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Hypusination of Eukaryotic Initiation Factor 5A via cAMP-PKA-ERK1/2 Pathway is required for Ligand-induced Downregulation of LH receptor mRNA Expression in the Ovary

Thippeswamy Gulappa[a], **Bindu Menon**[a], and **K. M. J. Menon**[a],*

[a]Departments of Obstetrics/Gynecology and Biological Chemistry, University of Michigan Medical School, Ann Arbor, MI - 48109–0617

Abstract

Luteinizing hormone receptor (LHR) mRNA expression in the ovary is regulated post-transcriptionally by an LH receptor mRNA binding protein (LRBP). Eukaryotic initiation factor 5A (EIF5A), identified as an LRBP-interacting protein plays a crucial role in LHR mRNA expression. In this study, we have demonstrated that during hCG-induced LHR downregulation, a significant upregulation of eIF5A mRNA expression and hypusination of eIF5A protein occurs in a time dependent manner. Pretreatment with H89, a specific inhibitor of PKA, and U0126, a specific inhibitor of ERK1/2 significantly inhibited both hCG-induced eIF5A mRNA expression and hypusination of eIF5A protein. Pretreatment with GC7, a specific inhibitor of eIF5A hypusination significantly abolished hCG-induced LRBP mRNA and protein expression. Furthermore, GC7 pretreatment significantly inhibited hCG-induced interaction of LRBP with LHR mRNA as assessed by RNA electrophoretic mobility gel shift assay (REMSA). GC7 treatment also reversed LHR mRNA downregulation. Taken together, these results suggest that hCG-induced LHR mRNA downregulation is mediated by cAMP-PKA-ERK1/2 signaling leading to activation of eIF5A hypusination.

1. Introduction

Luteinizing hormone-human choriogonadotropin (LH/hCG) receptor (LHR), a member of the glycoprotein hormone receptor sub family of the large G-protein coupled receptor family is crucial for mammalian reproduction. LHR is expressed primarily in the testis and ovary [1,2]. During normal ovarian cycle, LHR expression undergoes remarkable changes, as revealed by the acquisition of LHR by the growing follicles in response to the combined

*Correspondence to, K.M.J. Menon, 6428 Medical Sciences Building I, 1301, Catherine Street, Ann Arbor, MI 48109–0617; Fax: +1 734–936- 8617, Tel: +1 734–764-8142; kmjmenon@umich.edu.

actions of FSH and estradiol [3] followed by a transient downregulation of LHR in response to the preovulatory LH surge. The latter phenomenon can be mimicked by administration of pharmacological dose of hCG to superovulated rats [4,5]. Studies from our laboratory have elucidated that the transient ligand-induced down-regulation of LHR mRNA occurs through post-transcriptional mechanism involving a specific mRNA binding protein designated as LHR mRNA binding protein (LRBP) leading to accelerated LHR mRNA degradation [6,7]. Since LRBP is devoid of mRNAse activity, we performed yeast two hybrid screening assay and identified eukaryotic initiation factor 5A (eIF5A) as one of the proteins that interact with LRBP [8]. EIF5A is a highly conserved, 17 kDa protein that is expressed ubiquitously in all cells. It undergoes hypusination, a unique post-translational modification by the addition of hypusine residue, an unusual amino acid from spermidine, to the ε-amino group of lysine and this modification of eIF5A is essential for its function [9]. Other known actions of eIF5A include its role as an elongation factor in protein translation [10–12] as well as shuttling proteins regulating mRNA transport [13], and regulation of cell proliferation [14], inflammation and apoptosis [15,16]. Recent studies from our laboratory have shown that hypusinated eIF5A interacts with LHR mRNA-LRBP complex and this interaction is critical for ligand-induced downregulation of LHR mRNA [17]. In the present study, we investigated the upstream signaling pathways of eIF5A hypusination and the role of hypusinated eIF5A in LH/hCG-induced downregulation of LHR mRNA in rat ovaries. Our results show that hCG-induced LHR downregulation is downstream of hCG-induced cAMP/PKA/ERK1/2 signaling pathway leading to eIF5A hypusination culminating in the downregulation of LHR mRNA.

2. Materials and Methods

2.1. Materials

Highly purified human chorionic gonadotropin (hCG; CR127) was purchased from Dr. A.F. Parlow (National Hormone and peptide program, Torrance, California). Pregnant mare serum gonadotropin was obtained from Calbiochem. EDTA-free protease inhibitor cocktail tablets and RNAse inhibitor were products of Roche Applied Science and Promega Corp., respectively. Real-time PCR Primers specific for LH receptor, LRBP, and 18S rRNA (TaqMan Assay-on-Demand Gene Expression Product) and Multiscribe reverse transcriptase were supplied by Applied Biosystems (Foster City, CA). Real-time PCR Primers specific for rat eIF5A and GAPDH were obtained from Qiagen. Anti-N-terminal mevalonate kinase IgG was raised against the first 15 N-terminal amino acids of mevalonate kinase (MLSEVLLVSAPGKVI) and this antibody is referred to as the LRBP antibody in the text. Recombinant eIF5A was purchased from Prospec Bio (Rehovot, Israel) and [^{3}H]-spermidine from Perkin Elmer (Waltham, MA). MEK inhibitor (U0126) was purchased from Promega and PKA inhibitor (H89) was obtained from Calbiochem (La Jolla, CA). EIF5A hypusination inhibitor, N1-Guanyl- 1,7 diaminoheptane (GC7) was purchased from BIOSEARCH Technologies, Petaluma, CA. Monoclonal mouse Anti-eIF5A antibody and anti-β-tubulin antibody were obtained from Sigma Aldrich (St. Louis, MO). The Super Signal West Femto chemiluminescence kit and anti-rabbit/anti-mouse IgG conjugated to horseradish peroxidase were obtained from Pierce (Rockford, IL). Quick spin columns (G-50-Sephadex) for radiolabeled RNA purification were purchased from Roche Molecular

Biochemicals. [α-^{32}P]-UTP was obtained from PerkinElmer (Santa Clara, CA), and MAXIscript T7 Kit was the product of Ambion (Austin, TX). All other chemicals and reagents used were conventional commercial products.

2.2. Animals and treatments

Sprague-Dawley female rats (23 day old) were purchased from Charles River Laboratories (Wilmington, MA). All the experimental protocols used in this study were approved by the University Committee on the Use and Care of Animals. Animals were housed in a temperature-controlled room with proper dark-light cycles as per the guidelines provided by the University Committee on the Use and Care of Animals. Superovulation was induced in 23-day-old female rats by subcutaneous injection of 50 IU of pregnant mare serum gonadotropin followed by 25 IU of human chorionic gonadotropin 56 h later. Five days after inducing superovulation, the animals were injected (s.c.) with single dose of hCG (50 IU) to downregulate LHR mRNA expression. The animals were euthanized by CO_2 asphyxiation and the ovaries were collected at 0, 1, 2, 4 and 6 hours after hCG injection and were immediately frozen in liquid nitrogen until further use. To block PKA and ERK1/2, the superovulated rats were treated (s.c.) with H-89 (100 mg/kg body weight) or UO126 (10 mg/kg body weight) 1 hour before the second hCG treatment. To block eIF5A hypusination, the superovulated rats were treated with GC7 (16 mg/kg body weight, i, p.) 2 hour before the second dose of hCG.

2.3. Preparation of tissue extracts and Western blot analysis

Ovaries were homogenized in Radioimmunoprecipitation assay (RIPA) buffer containing EDTA-free protease inhibitor cocktail followed by brief periods of sonication. The samples were centrifuged at $14,000 \times$ g for 15 min at 4° C and total protein content of the supernatants was measured by Bicinchoninic acid assay (BCA) reagent method. Proteins (60 μg/lane) were separated by electrophoresis using 12% SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). The membranes were blocked in 5% fat-free skimmed milk in TBS-tween-20 buffer (TBST), pH-7.4 for 1h at room temperature and then incubated overnight at 4° C with primary antibody in 5% fat free milk/TBST. The membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies for 1h at room temperature. The membrane bound antibodies were detected with the west Femto-Supersignal substrate system Western blotting detection kit (Pierce). Protein loading was monitored by stripping and re-probing the same blots with appropriate antibodies (internal control) as indicated in the figure legends.

2.4. *In vitro* hypusination assay

In vitro hypusination assay was performed using our previously published procedure [17]. Briefly, ovarian S10 fractions (40 g protein) were added to assay mixture containing 50 mM glycine, pH 8.3, 20% glycerol, 2 mM DTT, 150 mM KCl, 10 mM $MgCl_2$, 0.1 mg/ml BSA, 0.1 mM NAD^+, 20 ng of recombinant eIF5A protein and 2.0 μCi of [^{3}H]-spermidine in a final volume of 25 μl. The reaction mixture was incubated at 37°C for 2h and terminated by the addition of 5μL of SDS-PAGE sample buffer. The proteins were separated on a 12% SDS-PAGE gel, transferred to nitro-cellulose membrane and subjected to flurography using

Kodak BioMax TranScreen LE and BioMax MS film at −70°C for 72 hours. The images were then scanned and quantitated.

2.5. RNA isolation and Real-time PCR

Total RNA was extracted from the ovaries using TRIzol reagent following the manufacturer's instructions (Life Technologies, Grand Island, NY). Aliquots of total RNA (100 ng) extracted from each sample were reverse-transcribed in a reaction volume of 20 μl using 2.5μM random hexamer, 500μM deoxynucleotide triphosphates, 5.5 mM $MgCl_2$, 8U ribonuclease inhibitor, and 25U multiscribe reverse transcriptase (Applied Biosystems). The resulting cDNA's were diluted with nuclease free water. The real-time PCR quantitation was then performed using 5 μl of the diluted cDNAs in triplicate using predesigned primers and probes. Reactions were carried out in a final volume of 25 μl using Applied Biosystems 7300 Real-Time PCR system. The fold change in gene expression was calculated using the standard curve method with 18S rRNA or GAPDH as the internal control using the Ct method [18].

2.6. RNA electrophoretic mobility shift assay (REMSA)

Ovaries were collected from superovulated rats treated with GC7 or saline, followed by s.c. injection with 50 IU hCG and ovaries collected 8h later as described in the section "Animals and treatments". Ovaries were homogenized in assay buffer (10 mM HEPES pH 7.9, 0.5 mM $MgCl_2$, 50 mM EDTA, 5 mM DTT and 10% glycerol) containing 50 mM KCl and protease inhibitor cocktail at 4° C. The homogenates were centrifuged at $105,000 \times g$ for 90 min at 4° C. The supernatants containing the cytoplasmic proteins (S100) were collected. REMSA was performed by incubating S100 cytosolic fractions with [α-^{32}P]-UTP-labeled LBS, as described previously [6,19,20]. The [α-^{32}P]-labeled RNA for the binding assay was prepared using the Maxiscript kit. The RNA-protein complexes were resolved by 5% native polyacrylamide (70:1) gel electrophoresis and analyzed by autoradiography, as described previously [19].

2.7. Statistical analysis

Statistical analysis was carried out using GraphPad Prism software. The data were analyzed using one-way ANOVA followed by the Tukey multiple comparison test. Values were considered statistically significant for $p<0.05$. Each experiment was repeated at least 3 times with similar results. Western blots and autoradiograms shown are representative of a minimum of 3 experiments.

3. Results

3.1. hCG induces eIF5A mRNA and protein expression

Since our previous study identified that eIF5A interacts with LHR mRNA-LRBP complex during hCG-induced LHR downregulation [17], we examined whether hCG has any effect on eIF5A expression. To examine this, RNA and proteins were extracted from ovaries of superovulated rats treated with hCG for 0, 1, 2, 4 and 6h representing the time periods prior to inducing complete downregulation. The extracts were analyzed for eIF5A mRNA and protein expression using real time PCR and Western blot analysis, respectively. The results

showed that hCG treatment causes significant upregulation of eIF5A mRNA expression in a time dependent manner starting with a 1.3 fold increase at 2h, 2.6 fold at 4h and 5 fold at 6h compared to control (Fig. 1A). Furthermore, Western blot results showed that hCG treatment significantly induced eIF5A protein expression in a time dependent manner (Fig. 1B). The unhypusinated (precursor) form of eIF5A (17 kDa) migrates faster in the gel and appears as a faint band directly below the hypusinated form in the Western blot in hCG treated samples (Fig. 1B, upper panel, lanes 1, 2 and 3). At 4h and 6h time points, the unhypusinated precursor form was not visible, however, the intensity of the hypusinated eIF5A band showed significant increase upon hCG treatment (Fig. 1B, upper panel, lanes 4 and 5). These results suggest that hCG causes upregulation of eIF5A expression which subsequently undergoes hypusination.

3.2. Pretreatment with PKA and ERK1/2 inhibitors abrogates hCG-induced eIF5A expression and hypusination

We then tested the signaling pathway that leads to increase in eIF5A mRNA expression and hypusination of eIF5A protein in response to hCG treatment. Specifically, we examined the role of cAMP/PKA/ERK1/2 signaling pathway, since our previous studies have shown that hCG-induced downregulation of LHR mRNA expression is mediated by this pathway [21]. To examine this, superovulated rats were treated with PKA inhibitor (H-89; 100 mg/kg body weight), ERK1/2 inhibitor (U0126; 10 mg/kg body weight) 1 hour before hCG treatment or eIF5A hypusination inhibitor (GC7; 16 mg/kg body weight) 2 hour before hCG treatment. Total RNA was extracted from the ovaries and eIF5A mRNA expression was analyzed by real time PCR. The results showed that, as expected, hCG significantly increased eIF5A mRNA expression. Conversely, pretreatment with PKA and ERK1/2 inhibitors significantly inhibited the hCG-induced upregulation of eIF5A mRNA (Fig. 2A). Western blot analysis of the cytosolic extracts indicated that pretreatment with PKA inhibitor or ERK1/2 inhibitor significantly reduced hCG-mediated eIF5A protein expression and its hypusination. As well as expected, treatment with the hypusination inhibitor, GC7 had no effect on eIF5A mRNA expression (Fig. 2B, middle panel). We also performed hypusination activity assay *in vitro* by incubating the ovarian cytosolic extracts with [^{3}H]-spermidine and recombinant eIF5A protein. Western blotting followed by fluorography analysis results showed that while hCG treatment increased the incorporation of [^{3}H]-spermidine into eIF5A, pretreatment with PKA inhibitor, ERK1/2 inhibitor and hypusination inhibitor separately produced significant inhibition of hypusination (Fig. 2B. top panel). A summary of the effect of various inhibitors on eIF5A mRNA, protein expression and hypusination is presented in Table -1. Collectively, these results suggest that eIF5A hypusination occurs through hCG-induced activation of cAMP/PKA/ERK1/2 pathway.

3.3. Inhibition of eIF5A hypusination by GC7 abrogates hCG-induced LRBP mRNA and protein expression

The role of eIF5A hypusination on the induction of LRBP was then examined by eIF5A hypusination inhibitor treatment. LRBP mRNA and protein expression were examined by real time PCR and Western blot analyses, respectively. The results showed that treatment with hCG produced a 2.5 fold increase in LRBP mRNA at 4h and 4 fold increases at 6h. GC7 pretreatment significantly inhibited the hCG-induced LRBP mRNA expression (Fig.

3A). A significant induction of LRBP protein was also seen in response to hCG treatment and this response was significantly inhibited by inhibition of eIF5A hypusination by GC7 (Fig. 3B). These results demonstrate that eIF5A hypusination is required for hCG-induced LRBP expression.

3.4. Inhibition of eIF5A hypusination by GC7 inhibits hCG-induced LRBP activity and LHR downregulation

Furthermore, we have previously shown that the hCG-induced inhibition of LHR mRNA expression is mediated by LRBP [7,22]. Since eIF5A hypusination inhibition blocks LRBP mRNA and protein expression as shown in this study, we therefore examined whether this hypusination inhibition would lead to reduced binding of LHR mRNA to LRBP. To test this, RNA electrophoretic mobility gel shift assay (REMSA) was performed with [^{32}P]-labeled rat LHR mRNA fragment that contain LRBP binding sequence (LBS) and S100 fractions from extracts of superovulated ovaries pretreated with vehicle or GC7 followed by hCG treatment. The results showed that hCG treatment showed an increase in the binding activity of LHR mRNA to LRBP compared to control. As expected, there was a significant increase in the intensity of the band in the hCG treated samples when compared to control (Fig. 4A, lane 1 and 2). GC7 pretreatment produced significant decrease in the binding activity as reflected in the reduction in the amounts of the band (Fig. 4A, lane 3) suggesting a role for eIF5A hypusination in hCG-induced increase in the binding of LHR mRNA to LRBP. We also examined the effect of GC7 treatment on LHR mRNA expression by Real time PCR. The results showed that GC7 pretreatment abrogated hCG-induced downregulation of LHR mRNA at 4 and 6h (Fig. 4B) again confirming the role of eIF5A hypusination in the downregulation of LHR mRNA.

4. Discussion

Previous studies from our laboratory have shown that LHR expression in the ovary is downregulated in response to preovulatory LH surge or by the administration of a pharmacological dose of hCG to superovulated rats through a posttranscriptional mechanism [22]. Using a rodent model system, we identified a protein designated as LHR mRNA binding protein (LRBP) in the downregulated rat ovary that binds specifically to the polypyrimidine-rich sequence in the coding region of LHR mRNA and accelerates its degradation [6], [22]. Further studies using yeast two hybrid screening, we identified eukaryotic initiation factor 5A (eIF5A), an 18 kDa protein, that interacts with LRBP during LHR downregulation [8]. Furthermore, we demonstrated that eIF5A functionally associates with LHR mRNA-LRBP complex during downregulation of LHR mRNA. Our recent study showed that eIF5A undergoes hypusination and this is necessary for its function [17]. The current study focused on the upstream signaling pathways of eIF5A hypusination and extends its role in LRBP-mediated downregualtion of LHR mRNA. The results clearly show that hCG also induces eIF5A mRNA and protein expression in addition to stimulating its hypusination. This hCG-induced response was blocked by the PKA inhibitor, H89 and ERK1/2 inhibitor, U0126 suggesting that hypusination of eIF5A occurs through hCG-induced activation of cAMP/PKA/ERK1/2 signaling pathway. Further, eIF5A hypusination

inhibitor GC7 blocked LRBP expression and its LHR-mRNA binding activity suggesting that eIF5A hypusination is essential for LRBP-mediated LHR downregulation.

It is known that hypusination of eIF5A protein occurs readily after translation [23]. The unhypusinated eIF5A precursor protein accumulates only when the deoxyhupusine synthesis is blocked by inhibitors of deoxyhupusine synthase, an enzyme involved in the first step of hypusination reaction or by deprivation of spermidine [24,25]. Since eIF5A interacts with LRBP-LHR mRNA complex during the downregulation of LHR mRNA, we hypothesized that hCG treatment might trigger eIF5A synthesis and facilitate its hypusination. The results presented here support this notion by demonstrating that the expression of both eIF5A mRNA and protein increases during downregulation of LHR mRNA. The LH/hCG-mediated upregulation of eIF5A mRNA and protein expression is mediated by cAMP/PKA/ ERK1/2 signaling pathway. PKA inhibitor H89 as well as ERK1/2 inhibitor U0126 blocked the expression of eIF5A in response to LH/hCG stimulation. This is consistent with the previous study by Wu et al. who examined the expression of forskolin-induced genes in an ovarian granulosa-like tumor cell line and found a 4.9 fold induction of eIF5A mRNA expression [26]. Likewise, in epithelial cells, eIF5A mRNA and protein expression have been shown to be induced in response to treatment with epidermal growth factor [27]. Thus our results are in agreement with findings in other systems.

We have previously shown that LH/hCG-activated cAMP/PKA/ERK1/2 signaling plays an important role in LHR downregulation by increasing LRBP expression [21]. The present study extends this further showing that this pathway is also instrumental in increasing the hypusination of eIF5A. As shown in Fig 2B, inhibition of PKA and ERK 1/2 abrogated the increases in the expression of hypusinated form of eIF5A seen during hCG treatment. It is possible that hCG might increase hypusination reaction via cAMP/PKA/ERK1/2 pathway by activating deoxy hypusine synthase activity which is an early step in the hypusination reaction. Additionally, the possibility that cAMP/PKA/ERK1/2 pathway increases eIF5A expression, which then results in increased accumulation of the hypusinated form of eIF5A cannot be ruled out. Identification of specific steps that is activated by the cAMP/PKA/ ERK1/2 signaling pathways leading to increased hypusination of eIF5A needs additional investigation.

Our previous studies have established that LRBP is a key component of the post-transcriptional mechanism of LH receptor expression by causing accelerated degradation of LHR mRNA during LHR downregulation [21,22]. The present findings extend this by demonstrating that inhibition of eIF5A hypusination by GC7 reduces the binding of LHR mRNA to LRBP as presented in Fig. 4A. The abolishment of LH/hCG-induced increases in LRBP mRNA and protein expression by inhibition of eIF5A hypusination as shown in the present study is consistent with previous reports using other systems showing the effect of hypusination inhibition on transcription and translation. For example, de Almeida et al. have reported that inhibition of eIF5A hypusination by GC7 inhibits mRNA expression of inflammatory mediators [28] and Li et al [11] have shown that eIF5A affects translation elongation in U2OS (human osteosarcoma), COS7, and RDG3 cells.

In conclusion, the present study demonstrates that LH/hCG-induced LHR downregulation in the ovary is proceeded by the activation of cAMP/PKA/ERK1/2 pathway and induction of eIF5A protein and its hypusination. Inhibition of eIF5A hypusination inhibits LRBP expression culminating in LHR downregulation. Thus, hypusination of eIF5A plays an important role in the regulation of LH receptor expression.

Acknowledgments

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Highlights

- LH/hCG increases eIF5A expression and hypusination during LHR downregulation.

- PKA and ERK1/2 inhibitor reduces hCG-induced eIF5A hypusination.

- EIF5A hypusination inhibitor GC7 blocks LRBP expression and LRBP-LHR mRNA binding activity.

- LH/hCG-induced LHR mRNA downregulation was preceded by activation of cAMP/PKA-ERK1/2-mediated hypusination of eIF5A.

- Hypusinated eIF5A regulates LHR expression.

A

B
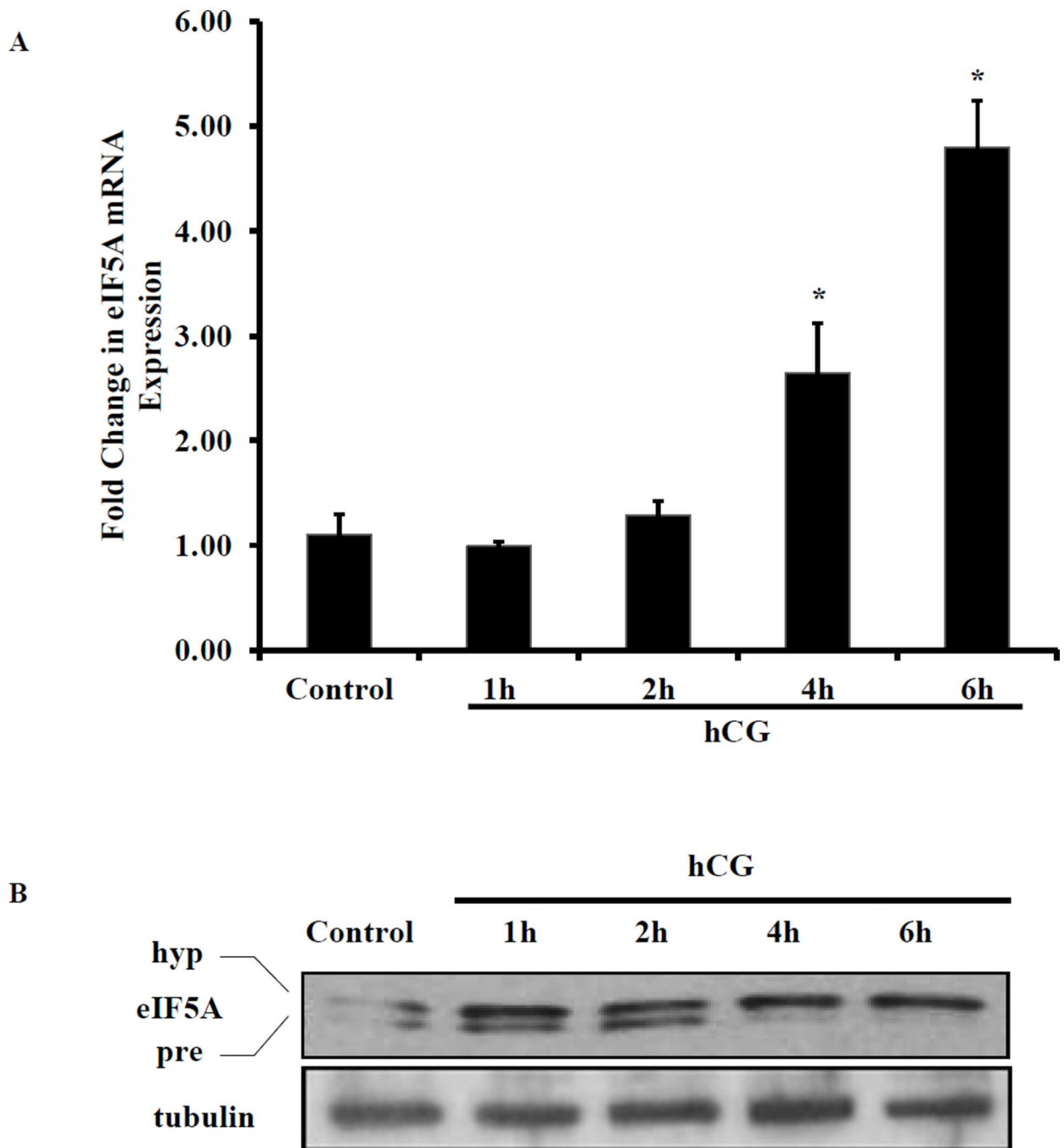
Fig. 1. hCG treatment for LHR downregulation induces expression and hypusination of eIF5A
(A) Superovulated rats were injected on day 5 with saline or hCG (50 IU) to induce LHR
downregulation. The ovaries were collected at 0, 1, 2, 4 and 6 hours later. RNA was isolated
and reverse transcribed and the resulting cDNA were subjected to real time PCR
quantitation using specific primers and probes for eIF5A and GAPDH. The graphs represent
changes in eIF5A levels normalized to GAPDH and shown as fold change vs control. Error
bars represent mean ± SE; *$p<0.05$ vs control. **(B)** Ovary lysates were prepared in RIPA
buffer by homogenization followed by brief sonication. The samples were centrifuged and

supernatants were collected. Equal amounts of protein from each sample were separated on 12% SDS-PAGE. The proteins were transferred to nitrocellulose membrane and probed with eIF5A antibody. The membranes were stripped and reprobed for β-tubulin antibodies. The blots shown are representative of three independent experiments.
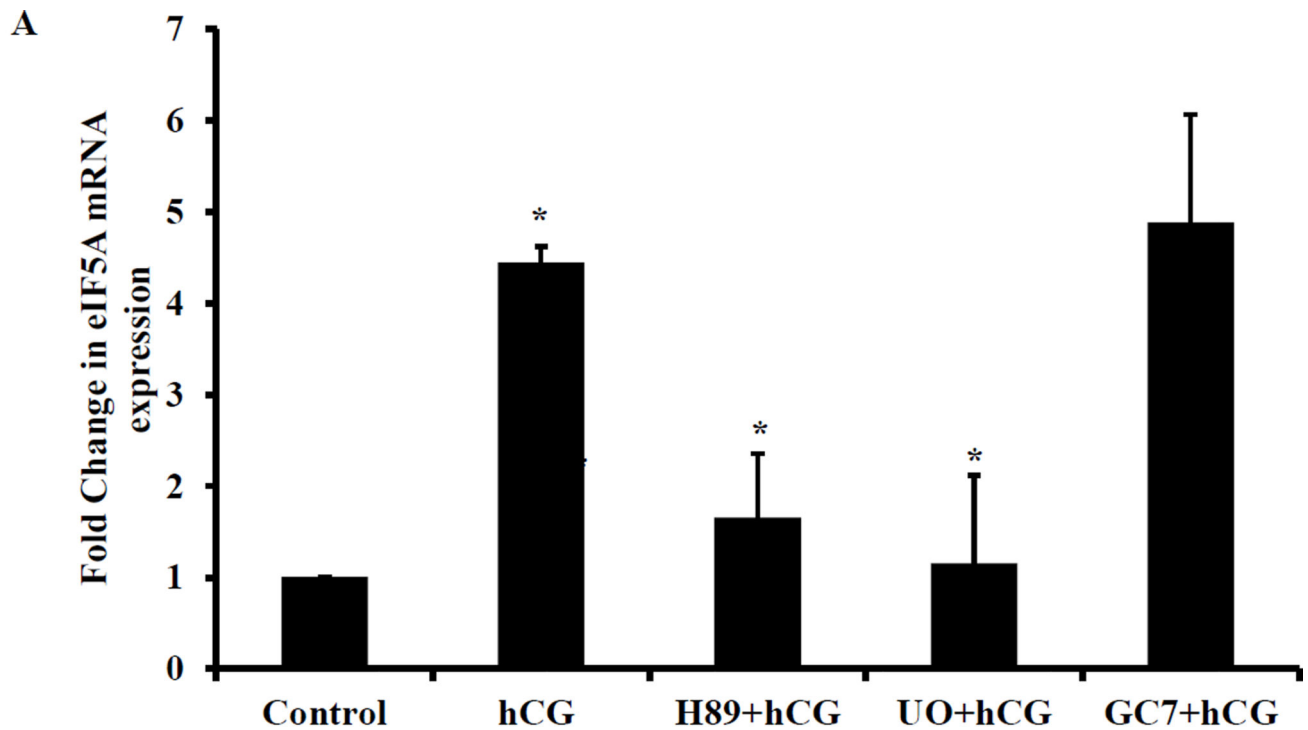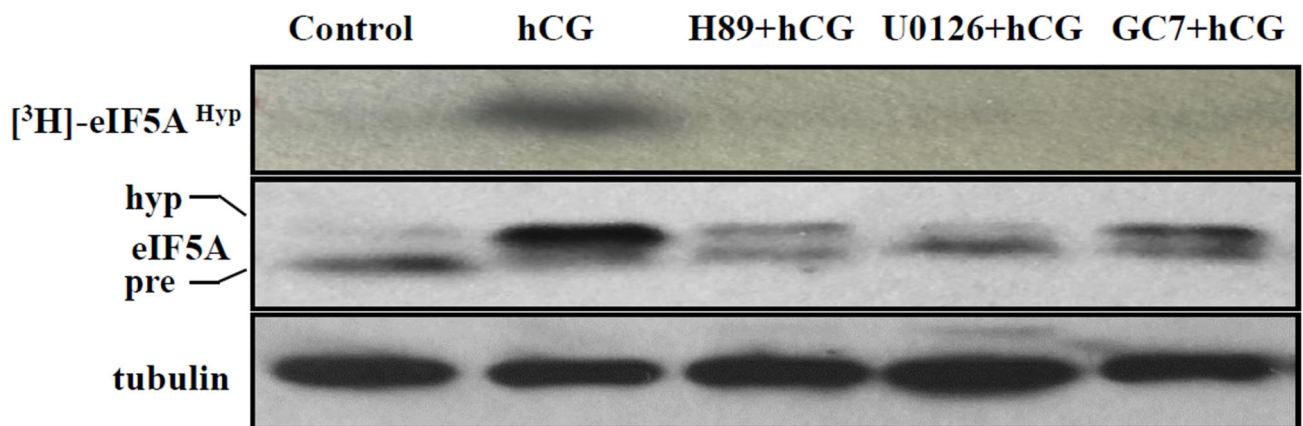
Fig. 2. Pretreatment with PKA inhibitor and ERK1/2 inhibitor inhibits hCG-induced eIF5A expression and hypusination

(**A**) Superovulated rats were injected with vehicle or PKA inhibitor H-89 (100 mg/kg body weight, s.c) or ERK1/2 inhibitor U0126 (10 mg/kg body weight, s.c) 1 hour before the second hCG treatment. The other set of rats were treated with GC7 (16 mg/kg body weight, i, p.) 2 hour before the second dose of hCG treatment. The ovaries were collected 6 hours later. The RNA was isolated from the ovaries and reverse-transcribed and the resulting cDNA were subjected to real time PCR quantitation using specific primers and probes for eIF5A and GAPDH. The graphs represent changes in eIF5A levels normalized to GAPDH

and shown as fold change vs control. Error bars represent mean ± SE; *p<0.05 vs control. **(B)** Ovaries were homogenized and S10 fractions were prepared and equal amounts of protein from each sample were subjected to *in vitro* hypusination assay as described in methods section. The fluorography image is shown in the top panel. Western blot analyses of the same samples for eIF5A and tubulin are shown in the bottom panels. The blots shown are representative of three independent experiments.
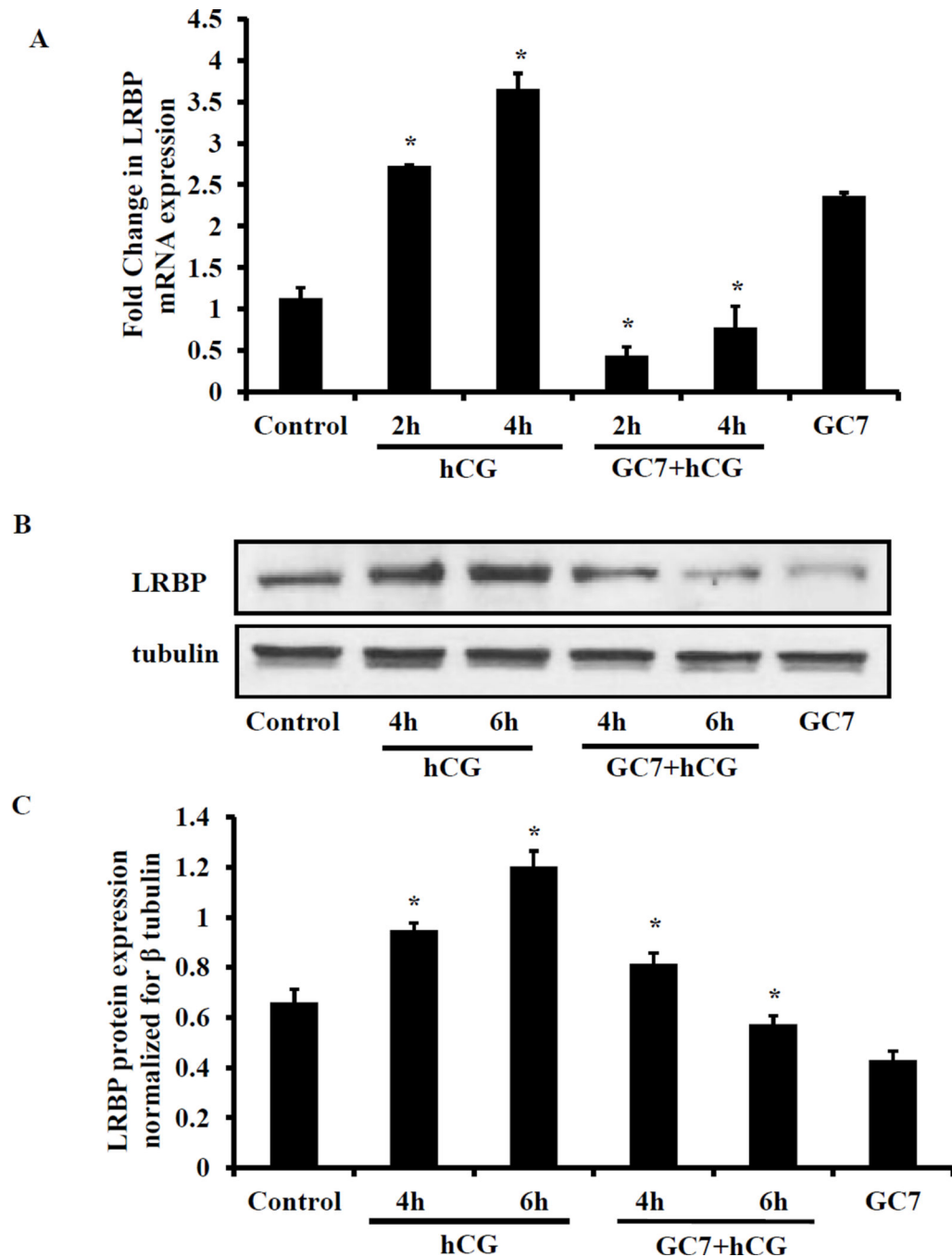
Fig. 3. Inhibition of eIF5A hypusination abrogated hCG-induced increase in LRBP expression
(**A**) GC7 (16 mg/kg body weight, i.p) was injected to superovulated rats on day 5 and 2
hours later the rats were injected with hCG (50 IU, s.c.) to induce LHR downregulation.
Ovaries were collected and processed for RNA isolation. The RNA was reverse transcribed
and the resulting cDNAs were subjected to real-time PCR quantitation using specific
primers and probes for LRBP and 18S rRNA. The graphs represent changes in LRBP levels
normalized to 18S rRNA and shown as fold change vs control. Error bars represent mean ±
SE; *p<0.05 vs control. (**B**) The ovaries were homogenized in RIPA buffer containing

protease inhibitor cocktail and S10 fractions were prepared. Equal amounts of protein from control, hCG-treated or GC7+hCG-treated S10 fractions were subjected to Western blot analysis using LRBP antibody. The membranes were stripped and reprobed for β-tubulin antibody. The bar graph represents the densitometry scanning of LRBP signals normalized with β-tubulin blots and expressed as fold change vs control. The blots shown are representative of three independent experiments and the results of the bar graphs are SEM of three experiments. *P<0.05 vs control.
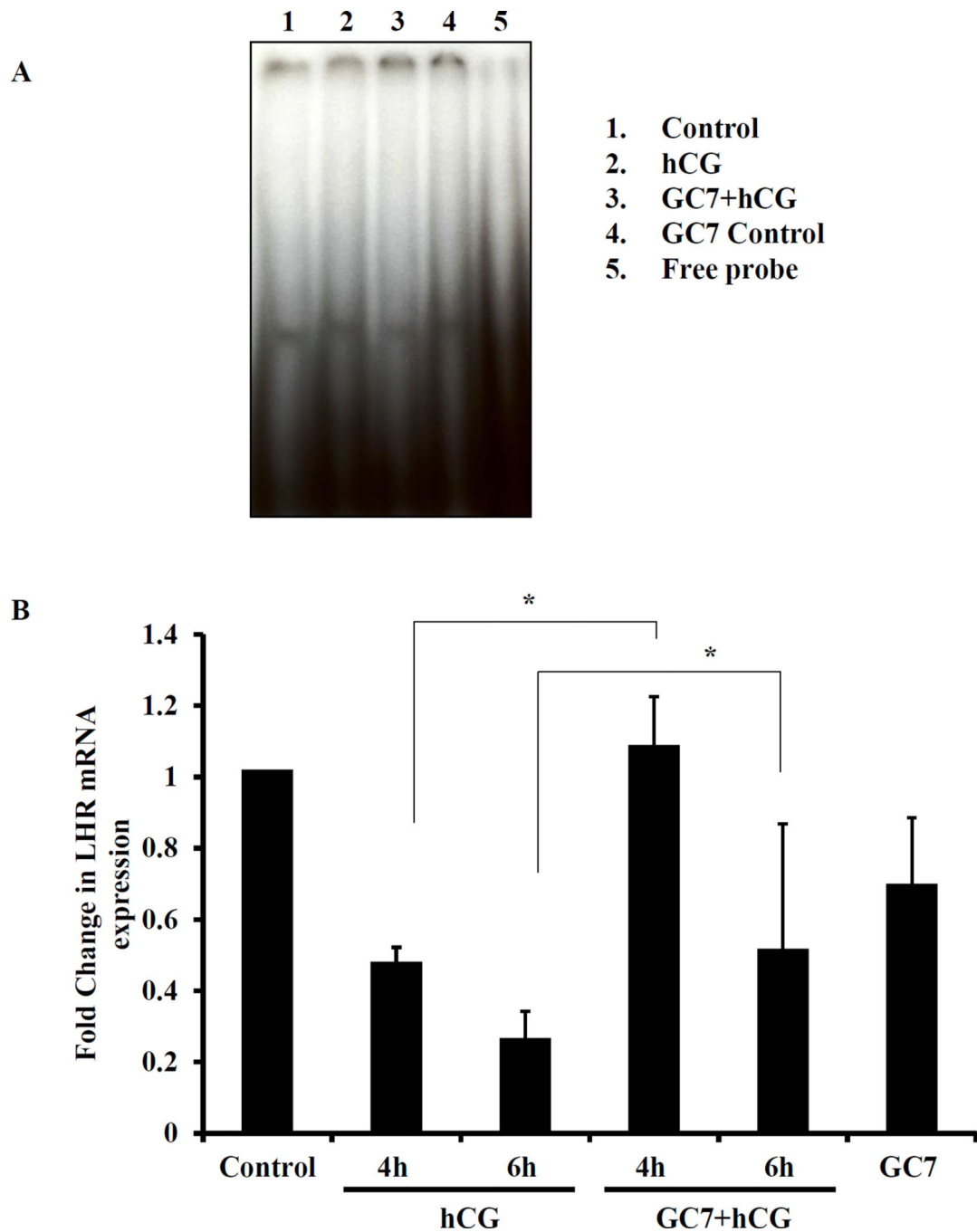
Fig. 4. Inhibition of eIF5A hypusination by GC7 inhibits hCG-induced LRBP activity and LHR downregulaton

Superovulated rats were injected with GC7 (16mg/kg body weight, i.p.), followed 2h later with hCG (50IU, s.c) and the ovaries were collected at different time intervals. **(A)** S100 fractions were prepared from the ovaries using 1× REMSA buffer. Equal amounts of protein from control, hCG or GC7+hCG-treated S100 fractions were subjected to RNA electrophoretic mobility shift assay as described in methods section. **(B)** The total RNA was isolated from superovulated rats with hCG or saline (with or without GC7 pretreatment).

cDNA was synthesized by reverse transcription PCR and Real-Time PCR was performed using specific primers and probes for rLHR and 18S rRNA. The graphs represent changes in LRBP levels normalized to 18S rRNA and shown as fold change vs control. Error bars represent mean ± SE; *p<0.05 vs control.

TABLE-1

Effect of various inhibitors on hCG-induced eIF5A mRNA, protein expression and eIF5A hypusination.

Inhibitors	eIF5A mRNA	eIF5A protein	eIF5A hypusination
H-89	Decrease	Decrease	Decrease
U0126	Decrease	Decrease	Decrease
GC7	No change	No change	Decrease