Role of Mitogen-activated Protein Kinase/Extracellular Signal-regulated Kinase Cascade in Gonadotropin-releasing Hormone-induced Growth Inhibition of a Human Ovarian Cancer Cell Line

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ABSTRACT

Although gonadotropin-releasing hormone agonists (GnRHa) have been used in the therapy of the endocrine-dependent cancers, their biological mechanism remained obscure. We have studied the roles of mitogen-activated protein kinase family in the antiproliferative effect of GnRH on the Caov-3 human ovarian cancer cell line. Reverse transcription-PCR assays confirmed mRNA for GnRH receptor in Caov-3 cells. In the presence of 1 μM GnRHa, the proliferation of cells was significantly reduced to 76% of controls after 24 h, and the effect was sustained up to 4 days. Although GnRHa had no effect on the activation of the Jun N-terminal kinase (JNK), treatment of Caov-3 cells with GnRHa activated extracellular signal-regulated protein kinase (ERK), and its effect was more than that induced by GnRH. Activation of ERK by GnRHa occurred within 5 min, with the maximum occurring at 3 h and sustained until 24 h. GnRHa also activated ERK kinase (mitogen-activated protein/ERK kinase) and resulted in an increase in phosphorylation of serine of sevenless (Sos), and Shc. Furthermore, we examined the mechanism by which GnRHa induced ERK activation. Both pertussis toxin (10 ng/ml), which inactivates Gα/Go proteins, and expression of a peptide derived from the carboxyl terminus of the β-adrenergic receptor kinase I, which specifically blocks signaling mediated by the βγ subunits of G proteins, blocked the GnRHa-induced ERK activation. Phorbol 12-myristate 13-acetate (PMA) also induced the ERK activity, but pretreatment of the cultured cells with PMA to down-regulate protein kinase C did not abolish the activation of ERK by GnRHa. Elimination of extracellular Ca2+ by EGTA also did not abolish the activation of ERK by GnRHa. To examine the role of ERK cascade in the antiproliferative effect of GnRHa, PD98059, an inhibitor of mitogen-activated protein/ERK kinase, was used. This inhibitor canceled the antiproliferative effect of GnRHa and apparently reversed the GnRH-induced dephosphorylation of the retinoblastoma protein, the hyperphosphorylation of which is a hallmark of G0-S transition in the cell cycle. These results provide evidence that GnRH stimulation of ERK activity may be mediated by Gβγ protein, not by PMA-sensitive protein kinase C or extracellular Ca2+ in the Caov-3 human ovarian cancer cell line, suggesting that this cascade may play an important role in the antiproliferative effect of GnRHa.

INTRODUCTION

In addition to its function as a key hormone in the regulation of pituitary-gonadal axis, GnRH2 probably affects human extraptuitary tissues (1, 2). GnRHas have been used in the therapy of the some sex hormone-dependent cancers, including breast (3–5), prostate (6–10), pancreatic (11, 12), endometrial (13), and ovarian (14, 15) cancers. Although this effect may be mediated by an indirect mechanism based on the reduction in sex hormone secretion, there are indications that GnRHa suppresses the growth of the cancer cells in vitro (16–20) and that the specific binding sites for GnRH are demonstrated in certain tumors responsive to GnRHa (16, 21–30). Moreover, GnRHa activates GnRH signal transduction pathways in breast cancer cells (31). These findings suggest direct inhibitory effects of GnRHa on the tumor growth. However, their mechanism of action remains unknown.

GnRH binds to a G protein-coupled membrane receptor in gonadotropes (32–35) and results in activation of multiple signaling pathways (36). The initial phase of GnRH action involves G protein-mediated stimulation of phospholipase C, leading to the formation of inositol 1,4,5-trisphosphate and diacylglycerol. Subsequently, inositol 1,4,5-trisphosphate induces the release of intracellular calcium, and diacylglycerol activates PKC, resulting in multiple cellular responses to GnRH.

Intracellular transmission of extracellular signals is mediated in large part by several groups of sequentially activated protein kinases, which are collectively known as the MAPK cascades (37, 38). In the growth factor signaling, the key elucidated MAPK cascade is the ERK (39). Recent evidence indicates that some G protein-coupled receptors can activate ERK cascade (40–43). The signals transmitted through the ERK cascade lead to activation of a set of regulatory molecules that eventually initiates cellular responses such as growth and differentiation (44–46). Recently, it has been shown that GnRHa is capable of activating ERK in the pituitary organ culture (47) and the αT3-1 gonadotroph cell line (48, 49) and that ERK is involved in regulation of gene expression of the gonadotropin α-subunit (48). However, the ERK cascade is not the only link between membranal receptors and their intracellular targets, and in the past few years, several other ERK-like cascades have been identified (45). One of the most studied of these cascades is JNK (also known as stress-activated protein kinase [50, 51]) cascade, which is known to be activated in response to cellular stress such as apoptosis (50, 52). ERK, JNK, and p38 (53) are members of the MAPK family. Recent data suggest that GnRH is capable of activating JNK in the αT3-1 gonadotroph cell line (54).

It was reported that the signaling cascade by which GnRH acts in peripheral tumors is distinct from that in the anterior pituitary (55, 56). In addition, the ERK cascade has been implicated in both cell proliferation (57–59) and growth arrest (60, 61). In particular, ERK is reported to be involved in G0-specific cell cycle arrest of human breast cancer cells (62), NIH 3T3 murine fibroblasts (63), and human myeloblastic leukemia cells (64).

D Dephosphorylation of a tumor suppressor gene product, pRB, seems to be a target of the extracellular signals that induce cell cycle arrest and differentiation (65, 66). In Go/Gi, pRB is underphosphorylated and complexed to the E2F transcription factor. This prevents the activation of some E2F-regulated genes required for DNA repli-
cation (67, 68). Phosphorylation of pRB, during mid to late G1, by cyclin D- and cyclin E-associated kinases (69, 70), is accompanied by release of E2F and activation of transcription of E2F-regulated genes, resulting in entry into S phase.

These findings led us to examine whether GnRHa has a direct antiproliferative effect and stimulates ERK cascade and JNK using Caov-3 human ovarian cancer cells, which express GnR receptor. Moreover, we compared the GnRHa-induced signaling cascade between Caov-3 and αT3-1 gonadotroph cell lines and examined the effect of GnRHa on the phosphorylation of pRB and the role of ERK cascade in the direct antiproliferative effect of GnRHa.

MATERIALS AND METHODS

Materials. PMA and MBP were purchased from Sigma Chemical Co. (St. Louis, MO). GnRHa was obtained from Peninsula Laboratories (Belmont, CA). GnRHa agonist, [α-Leu6, Po4-2HET] LHRRH (leuprolide), was a gift from Takeda Pharmaceutical Co. (Osaka, Japan). Cisplatin was a gift from Nichiyaku Co. (Tokyo, Japan). ECL Western blotting detection reagents were obtained from Takeda Pharmaceutical Co. (Osaka, Japan). Tri-Reagent was obtained from Molecular Research Center Inc. (Cincinnati, OH). The CellTiter 96 cell proliferation assay was obtained from Promega (Madison, WI).

Cell Cultures. Human ovarian papillary adenocarcinoma cell line (Caov-3) was obtained from American Type Culture Collection (Manassas, VA). αT3-1 was kindly provided by Dr. P. L. Mellon (University of California, San Diego, CA, Ref. 72). Both Caov-3 and αT3-1 clones were cultured at 37°C in DMEM with 10% FBS in a water-saturated atmosphere of 95% O2 and 5% CO2.

RT and PCR. Total cellular RNA was isolated (73) using the Promega Access RT-PCR System. PCR primers were synthesized of GnRHa (2.5 × 10−3 M) in DDW, resulting in a final GnRHa concentration of 10−6 M, or of DDW (controls) was added. These additions of GnRHa or vehicle were repeated every 24 h for 4 days. Every day, the number of surviving cells was determined by determination of A590 nm of the dissolved formazan product after addition of MTS for 1 h as described by the manufacturer (Promega). All experiments were carried out in quadruplicate and the viability is expressed as the ratio of the number of viable cells with GnRHa treatment to that without treatment.

Construction of Expression Plasmids. Myc-tagged p42mapk expression plasmid (pSG2) was obtained from C. J. Marshall (Institute of Cancer Research, London, United Kingdom; Ref. 76). The βARKct peptide-encoding minigene, containing cDNA encoding the carboxyl-terminal 195 amino acids of βARK1, was prepared as described previously (77, 78).

Assay of ERK Activity. Cells were incubated in the absence of serum overnight and then treated with various materials. They were then washed twice with PBS and lysed in ice-cold HNTG buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl2, 1 mM EDTA, 10 mM sodium P2, 100 μM sodium orthovanadate, 100 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride; Ref. 79). The extracts were centrifuged to remove cellular debris, and the protein content of the supernatants was determined using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Richmond, CA). Erk1 rabbit polyclonal antibody was bound to protein A-Sepharose beads, and 300 μg of protein from the lysate samples were immunoprecipitated at 4°C for 2 h. The immunoprecipitated products were washed once in HNTG buffer, twice in 0.5 M Tris, pH 8.0, and once in kinase assay buffer (25 mM HEPES, pH 7.2–7.4, 10 mM MgCl2, 10 mM MnCl2, and 1 mM DTI). Samples were resuspended in 30 μl kinase assay buffer containing 10 μg of MBP and 40 μg of [γ-32P]ATP (1 μCi) as described previously (48). The kinase reaction was allowed to proceed at room temperature for 5 min and stopped by the addition of Laemmli SDS sample buffer (80). Reaction products were resolved by 15% SDS-PAGE.

Assay of 42-kDa ERK Activity Using a Transient Expression System. Caov-3 or αT3-1 cells cultured in 100-mm dishes were transfected with Myc-tagged p42mapk expression plasmid (1 μg of pEXV-Erk2-tag) in combination with 9 μg of pRK or pRK-βARK1 using LipofectAMINE Plus (Life Technologies, Inc., Gaithersburg, MD). At 72 h after transfection, serum-deprived cells were incubated with 1 μg GnRHa for 30 min in Caov-3 cells or for 5 min in αT3-1 cells, and expressed Myc-tagged pl42mapk was immunoprecipitated with 1 μg of antibody 9E10. The ERK activity in the immunoprecipitate was measured as described above. The transfection efficiency of each experiment was 8–10% as assessed by β-galactosidase staining after transfection of a β-galactosidase containing expression plasmid.

Assay of MEK Activity. Cells (100-mm dishes) were serum deprived for 16 h. After treatments, cells were lysed in HNTG buffer, and lysates were immunoprecipitated with anti-MEK antisier (1:200 dilution) for 2 h at 4°C. This antisier precipitates both MEK1 and MEK2. Immunoprecipitates were mixed with protein A-Sepharose beads for 30 min, and the beads were washed twice with 1 ml of HNTG buffer. The sample was then resuspended in 100 μl of reaction buffer, containing 20 mM Tris-HCl, pH 7.4, 10 mM MgCl2, 1 mM MnCl2, 1 mM EGTA. Reactions were initiated by the addition of 10 μCi of [γ-32P]ATP (50 μCi) and 10 μg of a GST-fusion protein containing p44 MAPK with a lysine to alanine mutation at position 71 (MAPK/KA; Ref. 81). This mutation eliminates the kinase activity of MAPK, so only the kinase activity attributed to the added MEK remains. After a 15-min incubation at 25°C, reactions were stopped with 20 μl of Laemmli sample buffer, and phospho-MAPK/KA was detected by SDS-PAGE followed by autoradiography.

Immunoblots. Cells were grown in 100-mm dishes. After treatment, the cells were washed once with ice-cold PBS before the addition of 1 ml of HNTG buffer. Lysates were centrifuged at 10,000 × g for 10 min. Supernatants were incubated for 1 h with the indicated antisier. Immunocomplexes were precipitated with protein A-Sepharose protein G plus/protein A-agarose and washed three times with HNTG buffer, and samples were resolved on the indicated percentage of SDS-PAGE, followed by immunoblotting with the indicated antisier.

Assay of JNK Activity. JNK activity was precipitated from 250 μg of whole-cell lysates by incubating with 2 μg of GST-c-Jun(1–89) fusion protein/GSH-Sepharose beads for 18 h at 4°C (New England BioLabs; Refs. 50 and 82). c-Jun(1–89) contains a high affinity binding site for JNK, close to the N-terminal, which is the two phosphorylation sites at Ser-63 and Ser-73. The AMPK was examined after SDS-PAGE and immunoblotting with anti-phosphotyrosine (PY20), anti-Shc, and anti-Sos1 antisier.

RESULTS

Expression of GnRHa Receptor in Caov-3 Cells. To clarify whether GnRHa receptor is expressed in Caov-3 cells, RT-PCR for GnRHa receptor was performed using RNA samples from Caov-3...
GnRH STIMULATES ERK CASCADE IN Caov-3 CELLS

Effect of GnRHα on the Proliferation of Caov-3 Cells. To examine whether GnRHα has any effect on the proliferation of Caov-3 cells, 4 μl of GnRHα (2.5 × 10⁻⁵ M) was added daily to the 100 μl of culture medium followed by analysis of cell proliferation by MTS assay. The cell proliferation was significantly reduced to 76% of that in control cultures after 24 h. This antiproliferative effect sustained up to 4 days (Fig. 2).

Effect of GnRHα on the Activity of ERK and JNK. To determine whether GnRHα affects ERK activity, cell lysates were immunoprecipitated with anti-ERK antibody, and ERK activity was examined by assaying the incorporation of 32P into MBP, followed by SDS-PAGE and autoradiography (Fig. 3A). We found that GnRHα activated ERK. Moreover, it appeared that ERK activity in response to GnRHα was more than that induced by GnRH.

To determine whether JNK activity is affected by GnRHα, we used a GST-c-Jun(1–89) fusion protein bound to GSH-Sepharose beads to precipitate JNK activities from Caov-3 or αT3-1 cell lysates. The precipitated complex was subjected to Western blotting with antiphospho (Ser-63) c-Jun antibody. JNK activation was detected 5 min after the initiation of the GnRHα treatment, peaked by 30 min, and decreased over the next 24 h in αT3-1 cells, showing a profile similar to that in a previous report (54). However, GnRHα had no effect on JNK activity in Caov-3 cells (Fig. 3B) whereas the JNK activity was induced by the DNA-damaging agent cisplatin (Fig. 3C).

Prolonged Activation of ERK by GnRHα. Prolonged activation of ERK is often correlated with cell cycle progression. GnRHα played an antiproliferative role in Caov-3 cells that is different from that in pituitary cells, in which GnRHα induces the synthesis and release of the pituitary gonadotropin LH and FSH. We investigated whether this is due to the differences in the profile of ERK activation by GnRHα. Cells were treated with 1 μM GnRHα for the indicated times or with 10 nm EGF for 5 min as a positive control (Fig. 4A). EGF induced the ERK activation. A long-lasting (>24 h) activation of ERK was induced by GnRHα in Caov-3 cells, whereas GnRHα gave a transient (<30 min) activation of ERK in αT3-1 cells (Fig. 4B).

Gβγ-Mediated GnRHα-induced ERK Activation. It has been shown that GnRH induces interaction of the receptor with the heterotrimeric Gq protein that leads to elevation of Ca²⁺ and activation of phospholipases, PKC, and ERK in pituitary cells. We compared the mechanism of GnRH-induced ERK activation between Caov-3 and αT3-1 cells. To determine which type of G-protein couples to this receptor, we preincubated cells for 4 h with 100 ng/ml PTX, which inactivate Gi and Go-proteins, followed by incubation with 1 μM GnRH (Fig. 5A). Although PTX had no effect on GnRH-induced ERK activation in αT3-1 cells (Fig. 5A, Lane 6), PTX at 100 ng/ml completely blocked the GnRHα-induced ERK activation in Caov-3 cells (Fig. 5A, Lane 3). It was reported that the carboxyl terminus of the β-adrenergic receptor kinase, containing the Gβγ-binding domain, is a cellular Gβγ antagonist capable of specifically distinguishing Go- from Gβγ-mediated processes (84, 85). To examine the effect by the Gβγ subunit-sequester βARKct peptide on GnRHα-induced exogenous ERK activity, a Myc-tagged p42mapk expression plasmid was used to distinguish exogenous ERK from endogenous ERK. We transfected cells with pRK or pRK-βARK1 together with Myc-tagged p42mapk expression plasmid (pEXV-Erk2-tag) and after 72 h stimulated them with 1 μM GnRHα (Fig. 5B). Cell lysates were immunoprecipitated with antibody against Myc epitope and examined for the exogenous ERK activity by assaying the incorporation of 32P into MBP, and the level of phosphorylation was normalized by the amount of Myc-tagged p42mapk. Transfection of pRK-βARK1 attenuated the GnRHα-induced ERK activation in Caov-3 cells (Fig. 5B, Lane 4) but not in αT3-1 cells (Fig. 5B, Lane 8). These results suggest that GnRHα-induced ERK activation is mediated by Gβγ in Caov-3 cells.

Effect of PKC on GnRHα-induced ERK Activation. Activation of ERK by GnRHα requires PKC in αT3-1 cells. Therefore, the role of PKC in GnRH-induced ERK activation was examined in Caov-3 cells (Fig. 6). Exposure of Caov-3 or αT3-1 cells to PMA caused a stimulation of ERK activity (Fig. 6, Lane 1). However, the ability of PMA to induce the activation of ERK does not necessarily mean that the PKC pathway is involved in the ERK signaling pathway, as in the case of norepinephrine-induced ERK activation in adipocytes (86) and GT-1 GnRH neuronal cell lines (87). Whether PKC is indeed involved in GnRH signaling was determined using PKC depletion. Pretreatment by 1 μM PMA for 16 h, which depletes most PKC isozymes, attenuated the PMA-induced ERK activation in both Caov-3 and αT3-1 cells (Fig. 6, Lane 4). Although there was a significant inhibition of the GnRH-induced ERK activity in αT3-1 cells (Fig. 6B,
Fig. 3. Effect of GnRHa on the activity of ERK (A) and JNK (B and C). A, Caov-3 cells grown in 100-mm dishes were treated with 1 μM GnRH (Lane 2) or 1 μM GnRHa (Lane 3) for 30 min. Lane 1, control (C). Lysates of cells were subsequently immunoprecipitated with anti-ERK antiserum, and the immunoprecipitates were incubated with MBP in the presence of [γ-32P]ATP, as described in “Materials and Methods”. After the reactions were stopped with Laemmli sample buffer, samples were subjected to 15% SDS-PAGE and autoradiography. Experiments were repeated three times with essentially identical results. B, Caov-3 cells (Lanes 1–5) and αT3-1 cells (Lanes 6–10), grown in 100-mm dishes, were treated with 1 μM GnRHa for the indicated times (Lanes 2–5 and 7–10). C, Caov-3 cells, grown in 100-mm dishes, were treated with 1000 μM cisplatin for 3 h (Lane 2). Lysates of cells were subsequently incubated with the GST-c-Jun fusion protein/GSH-Sepharose beads followed by 10% SDS-PAGE and immunoblotting with antiphospho (Ser-63) c-Jun antibody, as described in “Materials and Methods”. Experiments were repeated three times with essentially identical results.

Lane 5), it was not observed in Caov-3 cells under PKC depleted conditions (Fig. 6A, Lane 5). These results suggest that activation of ERK by GnRHa in Caov-3 cells is not mediated by PMA-sensitive PKC.

Effect of Extracellular Ca2+ on GnRHa-induced ERK Activation. Because it was reported that GnRHa-induced ERK activation also depends on Ca2+ influx (49), we evaluated the effect of Ca2+ influx on GnRHa-induced ERK activation in Caov-3 cells (Fig. 7). Elimination of extracellular Ca2+ by EGTA completely blocked the GnRHa-induced ERK activation in αT3-1 cells (Fig. 7B), whereas the inhibition was not observed in Caov-3 cells (Fig. 7A).

GnRHa Stimulation of Sos and Shc Phosphorylation. Receptor tyrosine kinase-mediated mitogenic signaling involves a series of SH2- and SH3-dependent protein-protein interactions among tyrosine-phosphorylated receptor, Shc, Grb2, and Sos, resulting in p21ras- and p74raf-1-dependent ERK activation (88). To examine the effect of GnRHa on Sos phosphorylation, cells were treated with 1 μM GnRHa for the indicated times or 10 mM EGF for 5 min (positive control; Fig. 8A). GnRH (Fig. 8A, Lanes 3–8) and EGF (Fig. 8A, Lane 1) stimulation resulted in a significant retardation of mobility of Sos on SDS-PAGE, reflecting Sos phosphorylation. This occurred first within 1 min of stimulation and was maximal by 2.5 min, reaching a plateau at 30 min. Next, we examined the effects of GnRHa on Shc-Sos association. Cells were treated with 1 μM GnRHa for the indicated times (Fig. 8B). Cell lysates were immunoprecipitated with anti-Shc antibody, followed by immunoblot with anti-Sos antibody. The association of Sos with Shc was detected 5 min after the GnRHa treatment, peaked by 30 min, and decreased over the next 3 h.

We also measured tyrosine phosphorylation of Shc after stimulation with 1 μM GnRHa for the indicated times or EGF (Fig. 8C). Cell lysates were immunoprecipitated with anti-Shc antibody, followed by immunoblot with anti-phosphotyrosine antibody. Shc exists in three isoforms of 46, 52, and 66 kDa. All three Shc proteins were phosphorylated by EGF (Fig. 8C, Lane 1), whereas pp52Shc was clearly phosphorylated by GnRHa within 5 min of stimulation, and the phosphorylation was maximal by 15 min (Fig. 8C, Lanes 3–6). These results suggest that GnRHa stimulates the tyrosine phosphorylation of Shc and the subsequent association with Shc/Sos complex, leading to MEK-ERK activation, a cascade known to play an important role in regulation of cell proliferation.

GnRHa Stimulation of MEK Activity. ERK was phosphorylated and activated by an immediately upstream activating kinase, MEK. Caov-3 cells were treated with 1 μM GnRHa or EGF (positive control) for 5 min. Cell lysates were immunoprecipitated with anti-MEK antibody and assayed for MEK activity by examining the incorporation of [32P] into GST-ERK fusion protein (Fig. 9). Both GnRHa and EGF produced a marked increase in [32P] incorporation into GST-ERK fusion protein suggesting that GnRHa activated MEK.

Reverse of GnRHa-induced Growth Inhibition and pRB De-phosphorylation by PD98059. To examine the hypothesis that the growth inhibition of Caov-3 cells by GnRHa is the result of ERK cascade activation, PD98059, an inhibitor of MEK, was used in a MTS assay. This compound is relatively specific for MEK, with no inhibitory activity against a number of other serine/threonine and tyrosine kinases (81, 89, 90). PD98059 completely attenuated the
Fig. 5. Gp9 mediates GnRH-induced ERK activation. A, cells grown in 100-mm dishes were pretreated with (Lanes 3 and 6) or without (Lanes 2 and 5) 100 ng/ml Ptx for 24 h, followed by treatment with 1 μM GnRHa for 30 min (Lanes 2 and 3) or 5 min (Lanes 5 and 6). Lysates of cells were subsequently immunoprecipitated with an anti-ERK antiserum, and the immunoprecipitates were incubated with MBP in the presence of γ-32P[ATP, as described in “Materials and Methods”. After the reactions were stopped with Laemmli sample buffer, SDS-PAGE and autoradiography were performed. An autoradiogram of 32P-labeled MBP (ERK activity) in immunoprecipitates with antibody against the Myc epitope is shown in the bottom panel, with the density of the control bands set arbitrarily at 1.0. Values shown represent mean ± SE from three separate experiments. Significant differences are shown by asterisks: **, P < 0.01. B, cells were transfected with pRK (Lanes 1, 2, 5, and 6) or pRK-BARK1 (Lanes 3, 4, 7, and 8) together with Myc-tagged p42 mapk expression plasmid (pEXV-Erk2-tag) and, after 72 h, were stimulated with 1 μM Myc-tagged p42 mapk expression plasmid (pEXV-Erk2-tag) and, after 72 h, were stimulated with 1 μM GnRHa (Lanes 2, 4, 6, and 8) for 30 min (Lanes 2 and 4) or for 5 min (Lanes 6 and 8). An autoradiogram of 32P-labeled MBP (ERK activity) in immunoprecipitates with antibody against the Myc epitope is shown in the bottom panel. Relative densitometric units of the MBP bands are shown in the top panel, with the density of the control bands set arbitrarily at 1.0. Values shown represent the mean ± SE from three separate experiments. **, P < 0.01.

GnRHa-induced ERK activation (Fig. 10A). In the presence of PD98059, GnRHa lost most of its ability to induce growth inhibition (Fig. 10B).

To investigate whether the GnRHa-induced growth inhibitory effects could be the results from the cell-cycle arrest at the late G1 phase, we examined the effect of GnRHa on the phosphorylation of pRB, because this phenomenon is a crucial milestone for the cell to advance into the S phase. Hyperphosphorylation of pRB allows transcription factors of the E2F family to become active and triggers the expression of genes that enable cell cycle progression in the absence of growth factor (91). Stimulation of the cells for 16 h with 1% FBS yields a significant retardation of mobility of pRB (Fig. 11, Lane 2). The addition of PMA, which is known to inhibit the transition from G1 to S phase in a variety of cell species (92, 93), completely attenuated the induction of hyperphosphorylated form of pRB by 1% FBS (Fig. 11, Lane 1). Similarly, the addition of GnRHa to 1% FBS leads to an apparent repression of hyperphosphorylated form of pRB, indicating hypophosphorylation of pRB (Fig. 11, Lane 3). In the presence of PD98059, however, GnRHa was not able to inhibit the induction of pRB phosphorylation by 1% FBS (Fig. 11, Lane 4). These results demonstrate that the ERK cascade is involved in signals leading to hypophosphorylation of pRB and growth inhibition in cells stimulated with GnRHa.

**DISCUSSION**

The presented results show that GnRHa caused ERK activation and that ERK activation was needed for GnRHa to ultimately induce the hypophosphorylation of pRB and growth inhibition in Caov-3 cells that expressed the GnRH receptor. Treatment of cells with GnRHa caused prolonged ERK activation up to 24 h. Suppression of ERK activation by PD98059, which binds MEK, blocked GnRHa-induced growth inhibition as well as hypophosphorylation of pRB.

The present results motivate the question of how GnRHa converted the outcome of ERK signaling from mitogenesis to growth inhibition. ERK activation was sustained in Caov-3 cells, whereas it was rapid and transient in αT3-1 cells (Fig. 4), as was reported previously (48, 51, 156, 244, 245, 318). The present results are consistent with the outcome of ERK signaling from mitogenesis to growth inhibition. The hypophosphorylation of pRB and growth inhibition were observed in Caov-3 cells treated with GnRHa and PD98059, whereas these effects were not observed in αT3-1 cells treated with GnRHa and PD98059. These results indicate that the ERK cascade is involved in signals leading to hypophosphorylation of pRB and growth inhibition in cells stimulated with GnRHa.
The potential ability of signal duration to affect growth factor signal outcome has been suggested in different contexts (46, 59, 94). For example, sustained activation of ERK has been associated with cell differentiation and growth arrest by nerve growth factor in PC12 cells, whereas transient activation of ERK by EGF leads to cell proliferation rather than differentiation. The duration of ERK activity seems important in determining cell cycle progression. Thus, there is a possibility that prolonged activation of ERK by GnRHa might mediate the mechanism to inhibit cell growth and to slow the cell cycle in Caov-3 cells, consistent with the findings of Thompson (95). Transition of cells from G1 to S phase in the cell cycle is controlled by pRB activity (96). In G1, pRB is hypophosphorylated and exerts its antiproliferative function. Hyperphosphorylation of pRB by cyclin D-Cdk4 and cyclin E-Cdk2 complexes inactivates it at the G1-S transition, allowing the cells to proceed into S phase (69, 70, 97–99). GnRHa induced the dephosphorylation of pRB, suggesting that GnRHa-induced growth inhibition of Caov-3 cells might be due to G1 arrest. Both GnRHa and PMA induced the ERK activation (Fig. 6) and PD98059 attenuated both GnRHa-induced (Fig. 10A) and PMA-induced (data not shown) ERK activation. Moreover, PD98059 reversed the dephosphorylation of pRB induced by GnRHa (Fig. 11).

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and PMA (data not shown). These results suggested that GnRHa activates the ERK cascade, which in turn leads to dephosphorylation of pRB, followed by G1 arrest.

Distinct pathways of Gi- and Gq-mediated ERK activation were reported (78). In the case of Gi-coupled receptors, such as thrombin (100), oxytocin (41), prostaglandin F2α (42), and endothelin-1 (101), activation by these appears to be PTX sensitive and PKC independent. In addition, Gi-mediated ERK activation is initiated by phosphatidylinositol 3-kinase activity followed by a pathway common to tyrosine-kinase receptors (102). This involves a series of SH2- and SH3-dependent protein-protein interactions among tyrosine-phosphorylated receptors, Shc, Grb2, and Sos, resulting in a Ras-dependent activation of Ras, ERK kinase kinase (which may be Raf-1), MEK kinase, and tyrosine residues of the enzyme as a result of successive stimulation of Ras, ERK kinase kinase (which may be Raf-1), MEK kinase or an alternative kinase, and MEK (44, 45). PKC is also an important serine/threonine kinase in GnRH postreceptor signaling (34–36). PKCα activates Raf-1 by direct phosphorylation (104). We, therefore, studied which pathway mediates the GnRHa-induced ERK activation in Caov-3 cells. Down-regulation of PKC by a prolonged incubation with PMA attenuated the stimulation of ERK activity by GnRHa in Caov-3 cells. Moreover, like oxytocin (41), prostaglandin F2α (42), and ET-1 (101), GnRHa stimulated the phosphorylation of Sos, the ras nucleotide exchange factor (Fig. 9). Thus, GnRHa stimulation of ERK activity in Caov-3 cells is likely to be mediated by Gi or Go-Sos, not by Gq-PKC.

Binding of GnRH to its membranal receptor elicits a series of signaling pathways (34–36), including activation of the ERK cascade (47–49). However, the ERK cascade is not the only route by which GnRH communicates with the nucleus. Although it is reported that GnRH induces JNK activation in αT3-1 cells (54), GnRHa failed to induce JNK activation in Caov-3 cells (Fig. 3), indicating the addi-

![Fig. 10. Effect of PD98059 on the activity of ERK (A) and on the GnRHa-induced growth inhibition (B). A, cells, grown in 100-mm dishes, were pretreated with (Lane 3) or without (Lane 2) 20 μM PD98059 for 30 min and then treated with 1 μM GnRHa for 30 min. Lysates of cells were subsequently immunoprecipitated with anti-ERK antisera, and the immunoprecipitates were incubated with MBP in the presence of \( \gamma^{-32P} \)ATP, as described in “Materials and Methods”. After the reactions were stopped with Laemlli sample buffer, samples were subjected to SDS-PAGE and autoradiography. Experiments were repeated three times with essentially identical results. B, cells were daily pretreated with (○) or without (□) 20 μM PD98059 for 30 min, and then GnRHa (4 μl of a 2.5 × 10^{-7} M solution in DDW) was added to the cultures, followed by analysis of cell proliferation by MTS assay. The cell number is expressed as a percentage of the respective controls (100%). Data points, means ± SE of three independent experiments performed in quadruplicate in three different passages of the cell lines. **, \( P < 0.01 \).

![Fig. 11. Effect of PD98059 on pRB phosphorylation. Cells, grown in 100-mm dishes, were pretreated with (Lane 4) or without (Lanes 1–3) 20 μM PD98059 for 30 min and then treated with 1 μM PMA for 16 h (Lane 1) or with 1 μM GnRHa for 24 h (Lanes 3 and 4) in DMEM containing 1% FBS. Lysates of cells were subsequently immunoprecipitated with anti-pRB antisera, and the immunoprecipitates were subjected to SDS-PAGE, followed by immunoblotting with anti-pRB antisera. An autoradiogram is shown in the bottom panel. Relative densitometric units of the phosphorylated pRB (ppRB) bands are shown in the top panel, with the density of the control bands set arbitrarily at 1.0. Values shown represent mean ± SE from three separate experiments. **, \( P < 0.01 \).
tional proof that post-GnRH receptor signaling cascade is different between Caov-3 cells and αT3-1 cells. We confirmed that JNK activation was induced by the DNA-damaging agent cisplatin in Caov-3 cells (Fig. 3C).

Why is the mechanism of GnRHa-induced ERK activation in Caov-3 cells different from that of GnRHa-induced ERK activation in αT3-1 cells? In the case of endothelin, its receptor couples with the Gi or Go families of PTX-sensitive G-proteins in the rat pulmonary uterine myometrial (101), the mesangial (105) cells, and rat ventricular myocytes (106), where PTX suppresses ET-1-induced ERK activation, inositol phosphate production, and positive ionotropic effect, respectively. However, contradictory findings have also been reported in rat myometrial tissue (107) and cultured vascular smooth muscle cells (108), showing the PTX-insensitive coupling of ET-1 to phospholipase C. Our data also suggest that PTX-sensitive G-protein stimulation by the GnRH receptor may participate in gonadotropin release or synthesis in αT3-1 cells. Thus, the postreceptor signaling cascade might be different depending on the cell, although the receptor is same.

In summary, we demonstrated here the activation of the ERK cascade in response to GnRHa that proceeded through She, SOS, and MEK. The ERK activation was mediated by Gβγ, not by PMA-sensitive PKC or by extracellular Ca2+ in Caov-3 cells. This cascade might have a role in GnRHa-induced antiproliferative effect. Furthermore, post-GnRH receptor signaling cascade in ovarian cancer cells seems to be different from that in pituitary cells.

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Role of Mitogen-activated Protein Kinase/Extracellular Signal-regulated Kinase Cascade in Gonadotropin-releasing Hormone-induced Growth Inhibition of a Human Ovarian Cancer Cell Line

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