Luteinizing Hormone-Releasing Hormone Induces Nuclear Factor κB-Activation and Inhibits Apoptosis in Ovarian Cancer Cells

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ABSTRACT

More than 80% of human ovarian cancers express LHRH and its receptor as part of a negative autocrine mechanism of growth control. This study was conducted to investigate whether LHRH affects apoptosis in ovarian cancer. EFO-21 and EFO-27 ovarian cancer cells were treated with LHRH agonist Triptorelin or with cytotoxic agent Doxorubicin in the absence or presence of Triptorelin. Apoptotic cells were quantified by flow cytometry. Expression of nuclear factor kappa B (NFκB) was assessed by RT-PCR and immunoblotting. Determination of Triptorelin-induced NFκB activation, cells were transfected with a NFκB-secreted alkaline phosphatase reporter gene plasmid (pNFκB-SEAP) and cultured for 96 h with or without Triptorelin. The causal relation between Triptorelin-induced NFκB and Triptorelin-induced protection against apoptosis was investigated using SN50, an inhibitor for nuclear translocation of activated NFκB. Apoptosis induction by Triptorelin was never observed. Treatment with Doxorubicin (1 nmol/L) for 72 h increased the percentage of apoptotic cells in EFO-21 and EFO-27 ovarian cancer cell lines to 31% or 34%, respectively. In cultures treated simultaneously with Triptorelin (100 nmol/L), the percentage of apoptotic cells was reduced significantly, to 17% or 18%, respectively (P < 0.001). RT-PCR and immunoblotting experiments showed that NFκB subunits p65 and p50 were expressed by ovarian cancer cell lines EFO-21 and EFO-27. When EFO-21 or EFO-27 cells were transfected with pNFκB-SEAP and subsequently treated with Triptorelin (100 nmol/L), NFκB-induced SEAP expression increased 5.3-fold or 4.7-fold, respectively (P < 0.001). Triptorelin-induced reduction of Doxorubicin-induced apoptosis was blocked by SN50-mediated inhibition of NFκB translocation into the nucleus. We conclude that LHRH induces activation of NFκB and thus reduces Doxorubicin-induced apoptosis in human ovarian cancer cells. This possibility to protect ovarian cancer cells from programmed cell death is an important feature in LHRH signaling in ovarian tumors, apart from the inhibitory interference with the mitogenic pathway. (J Clin Endocrinol Metab 85: 3815–3820, 2000)


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onist Triptorelin is able to reduce Doxorubicin-induced apoptosis. In addition, we assessed whether the putative antipoptotic effects of LHRH-agonists might be mediated through an increased activation of NFκB.

Subjects and Methods

Cell lines and culture conditions

The human ovarian cancer cell lines used were derived from a poorly differentiated serous adenocarcinoma (EFO-21) and a mucinous papillary adenocarcinoma of intermediate differentiation (EFO-27) (15). The cells were cultured as described in detail previously (8).

To show that LHRH agonist Triptorelin ([D-Trp⁶]-LHRH, Ferring Pharmaceuticals Ltd., Kiel, Germany) does not induce apoptosis, cells were cultured for 72 h in the absence or presence of LHRH agonist Triptorelin (1 nmol/L–10 μmol/L). In a positive control experiment, cells were cultured in the presence of 1 nmol/L cytotoxic agent Doxorubicin (Sigma, Deisenhofen, Germany). To analyze whether LHRH agonist Triptorelin reduces Doxorubicin-induced apoptosis, the cells were cultured for 72 h in the presence of 1 nmol/L Doxorubicin, with or without 100 nmol/L Triptorelin. In a second set of experiments, cells were cultured for 1 h, in the presence of 100 nmol/L Doxorubicin, with or without pretreatment with Triptorelin (100 nmol/L), for 3 h. In a third set of experiments, the cells were cultured for 72 h in the presence of 1 nmol/L Doxorubicin, with or without 100 nmol/L Triptorelin, with or without 18 μmol/L SN50 (Calbiochem, Bad Soden, Germany), a cell membrane-permeable inhibitor peptide that blocks the nuclear translocation of the activated NFκB complex into the nucleus (16). In control experiments, we used 18 μmol/L SN50 M (Calbiochem), an inactive control peptide for SN50, instead of SN50 (16). After Doxorubicin treatment, the cells were cultured in the absence of Triptorelin and Doxorubicin for 72 h. Attached and floating cells both were collected by gentle centrifugation and washed twice with PBS.

To assess Triptorelin-induced NFκB activation, cells were transfected with pNFκB-SEAP (see below). Subsequently, these cells were cultured for 96 h in the absence of FCS and phenol red, or in the presence of 1% FCS with or without 100 nmol/L Triptorelin. Every 24 h, 100 μL of the media were collected and analyzed for SEAP activity (see below).

Flow cytometry

To quantify cells with advanced DNA degradation, we used a procedure similar to that described by Nicoletti et al. (17). A pellet containing 1 × 10⁶ cells (see above) was gently resuspended in 500 μL of hypotonic fluorochrome solution containing 0.1% Triton X-100 (Sigma), 0.1% sodium-citrate (Sigma), and 50 μg/mL propidium-iodide (Sigma). Cell suspensions were kept at 4 °C in the dark, overnight, before flow cytometry. Analysis of cellular DNA content was performed using a FACScan (Becton Dickinson and Co., Mountain View, CA).

Isolation of RNA and complementary DNA (cDNA) synthesis

Total RNA was prepared, from cells grown in monolayer, using the RNeasy protocol (QIAGEN, Hilden, Germany). The concentration of RNA in each sample was determined by photospectroscopy. First-strand cDNA was generated by RT of 4 μg of total RNA using p(dT)₁₅ primers (Boehringer, Mannheim, Germany) with Moloney murine leukemia virus reverse transcriptase, according to the instructions of the suppliers (Life Technologies, Karlsruhe, Germany). After determining the concentration of the cDNAs, the samples were used for semiquantitative PCR analysis. The integrity of the cDNAs was tested by RT-PCR of the house keeping gene GAPDH (forward primer: 5′ CAT CAC CAT CTT CCA GGA GGC GAG 3′, backward primer: 5′ GTG TTC TGG GTG GCA GTC GTG 3′).

PCR

The cDNAs (2 ng) were amplified in a 50-μL reaction vol 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 deoxynucleotide triphosphates, 1 μmol/L of the appropriate primers (p50: forward primer: 5′ CAC CTA GCT GCC AAA GAA GG 3′, backward primer: 5′ AGG CTC AAA GTT CTC CAC CA 3′; p65: forward primer: 5′ TCA ATG GCT ACA CAG GAC CA 3′, backward primer: 5′ CAC TGT CAC CTG GAA CCA GA 3′), and 1.25 U Taq polymerase (Boehringer) in a Perkin-Elmer Corp. (Weiterstadt, Germany) DNA thermal cycler 2400. Thirty cycles of amplification were carried out: denaturation at 94 °C for 30 sec, annealing at 56 °C for 30 sec, followed by extension at 72 °C for 60 sec. The PCR products amplified with the p50 or the p65 primers had a total length of 399 bp or 308 bp, respectively. PCR products were separated by gel electrophoresis in 1.5% agarose, and bands were visualized by ethidium bromide staining on a ultraviolet transilluminator.

Antibodies and immunoblotting

For immunoblots, polyclonal rabbit antihuman p50 and rabbit antihuman p65 (Serotec, Oxford, UK) were used in an 1:1000 dilution, followed by a peroxidase-conjugated antirabbit IgG (Amersham Pharmacia Biotech, Buckinghamshire, UK) in an 1:1000 dilution. Proteins were detected with ECL reagents (Amersham Pharmacia Biotech).

Plasmids and transfection

pNFκB-SEAP (CLONTECH Laboratories, Inc., Palo Alto, CA) is designed to monitor the activation of NFκB and NFκB-mediated signal transduction pathways. pNFκB-SEAP contains four tandem copies of the κB enhancer fused to the HSV-TK promoter. pTK-SEAP (CLONTECH Laboratories, Inc.) was used as a negative control to determine the background signals associated with the culture medium.

Chemiluminescence

Chemiluminescence detection of SEAP activity was performed according to the manufacturer’s instructions (CLONTECH Laboratories, Inc.) using a plate fluorometer (Berthold, Bad Wildbad, Germany).

Statistical analysis

All experiments were reproduced four times in different passages of the EFO-21 and EFO-27 cell lines. Data were tested for significant differences using a Mann-Whitney U-Test.

Results

Ovarian cancer cell lines EFO-21 and EFO-27, treated either with authentic LHRH or LHRH agonist Triptorelin (concentrations of 1 nmol/L–10 μmol/L), showed no morphologic signs of programmed cell death (not shown). The absence of apoptosis induction by LHRH agonist Triptorelin was also assessed by flow cytometry (Fig. 1). The percentage of living and apoptotic cells in the LHRH agonist-treated groups (94.0% and 5.9%, respectively; Fig. 1A) were in the same range as in the untreated controls (93.0% and 6.2%, respectively; Fig. 1B). In positive controls treated with Doxorubicin (1 nmol/L), the percentage of apoptotic cells was increased to 31% (Fig. 2A).

When EFO-21 cells were treated with the cytotoxic agent Doxorubicin (1 nmol/L) for 72 h, 31% of the cells showed all morphologic signs of apoptosis, including DNA fragmentation. Vacuoles in the cytoplasm, rounding-up of cells, apoptotic bodies, and bleb formation were the most characteristic signs of the apoptotic process (not shown). The percentages of living cells and apoptotic cells, assessed by flow cytometry, were 64.0% and 31.0%, respectively (Fig. 2A). If the cells were simultaneously treated with LHRH
agonist Triptorelin (100 nmol/L) for 72 h, the percentage of apoptotic cells was reduced significantly, to 17.0% (P < 0.001; Fig. 2B). When EFO-21 cells were treated for 1 h with Doxorubicin (100 nmol/L), previous incubation with LHRH agonist Triptorelin (3 h, 100 nmol/L) reduced the percentage of apoptotic cells from 83% (no LHRH pretreatment) to 52% (LHRH pretreatment; P < 0.001; not shown). Experiments using ovarian cancer cell line EFO-27 gave results comparable with those of ovarian cancer cell line EFO-21 (cf. Fig. 5B).

To check whether ovarian cancer cell lines EFO-21 and EFO-27 express NFκB, we have assessed the presence of NFκB subunits p50 and p65 mRNA and immunoreactivity. Both NFκB subunits were expressed in both ovarian cancer cell lines (Fig. 3, A and B).

To examine whether NFκB plays a role in LHRH-mediated protection against apoptosis, we transiently transfected EFO-21 and EFO-27 ovarian cancer cells with a reporter vector containing a κB4 cis-acting DNA sequence (response element) 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 EFO-21 or EFO-27 cells, under serum- and phenol-red-free conditions, LHRH agonist Triptorelin treatment resulted in a 3.4-fold (Fig. 4A) or in a 2.9-fold (data not shown) increase of NFκB-induced SEAP expression, respectively (P < 0.001).

Under the same conditions, including 1% FCS, the LHRH agonist Triptorelin treatment resulted in a 5.3-fold (Fig. 4B) or in a 4.7-fold (not shown) increase of NFκB-induced SEAP expression, respectively (P < 0.001).

To study the causal relationship between Triptorelin-induced NFκB activation and Triptorelin-induced protection against Doxorubicin-induced apoptosis, we used SN50, an inhibitor of nuclear translocation of activated NFκB. When EFO-21 (Fig. 5A) or EFO-27 (Fig. 5B) cells were treated with Doxorubicin (1 nmol/L) for 72 h, the percentages of apoptotic cells, assessed by flow cytometry, were 31.0% or 34.0%, respectively. If the cells were simultaneously treated with LHRH agonist Triptorelin (100 nmol/L) and with NFκB nuclear translocation inhibitor SN50 for 72 h, the percentage of apoptotic cells was increased to 28.0% or 31.0%, respectively (P < 0.001). The inactive control peptide SN50 M had no effects.

Discussion

Looking for apoptosis induction by authentic LHRH or LHRH agonists, we found no signs for an increase of pro-
The activity of NFκB is strictly regulated by an inhibitor, IκB, that forms a complex with NFκB and keeps NFκB in the cytoplasm. When cells receive signals that activate NFκB, IκB is phosphorylated and degraded through a ubiquitin/proteasome pathway. The degradation of IκB triggers the translocation of NFκB from the cytoplasm into the nucleus, where it regulates the transcription of NFκB-responsive genes by interacting with κB binding sites (19).

The role of NFκB in apoptosis seems to be complex, because it has been found to depend on the cell type. Some studies have implied that NFκB promotes apoptosis in certain cells, such as neurons (20), Schwann cells (21), and embryonic kidney cells (22). In contrast, several recent reports provided convincing evidence that NFκB is involved in apoptosis inhibition. Cells from transgenic mice deficient in NFκB subunit p65 are highly susceptible to TNF-α-induced apoptosis, and this susceptibility is reversed by transfection of the cells with the wild-type p65 gene (23). Inhibition of NFκB induces apoptosis in murine B cells, a cell type expressing constitutively active NFκB (24). Inhibition of NFκB potentiates amyloid β-mediated neuronal apoptosis (25). In Chinese hamster ovary cells overexpressing wild-type insulin receptors, NFκB plays an important role in the antiapoptotic function of insulin (26).

As shown by RT-PCR and Western blotting, both NFκB subunits were expressed in the ovarian cancer cell lines EFO-21 and EFO-27. Several other ovarian cancer cell lines were found to express NFκB, including OVCAR-3 (27), CA-OV-3 (28), and UT-OC-5 (29).

One important pathway involved in initiating apoptosis is activated by stress inducers, including chemotherapeutic drugs (e.g., Doxorubicin) or ionizing radiation. These inducers damage mitochondria by an unknown mechanism, leading to the release of cytochrome c from mitochondria into the cytosol (30–32). Cytochrome c and different other factors recruit and process procaspase 9 (31). The active caspase 9 activates the effector caspases, such as caspase 3, to induce apoptosis (31, 33, 34). The inducible transcription factor NFκB plays an important role in inhibiting chemotherapy-induced apoptosis (23, 24, 35–37). NFκB activation blocks caspase cleavage and cytochrome c release, indicating that NFκB suppresses the earliest signaling components of the caspase cascade (38).

Using a NFκB-SEAP reporter gene construct, a significant increase in NFκB activation was found in LHRH agonist-treated EFO-21 and EFO-27 ovarian cancer cells. To show the link between the inhibitory effect of Triptorelin on Doxorubicin-induced apoptosis and the Triptorelin-induced activation of NFκB, we used a synthetic peptide (SN50), which inhibits the nuclear translocation of activated NFκB (16). SN50 is a cell-permeable peptide constructed to control nuclear translocation of NFκB in intact cells. If the cells were treated simultaneously with LHRH agonist Triptorelin and
with SN50 peptide, the LHRH-induced reduction of apoptosis in Doxorubicin-treated cells was virtually blunted. These experiments clearly suggest that LHRH agonist Triptorelin inhibits Doxorubicin-induced apoptosis via activation of NFκB. Because the antiapoptotic effects of Triptorelin (45%) are much greater than its antiproliferative effects (20%) (7), it is unlikely that the antiapoptotic activity of Triptorelin is just a direct shot off of its antiproliferative effects. In addition, the experiments with SN50 clearly support the concept that the activation of NFκB induced by Triptorelin is the crucial mechanism mediating its antiapoptotic activity.

The LHRH agonist-induced activation of NFκB is presumably mediated by G protein i, because it can be inhibited by pertussis toxin (unpublished data). However, this effect seems to be independent of the LHRH-induced activation of tyrosine-phosphatase, because the phosphatase inhibitor vanadate does not have any influence on the LHRH agonist-induced NFκB activation (unpublished data). Therefore, the LHRH-induced activation of NFκB seems to be independent from interaction between LHRH agonists and the signal transduction of the EGF-receptor. In ovarian cancer cells, LHRH seems to have two opposite activities: 1) the anti-mitotic activity through inhibition of signal transduction of mitogens such as EGF (5); and 2) the inhibition of Doxorubicin-induced apoptosis via activation of NFκB as shown here.

NFκB plays a negative role in chemotherapy-mediated apoptosis, and LHRH is able to induce NFκB activation, as shown by reporter gene assay. Inhibition of nuclear translocation of activated NFκB abolishes the antiapoptotic effect of the LHRH agonist. We therefore believe that the LHRH-induced mechanism reducing apoptosis is likely to be important in blocking cell death induced by Doxorubicin therapy. We here present data showing, for the first time, a mechanism based on LHRH that suppresses chemotherapeutic drug-induced apoptosis, possibly mediated through...
NFkB activation. The knowledge about this antiapoptotic mechanism ought to be deepened by further research and might open new therapeutic options.

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References


