EXPERIMENTAL STUDY

Protein kinase C-independent stimulation of activator protein-1 and c-Jun N-terminal kinase activity in human endometrial cancer cells by the LHRH agonist triptorelin

Carsten Gründker, Lars Schlotawa, Volker Viereck and Günter Emons
Department of Gynaecology and Obstetrics, Georg-August-University, D-37070 Göttingen, Germany
(Correspondence should be addressed to G Emons, Department of Gynaecology and Obstetrics, Robert-Koch-Street 40, D-37075 Göttingen, Germany; Email: emons@med.uni-goettingen.de)

Abstract

Objective: The expression of luteinizing hormone-releasing hormone (LHRH) and its receptor as a part of an autocrine regulatory system of cell proliferation has been demonstrated in a number of human malignant tumours, including cancers of the endometrium. The signalling pathway through which LHRH acts in endometrial cancer is distinct from that in pituitary gonadotrophs. The LHRH receptor interacts with the mitogenic signal transduction of growth factor receptors via activation of a phosphotyrosine phosphatase, resulting in down-regulation of cancer cell proliferation. In addition, LHRH activates nucleus factor κB (NFκB) and protects the cancer cells from apoptosis. This study was conducted to investigate additional signalling mechanisms of the LHRH receptor cooperating with NFκB in endometrial cancer cells.

Design: The LHRH agonist triptorelin-induced activator protein-1 (AP-1) activation was analysed using a pAP-1-SEAP reporter gene assay. Expression of c-jun mRNA was quantified using quantitative reverse transcription (RT)-PCR. c-Jun N-terminal kinase (JNK) activity was measured by quantification of phosphorylated c-Jun protein.

Results: Treatment of Ishikawa and Hec-1A human endometrial cancer cells with 100 nM triptorelin resulted in a 3.1-fold and 3.5-fold activation of AP-1 respectively (P < 0.05). If the cells had been made quiescent, treatment with triptorelin (100 nM) resulted in a 41.7-fold and 48.6-fold increase of AP-1 activation respectively (P < 0.001). This effect was completely blocked by simultaneous treatment with pertussis toxin (PTX). A 17.6-fold and 17.3-fold increase of c-jun mRNA expression respectively (P < 0.001) was obtained after 20 min of stimulation with triptorelin (100 nM). Treatment with 1 nM triptorelin resulted in a 12.5-fold or an 11.9-fold increase, and treatment with 10 pM triptorelin resulted in a 6.5-fold or a 5.2-fold increase of maximal c-jun mRNA expression respectively (P < 0.001). Maximal c-Jun phosphorylation (68.5-fold and 60.2-fold, respectively, P < 0.001) was obtained after 90 min incubation with triptorelin (100 nM).

Conclusions: These results suggest that the LHRH agonist triptorelin stimulates the activity of AP-1 in human endometrial cancer cells mediated through PTX-sensitive G-protein αi. In addition, triptorelin activates JNK, known to activate AP-1. In earlier investigations we have shown that triptorelin does not activate phospholipase and protein kinase C (PKC) in endometrial cancer cells. In addition, it has been demonstrated that triptorelin inhibits growth factor-induced mitogen activated protein kinase (MAPK, ERK) activity. Thus triptorelin-induced activation of the JNK/AP-1 pathway in endometrial cancer cells is independent of the known AP-1 activators, PKC or MAPK (ERK).

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Introduction

The hypothalamic decapeptide luteinizing hormone releasing hormone (LHRH) plays a key role in the control of mammalian reproduction (1–3). The neuropeptide specifically binds to high-affinity receptors in pituitary gonadotrophs, which are coupled to the pertussis toxin (PTX)-insensitive G-protein αq/11 and to the phospholipase C (PLC) signalling pathway (4–6). In addition to these well documented hypophysiotropic actions, LHRH is present in the brain and a variety of peripheral organs, both normal and tumoural, where it probably acts in an autocrine/paracrine fashion (7–16). In endometrial, ovarian, breast and prostate cancer the in vitro proliferation can be inhibited by agonistic and/or antagonistic analogs of LHRH in a dose- and time-dependent manner (11, 12, 17). The classical LHRH receptor signal-transduction mechanisms, known to
operate in the pituitary (6, 18), are not involved in the mediation of the antiproliferative effects of LHRH analogs in cancer cells (19). Although we could clearly demonstrate the activation of PLC, protein kinase C (PKC) and adenyl cyclase in the tumour cells by pharmacological stimuli, triptorelin, at concentrations that are clearly inhibitory on proliferation, had no effects on the activity of these signalling systems (19). LHRH analogs rather interfere with the mitogenic signal transduction of growth-factor receptors presumably through activation of a phosphotyrosine phosphatase mediated through G-protein α. This results in inhibition of growth factor-induced mitogen activated protein kinase (MAPK) activity (11, 19) and down-regulation of growth factor-induced c-fos expression (20).

Recently we found another LHRH signal transduction pathway acting in ovarian and endometrial cancer cells. triptorelin induces activation of nuclear factor-κB (NFκB) and thus reduces doxorubicin-induced apoptosis in human ovarian (21) and endometrial cancer cells (unpublished results).

Activator protein-1 (AP-1) is predominantly a heterodimeric complex of c-Fos and c-Jun proteins (22). Only the members of the Jun family, which includes c-Jun, JunB and JunD, can form homodimeric complexes (23). It is well established that modulation of AP-1 activity is one of the most critical steps in the control of cell proliferation and transformation. The Jun family responds to a variety of extracellular stimuli by changing their expression levels, by altering their phosphorylation status, or by forming homodimeric complexes or heterodimeric complexes with Fos proteins. Because Fos proteins cannot form homodimers and therefore cannot bind DNA without Jun participation, Jun proteins are more important than Fos proteins in gene regulation because they are required absolutely for the functioning of the AP-1 transcription factors (23).

It was recently reported that JunD, which is usually constitutively expressed in cells, might function as a negative regulator of cell proliferation as overexpression of JunD slowed cell growth and resulted in down-regulation of growth factor-induced c-fos expression (20).

Materials and methods

Cell lines and culture conditions

The human endometrial cancer cell lines used were derived from an endometrial adenocarcinoma (Ishikawa) (28) or a moderately differentiated papillary adenocarcinoma (HeLa-1A) (29). The cells were cultured as described in detail previously (30). To assess triptorelin-induced AP-1 activation, cells were transfected with pAP-1-SEAP (see below). Subsequently these cells were cultured for 96 h in the absence of foetal calf serum (FCS) and phenol red with or without 100 nM triptorelin in the presence or absence of PTX (2 μg/ml). Every 24 h, 200 μl of the media were collected and analysed for SEAP activity (see below).

Plasmids and transfection

pAP-1-SEAP (Clontech, Palo Alto, CA, USA) is designed to monitor the induction of AP-1 and the stress-activated protein kinase/Jun N-terminal kinase (SAPK/JNK) signal transduction pathway (31). pAP-1-SEAP contains six tandem copies of the AP-1 enhancer fused to HSV-TK promoter. pTK-SEAP (Clontech) was used as a negative control to determine the background signals associated with the culture medium. The enhancerless 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 using the Superfect liposome reagents and following the manufacture’s instructions (Qiagen, Hilden, Germany) and cells were treated as described above. Chemiluminescence detection of SEAP activity was performed according the manufacturer’s instructions (Clontech) using a plate fluorometer (Berthold, Bad Wildbach, Germany).

Isolation of RNA and cDNA synthesis

For determination of triptorelin-induced c-jun mRNA expression, quiescent cells grown in monolayer were incubated with or without decreasing concentrations (100 nM, 1 nM, and 1 pM) of the agonist. Total RNA was prepared using the RNaseasy protocol (Qiagen). The concentration of RNA in each sample was determined by photospectroscopy. First-strand cDNA was generated by reverse transcription (RT) of 4 μg total RNA using p(dT)15 primers (Boehringer Mannheim, Mannheim, Germany) with MMLV-reverse transcriptase according to the instructions of the suppliers (Gibco BRL, Karlsruhe, Germany). After determining the concentration of the cDNAs, the samples were used for semiquantitative PCR analysis. The integrity of the samples was tested by RT-PCR of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward primer, 5’ ATT CCA CCC ATG GCA AAT TC 3’; backward primer, 5’ AGT GAT GGC ATG GAC TGT GG 3’).
**Semiquantitative PCR amplification**

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 of the appropriate primers (c-jun: forward primer, 5’ TCC TAT GAC GAT GGC CTC AAC 3’; backward primer, 5’ GTG TTC TGG CTG TGC AGT TC 3’; GAPDH, forward primer, 5’ ATT CCA CCC ATG GCA AAT TC 3’; backward primer, 5’ AGT GAT GCC ATG TGT GG G 3’), and 1.25 U Taq polymerase (Boehringer Mannheim) in a Perkin Elmer DNA thermal cycler 2400 (Weiterstadt, Germany). Twenty-five cycles of amplification representing the exponential phase of the PCR were carried out: denaturation at 94 °C for 30 s; annealing at 61 °C for 30 s; followed by extension at 72 °C for 60 s. The PCR product amplified with the c-jun primers had a total length of 353-bp. The PCR product amplified with the GAPDH primers has a total length of 398-bp. The PCR products were separated by gel electrophoresis in 1.5% agarose. The respective DNA products were run on the same gel. For quantification of phosphorylated c-Jun protein (JNK activity)

For the determination of triptorelin-induced JNK activity, agonist-induced phosphorylation of c-Jun protein was quantified. The cells were plated at a density of 10⁶ cells in 100 mm dishes and grown under standard conditions. After 2 days, culture media were changed to FCS-free and phenol red-free medium and the quiescent cells were incubated with or without 100 nM triptorelin. After incubation the cells were detached immediately with 1 ml of a solution containing 0.5 g trypsin (Biochrom, Berlin, Germany) and 5 mmol EDTA in 1 l phosphate-buffered saline (PBS)/bovine serum albumin (BSA) and counted. The cells were lysed using a buffer containing 200 mM dithiothreitol (DTT; Sigma, Deisenhofen, Germany), 2% sodium dodecyl sulphate (SDS), 10% glycerol, 0.004% bromphenol blue in 160 mM Tris–HCl, pH 6.8. The cell lysates were electrophoresed on SDS-PAGE (15%) under reducing conditions and transferred to nitrocellulose. The nitrocellulose membranes were blocked in 5% BSA (Amersham, Bucks, UK) in PBST (10 mM PBS, pH 7.0, 1% Tween 20) for 1 h, incubated with polyclonal rabbit antibody against human c-Jun phosphorylated at Ser⁶³ (Calbiochem, Bad Soden, Germany) in a 1:1000 dilution in 1% BSA in PBST for 1 h, and then, following washings, incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Amersham) at an 1:1500 dilution in 1% BSA in PBST for 1 h. After washings, specifically bound antibody was detected using the enhanced chemiluminescence kit (ECL; Amersham). The bands were analysed using the Kodak 1D image system (Kodak).

**Statistical analysis**

All experiments were reproduced three times with different passages of the Ishikawa and Hec-1A endometrial cancer cell lines. Results were expressed as percentages of the respective controls and were pooled before they were tested by one-way ANOVA followed by a Newman Keuls’ test for the comparison of individual groups, after a Bartlett test had shown that variances were homogenous. Results are given as means±s.e.

**Results**

**Induction of AP-1 activation by triptorelin in endometrial cancer cells**

To examine whether or not AP-1 plays a role in LHRH signalling, we transiently transfected Ishikawa and Hec-1A endometrial cancer cells with a reporter vector containing an AP-1 enhancer fused to HSV-TK promoter and the SEAP reporter gene or the pTK-SEAP vector as a negative control. SEAP activity was detected using a chemiluminescence assay. During culture of the transfected cells under serum- and
phenol red-free conditions. Treatment with triptorelin (100 nM) resulted in a 48.6-fold (Fig. 1A) or in a 41.7-fold (Fig. 1B) increase of AP-1-induced SEAP expression respectively. Using medium containing FCS, treatment of the endometrial cancer cells with triptorelin (100 nM) resulted in a 3.1-fold or 3.5-fold activation of AP-1 respectively (P < 0.05, not shown). This effect was completely blocked by simultaneous treatment with PTX (2 μg/ml, not shown).

Induction of c-jun mRNA expression by LHRH agonist triptorelin in endometrial cancer cells

AP-1 consists of homo- or heterodimeric protein complexes formed by the related jun and fos gene family members. Because triptorelin down-regulates growth factor-induced c-fos expression in human ovarian and endometrial cancer cells (20), we examined the effects of triptorelin on c-jun gene expression. The levels of c-jun mRNA in triptorelin-treated cells were estimated by semiquantitative RT-PCR in comparison with the mRNA expression levels of the housekeeping gene GAPDH. Treatment of quiescent cells with triptorelin resulted in a marked increase of the c-jun mRNA expression levels in both endometrial tumour cell lines. In the Ishikawa (Fig. 2A, B) and the Hec-1A (Fig. 2C, D) cancer cells, maximal c-jun mRNA expression levels (17.6-fold or 17.3-fold increases respectively, P < 0.001) were reached 20 min after triptorelin treatment. Quantitative data obtained from three independent experiments run in duplicate in three different passages of each cell line are shown in the graphics (B and D). a, P < 0.001 vs control; b, P < 0.05 vs control (one-way ANOVA followed by Newman Keuls test).

**Figure 2** PCR amplification (25 cycles) of first-strand cDNA from the endometrial cancer cell lines Ishikawa (A and B) and Hec-1A (C and D). Oligonucleotide primers for human c-jun were used. Gel was stained with ethidium bromide, and bands were visualized with u.v. light. Upper bands represent c-jun amplification, lower bands represent GAPDH amplification. Quiescent cells were incubated in the absence (control) or presence of triptorelin (100 nM). After treatment, a significant increase of c-jun mRNA expression is observed. Maximal c-jun mRNA expression (17.6-fold or 17.3-fold increases respectively, P < 0.001) was obtained 20 min after triptorelin treatment. Quantitative data obtained from three independent experiments run in duplicate in three different passages of each cell line are shown in the graphics (B and D). a, P < 0.001 vs control; b, P < 0.05 vs control (one-way ANOVA followed by Newman Keuls test).
Induction of c-Jun phosphorylation (JNK activity) by triptorelin in endometrial cancer cells

It has been shown that JNK activation is responsible for phosphorylating the transactivating domain of the c-Jun protein (32) and, in turn, phosphorylated c-Jun homodimers have potent AP-1 activity, which regulate the expression of a number of genes including c-jun itself (23, 33). Therefore we tested the effect of triptorelin on JNK activity by quantification of c-Jun protein phosphorylation using an antibody against phosphorylated c-Jun protein. Treatment of quiescent cells with 100 nM triptorelin resulted in a marked increase of the phosphorylated c-Jun protein in both endometrial tumour cell lines. In the Ishikawa (Fig. 4A) and the Hec-1A (Fig. 4B) cancer cells, maximal levels of phosphorylated c-Jun protein (68.5-fold or 60.2-fold respectively, \( P < 0.001 \)) were reached 90 min after treatment.

Discussion

AP-1 is activated in response to many growth factors, hormones and other agents, and it controls cell growth, apoptosis and the transcription of TPA (12-O-tetradecanoylphorbol-13-acetate)-responsive element (TRE)-containing genes, e.g., transforming growth factor-β, endothelin-1, insulin-like growth factor II, pro-α1 collagen, collagenase type I, and c-jun (34).

In pituitary gonadotrophs, the JNK pathway is activated significantly in response to LHRH (35). The JNK cascade utilizes a sequential activation of p21-activated kinase/mixed lineage kinase (PAK1/MLK), mitogen-activated kinase kinase kinase 1 (MEKK1), stress-activated protein kinase kinase 1/mitogen-activated protein kinase kinase 7 (SEK1/MKK7), and JNK1/2 to activate transcription factors such as c-Jun, AFT2, and Elk 1 (36, 37). The stimulation of JNK activity is mediated by a unique pathway that includes activation of PKC (35).

In human cancers the mechanisms of actions of LHRH are completely different. The antiproliferative effects of LHRH analogs are mediated through interaction with growth factor-induced mitogenic signalling, as LHRH analogs antagonized growth factor-induced proliferation, tyrosine phosphorylation, activity of MAPK, and c-fos expression (11, 19, 20) probably through activation of PTP (11). Comparable data were obtained by Moretti et al. (38) in the human prostatic cancer cell lines LNCaP and DU 145. In the tumour system, typically higher LHRH agonist concentrations (1 nM–10 μM) than in pituitary gonadotrophs are
required to achieve measurable effects. In this study we used 100 nM triptorelin, which has been shown to induce clearly measurable effects (11, 20). In addition lower concentrations (1 nM, 10 pM) were tested to assess the dose–response relation.

In this study, we show that triptorelin is a potent stimulator of JNK and AP-1 activity in human endometrial cancer cells. Treatment with 100 nM resulted in a 41.7–48.6-fold increase of AP-1 activation in quiescent human endometrial cancer cells. This effect was completely blocked by pertussis toxin indicating that it is mediated by G-protein αi, which is known to be coupled to the LHRH receptor in endometrial cancers. Recently G-protein αi-dependent activation of JNK was shown in human embryonal kidney 293 cells (39). Triptorelin also activates JNK, known to activate AP-1. Depending on the method and conditions of determining activity, JNK is activated 20–50-fold by LHRH in αT3-1 pituitary cells (35, 40). We found in this study that treatment of quiescent endometrial cancer cells with 100 nM triptorelin resulted in a 60.2–68.5-fold increase of JNK activity measured as c-Jun protein phosphorylation. In the pituitary cells, the maximum was reached approximately 30 min after LHRH treatment (40). In the tumour cells, maximal JNK activity was detected 90 min after triptorelin treatment. A 17.3–17.6-fold increase of c-jun mRNA expression was obtained after 20 min of triptorelin treatment. In contrast to the pituitary gonadotrophs the activation of JNK in endometrial cancer cells is PKC-independent. In earlier studies we showed that LHRH analogs have no effects on PKC, PLC and adenylyl cyclase in ovarian and endometrial tumour cells although the activation of these factors by pharmacological stimuli could be clearly demonstrated (19). In Swiss 3T3 and HeLa cells the stimulation of JNK and AP-1 activity by lysophosphatidylcholine is also PKC-independent (41). In pituitary gonadotrophs, depletion of PKC or the use of PKC inhibitors prevented only ~70% of the LHRH-JNK signals, indicating the existence of a minor PKC-independent signalling component in pituitary gonadotrophs (35).

Because LHRH analogs inhibit growth factor-induced mitogen activated protein kinase (MAPK, ERK) activity in endometrial cancer cells (11, 19), triptorelin-induced activation of AP-1 is presumably independent on the AP-1 activator MAPK (ERK). In other cell types activation of G-protein-coupled receptors (GPCRs) was found to induce a clearly distinct pattern of expression of immediate early genes, including those of the jun and fos family (42). In particular, activation of GPCRs but not tyrosine kinase receptors led, in NIH 3T3 cells, to a remarkable expression of c-jun (42). This response did not correlate with MAPK activation (42), thus suggesting that GPCRs control a distinct biochemical route regulating gene expression. Furthermore recent work demonstrated that a novel family of enzymes closely related to MAPK, named JNKs (43), selectively phosphorylated and regulated the activity of the c-Jun protein. Recently, it was found that the Ras-related small GTP-binding proteins Rac1 and Cdc42 initiate an independent kinase cascade regulating JNK activity (44) and Rac1 and Cdc42 are an integral part of the signalling route linking many cell surface receptors, including GPCRs, to JNK (45). More recent work has identified many components of this pathway and has shown that JNK is potently activated by several naturally occurring human oncogenes (46). Further examinations of the G-protein subunits linking GPCRs to JNK provided evidence that free βγ dimers (45) and, in some cellular systems, Goα12 (47) transfer signals from this class of receptors to JNK.

Recently Yamauchi et al. (48) found that JNK is involved in inhibition of cell proliferation induced by α1B-adrenergic receptors in human embryonic kidney cells. In a study on rats, c-jun mRNA depression and endometrial epithelial cell proliferation were suggested to be linked (49). In UT-OC-3 ovarian cancer cells cytokines have inhibitory effects on cell proliferation and activate AP-1 and NFkB (50). Because triptorelin activates the JNK/c-jun pathway and JNK/c-jun was found to be involved in down-regulation of cell proliferation in different systems, it seems reasonable to speculate that the JNK/c-jun pathway is involved in the antiproliferative actions of this agonist.

Recently we found that triptorelin induces activation of NFκB and thus reduces doxorubicin-induced apoptosis in human ovarian (21) and endometrial cancer cells (unpublished results). This possibility of protecting ovarian and endometrial cancer cells from programmed cell death is a new feature in LHRH signalling in ovarian and endometrial tumours separate from the inhibitory interference with the mitogenic pathway. Recently Wisdom et al. found that c-Jun protects human fibroblast cells from UV-induced apoptosis and cooperates with NFκB to prevent apoptosis induced by tumour necrosis factor-α (51). The molecular mechanisms by which c-Jun protects cells from apoptosis are unclear. One possibility is that c-Jun might participate in a checkpoint function, mediating a growth arrest that permits repair of damaged DNA. A second possibility is that c-Jun induces the expression of genes that block apoptosis. This would be comparable to the mechanism by which NFκB protects cells from apoptosis, which involves transcriptional activation of anti-apoptotic genes.

In endometrial cancer cells LHRH analogs mediate antiproliferative actions through inhibition of growth factor-induced mitogenic signal transduction. Triptorelin protects cancer cells from apoptosis via activation of NFκB, and it stimulates AP-1 and JNK activity. It might be interesting to investigate the basis of the cooperation of AP-1 with NFκB, as well as the molecular basis of the anti-apoptotic functions. Further investigations are required to analyse whether or not...
the triptorelin-induced increase in AP-1 activity is involved in the antiproliferative action of LHRH analogs possible via increasing the percentage of cells in the G0/G1 phase of cell cycle.

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