The Luteinizing Hormone-Releasing Hormone Receptor in Human Prostate Cancer Cells: Messenger Ribonucleic Acid Expression, Molecular Size, and Signal Transduction Pathway*

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ABSTRACT

Evidence has accumulated indicating that LHRH might behave as an autocrine/paracrine growth inhibitory factor in some peripheral tumors. However, LHRH receptors in tumor cells have not been fully characterized, so far. The present experiments were performed to analyze: 1) the messenger RNA expression; 2) the molecular size; and 3) the signal transduction pathway of LHRH receptors in prostate cancer. For these studies, the human androgen-dependent LNCaP and androgen-independent DU 145 prostate cancer cell lines were used. 1) By RT-PCR, a complementary DNA product, which hybridized with a 32P-labeled oligonucleotide probe specific for the pituitary LHRH receptor complementary DNA, was found both in LNCaP and in DU 145 cells. 2) Western blot analysis, using a monoclonal antibody raised against the human pituitary LHRH receptor, revealed the presence of a protein band of approximately 64 kDa (corresponding to the molecular mass of the pituitary receptor) in both cell lines. 3) In LNCaP and DU 145 cells, pertussis toxin completely abrogated the antiproliferative action of a LHRH agonist (LHRH-A). Moreover, LHRH-A substantially antagonized the pertussis toxin-catalyzed ADP-ribosylation of a Gαq protein. Finally, LHRH-A significantly counteracted the forskolin-induced increase of intracellular cAMP levels in both cell lines. These data demonstrate that the LHRH receptor, which is present in prostate cancer cells, independently of whether they are androgen-dependent or not, corresponds to the pituitary receptor, in terms of messenger RNA expression and protein molecular size. However, at variance with the receptor of the gonadotrophs, prostate cancer LHRH receptor seems to be coupled to the Gαq protein-cAMP signal transduction pathway, rather than to the Gαq11-phospholipase C signaling system. This might be responsible for the different actions of LHRH in anterior pituitary and in prostate cancer. (Endocrinology 140: 5250–5256, 1999)

THE HYPOTHALAMIC LHRH, by stimulating gonadotropin synthesis and release, is the key hormone in the regulation of reproduction (1–4). The neurohormone specifically binds to high-affinity pituitary receptors, which belong to the seven-transmembrane domain family and are coupled to the pertussis toxin (PTX) insensitive Gαq/G11 protein and to the phospholipase C signaling system. This might be responsible for the different actions of LHRH in anterior pituitary and in prostate cancer levels in both cell lines. These data demonstrate that the LHRH receptor, which is present in prostate cancer cells, independently of whether they are androgen-dependent or not, corresponds to the pituitary receptor, in terms of messenger RNA expression and protein molecular size. However, at variance with the receptor of the gonadotrophs, prostate cancer LHRH receptor seems to be coupled to the Gαq protein-cAMP signal transduction pathway, rather than to the Gαq11-phospholipase C signaling system. This might be responsible for the different actions of LHRH in anterior pituitary and in prostate cancer.

Materials and Methods

Materials

The LHRH agonist Zoladex [D-Ser(tBu)6Aza-Gly-LHRH, LHRH-A] was kindly donated by Zeneca Pharmaceuticals Divisione Farmaceutici (Milan, Italy). PTX and GTP were purchased from Sigma Chemical Co. (St. Louis, MO). 32P-NAD was obtained from NEN Life Science Products (Boston, MA).
Cell cultures

The cells line LNCaP-FGC (Lymph Node Carcinoma of the Prostate-Fast Growing Colony) and DU 145 were obtained from American Type Culture Collection (Rockville, MD). Both cell lines (passages 27-35 for LNCaP and 60-70 for DU 145) were routinely grown in RPMI-1640 medium (Seromed, Biochrom KG, Berlin, Germany) supplemented with FBS (Life Technologies, Paisley, Scotland) (10% for LNCaP and 5% for DU 145 cells), glutamine (1 mM) and antibiotics (100 IU/ml penicillin G sodium, 100 μg/ml streptomycin sulfate) in a humidified atmosphere of 5% CO2-95% air. In these culture conditions, the duplication period is 48 h for LNCaP and 36 h for DU 145 cells, respectively.

Animals

Because of the impossibility of obtaining normal human pituitary in our country, male rats have been used as positive (pituitary) and as negative (skeletal muscle) controls in Western blot analysis of LHRH receptors.

Adult male Sprague Dawley rats were obtained from Charles River Laboratories, Inc. (Calco, Como, Italy). Animals were maintained on a 14-h light, 10-h dark schedule, with standard pellet food and water available ad libitum. All rats were killed by decapitation; tissues were quickly collected and frozen at −70°C until Western blot analysis.

RT-PCR analysis of LHRH receptor mRNA

Total RNA from LNCaP and DU 145 cell lines was prepared according to a modification of the guanidinium thiocyanate/cesium chloride method (26). RNA (2 μg) from each sample was used in a RT reaction. Complementary DNA (cDNA) synthesis was performed using the Gene Amp kit (Perkin-Elmer Corp. Cetus, Norwalk, CT), with an oligo(dT)16 primer as a primer for the reverse transcriptase. Samples containing cDNAs obtained from prostate tumor cells were then amplified in a 100-μl solution containing PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 2 mM MgCl2, 0.15 μM of a pair of specific primers, and 2.5 U Taq polymerase. Amplification was carried out for 35 cycles (1-min denaturation at 94°C, 1-min primer annealing at 50°C, and 3-min primer extension at 72°C). The primers were synthesized, based on the reported sequence of the human pituitary LHRH receptor cDNA (27). The primers used were: sense 9'-GCTTGAAGCTCTGTCCTGGGA-3' (25 to 5') and antisense 5'-CCTAGGACATAGTAGGG-3' (844 to 860). These primers have been previously used by Kakar et al. (14) to detect the presence of LHRH receptor mRNA in human extrapituitary tissues. After PCR, the amplified cDNA products were separated on a 1.5% agarose gel and stained with ethidium bromide. Southern blot analysis was performed as described (28). Blots were hybridized with a synthetic 32P-labeled oligonucleotide probe (19 mer) specific for a sequence (nucleotides 392/410) of the human pituitary LHRH receptor cDNA.

Western blot analysis of the LHRH receptor

Membrane fractions were prepared from rat pituitaries and skeletal muscle, from LNCaP, and from DU 145 cells, according to the protocol reported by Karande et al. (29), with some modifications. Samples were homogenized in 10 mM Tris-HCl (pH 7.6) buffer containing 1 mM dithiothreitol on ice. The homogenates were centrifuged twice for 10 min each at 800 × g to remove cellular debris, and the resulting supernatants were centrifuged at 18,000 × g to pellet down the membrane fractions. The pellets were solubilized in RIPA buffer [50 mM Tris-HCl (pH 7.7), 150 mM NaCl, 0.8% Triton X-100, 0.8% sodium deoxycholate, 0.08% SDS, 10 mM ethylenediamine tetraacetate, 100 μM Na3VO4, 50 mM NaF, 0.3 mM phenylmethylsulfonyl fluoride, and 5 mM iodoacetic acid] and electrophoresed on 10% polyacrylamide gel under reducing conditions. Proteins were transferred onto a nitrocellulose filter, in 25 mM Tris-HCl (pH 8.3), 92 mM glycine, and 20% methanol at 30 V overnight. Filters were probed with the FIG4 monoclonal antibody raised against the human pituitary LHRH receptor (kindly provided by Dr. A. A. Karande, Department of Biochemistry, Indian Institute of Science, Bangalore, India), followed by incubation with an antimouse IgG. Antibody bound to the LHRH receptor was detected with the ECL-Western blotting detection system after a 5- to 10-min exposure to a Hyperfilm-ECL x-ray film (Amerham Pharmacia Biotech, Milan, Italy), at room temperature (30). The specificity of FIG4 antibody for the human pituitary LHRH receptor has been previously demonstrated (29).

Cell proliferation studies

LNCaP and DU 145 cells were plated at a density of 5,000 and 500 cells/cm², respectively, in 6-mm dishes in standard culture medium. Cells were allowed to attach and start growing for 3 days; the seeding media were then changed to experimental media. Cells were treated, for 7 days, with LHRH-A (1 μM) either in the absence or in the presence of PTX (25 ng/ml for LNCaP and 50 ng/ml for DU 145 cells); the medium was changed every 2 days. At the end of the treatment, cells were collected and counted by hemocytometer. The dose of LHRH-A selected for this and the following experiments has been chosen on the basis of previous papers from our laboratory analyzing the interaction between LHRH and stimulatory growth factors in prostate cancer cells (31, 32).

ADP-ribosylation

ADP-ribosylation was carried out as described (33). Briefly, isolated plasma membranes (0.5 mg/ml) from LNCaP and DU 145 cells were incubated with FITA (2 μg/ml) in 20 mM Tris-HCl, pH 7.5, containing 1 mM ATP, 0.1 mM GTP, 1 mM EDTA, 1 mM dithiothreitol, 10 mM thymidine, 10 μM [32P]-NAD (5 × 10⁶ cpm/nmol), either in the absence or in the presence of LHRH-A (1 μM), in a final vol of 200 μl. After incubation for 30 min at 37°C, the reaction was terminated by the addition of 1 ml ice-cold 20 mM Tris-HCl, pH 7.5. Membranes were pelleted by centrifugation and washed twice with the same buffer. Membrane proteins were solubilized in Laemmli’s SDS sample buffer and resolved by 12% PAGE. After electrophoresis, gels were dried, and ADP-ribosylated proteins were detected by autoradiography.

Western blot analysis of Goα11 subunit protein

The polyclonal rabbit antiserum IIC, directed toward a synthetic peptide corresponding to aminos acids 160–169 (only present in the Goα11 subunit) was kindly donated by C. Milligan (Division of Biochemistry and Molecular Biology, University of Glasgow, Glasgow, Scotland, UK) (34). Membrane preparations, protein separation, and electrophoretic transfer to nitrocellulose filters were performed as described under Western blot analysis of LHRH receptors. Blots were incubated overnight at 4°C in TBST buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) containing 5% powdered skim milk. After five washes with TBST, membranes were incubated for 3 h at room temperature with IIC primary antibody diluted in milk/TBS and for 1.5 h with horseradish peroxidase-conjugated goat antirabbit IgG. Proteins were detected using the SuperSignal detection kit (Pierce Chemical Co., Rockford, IL).

cAMP determination

LNCaP and DU 145 cells were plated at a density of 30,000 cells/cm² in 24-multiwell plates. After 2 days, cells were washed with 1 ml serum-free medium, pretreated with 3-isobutyl-1-methylxantine (0.5 mM) (Sigma Chemical Co.) for 15 min at 37°C and then treated with forskolin (FSK, 5 μM), either alone or in the presence of LHRH-A (1 μM), for 15 min at 37°C. After the treatment, cells were extracted with ethanol 65% at 4°C overnight. The supernatants were collected, dried, and stored at −20°C. cAMP content in each sample was determined by the 3H-cAMP assay system (Amerham Pharmacia Biotech), according to the manufacturer’s instructions.

Statistical analysis

The data from experiments involving cell proliferation and cAMP determination were analyzed according to Dunnnett’s test (35) after one-way ANOVA.

Results

Expression of the LHRH receptor

The results obtained, by RT-PCR with the appropriate probes, demonstrate that the predicted 885-bp cDNA frag-
ment (Fig. 1A) was obtained both in LNCaP (lane 1) and in DU 145 (lane 2) cells. Negative control samples without RT did not give rise to any detectable product (lane 3), ruling out the possibility of genomic DNA contamination. Gels were Southern blotted and hybridized with a synthetic $^{32}$P-labeled oligonucleotide probe specific for the pituitary LHRH receptor cDNA. The cDNA fragments obtained from LNCaP and DU 145 cells specifically hybridized with the labeled probe (Fig. 1B, lanes 1 and 2). No hybridization signal was found in the negative control (Fig. 1B, lane 3).

**Molecular size of the LHRH receptor protein**

The presence of a protein corresponding to the LHRH receptor has been investigated by Western blotting in prostate tumor cells by means of the F1G4 monoclonal antibody specifically raised against the human pituitary LHRH receptor. As shown in Fig. 2, a major protein band of approximately 64 kDa molecular mass is identified by the antibody both in LNCaP and in DU 145 cells (lanes 1 and 2, respectively). This molecular size corresponds to that reported for the human pituitary LHRH receptor (29, 36). Fig. 3 (lanes 3 and 4) confirms the presence of a 64-kDa protein binding the F1G4 antibody in prostate cancer cell lines. Moreover, Fig. 3 (lane 1) also shows that, in rat pituitary protein preparations, the antibody identified a band of approximately 60 kDa; this molecular mass corresponds to that previously described by Wormald *et al.* (36) for the rat pituitary LHRH receptor. No binding of the F1G4 antibody to protein preparations from rat skeletal muscle samples could be detected (Fig. 3, lane 2).

**Effect of PTX on the antiproliferative action of LHRH-A**

Preliminary experiments performed in our laboratory had indicated that the treatment of prostate cancer cells with LHRH-A did not affect phosphoinositide turnover or intracellular Ca$^{2+}$ levels (unpublished observations), suggesting that, in these cells, LHRH receptors might not be coupled to the Go$_{s/11}$-phospholipase C system as occurs at the pituitary level. Therefore, we hypothesized that, in these cells, the antiproliferative action of LHRH agonists might be mediated by the Go$_{i}$-cAMP signal transduction pathway. It is known that PTX, through ADP-ribosylation of Go$_{i}$ proteins, impairs the receptor-effector interaction (37). Therefore, we have
studied whether PTX might interfere with the antimitogenic action of LHRH-A. As expected, the LHRH agonist significantly inhibited LNCaP cell growth (Fig. 4A), around 30% in the experiments here reported. In previous studies, we have demonstrated that this antiproliferative action is specific, because it can be blocked by a second-generation antagonist (Nal-Arg-LHRH) (18, 19). More recently, third-generation antagonists have been reported to act as agonists in this and in similar experiments (38, 39). Fig. 4 also shows that the treatment with PTX alone did not affect cell proliferation. On the other hand, when PTX and LHRH-A were given together, PTX completely prevented the antiproliferative action of the LHRH agonist (Fig. 4A). Similar results were obtained in DU 145 cells (Fig. 4B).

Effect of LHRH-A on PTX-induced ADP-ribosylation

The preceding observation that PTX significantly counteracts the antiproliferative action of LHRH-A suggests that the LHRH agonist might act through a Goi protein. To further confirm this hypothesis, we investigated whether LHRH-A might affect PTX-induced ADP-ribosylation of the Goi protein. As expected, incubation of prostate cancer cell membranes with PTX, in the presence of 32P-NAD, brought about ADP-ribosylation of a 41-kDa Goi protein in both LNCaP and DU 145 cells (Fig. 5, A and B; lane 1). LHRH-A substantially counteracted the transfer of 32P-ADP-ribose to the Goi protein in the two cell lines (Fig. 5, A and B; lane 2).

Detection of Goi proteins

The presence of Goi subunit proteins in prostate cancer cells was evaluated by Western blot analysis. Fig. 6 shows that, after immunoblotting with the specific antibody, the 41-kDa Goi1 subunit could be detected in LNCaP as well as in DU 145 cells and suggests that this specific Goi protein subunit might mediate LHRH activity in prostate cancer cells. In these studies, Western blot analysis has been performed with an antibody specifically recognizing the 41-kDa Goi1 protein subunit. Obviously, the possibility that additional Goi subunits might be present (and/or coupled to the LHRH receptor) in prostate cancer cells cannot be ruled out.

It is not surprising to find that the amounts of Goi1 protein seem different in LNCaP and in DU 145 cells. This and other G proteins are not exclusively linked to the LHRH receptor; they may represent the beginning of the signaling pathways for other growth regulatory mechanisms that have not been investigated in the present study.

Effects of LHRH-A on cAMP accumulation

Activation of Goi subunit proteins is negatively correlated with cAMP production. To further confirm that, in prostate cancer cells, LHRH receptors might be coupled to Goi proteins, we studied the effects of LHRH-A on FSK-induced cAMP accumulation, both in LNCaP and in DU 145 cells. In LNCaP cells (Fig. 7A), LHRH-A, when given alone, did not affect cAMP levels. FSK, as expected, substantially stimulated cAMP accumulation. LHRH-A significantly counter-
acted the increase in cAMP levels induced by FSK, an effect which could be blocked by a second-generation antagonist (Nal-Arg-LHRH, $10^{-8}$ m) (data not shown). Similar results were obtained in DU 145 cells (Fig. 7B).

Discussion

The properties of LHRH receptors have been analyzed in two prostate tumor cell lines (one androgen-dependent, LNCaP, and the other one androgen-independent, DU 145) by evaluating their mRNA expression, their molecular size, and their signal transduction pathway(s).

The data obtained show, by RT-PCR, that a mRNA coding for the LHRH receptor can be detected in LNCaP as well as in DU 145 cells. In line with this observation, the expression of the mRNA for LHRH receptors has been previously reported in some peripheral tumors, such as breast, endometrial, and ovarian cancers (13–17).

In LNCaP and in DU 145 cells, the mRNA is further translated into a receptor protein. This is based on the observation that both cell lines, when analyzed by Western blot using a monoclonal antibody raised against the human pituitary LHRH receptor, reveal the presence of a protein of the same molecular size of the receptor found in normal human gonadotrophs (64 kDa) (29, 36).

The present data, obviously, do not exclude that the gene coding for this protein might have undergone a mutation and that, consequently, the protein, even with the same molecular size, might present some deviation from the classical receptor. This would be in line with the reports from us and from others showing that the binding affinity of the receptor in prostate and in other tumoral tissues might be significantly lower than that observed in the pituitary (18, 19, 22, 40, 41).

Because of the findings by Kakar et al. (14) and those here reported, which have used the same methodology, one would expect the eventual mutation to reside outside the 885-bp fragment amplified by Kakar et al. (14) and in the present work.

Some doubts have been raised on the ability of the antibody used in the experiments here reported to recognize the authentic LHRH receptor protein. This argument may be counteracted by the following considerations. First, this antibody fully recognizes the 29-amino acid peptide, corresponding to the N-terminal portion of the LHRH receptor protein, which has been used to raise it (29). Moreover, in immunohistochemical studies, the antibody stains clusters of cells in the human pituitary. This staining is specific, because the reaction can be stopped by a preincubation of the antibody with an excess of the antigenic peptide before incubation with the tissue section (29). By Western blot analysis, the antibody will recognize the typical 64-kDa band of the LHRH receptor in human pituitary (29), as well as in human placenta and in breast carcinoma (A. A. Karande, personal communication). The same band will also bind a $^{125}$I-labeled LHRH, showing its ability to act as a full receptor. Finally, by immunohistochemistry, COS-7 cells transfected with the LHRH receptor cDNA show a specific positive staining to the monoclonal antibody (A. A. Karande, personal communication).

At the pituitary level, LHRH receptors are coupled, via the $G_{o1}$ group of G proteins, to PLC (5–7). The data here reported indicate that, in prostate tumor cells, the LHRH receptor is linked to $G_{o}$ proteins which, through the inhibition of cAMP accumulation, probably mediate the antiproliferative action of the peptide. These conclusions are
based on the following observations: 1) treatment with PTX counteracts the antiproliferative action of LHRH agonists; 2) LHRH agonists cause a profound reduction in the PTX-mediated ADP-ribosylation of a membrane protein of relative molecular mass 41 kDa corresponding to $G_{a1}^i$, as revealed by Western blot analysis, performed using a specific antibody; 3) LHRH agonists significantly counteract FSK-induced increase of intracellular cAMP levels. In the authors’ opinion, this is the first demonstration of the coupling of the LHRH receptor to the $G_{a1}^i$-cAMP signal transduction pathway in prostate cancer, in addition to the identification of the molecular size of the LHRH receptor protein (see above). In line with the present data, Imai and co-workers (42) have recently reported that, in tumors of the human female reproductive tract, LHRH receptors are coupled to a 41-kDa $G_{a1}^i$ protein; these authors suggested that this protein might mediate the LHRH-induced phosphotyrosine phosphatase activity in tumor cells (42, 43).

We failed to observe any change in phosphoinositide metabolism and/or $Ca^{2+}$ levels in prostate cancer cells after the treatment with LHRH agonists (unpublished observations), which inhibit cell proliferation; this suggests that the LHRH receptor might not be linked to the $G_{a11}$-PLC system in these cells. In agreement with our observations, Emons and co-workers (44) have shown that, in human ovarian and endometrial cancer cells, the LHRH agonist triptorelin, at concentrations that are clearly inhibitory on cell proliferation, does not affect PLC or PKC activity. However, at variance with these observations, LHRH agonists have been reported to stimulate PLC activity in other types of tumors, such as rat (45) and human (46) mammary tumors, as well as human ovarian cancers (47). Therefore, the possible involvement of the $G_{a11}$ signaling system in the antimitogenic action of LHRH still remains an open question and, certainly, needs further studies.

Taken together, the present data strongly indicate that, in prostate tumor cells, the LHRH receptor is expressed and corresponds to the pituitary LHRH receptor, in terms of molecular weight. Interestingly, the signal transduction pathway of this receptor ($G_{a1}^i$-cAMP pathway) seems to be different from that of the same receptors at pituitary level ($G_{a11}$-PLC system) (Fig. 8). This fact might be responsible for the different actions of LHRH in peripheral tumors and in the anterior pituitary. In gonadotrophs, LHRH receptor-coupled $G_{a11}$ proteins mediate the stimulatory action of the hypothalamic hormone on gonadotropin synthesis and release (5–7). In prostate cancer cells, on the contrary, LHRH may behave as an inhibitory autocrine/paracrine factor, which exerts its antimitogenic action through the activation of $G_{a1}^i$ proteins, negatively coupled to the c-AMP intracellular signaling pathway. It is noteworthy, in this context, that LHRH receptor mRNA, molecular size, and signal transduction pathway seem to be the same in androgen-dependent and androgen-independent prostate cancer cells.

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References


