Gonadotropin-Releasing Hormone (GnRH) Antagonists Promote Proapoptotic Signaling in Peripheral Reproductive Tumor Cells by Activating a \( \gamma \)-Coupling State of the Type I GnRH Receptor

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ABSTRACT

Gonadotropin-releasing hormone (GnRH) receptor agonists are extensively used in the treatment of sex hormone-dependent cancers via the desensitization of pituitary gonadotropes and consequent decrease in steroid sex hormone secretion. However, evidence now points to a direct inhibitory effect of GnRH analogs on cancer cells. These effects appear to be mediated via the \( \gamma \)-type \( G \) protein, in contrast to the predominant \( \alpha \)-type \( G \) protein in gonadotropes. Unlike \( \alpha \)-type \( G \) coupling, \( \gamma \)-type \( G \) coupling of the GnRH receptor can be activated by both agonists and antagonists. This unusual pharmacology suggested that the receptor involved in the cancer cells may not be the classical gonadotrope type I GnRH receptor. However, we have previously shown that a functional type II GnRH receptor is not present in man. In the present study, we show that GnRH agonists and selective GnRH antagonists exert potent antiproliferative effects on JEG-3 choriocarcinoma, benign prostate hyperplasia (BPH-1), and HEK293 cells stably expressing the type I GnRH receptor. This antiproliferative action occurs through a \( \gamma \)-mediated activation of stress-activated protein kinase pathways, resulting in caspase activation and transmembrane transfer of phosphatidylserine to the outer membrane envelope. Structurally related antagonistic GnRH analogs displayed divergent antiproliferative efficacies but demonstrated equal efficacies in inhibiting GnRH-induced \( \alpha \)-based signaling. Therefore, the ability of GnRH receptor antagonists to exert an antiproliferative effect on reproductive tumors may be dependent on ligand-selective activation of the \( \gamma \)-coupled form of the type I GnRH receptor.

INTRODUCTION

Gonadotropin-releasing hormone (GnRH) is the central regulator of the reproductive hormonal cascade and was first isolated from mammalian hypothalamus (1–3). This decapeptide is synthesized and released by hypothalamic secretory neurons and is delivered to the pituitary gland via the hypophyseal portal blood system. Interaction of the GnRH decapeptide with heptahelical GnRH receptors on pituitary gonadotrope cells induces the release of the pituitary gonadotropin hormones. GnRH has also been found in extrahypothalamic regions of the central nervous system (4) and in nonneuronal tissues such as placenta (5), ovary (6), mammary gland (7), and lymphoid cells (8). The heptahelical type I GnRH receptor is also expressed in these tissues (9). In addition, GnRH I ligand and the type I GnRH receptor are expressed in a number of malignant tumors and cell lines, including cancers of the breast, ovary, endometrium, and prostate (10). The specific function of GnRH I and its receptor in these extrapituitary sites is unclear. However, an autocrine/paracrine function has been suggested (11, 12). Linked to this hypothesis is the well-documented observation that direct application of GnRH analogs to peripheral reproductive tumor cells results in an attenuation of cellular proliferation and activation of cell death mechanisms (refs. 11–17; for review, see ref. 18).

The ability of both GnRH agonists and antagonists to inhibit tumor cell growth suggested that the effects may be mediated by a novel “type II” GnRH receptor, distinct from the cloned pituitary type I receptor at which GnRH agonists stimulate \( \alpha \)-and \( \gamma \)-coupling and the production of inositol trisphosphate and diacylglycerol that consequently mediate intracellular calcium and activate protein kinase C. This notion was reinforced by the observation that classical antagonist GnRH analogs induce antiproliferative actions on tumor cells that are mediated by \( \gamma \)-activation (for review, see refs. 19–22). Although a type II GnRH receptor has been cloned from some primates (23, 24), a functional type II GnRH receptor is not present in man (for reviews, see refs. 25 and 26). Moreover, the only functional GnRH receptor transcripts present in human peripheral tissues and tumor cells are identical to type I GnRH receptor sequence expressed in the pituitary (19, 27, 28). Thus, it appears that the difference in cellular milieu between the pituitary gonadotropes and the peripheral sites of type I GnRH receptor expression may be responsible for the differences in GnRH-mediated signaling. The distinctions in GnRH receptor-ligand pharmacology demonstrated in peripheral cells for the activation of \( \gamma \)-coupling, as opposed to \( \alpha \)-coupling, also suggest that the ligand stabilizes the receptor in a \( \gamma \)-coupling conformation that is different from the conformation mediating \( \alpha \)-coupling.

The principle of agonist-directed trafficking of receptor signaling [or “ligand-selective signaling,” as we prefer to term it (9, 29)] predicts that when a receptor signals through more than one independent signal transduction pathway, the relative efficacies/potencies of a series of analogs may differ for the respective pathways (30, 31). This hypothesis builds on the concept that a heptahelical rhodopsin-like \( G \)-protein-coupled receptor can exist in distinct states (or conformations) and that the ability of those states to activate different \( G \) protein types may differ. Recently, several examples of such ligand-selective signaling have been demonstrated in biogenic amine of \( G \) protein-coupled receptors (32–35). Because there appears to be a specific divergence with respect to the pharmacological profile between the peripheral sites of GnRH action and those in the pituitary, we set out to determine the relationship between the nature of GnRH analogs and their specific effects on differential signal activation.

In this study, we have demonstrated that analogs of GnRH that exert a classical antagonist action on cell systems in which the type I GnRH receptor stimulates \( \gamma \)-type mechanisms can differ in their ability to stabilize the specific \( \gamma \)-type \( G \) protein-coupling state of the type I GnRH receptor. Thus, we have identified some specific molecular properties of two structurally similar GnRH receptor peptide ligands that can determine the capacity of signaling through a specific class of downstream \( G \) protein-linked systems. Our discoveries are particularly pertinent in that the predominant current thera-

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pies for hormone-dependent cancers, such as prostate cancer, use GnRH analogs that inhibit sex hormone production. Although this therapy provides amelioration of the disease in the short term, this is often followed by aggressive recurrence in the form of sex hormone-independent cancers. Thus, the identification of GnRH analogs with direct effects on cancer cells offers the opportunity of targeting them in conjunction with, or independently from, hormone depletion.

**MATERIALS AND METHODS**

**Reagents.** The p38/c-Jun NH₂-terminal kinase (JNK) inhibitor SB203580 and the MAPK-kinase 1(2) (MEK1/2) inhibitor PD98059 were obtained from Calbiochem (La Jolla, CA) and prepared in dimethyl sulfoxide (DMSO; final DMSO concentration, 0.1% in cell treatments). Fluorescein isothiocyanate (FITC)-conjugated annexin V and anti-myc sera were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-cleaved and pro-caspase-3, anti-active extracellular signal-regulated kinase (ERK) 1/2, ERK5, JNK, and p38 sera were all obtained from New England Biolabs (Beverly, MA). Pertussis toxin (PTX) was obtained from Affiniti Research Products (Biomol, Plymouth Meeting, PA). GnRH I/II, lysophosphatidic acid (LPA), and forskolin (FSK) were obtained from Sigma (St. Louis, MO). Antagonists 135-18, 135-25, 6, and 21 were generously supplied by Roger Roeske (University of Indiana, Indianapolis, IN). The myc-tagged mitogen-activated protein kinase (MAPK) cDNA isofoms JNK and p38α were generously supplied by Eisuke Nishida (Kyoto University, Kyoto, Japan).

**Cell Culture and Transfection.** Human benign prostate hyperplasia (BPH-1), human JEG-3 choriocarcinoma (American Type Culture Collection, Manassas, VA), HEK293 cells stably expressing the type I GnRH receptor (designated as SCL60; ref. 36) and αT4-gonadotropes (obtained from Pamela Mellon, University of California at San Diego, San Diego, CA) stably expressing the marmoset type II GnRH receptor (designated as MELLON; University of California at San Diego, San Diego, CA) were maintained in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal bovine serum, 2% glutamine, and 1% penicillin (10,000 units/mL)/streptomycin (10,000 μg/mL) at 37°C in a humidified 5% CO₂ atmosphere. Where required, cells were serum-deprived by incubation for 16 hours in Dulbecco’s modified Eagle’s medium supplemented with only glutamine and penicillin/streptomycin. Ligands were applied to cells at 37°C for the time periods specified in figure legends. Chemical inhibitors were preincubated with the cells before agonist stimulation for the time periods specified in the figure legends. Transient transfections of JEG-3 or BPH-1 cells were performed using Superfect (Quagen, Valencia, CA) according to the manufacturer’s instructions.

**Immunoprecipitation and Immunoblotting.** After stimulation, cytoplasmic proteins were extracted as described previously (37). Proteins were resolved by SDS-PAGE for confirmation of plasmid expression or determination of intracellular protein activation by immunoblotting. Immunoprecipitation of myc epitope-tagged proteins was achieved by addition of 25 μL of a 30% slurry of anti-myc agarose preconjugated antibodies to the clarified cell lysate (Santa Cruz Biotechnology) with agitation for 16 hours at 4°C. Immunocomplexes were collected by centrifugation (10,000 × g, 10 minutes) and washed twice in ice-cold Nonidet P-40–based solubilization buffer (37) before addition of 25 μL of Laemmli sample buffer. Immunoprecipitates were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membrane (NEN Life Sciences, Boston, MA) for protein immunoblotting. Polyvinylidene difluoride membranes were blocked in a 4% bovine serum albumin, 50 mmol/L Tris-HCl (pH 7.0), 0.05% Tween 20, and 0.05% Nonidet P-40 blocking solution.

Immunoblotting of the active forms of ERK1/2, p38, and JNK was performed as described previously by Millar et al. (23). Phosphorylation of ERK1/2, JNK, p38, or ERK5 was detected with a 1:1,000 dilution of anti-phospho-specific ERK1/2, JNK, p38, or ERK5 rabbit polyclonal antibodies, respectively (New England Biolabs). The extent of MAPK activation was assessed and normalized by subsequently applying antisera (1:1,000 dilution) against the unphosphorylated forms of ERK2, JNK, or p38 (New England Biolabs) to primary antibody-stripped immunoblots. An alkaline phosphatase-conjugated IgG (Sigma) was used as a secondary antibody for anti-active ERK1/2/JNK/p38 and unphosphorylated ERK1/2/JNK/p38. Visualization of alkaline phosphatase-labeled proteins was performed using enzyme-linked chemiluminescence Amersham Pharmacia Biotech (Piscataway, NJ) and quantified using a Molecular Dynamics (Sunnyvale, CA) Storm 860 PhosphorImager.

**Cell Proliferation.** The proliferation of SCL60, BPH-1, and JEG-3 cells was measured by counting the number of viable trypan blue-excluding cells after 5 days of continuous GnRH receptor-interacting ligand exposure. Cells were plated at an initial minimal confluence (10–20%) to allow for 5 days of continual growth that would not result in 100% cell confluence by day 5. Cells were replenished with new ligand every 12 hours, and the number of viable cells compared with vehicle-treated control cells was measured. Annexin V-FITC staining was performed on cells treated for various time periods with GnRH by immersing live cells in starving media supplemented with a 1:100 dilution of annexin V-FITC for 30 minutes before fixing the cells in 100% ethanol (34). Viable cells were counted in triplicate at the end of the 5-day period. The data represent the mean ± SE of four experiments.

![Fig. 1. Antiproliferative effects of GnRH receptor-interacting ligands on human choriocarcinoma cells (JEG-3), human benign prostate hyperplastic cells (BPH-1), and type I GnRH receptor-expressing human embryonic kidney cells (SCL60). JEG-3 (A), BPH-1 (B), and SCL60 (C) cells were treated continuously for 5 days with the indicated doses of GnRH I (○), GnRH II (△), antagonist 135-18 (▼), or antagonist 135-25 (▲) added directly to the growth medium. Ligands were replenished every 24 hours. Viable cells were counted in triplicate at the end of the 5-day period. The data represent the mean ± SE of four experiments.](image-url)
methanol for 10 minutes at –20°C and mounting them in Permafluor (Immuno-techn, Marseilles, France). Annexin V-FITC–reactive cells were observed using a Zeiss LSM510 confocal scanning laser microscope. The GnRH-induced expression of cleaved and pro-caspase-3 was assessed by specific immunoblot. The expression of caspases was measured by immunoblotting for the specific cleaved or pro-caspase forms with rabbit polyclonal antisera (1:1,000 dilutions; New England Biolabs) as a secondary antibody. Proteins were detected by enzyme-linked chemifluorescence measured with a Molecular Dynamics Storm 860 PhosphorImager.

Phosphatidylinositol Hydrolysis. Inositol phosphate production was assayed as described previously by prelabeling cells with myo-[3H]inositol (Amersham Pharmacia Biotech) and measuring [3H]inositol phosphates after GnRH stimulation (23, 24).

Intracellular Cyclic AMP Measurement. Intracellular cyclic AMP (cAMP) concentration in BPH-1 or JEG-3 cells was measured using a proprietary fluorescent cAMP assay kit according to the manufacturer’s instructions (Biomol). GnRH pretreatments (for the time periods specified in the appropriate figure legends) were made before the standard 15-minute FSK application to the cells (1 μmol/L, JEG-3 cells; 3 μmol/L, BPH-1 cells).

RESULTS

GnRH and GnRH Analogs Mediate Antiproliferative Effects on JEG-3, BPH-1, and HEK293 Cells. Continuous treatment of JEG-3 human choriocarcinoma with GnRH I, GnRH II, and the antagonist 135-25 [Ac-α-Nal(2)-α-4-ClPhe-α-Pal-Leu-IsopropylLys-Pro-α-AlaNH2] resulted in a dose-response inhibition of cellular growth. Antagonist 135-18 [Ac-α-Nal(2)-α-4-ClPhe-α-Pal-Ser-Ile-α-IsopropylLys-Leu-IsopropylLys-Pro-α-AlaNH2], despite a single amino acid difference in position 5 of the decapeptide, failed to demonstrate an antiproliferative effect of a magnitude similar to that generated by antagonist 135-25, GnRH I, and GnRH II (Fig. 1A). The hyperplastic prostate cell line BPH-1 exhibited a similar profile of antiproliferative response to the same panel of GnRH analogs (Fig. 1B).

We further investigated the nature of the ligand-receptor specificity of the antiproliferative effects of GnRH receptor systems by studying the effects of the same panel of ligands on a model cell background, i.e., SCL60 cells. After continuous treatment of the SCL60 cells with GnRH I (Fig. 1C), there was a dramatic reduction in the growth of the cells and a substantial decrease in total cell number at the end of the experimental period. As with the JEG-3 and BPH-1 cells, GnRH II demonstrated a capacity similar to that of GnRH I when inhibiting cell growth. The two GnRH receptor antagonists behaved in a similar manner as in the JEG-3 and BPH-1 cells, in that antagonist 135-18 proved to be relatively ineffective at inhibiting the SCL60 cell proliferation, whereas antagonist 135-25 was nearly as effective as GnRH I and GnRH II. Compared with either JEG-3 or BPH-1 cells, there was considerably greater inhibition of cell growth and a greater degree of detectable cell death apparent from 48 hours onward of GnRH stimulation. We attribute this greater effect of the GnRH receptor ligands on SCL60 cells to the much greater level of receptor expression in these cells. Only minimal levels of cell surface receptor expression were noted in JEG-3 and BPH-1 cells (not in excess of 200 specific cpm for 125I-His5-α-Tyr6-GnRH I; data not shown), whereas as much as a 10- to 20-fold greater expression level was demonstrated in SCL60 cells.

Recent reports have suggested that the atypical GnRH receptor...
pharmacology observed in peripheral reproductive tumor lines is due to the expression of a type II human GnRH receptor, similar to that originally cloned by Millar et al. (23). However, the data above suggest that it is unlikely that the antiproliferative GnRH effects observed occur via a type II GnRH receptor because antagonist 135-18 possess a high degree of agonistic activity on type II GnRH receptors cloned from several species (23, 38). In the presence a type II receptor, antagonist 135-18 would be expected to exert a profound antiproliferative effect; however, it had the lowest effective antiproliferative capacity observed among the four GnRH analogs. Moreover, a full-length type II receptor cannot be transcribed from the human gene due to a frameshift and premature stop codon (39).

Antagonist 135-25 Demonstrates a Selective Type I Gonadotropin-Releasing Hormone Receptor Activation Profile. We have demonstrated that despite a high degree of similarity between antagonist 135-18 and antagonist 135-25, there is a significant difference in their capacity to stimulate certain forms of GnRH receptor activity, i.e., an antiproliferative action on both tumorous and hyperplastic cell lines. When compared in SCL60 cells expressing a type I GnRH receptor, both antagonists 135-18 and 135-25 demonstrate no agonistic activity (Fig. 2A and B). Both agents can also efficiently inhibit GnRH-mediated accumulation of inositol phosphates at the type I GnRH receptor (data not shown). When the two antagonists are compared in a cellular background solely expressing the marmoset type II GnRH receptor, i.e., αT4-II cells (23), antagonist 135-18 demonstrated considerable agonistic activity (Fig. 2C), whereas antagonist 135-25 showed no activity at all (Fig. 2D). If the antiproliferative actions of GnRH I or antagonist 135-25 are occurring via stimulation of a human Type II-like GnRH receptor.

Gonadotropin-Releasing Hormone Activates G<sub>i</sub>-Mediated Receptor Signaling Pathways in JEG-3 and BPH-1 Cells. It has been shown in several reports that the G protein coupling of GnRH receptors expressed in peripheral tumor tissues differs from that of the anterior pituitary. In the pituitary, the primary G protein coupling event of the stimulated GnRH receptor is via the G<sub>αq</sub>-type G protein. In contrast, in peripheral tissues, the primary GnRH receptor G protein coupling event appears to be via the PTX-sensitive G<sub>αi</sub>-type G protein pathway. We tested whether GnRH induced the activation of G<sub>αi</sub>-mediated signaling pathways in our experimental paradigms. FSK stimulation of JEG-3 (1 μmol/L) and BPH-1 (3 μmol/L) cells (inducing a 50% R<sub>max</sub> cAMP response in each case) was blunted with extended cellular pretreatment times (10–60 minutes) with either 100 nmol/L GnRH I or antagonist 135-25 [Fig. 3A (JEG-3) and B (BPH-1)]. In Fig. 3C (JEG-3) and D (BPH-1), the ability of the 60-minute pretreatment to attenuate FSK-induced cAMP accumulation is inhibited by preincubation of the cells with PTX (200 ng/mL, 16 hours). E (JEG-3) and F (BPH-1) demonstrate differential ability of GnRH I, antagonist 135-25, and antagonist 135-18 preincubation (100 nmol/L, 60 minutes) to attenuate FSK-induced cAMP accumulation. Each histogram in A–F depicts the mean ± SE of three to four experimental replicates of the cAMP accumulation assay.
GnRH I or antagonist 135-25 pretreatments to inhibit the FSK-mediated cAMP accumulation was attenuated by a 16-hour pretreatment with 200 ng/mL PTX. It therefore appears that both ligands can efficiently activate the adenylate cyclase inhibitory activity of G\(_\alpha_i\) in both cell models tested. However compared with antagonist 135-25 and GnRH I, antagonist 135-18 was significantly less able to inhibit FSK-induced cAMP accumulation in both JEG-3 (Fig. 3E) and BPH-1 cells (Fig. 3F).

The GnRH activation of MAPKs in peripheral tumor cells has been reported to be accompanied by a lack of inositol phosphate turnover. Our studies also demonstrated that there was no appreciable inositol phosphate turnover, even at high doses (0.1–1 \(\mu\)mol/L) of GnRH and its analogs. However, at even higher doses (10–50 \(\mu\)mol/L), GnRH I, GnRH II, and antagonist 135-25 all displayed a small capacity to induce inositol phosphate accumulation [Fig. 4A–D (JEG-3) and F–I (BPH-1)]. The level of doses required to accomplish this suggests that activation of inositol turnover was being mediated by \(G_{\beta\gamma}\) subunits of another G protein, e.g., G\(_{\alpha_i}\) rather than G\(_{\alpha_q}\) activation of phospholipase C-\(\beta\). In Fig. 4I (JEG-3) and J (BPH-1), we demonstrated that the minimal inositol phosphate turnover induced by GnRH was sensitive to pretreatment with PTX, whereas the more robust inositol phosphate turnover induced by LPA treatment (activating the endogenous G\(_{\alpha_q}\)-coupled endothelial differentiation gene receptor) was completely insensitive to the PTX. Thus it appears that there is negligible inositol phosphate turnover induced in peripheral tumor cells by submicromolar concentrations of GnRH. However, at much higher doses, there is a PTX-sensitive capacity to minimally stimulate inositol phosphate turnover, presumably via the \(G_{\beta\gamma}\)-mediated activation of phospholipase C-\(\beta\). Exposure of either JEG-3 or BPH-1 cells to 1 \(\mu\)mol/L LPA, potently stimulating ERK1/2 via the endogenous PTX-insensitive G\(_{\alpha_i}\) signaling pathway, failed to significantly attenuate cell growth. In contrast to GnRH receptor activation, an elevation in cell number after 5 days of continuous LPA treatment occurred (data not shown). Thus, it appears that the downstream effects of ERK-mediated pathways are not directly linked to the eventual anti-proliferative effects.

**Gonadotropin-Releasing Hormone Activates Stress-Activated Protein Kinase Pathways in JEG-3 and BPH-1 Cells.** Several groups have demonstrated that GnRH treatment of tumor cell lines induces a potent stimulation of the ERK isoforms of the MAPK family (40, 41). We observed a protracted activation of ERK1/2 in JEG-3 cells (Fig. 5A) and BPH-1 cells (Fig. 5B). We additionally assessed whether GnRH stimulation of either JEG-3 or BPH-1 cells resulted in significant activation of any other of the MAPK isoforms. We observed no GnRH-specific activation of ERK5/Big-MAPK isoform of MAPK (data not shown). However, there was a distinct GnRH-induced activation of JNK in JEG-3 cells and a GnRH-induced activation of p38 in BPH-1 cells. It therefore appeared that there was

![Fig. 4. Induction of inositol phosphate accumulation by GnRH receptor-interacting ligands.](image-url)
a degree of cell specificity of MAPK stimulation with GnRH. To further investigate these stress-activated protein kinase (SAPK) activation events, we transfected the tumor cell lines with myc-tagged JNK2 or p38 MAPK isoforms. GnRH caused a time-dependent and protracted activation of the immunoprecipitated JNK2 in JEG-3 cells (Fig. 5C), and p38 in BPH-1 cells (Fig. 5D) that was considerably delayed (30 minutes) in contrast to ERK activation (20 minutes). Recent data have demonstrated that an inhibitory effect on epidermal growth factor receptor activity may underlie the antiproliferative action of GnRH analogs (19). However no demonstrable GnRH-induced reduction in the phosphorylation status of the epidermal growth factor receptor in either JEG-3 or BPH-1 cells was observed (data not shown).

We have demonstrated that continuous stimulation with either GnRH I or antagonist 135-25 retarded the growth of both JEG-3 and BPH-1 cells (Fig. 1). However, when these experiments were performed using antagonist 135-18, which differs from antagonist 135-25 by only one amino acid, the antiproliferative effect was minimal (Fig. 1). We investigated whether this phenomenon of low antiproliferative potency of antagonist 135-18 resided in its capacity (or incapacity) to activate the Goi-SAPK pathways. GnRH I, GnRH II, and antagonist 135-25 all activated ERK1/2 to a substantially greater extent than antagonist 135-18 (all 1 μM, 10 minutes) in JEG-3 cells (Fig. 6A) and BPH-1 cells (Fig. 6B). When tested for its capacity to activate either JNK2 in JEG-3 cells or p38α in BPH-1 cells, antagonist 135-18 demonstrated a dramatically lower efficacy than GnRH I or antagonist 135-25 with respect to the activation of SAPK isoforms [Fig. 6C (JEG-3) and D (BPH-1)].

By using transfected myc-JNK in JEG-3 cells or myc-p38α in BPH-1 cells, we investigated whether the previously demonstrated GnRH/antagonist 135-25-induced SAPK activation was mediated through a Goi-dependent mechanism. As demonstrated in Fig. 7, treatment with either GnRH I (100 nmol/L, 40 minutes) or antagonist 135-25 (100 nmol/L, 40 minutes) led to JNK or p38α activation in JEG-3 (Fig. 7A) or BPH-1 cells (Fig. 7D), respectively, which was inhibited by PTX. This suggests that both ligands activate SAPKs through Goi-type G protein pathways (JEG-3, Fig. 7B and C; BPH-1, Fig. 7E and F).

Antagonist 135-25 but not Antagonist 135-18 Potently Stimulates the Activation of Goi–Stress-Activated Protein Kinase Pathways. When we screened a panel of classical GnRH receptor Goi antagonists for their ability to stimulate the SAPK pathway and inhibit BPH-1 cell growth, we noted that their ability to stimulate p38 activation (Fig. 8A) was related to their capacity to inhibit BPH-1 cell proliferation (Fig. 8B). It therefore appears that antagonist 135-25, like GnRH I, can adequately activate the Goi-type pathway in JEG-3 or BPH-1 cells, whereas the chemically related antagonist 135-18 has a much lower potency with respect to this form of atypical GnRH receptor activation. We suggest that this inability of antagonist 135-18 (and other antagonists) to induce a productive coupling between the GnRH receptor and the...
Go_1-type G protein pathway in these model cells may account for its poor antiproliferative efficacy.

**Gonadotropin-Releasing Hormone Induces the Generation of Proapoptotic States in JEG-3 and BPH-1 Cells.** To determine whether GnRH induced apoptosis, we measured the effects of GnRH on the structural integrity of the cells’ plasma membrane. A well-documented early event in apoptosis is the reversal in polarity of plasma membrane constituents such as phosphatidylserine (PS) [42]. Using FITC-conjugated annexin V protein, which has a high affinity for exposed PS, we tested whether exposure of JEG-3 or BPH-1 cells to GnRH resulted in the expression of PS on the membrane outer envelope. After 24 hours of subculture of JEG-3 cells in the absence or presence of GnRH (100 nmol/L), no annexin FITC binding could be demonstrated (Fig. 9A, 1–6), but after 48 hours of GnRH exposure, a considerable amount of external membrane annexin V-reactive PS was evident (Fig. 9A, 7–12). Cells not exposed to GnRH failed to exhibit any external membrane annexin V-FITC staining even after 48 hours (Fig. 9A, 13–15). Similar results were obtained from BPH-1 cells for the same period of GnRH I stimulation (Fig. 9A, 16–20). In addition to the generation of early plasma membrane PS reversal, we demonstrated the generation of proapoptotic caspase enzymes that have been shown to be involved in cell degradation in many tissues (for review, see ref. 43). In JEG-3 cells continuously exposed to GnRH I (100 nmol/L, 24–96 hours), there was a substantial elevation in the cellular levels of pro-caspase 3 (Fig. 9B and D). GnRH I elevation of active caspase-3 levels took longer to emerge and was only substantially evident between 48 and 72 hours of incubation (Fig. 9B and C). A similar pattern of time-dependent increases in pro-caspase and cleaved caspase-3 was evident in BPH-1 cells after GnRH I treatment (Fig. 9E–G).

**Gonadotropin-Releasing Hormone-Induced Activation of Stress-Activated Protein Kinase Pathways Is Involved in the Induction of a Proapoptotic State.** We investigated whether there was a connection between the capacity of GnRH to activate the SAPK pathways and the observed generation of the early signs of apoptosis, e.g., the PS transfer from the internal face of the plasma membrane envelope to the external face. To this end, we used the SAPK inhibitor SB203580, which at low doses (1 μmol/L) acts as a potent inhibitor of p38 SAPK activity and at higher doses (20 μmol/L) exerts an additional inhibitory activity on the JNK family of SAPK proteins [44]. Coincubation of JEG-3 cells with 20 μmol/L SB203580 and GnRH I (100 nmol/L) for 48 hours resulted in a...
significant reduction in the degree of annexin V-FITC staining of the external aspect of the plasma membrane (Fig. 10A, compare 5 with 8). There was no significant difference in the general growth patterns and gross morphology of the cells treated with SB203580 compared with those treated with GnRH alone or unstimulated cells (data not shown). Similarly in BPH-1 cells treated with SB203580 (1 μmol/L) for 48 hours, the GnRH-mediated induction of annexin V-FITC reactivity on the outer plasma membrane envelope was almost completely abolished (Fig. 10B, 8). As in the JEG-3 cell experiment, there was no significant observable change in cell morphology or growth rates in either the SB203580-treated cells or the DMSO vehicle-treated cells. In addition, we demonstrated that the GnRH-induced generation of annexin V-FITC–reactive cells was unaffected by continuous treatment with 10 μmol/L PD98059 (an inhibitor of MEK1/2), which completely inhibited the capacity of all of the GnRH analogs to activate ERK1/2 in both cell types (data not shown). Thus, it appears that inhibition of the GnRH-induced SAPK pathways in JEG-3 cells and BPH-1 cells can attenuate the capacity of GnRH to induce apoptotic signs in these two cell lines. Because SB203580 is not highly specific, confirmatory studies involving the generation of stable JEG-3 and BPH-1 cell lines expressing dominant negative JNK and p38 or small interfering RNAs are needed.

**DISCUSSION**

In this study we have demonstrated that some classical pituitary, \( \text{GnRH}_q \) signal-inhibiting, GnRH receptor antagonists can act in the same...
manner as classical agonists in inhibiting tumor cell proliferation. The ability of these antagonists to exert antiproliferative effects on tumor cells is related to the altered pharmacological profile of GnRH receptor signaling in these cells. The differences in efficacy between antagonist 135-25 and antagonist 135-18 with respect to their antiproliferative action appear to be due to the relative abilities of the two peptides to stabilize an active form of the receptor that is capable of coupling productively to $G_{\alpha_i}$. Therefore, the designation of antagonist 135-25 as a GnRH receptor "antagonist" is somewhat spurious because this only describes its ability to stabilize the $G_{\alpha_i}$-preferring form/state of the type I GnRH receptor, which is the predominant signaling mechanism in pituitary gonadotrophs. Hence we have demonstrated that antagonist 135-25 exerts a potent antiproliferative action in JEG-3 and BPH-1 cells due to its ability to activate a $G_{\alpha_i}$-coupling form of the type I GnRH receptor while being unable to induce GnRH receptor $G_{\alpha_q}$ coupling. We have demonstrated that the two endogenous forms of GnRH (GnRH I and GnRH II) and also some classical antagonists can attenuate cell growth (Fig. 1) and that this antiproliferative effect is coincident with the induction of a proapoptotic state of the cells, as demonstrated physically by PS membrane translocation and the activation of proteolytic caspases (Figs. 8 and 9). Activation of the GnRH receptor appears to mediate these effects via a potent stimulation of members of the SAPK pathway, i.e., JNK and p38 (Figs. 5 and 6), through a $G_{\alpha_i}$-dependent mechanism (Figs. 3, 4, and 9). Thus, certain GnRH analogs are able to stabilize a specific active conformation of the GnRH receptor that will potently convey an antiproliferative effect in peripheral tissues, but not in the pituitary. Whereas the studies here have focused on the apoptotic pathway associated with the $G_{\alpha_i}$-mediated antiproliferative effects of GnRHs and analogs, we have also shown an inhibition of thymidine incorporation and cell cycle arrest (data not shown) as has been described previously (45). The relative contributions of these mechanisms to the net antiproliferative effects of GnRH and analogs have not been determined.

Initial hypotheses concerning the nature of the divergence in signaling between GnRH-responsive sites in the pituitary and those in...
A similar experimental approach was used for BPH-1 cells. As with A, SB203580 cotreatment with GnRH I abolished the GnRH-induced generation of annexin V-FITC reactivity (compare 4–6 with 7–9).

Peripheral reproductive tissues suggested that the receptor present in the peripheral sites was different from that in the gonadotrope. Recent evidence, however, has suggested that the GnRH receptors present at these two sites are indeed the same, despite their different signaling behavior (46–48). A distinction between GnRH signaling in peripheral compared with pituitary sites also extended to the effects of GnRH agonist and antagonist analogs. Hence, proliferation of both endometrial and ovarian cancer cells can be inhibited by both agonistic and certain antagonistic analogs of GnRH (10). A solution to this problem was proposed by Imai et al. (21), who speculated that Goq coupling of the GnRH receptor to its effectors may be responsible for the differences in GnRH agonist/antagonist response between peripheral tumors and the anterior pituitary. Our findings support this conclusion. Interestingly, we have shown that a functional LPA-mediated Goq-coupling activity is extant in these tumor cells; thus, a pathophysiologic loss of Goq protein cannot explain the paradoxical change in GnRH receptor signaling. In the present study, we observed negligible GnRH-mediated activation of Goq in JEG-3 and BPH-1 cells. However, other reports have demonstrated that in other reproductive tumor lines, such as Ishikawa cells, GnRH can induce coupling to Goq (19). In the present study, Goq signaling was clearly not involved in the antiproliferative effect of GnRH because LPA-mediated activation of Goq failed to inhibit cell growth. Thus, it is probable that the functional signaling complexes associated with the GnRH receptors in peripheral tumor sites are able to cohere the receptor into specific Gq coupling and that such specific complexes are not present in gonadotropes because LBT2 cell proliferation was not inhibited by continuous GnRH exposure (data not shown). Whatever the nature of the protein intermediates responsible for this shift in functionality, it is clear that additional receptor-interacting factors can dramatically alter the way in which a given ligand can direct its signal to the intracellular environment and eventually induce distinct physiologic end points. This cell environment-specific differential coupling of the receptor therefore necessitates a reevaluation of terminology with respect to the nature of ligand interaction with the GnRH receptor at these peripheral tumor sites. Thus we have shown that whereas antagonist 135-25 can be adequately described as an antagonist at the anterior pituitary level with respect to GnRH-induced activation of the Goq-based signaling mechanisms, it behaves as an agonist in the peripheral cells because it is almost equally as effective as GnRH in stimulating the endogenous Gq-coupled type I GnRH receptors to inhibit cell growth. In addition, we have demonstrated that a separation between these two effects at the periphery and the pituitary can be engineered by alteration of the primary sequence of the GnRH peptide ligand. Therefore substitution of the single amino acid in Ile in antagonist 135-18 to 1-MePal in antagonist 125-25 resulted in a dramatic elevation of potency at the Gq-coupled peripheral GnRH receptor but did not change its ability to functionally inhibit the action of GnRH at the pituitary Gq-coupled receptor.

Both agonist and antagonistic GnRH analogs are now widely used as therapeutics in gynecology, reproductive medicine, and oncology. The mechanisms of action of the majority of these therapeutics are based on a continuous treatment regime, causing an anterior pituitary loss of sensitivity to endogenous GnRH. This causes a reduction in gonadotropin secretion, leading to a diminution of circulating sex steroids. Classical antagonistic GnRH receptor ligands have an advantage over GnRH agonistic peptides due to the fact that they inhibit the secretion of gonadotropins and reduce sex steroids immediately after first application, achieving more rapid therapeutic effects than GnRH agonists (49). The repeated exposure to agonistic agents is required to induce a functional desensitization of the anterior pituitary gonadotrope. These agonists initially stimulate the reproductive system, followed by functional desensitization, which takes days to weeks to occur. For conditions such as prostate cancer, GnRH classical antagonist molecules are therefore preferable to agonists because they avoid the so-called “flare” of the disorder that occurs in approximately 10% to 20% of patients when agonists are given as single agents (50). Preexisting antagonistic therapies for reproductive tumors involve the administration of cetrorelix, which has been demonstrated in some circumstances to attenuate the growth of androgen-dependent prostate cancers (51, 52). Interestingly, higher doses of cetrorelix have been reported to attenuate the growth of androgen-resistant tumor cells (53, 54), implicating a direct antiproliferative effect, which requires a higher dose than that required to inhibit anterior pituitary.

\[ \text{GnRH RECEPTOR-MEDIATED INHIBITION OF PROLIFERATION} \]

\[ \text{Annexin V-FITC} \]

\[ \text{merge} \]

\[ \text{GnRH RECEPTOR-MEDIATED INHIBITION OF PROLIFERATION} \]

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\[ \text{Annexin V-FITC} \]

\[ \text{merge} \]
gonadotropin release, for the classical mode of action of depriving the androgen-sensitive tumor of steroid. Although deprivation of androgen by GnRH analogs is generally beneficial to patients with androgen-dependent prostatic cancer, the tumors can often “escape” and return as aggressive androgen-independent forms. Thus, there would appear to be value in therapies involving GnRH analogs with direct antiproliferative effects. In our hands, cetrorelix was significantly less potent than antagonist 135-25 when we compared their ability to stimulate PTX-sensitive/Gqα-dependent MAPK isofrome activation (Fig. 8). In addition, it appears that cetrorelix may not possess a particularly profound antiproliferative effect on all reproductive tumors expressing GnRH receptors, e.g., the antiproliferative effect of triptorelin (GnRH agonist) on LNCaP prostate cells was actually inhibited in the presence of cetrorelix (55). Thus, it is possible that cetrorelix, which possesses a significantly lower antiproliferative potency than triptorelin, acted as a functional antagonist of the agonist action. In additional experimental paradigms, other classical GnRH antagonists, e.g., antide, have been shown to functionally inhibit the antiproliferative effects of classical GnRH receptor agonists (56).

Thus, it is possible that the majority of existing GnRH antagonist therapeutic potencies may not have a significantly potent direct antitumor effect, which would be desirable for steroid-resistant neoplasms. Their poor potency may be due to their poor ability to stabilize/induce the Gqα-prefering conformation/state of the type I GnRH receptor. We have therefore shown that antagonists can be identified that have enhanced direct antiproliferative activity via their ability to potently activate the Gqα-type of GnRH receptor signaling seen in peripheral reproductive tumors (Figs. 3 and 7). An agent such as antagonist 135-25 would theoretically display several properties making it superior to current GnRH-based peptide treatment of reproductive neoplasms: Firstly, because it is not a pituitary agonist, there is no initial disease flare (49), whereas its inhibitory action at the pituitary will decrease serum levels of sex steroids, thereby attenuating steroid-dependent prostatic cancer, the tumors can often ‘escape’ and return as aggressive androgen-independent forms. Thus, there would appear to be value in therapies involving GnRH analogs with direct antiproliferative effects. In our hands, cetrorelix was significantly less potent than antagonist 135-25 when we compared their ability to stimulate PTX-sensitive/Gqα-dependent MAPK isofrome activation (Fig. 8). In addition, it appears that cetrorelix may not possess a particularly profound antiproliferative effect on all reproductive tumors expressing GnRH receptors, e.g., the antiproliferative effect of triptorelin (GnRH agonist) on LNCaP prostate cells was actually inhibited in the presence of cetrorelix (55). Thus, it is possible that cetrorelix, which possesses a significantly lower antiproliferative potency than triptorelin, acted as a functional antagonist of the agonist action. In additional experimental paradigms, other classical GnRH antagonists, e.g., antide, have been shown to functionally inhibit the antiproliferative effects of classical GnRH receptor agonists (56).


Gonadotropin-Releasing Hormone (GnRH) Antagonists Promote Proapoptotic Signaling in Peripheral Reproductive Tumor Cells by Activating a $\alpha_i$-Coupling State of the Type I GnRH Receptor

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