Mechanism of gonadotropin-releasing hormone (GnRH)-I and -II-induced cell growth inhibition in ovarian cancer cells: role of the GnRH-I receptor and protein kinase C pathway

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Abstract

In our previous studies, we demonstrated that ERK1/2 (extracellular signal-regulated protein kinase) and p38 MAPK (mitogen-activated protein kinase) are required for gonadotropin-releasing hormone (GnRH)-II-induced anti-proliferation of ovarian cancer cells. In the present study, we examined the role of the GnRH-I receptor, as well as the activation of protein kinase C (PKC), in the anti-proliferative effect induced by GnRH-I or II in ovarian cancer cells. Our results demonstrated that Antide, a GnRH-I antagonist, reversed the activation of ERK1/2 induced by GnRH-I or II and abolished the anti-proliferative effect of GnRH-I and II in ovarian cancer cells. Transfection of short-interfering RNA to abrogate the gene expression of the GnRH-I receptor reversed GnRH-I and II-induced anti-proliferation. These results indicate that GnRH-I or II induce anti-proliferation through the GnRH-I receptor in ovarian cancer cells. In addition, the activation of ERK1/2 by GnRH-I or II was mimicked by phorbol-12-myristate 13-acetate, a PKC activator. Pretreatment with GF109203X, an inhibitor of PKC, blocked GnRH-induced ERK1/2 activation and anti-proliferation. These results suggest that the activation of PKC is responsible for GnRH-induced ERK1/2 activation and anti-proliferation in ovarian cancer cells. Taken together, these results indicate that binding of GnRH-I and II to the GnRH-I receptor activates ERK1/2 through a PKC-dependent pathway and is essential for GnRH-induced anti-proliferation of ovarian cancer cells.

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Introduction

Gonadotropin-releasing hormone (GnRH) is a key regulator in the hypothalamus–pituitary–gonadal axis which regulates the reproduction of vertebrates. In addition to its well known physiological role in the regulation of gonadotropin secretion from the pituitary, GnRH has been shown to exert direct anti-proliferative effect on ovarian cancer cells (Choi et al. 2001a, Grundker et al. 2002, Grundker & Emons 2003, Rzepka-Gorska et al. 2003). Recently, a novel form of GnRH (referred to GnRH-II, to distinguish it from the classical mammalian form of GnRH, i.e., GnRH-I) has been identified in the human (White et al. 1998). There is some preliminary evidence that GnRH-II induces a stronger anti-proliferative effect than GnRH-I in ovarian cancer cells (Grundker et al. 2002).

The GnRH-I receptor is a member of the G protein-coupled receptors (GPCR) family and its mRNA is expressed in most primary ovarian tumors (Irmer et al. 1995, SealFon et al. 1997). The human GnRH-II receptor mRNA is also expressed in various tissues (Neill et al. 2001). Furthermore, it has been suggested that the signal transduction pathways coupled to the GnRH-II receptor may be different from those triggered by activation of the GnRH-I receptor (Millar et al. 2001, Neill et al. 2001). Nevertheless, direct evidence demonstrating the existence of a
full-length, functional GnRH-II receptor RNA transcript in human tissues is lacking and the issue of whether the GnRH-I receptor mediates the effects of both GnRH-I and II remains unresolved.

Our previous studies showed that the anti-proliferative effect of GnRH-II are mediated by the ERK1/2 (extracellular signal-regulated protein kinase) and p38 MAPK (mitogen-activated protein kinase) pathways (Kim et al. 2004, 2005). MAPKs are activated in response to mitogens and growth factors, and play an important role in the control of cell growth and differentiation. Diverse mechanisms have been proposed for MAPK activation through GPCRs (Crespo et al. 1994, Ohmichi et al. 1994) and GnRH-I activates MAPK in various cell types including ovarian cancer cells (Kraus et al. 2001, Grundker & Emons 2003). However, there is little information on the mechanism of MAPK activation by GnRH in ovarian cancer.

The activation of PKC and MAPK in response to diverse stimuli is known to regulate cell growth and differentiation. Many GPCRs can activate ERK1/2 and the activation of PKC is an important event in the activation of ERK1/2 by GnRH in pituitary cells (Harris et al. 1997). In addition, GnRH-I-stimulated ERK1/2 activation was shown to be mediated through PKC activation in human granulosa-luteal cells (Kang et al. 2001b). However, it has also been reported that GnRH signaling is independent of PKC in COS7 cells (Kimura et al. 1999), suggesting that GnRH-I signaling may vary by cell type. A better understanding of the mechanism of action of GnRH and the elucidation of the signal transduction pathways controlling anti-proliferation and apoptosis are essential to the development of novel and efficient therapies for ovarian cancer. Considering that the intracellular signals generated by GnRH are still poorly understood in ovarian cancer, we investigated the role of the GnRH-I receptor and PKC in GnRH-I and II-induced ERK1/2 activation and anti-proliferation in ovarian cancer cells.

Materials and Methods
Cell culture
Human ovarian adenocarcinoma cell lines, OVCAR-3 and SKOV-3, were cultured as previously described (Kang et al. 2000b, Choi et al. 2001b, Kim et al. 2004). Briefly, the cells were cultured in medium 199:MCDB 105 (1:1, Sigma, St Louis, MO, USA) supplemented with 10% FBS (Hyclone, Logan, UT, USA), 100 U/ml penicillin G and 100 μg/ml streptomycin (Life Technologies, Inc., Rockville, MD, USA) in a humidified atmosphere of 5% CO2-95% air at 37°C. Cells were washed once with medium and serum starved overnight before treatment with GnRH-I agonist, (D-Trp6)-GnRH (Sigma), GnRH-II analog, D-Arg(6)-Azagly(10)-NH2 (Peninsula laboratories, Belmont, CA, USA) or phorbol 12-myristate 13-acetate (TPA; Sigma).

Immunoblot analysis
To investigate the involvement of the GnRH-I receptor in GnRH-I and II-induced ERK1/2 activation, cells were seeded at a density of 2 × 10^5 cells in 35 mm dishes and cultured for 2 days. The cells were pre-treated with Antide (100 nM) for 15 min and then treated with GnRH-I or II (100 nM) for 10 min. Immunoblot analysis was carried out as previously described (Kang et al. 2001a, Kim et al. 2004). Briefly, 35 μg of total protein was run on a 10% SDS–PAGE gel and transferred to nitrocellulose membrane. The membrane was immunoblotted using a mouse monoclonal antibody for phosphorylated ERK1/2 (P-ERK1/2; New England Biolabs, Inc., Pickering, ON, Canada). Alternatively, the same membrane was probed with a rabbit polyclonal antibody for ERK1/2 to detect total ERK1/2 (T-ERK1/2; phosphorylation-state independent). Signals were detected with horseradish peroxidase (HRP)-conjugated secondary antibody and visualized using the enhanced chemiluminescence kit (ECL; Amersham Pharmacia Biotech Inc., Oakville, ON, USA). The intensity of signals was quantified by densitometry (BioDocAnalyze, Biometra, Göttingen, Germany). The activity of ERK1/2 is presented as a ratio of P-ERK1/2 to T-ERK1/2. To explore the role of PKC activation in GnRH-I or II-induced ERK1/2 activation, cells were pretreated with the PKC specific inhibitor, GF109203X (3 μM) for 30 min, followed by treatment with GnRH-I or -II (100 nM).

In vitro transfection with small interfering RNAs (siRNAs) targeting the GnRH-I receptor
GnRH-I receptor siRNAs were synthesized by Invitrogen (Burlington, ON, Canada), and the sequences were as follows: hGnRH-I-WS (5'-GCUCU CUGCG ACCUU UAAU-3'), and hGnRH-I-WA (5'-AUUAAG AGGUC GCAGA GAGC-3'). In addition, a non-specific scrambled siRNA was purchased from Invitrogen used as a control and the sequences were as follows: S (5'-GCUCU CGAGC CUUUC UAAU-3') and A (5'-AUUAAG AAAGG CUGC CAGC-3'). The siRNA transfection was performed according to the manufacturer’s suggested procedure (Invitrogen). In brief, 1 day before transfection, 2 × 10^4 cells per well
of a 6-well plate were seeded in 2 ml of culture medium or 4 x 10⁴ cells per well of a 24-well plate were seeded in 500 µl. The cells were transfected with GnRH-I receptor (12.5 pM final concentration) or scrambled siRNA with 1 µl lipofectamine 2000 reagent, following the manufacturer’s protocol. Then, the cells were challenged with GnRH-I or -II for 24 h. To monitor the siRNA transfection efficiency, immunoblot analysis was performed using GnRH-I receptor antibody (Neomarkers, Fremont, CA, USA).

**Thymidine incorporation assay**

Cell proliferation was monitored using [³H]thymidine incorporation as previously described (Choi et al. 2001a, Choi et al. 2001b, Kang et al. 2001a). Briefly, 4 x 10⁴ OVCAR-3 or SKOV-3 cells were plated in 24-well dishes in 0.5 ml of medium as described above. After 24 h of incubation the cells were treated with a final concentration of 100 nM GnRH-I or II for 24 h. Following treatment, the cells were then incubated with medium containing 1 µCi [³H]thymidine (0.5 Ci/ mmol; Amersham Pharmacia Biotech Inc.) and collected 6 h later. In order to block the activation of GnRH-I receptor or PKC, the cells were pretreated with Antide (100 nM) or GF109203X (0.03, 0.3 or 3 µM) for 15 min. The cells were transfected with siRNA targeting the GnRH-I receptor 24 h prior to treatment with GnRH-I or -II.

**Statistical analysis**

The data from three separate experiments are presented as the mean ± s.d. Each individual experiment was performed in duplicate or triplicate. Statistical analysis was performed by one-way ANOVA followed by Tukey’s multiple comparison test. *P* < 0.05 was considered statistically significant.

**Results**

**Effect of a GnRH-I antagonist on GnRH-I and II-induced ERK1/2 activation and cell proliferation**

OVCAR-3 and SKOV-3 cells were treated with GnRH-I or II (100 nM) and ERK1/2 phosphorylation was measured after 10 min. The phosphor-specific antibody to ERK1/2 was employed to estimate alterations in ERK1/2 activity induced by GnRH-I and II treatment. GnRH-I and II induced a 4-fold increase in phosphorylation of ERK1/2 (Fig. 1A). To determine the role of the GnRH-I receptor in ERK1/2 activation, cells were pretreated with Antide (100 nM) for 15 min, followed by GnRH-I or II for 10 min. Pretreatment with Antide significantly reversed GnRH-I or II-induced ERK1/2 activation, whereas Antide alone did not alter the phosphorylation of ERK1/2 as seen in Fig. 1A.

To evaluate the role of the GnRH-I receptor in GnRH-induced anti-proliferation, cells were pretreated with Antide, a GnRH-I receptor antagonist, followed by treatment with GnRH-I or II. As expected, treatment with GnRH-I or -II induced a significant decrease of cell proliferation in both SKOV-3 and OVCAR-3 cells. In addition, it is of interest that co-treatment with Antide reversed the anti-proliferative effect of GnRH-I and II in these cells (Fig. 1B), suggesting that GnRH-I or GnRH-II-induced anti-proliferation may be mediated by the GnRH-I receptor.

**Anti-proliferative effects of GnRH-I and II mediated by the GnRH-I receptor**

To confirm the role of the GnRH-I receptor, the cells were transfected with siRNAs targeting the GnRH-I receptor. The efficiency of siRNA-mediated down-regulation of the GnRH-I receptor was monitored by immunoblot analysis. Treatment with GnRH-I receptor siRNA resulted in a significant decrease in GnRH-I receptor expression at the protein level in both SKOV-3 and OVCAR-3 cells (Fig. 2A). In addition, transfection with GnRH-I receptor siRNA blocked the activation of ERK1/2 induced by GnRH-I or II, and scrambled RNA had no significant effect on GnRH-I or-II-induced activation of ERK1/2 (Fig. 2B). Treatment with siRNA of GnRH-I receptor completely reversed the anti-proliferative effects of GnRH-I or -II (Fig. 2C), indicating that the GnRH-I receptor is required for both GnRH-I and -II-induced growth inhibition in these ovarian cancer cells.

**GnRH-I and -II induced cell activated ERK1/2 in a PKC-dependent manner**

To determine the role of the PKC pathway in GnRH-I and -II-induced ERK1/2 activation, cells were pretreated with GF109203X, a PKC inhibitor, followed by GnRH-I or II. As shown in Fig. 3A, activation of ERK1/2 by GnRH-I and -II was blocked by GF109203X, indicating that GnRH-induced ERK1/2 activation is PKC-dependent. Treatment with a PKC-activating phorbol ester (TPA) induced the phosphorylation of ERK1/2. Pretreatment with GF109203X for 15 min completely abolished the activation of ERK1/2 by TPA.
Figure 1  Effect of GnRH-I and II on ERK1/2 activation and growth inhibition in the presence or absence of Antide. (A) OVCAR-3 and SKOV-3 cells were pretreated with Antide (100 nM) for 15 min prior to treatment with GnRH-I (GI) or II (GII) at the concentration of 100 nM for 10 min. The T-ERK1/2 and P-ERK1/2 levels were analyzed by immunoblot assay. P-ERK1/2 levels are expressed as a fold change relative to basal level. Data were analyzed by ANOVA followed by Tukey’s multiple comparison test. Values are presented as the mean ± s.d. of three individual experiments. (B) The cells were treated with GnRH-I (GI) or II (GII) at the concentration of 100 nM in the presence or absence of Antide (100 nM), a GnRH antagonist. A [³H]-thymidine incorporation assay was performed to quantify s-phase growing cells. The data from three individual experiments are presented as the mean ± s.d.; a, P<0.05 versus control (Cnt); b, P<0.05 versus treatment with GnRH-I or II alone.
Effect of a PKC inhibitor on GnRH-I and II-induced anti-proliferation

To elucidate the relevance of the PKC pathway in the proliferation of ovarian cancer cells, SKOV-3 and OVCAR-3 cells were treated with GnRH-I or II (100 nM) for 24 h in the presence or absence of GF109203X (0.03, 0.3 or 3 μM). GnRH-I and II-induced anti-proliferation was abolished by GF109203X at all concentrations tested, however...
Figure 3 Effect of a PKC inhibitor on GnRH-I- and -II-induced ERK1/2 activation and growth inhibition. (A) The cells were pretreated with GF109203X (GF; 3 µM) for 30 min, followed by stimulation with GnRH-I (GI; 100 nM) or GnRH-II (GII; 100 nM) in the absence and presence of phorbol 12-myristate 13-acetate (TPA; 160 nM) for 10 min. Control cells were treated with vehicle. T-ERK1/2 and P-ERK1/2 levels were analyzed by immunoblot assay. Values are presented as the mean ± S.D. of three individual experiments. a, P < 0.05 versus control (Cnt); b, P < 0.05 versus treatment with GnRH-I or II alone; c, P < 0.05 versus TPA alone.

(B) The cells were treated with GI or GII (100 nM) for 24 h in the presence or absence of GF109203X (GF; 0.03, 0.3 or 3 µM). A [3H]thymidine incorporation assay was performed to quantify S-phase growing cells. Data are shown as the mean ± S.D. of three individual experiments. a, P < 0.05 versus control (Cnt); b, P < 0.05 versus treatment with GnRH-I or II alone; c, P < 0.05 vs. treatment with TPA alone.
higher concentrations also resulted in a significant decrease in the basal level of thymidine uptake (Fig. 3B), suggesting that the PKC pathway mediates the anti-proliferative effect of GnRH-I and II in these cells.

Discussion

GnRH-I analogs have long been shown to have direct anti-proliferative effect on ovarian cancer cells (Balbi et al. 2004). Furthermore, it has been reported that GnRH-II has a stronger anti-proliferative effect than GnRH-I in ovarian cancer cell lines, suggesting that GnRH-II could be considered as a novel target for anti-proliferative therapeutic approaches (Grundker et al. 2002). Our previous studies have shown that the ERK1/2 pathway is involved in the anti-proliferative effect of GnRH-II in ovarian cancer cells (Kim et al. 2005). However, the intracellular signaling generated by GnRH in order to induce the activation of MAPK and anti-proliferation of cancer cells is still poorly understood. In this context, the elucidation of the signaling pathways involved in the anti-proliferative effects of GnRH could provide new therapeutic insights for ovarian cancer prevention and/or treatment. Previous studies have shown that OVCAR-3 and SKOV-3 cells, ovarian adenocarcinoma cell lines, express the mRNA for GnRH-I receptor (Yin et al. 1998, Ngan et al. 1999) and respond to GnRH I (Kim et al. 1999, Kang et al. 2000c). Although the expression of the GnRH-I receptor in SKOV-3 cells is still debated (Grundker et al. 2002), we did not observe any significant difference in the anti-proliferative effect of GnRH-I and II, which prompted the study investigating the role of the GnRH-I receptor.

The GnRH-I receptor is a member of the G protein-coupled receptor (GPCR) family. GPCRs are characterized by the presence of seven transmembrane (7 TM) domains and transmit their signals through multiple G protein subunits (Gαq, Gαs, and Gαi), often activating multiple signaling pathways. The mammalian GnRH-I receptor is unique among GPCRs because it lacks the cytoplasmic carboxy-terminal tail that is known to be responsible for internalization and desensitization following ligand stimulation (Millar et al. 2004). A search of the human genome database has revealed a putative GnRH-II receptor gene located on chromosome 1 which shares 40% identity with the GnRH-I receptor gene (Neill et al. 2001). It is predicted that the GnRH-II receptor may contain a cytoplasmic tail and only five transmembrane domains (5 TM lacking TM I and II) (Neill 2002, Millar 2003). However, a human GnRH-II receptor protein has not been identified since the human GnRH-II receptor transcript has a frame-shift resulting in a premature stop codon (Morgan et al. 2003). Thus, the issue of whether this transcript encodes a functional receptor protein in any human tissue and the potential roles of the GnRH-II receptor in mediating the effects of GnRH-I and II remains obscure. However, there is a discrepancy among the previous reports regarding the role of GnRH-I and II receptors. Enomoto et al. (2004) showed that GnRH-II receptor is required to mediate the effect of GnRH-II, while Grundker et al. (2004) reported that the anti-proliferative effect induced by GnRH-II is not mediated through GnRH-I receptor. On the other hand, a recent study demonstrated that transient transfection with GnRH-II receptor-reliquum inhibited the expression of GnRH-I receptor at the cell surface and impaired signaling via the GnRH-I receptor by reduction of GnRH-induced inositol phosphate accumulation, indicating that GnRH-I receptor may be a common receptor that mediates the effects of both GnRH-I and GnRH-II in ovarian cancer cell lines (Pawson et al. 2005). This discrepancy suggests that the role of GnRH I and II receptors may vary considerably in different cell types or conditions. In the present study, we investigated the role of the GnRH-I receptor and PKC signaling pathway in GnRH-induced MAPK activation and growth inhibition of ovarian cancer cells. In this study, the expression of GnRH-I receptor was monitored by immunoblot assay and the GnRH-I antagonist, Antide, was used to block GnRH-I receptor-induced signaling. Antide is known to exert its antagonistic action in ovarian cancer cells (Li et al. 1994) and pretreatment with Antide reduced GnRH-I-induced ERK1/2 activation in endometrial epithelial cells (Luo et al. 2004).

Although there is a previous report that GnRH-I does not activate MAPK (Emons et al. 1998), others have documented that GnRH-I activates MAPK in various cell types including ovarian cancer cells (Kimura et al. 1999, Kraus et al. 2001). In addition, our previous studies have shown that GnRH-II activates ERK1/2 (Kim et al. 2005) and that this pathway is involved in the effect of GnRH-II on anti-proliferation. In the present study, we found that Antide blocked the activation of ERK1/2 by GnRH-I, as well as GnRH-II, in OVCAR-3 and SKOV-3 cells. Furthermore, combined treatments of GnRH-I and GnRH-II did not have an additive effect on the activation of ERK1/2 (data not shown). Our present and previous studies have showed that GnRH-I and -II have anti-proliferative effects on...
ovarian cancer cells (Kang et al. 2000a, Kim et al. 2004b) and Antide was used to investigate the involvement of the GnRH-I receptor in GnRH-I- and -II-induced anti-proliferation. Co-treatment with Antide prevented GnRH-I and II-induced anti-proliferation. These results are consistent with the hypothesis that the GnRH-I receptor mediates GnRH-I and II-induced ERK1/2 activation and anti-proliferation in ovarian cancer.

The technique of RNA interference was employed to further examine the role of the GnRH-I receptor in mediating the effects of GnRH-I and II. SKOV-3 and OVCAR-3 cells were transfected with siRNAs targeted against the GnRH-I receptor gene. Immunoblot analysis was used to examine GnRH-I receptor gene expression in transfected cells. The GnRH-I receptor was expressed in the cells treated with vehicle and scrambled siRNA, whereas its expression was significantly reduced in siRNA-transfected cells, indicating that the transcription and translation of the GnRH-I receptor is reduced in these cells. The proliferation of cells transfected with siRNAs was monitored by thymidine incorporation assay. Transfection with siRNAs targeted to the GnRH-I receptor blocked the anti-proliferative effect of GnRH-I and II. These data, together with the effects of Antide, indicate that the anti-proliferative effect of GnRH-I and II on ovarian cancer cells is dependent on the GnRH-I receptor.

The signal transduction pathway activated following the binding of GnRH-I to the GnRH-I receptor have been studied extensively (Millar et al. 2004). The receptor is coupled to the Goq protein that activates phospholipase Cβ, leading to the activation of PKC and various downstream signal transduction cascades, including the MAPK pathways (Kraus et al. 2001). Activation of the PKC pathway has been well documented in response to GnRH-I stimulation (Andrews & Conn 1986, Zheng et al. 1994), and GnRH-I induces the translocation of PKC and stimulates enzyme activity (Farshori et al. 2003). Activation of PKC appears to be an important second messenger mediating GnRH-I-induced ERK activation in pituitary cells (Harris et al. 1997) and ovarian cancer cells (Chamson-Reig et al. 2003). In contrast, the GnRH-II signaling mechanism is poorly understood. Our results demonstrate that treatment of OVCAR-3 and SKOV-3 cells with GnRH-I or II for 10 min resulted in activation of ERK1/2. Furthermore, pretreatment with GF109203X, which inhibits PKC by competing with ATP (Barent et al. 1998), abolished the activation of ERK1/2 induced by GnRH-I, II or TPA. Our results are consistent with the finding that GnRH-I activates the PKC pathway in ovarian tumor cells (Chamson-Reig et al. 2003) and pituitary cells (Sundaresan et al. 1996). Although the PKC isozyme involved in the activation of ERK1/2 by GnRH-I and II in these cells is not yet known, these results suggest that both GnRH isoforms activate ERK1/2 through a PKC-dependent pathway, possibly coupled to the Goq protein. In contrast, Kimura et al. showed that the activation of ERK1/2 by GnRH-I appears to be independent of PKC in Caov-3 cells (Kimura et al. 1999). In addition, activation of the c-Jun N-terminal kinase (JNK) pathway was independent of PKC in ovarian and endometrial cancer cells (Grundker & Emons 2003). This demonstrates that involvement of the PKC pathway is dependent on the cell type and the expression of the required signaling components. To further investigate the role of PKC in GnRH-I-induced anti-proliferation, we treated ovarian cancer cells with GF109203X in a dose-dependent manner. Inhibition of PKC reversed the anti-proliferative effects of GnRH-I and -II, further supporting the role of activated PKC in the regulation of cellular functions in response to both forms of GnRH in ovarian cancer cells.

In summary, we demonstrated that Antide and GF109203X blocked the activation of ERK1/2 and anti-proliferation induced by GnRH-I and II. Furthermore, siRNA targeting the GnRH-I receptor abolished GnRH-I and II-induced anti-proliferation. Taken together, our results indicate that the GnRH-I receptor and PKC play an important role in mediating the cellular responses induced by GnRH-II, as well as GnRH-I, in ovarian cancer cells.

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