate antisense probe for *Enpp3* mRNA. Representative bright-field (A) and corresponding dark-field (B) photomicrographs are depicted. Sections hybridized with the negative control sense probe (Neg) are shown. Scale bars, 500 μ M. *Enpp3* indicates ectonucleotide pyrophosphatase/phosphodiesterase; F, follicle; hCG, human chorionic gonadotropin; mRNA, messenger RNA; nCL, newly formed corpus luteum.

that occur during the ovulatory process are mediated by ENPP3. For example, in the inflammatory cascade, ENPP3 has been shown to be present on basophils and mast cells and regulate the response of these cells to inflammatory mediators by suppressing ATP-dependent inflammation in response to an allergic stimulus.^{8,10} Physiologic stimuli induce the secretion of extracellular ATP, which acts in an autocrine or paracrine manner to modulate cellular functions, including the activation of basophils and mast cells. ENPP3 acts to hydrolyze extracellular ATP, thereby suppressing basophil and mast cell activity, which has been shown to be important in the release of histamine during the ovulatory process.^{10,30}

During the ovulatory process, there are marked changes in the differentiation, proliferation, and reorganization of the ruptured follicle as it transforms to the CL. Ectonucleotidases play a pivotal role in the regulation of the extracellular levels of the nucleotide effectors, which can have a profound influence on cell proliferation and differentiation processes.^{29,31} For example, overexpression of ENPP3 in mouse fibroblasts and rat glioma cells, which do not normally express ENPP3, resulted in the expression of proteins associated with differentiation as well as a change toward an invasive phenotype.⁷ Likewise, in neuro-2a neuroblastoma cells, the levels of ENPP1 and ENPP3 were associated with differentiation into a neuronal-like phenotype.³¹ This role in differentiation may be important in the transition from a granulosa cell into a luteal cell phenotype. It has been shown that *Enpp3* expressed significantly higher in floating granulosa cells compared to cumulus granulosa cells obtained from women stimulated antral follicles.³² The data highly suggested that *Enpp3* was involved in the folliculogenesis and ovulation.

The findings of the present study demonstrate that *Enpp3* is highly induced in both granulosa cells and theca-interstitial cells by hCG both in vivo and in vitro. The induction of *Enpp3* in the granulosa cells is much higher than that in theca-interstitial cell compartment, and the significance of this difference in expression is unclear. Interestingly, the localization studies revealed that *Enpp3* mRNA expression was initially detected in theca-interstitial cells at 4 hours after hCG and remained elevated until 12 hours after hCG. The expression of *Enpp3* mRNA then gradually increased in the granulosa cell compartment from 8 to 12 hours after hCG, and expression of *Enpp3* mRNA was also detected in the forming CL. The high expression in the forming CL suggests a potential role of the *Enpp3* in luteal function. Our observations support a role for *Enpp3* in follicular differentiation and remodeling during follicular rupture and/or the redistribution of granulosa and theca cells to achieve luteal differentiation.

The preovulatory LH/hCG stimulus is known to activate both the PKC and PKA signaling pathways in preovulatory granulosa cells.³³ The current results suggested that hCG acts to stimulate *Enpp3* mRNA expression through the PKC but not the PKA pathway, as PMA mimicked the hCG induction of *Enpp3* mRNA whereas FSK had no effect. Further support for

Figure 3. Induction of ectonucleotide pyrophosphatase/phosphodiesterase (*Enpp3*) messenger RNA (mRNA) expression by human chorionic gonadotropin (hCG) or forskolin + phorbol 12 myristate 13-acetate (FSK + PMA) in rat granulosa cells in vitro. Real-time polymerase chain reaction (PCR) analysis shows the expression of *Enpp3* messenger RNA (mRNA) in granulosa cells obtained from rat preovulatory ovaries (48 hours post-pregnant mare serum gonadotropin [PMSG]) and cultured in medium alone (control), hCG (1 IU/mL; A), or with FSK (10 μ M) + phorbol 12 myristate 13-acetate (PMA, 20 nM; B) for different time points. Relative levels of mRNA for *Enpp3* were normalized to the *Rpl32* in each sample (mean $\pm$ standard error of the mean [SEM]; $n = 3$ independent culture experiments). All the 0 hour time points were standardized to "1." Bars with no common superscripts are significantly different ($P < .05$).

Figure 4. Regulation of ectonucleotide pyrophosphatase/phosphodiesterase (*Enpp3*) messenger RNA (mRNA) expression in granulosa cells in vitro. Granulosa cells from rat preovulatory ovaries (48 hours post-pregnant mare serum gonadotropin [PMSG]) were cultured in medium alone (Ctrl), forskolin (FSK; F, 10 μ M), or with phorbol 12 myristate 13-acetate (PMA; P, 20 nM) for 12 hours. Relative levels of mRNA for *Enpp3* were normalized to the *Rpl32* in each sample and expressed as a fold change relative to the untreated control (mean $\pm$ standard error of the mean [SEM]; $n = 4$ independent culture experiments). Bars with no common superscripts are significantly different ($P < .05$).

the regulation of *Enpp3* mRNA by the PKC pathway was evident by experiments where the induction of *Enpp3* mRNA was blocked after administration of the PKC inhibitor GF109203X, whereas both the PKA inhibitor H89 and the PKA pathway downstream mediator MEK inhibitor PD98059 had no effect on the stimulation of *Enpp3* mRNA. An LH/hCG stimulus initiates other kinase signaling pathways such as the cyclic adenosine monophosphate-dependent, PKA-independent activation of PI3 kinase.³⁴ The administration of the PI3 kinase inhibitor LY294002 reduced the late expression of *Enpp3*

Figure 5. Regulation of ectonucleotide pyrophosphatase/phosphodiesterase (*Enpp3*) messenger RNA (mRNA) expression by inhibitors of intracellular signaling pathways in granulosa cells in vitro. Granulosa cells were cultured with medium alone (Ctrl), inhibitors of various signaling molecules (an inhibitor of protein kinase A [PKA; H89, 10 μ M], protein kinase C [PKC; GF109203X [GF], 1 μ M], phosphatidylinositol 3-kinase [LY294002 [LY], 25 μ M], MEK [PD98059 [PD], 20 μ M]), human chorionic gonadotropin (hCG), or hCG + inhibitors of various signaling molecules for 12 hours. Relative levels of mRNA for *Enpp3* were normalized to the *Rpl32* in each sample (mean $\pm$ standard error of the mean [SEM]; $n = 3$ independent culture experiments). Bars with no common superscripts are significantly different ($P < .05$).

mRNA. This result demonstrated that the PI3 kinase pathway was also involved in regulating *Enpp3* mRNA expression.

As a series of downstream events induced by LH surge are crucial for ovulation,³⁵ we further examined the impact of

Figure 6. Hormonal regulation of the human chorionic gonadotropin (hCG)-induced ectonucleotide pyrophosphatase/phosphodiesterase (*Enpp3*) messenger RNA (mRNA) expression in cultured rat granulosa cells. The cells were cultured in medium alone (Ctrl), with the progesterone receptor antagonist RU486 (RU, 1 μ M), the epidermal growth factor (EGF) receptor tyrosine kinase selective inhibitor AG1478 (AG, 1 μ M), the prostaglandin-endoperoxide synthase 2 inhibitor NS-398 (NS, 1 μ M), the new protein synthesis inhibitor cycloheximide (CHX, 1 μ g/mL), hCG (1 IU), or hCG + the inhibitors of various hormonal signaling molecules for 12 hours. Relative levels of mRNA for *Enpp3* were normalized to the *Rpl32* in each sample and expressed as a fold change relative to the untreated control (mean $\pm$ standard error of the mean [SEM]; $n = 3$ independent culture experiments). Bars with no common superscripts are significantly different ($P < .05$).

progesterone, prostaglandins, and the EGF signaling pathway on the regulation of *Enpp3* mRNA expression. *Enpp3* mRNA expression was partly blocked by the EGF receptor tyrosine kinase inhibitor AG1478 or the prostaglandin synthesis inhibitor NS398. There was no effect of PGR antagonist RU486 on hCG-induced expression of *Enpp3* mRNA. The present findings are in agreement with observations that prostaglandins, particularly prostaglandin D2 and to a lesser effect prostaglandin F2- α , regulate ENPP3 expression in basophils.³⁶ There are no reports of the regulation of *Enpp3* mRNA by progesterone or the EGF pathway. The current findings demonstrate an inhibitory effect of AG1478 and NS398, suggesting that the EGF pathway and prostaglandins play important roles in regulating *Enpp3* mRNA.

In summary, the present results demonstrate an induction of *Enpp3* expression in granulosa cells and theca-interstitial cells after hCG treatment. Human chorionic gonadotropin stimulated the expression of *Enpp3* through the PKC pathway. Furthermore, *Enpp3* induction is dependent on new protein synthesis, and the EGF pathway and prostaglandins mediate the stimulation of *Enpp3* expression by hCG. Our findings support the concept that *Enpp3* may play a role in the morphological changes and inflammatory response during ovulation and luteinization.

Authors' Note

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Declaration of Conflicting Interests

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