

THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

**Expression of human type-I LHRH receptors and type-I LHRH in
human benign prostatic hyperplasia**

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Introduction

Benign prostatic hyperplasia (BPH) is the nonmalignant enlargement of the prostate resulting from over-proliferation of the stromal and glandular elements of the prostate. BPH is a disease of the ageing male population and a frequent cause of bothersome lower urinary tract symptoms (LUTS), which can impact on the patient's quality of life (QoL). Although the incidence of BPH-related mortality is very low ($< 0.5/100,000$), this disease significantly affects the QoL with an estimated 25% of the elderly needing surgical treatment. It is one of the most common conditions affecting men, with incidence increasing dramatically after the age of 50 years. The incidence is $\sim 70\%$ at 70 years of age and becomes nearly universal with advancing age. LUTS is associated with obstructive/voiding symptoms (e.g., weak stream, hesitancy, intermittency and terminal dribbling) and irritative/storage/filling symptoms (e.g., frequency, nocturia, urgency, urge incontinence and possibly dysuria), which adversely affect the QoL. The pathogenesis of clinical BPH typified by LUTS is multifactorial and poorly understood.

The nodular enlargement of the prostate (benign prostatic enlargement [BPE]) physically impinges on the free flow of fluids through the male urethra and leads to a varying degree of bladder obstruction (bladder outlet obstruction [BOO]). This component has been referred to as the static component, which represents a mechanical obstruction of the bladder neck by the prostate and is characterised by increased cell proliferation and/or impaired apoptosis. Surgery and several therapeutic interventions target the static component to treat BPH. In addition to the static component, the dynamic component, due to the contractile properties of the prostate, bladder neck and urethra, is also involved. This component contributes to $\sim 40\%$ of all outflow obstruction. The tension in smooth muscle is maintained by the autonomic nervous system via $\alpha 1$ -adrenoceptor-mediated signalling. The antagonism at $\alpha 1$ -adrenoceptors relaxes the smooth muscle tone-regulated dynamic component and this forms the basis for the development of $\alpha 1$ -blockers for the treatment of BPH and associated LUTS. The treatment options for symptomatic BPH

include medical treatment and surgical interventions. The aim of all of the treatment modalities lies in maintaining unobstructed urine flow, thereby improving the QoL.

(Thus, the overall disease process that leads to the production of symptomatic BPH is very complex.) Today, medical therapy is the preferred first-line approach to treating BPH because of the probability of clinical improvement and the patients' concern about surgery or other invasive treatments. Alpha 1 adrenergic receptor blockers and 5- α reductase inhibitors are the two classes of drugs currently approved by the FDA for the treatment of symptomatic BPH, but these drugs do not offer long term remission of LUTS after discontinuation and can be used only in a selected population of patients.

Testosterone production is controlled by certain higher brain centres in which the hypothalamus and pituitary are of paramount importance. Pulsatile release of gonadotropin-releasing hormone (GnRH), also known as luteinising hormone (LH)-releasing hormone (LHRH), stimulates the pituitary secretion of LH and follicle-stimulating hormone (FSH), which subsequently control the hormonal and reproductive function of the gonads. Therefore, among other antiandrogen approaches, targeting the upper brain centres was sought as an option to suppress the androgen-stimulated growth of the prostate. These approaches involve LHRH agonists and antagonists.

The hypothalamic decapeptide luteinizing hormone-releasing hormone (LH-RH; Gln-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH₂), also called gonadotropin-releasing hormone (GnRH), was isolated from hypothalamic extracts and its amino acid sequence was established by Andrew V. Schally and his colleagues (1971) (Schally's group was the first to accomplish its isolation, elucidation of structure and synthesis.).

Most vertebrates express at least two LHRH isoforms. In the human genome only LHRH-I (mammalian LHRH) and LHRH-II (chicken LHRH-II) genes were identified. LHRH-II has been likewise found in tumors such as breast carcinoma, but the functional receptor for LHRH-II is absent in humans. The human LHRH-I gene is composed of four exons separated by three introns and is present as a single gene copy on chromosome 8p11.2-p21. Three LHRH receptors or receptor-like genes were demonstrated earlier. The well-

established type-I LHRH receptor (LHRH-R-I) gene has been identified as a single copy on chromosome 4q21.2 and is composed of three exons separated by two introns . A vast literature indicates that the LHRH-I is expressed not only in the hypothalamus but also in peripheral tissues, both normal and tumoral. However, only limited findings are available on the presence of full length LHRH-R-I and its splice variants or transcript forms in human malignant and benign tissues. The LHRH-R-I belongs to the G protein-coupled receptor (GPCRs) family with seven transmembrane domains (TMs) connected by extracellular loops (ECLs) and intracellular loops (ICLs). LHRH-R-I is expressed on the surface of pituitary gonadotrope cells. This receptor is characterized by the unique absence amongst GPCRs of a carboxyl-terminal tail. LHRH-R-I has much higher affinity for LHRH-I than for LHRH-II. Based on their ligand binding properties LHRH receptors appear to be quite similar in the human pituitary, human normal extrapituitary tissues and human cancers, but their signalling pathways are different. In the past 25 years several thousand LHRH analogs have been synthesized. Many analogs were shown to have important clinical applications in gynecology, oncology and urology.

LHRH agonists for the management of BPH were conceptualized on the fact that continuous LHRH administration causes an initial gonadotropin hypersecretion (known as disease flare up), which is rapidly followed by pituitary desensitisation and hence arrest of gonadotropin release. The mechanism is not well understood, except that receptor downregulation may play a central role. The synthetic LHRH agonists were successful over native LHRH in terms of their prolonged half-life and bioavailability due to increased lipophilicity. All seven agonists (leuprorelin, buserelin, goserelin, histrelin, deslorelin, triptorelin and nafarelin) have been approved for prostate cancer, endometriosis or breast tumour; however, none of the agonists have been indicated for the treatment of BPH. The reason lies in the differences in the efficacy:tolerability ratio for patients suffering from BPH versus prostatic cancer, in which survival is recognised as a key end point and a difference in outcome of only 2 – 3% may be of considerable clinical importance.

LHRH antagonists are competitive antagonists of the LHRH receptor, which cause an immediate and rapid, reversible suppression of gonadotropin secretion. Unlike agonists, LHRH antagonists do not induce an initial stimulation of gonadotropin release (clinical flare up). The drug designing for LHRH antagonists was more challenging compared with the synthesis of agonists; the frequent failures occurred due to insufficient potency, lack of solubility and unacceptable anaphylactic reactions due to the increased histamine-releasing activity. These limitations were overcome in the third-generation antagonists such as Cetrorelix (ASTA Medica AG), abarelix and ganirelix.

Cetrorelix is a decapeptide which was originally synthesized at Tulane University, New Orleans, USA, Bokser et al. (1990). Cetrorelix has a highly modified LHRH sequence, comprising 10 amino acid, five out of which are in a non-natural D-configuration. The C- and N-terminal protecting groups (acetyl, amide) provide stability and are mandatory for complete antagonistic activity.

The administration of LHRH antagonists results in the suppression of serum LH (51 – 84%) and FSH (17 – 42%) levels within 8 – 24 h after initial dose. As FSH has been implicated as an additional factor in prostate overgrowth, the significant long-term (≤ 169 days) suppression of FSH is an additional advantage over agonists.

Cetrorelix and teverelix (Ardana) are indicated for the treatment of BPH, and are in Phase II clinical trials for BPH. In a randomised, double-blind, placebo-controlled clinical trial, Cetrorelix 5 mg b.i.d. for 2 days followed by 1 mg b.i.d. for 2 months resulted in a rapid reduction in prostate volume (~ 27%) and a 53% reduction of symptoms with improvement in the QoL.

Cetrorelix is one of the potent modern LHRH antagonists that induce an immediate inhibition of the pituitary–gonadal axis and is the only LHRH antagonist that has been extensively studied in men with BPH. Accumulating clinical experience with Cetrorelix in men with BPH has shown that this drug is associated with a dose-dependent improvement of International Prostate Symptom Score (IPSS) and a reduction in prostate volume. Recent studies show that the action of Cetrorelix on BPH is likely mediated through LHRH receptors and involves inhibitory effects on growth factors as well as down-

regulation of α -1A adrenergic receptors and LHRH receptors. Human prostate and prostate cancer specimens have been shown to express LHRH receptors. These observations indicated the need for a more thorough investigation of LHRH receptors in surgical specimens of human BPH.

Aims

I. Our aim was to investigate the expression of mRNA for human type-I LHRH ligand and human type-I LHRH receptor and its transcript forms

- in specimens of human BPH and
- in human pituitary using as a positive control by RT-PCR.

II. We evaluated the localization of the LHRH receptor protein

- in BPH specimens obtained from surgery or transurethral resection (TUR) and
- in human pituitary using as a positive control obtained from autopsy by immunohistochemistry.

III. We evaluated the characteristics of the LHRH receptor protein in BPH specimens obtained from surgery or transurethral resection (TUR) by RIA.

Materials and methods

1. Tissue Samples From Patients

Human BPH specimens were obtained from patients 50–83 years of age at the time of initial open surgical treatment or TUR at Veterans Affairs Medical Center, New Orleans, LA; Department of Urology, University of Debrecen, Debrecen, Hungary and Department of Urology, University of Miami, Miami, FL. The local Institutional Review Boards approved the collection and use of these specimens for the current study. All analyses were first conducted to meet the primary clinical requirement for patient management, and only residual tissue was used for this study. After surgical removal, select portions of the

prostate tissues were flash-frozen in liquid nitrogen and transported on dry ice. Histopathological examination of each specimen was undertaken to confirm the presence of BPH before the molecular biological studies. The intact specimens and their membrane fractions were stored at -80°C until analyses of LHRH binding sites and molecular biology studies were carried out.

2. RNA isolation and RT-PCR

Tissues were homogenized with a Mikro-Dismembrator-U (*Sartorius B.Braun Biotech, Melsunge, Germany*) and RNA was extracted with Nucleospin Total RNA Isolation Kit (*Macherey-Nagel, Germany*). RNA concentration and purity were determined using the Nanodrop ND-1000 UV Spectrophotometer (*Nanodrop Technologies, Wilmington, DE, USA*). Total RNA (100-250 ng) was reverse transcribed to cDNA with MMLV Reverse Transcriptase (*Promega Co., Madison, WI, USA*) according to the manufacturer's instructions. For amplification from first strand cDNA gene-specific primers for LHRH-R-I (forward) F1/ (reverse) R1: F1:5'-GACCTTGCTGGAAAGATCC-3' (exon 1 1844-1863) and R1: 5'-TGATGGTGGTGATCAGCCTG-3' (exon 1 2143-2162), LHRH-R-I F2/R3: F2: 5'-AGC AGACAGCTCTGGACAGACAAAA-3' (exon 2 2300-2322) and R3: 5'-TGTCTGCTGGACTCCCTACTATGT-3' (exon 3 2582-2605), for LHRH-I: sense 5'-CTACTGACTTGGTGCGTGGA-3' and antisense: 5'-CTGCCCAGTTTCCTCTTCAA-3' and beta actin (bACT) housekeeping gene F3/R4: F3: 5'-GGCATCCTCACCTGAAGTA-3' (exon 3) R4: 5'-GGGGTGTGAAGGTCTCAA-3' (exon 4) were described earlier. Primers for LHRH-R-I F1'/R2': F1': 5'-TAGTGTCTTTGCAGGACCACA-3' (exon 1 2262-2273) and R2': 5'-AATCATCTTCACCCTGACACG-3' (exon 2 2460-2480) were designed using Primer3 (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). For LHRH-R-I F1'/R1, LHRH-R-I F1'/R2' and bACT, the PCR reaction mixture contained 2 µl cDNA, 1.5 mM MgCl₂, 0.5 µM of each primer (*Invitrogen*), 1X PCR buffer, 200 µM of each dNTP, and 1 U Taq Polymerase (*Invitrogen*) in a final volume of 25 µl. After denaturation (3 min at 94°C) cDNA was amplified for 45 cycles (45 sec at 94°C; 30 sec at 60°C; and 90 sec at

72°C), bACT was amplified with 30 cycles, then a final elongation step at 72°C for 10 min was applied. For LHRH-R-I F2/R3 and LHRH-I the PCR reaction mixture contained 2 µl cDNA, 3 mM MgCl₂, 0.5 µM of each primer (*Invitrogen*), 1X PCR buffer, 200 µM of each dNTP, and 1 U Taq Polymerase (*Invitrogen*) in a final volume of 25 µl. After denaturation (3 min at 94°C) cDNA was amplified for 45 cycles (45 sec at 94°C; 30 sec at 66°C; and 90 sec at 72°C) and then a final elongation step at 72°C for 10 min was applied. PCR products were separated electrophoretically on 1.5% agarose gel and stained with ethidium bromide.

3. Immunohistochemistry

Human BPH specimens from 10 patients were collected at the Department of Urology, University of Debrecen, Debrecen, Hungary. Approximately 8-10 mm³ of each tissue was used for molecular biology studies. Two normal pituitary samples were collected at autopsy from the Department of Pathology, University of Debrecen and were used as positive controls. Immediately after removal, the tissue samples were fixed in 4 % buffered formalin (24 h) and embedded in paraffin wax. Sections 2-3 µm thick were cut the type of MICROM HM 335 E microtome from both the the formalin-fixed, paraffin-embedded tissue samples on silanised (APES, 3-aminopropyltriethoxysilane) slides. The tissue sections were either incubated high temperature at high pressure (in a pressure cooker) for 3 min in 0.1 M citrate buffer pH 9.0 (*Target Retrieval Solution, pH 9 10X Dako*). For the immunreaction was using Bond TM automated system and Bond TM Polymer Refine Detection Kit according to the manufacturer's instructions (*NovoCastr Visionbiosystems Bond TM, Novocastra Laboratories Ltd.*). The tissue sections were incubated Gonadotropin-Releasing Hormone Receptor mouse monoclonal antibody (*NCL-GnRHR A9E4 clone Novocastra Laboratories Ltd.*). The antibody raised in mouse targeted the human Gonadotropin-Releasing Hormone Receptor terminal region. The specificity of the 1-29 NH₂ terminal region of GnRHR was checked using the basic local alignment search tool (BLAST) at the National Center for Biotechnology Information (NCBI), National Library of Medicine (<http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/nph->

[newblast](#)). The BLAST protein database search program indicated that the amino acid sequence 1-29 of human GnRHR does not show any structural homology with other human pituitary hormones and pituitary hormone receptors. The antibody dilution range was 1:20. The immunoreaction was visualised using a diamino-benzidine (DAB) kit (*Vector Laboratories, CA, USA*). In most of the cases, counterstaining (haematoxylin) was also performed. All immunoreactions were investigated using a Olympus BX 51 microscope and Olympus DP50 digital camera (*Olympus Europe, Hamburg, Germany*) connected to a computer. The microscope used the following objective lenses: Plan 4X/0.1 numerical aperture (NA), Plan 10X/0.25 NA, Plan 40X0.65 NA, and Plan FI 100X1.30 NA (oil).

4. Preparation of Membranes and Radioligand Binding Studies

Peptides and Chemicals: LHRH antagonist [D-Trp⁶]LHRH was supplied by Debiopharm S.A. (*Lausanne, Switzerland*). LHRH antagonist, Cetrorelix, first synthesized at Tulane University, New Orleans, USA, Bokser et al. (1990) and was supplied by Aeterna Zentaris GmbH (*FrankfurtamMain, Germany*). Radioisotope ¹²⁵I-labeled sodium was purchased from Amersham Pharmacia Biotech (*Piscataway, NJ*). All other chemicals were obtained from Sigma (*St. Louis, MO*), Bachem (*Torrance, CA*), R&D Systems (*Minneapolis, MN*), or California Peptide Research, Inc. (*Napa, CA*).

Preparation of membranes for receptor studies was performed as described previously. Briefly, the samples were thawed and cleaned, and then homogenized in 50mM Tris-HCl buffer (pH 7.4), supplemented with protease inhibitors (0.25mM phenylmethylsulfonyl fluoride, 0.4% (v/v) aprotinin, and 2 mg/ml pepstatin A) using an Ultra-Turrax tissue homogenizer (*IKA Works, Wilmington, NC*) on ice. The homogenate was centrifuged at 500g for 10 min at 4°C to remove nuclear debris and lipid layer. The supernatant containing the crude membrane fraction was ultracentrifuged (*Beckman L8-80 M*) twice at 70,000g for 50 min at 4°C after resuspending in fresh buffer. The final pellet was resuspended in homogenization buffer and stored at -80°C until assayed. Protein

concentration was determined by the method of Bradford using a Bio-Rad protein assay kit (*Bio-Rad Laboratories, Hercules, CA*). Radioiodinated derivatives of [D-Trp6]LHRH were prepared by the chloramine-T method and purified by reverse-phase HPLC in our laboratory. LHRH receptor binding assays were carried out as reported using in vitro ligand competition assays based on binding of [¹²⁵I][D-Trp6]LHRH as radioligand to BPH membrane fractions. This radioligand has been well characterized previously and shows high-affinity binding to human and rat pituitaries and human breast, prostate, and other cancers. In brief, membrane homogenates containing 50–160 mg protein were incubated in duplicate or triplicate with 60–80,000 cpm [¹²⁵I][D-Trp6]LHRH and increasing concentrations (10^{-12} - 10^{-6} M) of non-radioactive peptides as competitors in a total volume of 150 ml of binding buffer. At the end of the incubation, 125 ml aliquots of suspension were transferred onto the top of 1ml of ice-cold binding buffer containing 1.5% bovine serum albumin in siliconized polypropylene microcentrifuge tubes (*Sigma*). The tubes were centrifuged at 12,000g for 3 min at 4°C (*Beckman J2-21M*). Supernatants were aspirated and the bottoms of the tubes containing the pellet were cut-off and counted in a gamma counter (*Micromedic System, Huntsville, AL*). Preliminary experiments were performed with membrane protein concentrations ranging from 20–250 mg/tube in order to determine the minimal amount of protein required to assess specific binding at a satisfactory level. Our work showed that accurate results can be obtained over a range of 40–180 mg membrane protein in an incubation volume of 150 ml.

5. Data Analysis

Specific ligand-binding capacities and affinities were calculated by the Ligand-PC computerized curve-fitting program of Munson and Rodbard. To determine the type of receptor binding, equilibrium dissociation constants (K_d values), and the maximal binding capacity of receptors (B_{max}), LHRH binding data were also analyzed by the Scatchard method.

Results

Molecular Biology Analysis

To investigate whether high-affinity binding sites for LHRH, present in the membranes of human BPH tissue, are the products of mRNA expression of type-I LHRH receptors and type-I LHRH ligand, we performed RT-PCR analysis on all samples collected for the study. Negative controls yielded no detectable signals, indicating that PCR products were generated from cDNA and not from genomic DNA. PCR amplification with b-actin-specific primers produced a single product from all samples, confirming that there was no degradation in the RNA preparation.

1. Expression of mRNA for human type-I LHRH receptors in human benign prostatic hyperplasia

RT-PCR analysis was carried out to investigate the expression of different LHRH-R transcript forms in human BPH tissue samples. We used LHRH-R-I F1/R1 primers encompassing the open reading frame (ORF) from exon 1 to exon 1, LHRH-R-I F2/R3 from exon 2 to exon 3 and LHRH-R-I F1'/R2' primers from exon 1 to exon 2. The predicted size of the PCR amplified LHRH-R F1/R1 cDNA was 319 bp for the full length transcript (sb1), for the partial exon 2-deleted variant (sb2) and for the exon 2-lacking (sb3). The predicted size of the PCR-amplified LHRH-R F2/R3 cDNA was 309 bp for the full-length transcript (sb1). The predicted size of the PCR-amplified LHRH-R F1'/R2' cDNA was 219 bp for the full-length transcript (sb1) and 91 bp for the partial exon 2-deleted variant (sb2). PCR products for LHRH-R-I F1/R1 were detected in 39 of 55 specimens examined (71%) human BPH specimens.

PCR products for LHRH-R-I F2/R3 were detected in 5 of 35 (14%) BPH specimens. We were not able to demonstrate PCR products for LHRH-R-I F1'/R2' in any of the BPH samples investigated. Although LHRH-R-I transcripts have been previously detected in extensive series of human tissues, in our study we were not able to find the mRNA for the full length LHRH-R-I by RT-PCR. PCR amplification with human bACT gene specific

primers generated a single product of the expected size of 203 bp in all samples investigated.

2. Expression of mRNA for human type-I LHRH receptors in human pituitary tissue.

In human pituitary tissue samples, used as a positive control, we were able to detect the mRNA for LHRH-R-I transcript forms with all the three primer sets applied in our study. We could not detect the partial exon 2-deleted variant (sb2) using LHRHR-I F1'/R2' primers in the human pituitary samples investigated. We did not use specific primers to detect the LHRH-R-I Sb3 that is also a variant lacking exon 2.

3. Expression of mRNA for human type-I LHRH ligand in human benign prostatic hyperplasia and human pituitary tissue.

To examine the mRNA expression of LHRH-I ligand, we also performed RT-PCR analyses on 35 samples of human BPH obtained from the University of Debrecen. In 18 of 35 specimens examined (51%), RT of RNA followed by PCR amplification with specific primers produced a fragment of expected size (230 bp) for LHRH-I.

In human pituitary tissue samples, used as a positive control, we were able to detect the mRNA for LHRH-I in all human pituitary tissue samples.

4. Immunohistochemistry of LHRH Receptors in human pituitary tissue and benign prostatic hyperplasia

In human pituitary tissue samples, used as a positive control, we were able to detect the LHRH-R-I with immunohistochemistry. The acidophil cells of adenohypophysis demonstrated strong LHRH-R-I specific immunolabelling.

Altogether 10 human BPH specimens were obtained and examined by immunohistochemistry. A positive reaction in the form of brown granules was observed

with antibody in the stromal smooth muscle cells of BPH. All BPH specimens stained expressed the LHRH receptor at a moderate level. The positive immuno-staining of LHRH receptors in BPH specimens was not related to the patients' age or other clinical and pathological findings.

5. Radioligand Binding Studies

The presence of specific LHRH binding sites and characteristics of binding of [¹²⁵I][D-Trp6]LHRH to membrane receptors on human BPH tissue were determined using ligand competition assays. Of the 20 specimens examined, 18 showed LHRH receptor binding (90%). Analyses of the typical displacement of radiolabeled [D-Trp6]LHRH by the same unlabeled peptide revealed that the one-site model provided the best fit, indicating the presence of one class of high-affinity LHRH receptors in crude membranes derived from human BPH specimens. The computerized nonlinear curve-fitting and the Scatchard plot analyses of the binding data in 18 receptor positive BPH specimens indicated that the single class of binding sites had a mean dissociation constant (K_d) of 4.04nM (range, 1.09–7.26 nM) with a mean maximal binding capacity (B_{max}) of 527.6 fmol/mg of membrane protein (range, 344.7–900.2 fmol/mgprotein). Biochemical parameters essential to establish the identity of specific binding sites were also determined. Thus, the binding of [¹²⁵I][D-Trp6]LHRH was found to be reversible, time- and temperature-dependent, and linear with protein concentration in human BPH specimens examined (data not shown). The specificity of LHRH binding was demonstrated by competitive binding experiments using several peptides structurally related or unrelated to LHRH. The binding of radiolabeled [D-Trp6]LHRH was completely displaced by increasing concentrations (10^{-12} – 10^{-6} M) of LHRH agonist buserelin and LHRH antagonist Cetrorelix. Cetrorelix displaced radiolabeled [D-Trp6]LHRH with an IC_{50} value of 0.94 ± 0.07 nM indicating its high affinity binding to LHRH receptors expressed in BPH tissues. None of the structurally and functionally unrelated peptides tested such as somatostatin-14, human growth hormone-releasing hormone, EGF, [Tyr4]bombesin and

insulin-like growth factor-I inhibited the binding of [¹²⁵I][D-Trp6]LHRH at concentrations as high as 1 mM. The expression of mRNA for LHRH receptors was accompanied by ligand binding in all samples examined. Two of 20 BPH specimens did not exhibit mRNA expression for LHRH receptors, or show ligand binding; conversely, all receptor-positive specimens expressed a detectable amount of the receptor gene. There was no correlation between receptor binding characteristics or mRNA expression and clinical and pathological findings.

Discussion

BPH is a condition that will affect most men should they live long enough. BPH is defined histologically by hyperproliferation of stromal and epithelial cells of the prostate, caused by complex cellular alterations including changes in proliferation, differentiation, and apoptosis. However, the pathogenesis and the complex mechanism of evolution of BPH are still incompletely understood. Medical therapy is usually recommended first because of the probability of clinical improvement and the patients' concern about surgery or other invasive treatment. Inhibitors of 5 α -reductase or α 1-adrenergic receptor antagonists do not offer long-term remission of urinary symptoms after discontinuation and can be used only in a selected population of patients. The clinical application of LHRH agonists in the treatment of human prostatic carcinoma is based on their ability to suppress testosterone secretion through the desensitization of the gonadotropes, downregulation of LHRH receptors in the pituitary, and a reduction in the levels of their mRNA. A repeated administration of LHRH agonists is required to continue the suppression of LH and FSH release and the reduction in the levels of sex steroids. This is clinically achieved by the periodic administration of depot preparations of LHRH agonists. The initial testosterone "flare" may be prevented by pretreatment with estrogens or antiandrogens. The side effects of LHRH agonists, including hot flashes, loss of libido, and erectile dysfunction, are acceptable when LHRH agonists are used for treatment of metastatic prostate cancer. LHRH agonists were tested as a treatment for

BPH, but the initial shrinkage of the prostate, and improved urinary flow were reversed by 6 months after cessation of treatment. Poor efficacy and unacceptable side effects also discouraged the use of LHRH agonists for treatment of BPH. The advantage in the use of LHRH antagonists for the treatment of both BPH and prostate cancer is based on direct inhibition of LHRH receptors and their ability to thus lower LH, FSH, and sex steroid secretion immediately after a single injection, thereby reducing the time to the onset of therapeutic effect and avoiding a flare in disease. A crucial therapeutic edge in the use of LHRH antagonists instead of agonists for the treatment of BPH is the capability to better control the level of testosterone suppression. In a clinical setting, low doses of Cetrorelix or other LHRH antagonists, released from depot preparation induce only a partial and transient inhibition of the pituitary-gonadal axis without a marked down-regulation of pituitary LHRH receptors avoiding medical castration. Cetrorelix, a highly active modern LHRH antagonist is the only LHRH antagonist that has been extensively evaluated in men with BPH. The principles of effective treatment of patients with BPH have been obtained from various uncontrolled and controlled clinical trials using daily injections of Cetrorelix acetate. Gonazales-Barecena et al. reported an open label study of 11 men with BPH who received 0.5mg Cetrorelix acetate s.c. twice daily for 28 days. A significant reduction in mean prostate volume, in LUTS and in mean peak urinary flow rate was observed. These clinical outcomes were maintained well beyond the 1-month active treatment phase. Similar results were obtained in a second open-label study on BPH demonstrating, by subjective and objective parameters, including a lowering in IPSS, the long-term benefits of cetrorelix. Lepor et al. reported the first multicenter, randomized, placebo-controlled clinical trial evaluating the safety and efficacy of Cetrorelix acetate in men with symptomatic BPH. Debruyne et al. recently reported the results of another multicenter, randomized, double-blind, placebo-controlled study of cetrorelix gluconate in men with BPH.

All active-treatment groups experienced significant improvements in the mean AUA symptom score and mean peak flow rate relative to placebo at 12 weeks. Debruyne also carried out a study with Cetrorelix pamoate in BPH patients and obtained significant

lowering of IPSS with cetorelix, but not with placebo. The mechanism for Cetorelix-mediated improvement in LUTS associated with benign prostatic enlargement is incompletely understood. This is due in part to the fact that the pathophysiology of LUTS in the aging male is poorly understood. Ongoing studies on the mechanism of action of Cetorelix in BPH indicate that its effects are mediated through the LHRH receptor and involve inhibition of growth factors and their receptors as well as α 1-A adrenergic receptors.

We examined the expression of LHRH-R-I gene in 55 human BPH specimens. Our work represents the first demonstration of the presence of LHRH-R-I transcripts in human BPH tissue samples obtained after radical prostatectomy. We were able to detect that the transcripts of LHRH-R-I were widely distributed in human BPH samples. In our study, sequences of LHRH-R-I from exon 1 to exon 1 were amplified in 39 of 55 the specimens and sequences from exon 2 to exon 3 were detected in 14% of the human BPH tissue samples investigated. However, we could not find sequences from exon 1 to exon 2 that correspond to the full-length LHRH-R-I sequences. Our results could be explained by the fact that LHRH-R-I gene may have more than two splice variants or other, uncharacterised transcript forms. We also confirmed the expression of the LHRH-R-I gene transcripts in human pituitary samples. By alternative splicing, multiple transcripts can be generated from a single gene. This represents an important key molecular mechanism of gene regulation in physiological and pathophysiological processes. It is important to note that different transcript forms of mRNA for LHRH-R-I were expressed in human BPH specimens, but these transcripts are not the same variants lacking exon 2. Finch *et al.* suggests that LHRH-R-I splicing may be regulated in a tissue-specific manner, or under specific hormonal or metabolic conditions. Generation of protein isoforms by alternative splicing is known to occur in numerous members of the G-protein-coupled receptors family, correlating with differences in affinity, potency, coupling efficiency, specificity and sensitivity to desensitization of the receptors. Differential splicing could also have a physiological significance, as production of a short transcript was shown to downregulate the level of a wild-type (WT) transcript. Splice variants of

peptide hormone receptors can differ fundamentally from their WT receptor counterparts in pharmacological and functional characteristics, in their distribution in normal and malignant human tissues, and in their potential use for clinical applications. It is well established that regulation of splicing may provide another control mechanism in the expression of the LHRH-R-I gene in the human pituitary and extrapituitary tissues. Although LHRH-I receptor transcripts were detected in an extensive series of human tissues, including those of the breast, uterus, endometrium, prostate and ovary, we have been unable, so far, to find full length LHRH-R-I using RT-PCR analyses. Although pituitary and extrapituitary LHRH-R-I transcripts appear identical, their functional characteristics may differ. In the pituitary, LHRH-R-I are coupled via $G_{q/11}$ to phospholipase C (PLC), causing an IP_3 -mediated mobilization of Ca^{2+} and a protein kinase C-mediated activation of MAP kinases. However, there is little evidence for PLC activation by endogenous extrapituitary LHRH-R-I. Instead, G_i -mediated activation of protein phosphatase and inhibition of MAP kinase activity may be the basis of some of the antiproliferative effects mediated by LHRH-R-I. Investigation of the expression of extrapituitary LHRH-R-I revealed major functional differences between LHRH-R-I in human pituitary and extrapituitary sites, in spite of the expression of identical receptor transcripts.

We were also able to demonstrate that 18 of the 35 BPH samples investigated (51%) expressed mRNA for type-I LHRH ligand. The presence of both type-I LHRH and its receptor in human BPH implies the existence of an autocrine mitogenic loop in accord with other findings.

Furthermore, using ligand competition assay we examined the binding of [^{125}I][D-Trp6]LHRH to membrane preparations of 20 BPH specimens. We found that 90% of human BPH samples investigated possessed specific LHRH receptors with a mean K_d of 4.04nM and with a mean B_{max} of 527.6 fmol/mg membrane protein. Cetrorelix displaced radiolabeled [D-Trp6]LHRH with an IC_{50} value of 0.94 ± 0.07 nM indicating its high affinity binding to LHRH receptors expressed in BPH tissues. It is also important to note

that all receptor positive specimens expressed a detectable amount of the receptor gene. The receptor protein encoded by mRNA for LHRH receptors was also demonstrated by immunohistochemistry in 10 BPH specimens. All BPH specimens stained expressed the LHRH receptor at a moderate level. The positive immuno-staining of LHRH receptors in BPH specimens was not related to the patients' age or other clinical and pathological findings.

Our findings support the merit of further investigation of the expression of LHRH-I and LHRH-R-I and their transcript forms in human benign prostatic hyperplasia. The results of our study demonstrate, for the first time, that human BPH specimens obtained from surgery express LHRH and its receptors. Our findings that a high percentage of human BPH specimens express receptors for LHRH support the view that LHRH antagonists could be used for an effective treatment of BPH. Since the LHRH receptors and mRNAs for receptor subtypes and transcript forms are variably expressed in BPH, a precise determination of LHRH receptors in samples of human BPH is necessary before therapy with LHRH analogs. Some BPH specimens may not have LHRH receptors and therefore would not respond to therapy with an antagonistic analog such as Cetrorelix. The response of individual patients with BPHs to LHRH analogs might be predicted by evaluating the LHRH receptors in their specimens. Thus, biopsy samples of BPH should be subjected to ligand competition assays for protein or RT-PCR analyses for mRNA expression of receptors for LHRH. A rational therapy with LHRH antagonistic analog could be then implemented. The receptors could also be monitored during therapy to detect any changes in their levels and expression.

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