Gonadotropin-releasing hormone (GnRH) agonist triptorelin inhibits estradiol-induced serum response element (SRE) activation and c-fos expression in human endometrial, ovarian and breast cancer cells

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Abstract

Background and methods: The majority of human endometrial (>80%), ovarian (>80%) and breast (>50%) cancers express GnRH receptors. Their spontaneous and epidermal growth-factor-induced proliferation is dose- and time-dependently reduced by treatment with GnRH and its agonists. In this study, we demonstrate that the GnRH agonist triptorelin inhibits estradiol (E2)-induced cancer cell proliferation.

Results: The proliferation of quiescent estrogen receptor α (ERα)/ERβ-positive, but not of ERα-negative/ERβ-positive endometrial, ovarian and breast cancer cell lines, was significantly stimulated (P < 0.001) (ANOVA) after treatment with E2 (10^{-8} M). This effect was time- and dose-dependently antagonized by simultaneous treatment with triptorelin. The inhibitory effect was maximal at 10^{-5} M concentration of triptorelin (P < 0.001). In addition, we could show that, in ERα/ERβ-positive cell lines, E2 induces activation of serum response element (SRE) and expression of the immediate early-response gene c-fos. These effects were blocked by triptorelin (P < 0.001). E2-induced activation of estrogen-response element (ERE) was not affected by triptorelin.

Conclusions: The transcriptional activation of SRE by E2 is due to ERα activation of the mitogen-activated protein kinase (MAPK) pathway. This pathway is impeded by GnRH, resulting in a reduction of E2-induced SRE activation and, in consequence, a reduction of E2-induced c-fos expression. This causes downregulation of E2-induced cancer cell proliferation.

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Introduction

The expression of gonadotropin-releasing hormone (GnRH) and its receptor as a part of a negative autocrine regulatory system of cell proliferation has been demonstrated in a number of human malignant tumors, including those of the endometrium, ovary and breast (1). Dose-dependent antiproliferative effects of GnRH agonists in cell lines derived from these tumors have been observed by various investigators (1). GnRH antagonists also have marked antiproliferative activity in most endometrial, ovarian and breast cancer cell lines tested, indicating that the dichotomy of GnRH agonists and antagonists might not apply to the GnRH system in cancer cells (1). The classical GnRH receptor signal-transduction mechanisms, known to operate in the pituitary, are not involved in the mediation of antiproliferative effects of GnRH analogs in cancer cells. The GnRH receptor instead interacts with the mitogenic signal transduction of growth-factor receptors and related oncogene products associated with tyrosine kinase activity via activation of a phosphotyrosine phosphatase (PTP), resulting in downregulation of the mitogen-activated protein kinase (MAPK) pathway and therefore in downregulation of cancer cell proliferation (2).

The c-fos proto-oncogene plays an important role in regulation of normal cell growth and differentiation, and cellular transformation processes (3–5). c-fos is an immediate early-response gene that is rapidly induced in response to diverse extracellular stimuli, including various growth factors and steroid hormones, during the early phases of recruitment of quiescent (G_{0}) cells in the cell cycle (3–5). Transcriptional regulation of c-fos is modulated, in part, by interactions of nuclear proteins with multiple cis-elements in the c-fos gene promoter (6–10). One of these cis-elements is serum response element (SRE), which mediates c-fos induction by growth factors, cytokines and other extracellular stimuli that activate MAPK pathways (6–10). Several studies have demonstrated that estrogen receptor α (ERα) mediates 17β-estradiol (E2)-activated expression of c-fos, which is induced as...
an immediate early-response gene in ERα-positive breast cancer cell lines (11–18). ERα activates SRE in MCF-7 breast cancer cells through MAPK-dependent phosphorylation of Elk-1 (19, 20). Duan et al. (19) have demonstrated that both E2 (ERα-dependent) and growth factors (ERα-independent) activate SRE in breast cancer cells via the Ras/MAPK pathway.

GnRH agonists antagonize epidermal growth factor (EGF)-induced proliferation and c-fos gene expression via Ras/MAPK signaling. We investigated whether or not the GnRH agonist triptorelin inhibits E2-induced SRE activation and c-fos expression in ERα-positive human endometrial, ovarian and breast cancer cells and reduces E2-induced cell proliferation.

Materials and methods

Cell lines and culture conditions

The human endometrial cancer cell lines KLE, HEC-1-A, Hec-1B and Ishikawa; the ovarian cancer cell lines EFO-21, OVCAR-3 and SK-OV-3; and the breast cancer cell lines MCF-7 and T-47-D were obtained from either the American Type Culture Collection (ATCC, Manassas, VT, USA) or the sources detailed previously (21). The cells were cultured at 37°C in a humidified atmosphere of 5% CO2 in air, as previously described (21). Except for the SK-OV-3 ovarian cancer cell line, all the above mentioned cancer cell lines express receptors for GnRH (22).

Hormones

E2 was purchased from Sigma (Deisenhofen, Germany). The GnRH agonist [d-Trp6]GnRH (triptorelin) was kindly provided by Ferring Pharmaceuticals (Copenhagen, Denmark).

Isolation of RNA and cDNA synthesis

Total RNA was prepared from cells grown in monolayer by the RNeasy protocol (Qiagen, Hilden, Germany). The concentration of RNA in each sample was determined by photospectroscopy. First-strand cDNA was generated by reverse transcription of 1 μg poly(A) mRNA, using p(dT)15 primers (Roche Diagnostics, Mannheim, Germany) with MMLV-reverse transcriptase, according to the instructions of the suppliers (Life Technologies, Karlsruhe, Germany). After determination of the concentrations of the cDNAs, the samples were used for PCR analysis. The integrity of the samples was tested by RT-PCR of the housekeeping gene GAPDH.

ERα and ERβ expression in human endometrial, ovarian and breast cancer cell lines

The cDNAs (2 ng) were amplified in a 50 μl reaction volume containing 10 mM Tris–HCl (pH 8.3), 50 mM potassium chloride, 1.5 mM magnesium chloride, 200 μM of each of the dNTPs, 1 μM primers specific for human ERα mRNA (forward primer: 5’ AAT TCA GAT AAT CGA CGC CAG 3’; backward primer: 5’ GTG TTT CAA CAT TCT CCC TGC TC 3’) and human ERβ mRNA (forward primer: 5’ TAG TGG TCC ATC GCC AGT TAT 3’; backward primer: 5’ GGG AGC CAC ACT TCA CCA T 3’), and 1.25 U AmpliTaq Gold polymerase (Applied Biosystems, Weiterstadt, Germany) in an Applied Biosystems DNA thermal cycler 9600. Thirty (ERα) or 33 (ERβ) cycles of amplification of PCR were carried out: denaturation at 95°C for 30 s and annealing at 56°C for 15 s, followed by extension at 72°C for 60 s. The PCR products were separated by gel electrophoresis in 1.5% agarose and visualized by ethidium bromide staining on an UV transilluminator.

Effects of triptorelin on E2-induced proliferation of endometrial, ovarian and breast cancer cells

The time-course and dose–response proliferation experiments were performed as described in detail previously (23, 24). Briefly, 20 000 cells of the above mentioned lines were plated in multiple four-well cluster dishes (Nunc, Roskilde, Denmark) in 1 ml standard medium, containing insulin, phenol red and 10% FCS, and allowed to attach to the dishes for 24 h. Then they were washed twice with medium, which contained 1 g/l of BSA (Sigma), but not insulin, phenol red or fetal calf serum (FCS) (serum/phenol red-free medium). Cells were then incubated in serum/phenol red-free medium in the absence or presence of E2 (10−8 M) with or without appropriate dilutions of triptorelin, resulting in final concentrations of 10−11 to 10−3 M. After 24-h incubation, the medium was changed. After 2, 4 and 6 days, the cells were counted in a Neubauer hemocytometer (23, 24).

Quantification of SRE induction

To assess the effects of triptorelin in E2-induced SRE activation, cells were transfected with pSRE-secreted alkaline phosphatase (SEAP) (see below). Subsequently, these cells were treated in the absence of FCS and phenol red with or without E2 (10−8 M) with or without 10−7 M triptorelin for 24 h, and they were cultured for an additional 72 h to observe SEAP expression. Every 24 h, 100 μl medium were collected and analyzed for SEAP activity.

pSRE-SEAP (Clontech, Palo Alto, CA, USA) is designed to monitor the induction of SRE and the MAPK signal transduction pathway. pSRE-SEAP contains the SEAP reporter gene. This vector also contains three tandem copies of the SRE consensus sequence fused to a TATA-like promoter (PTAL) region from the herpes simplex virus thymidine kinase (HSV-TK) promoter. After transcription factors bind SRE, transcription...
is induced, and the reporter gene is activated. pTK-SEAP (Clontech) was used as a negative control to determine the background signals associated with the culture medium. The enhancer-less pTK-SEAP contains HSV-TK upstream of the SEAP coding sequence. A total of 200,000 cells were grown for 24 h on 30 mm plates. Transfections were carried out with Superfect liposome reagents according to the manufacturer’s instructions (Qiagen), and cells were treated as described above. Chemiluminescence detection of SEAP activity was performed according to the manufacturer’s instructions (Clontech) with a plate fluorometer (Berthold, Bad Wildbach, Germany).

Quantification of ERE induction

To assess the effects of triptorelin in E2-induced ERE activation, cells were transfected with pERE-TA-SEAP (see below). Subsequently, these cells were treated in the absence of FCS and phenol red with or without E2 (10^{-8} M) with or without 10^{-7} M triptorelin for 24 h, and they were cultured for an additional 72 h to observe SEAP expression. Every 24 h, 100 μl medium were collected and analyzed for SEAP activity. pERE-TA-SEAP (Clontech) is designed to monitor the induction of ERE. pERE-TA-SEAP contains two copies of the ERE enhancer element, located upstream of the minimal TA promoter, the TATA box from the herpes simplex virus thymidine kinase promoter (P_{TA}). Located downstream of P_{TA} is the SEAP reporter gene. Upon binding of the activated ER to the cis-acting ERE enhancer element, transcription is induced and the reporter gene (SEAP) is activated. A total of 200,000 cells were grown for 24 h on 30 mm plates. Transfections were carried out with Superfect liposome reagents according to the manufacturer’s instructions (Qiagen), and cells were treated as described above. Chemiluminescence detection of SEAP activity was performed according to the manufacturer’s instructions (Clontech) with a plate fluorometer (Berthold).

Quantification of c-fos mRNA expression

For determination of E2-induced c-fos mRNA expression, the cells were cultured for 72 h in the absence of FCS and phenol red. The quiescent cells were incubated with E2 (10^{-8} M) for 20 min with or without a previous incubation (15 min) with 10^{-7} M triptorelin. c-fos mRNA expression was determined after 30 min by semiquantitative RT-PCR.

Semiquantitative RT-PCR of c-fos was carried out as described in detail previously (25). Briefly, a 161 bp internal standard was generated by PCR containing synthetic DNA and c-fos-specific primer sites. The PCR product amplified with the c-fos primers (forward primer, 5′-GAG ATT GCC AAC CTG CTG AA-3′; backward primer, 5′-AGA CGA AGG AAG AGC TGT AA-3′) has a total length of 483 bp. For determining the optimal concentration of internal standard used in semiquantitative PCR, internal standard and target cDNA were added to the PCR tubes in inverse serial dilutions. PCR products were separated by gel electrophoresis in 1.5% agarose. PCR reactions yielding standard and target signals of identical intensity were used for PCR analysis for determination of c-fos expression levels. The respective DNA products were run on 1.5% agarose gels, bands were visualized by ethidium bromide staining on an UV transilluminator. The bands were quantified by the Kodak 1D image system in comparison with basal c-fos expression levels.

Statistical analysis

All experiments were repeated three times with different passages of the respective cell lines. Data were tested for significant differences by the Mann–Whitney U-Test. The data from the dose–response experiments were tested for significant differences by one-way analysis of variance followed by the Student–Newman–Keuls test for comparison of individual groups, after the Bartlett test had shown that variances were homogeneous.

Results

ERα and ERβ expression in human endometrial, ovarian and breast cancer cell lines

Primers used for RT-PCR of ERα and ERβ were derived from the N-terminal and the hinge region, with highly characteristic sequence inhomology between ERα and ERβ. After 30 and 33 cycles, PCR products 344 and 392 bp in length for ERα and ERβ respectively were generated. The amount of PCR product for both ERα and ERβ increased exponentially for up to 40 cycles, and increased linearly as a function of the amount of total RNA used (data not shown). No PCR product was observed with a negative control RT reaction (H₂O) containing all agents except reverse transcriptase, thus showing that the amplified product was mRNA specific. GAPDH served as a housekeeping gene.

To assess the estrogen responsiveness of the human endometrial, ovarian and breast cancer cell lines used, we first characterized the cell lines for ERα and ERβ gene expression. Using RT-PCR, we could demonstrate that the human endometrial cancer cell lines KLE and Hec-1B; the human ovarian cancer cell lines EFO-21, OVCAR-3 and SK-OV-3; and the human breast cancer cell lines MCF-7 and T-47-D were ERα- and ERβ-positive, whereas the human endometrial cancer cell lines Hec-1A and Ishikawa were ERβ-positive but ERα-negative (Fig. 1).
The proliferation of the ERα- and ERβ-positive cancer cell lines KLE, Hec-1B, OVCAR-3, SK-OV-3, MCF-7 and T-47-D, cultured in the absence of serum and phenol red, was significantly stimulated (P < 0.001) after treatment with E2 (10^{-8} M) for 6 days. The cell numbers after 6 days of treatment with E2 (10^{-8} M), expressed as percentages of control cultures (without E2 = 100%), were 189±14% (KLE), 153±11% (Hec-1B), 175±17% (EFO-21), 272±22% (OVCAR-3), 256±29% (SK-OV-3), 241±23% (MCF-7) and 229±19% (T-47-D) respectively. The proliferative effect of E2 on the GnRH receptor-positive cancer cell lines KLE, Hec-1B, EFO-21, OVCAR-3, MCF-7 and T-47-D was time-dependently antagonized by simultaneous treatment with triptorelin (10^{-5} M).

Figure 2A shows the effects of E2 treatment and of E2/triptorelin treatment on the cell proliferation of the ovarian cancer cell line OVCAR-3. In the ovarian cancer cell line SK-OV-3, which does not express the GnRH receptor, triptorelin treatment had no antiproliferative effect on E2-induced cell proliferation. In the endometrial cancer cell lines Hec-1A and Ishikawa, which express ERβ but not ERα, E2 had no effect on cell proliferation.

The proliferative effect of E2 on day 6 was also dose-dependently antagonized by simultaneous treatment with triptorelin (10^{-11} to 10^{-5} M) in the ovarian cancer cell line OVCAR-3 (Fig. 2B). Similar dose-response relationships for the antiproliferative effects of triptorelin were observed for the GnRH receptor-positive cell lines KLE, Hec-1B, EFO-21, MCF-7 and T-47-D (not shown).

Effects of triptorelin on E2-induced activation of SRE

To examine whether SRE plays a role in E2-induced cell proliferation and in its reduction by triptorelin, we transiently transfected the cancer cells with a reporter vector containing the SEAP reporter gene under the control of SRE. SEAP activity was detected by chemiluminescence assay. During culture of the transfected cells under serum- and phenol red-free conditions, treatment with E2 (10^{-8} M) for 24 h resulted in a five- to sevenfold increase of SRE-induced SEAP expression in the ERα- and ERβ-positive cancer cell lines KLE, Hec-1B, EFO-21, OVCAR-3 (Fig. 3B), SK-OV-3 (Fig. 3C), MCF-7 (Fig. 3D) and T-47-D. Cotreatment with triptorelin (10^{-7} M) resulted in a decrease of E2-induced SRE activation in the GnRH receptor-positive cancer cell lines KLE, Hec-1B, EFO-21, OVCAR-3 (Fig. 3B), MCF-7 (Fig. 3D) and T-47-D. In the ovarian cancer cell line SK-OV-3, which expresses ERα and ERβ, but not the GnRH receptor, triptorelin

Figure 1 Expression of estrogen receptor α (ERα) and ERβ mRNA in human endometrial cancer cell lines KLE, HEC-1A, Hec-1B and Ishikawa; ovarian cancer cell lines EFO-21, OVCAR-3 and SK-OV-3; and breast cancer cell lines MCF-7 and T-47-D by RT-PCR, using primers specific for human ERα and human ERβ. For further details, see Materials and methods.

Figure 2 (A) Time-course effects of 17β-estradiol (E2; 10^{-8} M) or the combination of E2 (10^{-8} M) and triptorelin (10^{-5} M) on the proliferation of quiescent OVCAR-3 human ovarian cancer cells. Cell number is expressed as a percentage of controls (100%). (B) Dose-response effects of triptorelin on E2-induced proliferation of quiescent OVCAR-3 human ovarian cancer cells. Cells were treated for 6 days with vehicle, E2 (10^{-8} M) or E2 (10^{-8} M) plus increasing concentrations of triptorelin (T; 10^{-11} to 10^{-5} M). Cell number is expressed as a percentage of E2-stimulated cells (100%). Each point represents the mean±S.E.M. of three independent experiments, performed with different passages of the cell line in quadruple determinations. a, P < 0.001 vs control; b, P < 0.05 vs E2 alone; c, P < 0.01 vs E2 alone; d, P < 0.001 vs E2 alone. Experiments using endometrial cancer cell lines KLE and Hec-1B, ovarian cancer cell line EFO-21 or breast cancer cell lines MCF-7 and T-47-D gave comparable results.
Treatment had no effect on E2-induced SRE activation (Fig. 3C). In the ERα-negative but ERβ-positive and GnRH receptor-positive endometrial cancer cell lines Hec-1A and Ishikawa (Fig. 3A), E2 did not induce SRE activation. Triptorelin had no effects on the basal SRE-induced SEAP expression.

**Effects of triptorelin on E2-induced activation of ERE**

To determine whether E2-induced activation of ERE is affected by triptorelin, we transiently transfected the cancer cells with a reporter vector containing the SEAP reporter gene under the control of ERE. SEAP activity was detected by chemiluminescence assay. During culture of the transfected cells under serum- and phenol red-free conditions, treatment with E2 (10^{-8} M) for 24 h resulted in a 15–20-fold increase of ERE-induced SEAP expression in the ERα- and ERβ-positive cancer cell lines KLE, Hec-1B, EFO-21, OVCAR-3 (Fig. 4B), SK-OV-3 (Fig. 4C), MCF-7 (Fig. 4D) and T-47-D. After cotreatment with triptorelin (10^{-7} M), E2-induced ERE activation was always the same. In the ERα-negative but ERβ-positive and GnRH receptor-positive endometrial cancer cell lines Hec-1A and Ishikawa (Fig. 4A), treatment with E2 (10^{-8} M) resulted in a seven- to ninefold increase of ERE-induced SEAP expression. Triptorelin treatment had no effect on E2-induced ERE activation.

**Effects of triptorelin on E2-induced c-fos expression**

To determine whether triptorelin can affect E2-induced c-fos expression, quiescent cells were kept for 15 min in the absence or presence of triptorelin (10^{-7} M) before they were incubated for 20 min with E2 (10^{-8} M). In the ERα- and ERβ-positive cancer cell lines KLE, Hec-1B, EFO-21, OVCAR-3 (Fig. 5B), SK-OV-3 (Fig. 5C), MCF-7 (Fig. 5D) and T-47-D, E2-induced c-fos expression was 7–11-fold higher than the basal c-fos expression levels (Fig. 5). After treatment with triptorelin, E2-induced c-fos expression remained on basal expression levels in the GnRH receptor-positive cancer cell lines KLE, Hec-1B, EFO-21, OVCAR-3 (Fig. 5B), MCF-7 (Fig. 5D) and T-47-D. In the ERα- and ERβ-positive but...
GnRH receptor-negative ovarian cancer cell line SK-OV-3 (Fig. 5C), triptorelin treatment had no effect on E2-induced c-fos expression. In the ERα-negative but ERβ-positive and GnRH receptor-positive endometrial cancer cell lines Hec-1A and Ishikawa (Fig. 5A), c-fos expression was not induced by E2 and remained on basal levels. Triptorelin had no effects on the basal c-fos expression.

Discussion

GnRH receptors in the human pituitary, normal extrapituitary tissues, and tumors appear to be quite similar as far as their ligand-binding properties are concerned (23, 24). Their signal-transduction mechanisms, however, seem to be different (1). The GnRH signal-transduction pathway operating in normal tissues seems not to be essential in cancer cells (26). In pituitary gonadotrophs, GnRH receptor signaling is essentially mediated through G-protein αq, leading to activation of phospholipase C (PLC) and the rapid hydrolysis of membrane phospholipids, the liberation of inositol phosphates, subsequent mobilization of intracellular Ca\(^{2+}\) and activation of protein kinase C (PKC) (27–29). In earlier studies, we could demonstrate the activation of PLC, PKC, and adenylyl cyclase in tumor cells by pharmacologic stimuli. However, triptorelin, at concentrations that are clearly inhibitory on proliferation, had no effect on the activity of these signaling systems (26). Instead, we found that the antiproliferative effects of GnRH analogs are mediated through interaction with growth factor-induced mitogenic signaling, as GnRH analogs antagonized growth factor-induced proliferation, activity of MAPK and c-fos expression (2, 26). GnRH receptor gene mutations, like splice variants, are not responsible for the different signaling in tumor cells from that in the pituitary (2). We could show that the tumor GnRH receptor instead activates phosphotyrosine phosphatase (PTP), an effect mediated through G-protein αi, counteracting EGF-induced tyrosine autophosphorylation of EGF receptors, and resulting in inhibition of mitogenic signal transduction and reduction of cell proliferation (2).

In 1984 and 1985, Rao and Reddy (30) and Reddy et al. (31) could demonstrate that GnRH and its agonists inhibit E2-induced ornithine decarboxylase
(ODC) and glucosamine-6-phosphate synthase activities in the rat uterus. These enzymes, which are markers for cell proliferation, are regulatory enzymes in the biosynthetic pathways of polyamines and glycoproteins respectively (30, 31). Despite the long time which has passed, the interactions between GnRH and E2 could not be completely elucidated until now. The interactions between peptide hormones, including GnRH, estrogens, growth factors and other mitogens in endometrial, ovarian and breast cancer cells are very complex and play an important role in the regulation of the cell cycle and cell proliferation (32).

Normally, estrogen influences gene transcription through activation of its nuclear receptors (33). In the classical pathway, estrogen-activated ERs bind as homo- or heterodimers to EREs found in the promoter of estrogen-sensitive genes, to activate or repress transcription (34–38). Estrogen has also been shown to alter transcription of estrogen-sensitive genes through nonclassical pathways (39–44). In these cases, activated ER does not bind to EREs in the promoter of the target gene (39–44). Instead, the ERs interact with other transcription factors bound to their response elements, resulting in enhancement or repression of transcription (39–44). Transcriptional factors shown to interact with the ER include steroidogenic factor-1 (SF-1) (39), specific factor-1 (Sp1) (40–42), nuclear factor-Y (42) and activator protein-1 (AP-1) (43, 44). In addition, it was shown that a membrane-impermeable estrogen, E2-BSA, can induce the rapid activation of the MAPK signal pathway in a human neuroblastoma cell line. This induces transcription of c-fos in these cells. Much evidence suggests that steroids exert rapid, nongenomic effects on cells (45). Nongenomic estrogenic action peaks minutes after stimulation in many cell types. Other characteristics of this mechanism include resistance to inhibitors of DNA transcription or protein synthesis (actinomycin D or cycloheximide) and recruitment of membrane- or cytosol-localized signaling components. These include the second messengers calcium and nitric oxide (NO); receptor tyrosine kinases, including the EGF receptor and IGF-1 receptor; G protein-coupled receptors (that is, GnRH receptor); and protein kinases, including phosphatidylinositol-3' kinase (PI3K), the serine-threonine kinase Akt, MAPK family members, the nonreceptor tyrosine kinase Src and protein kinases A and C (PKA and PKC respectively) (46–48).

Figure 5 Effects of 17β-estradiol (E2; 10⁻⁸ M), triptorelin (10⁻⁷ M) or the combination of these compounds on c-fos expression of quiescent Ishikawa (A) human endometrial cancer cells, OVCAR-3 (B), SK-OV-3 (C), and human ovarian cancer cells and MCF-7 (D) breast cancer cells. Quiescent cells were incubated with E2 (10⁻⁸ M) for 20 min with or without previous incubation (15 min) with 10⁻⁷ M of triptorelin. c-fos mRNA expression was determined after 30 min by semiquantitative RT-PCR. Quiescent cells without treatment (C = control); quiescent cells with triptorelin treatment (T); quiescent cells with E2 treatment (E); quiescent cells with cotreatment (E/T). Columns represent means ± S.E.M. of data obtained from three independent experiments run in duplicate in three different passages of each cell line. a, $P < 0.001$ vs control; b, $P < 0.001$ vs E2 treatment. Experiments using endometrial cancer cell lines KLE and Hec-1B or ovarian cancer cell line EFO-21 gave comparable results to those shown for the OVCAR-3 cell line (B). Experiments using endometrial cancer cell line Hec-1A gave comparable results to those shown for the Ishikawa cell line (A). Experiments using breast cancer cell line T-47-D gave comparable results to those shown for the MCF-7 cell line (C).
It is possible that ERα might direct the activation of more receptor-proximal signaling complexes located at the plasma membrane. When overexpressed in cells, ligand-bound ERα induces the rapid phosphorylation of IGF-1 receptor and the activation of ERK1/2. Because these receptors co-immunoprecipitate in a ligand-dependent manner, a direct physical interaction between ERα and IGF-1 receptor could conceivably mediate the activation of ERK1/2 (49). In breast cancer cell lines, ligand-bound ERα promotes the rapid phosphorylation of the proteins Src and Shc, leading to downstream activation of Ras, Raf and MAPK (50). Similarly, in both breast and prostate cancer cells, E2 treatment induces the association of ERα phospho-Tyr537 with the Src Sh2 (Src homology 2) domain, leading to downstream activation of Ras, Raf and MAPK (51, 52). Additionally, in breast cancer cells, Src modulates PI3K-Akt signaling through a reversible cross-talk mechanism whereby the ligand-bound ERα forms a ternary complex composed of ERα, PI3K and Src (53).

Since E2 promotes mitogenic signal transduction, and GnRH and its agonists counteract the mitogenic effects of growth factors (such as insulin-like growth factor (IGF) and EGF) in human endometrial, ovarian and breast cancer cells, indicating an interaction with the mitogenic signal transduction pathway (1), we asked whether GnRH would also affect E2-induced mitogenic signaling.

Here we show that triptorelin inhibits E2-induced cell proliferation of human endometrial, ovarian and breast cancer cells. In addition, E2-induced activation of the SRE and the E2-induced expression of the immediate early-response gene c-fos was blocked by triptorelin treatment. In contrast, triptorelin had no effect on E2-induced activation of the ERE.

In investigating the estrogen responsiveness of the human endometrial, ovarian and breast cancer cell lines used, we first characterized the cell lines for ERα and ERβ gene expression. We found that the endometrial cancer cell lines KLE and Hec-1B; the ovarian cancer cell lines EFO-21, OVCAR-3 and SK-OV-3; and the breast cancer cell lines MCF-7 and T-47-D were ERα- and ERβ-positive, whereas the endometrial cancer cell lines Hec-1A and Ishikawa human breast cancer cell lines were ERα-negative. Because endometrial carcinomas tend to be ERα-dominant, it was a little surprising to find ERα-negative endometrial cancer cell lines. However, in our hands, the Hec-1A and Ishikawa human endometrial cancer cell lines were ERα-negative. In addition, these cancer cell lines are notorious for laboratory-to-laboratory variation in their expression of several differentiation markers, particularly the ERs.

The proliferation of all ERα- and ERβ-positive cancer cell lines analyzed was increased after treatment with E2. This proliferative effect was time- and dose-dependently antagonized by simultaneous treatment with triptorelin. The maximal effect was observed at 10^{-7} M concentration of triptorelin. This is a relatively high concentration. However, the lowest concentration of triptorelin showing significant inhibitory effects on E2-induced proliferation was 10^{-9} M. The effective concentrations of triptorelin in pituitary gonadotrophs, on the one hand, and gynecologic and breast cancers, on the other hand, are different because of the completely different signal transduction pathways in these cells (1). In experiments using endometrial cancer cell lines Hec-1A and Ishikawa, which do not express ERα, E2 had no promoting effect on cell proliferation. In experiments using ovarian cancer cell line SK-OV-3, which expresses ERα but not GnRH receptor, triptorelin had no inhibitory effect on E2-induced proliferation. In an earlier study using GnRH receptor knockout experiments, we could show that the antiproliferative effect of GnRH agonists is clearly mediated through the GnRH receptor (54).

After E2 treatment, a significant activation of the SRE was observed in all ERα-positive cell lines. Treatment with triptorelin alone shows no effect on basal SRE activity. After treatment with triptorelin (10^{-7} M), the E2-induced SRE activation was decreased in the GnRH receptor-positive cancer cell lines, but not in the GnRH receptor-negative cell line SK-OV-3. In the ERα-negative but ERβ-positive and GnRH receptor-positive endometrial cancer cell lines, E2 did not induce SRE activation. Therefore, the E2-induced expression of the immediate early gene c-fos, a mechanism further downstream in the mitogenic signal transduction, was abrogated in all cancer cell lines that express GnRH receptors by treatment with triptorelin. After E2 treatment, a significant increase of c-fos expression was observed in all ERα-positive cell lines. Treatment with triptorelin alone showed no effect on basal c-fos expression. After treatment with triptorelin, the E2-induced c-fos expression was decreased in the GnRH receptor-positive cancer cell lines, but not in the GnRH receptor-negative cell line SK-OV-3. In the ERα-negative but ERβ-positive and GnRH receptor-positive endometrial cancer cell lines, E2 did not induce c-fos expression.

The effects on the ERE were different. After E2-treatment, a significant increase of ERE activity was observed in all ERα- and ERβ-positive cell lines. Treatment with triptorelin never had any effect on basal or on E2-induced ERE activation in GnRH receptor-positive cancer cell lines, nor in the GnRH receptor-negative cell line SK-OV-3, indicating that no interactions between GnRH and estrogens exist in this E2-induced pathway. In the ERα-negative but ERβ-positive endometrial cancer cell lines Hec-1A and Ishikawa, E2-induced activation of ERE was much lower, as observed in the ERα- and ERβ-positive cell lines. The activation of ERE in the ERα-negative cell lines Hec-1A and Ishikawa indicates a putative Ero independence of ERE activation in these cell lines. Comparable data were shown by Jang et al. (55). They could demonstrate the upregulation of...
ERE-driven transcription after upregulation of ERβ in ERα-negative breast cancer cells.

Kang et al. (56) recently have demonstrated that OVCAR-3 ovarian cancer cells and normal human ovarian surface epithelium cells (hOSE) express ERα and ERβ, and that estrogen downregulates GnRH mRNA level in OVCAR-3 cells and GnRH receptor mRNA levels in OVCAR-3 and hOSE cells. They could further show that GnRH inhibits OVCAR-3 and hOSE cell proliferation, whereas E2 has a stimulatory effect on OVCAR-3, but not hOSE, cell proliferation. In addition, E2 antagonizes the antiproliferative activity of GnRH in OVCAR-3, but not in hOSE, cells (55). These data accord with our present data and demonstrate the strong interactions between peptide hormones, including GnRH, estrogens and growth factors in endometrial, ovarian and breast cancer cells.

In summary, we have demonstrated that triptorelin inhibits E2-induced cell proliferation of human endometrial, ovarian and breast cancer cells. Activation of tumor cell GnRH receptor signaling counteracts the E2-induced activation of SRE and therefore the E2-induced expression of the immediate early-response gene c-fos was blocked, resulting in downregulation of E2-induced cell proliferation. E2-induced activation of ERE was not affected by triptorelin.

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