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In-vitro study of gonadotrophin signaling pathways in human granulosa cells in relation to progesterone receptor expression

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KEY MESSAGE

In this study, a differential and newly identified progesterone receptor-dependent effect of HIF1A as a mediator of human follicular rupture was analysed. This finding improves our knowledge of LH–progesterone-receptor-signalling pathways involved in human ovulation.

A B S T R A C T

In humans, data on gonadotrophin-activated (LH, HCG and FSH) progesterone receptor expression and signalling pathways involved in matrix metalloproteinases (MMPs) expression presumably linked to the follicle rupture, are limited. Our hypothesis is LH, HCG and FSH increase progesterone receptor expression in granulosa cells through different signalling pathways, leading to an increased expression of ADAMTS-1 and MMP3/10, which may mediate follicular rupture through the transcription factor, HIF1A. Human granulosa cells were isolated from follicular aspirates obtained from 22 healthy women participating in our IVF programme for male-factor infertility. Progesterone receptor and HIF1A expression was assessed by immunofluorescence, and PKA-PKC-PI3K- ERK1/2, ADAMTS-1 and MMP3/10 expression by Western blot in pre-ovulatory and in cultured granulosa cells. Results show that HCG, LH and FSH regulate progesterone receptor expression and activate PKA, PKC, PI3K and ERK1/2 signalling pathways in granulosa cells but progesterone receptor expression is only mediated by PKA, PKC and ERK pathways. HCG, FSH and LH regulated MMPs expression through progesterone receptors. Moreover, HCG-progesterone-receptor-dependent HIF1A expression stimulated MMP3/10 expression but not that of ADAMTS-1. These results suggest differential downstream progesterone receptor signalling, as progesterone receptor regulates MMP3/10 expression via HIF1A, which is not involved in ADAMTS-1 expression.

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Introduction

Ovulation is a complex endocrine and molecular process, leading to the release of the oocyte from the dominant follicle. The process begins with a pre-ovulatory LH and follicle FSH surge that stimulates the expression of a number of genes that trigger various physiological, events such as cumulus expansion, granulosa cell luteinization and follicular rupture (Espey and Richards, 2002; Richards et al., 2002; Stouffer, 2003). The binding of progesterone to the progesterone receptor is an essential factor in ovulation. This finding is supported by numerous studies in rodents and a primate showing that administration of anti-progestins, such as mifepristone, inhibits ovulation (Loutradis et al., 1991; Richards, 2005). Treatment of progesterone-receptor-knockout mice with exogenous gonadotropins results in normal follicular development, but follicular rupture does not occur despite the luteinization of the granulosa cells. This finding indicates that progesterone receptor is essential for LH-dependent follicular rupture, but is not involved in granulosa and luteal cell differentiation for progesterone biosynthesis (Lydon et al., 1995; Robker et al., 2009). Progesterone receptor mediates the expression of several matrix metalloproteinases (MMPs), including metalloproteinases 3 and 10 (MMP3/10) and members of the desintegrin and metalloproteinase with thrombospondin motifs family (ADAMTS-1), which remodel the extracellular matrix of the follicle wall, ultimately resulting in rupture. All of these MMPs are present in the ovarian granulosa cells of rodents, sheep and humans after the LH surge (Espey et al., 2000; McCord et al., 2012; Robker et al., 2000; Yung et al., 2010). In mice, hypoxia-inducible factor 1-alpha (HIF1A) mRNA and protein expression are also induced through the progesterone receptor. Inhibiting HIF1A transcriptional activity with echinomycin suppresses ovulation by preventing follicular rupture, in part via effects on ADAMTS-1 expression (Kim et al., 2009).

Previous reports from our laboratory provided evidence of transient progesterone receptor expression in human granulosa cells during the peri-ovulatory period, disappearing after the LH surge (García et al., 2012). In addition, in-vitro studies on human granulosa cells showed that progesterone receptor mRNA expression significantly increases 6 h after HCG stimulation. This increase is blocked by a PKA inhibitor (García et al., 2012). Interestingly, in mice, LH can increase progesterone receptor expression through other signalling pathways, such as PI3K, PKC and ERK1/2 (Robker et al., 2009). These findings have not been confirmed in human granulosa cells.

Few data are available on the various gonadotrophin-activated (LH, HCG and FSH) signalling pathways involved in triggering the rupture of the dominant follicle. On the basis of our previous in-vivo findings of transient progesterone receptor expression in pre-ovulatory granulosa cells, the present study was carried out in order to determine progesterone receptor expression and localization in primary granuloa cell cultures treated with HCG, LH and FSH; (2) evaluate the roles of the various HCG-LH-FSH-activated signalling pathways (PKA, PKC, ERK1/2, AKT, or both) in progesterone receptor expression; (3) determine ADAMTS-1 and MMP3/10 expression in granuluosa cells in the pre-ovulatory follicle after HCG administration in IVF cycles; and (4) to assess the gonadotrophin-progesterone-receptor-dependent role of HIF1A in the expression of ADAMTS-1 and MMP3/10 in granulosa-cell cultures.

Materials and methods

Participants

Follicular aspirates from 22 healthy women (25–32 years old) undergoing ovarian stimulation for IVF for treatment of male factor infertility, were aspirated using a KITAZATO G -21 needle 36 h after HCG administration (10,000 IU). The aspiration used a pump setting of 150 mm Hg. This procedure setting removes granulosa cells with minimal aspiration of theca and endothelial cells. The granulosa cells were obtained after centrifugation of follicular fluid at 400 x g for 5 min. The pellet was suspended, and red blood cells and detritus were removed using a Histopaque (Sigma-Aldrich) gradient. Macrophages were eliminated by pre-plating for 30 min at 37°C, and cells were cultured as previously described (Olivero et al., 2008). The purity of the granulosa cell cultures was assessed by immunodetection of the steroidogenic acute regulatory protein. This protein is expressed in granulosa cell after the LH peak in normal cycles or after HCG administration in ovarian stimulation cycles (Henríquez et al., 2016). A portion of the granulosa cells obtained 36 h after HCG administration was used immediately to determine MMPs expression by Western blotting.

The Institutional Review Board of School of Medicine, University of Chile, approved the study on 8 September 2015, and signed informed consent was obtained from all women's participating in the study.

Granulosa-lutein cell cultures

Granulosa cells were cultured for 72 h in growth medium (M199) supplemented with 10% fetal bovine serum (FBS) followed by 24 h in serum-free medium (M199, Sigma-Aldrich, Missouri, USA). Progesterone receptor was detected in granulosa cells using temporal course of 6, 8, 12, 18 and 24 h under basal conditions and in the presence and absence of HCG (10 IU/ml, Sigma-Aldrich, Missouri, USA), LH (10 IU/ml, Luveris, Merck-Serono, Darmstadt, Germany), FSH (10 IU/ml, GONAL-f, Merck-Serono), H-89 (10 μM, Calbiochem, Merck millipore, Darmstadt, Germany), AKT inhibitor (LY 294002) (20 μM, Calbiochem, Merck millipore, Darmstadt, Germany), UO126 (10 μM, Calbiochem), bisindolylmaleimide I (BIM) (2,5 μM, Calbiochem, Merck millipore, Darmstadt, Germany). The concentrations of gonadotrophins and inhibitors were selected for maximal stimulation (gonadotrophins) or minimum inhibitory concentrations (inhibitors) based on dose-response curves from our previous studies and published data (García et al., 2012; Kong et al., 2005). Under these culture conditions, the granulosa cells do not express progesterone receptor (basal state), but they do recover the ability to express progesterone receptor and MMPs after gonadotrophin stimulus.

Protein extracts from granulosa cells obtained immediately after follicular aspiration after HCG administration were used to determine MMP expression *ex vivo*.

Gonadotrophin-dependent signalling pathways in granulosa cells were determined immediately after 24 h FBS deprivation. The granulosa cells were cultured for 5, 15 and 30 min under basal conditions and in the presence of HCG (10 IU/ml), LH (10 IU/ml) or FSH (10 IU/ml) to determine the levels of ERK1/2, AKT and CREB (protein phosphorylated by PKA). MARCKS (a protein phosphorylated by PKC) levels were determined only in the presence of HCG (10 IU/ml) and FSH (10 IU/ml). ADAMTS-1 and MMP3/10 levels were quantified in extracts of granulosa cells cultured for 24 h under basal conditions and in the presence or absence of HCG (10 IU/ml), LH (10 IU/ml) and FSH

(10 IU/ml), RU 486 (100 nM, Sigma-Aldrich, Missouri, USA) or echinomycin (0.5 uM, Sigma-Aldrich, Missouri, USA).

The cellular viability was evaluated at the end of the culture with MTS assay, a colorimetric method for determining the number of cell viable, described by the manufacturer (The CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega Corp, Wisconsin, USA).

Immunofluorescence

Progesteron receptor was detected by immunofluorescence in granulosa cells (50,000 cells per cover slip) cultured in the conditions described above. The coverslips were methanol-fixed and blocked with 3% bovine serum albumin in phosphate-buffered saline for 1 h at room temperature, followed by overnight incubation at 4°C with a primary monoclonal antibody capable of recognizing both progesterone receptor isoforms (progesterone receptor A/B antibody, Cell Signaling Technology, Massachusetts, USA, diluted 1:100). Subsequently, cells were incubated with anti-mouse secondary antibody conjugated with FITC (Sigma-Aldrich, Missouri, USA) for 1 h at 37°C, diluted 1:1000. The cellular localization of HIF1A in granulosa cells was carried out by time courses at 6, 12, 18 and 24 h under basal conditions, and in the presence and absence of HCG (10 IU/ml) and mifepristone (100 nM RU486). HIF1A was detected by overnight incubation at 4°C with anti-HIF 1A, rabbit polyclonal antibody (Santa Cruz Biotechnology, Texas, USA, diluted 1:100) and incubated with anti-rabbit secondary antibody conjugated with Alexa Fluor 488 (Molecular Probes, Inc., Oregon, USA) for 1 h at 37°C, diluted 1:1000. The primary antibody was omitted in the negative control. The cells were embedded and mounted for microscopy with ProLong Gold mounting medium with DAPI, a DNA-intercalating agent 4',6-diamidino-2-phenylindol dihydrochloride, for detection of nuclear DNA (Molecular Probes, Inc., Oregon, USA). Immunofluorescence was documented with an epifluorescence microscope (Olympus BX-51TF; Olympus Optical Co. Ltd, Tokyo, Japan) and CoolSNAP-Pro camera (Media Cybernetics, Maryland, USA).

Western blotting

The levels of ERK1/2, AKT, CREB (proteins phosphorylated by PKA) and MARCKS (a protein phosphorylated by protein kinase C) were measured using Western blotting in homogenates of granulosa cells. Gel electrophoresis was carried out in 10% SDS-PAGE. Total protein (30 µg), determined by the dye-binding assay (Bio-Rad Lab. Inc., California, USA), was loaded in each lane and were transferred onto nitrocellulose membranes (Thermo Fisher Scientific, Massachusetts, USA) as previously reported (Kohen et al., 2013). After the transfer, blots were blocked in 3% bovine serum albumn in T-TBS (20 mM Tris, 500 mM NaCl and 0.01% Tween 20) for 1 h and incubated overnight at 4°C with primary antibody: (1) anti-phospho or total ERK1/2 (Mouse monoclonal antibody, Santa Cruz Biotechnology, Texas, USA, diluted 1:3000); (2) anti-phospho or total AKT (Rabbit polyclonal antibody, Cell Signaling Technology, Massachusetts, USA, diluted 1:1000); (3) anti-phospho or total CREB (Rabbit polyclonal antibody, Cell Signaling Technology, diluted 1:500); (4) anti-phosphor or total MARCKS (Rabbit polyclonal antibody, Cell Signaling Technology, Massachusetts, USA, diluted 1:1000); (5) anti-ADAMTS-1 (H-60) rabbit polyclonal antibody for the mature 85 kDa isoform (Santa Cruz Biotechnology, Inc, Texas, USA, diluted 1:500); (6) anti-MMP3/10 (F-10) mouse monoclonal antibody (Santa Cruz Biotechnology, Inc, Texas, USA, diluted 1:300); or (7) anti-β-actin (Mouse monoclonal antibody, Sigma-Aldrich, Missouri, USA, diluted 1:10000). Horseradish peroxidase-conjugated secondary antibody: Peroxidase-conjugated affiniPure Goat Anti-Rabbit IgG (Jackson Immuno Research, Laboratories, Inc., Pennsylvania, USA, diluted 1:5000) and Peroxidase-conjugated affiniPure Goat Anti-Mouse IgG (Jackson Immuno Research, Laboratories, Inc. diluted 1:5000) were incubated for 1 h at 37 C and detected by chemiluminescence (Western Lightning Plus-ECL, Perking Elmer, Inc., Pennsylvania, USA) using an Ultra Quant 6.2 Image Reader and analyzed by Image Quant 5.2 software (Molecular Dynamic Inc., Sunnyvale, CA, USA). Densitometric analyses of immunoreactive bands were normalized to beta-actin or by total protein when referring to the phosphorylated protein.

Statistical analysis

The statistical analysis of the densitometric data from Western blotting ($n = 4$) was carried out using a non-parametric method: Kruskal–Wallis (one-way analysis of variance) followed by a Dunn's Multiple Comparison post-hoc test with GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA). Previously, the data were checked for normality with the Shapiro–Wilk test, $P < 0.05$, indicating that our data do not have normal distribution. Significance was defined as $P < 0.05$. Data are presented as means ± SEM.

Results

Progesterone receptor expression and localization in human granulosa cells

PR-A/B immunofluorescence in primary cultures of human granulosa cells under basal conditions (A) and after 12 h treatment with HCG, LH and FSH (C, D and E) are shown in **Figure 1**. Progesterone receptor staining was weak in the absence of gonadotrophins (2–8% positive nuclei for progesterone receptors) (A). In contrast, the progesterone receptor signal increased after 8 h of HCG, LH and FSH treatment, between 20 and 40% compared with the baseline condition (data not shown), peaking 12 h after treatment (66–88%). Immunodetection of total progesterone receptor revealed higher intensity (green), and mostly nuclear localization (C, D and E).

The signalling pathways activated downstream of HCG, LH or FSH in primary cultures of human granuulosa cells is shown in **Figure 2**. We selected 5, 15 and 30 min of gonadotrophin stimulation, as it has been reported that the intracellular signalling pathways are activated rapidly after the stimulation with gonadotrophins (Wood and Strauss, 2002). HCG and LH significantly stimulated ERK 1/2 phosphorylation after 5 min of treatment (A), wheras AKT and CREB phosphorylation were detectable after 15 min (B and C) ($P < 0.05$), a preliminary result showed that at 5 min there were no detected differences compared with time zero (data not shown). HCG also stimulated MARCKS phosphorylation after 5 min of treatment (D). Similarly, FSH significantly stimulated ERK 1/2, AKT, CREB and MARCKS phosphorylation after 5 min of treatment (A, B, C and D). These findings suggest that the ERK 1/2, AKT, PKA and PKC signalling pathways could play a role in HCG-, LH- and FSH-dependent progesterone receptor expression.

To clarify which of the pathways investigated above regulate progesterone receptor expression in granulosa cells, we used specific inhibitors of these pathways. **Figure 3** shows the in-vitro progesterone receptor immunofluorescence in human granulosa cell cultures stimulated for 12 h with HCG (10 IU/ml) in the presence or absence

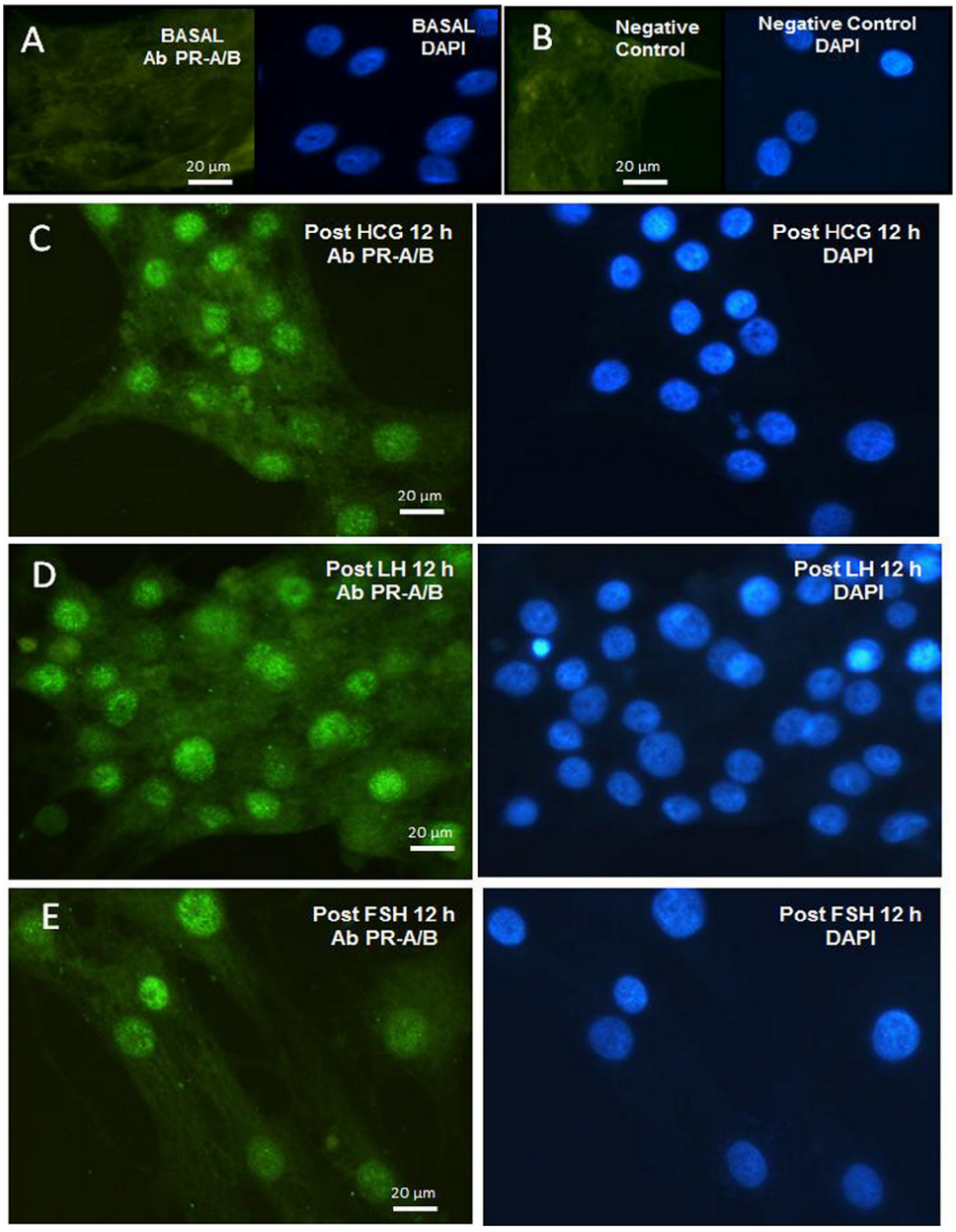

Figure 1 – Expression and localization of progesterone-receptor A/B in primary human granulosa cell cultures treated with HCG, LH and FSH. Panels A and B show immunofluorescence progesterone receptor under basal conditions and negative control. Panels C, D and E show the progesterone receptor after 12 h of HCG (10 IU/ml), LH (10 IU/ml) and FSH (10 IU/ml) stimulation, respectively. The green staining is localized inside the nucleus. DAPI represents the nucleus (blue). The figure represents *n* = 5 independent experiments. DAPI, 4',6-diamidino-2-phenylindole; PR, progesterone receptor. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of the specific inhibitor for each signalling pathway: H-89 (PKA inhibitor), BIM (PKC inhibitor), LY 294002 (PI3K inhibitor) and UO126 (ERK1/2 inhibitor). HCG treatment increased progesterone receptor immunodetection levels after 12 h (66–88% positive nuclei for progesterone receptors) (A). Conversely, the presence of H-89 (10 µM), BIM (2,5 µM) and UO126 (10 µM) significantly reduced the stimulatory effect of HCG on progesterone receptor expression (2–10% positive nuclei) (B, D and E) ($P < 0.05$). These findings suggest that HCG regulates progesterone receptor expression in granulosa cells through the PKA, PKC and ERK1/2 signalling pathways. AKT inhibition did not affect HCG-dependent progesterone receptor expression (C). The experiments with inhibitors of each signalling pathway were carried out for the three gonadotrophins (HCG, LH and FSH) with similar results obtained for each hormone. The results for LH and FSH stimulation are shown in **Supplementary Figure S1**.

The specificity of action of the inhibitors at the concentrations used was demonstrated by blockade of HCG-induced phosphorylation of the substrate of the targeted kinase, with no effect on the phosphorylation of substrates targeted by other kinases as shown in **Supplementary Figure S2**.

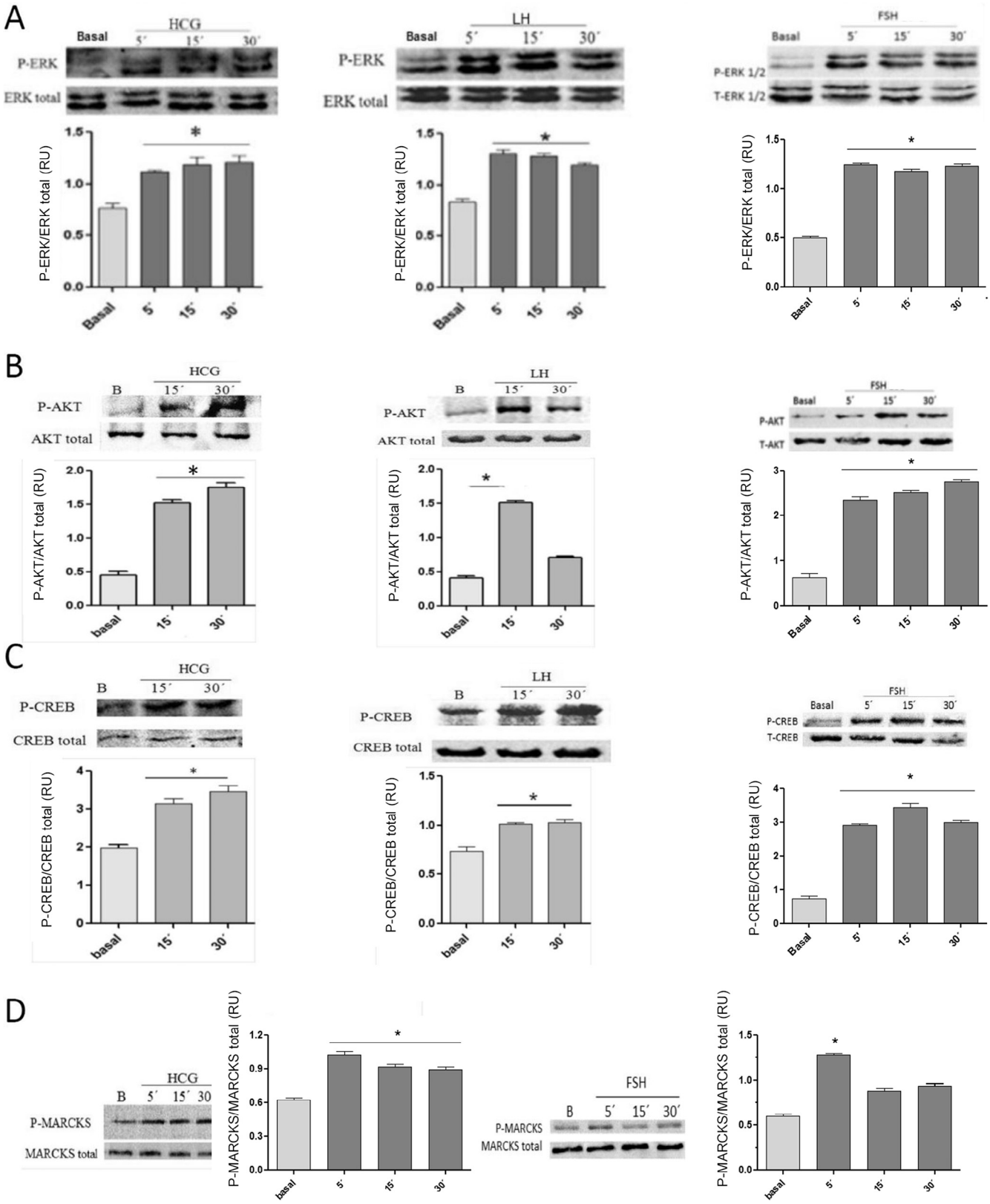

Figure 2 – ERK 1/2, AKT, CREB and MARCKS signalling pathways activated by HCG, LH and FSH in primary cultures of human granulosa cells. Panels A, B and C show the Western blot and densitometry analyses of the phosphorylated ERK 1/2, AKT and CREB, respectively, collected after HCG (10 IU/ml), LH (10 IU/ml) and FSH (10 IU/ml) stimulation (5, 15 and 30 min). Panel D: phosphorylated MARCKS levels were determined in the presence of HCG (10 IU/ml) and FSH (10 IU/ml). Densitometry relative units (RU) are normalized with total ERK1/2, AKT, CREB and MARCKS. Values are means ± SEM from four individual observations ($n = 4$); *$P < 0.05$. DAPI, 4′,6-diamidino-2-phenylindole; PR, progesterone receptor.

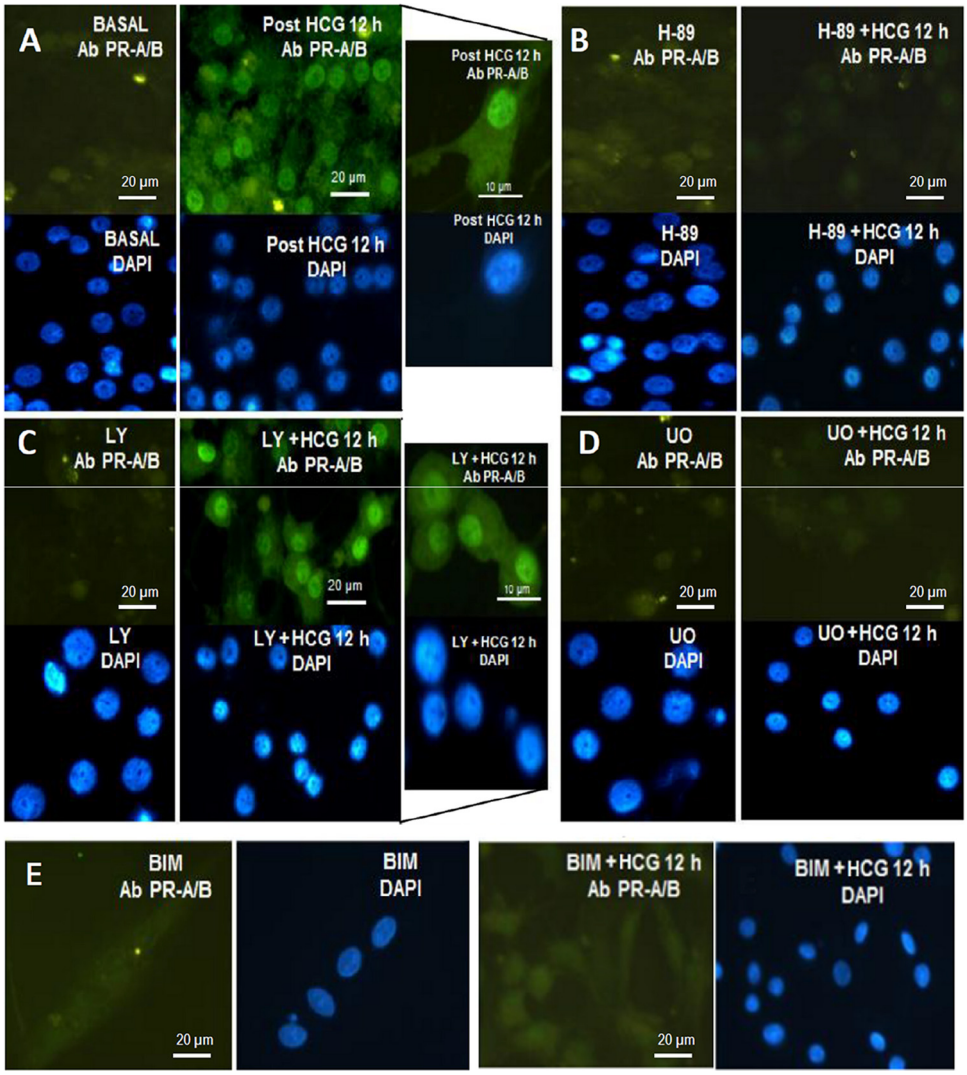

Figure 3 – Progesterone receptor expression activated by HCG signalling pathways in primary cultures of human granulosa cells. Panel A represents the increased immunodetection of progesterone receptor after 12 h of incubation with HCG (10 IU/ml). Panels B, D and E show that the PKA inhibitor (H-89, 10 µM), ERK1/2 inhibitor (UO126, 10 µM) and PKC inhibitor (BIM, 2.5 µM) abolished the stimulatory effect of HCG on progesterone receptors. Panel C shows the AKT inhibitor (LY 294002, 20 µM) had no effect on progesterone receptors. Images are representative of four separate experiments. BIM, bisindolylmaleimide I; DAPI, 4',6-diamidino-2-phenylindole; PR, progesterone receptor.

ADAMTS, MMP3/10 and HIF1A expression in human granulosa cells

Changes in oxygen concentration during follicular development can induce hypoxic conditions, stimulating HIF1A expression, which could play a role in the ovulatory process (Basini et al., 2004; Zhang et al., 2012). We carried out time courses for HIF-1A with sampling at 6, 12, 18 and 24 h and observed a strong signal at 18 h compared with the other times (data not shown). The HIF1A signal increased significantly in HCG-stimulated granulosa cells, with 80–90 % positive nuclei (18 h) ($P < 0.05$), and diminished significantly in the presence of RU486, indicating that it is regulated by progesterone receptor ($P < 0.05$). ADAMTS-1 and MMP3/10 expression were also examined in cultured human granulosa cells (**Figure 4**). We also carried out time courses for ADAMTS-1 and MMP3/10, from 6, 12, 18 and 24 h and observed that at 18 h there was a weak signal, which increased in intensity to 24 h (data not shown). ADAMTS-1 and MMP3/10 protein levels increased after HCG stimulation 1.7 and 1.2-fold, respectively (24 h) and decreased in the presence of RU486 ($P < 0.05$) (**Figures 4B and 4C**). These observations suggest that the progesterone receptor regulate the levels of these metalloproteinases in human granulosa cells. Similar findings were observed with granulosa cells stimulated with LH and FSH (**Figure 4D**), showing that ADAMTS-1 and MMP3/10 protein levels increased after gonadotrophin stimulation, two-fold with LH and 1.8-fold with FSH (24 h), but the gonadotrophin-induced increases were inhibited in the presence of RU486 ($P < 0.05$). granulosa cells obtained immediately after follicular aspiration (36 h after HCG administration) showed that MMPs levels are higher at the time of follicular aspiration compared with levels after 24 h of basal culture. **Supplementary Figure S3** shows the in-vitro stimulation with a combination of LH and FSH on protease expression in granulosa cell culture. The expression levels of proteases were similar to that observed when cells were stimulately separately with LH and FSH.

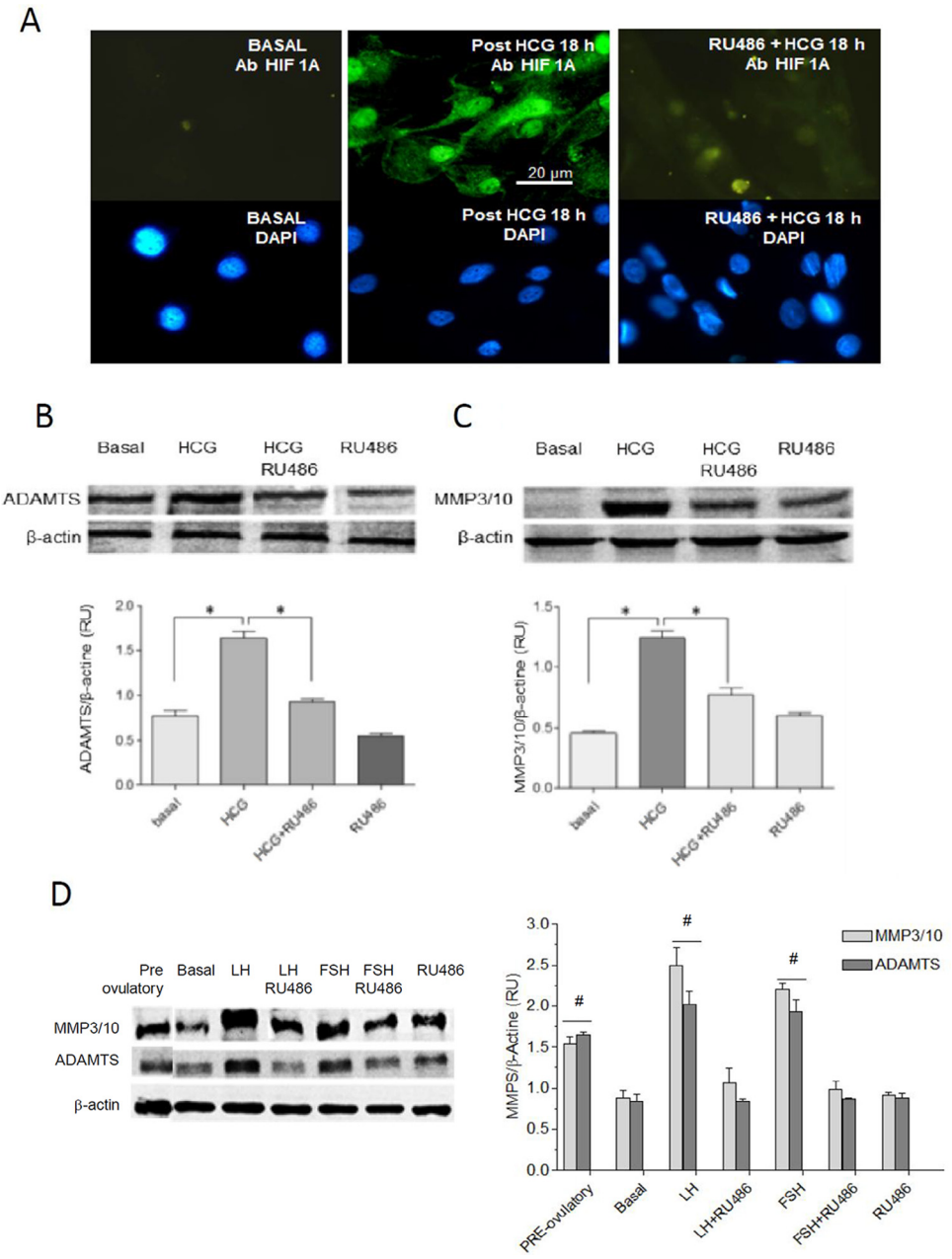

Figure 4 – Progesterone receptor modulates HIF1A, ADAMTS-1 and MMP3/10 expression in human granulosa cell cultures after HCG stimulation. Panel A shows the immunodetection of HIF1A (green signal) after HCG stimulation. This signal decreased in the presence of HCG (10 IU/ml) plus RU486 (100 nM). Images represent four experiments. Panels B and C show the effects of HCG (10 U/ml) and Panel D show the effect of LH (10 IU/ml) and FSH (10 IU/ml) on ADAMTS-1 (85 kDa) and MMP3/10 (58 kDa) expression. This stimulatory effect is eliminated in the presence of RU486. The level of ADAMTS-1 and MMP3/10 in granulosa cells obtained immediately after follicular aspiration (36 h after HCG administration) is shown in panel D. Densitometry relative units (RU) were normalized with beta-actin. Values are means ± SEM, $n = 4$, *$P < 0.05$, # $P < 0.05$ respect to basal. DAPI, 4',6-diamidino-2-phenylindole. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Role of HIF1A in MMP3/10 and ADAMTS expression

The role of HIF1A in the expression of ADAMTS-1 and MMP3/10 was also studied in the human granulosa cell cultures. The results for granulosa cells stimulated with HCG in the presence and absence of echinomycin (an HIF1A inhibitor) are shown in **Figures 5A and 5B**. ADAMTS-1 and MMP3/10 protein levels increased after HCG stimulation, 1.2- and 2.7-fold, respectively (24 h). MMP3/10 levels, however, were significantly reduced in the presence of echinomycin ($P < 0.05$). In contrast, ADAMTS-1 expression was not affected. These results show that inhibiting this transcription factor with echinomycin affects only HCG-PR-dependent MMP3/10 expression. This finding suggests different signalling pathways are involved in ADAMTS-1 regulation downstream of the progesterone receptor.

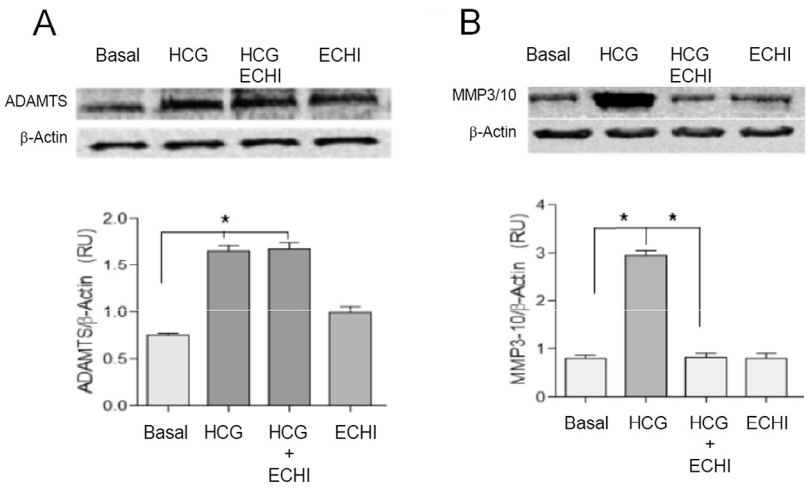

Figure 5 – HIF1A modulates ADAMTS-1 and MMP3/10 expression in human granulosa cell cultures after HCG stimulation. Panels A and B demonstrate the stimulatory effect of HCG (10 IU/ml) on ADAMTS-1 and MMP3/10 in the presence of the specific inhibitor of HIF1A, echinomycin (0.5 μM). MMP-3/10 levels are reduce in the presence of echinomycin, but not ADAMTS-1. ECHI, Echinomycin (HIF1A inhibitor). Densitometry relative units (RU) are normalized with beta-actin. Values are mean ± SEM, *n* = 4, **P* < .05.

The cellular viability was evaluated at the end of the culture with MTS assay and was no different to the initial time (data not shown).

Discussion

Ovulation is an orchestrated process largely synchronized by a mid-cycle surge of pituitary gonadotropins (LH and FSH). Physiologically, the LH and FSH surges occur simultaneously (Richards et al., 2002). These gonadotrophin surges initiate a series of biochemical events in the pre-ovulatory follicle, resulting in expansion of the cumulus oocyte complex, rupture of the dominant follicle and ultimately expulsion of the oocyte. The gonadotrophin cascade initiates expression of a number of specific transcription factors, steroids receptors, cytokines and metalloproteinases and induces progesterone synthesis. During induced ovulatory cycles, HCG administration is used as a substitute for the LH surge, resulting in follicular rupture and stimulating the final stages of ovum maturation (Espey and Richards, 2002; Zafeiriou et al., 2000). It has been postulated that proteinases digest the connective tissue matrix to allow for follicular rupture. The present study was designed to examine the various LH-, FSH-, and HCG-driven signalling pathways involved in progesterone receptor and MMP expression in primary cultures of human granulosa cells.

A previous study from our laboratory found that progesterone receptor mRNA was transiently up-regulated in human granulosa cell culture 6 h after HCG administration. This stimulation was achieved through the LH and HCG, cAMP, and PKA pathways (García et al., 2012). The present findings indicate that progesterone receptor expression in human granulosa cell culture becomes detectable with immunofluorescence after 12 h of treatment with LH, HCG and FSH. This stimulation occurs through the PKA, PKC and ERK1/2-signalling pathways. These results confirm the report by Robker et al. (2009) that, in the mouse and the rat, LH and HCG activation of the PKA, PKC or MAPK signalling pathways is required to stimulate progesterone receptor mRNA expression in granulosa cells. It is important to note that our experiments were conducted on granulosa cells obtained 36 h after HCG administration, when progesterone receptors are not expressed. Interestingly, these cells become sensitive to gonadotrophins after 72 h of culture followed by 24 h in serum-free medium, expressing progesterone receptor after 12 h gonadotrophin stimulation.

During the pre-ovulatory period, remodelling of the follicle wall extracellular matrix facilitates rupture and expulsion of the oocyte. These biological events depend on the induction and activation of various transcription factors and MMPs. The present study establishes that HCG operates through progesterone receptor to regulate HIF1A transcription factor expression along with ADAMTS-1 and MMP3/10 expression in human granulosa cell cultures. The antiprogestin RU486 abolished this regulation. These findings corroborate in-vivo studies showing that HIF1A is critical for follicular rupture in mice (Kim et al., 2009). Furthermore, these results are consistent with findings of hypoxic stress within the periovulatory follicle in mice, resulting in high levels of HIF1A associated with follicular growth and rupture (Basini et al., 2004; Zhang et al., 2012). Recent studies in human fibroblast culture indicate that MMP3 expression is exclusively regulated by HIF1A in response to inflammation that suggests that metalloproteinases expression is involved in follicular rupture (Ahn et al., 2008). It is well known that inflammation is a key component of the ovulation process (Boots and Jungheim, 2015; Espey et al., 1994).

In human granulosa cells, MMP3/10 expression is dependent of the progesterone receptor. It is tempting, however, to speculate that different mechanisms are involved in MMP3/10 expression, as the presence of the specific inhibitor of HIF1A, echinomycin, abolished the progesterone-receptor-dependent increase in MMP3/10. In contrast, progesterone-receptor-dependent induction of ADAMTS-1 was evidently not mediated through HIF1A. Importantly, MMP3/10 and ADAMTS-1 are expressed before follicular rupture, as they were detected in granulosa cells immediately after collection from the pre-ovulatory follicle. These results suggest the possibility of a differential and newly identified progesterone-receptor-dependent, HIF1A-mediated regulation mechanism involved in human follicular rupture.

Clinicians have recently been using GnRH agonist for triggering of ovulation (through an endogenous LH and FSH surge). This study shows that LH and FSH significantly stimulate the expression of both

proteases in a progesterone receptor-dependent manner, but the combined effects of LH and FSH together are similar to the effects of LH and FSH separately.

In conclusion, HCG, LH, and FSH activate intracellular signalling pathways involving PI3K, PKC, ERK 1/2 and PKA. Only PKC, ERK 1/2 and PKA, however, stimulate progesterone receptor expression in human granulosa cells. progesterone receptor activation regulates proteases involved in the ovulatory process, such as ADAMTS-1 and MMP3/10. Furthermore, progesterone receptor expression up-regulates HIF-1A, which increases MMP3/10 levels, but not ADAMTS-1. These observations provide a better understanding of the mechanisms by which LH and proesterone receptor signalling pathways mediate the ovulation process in humans.

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Appendix: Supplementary material

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