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GnRH Ⅰ及 GnRH Ⅱ在胎盤及子宮內膜細胞訊息傳遞路徑的探討

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GnRH I and II –stimulated AP-1 activity requires activation of the JNK and /or ERK signaling pathways in human trophoblasts

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Abstract

The spatiotemporal expression of GnRH I and II in the human placenta is believed to impact on the process of placenta invasion and embryo development. In particular, uPA/PAI-1 expression has been associated with GnRH I and II in human trophoblastic cells. Three MAPKs, extracellular signal-regulated kinase (ERK), p38 MAPK and c-Jun N-terminal kinase (JNK), which are simultaneously activated by GnRH I and II, mediated subsequent c-jun phosphorylation at Ser 73 and DNA binding of AP-1 at different magnitudes. Activation of MAPK pathways is followed by acquisition of the AP-1 DNA-binding and transactivation capacities. While inhibition of JNK mainly prevented expression and phosphorylation of c-Jun, inhibition of the ERK pathway suppressed only part of phosphorylation and expression of c-jun protein. Furthermore, only the activity of the JNK pathway is essential for the c-jun response. Conversely, p38 MAPK did not induce c-jun activation directly. Taken together, the results show that AP-1 is a nuclear target of the JNK/ERK-pathway and subsequently mediates uPA/PAI-1 expression human trophoblasts.

Introduction

Gonadotrophin releasing hormone (GnRH) I and II play important regulatory roles in the function and growth of the human placenta (1, 2). GnRH and gonadotrophin releasing hormone receptor (GnRHR) expression has been found in both the placenta and cultured trophoblasts (3). Previously, we reported that GnRH I is widely expressed among the distinct subpopulations of trophoblasts in the placenta, whereas the expression GnRH II is restricted to mononucleate villous and extravillous cytotrophoblasts (4).We also reported that both GnRH I and II are capable of exerting their effects on trophoblasts *in vitro* (5,6). These findings indicated that the placenta might possess GnRH I and II-mediated regulatory systems, presumably through autocrine and/or paracrine methods.

There is increasing evidence to suggest that the nature of the intracellular signaling initiated by GnRH binding to its receptor may vary from cell to cell. For instance, in pituitary-derived αT3 cells, GnRH-GnRHR interactions leads to the stimulation of two members of the family of GTP-binding proteins (G-proteins), Gq and/or G11 (7) whereas in other cell types, the GnRH-R may couple to other G proteins. In addition, GnRHR has also been shown to activate four distinct mitogen-activated protein kinase (MAPK) signaling cascades; ERK, JNK, p38MAPK and BMK (8). However, the JNK signaling cascade appears to be the main MAPK pathway involved in GnRH signaling in alpha-T3-1 cells. GnRH binds to its receptor and initiates an increase in calcium influx possibly mediated by PKC and an IP3-mediated release of calcium from internal stores and then stimulates JNK activity and subsequently JNK-related nuclear events in these cells (9). Activation of the MAPK cascades is also believed to play a role in the transcription of genes under the regulation of GnRH (10).

In order to show that trophoblastic cells, known to express GnRH-R, are sensitive to the actions of GnRH I and II, we have conducted experiments using cultured trophoblasts and elucidated the signaling transduction pathways of these cells. For the first time, we present an elucidation of the signaling transduction pathways in human trophoblasts, assessed using MAPK inhibitors.

Materials and Methods

Materials

GnRH I and II were obtained from Peninsula Laboratories. ERK1/2 inhibitor (PD 98059) and p38 inhibitor (SB203580) were obtained from A.G. Scientific, Inc. (San Diego, LA). JNK inhibitor (SP600125) was obtained from Tocris Cookson Inc. (Ellisville, MO, USA). Tissues

Tissue samples from first trimester placentas were obtained from women undergoing elective terminations of pregnancy. The use of these tissues was approved by the Committee for Ethical Review of Research Involving Human Subjects, Taipei Medical University Hospital. All patients provided informed written consent.

Cell Isolation and Culture

EVTs were propagated from first trimester placental tissue explants as described by Graham et al. (11). Briefly, chorionic villi were washed thoroughly in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Burlington, ON) containing penicillin and streptomycin (100 IU/ml and 100 ug/ml, respectively). The villi were finely minced and plated in 25cm^2 tissue culture flasks (Becton Dickinson, Franklin, NJ) containing DMEM supplemented with antibiotics and 10% heated-inactivated fetal bovine serum (FBS; Gibco BRL). The fragments of chorionic villi were allowed to adhere for 2-3 days, after which, any non-adherent material was removed. These tissue explants were cultured for a further 10-14 days with the culture medium being replaced every 2 days. EVTs were separated from the villous explants by a brief (2-3 min) trypsin digestion (0.125% v/v trypsin-EDTA/Ca^{2+}-, Mg^{2+}-free PBS) at 37^oC and plated in 60

mm^2 culture dishes (Becton Dickinson) containing DMEM supplemented with antibiotics and 10% FBS.

The purity of the EVT cultures was determined by immunostaining with a monoclonal antibody directed against cytokeratin 8 and 18 (Becton Dickinson) according to the methods of MacCalman et al. (12). Only cell cultures that exhibited 100% immunostaining for cytokeratin were included in these studies. All studies were performed using EVTs (passage 2) plated in 60 mm^2 culture dishes at a density of 1x 10^6 cells (Becton Dickinson) and grown to 80% confluency. FBS was removed from the culture medium 24 hours before each hormone treatment.

Hormone treated EVTs were cultured in the presence of GnRH I or GnRH II (100 nM) for 24 hours for 0, 5, 10, 30, 60 min for MAPK assays. In addition, cultures of EVTs were treated with a fixed concentration of GnRH I (100 nM) and 100nM GnRH II, Cetrorelix, for 24 hours. Cells treated with vehicle (0.1% ethanol) alone served as a control for all of these experiments.

ERK1/2, p38MAPK and JNK activity assay

The levels of p38MAPK phosphorylation at Thr^{180}/Tyr^{182}, JNK phosphorylation at Thr^{183}/Tyr^{185} and ERK1/2 (p44 and p42) phosphorylation at Thr^{185}/Tyr^{187} were determined by solid phase sandwich enzyme-linked immunosorbent assay (ELISA). ERK1/2, p38MAPK and JNK activity assay were carried out as described by the protocol of Biosource International Inc. In brief, EVTs were cultured in FBS free DMEM (37^oC, 5% CO_2) for 16 hours. The cells were washed once with phosphate-buffered saline (PBS) and the treatments with GnRH I or II were carried out for the amount of dose or time indicated in each figure before harvest. The cells were then washed with PBS and lysed in 200µl of cell extraction buffer(10 mM Tris, (pH 7.4), 100 mM NaCl, 1mM EDTA, 1mM EGTA, 1mM NaF, 20mM$Na_4P_2O_7$, Na_3VO_4, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5%deoxycholate, 1mM phenylmethylsulfonyl fluoride, Protease

inhibitor cocktail). The activities of ERK1/2, p38MAPK and JNK in the cell lysates were measured by ELISA. A monoclonal antibody specific for ERK1/2, p38MAPK and JNK was coated onto the wells of microtiter strips provided. The samples and controls were pipetted into these wells. During the first incubation, the ERK1/2, p38MAPK and JNK antigens bound to the immobilized antibody. After washing, antibodies specific for phosphorylated ERK1/2, p38MAPK and JNK were added to the wells. During the second incubation, these antibodies served as detection antibodies by binding to the immobilized ERK1/2, p38MAPK and JNK proteins captured during the first incubation. After removal of excess detection antibody, a horseradish peroxidase-labeled anti-rabbit IgG was added. This bound to the detection antibody to complete the four member sandwich. After a third incubation and washing to remove all the excess anti-rabbit IgG-HRP, a substrate solution was added, which is acted upon by the bound enzyme to produce color the intensity of which was determined through the absorbance of each well at wavelength 450 nm with MRX microplate reader (DYNEX Technologies, Chantilly, VA). ERK1/2, p38MAPK and JNK were detected by ELISA with a mean intra-assay and inter-assay coefficient of variation of 5.7% and 6.4%, 4.3% and 8.8%, 7.34% and 9.0%, respectively. All samples were assayed in duplicate.

Transfection, Stimulation, and Harvesting of EVTs

Transient transfections were performed using Lipofetamine^{TM} 2000 transfection reagent (Invitrogen life technologies Carlsbad, CA), following the manufacturer's protocol. To correct for different transfection efficiencies of the various luciferase constructs, the Rous sarcoma virus (RSV)-*lacz* plasmid was co-transfected into the cells with 1μg vectors that contained a specific cis-acting DNA sequence (enhancer element) and a sensitive reporter gene. Briefly, 4 x 10^5 of cells were seeded into six-well tissue culture plates before the day of transfection. One

microgram of the various luciferase constructs and 1 µg of RSV-*lacZ* vector were co-transfected into the cells under serum-free conditions. After 6 hours of transfection, 1 ml of medium containing 20% FBS was added, and the cells were further incubated for 24 hours. After incubation, the old medium was removed and the cells were cultured for another 24 hours with normal fresh medium containing 10% FBS. The treatments were carried out 40 hours after transfection and the cells were exposed in the presence of 100nM GnRH I or II for 6 hour.

Luciferase Reporter Assays

Cellular lysates were collected with 150µl reporter lysis buffer (Promega Corp., Nepean, Canada) and immediately assayed for luciferase activity with the Luciferase Assay System (Promega Corp. Nepean, Canada) at room temperature. The transfected cells were rinsed twice with PBS (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.47 M KH_2PO_4) and incubated for 15 min after addition of 200 贡l of cell lysis buffer. Cells were then collected by centrifugation (14000 rpm, 10 sec, 4°C). Fifty 贡l of cell lysates was mixed with 100 贡l each of Substrate A and Substrate B, and luminescence was immediately measured by Lumat LB9507 luminometer (EG&G Berthold, Bad Wildbad, Germany). Beta-galactosidase (刍-Gal) activity was also measured and used to normalize for varying transfection efficiencies. Promoter activity was calculated as luciferase activity/ß-galactosidase activity. A promoterless pGL2-Basic vector was included as a control in the transfection experiments.

Plasmid

PAP-1-Luc vector (Clontech, Polo Alto, CA, USA) was designed to monitor the induction of AP-1 signal transduction pathway and contains the firefly luciferase (*luc*) gene from *Photinus pyralis*. PAP-1-Luc contains an AP-1 response element, located upstream of the minimal TA promoter, the TATA box from the herpes simplex virus thymidine kinase promoter (P_{TA}),

located downstream of P_{TA} is the firefly luciferase reporter gene (*luc*). After transcription factors bind to the AP-1-response elements, transcription is induced and the reporter gene is activated. pTAL-Luc (Clontech) was used as a negative control to determine background signals associated with the cell lysates. The enhancerless pTAL-Luc contains HSV-TK upstream of the Luciferase coding sedquence.

Western Blot analysis Cells were treated with GnRH I or GnRH II in the presence or absence 100 nM Cetrorelix, SP600125,10μM PD 98059, 10μM SB203580 for 6 hours. Cells were lysed in 100 μl of lysis buffer (25 mM Tris-HCl, pH 7.6, 50 mM NaCl, 0.5% sodium deoxycholate, 2% Nonidet P-40, 0.2% SDS, 1 mM phenylmethylsulfonyl fluoride, 50 μg/ml aprotinin, and 50 μM leupeptin). Lysates were centrifuged (10 min,14,000 rpm) in an eppendorf microcentrifuge and 90 μl of the supernatants were mixed with 30 μl of 4× Laemmli sample buffer . Samples were boiled for 5 min. The prepared samples (20 μl) were electrophoresed through a 12% SDS-polyacrylamide gel and the separated proteins were then electrophoretically transferred onto nitrocellular paper (Hybond-C, Amersham-Pharmacia Biotech, Morgan, Canada). Membranes were incubated in blocking buffer (25 mM Tris-HCl, pH 8.0, 125 mM NaCl, 0.1% Tween 20, 2% bovine serum albumin, and 0.1% NaN_3) at 4 °C overnight; then monoclonal rabbit anti-phospho-c-jun (Ser 73) antibody or anti- total c-jun antibody (New England Lab 1:1000) was added to the blocking buffer, and blots were incubated for an additional 2 h at room temperature. The blots were washed three times in TBST (25 mM Tris-HCl, pH 8.0, 125 mM NaCl, 0.1 % Tween 20) and incubated with Horseradish Peroxidase (HRP)-conjugated anti-rabbit Ig (Amersham; 1:2,000 in TBST) for 1 h at room temperature. The blots were washed three times in TBST. The Amersham enhanced chemiluminescence system (ECL) was used for detection. Membranes were visualized by exposure to Kodak X-Omat film.

The radioautograms were then scanned and quantified with Scion Image-Released Beta 3b software (Scion Corporation, Maryland, USA).

Preparation of nuclear extracts

Nuclear extracts were prepared from EVTs as previously described (13). Briefly, cells at 80% confluency were lysed in 2.5ml of lysis Buffer, containing 20mM HEPES pH 7.6, 20% Glycerol, 10mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 0.1% Triton X-100, 1mM DTT, 1mM PMSF, 10贡g/ml leupeptin, 10贡g/ml pepstatin, and 100贡g/ml aproteinin. Cells were dislodged by scrapping and collected by centrifugation (10 min, 2000 rpm, 4^{o}C). Nuclei were resuspended at 2.5 x 10^7 nuclear per ml in nuclear extraction buffer (lysis buffer plus 500mM NaCl). Nuclei were gently rocked for 1h at 4^{o}C and centrifuged (10,000 rpm, 10 min). Resulting supernatants were aliquoted frozen quickly in liquid nitrogen, and stored at -80^{o}C.

c-jun transcription factor ELISA-based assay

c-jun-dependent transcription activity was assayed by using nuclear extract from primary cultures of EVTs. Monolayers of EVTs (80% confluent) were cultured in 100 mm culture plates containing 10% FBS DMEM (37 °C, 5% CO_2). Cells (100-mm dishes) were serum deprived for 16 hrs before treatment. The cells were treated with GnRH I or II and different concentrations of inhibitors for 6 hours. Nuclear extracts were prepared from these EVTs. c-jun-dependent transcription activity was measured by Active Motif's TransAM™ kits (Activemotif Inc, Carlsbad, CA). In brief, the cell extracts containing activated transcription factors were added into 96-well plates that contained immobilized cis-response element oligonucleotide containing a AP-1 consensus binding site (5'-TGAGCTCA-3'). Anti-c-jun antibody, which recognizes accessible epitopes on c-jun proteins upon DNA binding, was added and incubated (1 hour, room temperature). Anti-rabbit HRP-conjugated antibody was added to all wells. The colorimetric

read out was quantified by MRX microplate reader (Dynex Technologies, Inc. Chantilly, VA). The results are expressed as relative c-jun activity.

EMSA

Oligonucleotides containing the consensus AP-1 binding sequence (5'–CGCTTGATGAGTCAGCCGGAA-3') were purchased from Invitrogen and annealed to form double-strand DNA. Probes for EMSAs were end-labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase and separated from unincorporated radionucleotides by the G-50 Micro Columns (Amersham Pharmacia Biotech). Protein concentrations were determined with a modified Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA.). EMSAs were carried out in 20 µl containing 20 mM HEPES (pH 7.5), 50 mM NaCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, 10 % glycerol, 1 µg poly (dI:dC), 20 µg nuclear proteins and 50 fmol radiolabeled probe (30,000 cpm). For the competitive assays, competitor oligonucleotides (5'–CGCTTGA**G**GAGTC**G**GCCGGAA-3') were added simultaneously with the radiolabeled probes. The binding reaction was incubated (room temperature, 30 min) and then separated by a 5 % PAGE gel containing 0.5 × TBE (0.09 M tris-borate and 2 mM EDTA, pH 8.0) at constant 200 V and at 4 ^{o}C. Before loading of samples, the gel was pre-run (90 min, 100 V). After electrophoresis, the gel was then dried and exposed to a Kodak X-OMAT AR film (Eastman Kodak Co., Rochester, NY) at -70 ^{o}C.

Results

GnRH stimulation increases phosphorylation of p38MAPK, JNK and ERK1/2 in a time-dependent manner. A significant increase in p38MAPK (Figures 1 A & B) and ERK1/2 (Figures 1 E & F) levels were detected in EVTs cultured in the presence of 100 nM GnRH I (Figures 1A & E) and II (Figures 1B & F) for 60 min with maximum levels being observed after 5 min of culture under these experimental conditions. In contrast, a significant and progressive increase in JNK levels was also detected in these cell cultures until the termination of these studies at 60 min with maximum levels being observed after 60 min of culture under these experimental conditions.

GnRH I and II stimulation increases luciferase activity of AP-1 in a time and dose-dependent manner. Time dependent effect on luciferase activity of AP-1 was determined by culturing the cells in the presence or absence of GnRH I (A) or GnRH II (B) for 0-48 hours. Dose-dependent effects were determined by culturing the cells increasing concentrations of (0-100nM) of GnRH I (C) or GnRH II (D) for 6 hours. Data shown is representative of five individual experiments, and is presented as the mean ± SE. (a, $P<0.001$; b, $P<0.05$ vs untreated control).

GnRH I and II stimulation increases phospho-c-jun (Ser 73) protein level in the cell lysate. GnRH I (100 nM) (Panel A) or GnRH II (100 nM) (Panel B) GnRH II increased the levels of c-jun phosphorylation at serine 73 and total c-jun in EVTs for the times designated. GnRH II (Panel B) stimulated higher levels of c-jun phosphorylation in comparison to GnRH I (Panel A).

Inhibition of GnRH-induced c-jun phosphorylation at serine 73 by JNK inhibitor, (SP600125) and partially ERK1/2 inhibitor (PD98059), but not by p38 kinase inhibitor, (SB202580). To confirm the interaction of the GnRH-induced c-jun phosphorylation with specific MAPKs, we used specific MAPK inhibitors to determine which MAPKs mediate c-jun phosphorylation in EVT. Serum-starved EVTs were exposed to 100nM GnRH I (Panel A) or GnRH II (Panel B) and then cultured for 6 hours. Nuclear extracts were prepared from these cells and analyzed by Western blotting using c-jun phosphorylation at serine 73 antibody. Serum-starved EVTs were treated with SB203580 (100 μM), PD 98059 (100 μM), and SP600125 (10 μM) for 30 min before stimulation with GnRH I or GnRH II (100 nM). Selective blocking of the activity of MEK with 98059 (100 μM) or JNK with SP600125 (10 μM), inhibited the activation of c-jun phosphorylation at serine 73 and imply GnRH I or GnRH II –induced c-jun pathways mediated by either MEK or JNK independently. The current results show that c-jun activation is independent to p38 MAPK. In addition, MEK activation plays a minor role to stimulate the activity of c-jun in contrast to JNK. Both GnRH GnRH II and I induced c-jun phosphorylation at serine 73 are mediated by either MEK or JNK pathways without significant differences between these two GnRHs. Data are representative of three independent experiments.

Assessments Cetrorelix effects, and confirmation of the results with increasing concentration of SB203580, PD 98059 and SP600125 on activity level of c-jun by GnRH I and GnRH II by ELISA-based assays. To explore the effect of cetrorelix on GnRH I and GnRH II-induced c-jun phosphorylation and further confirm GnRH I and GnRH II-induced c-jun phosphorylation at serine 73 are mediated by either MEK or JNK pathways but not p38 MAPK, the c-jun-dependent transcription activity was assayed by using nuclear extract from primary cultures of EVTs. The c-jun-dependent transcription activity was measured by Active Motif's

TransAM™ kits (Activemotif Inc, Carlsbad, CA) according to the manufacturer's instructions. Cetrorelix inhibited the activity of c–jun in EVTs cultured in the presence of 100 nM GnRH I but not in GnRH II (Panels A). JNK inhibitor, SP600125 was capable of blocking GnRH I and II–induced c–jun activation in a dose dependent manner. ERK inhibitor PD 98059 inhibited the effect GnRH I-induced c–jun activation with a higher concentration (100μM) (Panel B) in contrast to a lower concentration (10μM) in the presence of GnRH II (Panel C). P38 MAPK specific inhibitor, SB203580 in different concentrations had no effect on levels of c-jun in EVTs cultured in the presence of 100 nM GnRH I (Panel B) or GnRH II (Panel C). Data shown is representative of five individual experiments, and is presented as the mean ± SE (a, $P<0.001$ vs 100nM GnRH, b, $P<0.05$ *vs* 100nM GnRH) in the bar graphs shown in the lower panels.

Identification and characterization of binding proteins from EVTs pretreated with GnRH antagonist, Cetrorelix, or SB203580, PD 98059 and SP600125 by electrophoretic mobility shift assay. EMSA was performed using end-labeled with [γ-^{32}P]ATP AP-1 probes and nuclear extracts from EVTs pretreated with GnRH antagonist, Cetrorelix, or SB203580, PD 98059 or SP600125 for 30 min. Serum-starved EVTs were then exposed to 100 nM GnRH I (A) or GnRH II (B) for 6h. To examine the direct effects of Cetrorelix, or SB203580, PD 98059 or SP600125 on GnRH I or GnRH II-induced AP-1 activity, EMSA was performed using [γ-^{32}P]ATP AP-1 probes containing the consensus AP-1 binding sequence (5'–CGCTTGATGAGTCAGCCGGAA-3'). As shown in Figure 6A, the nuclear proteins from EVT in the presence of GnRH I (lane 2) formed a major shifted band. The complex was abolished by addition of PD 98059 (100μM) (lane5) or a more suppression by SP600125 (100μM) (lane6). In contrast, when competitor oligonucleotides (5'–CGCTTGA**G**GAGTC**G**GCCGGAA-3') were added, it failed to abolish the binding (data not shown). Additionally, the amount of the complex

returned to normal levels by addition of Cetrorelix(100 nM) (lanes3), **suggested that Cetrorelix was a specific anatagonist of GnRH I**. The binding activity to the AP-1 was not abolished by SB203580, **suggesting that P38 MAPK does not involve the activity of AP-1** (lane 4). Results from EMSA experiments involving the nuclear extracts from EVTs in the presence of GnRH II (Figure 6B) showed a similar binding pattern as GnRH I (Figure 6A). However, Cetrorelix (Figure 6B) (lanes3) did not abolish the effects of GnRH II completely on AP-1, **suggesting that the cross binding of GnRH II with GnRH I receptor is minimal.** Furthermore, SP600125 (100μM) (lane 6) completely abolished the binding with increasing concentrations, **implying that GnRH II increases the binding depending on the JNK pathway.** The results were similar to those obtained with GnRH I. Nuclear extracts from EVTs pretreated with SB203580 in the presence of GnRH II did not increase the DNA-protein complex formation (lane 4). **These results suggest that GnRH I and II-induced the level of DNA binding activity are dependent on JNK mostly and ERK in part pathways in our experiments.**

We showed that activation of JNK and MEK/ERK1/2 by GnRH I and II, following interaction with their specific receptors (GnRH I and possibly II receptors, GnRHR I and GnRHR II) results in transcriptional activation of c-*jun*, whose activation/binding to activating protein (AP)-1 sites of promoters of their target gene results in differential transcription of target genes, i.e., uPA or PAI-1. Binding of GnRH I or/and GnRH II to GnRH receptor leads to activation of p38 MAPK, ERK 1/2 and JNK. Either activation of ERK 1/2 or JNK may result in phosphorylation of c-jun at 73, which subsequently regulates the down stream gene expression such as uPA and PAI-1 in human trophoblasts (Figure 7).

Discussion

Although GnRHa is widely used in IVF to inhibit the endogenous gonadotrophin, and it may be exposed during the process of placenta formation after the success of assisted reproductive technology (ART), however, the biological effects of GnRHa in human placenta remain unclear. In previous studies, we showed that GnRH I and II exerted their effects on the UPA/PAI-1 system and on the levels of MMP-2, MMP-9, and TIMP-1 expression in human trophoblasts and these protease systems have been assigned key roles in extracellular matrix remodeling and promoting the invasive capacity of cytotrophoblasts (5,6). The thorough molecular mechanisms involved in ligand binding, receptor activation, and intracellular signal transduction in human placenta remain poorly characterized.

We recently reported that GnRH I but not GnRH II was expressed in the outer multinucleated syncytial trophoblast layer of first trimester chorionic villi; in contrast, both GnRHs are expressed in the inner cytotrophoblast layer (4). GnRH I receptors are expressed in human trophoblasts (1, 2, 18). However, GnRH II specific binding site, a putative receptor gene on human chromosome 1(14), together with an apparent absence of a functional full-length type II receptor was detected in these cells (unpublished data). Thus, GnRHs expressed in these tissues and cells must act in an autocrine/paracrine manner, although exposure of these two classes of GnRH receptors to pharmacological doses during GnRHa therapy could result in their activation and generation of the necessary signaling (15).

GnRHs bind to specific receptors on trophoblastic cells to initiate signaling cascades leading to the expression of specific genes, increased synthesis and release of human chorionic gonadotrophin (hCG)(17,18,19). Additionally, GnRH-II is more effective than GnRH-I in stimulating leptin secretion (21). This difference could be explained by the existence of two different types of placental GnRH receptors or two different pathways of GnRH degradation (20).

Our previous study demonstrated the differential effects of GnRH I and II on the uPA / PAI-1 system in human endometrial stromal cells (16). However, the exact mechanisms for these differences remain unclear.

Many of the cellular actions of GnRH are dependent on the phosphorylation and activation of MAPKs, including ERK1/2, c-Jun N-terminal kinase, and p38 MAPK, through diverse signaling pathways (22). In this study, we demonstrated that GnRH I and II also employ MAPKs as part of their signaling pathway in trophoblasts. The effects of GnRH I and II on MAPKs induction in human trophoblast were relatively similar and moderate, possibly because of the presence of constitutively activated MAPKs, induced by many autocrine/paracrine growth factors and cytokines expressed by these cells. However, GnRH I and II may bind to the same receptor, at least in part, to elicit similar effects via similar pathways, including PKC, Ca^{2+}/CaM, MAPK pathway, such as p38 MAPK, ERK and c-Jun N-terminal kinase (JNK), that could serve as their main signaling pathways in human trophoblasts. However, the involvement of these signaling pathways and their crosstalk with MAPK in mediating GnRH I and GnRH II actions in human trophoblasts remains to be investigated.

GnRH I and II treatment resulted in MAPKs activation and in turn increasing c-*jun* activity in human trophoblasts. Unlike p38 MAPK, activation of JNK and ERK1/2 is capable of inducing c-jun phosphorylation at serine 73 in these cells. Furthermore, GnRH I and II, through this signaling pathway alter the expression of uPA /PAI-1 and MMPs in human trophoblasts in a cell- and gene-specific manner (5,6). As the products of these genes regulate diverse biological activities, specifically extracellular matrix turnover, their differential expression may play a critical role in placenta growth and regression (6).

However, the differential effects of these two GnRHs types on signal transduction pathways have not been elucidated. GnRH II may share GnRH I receptor to elicit similar effects with GnRH I, however, it may exert its effects via different pathways. Cetrorelix, the GnRH-I specific antagonist, is able to abolish the effect of GnRH I on c-jun activation, but not that of GnRH II , suggesting that GnRH II has its own unique receptor through which it exerts its effect on c-jun activation. This would explain the inability of Cetrorelix to completely block the effects of GnRH II. In this study, GnRH II appears to be a more potent ligand for MAPKs than GnRH II, suggesting that GnRH II not only mediates the original GnRH receptor pathway but also induces signaling through its own unique pathways. There is insufficient evidence to show that a functional GnRH II receptor is present in the human tissue. GnRH I and II signaling through the MAPK pathway is known to regulate the transcriptional activation of immediate early response genes and their target genes (23). GnRH I and II, through this pathway, regulated the expression of c-*jun* as well as uPA and PAI-1 mRNA in human trophoblasts (5). GnRH I and II -induced JNK was highly sensitive to SP600125, an effective JNK inhibitor; however, pretreatment with PD98059, a MEK1 inhibitor, with partially blocked GnRH I and II action on c-jun in human trophoblasts. Because GnRH I and II signaling is also mediated through p38 MAPK and their crosstalk interacts with MAPK/ERK, pretreatment with SB203580, a p38 MAPK inhibitor, had no effect on GnRH I and II -induced c-jun in human trophoblasts. Although the results further support that MAPKs signaling is involved in mediating GnRH I and II signaling in human trophoblasts, their cell-specific activation of this pathway and their target genes with the possibility of crosstalk with other pathways requires detailed investigation.

The major MAPK involving c-jun phosphorylation is JNK, however, ERK plays a synergistic role to assist JNK acting on c-jun. P38 MAPK and ERK were activated in both

GnRH I and GnRH II system, however, these activation were rapid and transient. They were activated within five minutes after GnRH I or II stimulation and recovered in ten minutes. In contrast, JNK was activated fifteen minutes after GnRH II stimulation or I and lasted for OVER sixty minutes. These observations implied that JNK may play a central role in the regulatation of c-jun activation and that ERK mediates this process. The actions of GnRH I and II are similar, at least in these cells, in that they activate p38 MAPK, ERK and JNK in a similar and time dependent manner. GnRH II appears to be more potent than GnRH I in stimulation of c-jun. JNK specific inhibitor, PD98059, is capable of abolishing the effects GnRH I and II on c-jun in a dose dependent manner, implying that JNK is necessary for mediating c-jun activation.

Heterodimerization of c-*fos* with members of the jun family of transcription factors form the AP-1 complex, which is critical in a wide range of cellular activities. Increased transcriptional activation and phosphorylation of c-*jun* through MAPK and/or several other signaling pathways leads to regulation of specific genes whose promoters contain activator protein (AP)-1 site( ). In LßT2 gonadotropes, GnRHa-induced nuclear accumulation of ERK and activation of c-*fos* resulted in transcriptional regulation of LH genes (24), whereas in GT1–7 cells, GnRHa caused a rapid induction of c-*fos* without nuclear translocation of ERK (25). In human trophoblasts, both GnRH I and II induce c-jun phosphorylation at Ser 73 via JNK activation leading to regulation of downstream genes uPA (26) and PAI-1(27). We demonstrated that the expression of uPA and PAI-1, whose promoters contain several AP-1 sites, are the targets for GnRH I and II actions in human trophoblasts.

Three distinct MAPKs, ERK, JNK, and p38, are activated during the TPA-induced megakaryocytic differentiation (28). Activation of MAPK pathways is followed by acquisition of the AP-1 DNA-binding and transactivation capacities. While inhibition of JNK mainly prevents

expression and phosphorylation of c-jun, inhibition of the ERK pathway suppresses only part of phosphorylation and expression of c-jun protein. Furthermore, only the activity of the JNK pathway is essential for the c-jun response. Three MAPKs, extracellular signal-regulated kinase (ERK), p38 MAPK and c-Jun N-terminal kinase (JNK), which were simultaneously activated by IL-1beta, mediated subsequent c-fos and c-jun mRNA expression and DNA binding of AP-1 at different magnitudes. GnRH stimulates gonadotropin subunit gene expression via GPCR which mediated by PKC and Ca signaling pathways. The transcription factors involved in these pathways are diverse in different cells. Ets-related factor and CREB protein have been proposed as targets of GnRH signaling on GTH α genes. SP-1 and early growth response protein 1 play pivotal roles in GnRH-stimulated LH-β gene expression in synergism with steroidogenic factor-1 and Ptx1. Here we reported AP-1 mediates GnRH I and II –induced JNK signaling to stimulate uPA gene expression.

Although ERK and JNK are activated in non-pituitary cells, the mechanism that induces this activation appears to vary and involve additional signaling components. The identification of distinct intracellular signaling pathways elicited by GnRHR may provide useful insights into the different biological effects observed for GnRH I and FnRH II placenta tissues.

Transcription of a specific gene is dependent upon an array of regulatory sequences known as the gene promoter which determines both the basal transcription of the gene and its response to specific stimuli. AP-1 activity is induced by a plethora of physiological stimuli and environmental insults. In turn, AP-1 regulates a wide range of cellular processes, including cell proliferation, death, survival and differentiation. AP-1 is a menagerie of dimeric basic region-leucine zipper (bZIP) proteins that recognize either 12-*O*-tetradecanoylphorbol-13-acetate (TPA)

response elements (5'-TGAG/CTCA-3') or cAMP response elements (CRE, 5'-TGACGTCA-3') (29).

In this study, we investigated the involvement of ERK1/2, p38 MAPK and JNK in the activation of c-jun in trophoblastic cells. We demonstrated that GnRH I and II induce JNK-mediated c-jun activity that ultimately phosphorylates ERK1/2 through GnRHR-dependent and -independent pathways. This leads to the induction of uPA secretion, cell motility, and ECM invasion through activation of AP-1, which is a prerequisite for proteolytic degradation and ECM remodeling.

In conclusion, we demonstrated that GnRH I and II mediate their actions in human trophoblasts through the JNK pathway, and in part the ERK pathway and transcriptional activation of c-*jun*, leading to differential expression of uPA and PAI-1. GnRH II may exert its effects through a distinct receptor but both GnRHs operate through similar pathways. Our data suggests that GnRH I and II signaling through this pathway, with possible interactions with other pathways activated by GnRH receptors, serve to mediate their diverse biological activities leading to placenta invasion and implantation.

Footnote

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