GnRH-II agonist [D-Lys^6]GnRH-II inhibits the EGF-induced mitogenic signal transduction in human endometrial and ovarian cancer cells

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Abstract. The majority of human endometrial and ovarian cancers express receptors for GnRH type I (GnRH-I). Their proliferation is time- and dose-dependently reduced by GnRH-I and its analogs. GnRH-I analogs activate a phosphotyrosine-phosphatase (PTP) and inhibit EGF-induced mitogenic signal transduction. Recently we found that GnRH type II (GnRH-II) and its agonist [D-Lys^6]GnRH-II also have antiproliferative effects on these tumor cells which are significantly greater than those of GnRH-I agonists. In a more recent study, we showed that the antiproliferative activity of GnRH-II on human endometrial and ovarian cancer cell lines is not mediated through the GnRH-I receptor. The underlying signal transduction mechanisms of GnRH-II are still unknown. In this study we showed that the mitogenic effects of growth factors in endometrial and ovarian cancer cell lines were counteracted by GnRH-II agonist [D-Lys^6]GnRH-II, indicating an interaction with the mitogenic signal transduction. We showed that [D-Lys^6]GnRH-II reduces EGF-induced auto-tyrosine-phosphorylation of EGF-receptors via activation of a PTP and that EGF-induced activation of mitogen-activated protein kinase was blocked in cells treated with [D-Lys^6]GnRH-II. Furthermore, EGF-induced expression of the immediate early gene *c-fos* was inhibited by treatment with [D-Lys^6]GnRH-II. After knock-out of GnRH-I receptor expression, GnRH-II agonist [D-Lys^6]GnRH-II still activated PTP and inhibited the EGF-induced mitogenic signal transduction. These data indicate, that the effects of GnRH-II are not due to a cross-reaction with the GnRH-I receptor. In conclusion these data suggest that the signaling of GnRH-II agonist [D-Lys^6]GnRH-II is comparable to that of GnRH-I analogs.

Introduction

The expression of gonadotropin releasing hormone (GnRH-I) and its receptor as a part of a negative autocrine/paracrine mechanism of cell proliferation has been demonstrated in a number of malignant tumors, including cancers of the endometrium, ovary and breast (1). In these cancers the *in vitro* proliferation can be inhibited by agonistic and/or antagonistic analogs of GnRH-I in a dose- and time-dependent manner (1-5). The classical GnRH-I receptor signal transduction mechanisms, known to operate in the pituitary, are not involved in the mediation of antiproliferative effects of GnRH-I analogs in cancer cells (1). The GnRH-I receptor rather 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) counteracting the EGF-induced auto-tyrosine phosphorylation of the EGF receptor. Thus, the activity of the extracellular regulated kinases 1 and 2 (ERK1/2) is decreased. Moreover the EGF-induced expression of the immediate early gene *c-fos* and its protein is reduced. As a result cell proliferation is decreased (6-8).

Recently, we could show that GnRH type II (GnRH-II) has antiproliferative effects in human endometrial and ovarian cancer cell lines that are significantly greater than those of the superactive GnRH-I agonist Triptorelin (9,10).

In the ovarian cancer cell line SKOV-3, which does not express GnRH-I receptors, GnRH-I agonist Triptorelin has no effects on cell proliferation (11), whereas GnRH-II has strong antiproliferative effects. It might be speculated that, in addition to the GnRH-I system, a second GnRH-system exists in human cancers. Recently we showed that in cell lines affected by both, GnRH-I agonist Triptorelin and GnRH-II agonist [D-Lys^6]GnRH-II, the effects of the former were abrogated after GnRH-I receptor knockout, whereas those of GnRH-II persisted (9). These findings suggest that the antiproliferative effects of GnRH-II are not mediated through the GnRH-I receptor. The mechanisms underlying the antiproliferative effects of GnRH-II are unknown. In this study we have assessed whether or not GnRH-II signal transduction is comparable to that of GnRH-I analogs.

Materials and methods

Cell lines and culture conditions. The human endometrial cancer cell lines used were derived from an endometrial adenocarcinoma (Ishikawa) (12) or a moderately differentiated papillary adenocarcinoma (Hec1A) (13). The human ovarian cancer cell lines used were derived from a poorly differentiated

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serous adenocarcinoma (EFO-21) (14) or an adenocarcinoma derived from ascites (SKOV-3) (15). They were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) or the sources as described previously (2,3). The cells were cultured at 37˚C in a humidified atmosphere of 5% CO_2 in air as described previously (2-4).

For determination of EGF-induced *c-fos* mRNA expression, the cells were cultured in the absence of fetal calf serum (FCS) and phenol-red for 72 h. The quiescent cells were treated with 100 nM human recombinant EGF (Sigma-Aldrich, Deisenhofen, Germany) for 30 min with or without previous treatment (1 h) with 10 μM of GnRH-II agonist [D-Lys^6]GnRH-II. *c-fos* mRNA expression was determined by semi-quantitative RT-PCR (see below). To analyze EGF-induced c-Fos protein synthesis, the cells were cultured as described above.

GnRH analogs. The GnRH-I agonist [D-Trp^6]GnRH-I (Triptorelin; pGlu-His-Trp-Ser-Tyr-D-Trp-Leu-Arg-Pro-Gly-NH_2) was kindly provided by Ferring Pharmaceuticals (Copenhagen, Denmark) and the GnRH-I antagonist Cetrorelix (SB-75; [Ac-DNal(2)^1, D-Phe(4Cl)^2, D-Pal(3)^3, D-Cit^6, D-Ala^{10}]GnRH-I) was kindly provided by Zentaris (Frankfurt, Germany). The GnRH-II agonist [D-Lys^6]GnRH-II (pGlu-His-Trp-Ser-His-D-Lys-Trp-Tyr-Pro-Gly-NH_2) was synthesized by Peptide Specialty Laboratories (Heidelberg, Germany).

Phosphatase assay. The cells were plated in 75-cm^2 dishes at a density of 5x10^5 cells and grown under standard conditions. After 3-4 days, when the confluence of each dish reached 90%, the cells were incubated with FCS-free and phenol-red-free medium for 48 h. Treatment with 100 pM, 1 nM, 100 nM or 10 μM of the GnRH-II agonist [D-Lys^6]GnRH-II (PSC Heidelberg) for 1 h followed. The reactions were stopped by washing with 5 ml of ice-cold PBS. After washing, the cells were detached immediately with 2.5 ml of a solution containing 0.5 g Trypsin (Biochrom, Berlin, Germany) and 5 mmol EDTA in 1 l PBS/BSA, counted and lysed in a buffer containing 60 mM sodium acetate, pH 5.2, 5 mM ß-mercaptoethanol and 1% Triton X-100. Endogenous phosphate was removed using Sephadex G-25 spin colums (Promega Corp., Madison, WI).

Tyrosine phosphatase activity was measured using a tyrosine phosphatase assay system (Promega Corp.), initially described by Harder *et al* (16). The synthetic PTP-specific phosphopeptide provided with this assay is END(pY)INASL (17). The assay was performed with 10 μg protein, initiated by the addition of the enzyme, and incubated at 37˚C for 15 min. The reaction was stopped by adding molybdate-malachite-green solution.

mRNA isolation and cDNA synthesis. mRNA was isolated from cells grown in a monolayer using the Qiagen RNeasy Mini kit (Qiagen, Hilden, Germany). The concentration of poly(A)mRNA in each sample was determined by photospectrometry. First-strand cDNA was generated by reverse transcription of 1 μg of mRNA using p(dT)15 primers (Roche Diagnostics, Mannheim, Germany) with a Superscript™ preamplification kit, according to the instructions of the supplier (Invitrogen, Life Technologies, Karlsruhe, Germany). After determining the concentration of the cDNAs, the samples were used for semi-quantitative PCR analysis. The integrity of the samples was tested by RT-PCR of the housekeeping gene *GAPDH* (forward primer: 5'-CAT CAC CAT CTT CCA GGA GCG AGA-3', backward primer: 5'-GTC TTC TGG GTG GCA GTG ATG G- 3').

PCR amplification. cDNAs (1 μg) were amplified in a 50-μl reaction volume containing 10 mmol/l Tris-HCl (pH 8.3), 50 mmol/l potassium chloride, 1.5 mmol/l magnesium chloride, 200 μmol/l of each of the NTPs, 200 μmol/l of primers specific for a 483-bp fragment of the human *c-fos* mRNA (forward primer, 5'-GAG ATT GCC AAC CTG CTG AA-3'; backward primer, 5'-AGA CGA AGG AAG ACG TGT AA-3') and 1.25 U Taq polymerase (Roche, Mannheim, Germany) (6). The reaction was performed in an Applied Biosystems DNA thermal cycler 2400 (Weiterstadt, Germany). Between 24 (EFO-21 and SKOV-3) and 26 (Ishikawa and Hec1A) cycles were carried out: denaturation 94˚C for 30 sec, annealing at 54˚C for 30 sec, followed by extension at 72˚C for 60 sec. To compare the expression levels of *c-fos*, PCR reactions using primers for the housekeeping gene *GAPDH* were performed (see above). Seventeen cycles were carried out: denaturation 94˚C for 60 sec, annealing at 60˚C for 60 sec, followed by extension at 72˚C for 60 sec. The PCR products of both reactions were separated by gel electrophoresis in 1.5% agarose and visualized by ethidium bromide staining on a UV transilluminator.

Western blotting. The cells were plated in 75-cm^2 dishes at a density of ~10^6 cells and grown under standard conditions. After achieving a confluence of ~70-80%, the culture medium was changed to FCS-free and phenol-red-free medium for 24 h (MAPK) or 48 h (c-Fos). To induce MAPK (ERK1/2) and c-Fos activity, cells were treated with 100 nM of human recombinant EGF (Sigma-Aldrich) for 30 or 15 min without or with previous treatment (15 min or 1 h) with 10 μM of the GnRH-II agonist [D-Lys^6]GnRH-II. After incubation, cells were detached with 2.5 ml of a solution containing Trypsin (0.5 g) (Biochrom) and 5 mmol EDTA in 1 l PBS/BSA. Protein per sample (5 μg) was used and diluted to a final volume of 40 μl with Laemmli-buffer, containing 519 mM DTT (Dithiothreitol, Sigma-Aldrich), 69 mM sodium dodecyl sulfate (SDS), 0.1% Triton X-100, 62.5 mM Tris/HCl pH 6.8 and 75 nM bromphenol blue. The cell lysates were separated on SDS-PAGE (10% acrylamide/N'N'-bis-methylene-acrylamide, AppliChem GmbH, Darmstadt, Germany) under reducing conditions and transferred to nitrocellulose membranes (Hybond™-ECL™ nitrocellulose membrane, Amersham Bioscience). The nitrocellulose membranes were blocked with 5% instant skimmed milk powder, spray-dried (Naturaflor, Töpfer GmbH, Dietmannsried, Germany) in TBST (137 mM NaCl, 2.7 mM KCl, 0.1% Tween-20, 25 mM Tris/Cl, pH 7.4) for 1 h at RT, washed with TBST and then incubated at 4˚C overnight with rabbit polyclonal anti-ACTIVE™ MAPK pAb, pTEpY (Promega Corp., Mannheim, Germany) or polyclonal rabbit anti-c-Fos (Abcam, Cambridgeshire, UK) in a 1:5000 or 1:200 dilution in TBST. After washing three times with TBST, specifically bound antibody was detected using the ECL™ plus Western blot analysis (Amersham Biosciences,

UK). The bands were analyzed using the Kodak 1D image system (Kodak, New Haven, CT).

GnRH-I receptor knockout. A 43-bp fragment of the human GnRH-I receptor cDNA was cloned in antisense orientation (50-CT AGA ACC ATG GAC TGT CCG ACT TTG CTG TTG CTT TTC AAA GC-30) into the *Nhe*I/*Sal*I sites of the eukaryotic expression vector, pIRES (Clontech, Palo Alto, CA, USA), to produce the pGnRH-I receptor-antisense vector. Cells were grown to ~50% confluence in 75-cm^2 dishes. Transfections were performed using Superfect liposome reagents and following the manufacturer's instructions (Qiagen). After 12 h, transfected cells and non-transfected control cells were treated with the GnRH-I agonist Triptorelin (100 nmol/l) to induce GnRH-I receptor protein internalization. Six hours later, the cells were incubated with FCS-free and phenol-red-free medium for 24 h followed by treatment with 100 pM, 1 nM, or 100 nM of the GnRH-II agonist [D-Lys^6]GnRH-II for 1 h. Reactions were stopped by washing with 5 ml of ice-cold PBS. PTP assays were conducted as described above.

Results

Effects of [D-Lys^6]GnRH-II on PTP activity. In previous studies it was shown that stimulation with GnRH-I agonist Triptorelin increased PTP activity in different ovarian and endometrial cancer cell lines (7). To assess whether GnRH-II agonist [D-Lys^6]GnRH-II has similar effects a set of experiments with increasing concentrations of [D-Lys^6]GnRH-II was performed.

Treatment of the ovarian cancer cell lines EFO-21 (Fig. 1A) and SKOV-3 (not shown) and the endometrial cancer cell lines Hec1A (Fig. 1B) and Ishikawa (not shown) with 10 pM, 1 nM, 100 nM or 10 μM of the GnRH-II agonist [D-Lys^6]GnRH-II for 1 h resulted in a dose-dependent increase of PTP activity.

After treatment of the ovarian cancer cell line EFO-21 with a 10-pM [D-Lys^6]GnRH-II concentration a slight increase of PTP activity to 110.93±4.81% of control (100%) was observed (Fig. 1A). At a 1-nM concentration of [D-Lys^6]GnRH-II, the increase of PTP activity was much higher (146.27±22.36% of control). The effects on PTP activity were maximal at 100-nM concentrations of [D-Lys^6]GnRH-II and corresponded to 260.27±85.06% of control. [D-Lys^6]GnRH-II at 10 μM had almost the same stimulatory effects on PTP activity (252.6±79.65% of control).

After treatment of the endometrial cancer cell line Hec-1A with a 10-pM [D-Lys^6]GnRH-II concentration a slight increase of PTP activity to 108.17±11.41% of control (100%) was observed (not significant) (Fig. 1B). At a 1-nM concentration of [D-Lys^6]GnRH-II, the increase of PTP activity was much higher (114.47±10.10% of control, not significant). The effects on PTP activity were maximal at 100-nM concentrations of [D-Lys^6]GnRH-II and corresponded to 165.33±7.86% of control (P<0.01 vs control). [D-Lys^6]GnRH-II at 10 μM had almost the same stimulatory effects on PTP activity (165.57±5.99% of control, P<0.01 vs control). Experiments using the ovarian cancer cell line SKOV-3 or the endometrial cancer cell line Ishikawa gave identical results (not shown).

Effects of [D-Lys^6]GnRH-II on EGF-induced MAPK (ERK1/2) activity. We analyzed the effects of [D-Lys^6]GnRH-II on the

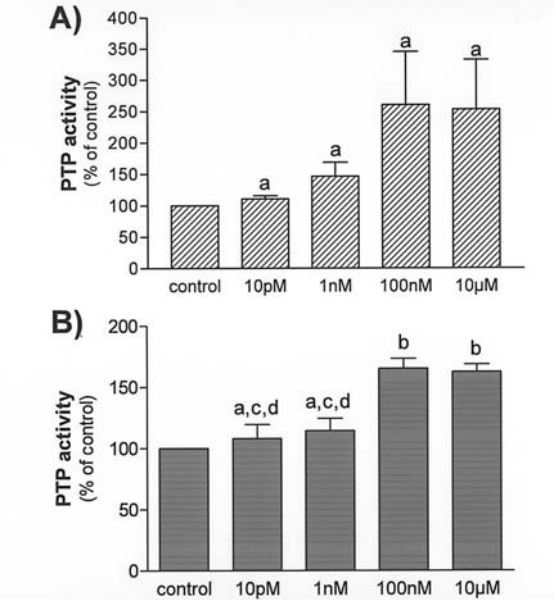

Figure 1. Effects of treatment with GnRH-II agonist [D-Lys^6]GnRH-II (1 h, 10 pM - 10 μM) on PTP-activity of quiescent EFO-21 ovarian cancer cells (A) and Hec1A endometrial cancer cells (B). a, ns vs control; b, P<0.01 vs control; c, P<0.01 vs 100 nM; d, P<0.01 vs 10 μM (one-way ANOVA followed by Newman Keuls). Experiments using ovarian cancer cell line SKOV-3 or endometrial cancer cell line Ishikawa show identical results. Data obtained from three independent experiments for each cell line.

EGF-induced activity of the extracellular regulated kinases 1 and 2 (ERK1/2).

To quantify phosphorylated ERK1/2, a polyclonal rabbit anti-ACTIVE™ MAPK pAb was used, which detects phosphorylated ERK1 (44 kDa) and ERK2 (42 kDa). By cultivation of the cells under serum- and phenol-red-free conditions for 72 h, MAPK activity was downregulated to basal expression levels (control = 100%) (Fig. 2). Treatment of the quiescent cells with EGF (100 nM) for 15 min resulted in a significant increase of MAPK activity in all cell lines tested (Fig. 2). After treatment with GnRH-II agonist [D-Lys^6]GnRH-II (10 μM) for 15 min, EGF-induced MAPK activity was abrogated (Fig. 2).

After EGF treatment (100 nM) for 15 min the activity of MAPK in the GnRH-I and GnRH-II receptor-positive endometrial cancer cell line Ishikawa was increased to 286.0±56.9% of control (P<0.05 vs control) (Fig. 2A). Additional treatment with GnRH-II agonist [D-Lys^6]GnRH-II (10 μM) for 15 min reduced the EGF-induced increase of MAPK activity to 133.6±21.5% (P<0.01 vs EGF). In the GnRH-I receptor-negative but GnRH-II receptor-positive ovarian cancer cell line SKOV-3 EGF treatment resulted in an increase of MAPK activity to 161.2±23.9% (P<0.05 vs control) (Fig. 2B). Additional treatment with GnRH-II agonist [D-Lys^6]GnRH-II (10 μM) for 15 min reduced the EGF-induced increase of MAPK activity to basal levels (99.1±10.2% of control, P<0.05 vs EGF).

Effects of [D-Lys^6]GnRH-II on EGF-induced c-fos mRNA expression. Previous experiments indicated that treatment of ovarian and endometrial cancer cells with GnRH-I agonist Triptorelin showed a decrease in expression of the early

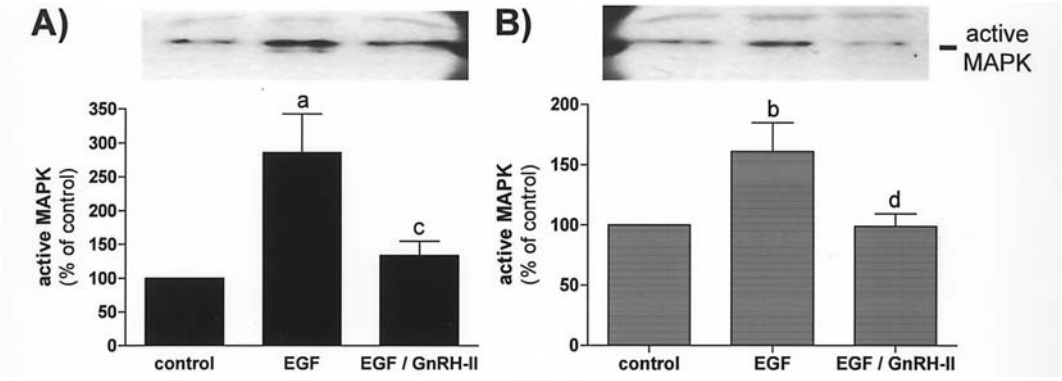

Figure 2. A, Immunoblotting of phosphorylated MAP-kinases ERK 1/2 in the endometrial cancer cell line Ishikawa (A) and in the human ovarian cancer cell line SKOV-3 (B). Cells were treated without or with EGF (100 nM) for 15 min, without or with previous treatment with [D-Lys^6]GnRH-II (10 μM) for 15 min. A polyclonal rabbit anti-ACTIVE™ MAPK pAb was used, which detects the phosphorylated active form of ERK1 (44 kDa) and ERK2 (42 kDa). The amount of activity is given in the absence (quiescent cells = control = 100%) or presence of [D-Lys^6]GnRH-II (10 μM for 15 min) and/or EGF (100 nM for 15 min). The data were obtained from three independent experiments run in duplicate in three different passages of each cell line. a, P<0.01 vs control; b, P<0.05 vs control; c, P<0.01 vs EGF; d, P<0.05 vs EGF (one-way ANOVA followed by Newman Keuls). Experiments using the endometrial cancer cell line Hec1A and the ovarian cancer cell line EFO21 gave identical results.

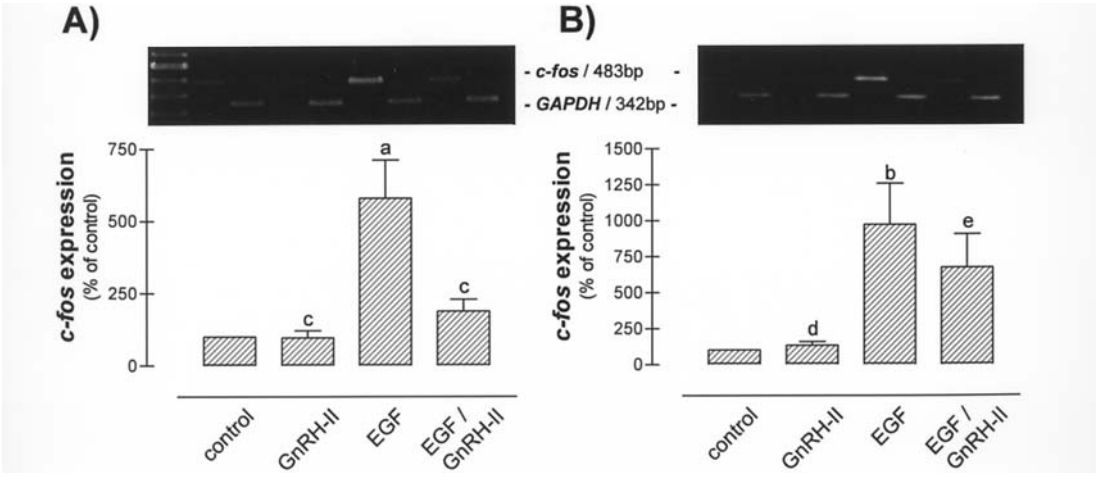

Figure 3. Expression of *c-fos* mRNA in the endometrial cancer cell line Ishikawa (A) and the ovarian cancer cell line SKOV-3 (B). Quiescent cells (control = 100%); cells treated with 10 μM [D-Lys^6]GnRH-II for 1 h (GnRH-II); cells treated with 100 nM EGF for 30 min (EGF) and cells treated with 100 nM EGF for 30 min with previous incubation with 10 μM [D-Lys^6]GnRH-II for 1 h (EGF/GnRH-II). The housekeeping gene *GAPDH* (342 bp) was used as standard. The data were obtained from three independent experiments run in duplicate in three different passages of each cell line. a, P<0.001 vs control; b, P<0.05 vs control; c, P<0.001 vs EGF; d, P<0.01 vs EGF; e, P>ns vs EGF (one-way ANOVA followed by Newman Keuls). Experiments using the endometrial cancer cell line Hec1A and the ovarian cancer cell line EFO-21 gave identical results to those obtained from the Ishikawa cell line.

response gene *c-fos* (6). After culture without FCS and phenol-red for 72 h the cells were treated with 100 nM EGF with or without a previous incubation with 10 μM [D-Lys^6]GnRH-II to analyze whether or not GnRH-II has comparable effects.

After cultivation of the cells under serum- and phenol-red-free conditions for 72 h, the *c-fos* expression of all analyzed cell lines was downregulated to basal expression levels (control = 100%) (Fig. 3). Treatment of the quiescent cells with 10 μM [D-Lys^6]GnRH-II alone had no effect on *c-fos* expression (Ishikawa: 97.4±23.5% of control, P>0.05 vs control; SKOV-3: 134.0±24.1% of control, P>0.05 vs control) (Fig. 3). Treatment of the quiescent cells with 100 nM EGF (30 min) resulted in a marked increase of *c-fos* expression levels in all cell lines tested (Ishikawa: 579.2±131.5% of control, P<0.001 vs control; SKOV-3: 972.4±284.0% of control, P<0.05 vs control) (Fig. 3). To analyze whether [D-Lys^6]GnRH-II is able to affect EGF-induced *c-fos* expression, quiescent cells were kept for 60 min in the absence or presence of the GnRH-II agonist [D-Lys^6]GnRH-II (10 μM) before they were incubated for 30 min with 100 nM EGF. The pre-treatment with [D-Lys^6]GnRH-II (10 μM) dramatically reduced the EGF-induced *c-fos* expression. In the GnRH-I and GnRH-II receptor-positive endometrium cancer cell line Ishikawa the EGF-induced *c-fos* expression was reduced from 579.2±131.5% of control to 188.1±41.2% of control (P<0.001) (Fig. 3A). In the GnRH-I receptor-negative but GnRH-II receptor-positive ovarian cancer cell line SKOV-3 the EGF-induced *c-fos* expression was reduced from 972.4%±284.0% of control to 675.0%±230.8% of control (not significant) (Fig. 3B).

To analyze whether GnRH-II agonist [D-Lys^6]GnRH-II treatment also affects c-Fos protein synthesis, quiescent cells

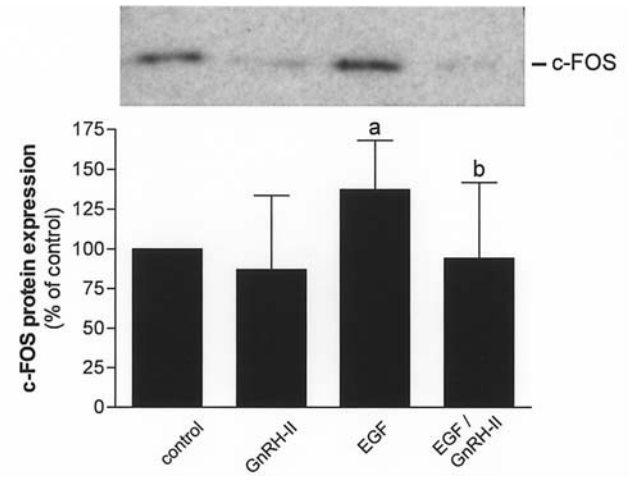

Figure 4. Immunoblotting of c-Fos protein in the endometrial cancer cell line Ishikawa using a polyclonal rabbit anti human c-Fos antibody. Quiescent cells (control = 100%)); cells treated with 10 μM [D-Lys^6]GnRH-II for 1 h (GnRH-II); cells treated with 100 nM EGF for 30 min (EGF) and cells treated with 100 nM EGF for 30 min with previous incubation with 10 μM [D-Lys^6]GnRH-II for 1 h (EGF/GnRH-II). The data were obtained from three independent experiments run in duplicate in three different passages. a, ns vs control; b, ns vs EGF (one-way ANOVA followed by Newman Keuls). Experiments using the endometrial cancer cell line Hec1A and the ovarian cancer cell lines EFO21 and SKOV3 gave identical results.

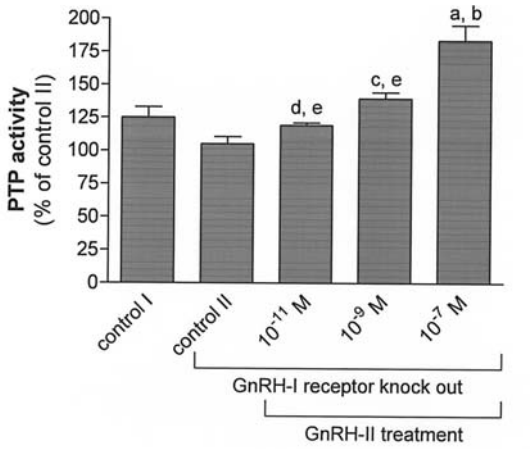

Figure 5. Effects of GnRH-II agonist [D-Lys^6]GnRH-II on PTP-activity of quiescent EFO-21 ovarian cancer cells after knockout of GnRH-I receptor expression. Control I (first bar) without GnRH-I receptor knockout. Control II (=100%; second bar) with GnRH-I receptor knockout without GnRH-II treatment. GnRH-I receptor knockout with treatment with 10 pM (third bar), 100 nM (fourth bar), or 1 nM (fifth bar) GnRH-II agonist [D-Lys^6]GnRH-II for 1 h. a, P<0.01 vs control II; b, P<0.01 vs control I; c, P<0.05 vs control II; d, ns vs control II; e, ns vs control I (one-way ANOVA followed by Newman Keuls). Data obtained from three independent experiments run in duplicate in three different passages. Experiments using the endometrial cancer cell lines Ishikawa and Hec1A gave identical results.

were treated as described above. The amount of c-Fos protein was evaluated by SDS-PAGE and Western blotting of the cell lysates. Fig. 4 shows that a protein band of 62 kDa, corresponding to c-Fos protein, is present in the cell lysates. In the serum-starved cells the amount of c-Fos protein was small (control = 100%). Treatment of the quiescent cells with 10 μM [D-Lys^6]GnRH-II alone had no effect on c-Fos protein expression. The amount of c-Fos protein was increased after EGF treatment (Fig. 4). Pre-treatment with [D-Lys^6]GnRH-II (10 μM) reduced the EGF-induced c-Fos protein expression (not significant).

Effects of [D-Lys^6]GnRH-II on PTP activity after GnRH-I receptor knockout. To assess whether the GnRH-II agonist [D-Lys^6]GnRH-II-induced signaling in endometrial and ovarian cancer cells is mediated through GnRH-I receptor, we analyzed the activation of PTP after knockout of GnRH-I receptor expression. Knockout of the GnRH-I receptor protein was controlled by immune cytochemistry using a monoclonal mouse anti-human GnRH-I receptor antibody and immunoblotting using a polyclonal rabbit anti-human GnRH-I receptor antiserum (18). After internalization of the GnRH-I receptor induced by the GnRH-I agonist Triptorelin, a high density of novel synthesis of GnRH-I receptor protein, seen as GnRH-I receptor antigenicity, was observed in non-transfected cells, whereas transfected cells showed only slight GnRH-I receptor antigenicity. As early as 1 day after knockout of GnRH-I receptor expression, GnRH-I receptor protein antigenicity was significantly reduced in comparison with the control. After 2 days, GnRH-I receptor protein was no longer detected.

After knockout of GnRH-I receptor expression, GnRH-II agonist [D-Lys^6]GnRH-II still activated PTP and inhibited the EGF-induced mitogenic signal transduction, whereas the effects of GnRH-I agonist Triptorelin were abrogated. Treatment of the GnRH-I receptor knockout cell line EFO-21 GnRH-I R$^-$ with 10 pM, 1 nM or 100 nM of the GnRH-II agonist [D-Lys^6]GnRH-II for 1 h resulted in a dose-dependent increase of PTP activity. At a 10-pM [D-Lys^6]GnRH-II concentration a slight increase of PTP activity to 119.3%±2.1% of control II (=100%) was observed (not significant vs control I; not significant vs control II). Treatment with 1 nM [D-Lys^6]GnRH-II showed a greater increase of PTP activity to 139.5%±4.8% of control II (P<0.05 vs control I; not significant vs control II). The effects on PTP activity were maximal at a 100 nM concentration of [D-Lys^6]GnRH-II (183.6%±11.6% of control II; P<0.01 vs control I; P<0.01 vs control II).

Discussion

Our data demonstrate that after the binding of GnRH-II to its receptor the EGF-induced signal transduction in human ovarian and endometrial cancer cells is inhibited. Direct activation of a phosphotyrosine-phosphatase (PTP) by GnRH-II agonist [D-Lys^6]GnRH-II counteracts the EGF-induced auto-tyrosine-phosphorylation of EGF receptors. Thus, the activity of the mitogen-activated protein kinases (MAPK) ERK 1/2 (extracellular regulated kinases 1 and 2) is decreased. Moreover the EGF-induced expression of the immediate early gene *c-fos* and its protein is reduced. The signal transduction mechanisms of GnRH-II agonist [D-Lys^6]GnRH-II mediating the antiproliferative effects seem to be comparable to that of GnRH-I analogs. However, in contrast to GnRH-I agonists, GnRH-II agonist [D-Lys^6]GnRH-II does not activate nucleus factor κB (NF-κB) and therefore does not protect the cancer cells from apoptosis (unpublished data).

Our results are supported by a recent study showing inhibition of cell proliferation in human breast cancer cells

after treatment with GnRH-I agonist Triptorelin or GnRH-II agonist [D-Lys^6]GnRH-II. EGF-induced auto-tyrosine-phosphorylation of EGF-receptors and activation of ERK-1/2 were blocked by GnRH-I and GnRH-II agonists (19).

Recently we showed that both, GnRH-II agonist [D-Lys^6]GnRH-II and GnRH-I antagonist Cetrorelix, had strong antiproliferative effects in the GnRH-I receptor negative human ovarian cancer cell line SKOV-3 (9,10). After knockout of GnRH-I receptor expression the antiproliferative effects of GnRH-I agonist Triptorelin on former GnRH-I receptor-positive endometrial and ovarian cancer cell lines were abrogated, whereas the growth inhibitory effects of the GnRH-I antagonist Cetrorelix and of GnRH-II agonist [D-Lys^6]GnRH-II were still the same as observed in non-transfected cells (9). We also demonstrated that treatment of GnRH-I receptor knockout cell lines with GnRH-I agonist Triptorelin could not reduce cell proliferation. After treatment with GnRH-II agonist [D-Lys^6]GnRH-II the cell number was decreased in all cell lines investigated ($P<0.001$ vs control) (9). Therefore, we assume that a functional GnRH-II receptor-ligand mechanism or a related mechanism, which is activated by GnRH-II agonist [D-Lys^6]GnRH-II or GnRH-I antagonist Cetrorelix, exists in human cancer cells. It is reasonable to speculate that this putative GnRH-II system might be comparable with the GnRH-I system of a negative autocrine mechanism of cell proliferation.

The mammalian GnRH-II receptor system is well characterized. The non-human primate GnRH-II receptor was cloned and detected in all tissues analyzed, organs and carcinomas (20,21). Functional analysis of the monkey GnRH-II receptor was performed. These investigations discovered that GnRH-II was active in the nanomolar range, which supported evidence that its receptor is functional (22). GnRH-II was ~400-fold more active than GnRH-I, which indicates the specificity of the interaction between GnRH-II and its receptor.

The existence of an independent GnRH-II receptor system in non-human primates might be an argument for the occurrence of a similar system in humans. But until now there has been neither success in cloning nor in sequencing the full-length human GnRH-II receptor, which is known to be a member of the 7 transmembrane (TM) receptor family (10,21,23,24). Probably, the functional GnRH-II receptor is expressed in a variety of splice variants (22). In this context there are two possibilities: firstly, that the GnRH-II receptor mRNA is expressed, encodes either 5 or 7 TM domains; or secondly, that a GnRH-II responsive complex is formed by the GnRH-I receptor and fragments derived from the GnRH-II receptor. All considerations lead to the conclusion that if a functional GnRH-II receptor is expressed in human tissues, it might be a 5 TM domain receptor, lacking the TM regions I and II (22). The existence of functional 5 TM G-protein coupled receptor (GPCR) mutants of chemokine receptors, where the N-terminal segment is connected directly to the transmembrane domain 3 as a result of a deletion of the transmembrane domains 1 and 2, is reported by Ling *et al* (26). Morgan *et al* discovered that the human GnRH-II receptor is also expressed in a variety of splice variants but a functional transcript could not be shown (25). This would support the results of the GnRH-II receptor mRNA detected and described in previous studies (10,25), where the receptor is suspected to be non-functional because of a stop codon in the second exon. Van Biljon *et al* have recently cloned a GnRH-II receptor transcript from human sperm, consisting of the three exons required for a full length receptor protein (27). This transcript also includes a stop codon and a frame shift. Although this would suggest that the gene is a transcribed pseudogene, there are several lines of evidence for a functional role of GnRH-II receptor in human sperm and testis.

Recent studies from our laboratory demonstrated the first clear evidence for the existence of a GnRH-II receptor-like protein (18). To identify the protein as a GnRH-II specific binding site we used the photo-affinity-labeling technique and to confirm our results, competition experiments were performed. These experiments and also proliferation assays made in previous studies (9,10) suggest the existence of a specific GnRH-II binding site in human cancer cells, which might be the putative GnRH-II receptor. Due to our present results that show inhibition of EGF receptor signal transduction after treatment with GnRH-II agonist [D-Lys^6]GnRH-II, even after knockout of GnRH-I receptor expression, it seems reasonable to speculate that an additional GnRH-II responsible receptor system exists in human endometrial and ovarian cancer cells. In contrast to our results, Kim *et al* (28) demonstrated that transfection of short-interfering RNA to abrogate GnRH-I receptor gene expression reversed GnRH-I- and -II-induced antiproliferative effects. These results indicate that GnRH-I and GnRH-II induce their antiproliferation through the GnRH-I receptor. We do not have an explanation for this discrepancy. However, it is possible that, in cells in which the GnRH-I receptor but not the putative GnRH-II receptor is expressed, both, GnRH-I and GnRH-II, activate GnRH-I receptor signal transduction. In addition it is possible that, in cells in which the GnRH-I receptor and the putative additional receptor for GnRH-II are coexpressed, a cross-talk between the signalling of both receptors might take place.

In conclusion, our findings show that in the cell lines used in the present study a putative GnRH-II receptor system might be similar to the well-known GnRH-I receptor system. The antiproliferative effects are mediated through an interaction with the growth factor-induced mitogenic signaling. We demonstrated that GnRH-II agonist [D-Lys^6]GnRH-II inhibits the EGF-induced auto-tyrosine-phosphorylation of EGF-receptors by activation of a PTP. Therefore, the proteins further downstream, such as ERK1/2 and c-Fos, are decreased in their activity or expression. Consequently, there is a reduction in cell proliferation. After knockout of GnRH-I receptor expression, GnRH-II agonist [D-Lys^6]GnRH-II still activates PTP and inhibits the EGF-induced mitogenic signal transduction. Therefore, the effects of GnRH-II are not due to a cross-reaction with the GnRH-I receptor.

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