

Transient expression of progesterone receptor and cathepsin-L in human granulosa cells during the periovulatory period

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Objective: To study in vivo the progesterone receptor (PR) expression levels in human granulosa cells (GCs) during the periovulatory period and the affect of the protein kinase A (PKA) pathway on PR expression and cathepsin-L expression-activation.

Design: Experimental study.

Setting: University research unit.

Patient(s): Twenty-five women of reproductive age.

Intervention(s): Follicular fluid and GCs obtained from spontaneous cycles before and during the normal luteinizing hormone surge, and samples obtained 36 hours after human chorionic gonadotropin (hCG) administration in patients undergoing in vitro fertilization.

Main Outcome Measure(s): To determine PR, cathepsin-L messenger RNA (mRNA) analysis via real-time polymerase chain reaction, and protein of PR, cathepsin-L, and PKA in human GCs.

Result(s): The Western blot analysis revealed that bands of PR (isoform A) were the most abundant and that mRNA (PR-A and PR-B) have a temporal pattern of expression throughout the periovulatory period. The protein levels of PR and cathepsin-L were up-regulated by hCG. The abundance of PR was diminished in the presence of PKA inhibitor, and cathepsin-L with PR receptor antagonist.

Conclusion(s): The transient expression of PR in human GCs of the preovulatory follicle suggests that PR and its ligand play a role in the activation of cathepsin-L, which is presumably involved in the degradation of the follicular extracellular matrix during human ovulation. (*Fertil Steril*® 2012;97:707–13. ©2012 by American Society for Reproductive Medicine.)

Key Words: Cathepsin -L, human ovulation, progesterone receptor

During the ovulation process, the oocyte is released from a dominant ovarian follicle. This event is associated with the preovulatory luteinizing hormone (LH) surge and expression of numerous genes that regulate luteinization, cumulus expansion, and follicle rupture (1–3). The progesterone receptor (PR) and its ligand is one of the key factors

involved in the ovulatory process (4, 5). In mouse and rat granulosa cells (GCs), PR messenger RNA (mRNA) generates two protein isoforms, PR-A (92 kd) and PR-B (116 kd) (6, 7). Both mRNA and protein are transiently expressed in vivo (8). Likewise, in primary GCs cultures, the PR isoforms are temporally expressed, reaching their peaks after 4 to 8 hours of human

chorionic gonadotropin (hCG) stimulus and their nadir at 12 hours after hCG treatment (9–12).

In monkeys and humans, PR is localized and expressed in GCs after the LH surge and the corpus luteum (CL) (13–16). In mice, PR signaling drives the induction of metalloproteinases such as ADAMTS-1 and cathepsin-L, promoting follicular rupture (17). Conversely, in human the molecular regulation of PR and metalloproteinases of preovulatory GCs is almost unknown. Our study was designed to determine the hormone concentration in follicular fluid (FF) and the localization and expression of PR in GCs during the periovulatory period. We hypothesized that PR has a temporal and transient expression pattern in mural GCs but plays a critical role in the physiology of human ovulation.

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MATERIALS AND METHODS

Patients

The endocrine status was assessed at different times throughout the periovulatory period. Blood, FF, and GC samples were obtained from women requesting tubal ligations or participants in our *in vitro* fertilization (IVF) program. Samples were obtained from spontaneous cycles before the LH surge ($n = 6$) and during the normal LH surge ($n = 5$). Participants underwent serial vaginal ultrasound scans and urine LH determinations to define the preovulatory period. Laparoscopies were scheduled when the leading follicle had reached a maximum of 15 mm in diameter in the absence of an LH surge for the pre-LH group), and when the leading follicle had reached 18 to 20 mm in diameter and the urine tested positively for LH for the LH surge group. Samples from patients undergoing ovarian stimulation for IVF due to male factor infertility were obtained 36 hours after 10,000 IU of intramuscular hCG administration for the post-hCG group ($n = 14$). The FF and GCs from the post-hCG group were collected at the time of oocyte retrieval, as reported previously elsewhere (18).

This study was approved by the local institutional review board of the Hospital Clínico San Borja-Arriarán. Signed, informed consent was obtained from all women participating in this study.

Isolation of Follicular Fluid and Granulosa Cells

The FF obtained from women undergoing tubal sterilization was centrifuged at $400 \times g$ for 5 minutes. The supernatant from the leading follicle was removed and frozen at -20°C for hormone determinations. The cell pellet was dispersed with 1 mM ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS), incubated for 10 minutes and centrifuged at $400 \times g$ for 5 minutes. Aliquots containing approximately 10,000 cells were centrifuged at $400 \times g$ for 8 minutes in a Cyto-spin system (Universal 32R; Hettich Zentrifugen) for immunofluorescence. The rest of the cells were stored at -80°C for real-time polymerase chain reaction (qRT-PCR) or Western blot analysis. The granulosa luteal cells (GLCs) were isolated and plated from aspirated follicles and culture, as previously reported elsewhere (19).

Primary Granulosa Luteal Cell Cultures

The GLCs were cultured for 76 hours in growth medium and 24 hours in serum-free Dulbecco's minimum essential medium (DMEM) supplemented with 0.1% bovine serum albumin (BSA), 20 nM insulin, 20 nM selenium, 20 nM apotransferrin, and 1 μM vitamin E in the presence or absence of several stimuli (hCG, forskolin, and mifepristone). Cultured cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2 . The medium was collected for progesterone (P4) determination, and the cells were stored for total RNA and protein extraction, and immunocytochemistry.

Viability of Cultured Granulosa Luteal Cells

The viability of GLCs was determined spectrophotometrically, assessing the uptake of crystal violet. The absorbance

was measured at 600 nm with a microplate reader (DNM-9602G; Bausch & Lomb). Cell viability was greater than 85%.

Hormone Levels in Follicular Fluid and Conditioned Culture Media of GLCs

The concentrations of estradiol (E_2), P4, and LH/hCG in the serum, FF, and media of cultured GLCs were determined in duplicate by radioimmunoassay (RIA) and immunoradiometric assay (IRMA), as previously reported elsewhere (20).

Western Blotting

The levels of PR, protein kinase A catalytic subunit (PKAc), and cathepsin-L in GCs were determined by Western blot analysis. A total protein homogenate (40 μg) was loaded onto 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences).

Immunoreactive proteins were detected with primary and POX-conjugated secondary antibodies and developed using a chemiluminescence kit (Amersham ECL Plus; GE Healthcare). The densitometric analysis was performed using the Image-ProPlus program (version 4.5.1.22; Media Cybernetics, Inc.). The antibodies used were anti-PKAc (BD Biosciences), anti-PR isoforms A and B (Santa Cruz Biotechnology), anti-PR isoform B (Lab Vision), and anti-cathepsin-L (Novus Biologicals).

Immunofluorescence and Immunocytochemistry

The cellular distribution of PR isoforms in GCs was determined by indirect immunofluorescence using an adapted methodology described elsewhere (21). Samples were blocked with 3% bovine serum albumin (BSA) in PBS at room temperature for 1 hour, followed by washings with PBS and an overnight incubation at 4°C with PR antibody (dilution 1:200). Subsequently, cells were incubated with fluorescein-conjugated anti-rabbit immunoglobulin G (Sigma-Aldrich) for 1 hour at 37°C (dilution 1:5,000). Immunofluorescence was documented with an epifluorescence microscope (Olympus BX-51TF; Olympus Optical Co. Ltd.) and CoolSNAP-Pro (Media Cybernetics).

The GLCs were grown on glass cover slips for immunocytochemical studies. The cover slips were incubated with a primary antibody against cathepsin-L (dilution 1:50) for 2 hours at 4°C . The primary antibody was absent in the negative control.

Progesterone Receptor RNA Extraction and Quantitative Real-time PCR

The total RNA extraction procedure was performed according to the manufacturer's instructions (Qiagen). The reverse transcription of RNA into complementary DNA (cDNA) was performed by use of the Improm II Reverse Transcription system (Promega) and the GeneAmp PCR system 9700 Thermal Cycler. Quantitative PCR experiments were performed with the ABI Prism 7700 Sequence Detection System (ABI) using

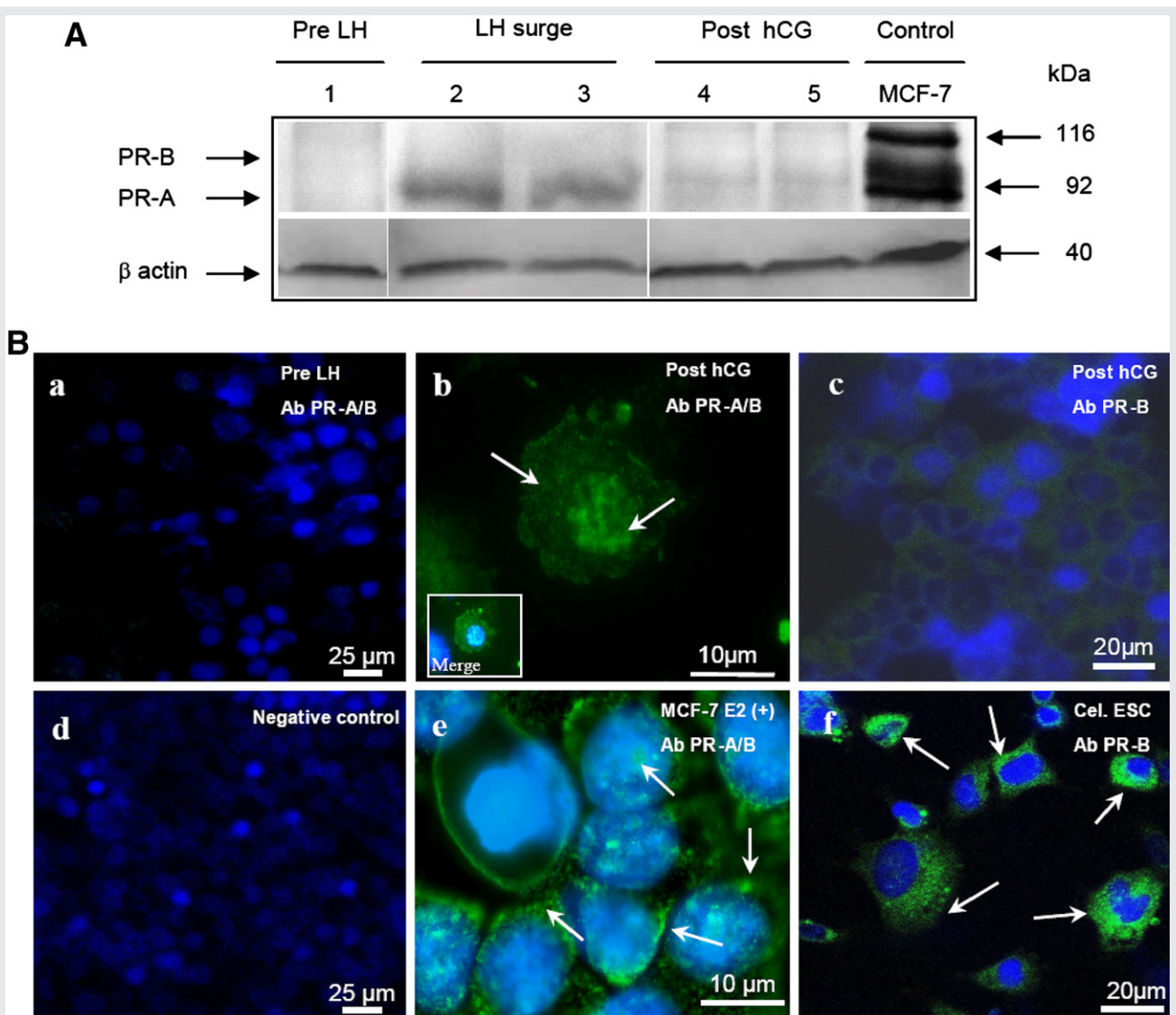
appropriate primers and GoTaq enzyme. The 18S RNA values were used for normalization. The relative quantification of the genes of interest was analyzed by the comparative threshold cycles (CT) method (22). The value was used to plot the expression level of the gene of interest using the expression $2^{-\Delta\Delta CT}$. The forward and reverse primer sequences (5' to 3') were as follows: PR total (293 bp), GTCAGTGGCA GATGCTGTA and TGTGAGCTCGACACAACCTCC; PR-B (196 bp), ACACCTGCCTGAAGTTTCG and CTGTCCTTTCTGGGG GACT; cathepsin-L (292 bp), GTGGACATCCCTAAGCAGGA

and CACAATGGTTTCTCCGGTCT; and 18S (343 bp), TAAC GAGGATCCATTGGAGG and CCCTCTAATCATGGCCTCA.

Statistical Analysis

All experiments were performed in duplicate and repeated at least three times. Experimental data are presented as the mean \pm standard error of the mean, and the number of experiments is indicated in the figure legends as n. The significance of the in vivo results was determined using Student's *t*-test. The

FIGURE 1



(A) Western blot of progesterone receptor (PR) isoform A (92 kd) and isoform B (116 kd) in human granulosa cells (GCs) collected from patients before and at the time of the luteinizing hormone (LH) surge and after human chorionic gonadotropin (hCG) administration in IVF stimulated cycles. The GCs collected at the time of LH surge showed the most abundant levels of PR-A isoforms. Molecular mass standard is indicated on the right; β -actin was used as loading control and MCF-7 cells as positive control. Data represent an individual patient. (B) Immunofluorescence of PR in human GCs obtained from patients before LH surge and after hCG administration: (a) PR isoforms not detected before LH surge. (b) Total PR (green) detected after hCG administration (white arrows). (c) PRB isoforms exhibiting a weak signal. Nuclei were staining with DAPI (blue). (d) Negative control. (e, f) MCF-7 cells and endometrial cells for PR-A/B and PR-B isoform used as a positive control, respectively. Images are representative of three experiments.

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in vitro results were analyzed by analysis of variance (ANOVA) followed by the Tukey's test for comparisons between control and treatment conditions; $P < .05$ was considered statistically significant.

RESULTS

Clinical and Endocrine Characteristics of Study Participants

There were no differences in the mean age and body mass index (BMI) between the three groups of women (Supplementary Table 1, available online). Throughout the LH surge and after hCG administration, the FF concentration of LH and hCG were statistically significantly higher than those obtained before the LH surge. The serum levels of P4 and E₂ at the time of the LH surge and after hCG administration were statistically significantly higher than those of the pre-LH group. The three groups displayed statistically significantly higher levels of LH in the serum than FF. Conversely, the levels of steroid hormones were statistically significantly higher in FF than in the serum in all groups.

Identification of PR in Human GCs

The PR protein levels were examined in vivo by Western blot and immunofluorescence in GCs (Fig. 1). The Western blot showed immunoreactive bands at 116 kd (PR-B) and 92 kd (PR-A). The PR-A was visualized only in patients with LH

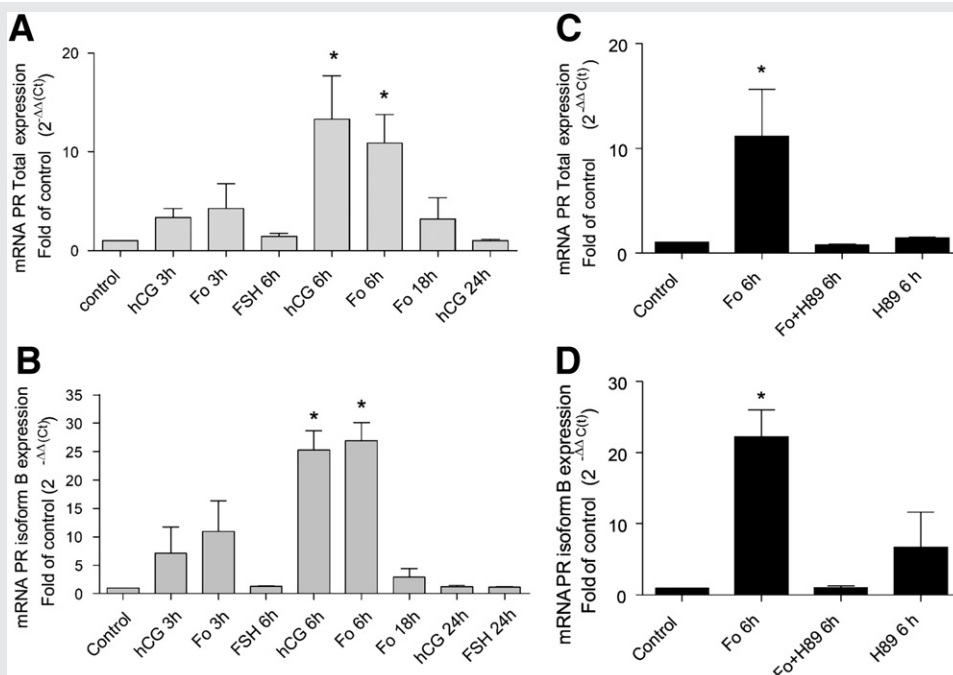
serum levels higher than 10 mIU/mL. The immunoreactive band for PR-B was not detected in any of the groups (Fig. 1A).

Figure 1B shows immunofluorescence of the PR in GCs of pre-LH (a) and post-hCG groups (b). This experiment was performed using an antibody that recognized both isoforms of PR (ab PR-A/B) and a supplementary antibody that recognized only PR-B (ab PR-B). In human GCs of the post-hCG group, the signal for PR-B was very weak (c). In GCs of the post-hCG group, ab PR-A/B revealed a higher intensity than observed in GCs of the pre-LH group, and the PR immunolocalization was predominantly nuclear (d, e, and f represent experimental controls).

Figure 2A and B depicts PR gene expression in GLCs cultured at different times in the presence of hCG (10 IU/mL) and forskolin (1 μ M). Both hCG and forskolin statistically significantly stimulated PR expression after 6 hours of treatment. However, no difference was observed between the basal levels and after 3, 12, and 24 hours of stimulation. These findings suggest that PR-A and PR-B have transient expression patterns in human GLCs.

Figure 2C and D illustrates the in vitro expression of PR mRNA in GLC cultures stimulated for 24 hours with forskolin in the presence or absence of the PKA inhibitor (H89). Forskolin treatment statistically significantly increased the levels of PR mRNA after 6 hours (PR total and isoform B). Conversely, GLCs cultured in presence of H89 (1 μ M) had a statistically significantly reduced forskolin response on PR mRNA

FIGURE 2



Progesterone receptor (PR) messenger RNA (mRNA) expression by quantitative real-time polymerase chain reaction in granulosa luteal cells (GLCs). (A, B) Total PR-A/B and PR-B messenger RNA (mRNA) increased after 6 hours incubation with human chorionic gonadotropin (hCG) (10 IU/mL) or Forskolin (1 μ M). (C, D) Protein kinase A (PKA) inhibitor (H89) abolished the stimulatory effect of hCG (10 IU/mL) and forskolin (1 μ M) of PR expression in GLCs culture. The values were expressed as mean \pm standard error of the mean (n = 6). * $P < .05$ compared with control and other experimental conditions.

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expression. These findings suggest that the LH-cAMP-PKA pathways regulate PR expression in GCs.

Furthermore, the analysis of the catalytic subunit of PKA (PKAc) was determined by Western blot in GCs obtained before the LH surge and after hCG administration. Figure 3 illustrates the protein expression of PKAc, which was higher in the post-hCG group than in the pre-LH group. These data indicate that the kinase is less abundant during the preovulatory stage, and its expression is up-regulated by LH/hCG.

Cathepsin-L in Human GCs

The expression of cathepsin-L was examined in preovulatory GCs and in cultured GLCs. The mRNA levels were not statistically significantly different between groups (data not shown). Figure 4A presents the protein levels of pro-cathepsin-L (43 kd) and active cathepsin-L (25 kd). Active cathepsin-L was more abundant in the post-hCG group. The pro-cathepsin-L signal was not detected in the pre-LH group. Figure 4B depicts GLCs that were stimulated with hCG in the presence and absence of mifepristone (RU486). The signal for pro-cathepsin-L and active cathepsin-L increased after the hCG stimulus, and was reduced in the presence of RU486. Dexamethasone (Dx), a drug that suppresses the corticosteroid action of RU486, did not change these findings. These observations suggest that PR modulates the protein levels of pro-cathepsin-L and active cathepsin-L in luteinizing human GCs.

Furthermore, active cathepsin-L and pro-cathepsin-L were immunolocalized in GLCs (Fig. 4D and E). Cathepsin-L

was observed mostly in the cytoplasm in a perinuclear pattern. The signal was stronger in hCG-stimulated GLCs, and diminished in the presence of mifepristone (Fig. 4F), suggesting that P4, at least, partially regulates its expression.

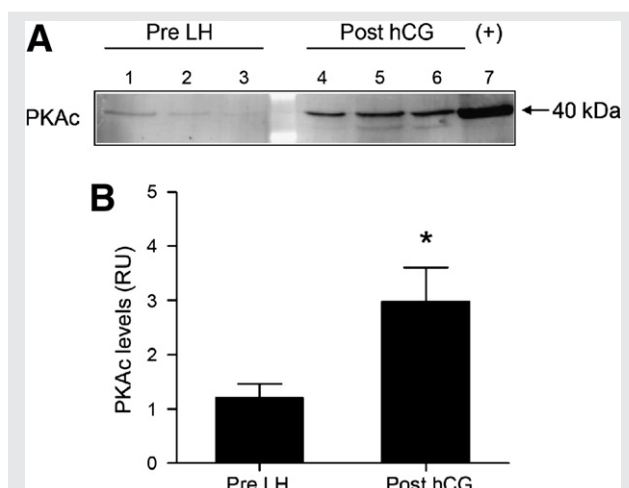
DISCUSSION

The results presented herein indicate that during the ovulatory process the steroid and gonadotropin levels in FF differed greatly among the pre-LH period, the LH surge, and after hCG. It is interesting that the levels of P4 before the LH surge in the FF were extremely high considering that the steroidogenic machinery in granulosa cells before the LH surge is limited. The steroidogenic acute regulatory protein (StAR) that is critical for P4 production is scarcely expressed in GCs of preovulatory follicles (23–25). These data suggest that the contribution of theca steroidogenesis may be critical to the follicular P4 during the pre-LH period. It is conceivable that the P4 ovulatory signal is downstream of the LH receptor and starts earlier than the LH surge, which is the main stimulus that induces ovarian PR expression in other mammalian models (26–28). We demonstrated by Western blot that PR-A expression in GCs begins to be visible with the LH surge, presumably triggered by the cAMP-signaling pathway. We did not detect PR-B by immunoblotting. The immunofluorescence studies were consistent with the Western blots, confirming the absence of PR-B signal in the pre-LH group. In vitro, mRNA PR-A and mRNA PR-B were transiently up-regulated in GLCs after hCG and forskolin stimulation. These findings confirm the results of other studies, namely, that PR expression in mammals exhibits a temporal, LH/hCG-dependent pattern (27, 28). The immunofluorescence analysis of PR indicated that PR-A is the predominant isoform expressed in human GCs during the LH surge. This is consistent with findings from different species, suggesting that PR-A is the most important PR isoform during ovulation.

In the rat and mouse, the activation of the G-protein coupled to LH-receptor in GCs in response to the LH surge stimulates cyclase. This increases intracellular cAMP, thereby activating protein kinase A and inducing expression of PR (29, 30). Our in vivo study also indicates that the PKA protein is activated by hCG and that in vitro H89 reduces significantly PR expression. These results might indicate that in our cellular type the LH-hCG/AMPC/PKA pathway is the most prevalent signal for inductions of PR expression. Nevertheless, a number of studies in the rat and mouse have shown that the LH surge via PKC activates MAPK, which is essential for the induction of PR mRNA and phosphorylation of ERK1/2 through the transcriptional factor Sp1 and Sp3 (31–34). Therefore, it is possible that the LH/hCG, PKA, PKC, or MAPK pathways can activate transcription factors such as Sp1 and Sp3 that are required for induction of PR mRNA expression in CGS, in contrast to other cell types (35, 36).

It is important to note that cathepsin-L mRNA levels were not different between the pre-LH and post-hCG groups. We believe that the lack of difference in cathepsin-L mRNA levels is due to the fact that its expression is not only LH dependent (17). Moreover, the expression levels of cathepsin-L in GCs cultures indicated that hCG up-regulates the

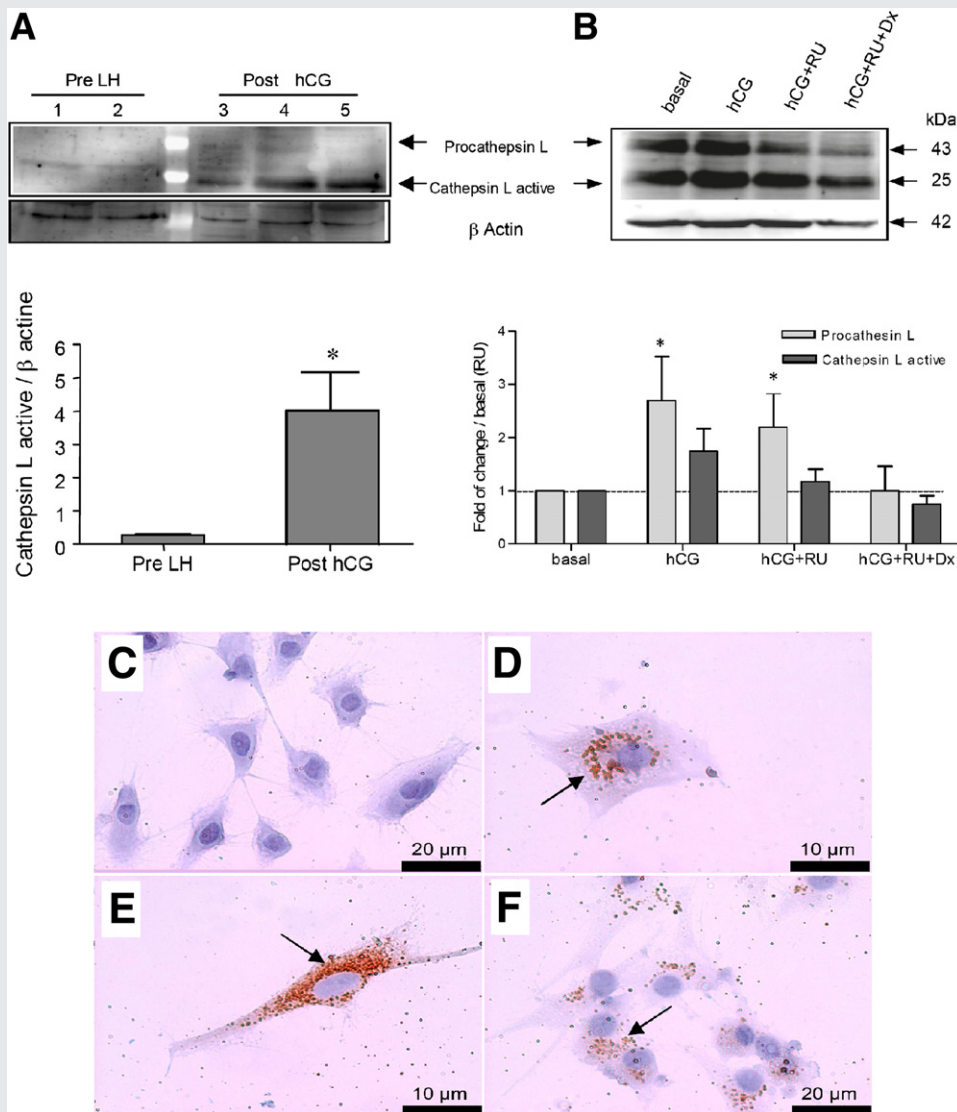
FIGURE 3



(A) Western blot and (B) densitometric analysis of the protein kinase A (PKA) catalytic subunit (40 kd) in human preovulatory granulosa cells (GCs) collected before the luteinizing hormone (LH) surge (lanes 1, 2, and 3) and after human chorionic gonadotropin (hCG) administration (lanes 4, 5, and 6). Total proteins (40 μ g) were loaded in each lane. Lane 7: homogenized rat brain used as a positive control. Densitometric relative units (RU) are normalized with Ponceau staining as loading control. Values are \pm standard error of the mean from three individual observations in each group ($n = 6$). * $P < .05$.

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FIGURE 4



(A) Immunoblots of pro-cathepsin-L (43 kD) and cathepsin-L active (25 kD) in human preovulatory granulosa cells (GCs) collected before the luteinizing hormone (LH) surge and after human chorionic gonadotropin (hCG) administration in the IVF cycle. Densitometric analysis shows that cathepsin-L active is statistically significantly expressed in the post-hCG group ($*P < .05$). (B) Effect of hCG (10 IU/mL) on cathepsin-L active and pro-cathepsin-L avoided when cells are incubated for 24 hours in the presence of antiprogesterin RU486 (1 μ M). Densitometric results are expressed as a fold of basal value for cathepsin-L active and pro-cathepsin-L in human granulosa luteal cells (GLCs). Values are expressed as mean \pm standard error of the mean ($n = 5$); $*P < .05$ compared with control and hCG + RU486 (mifepristone) + Dx (dexamethasone). (C–F) Immunolocalization of cathepsin-L in human GLCs in presence and absence of RU486. (C) GLCs negative control. (D) GLCs basal. (E) GLCs culture + hCG (10 IU/mL). (F) GLCs culture + hCG (10 IU/mL) and RU486 (1 μ M).

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expression levels of the proteases, but mifepristone reduces their expression levels, suggesting that PR modulates cathepsin-L protein levels in human GLCs, presumably via effects. To the best of our knowledge, these data are the first to show the expression of cathepsin-L in human GCs and to demonstrate two key points in its regulation, which are consistent with findings in rodents (37). It is thought that this protease plays an important function in remodeling the extracellular matrix (38), a critical process during ovulation.

Our in vivo and in vitro study enhances the understanding of molecular regulatory mechanisms presumably involved in human ovulation. These findings could have significant clinical implications on the development of cell- or tissue-specific treatments for human ovulation and contraceptive development.

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SUPPLEMENTARY TABLE 1

Demographic features of patients and serum and follicular fluid hormone levels throughout preovulatory stages and after human hCG administration.

	Before LH	LH surge	After hCG (36 h)
Age (y)	33.0 ± 1.96	35.3 ± 1.76	34.7 ± 0.66
BMI (kg/m ²)	28.0 ± 1.6	29.8 ± 4.1	31.0 ± 0.98
LH serum (mIU/mL)	8.06 ± 3.5 ^a	73.3 ± 22.3	40.1 ± 13.5
E ₂ serum (nmol/L)	1.03 ± 0.14	1.21 ± 0.18	7.46 ± 0.62 ^b
P4 serum (nmol/L)	0.93 ± 0.35	1.08 ± 0.36	3.88 ± 1.37 ^c
LH FF (mIU/mL)	1.33 ± 0.43 ^d	6.33 ± 2.4	7.0 ± 2.96
E ₂ FF (μmol/L)	5.76 ± 0.36	5.51 ± 0.63	6.19 ± 0.83
P4 FF (μmol/L)	7.25 ± 1.0 ^e	20.2 ± 1.6	38.5 ± 7.63

Note: Values are mean ± standard error of the mean. BMI = body mass index; E₂ = estradiol; FF = follicular fluid; hCG = human chorionic gonadotropin; LH = luteinizing hormone; P4 = progesterone.

^a P < .05 versus LH surge and after hCG.

^b P < .05 versus before LH and LH surge.

^c P < .05 versus before LH and LH surge.

^d P < .05 versus LH surge and after hCG.

^e P < .05 versus LH surge and after hCG.

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