Regulation of mRNA by GnRH through the RNA-binding protein HuR

Bhakti Gangapurkar, Class of 2012

University of California, San Diego School of Medicine

ISP advisor: Dr. Mark Lawson

ISP committee members: Dr. Jeffrey Chang and Dr. Shunichi Shimasaki

Regulation of mRNA by GnRH through the RNA-binding protein HuR

Abstract

The hypothalamic-pituitary-gonadal axis is a principal regulator of reproduction in mammals. GnRH, a pivotal component of this axis, regulates the synthesis and secretion of the gonadotropins luteinizing hormone (LH) and follicle stimulating hormone (FSH) by the gonadotropes of the anterior pituitary. LH synthesis is regulated at two different levels, the transcriptional and the post-transcriptional/ translational level. Previous studies have shown that GnRH activates cap-dependent translation promoting translation of mRNAs. Translational regulation by GnRH is specific to some mRNAs and occurs independently of transcription. Evaluation of polyribosome profiles in GnRH-stimulated LβT2 gonadotrope cells showed LHβ mRNA redistributes to the transiently paused pool of mRNA as part of the unfolded protein response (UPR). In contrast, the mRNA encoding *Dusp1*, a protein phosphatase critical in resolving activation of MAP kinases, redistributes to actively translating polyribosomes. *Dusp-1* mRNA is regulated and stabilized by various mRNA-binding proteins such as HuR (Human Antigen R, or ELAV1 in mice at its 3' UTR) and NF 90. *Dusp1* mRNA encodes a binding site for the ARE (AU Rich Element)-binding protein HuR. Under chemical insult, HuR interaction stabilizes *Dusp*1 mRNA and promotes its translation. This drew our attention to whether GnRH regulates the translation of *Dusp1* mRNA via HuR. Immunofluorescence experiments determined that treatment with GnRH promotes shuttling of HuR, to the nucleus and by blocking various signaling pathways it was determined that this translocation is MAP kinase dependent.

Introduction

The reproductive axis is controlled by the release of the decapeptide gonadotropin-releasing hormone (GnRH) from various nuclei in the hypothalamus. GnRH binds to G-coupled protein receptors on the gonadotrope cells in the anterior pituitary and activates the synthesis of LH and FSH. These hormones control ovulation and spermatogenesis. An important feature of the hypothalamic-pituitary-gonadal axis is the pulsatile release of GnRH, which is essential to the maintenance of normal physiology and proper functioning of the gonadotropes (1, 2). Non-pulsatile, continuous exposure to GnRH results in an inhibition of gonadotropin release. Elevation in GnRH pulse amplitude and frequency results in the LH surge required for ovulation (3).

Understanding the signaling and mechanism of the hypothalamic-pituitary-gonadal axis is important for understanding reproductive disorders, such as polycystic ovarian syndrome. Polycystic ovarian syndrome is characterized by irregular menstrual cycles, ovarian cysts, and is associated with infertility.

GnRH binds to the GnRH receptor in the pituitary gonadotropes and activates $G_{q/11}$ and phospholipase C (PLC), which in turn produces the second messengers inositol 1, 4, 5-trisphosphate (IP_3), diacylglycerol (DAG), and arachidonic acid (AA). These second messengers cause mobilization of Ca^{2+} and activate protein kinase C isoforms (PKCs). PKCs mediate the downstream activation of MAP kinase cascades such as extracellular signal-regulated kinase (ERK), jun-N-terminal kinase (JNK), and p38 MAPK. These activated MAP kinases phosphorylate cytosolic and nuclear proteins to initiate the transcriptional activation of the beta subunit of LH (4).

GnRH pulse frequency regulates the production of gonadotropins. The gonadotropes analyze and interpret the pulse frequency and amplitude of GnRH and produce specific activators and repressors in response. High pulse amplitude increases levels of LHβ mRNA more than lower pulse amplitudes. Distinct patterns of gene expression have been associated with each pulse frequency. For example, early growth response (*Egr1* and *Egr2*) mRNAs have been associated with higher pulse frequencies. EGR1 and EGR2 activate LHβ subunit gene (*Lhb*) promoter, thus inducing the production of LH. At lower frequencies Ngfi-A binding proteins (NAB1 and NAB2) are produced and these inhibit the actions of EGR1/2 (5). It has also been shown that the ERK pathway is involved in LHβ protein synthesis (6).

GnRH mediates LH production via the ERK/MAPK signaling pathway that controls the translation of the beta subunit of LH. In one study, a significant disparity was seen between the levels of *Lhb* mRNA and serum level of LH hormone following treatment with GnRH. This indicates that there is some GnRH control after transcription of *Lhb* mRNA, but before release of LH hormone, possibly at the translational level or the level of protein synthesis (5).

Studies have shown that the pulsatile release of GnRH alters the response of the ERK pathway, which is involved in LHβ production (7). Mitogen-Activated Protein Kinase Phosphatase 1/Dual Specificity Phosphatase 1 (MKP-1 / *Dusp1*) may be involved in this alteration. *Dusp1* has been shown to act on ERK in osteoblasts, and is important for regulation of osteoblast differentiation (8). *Dusps* work by dephosphorylating the tyrosine and threonine residues of ERK, thus inactivating the MAPKs. The phosphorylated (active) form of ERK is controlled by a balance between positive stimuli and activity of phosphatases such as *Dusp1* (9). It has been demonstrated that treatment of LβT2 cells with tonic GnRH results in increased levels of *Dusp-1* (10).Data suggests that ERK is activated differentially in response to different

pulse amplitudes, and these responses are regulated by *Dusp1*. The data shows that *Dusp1* mRNA increases at high GnRH pulse frequency and amplitude and tonic treatment in LβT2 cells. Overexpression of *Dusp1* was found to reduce the amount of ERK phosphorylation (6).

Studies have shown that *Dusp1* mRNA is stabilized and its translation is controlled by RNA-binding proteins such as HuR and NF 90. Lack of these binding proteins results in lowered *Dusp1* expression and therefore elevated phosphorylation of JNK and p38 (11). HuR is a ubiquitously expressed protein that is involved in the stabilization of *Dusp1* mRNA under chemical insult (11). This drew our attention to whether regulation of *Dusp1* mRNA translation by GnRH is mediated by HuR, and which signaling pathways are involved. Initial data from this lab suggested that tonic treatment of LβT2 cells with GnRH causes the movement of the mRNA binding protein HuR from the cytoplasm to the nucleus. Possible pathways that cause the phosphorylation and movement of HuR include extracellular signal regulated kinase (ERK), p38 MAPK, and protein kinase C (PKC). By individually blocking these pathways and the release of Ca^{2+} from the Endoplasmic reticulum, the pathway by which HuR is regulated was determined.

Summary of introduction

This study examines the role of the mRNA binding protein, HuR/ELAV1, and its translocation within the cell in response to GnRH. The signaling pathway by which this occurs was determined.

Results

To evaluate the cellular distribution of HuR within LβT2 cells (a pituitary gonadotrope cell line that is involved in production of luteinizing hormone) and its response to treatment with GnRH, immunofluorescence experiments were performed. In untreated cells, HuR is mainly localized to the nucleus, with some HuR translocating to the cytoplasm (figures 1(a)-3(a) panel A). Additionally, some cells appear to have no staining for HuR. Following treatment with 10nM GnRH for 30 minutes there appears to be an increase in relative amount of HuR in the nucleus (Figures 1(a)-3(a) panel B). There is also an increase in the amount of HuR protein in cytoplasm and the majority of cells stain for HuR (Figures 1(a)-3(a) panel B). Quantitative analysis, involving color intensity analysis of these images, was used to determine the green (representing HuR) to blue ratio (DAPI stain of nucleic acid) in the nuclei of these cells. This data suggests that this ratio (reflective of the amount of HuR in the nucleus) in cells tonically treated with GnRH is significantly different from the untreated control.

When LβT2 cells were treated with the 1μM U0126 (ERK inhibitor) followed by 10nM GnRH, the HuR translocation effects seen above are reversed (Figure 1(a) panel D), with no significant different in the HuR:DAPI ratio of cells treated with U0126 and GnRH compared to the vehicle.

Treatment of LβT2 cells with MAPK pathway inhibitor PD 98059, followed by treatment with 10nM GnRH, showed complete blockage of GnRH effect described above (Figure 2(a) panel D). Quantification of this effect revealed a significant difference in the HuR: DAPI ratio of cells treated with PD 98059 and GnRH versus those treated with GnRH alone.

Following treatment with 100nM of bisindolylmaleimide (BIS), which is a PKC inhibitor, in addition to GnRH, the translocation effects that were seen with GnRH alone were not completely reversed. There was a significant different in the HuR: DAPI ratio of cells treated with BIS and GnRH compared to the vehicle. The BIS inhibitors are less specific than the ERK/ MAPK inhibitors, so complete inhibition is not expected. (figure 3(a) panel D).

LβT2 cells were also treated with hydrogen peroxide, in order to determine the distributive effect on HuR in response to oxidative stress. It appears that treatment with 250μM hydrogen peroxide results in HuR translocation to the nucleus (Figure 4 panel C). Treatment with hydrogen peroxide and 10nM GnRH has a similar effect with an increase in relative amount of HuR in the nucleus (Figure 4(a) panel D). Statistical analysis of variance in this data (both transformed and untransformed) showed a significant different from the grand mean in the GnRH treatment group and hydrogen peroxide treatment group when accounting for other variables. Cross analysis of GnRH and hydrogen peroxide, showed a greater effect than each individual component alone, indicating a synergistic effect.

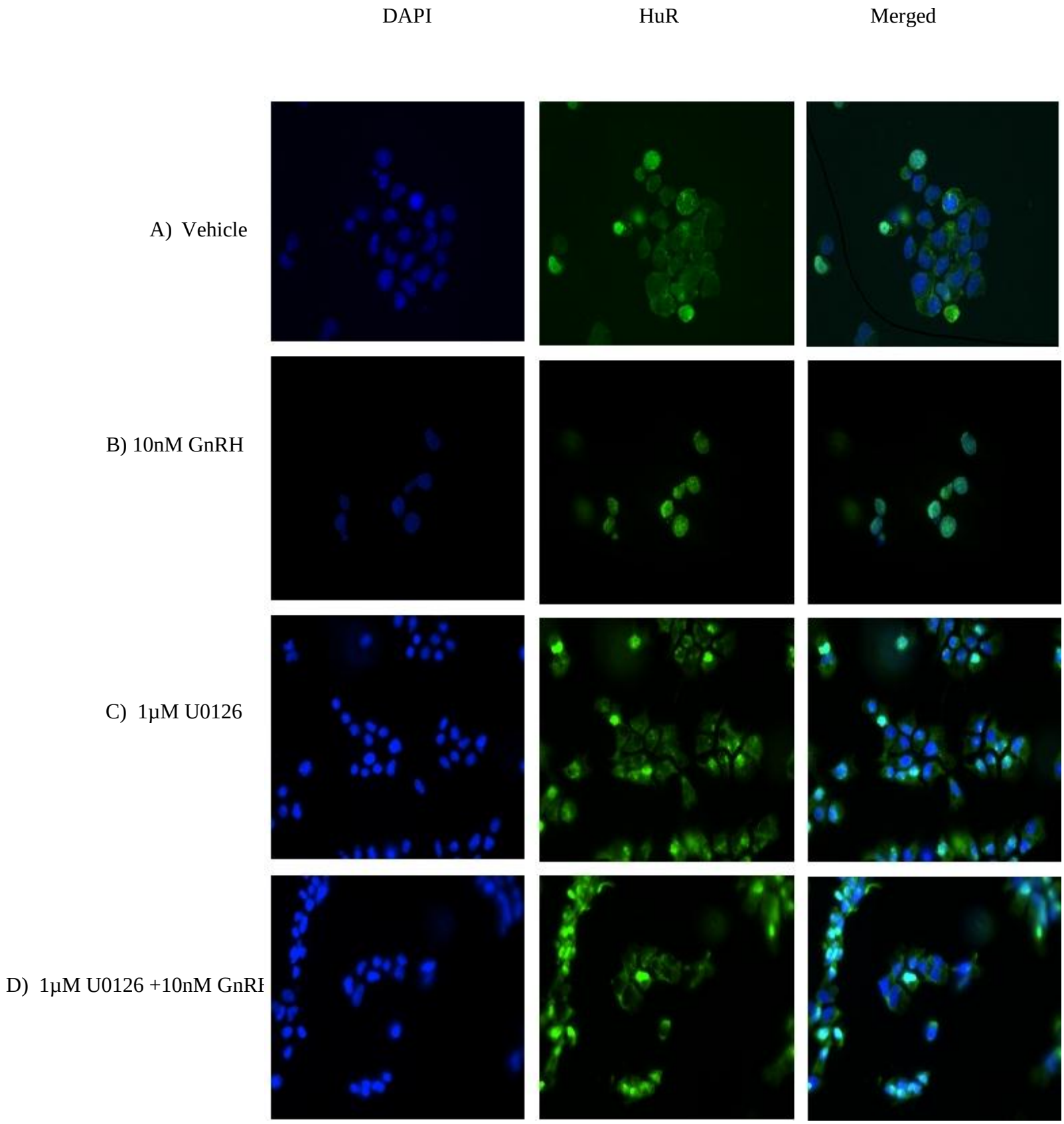
DAPI
HuR
Merged
A) Vehicle
B) 10nM GnRH
C) 1µM U0126
D) 1µM U0126 +10nM GnRH

Figure 1(a): LβT2 cells were serum starved overnight and treated with GnRH for 30 min, U0126, U0126+ GnRH, and vehicle at the indicated concentrations. The cells were then fixed, permeabilized, treated with primary antibodies, followed by treatment with secondary fluorescent antibodies. Immunofluorescence microscopy was used to assess HuR distribution (green) in cells that were untreated (A), treated with 10nM GnRH for 30min (B), treated with 1μM U0126 (positive control, C) and treated with 1μM U0126 in addition to 10nM GnRH (D). Nuclei were visualized using nucleic acid stain, DAPI. Merged, overlay of HuR and DAPI are shown in the last column.

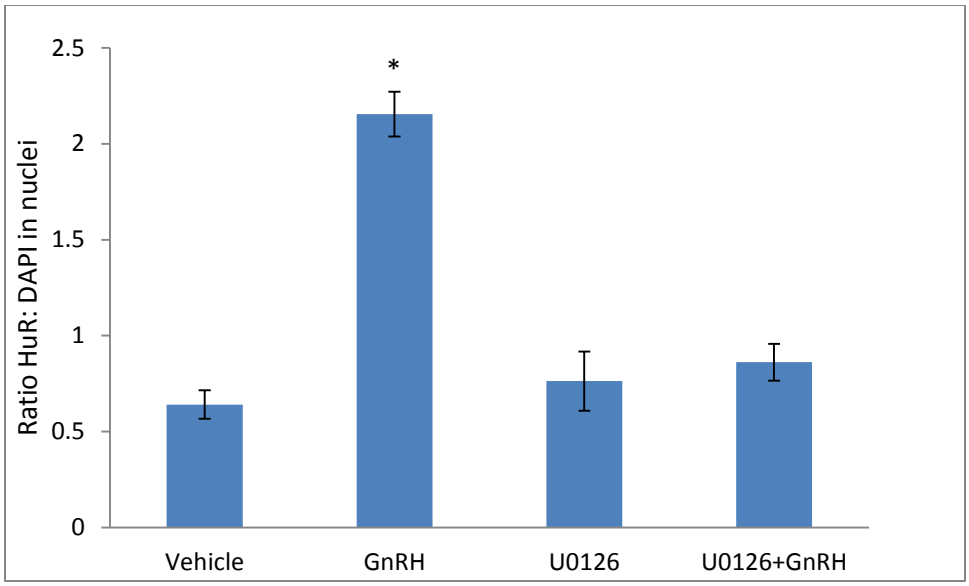

Figure 1 (b): The histogram represents quantitative analysis of three separate immunofluorescence studies. Data are expressed as the mean intensity ratio of HuR: DAPI in nuclei +/- SEM. Quantification of the immunofluorescence data was performed by intensity analysis of green (HuR) and blue (DAPI stain of nucleic acids) in the nuclei of cells. This shows increased HuR in the nucleus in response to GnRH with HuR to DAPI ratio of 2.15 versus 0.65 seen in untreated cells. This effect was blocked with addition of ERK inhibitor U0126, indicated by HuR: DAPI ratio of 0.86.

**marks significance*

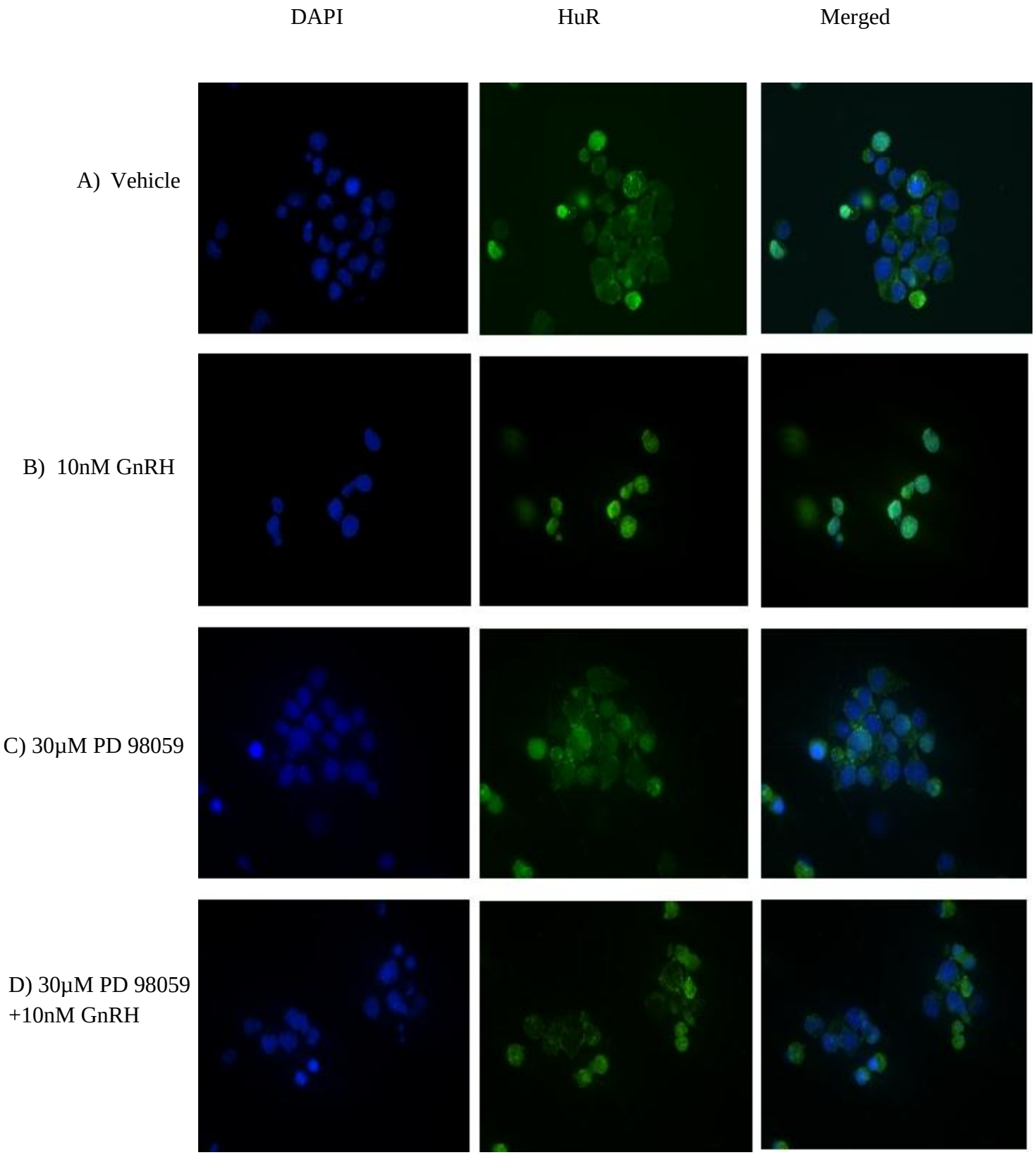
DAPI
HuR
Merged
A) Vehicle
B) 10nM GnRH
C) 30µM PD 98059
D) 30µM PD 98059
+10nM GnRH

Figure 2 (a): LβT2 cells were serum starved overnight and treated with GnRH for 30 min, PD 95059, PD 98059+ GnRH, and vehicle at the indicated concentrations. The cells were then fixed, permeabilized, treated with primary antibodies, followed by treatment with secondary fluorescent antibodies. Immunofluorescence microscopy was used to assess HuR distribution (green) in cells that were untreated (A), treated with 10nM GnRH for 30min (B), treated with 30μM PD 98059 (positive control, C) and treated with 30μM PD 98059 in addition to 10nM GnRH (D). Nuclei were visualized using nucleic acid stain, DAPI. Merged, overlay of HuR and DAPI are shown in the last column.

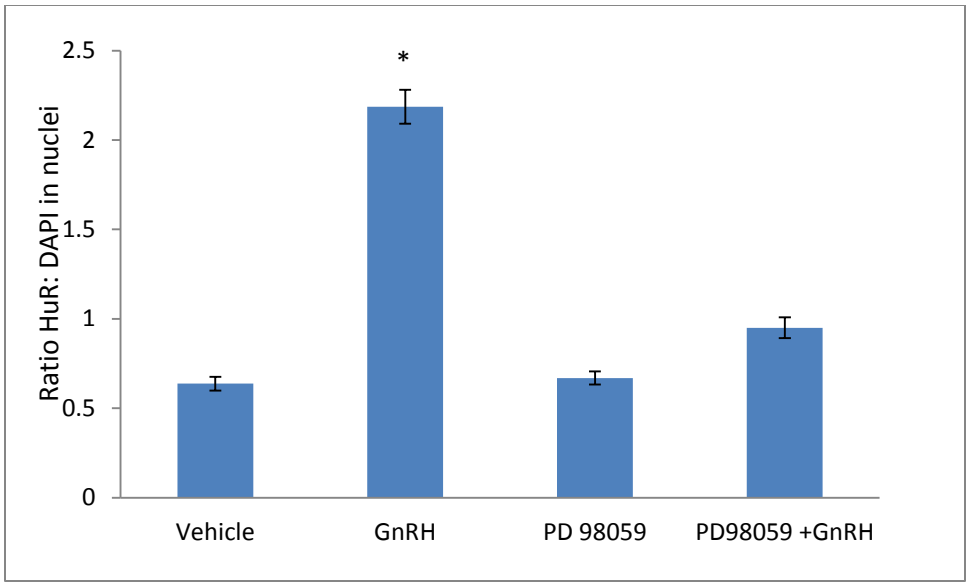

Figure 2 (b): The histogram represents quantitative analysis of three separate immunofluorescence studies. Data are expressed as the mean intensity ratio of HuR: DAPI in the nuclei +/- SEM. Quantification of the immunofluorescence data was performed by intensity analysis of green (HuR) and blue (DAPI stain of nucleic acids) in the nuclei of cells. This shows increased HuR in the nucleus in response to GnRH with HuR: DAPI ratio of 2.18 versus 0.63 seen in untreated cells. This effect was blocked with addition of MAPK inhibitor, PD 98059 indicated by HuR: DAPI ratio of 0.94.This suggests increased HuR in the nucleus in response to GnRH, with this shuttling being MAPK dependent (illustrated by reversal when treated with PD 98059).

**marks significance*

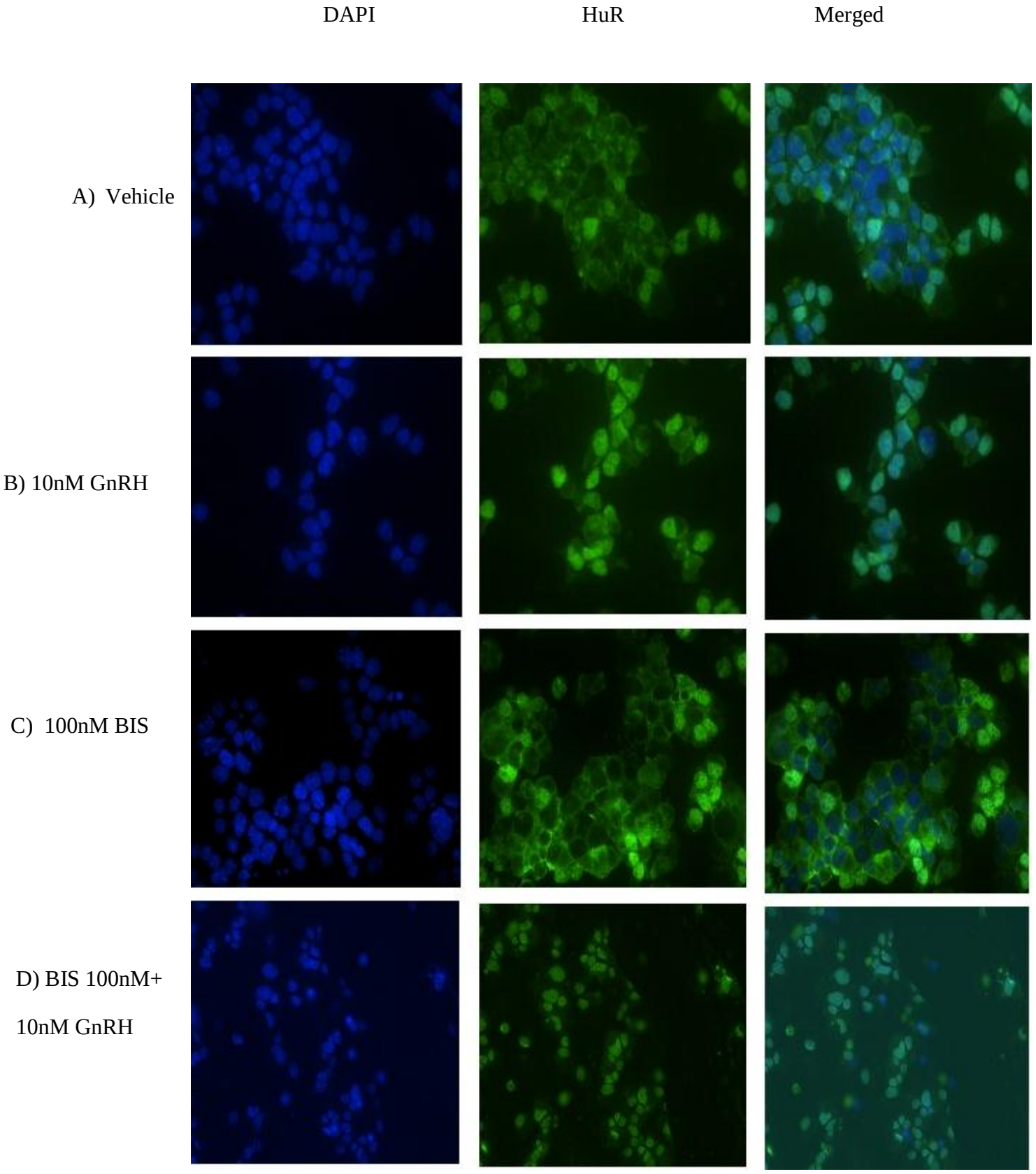
DAPI
HuR
Merged
A) Vehicle
B) 10nM GnRH
C) 100nM BIS
D) BIS 100nM+
10nM GnRH

Figure 3(a): LβT2 cells were serum starved overnight and treated with GnRH for 30 min, BIS, BIS+ GnRH, and vehicle at the indicated concentrations. The cells were then fixed, permeabilized, treated with primary antibodies, followed by treatment with secondary fluorescent antibodies. Immunofluorescence microscopy was used to assess HuR distribution (green) in cells that were untreated (A), treated with 10nM GnRH for 30min (B), treated with 100nM BIS (positive control, C) and treated with 100nM BIS in addition to 10nM GnRH (D). Nuclei were visualized using nucleic acid stain, DAPI. Merged, overlay of HuR and DAPI are shown in the last column.

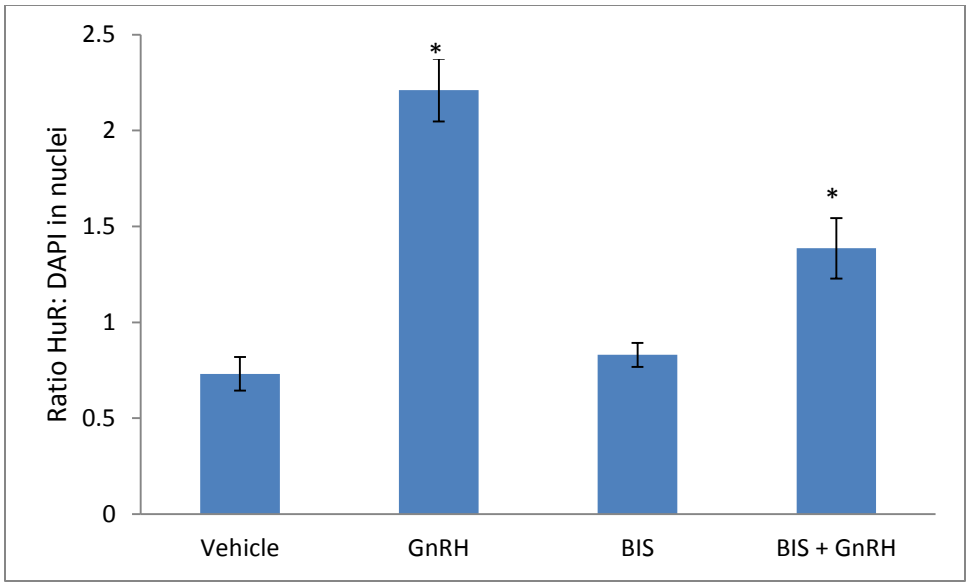

Figure 3 (b): Quantification of the immunofluorescence study with data representing the mean intensity ratio of HuR: DAPI in the nuclei +/- SEM. Quantification of the immunofluorescence data by performing intensity analysis of green (HuR) and blue (DAPI stain of nucleic acids) shows increased HuR in the nucleus in response to GnRH with HuR: DAPI ratio of 2.21 versus 0.73 seen in untreated cells. This effect was incompletely blocked with addition of PKC inhibitor bisindolylmaleimide (BIS) indicated by HuR: DAPI ratio of 1.38.

**marks significance*

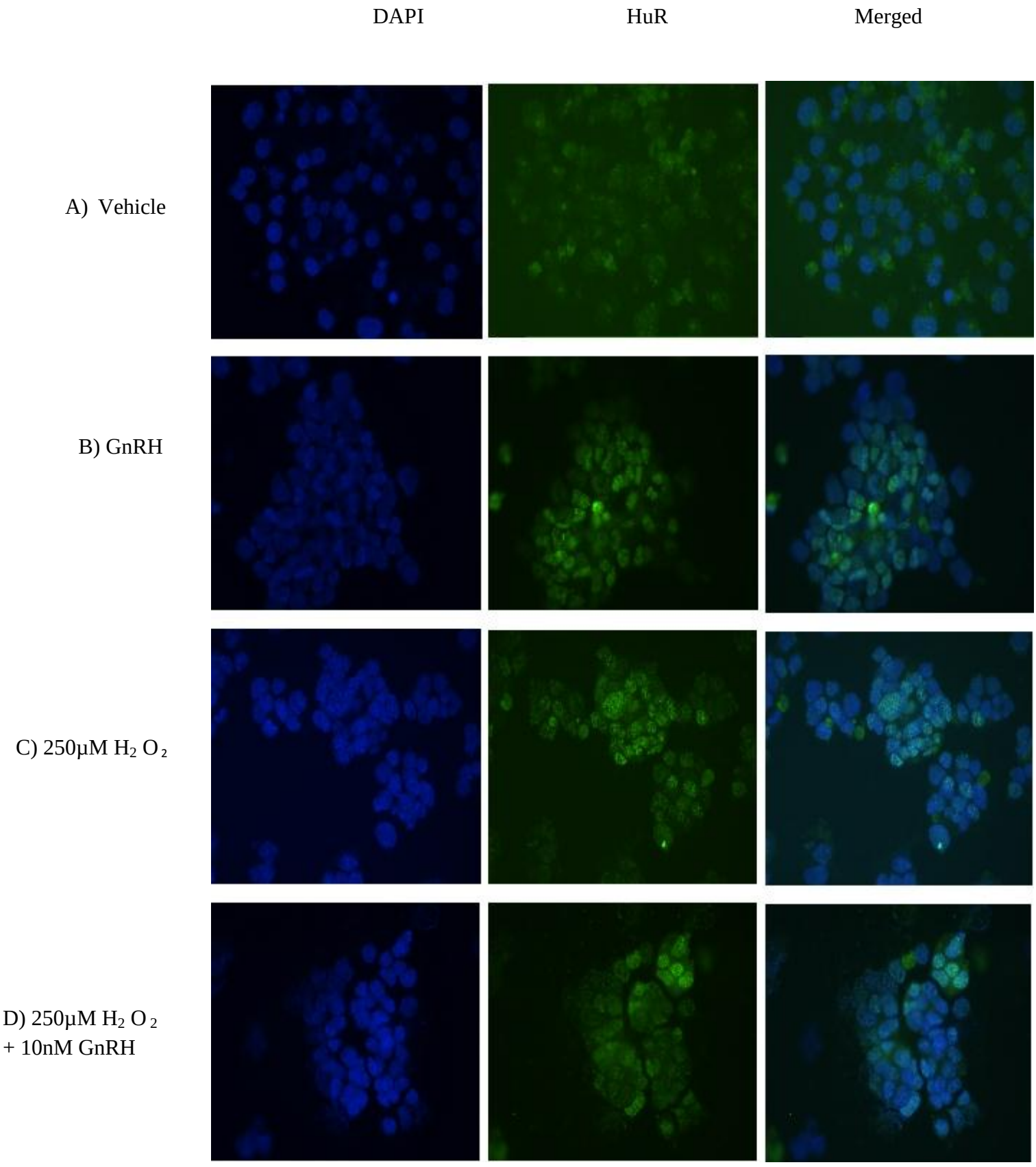
DAPI
HuR
Merged
A) Vehicle
B) GnRH
C) 250µM H_2O_2
D) 250µM H_2O_2 + 10nM GnRH

Figure 4(a): LβT2 cells were serum starved overnight and treated with GnRH for 30 min, H_2O_2, H_2O_2+ GnRH, and vehicle at the indicated concentrations. The cells were then fixed, permeabilized, treated with primary antibodies, followed by treatment with secondary fluorescent antibodies. Immunofluorescence microscopy was used to assess HuR distribution (green) in cells that were untreated (A), treated with 10nM GnRH for 30min (B), treated with 250µM H_2O_2 (positive control, C) and treated with 250µM H_2O_2 in addition to 10nM GnRH (D). Nuclei were visualized using nucleic acid stain, DAPI. Merged, overlay of HuR and DAPI are shown in the last column.

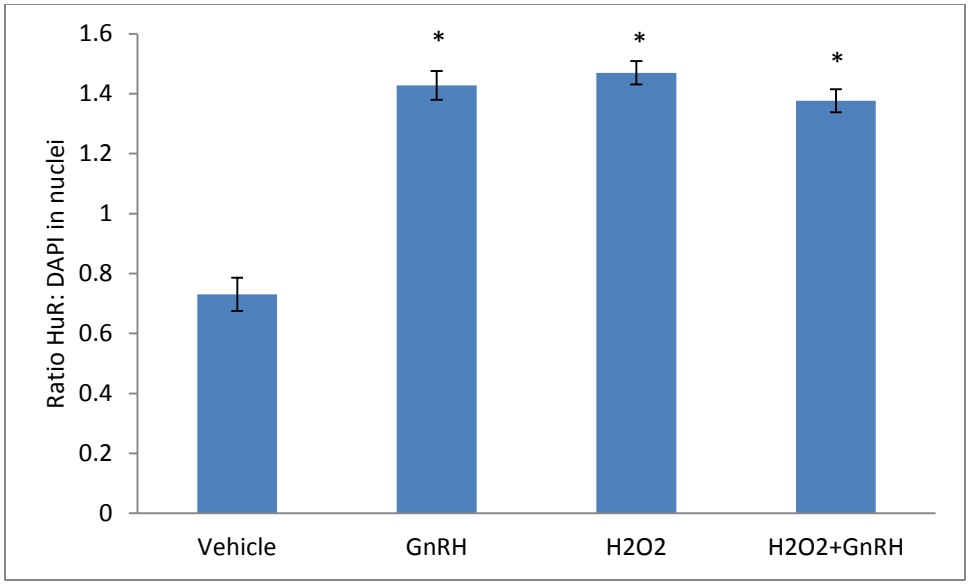

Figure 4 (b): The histogram represents quantitative analysis of three separate immunofluorescence studies. Data are expressed as the mean intensity ratio of HuR: DAPI in the nuclei +/- SEM. Quantification of the immunofluorescence data was performed by intensity analysis of green (HuR) and blue (DAPI stain of nucleic acids) in the nuclei of cells. This shows increased HuR in the nucleus in response to GnRH with HuR: DAPI ratio of 1.42 versus 0.73 seen in untreated cells. A similar effect was seen following treatment with 250µM H_2O_2 alone and 250µM H_2O_2 + GnRH indicated by HuR: DAPI ratios of 1.46 and 1.37 respectively.

**marks significance*

Discussion

It has been established by this laboratory that GnRH induces the unfolded protein response (UPR) in the LβT2 cell line. Transcription of certain mRNAs, such as *Dusp1*, is increased in response to GnRH. This is indicated by *Dusp1* mRNA associating more significantly with polysome fractions rather than RNP complexes (12). It has been shown in other cell models that oxidative stress increases *Dusp1* mRNA, which is stabilized by an RNA-binding protein HuR, due to the HuR AU-motif in the 3' UTR of *Dusp1* mRNA (8). In these models HuR condenses in the cytoplasm, stabilizes, and promotes translation of *Dusp*1 mRNA (11). Our data suggests that in contrast to the response to other inducers of the unfolded protein response (UPR) such as hydrogen peroxide (in the study above), in LβT2 pituitary-gonadotrope cell line, tonic treatment with GnRH results in a transient shift in location of HuR (which is predominantly a nuclear protein), causing it to translocate to the nucleus. This translocation was determined to be MAP kinase dependent, based on blockage of this effect following treatment with inhibitors U0126 (ERK inhibitor) and the more downstream inhibitor PD 98059 (MAPK inhibitor).

Inducing oxidative stress with hydrogen peroxide in LβT2 results has a similar effect to treatment with GnRH, with HuR translocation to the nucleus. It is possible that our results vary from the study described above due to use of a different cell line (HeLa vs. LβT2). A relatively low concentration of hydrogen peroxide (250μM) was used, making nuclear membrane breakdown as a cause of this effect unlikely. This effect was also seen in cells treated with 100μM of hydrogen peroxide. Statistical analysis of this data indicates a synergistic effect between GnRH and hydrogen peroxide. It is possible that hydrogen peroxide may be enhancing

the same MAPK signaling pathway involved in the translocation of HuR or it may be activating a signaling pathway that feeds into it, causing this synergistic effect.

From immunoprecipitation experiments, it was illustrated that HuR RNA-BP, does associate with *Dusp1* mRNA in the 3'UTR region (14). This is significant because it demonstrates the possibility of a regulatory mechanism for redistribution of *Dusp1* mRNA into actively translating polysomes. Further experiments in this laboratory involving ribosome fractionation of cells treated with GnRH were performed. Coincident redistribution of HuR in polysome profiles was studied and it was determined that in contrast to the response in other inducers of the unfolded protein response li *Dusp1* in polyribosome profiles. HuR was found to redistribute to temporarily paused or translationally inactive RNP fraction in response to GnRH treatment, which is opposite to *Dusp1* redistribution in to actively translating polysome fraction. This effect was also found to be blocked by PD 98059, an inhibitor of the MAPK signaling pathway (supporting the involvement of the MAPK pathway).

From the findings described, it can be concluded that HuR does not positively help in the translation of *Dusp1* mRNA in LβT2 cells, specifically in response to GnRH. HuR may work differently in LβT2 cells than what has been assumed in other models.

Future directions include fluorescently labeling HuR in order to actively track the movement of HuR within the cell in response to GnRH and to determine whether new HuR protein is synthesized to replace cytoplasmic HuR or HuR then re-locates to the cytoplasm as the response resolves. . In addition to this, HuR bound to mRNAs will be extracted via immunoprecipitation in order determine the various mRNAs that interact with and are stabilized by HuR. How these interactions change, and whether different mRNAs associate with HuR in

response to GnRH treatment will also be investigated. Finally, the various mRNA binding proteins other than HuR that are bound to the 3' UTR of *Dusp1* mRNA will be studied via mass spectroscopy to investigate which proteins may stabilize and impact the translation of *Dusp1* mRNA.

Conclusions:

HuR does not positively impact translation of *Dusp1* mRNA specifically in response to GnRH. In contrast to the response to inducers of the unfolded protein response (UPR) in other cell model systems treatment of the LβT2 pituitary-gonadotrope cell line with GnRH results in a transient shift in location of HuR, causing it to translocate to the nucleus. This translocation was determined to be MAP kinase dependent.

Materials and Methods

Cell Culture

The pituitary gonadotrope cell line, LBT2 was maintained in Dulbecco's Modified Eagle medium, supplemented with 4.5mg/ml Glucose, 10% fetal bovine serum and 5% penicillin/streptomycin and incubated in a humidified atmosphere of 5% carbon dioxide.

Immunofluorescence and Inhibitors

LβT2 cells were plated in chambered slides and washed with ice cold phosphate-buffered saline (PBS). LβT2 cells were treated with drugs that block various signaling pathways as indicated. Cells were treated with various concentrations of each inhibitor or no drug (2 sets of cells were plated at each concentration) for 0.5 hours. These inhibitors included PD 98059, U 0126 and bisindolylmaleimide (BIS). Cells were also treated with hydrogen peroxide in a similar manner. Following treatment with the drug, one set of the cells at each concentration was given a tonic dose of 10nm GnRH for 0.5 hours and the other set was not treated with GnRH. Following this,

the cells were fixed with 2% formaldehyde in PBS for 10min. Cells were then permeabilized using 0.1% Triton X-100, washed with ice-cold PBS, and blocked using 5% bovine serum albumin in PBS for 1 hr at 25 degrees Celsius. Cells were then incubated with a primary HuR mouse IgG monoclonal antibody overnight and underwent additional washes with ice-cold PBS followed by incubation with secondary fluorescent goat anti-mouse IgG antibodies (Alexa Fluor 488) for 0.5 hrs. After the slides were washed thoroughly, they were fixed with a drop of Vectashield (Hard set) mounting medium with 4'-6-Diamidino-2-phenylindole (DAPI) nucleic acid stain. Following staining immunofluorescence images of the cells were taken under the microscope at 40X. Separate pictures were taken for the DAPI stain (blue, nucleus) and fluorescence (green, HuR antibody). These pictures were then superimposed in order to determine the location of HuR protein with in the cell (nucleus versus cytoplasm). These images were used to determine the drug that successfully blocked the movement of HuR. Additionally, the proportion of cells demonstrating the movement (or lack of movement following blockade) was quantified by performing intensity analysis of green and blue in the nuclei of these cells. Thirty cells from each treatment group were randomly selected for this intensity analysis, giving a total N=90 from 3 samples.

Statistical Analysis

All statistical analysis was performed using JMP software (SAS Institute, Carey, NC). Data were expressed as means +/- standard error of the mean of at least three independent experimental samples per group. Results were analyzed for significant of differences by analysis of variance using untransformed data or data optimally transformed by the method of Box and Cox as implemented in JMP to correct for a non-normal distribution and variance. Post-hoc group

comparisons were made using Student's T-test or Tukey's HSD test. A $p < 0.05$ was the requirement for declaring significance.

References

1. Knobil E 1974 On the control of gonadotropin secretion in the rhesus monkey. Recent Prog Horm Res 30:1-46

2. Belchetz PE, Plant TM, Nakai Y, Keogh EJ, Knobil E 1978 Hypophysial responses to continuous and intermittent delivery of hypopthalamic gonadotropinreleasing hormone. Science 202:631-633

3. Moenter SM, Caraty A, Locatelli A, Karsch FJ 1991 Pattern of gonadotropinreleasing hormone (GnRH) secretion leading up to ovulation in the ewe: existence of a preovulatory GnRH surge. Endocrinology 129:1175-1182

4. Naor Z 2008 Signaling by G-protein-coupled receptor (GPCR): Studies on the GnRH receptor. Frontiers in Neuroendocrinology 30:10-29

5. Burger LL, Dalcon AC, Aylar KW, Haisenleder DJ, Marshall JC 2002 GnRH pulse frequency modulation of gonadotropin subunit gene transcription in normal gonadotropes-assessment by primary transcript assay provides evidence for roles of GnRH and follistatin. Endocrinology 143(9):3243-9.

6. Lawson MA, Tsutsumi R, Zhang H, Talukdar I, Butler BK, Santos SJ, Mellon PL, Webster NJ 2007 Pulse sensitivity of the luteinizing hormone beta promoter is determined by a negative feedback loop Involving early growth response-1 and Ngfi-A binding protein 1 and 2. Mol Endocrinol 21:1175-1191

7. Liu F, Austin DA, DiPaolo D, Mellon PL, Olefsky JM, Webster NJG 2002 GnRH activates ERK1/2 leading to the induction of c-fos and LHβ protein expression in LβT2 cells. Mol Endocrinol 16:419-434

8. Haisenleder DJ, Cox ME, Parsons SJ, Marshall JC 1998 Gonadotropin-releasing hormone pulses are required to maintain activation of mitogen-activated protein kinase: role in stimulation of gonadotrope gene expression. Endocrinology 139:3104-3111

9. Horsch K, de Wet H, Schuurmans MM, Allie-Reid F, Cato ACB, Cunningham J,Burrin JM, Hough FS, Hulley PA 2007 Mitogen-Activated Protein Kinase Phosphatase 1/Dual Specificity Phosphatase 1 Mediates Glucocorticoid Inhibition of Osteoblast Proliferation. Mol Endocrinol 21:2929-2940

10. Brondello JM, Brunet A, Pouyssegur J, McKenzie FR 1997 The dual specificity mitogen activated protein kinase phosphatase-1 and -2 are induced by the p42/p44MAPK cascade. The Journal of biological chemistry 272:1368-1376

11. Zhang T, Roberson MS 2006 Role of MAP kinase phosphatases in GnRH dependent activation of MAP kinases. J Mol Endocrinol 36:41-50

12. Kuwano Y, Kin H, Kotb A *et al* 2008 MKP-1 mRNA Stabilization and Translational Control by RNA-Binding Protein HuR and NF90. Molecular and Cellular Biology 28: 4562-4574

13. Do, M.H., S.J. Santos, and M.A. Lawson, *GNRH induces the unfolded protein response in the LbetaT2 pituitary gonadotrope cell line.* Mol Endocrinol, 2009. 23(1): p. 100-12.

14. Unpublished observations by Hiral Upadhyay and Andy Lim.