Regulation of Type II Luteinizing Hormone-Releasing Hormone (LHRH-II) Gene Expression by the Processed Peptide of LHRH-I, LHRH-(1–5) in Endometrial Cells

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Luteinizing hormone-releasing hormone (LHRH) was first isolated in the mammalian hypothalamus and shown to be the primary regulator of the reproductive system through its initiation of pituitary gonadotropin release. Since its discovery, this form of LHRH (LHRH-I) has been shown to be one of many structural variants with a variety of roles in both the brain and peripheral tissues. Enormous interest has been focused on LHRH-I, LHRH-II, and their cognate receptors as targets for designing therapies to treat cancers of the reproductive system. LHRH-I is processed by a zinc metalloendopeptidase EC 3.4.24.15 (EP24.15) that cleaves the hormone at the Tyr^5-Gly^6 bond. We have previously reported that the autoregulation of LHRH gene expression can also be mediated by its processed peptide, LHRH-(1–5). Given its importance in the brain, we have investigated the role of the specific processed peptide of LHRH-I, LHRH-(1–5), within Ishikawa cells, a human endometrial cell line. Using real-time polymerase chain reaction, we observed that LHRH-(1–5) upregulates LHRH-II mRNA expression in Ishikawa cells but does not exert any influence on LHRH-I mRNA levels. This is in contrast to the effects of LHRH-I, which affects the expression of LHRH-I mRNA. Our findings support a potential role for LHRH-(1–5) as a processed metabolite in the endometrium. Further investigations are needed to determine the role of this processed metabolite and to identify specific pathways involved in LHRH-(1–5) signaling. Exp Biol Med 232:146–155, 2007

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Introduction

Luteinizing hormone-releasing hormone (LHRH) is known primarily for its role as the central regulator of vertebrate reproduction. Produced and released from the hypothalamus, this neuropeptide acts *via* a specific G-protein-coupled receptor, the LHRH receptor (LHRHR), on pituitary gonadotropes to control the synthesis and release of luteinizing hormone and follicle-stimulating hormone (1). This hypophysiotropic form of the peptide (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) was the first discovered and has been designated LHRH (LHRH-I). Studies have since found that most vertebrate species express at least two forms of LHRH (2). Among these is a type originally isolated from the chicken brain (designated LHRH-II), which is conserved among taxa from jawed fishes to humans (2), although its expression in the rodent remains a subject of debate (3–7). The presence of a specific receptor with a high affinity for LHRH-II (LHRHR-II) was first reported in amphibians and more recently in marmoset, African green monkey, and rhesus monkey (8).

LHRH-I and LHRH-II are expressed in a variety of tissues, both neuronal and peripheral in humans. Outside of the brain, the reproductive system has been the focus of a great deal of research relating to the activity of both peptides. LHRH-I and its cognate receptor, LHRHR-I, have been found in the endometrium, placenta, breast, ovary, testis, and prostate (9–13), as well as several malignant tumors and cell lines (11). LHRH-II is even more broadly distributed and highly expressed than the type-I peptide throughout the periphery, including ovarian, endometrial, and breast tissue (14). LHRHR-II expression has also been reported in many nonneural reproduction-related tissues, such as mammary gland, prostate, endometrium, placenta, and gonads (2, 15–17). However, only truncated forms and not the fully functional LHRHR-II are known to exist in the

human (4). Functionally, the LHRH system is thought to have local effects, such as autocrine and paracrine mechanisms, in many reproductive tissues (9–17). For example, analogs of LHRH-I have been shown to serve as an autocrine negative regulatory factor on tumor growth progression and metastasization (11).

Our laboratory has been investigating the actions of a specific processed peptide of LHRH-I that comprises the first five amino acids. This pentapeptide, LHRH-(1–5), is formed when the zinc metalloendopeptidase EC 3.4.24.15 (EP24.15) cleaves the Tyr^5-Gly^6 peptide bond (18, 19). We recently reported that this processed peptide, LHRH-(1–5), may have differential effects from its parent peptide, LHRH (20, 21). We have shown that LHRH-(1–5) stimulates LHRH-I mRNA expression within the neuronal GT_{1-7} cell line and operates through a pathway that is distinct from that used by its parent molecule (20). LHRH-(1–5) mediates the ability of LHRH to facilitate lordosis in the female rat (21). Its apparent biological activity, along with the observed differences between LHRH-(1–5) and LHRH-I, lead us to hypothesize that LHRH-(1–5) is involved in regulating the LHRH-I and/or LHRH-II systems in peripheral reproductive tissues.

The accumulation of considerable evidence to suggest that LHRH might serve as an autocrine/paracrine growth inhibitory factor in some peripheral tumors (11) led us to examine the possible role of its processed metabolite LHRH-(1–5) in endometrial cells. In this initial study to contrast the possible difference in effects between LHRH-I and LHRH-(1–5), we used real-time polymerase chain reaction (PCR) to quantify the processed peptide's ability to regulate expression of LHRH-I and its respective receptor, LHRHR-I. Because LHRH-II and its receptor are expressed in Ishikawa cells, we also examined the effect of LHRH-(1–5) on its expression.This cell line has been used as a model for endometrial cancer as well as in studies that examine hormone-induced signal transduction pathways. In addition, the Ishikawa cells have also been used for studies on LHRH-I signaling and effects in endometrial cancer (22–24).

Our findings show that, unlike the GT_{1-7} cells, LHRH-(1–5) does not have a significant effect on the expression of the LHRH-I system in Ishikawa cells. It does, however, exert a regulatory influence on LHRH-II mRNA levels. As in GT_{1-7} cells, LHRH-(1–5) and LHRH-I had different effects. These results support our previous findings that significant differences exist between the regulatory roles of LHRH-I and its processed peptide LHRH-(1–5). Further elucidation of this processed peptide's role in regulating LHRH systems could reveal novel mechanisms for local regulation within tissues that express LHRH systems.

Materials and Methods

Cell Culture and Treatment. Ishikawa endometrial cancer cells (25) were grown in a 1:1 medium consisting of Dulbecco's modified Eagle's medium and Ham's F12 (Gibco, Carlsbad, CA) supplemented with 10% fetal calf serum (HyClone Laboratories, Logan, UT) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) and maintained at 37°C with 5% CO_2. Cells were passaged at 90%–100% in a 1:5 ratio in 10-cm dishes (Nunc, Rochester, NY). Prior to the experiment, one 10-cm dish of cells, at approximately 90%–100% confluency, was passaged to three 6-well dishes and grown to approximately 80%–90% confluency before treatment. The cells were changed to serum- and antibiotic-free medium 1 hr prior to treatment.

RNA Extraction and Reverse Transcription. Total RNA was extracted from cells using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. At the end of treatment, each plate of treated cells was placed on ice, washed with ice-cold phosphate buffered saline (PBS; 0.1 *M* phosphate buffer, pH 7.4 containing 0.87% sodium chloride) and extracted for total RNA using Trizol. Subsequent to extraction, total RNA was further treated with DNase I (Promega, Madison, WI) and then purified using standard ethanol precipitation and ethanol wash. After drying, the RNA sample was resuspended in deionized distilled water. The amount of RNA extracted for each sample was determined by measuring the absorbance at 260 nm and its purity determined using the 260/280 ratio. Samples were also randomly run on an agarose gel to affirm its purity and integrity. Only RNA samples showing a 260/280 ratio between 1.8 and 2.0 were used. First-strand cDNA synthesis was carried out in a reaction volume of 25 μl with 3 μg RNA, 10 units/μl AMV-RT (Promega), 5 μ*M* Oligo(dT), 500 μ*M* dNTPs, 0.4 units/μl RNase Inhibitor (Promega), and 1× AMV-RT buffer (Promega) per reaction. This reaction was maintained at 42°C for 60 mins.

Standard PCR. Two sets of primers for each gene were used to verify the expression of LHRH-I, LHRH-II, LHRHR-I and LHRHR-II mRNA using PCR followed by sequencing (AP 3100 DNA Sequencer; Applied Biosystems, Forest City, CA). The resulting sequence from each PCR primer pair was compared to the public database (NCBI gene sequence database) for final confirmation. The primers were those previously used to verify the expression of these genes using RT-PCR (26, 27; Table 1) as well as the primer pairs used for real-time PCR (see below). The PCR reactions included 0.5 μg cDNA, 250 n*M* forward and reverse primers, 1× Mg^{2+} free thermophilic DNA polymerase buffer (Promega), 0.2 m*M* dNTP (Promega), 2.5–3.0 m*M* $MgCl_2$, 0.625 units Taq polymerase (Promega), and distilled deionized water to 50 μl. Thirty-five cycles of amplification were preceded by an initial denaturation at 94°C for 2 mins, with each amplification cycle including a 30-sec denaturation at 94°C, a 30-sec annealing at 55°–62°C (primer pair specific), and a 30-sec extension at 74°C. At the end of the amplification cycles, the samples were subjected to a 10-min extension at 74°C.

Table 1. Identification of Gonadotropin-Releasing Hormone (GnRH)-I, GnRHR-I, GnRH-II, and GnRHR-II mRNA by RT-PCR Using Specific Primer Pairs that Were Previously Used in Published Literature (26, 27)

mRNA Measured	Primers (5′–3′);
LHRH-I	ATT CTA CTG ACT TGG TGC GTG (Forward) GGA ATA TGT GCA ACT TGG TGT (Reverse)
LHRH-II	GCC CAC CTT GGA CCC TCA GAG (Forward) CCA ATA AAG TGT GAG GTT CTC CG (Reverse)
LHRHR-I	AAT ATG GCA AAC AGT GCC TCT (Forward) GGA TAT TTT TCT CTG TGA TTG (Reverse)
LHRHR-II	CTG GCT GTG GAC ATC GCA TGT (Forward) ATG GCA GTC AGT GGC AGC AGA (Reverse)

Real-time PCR. Quantitative PCR (qPCR) was performed using the BioRad iCyCler iQ detection system (BioRad, Hercules, CA). For each reaction, 0.5 μg of cDNA was added to 1× SYBR Green Supermix (BioRad), along with 250 n*M* of each forward and reverse primer and water, for a final reaction volume of 50 μl. The primers used are as follows: LHRH-I forward 5′-ATC ACC AGC CAC AGA GAT CC-3′; LHRH-I reverse 5′-CAA GGG GGC CTC TAA TTT TC-3′; LHRH-II forward 5′-AGG AGC CAT CTC ATC CAC AG-3′; LHRH-II reverse 5′-GCT TTC CTC CAG GGT ACC AG-3′; LHRHR-I forward 5′-CAA GGC TTG AAG CTC TGT CC-3′; LHRHR-I reverse 5′-AAG GTC AGA GTG GGG AGG TT-3′; LHRHR-II forward 5′-TCG GAC ACT GAT GTT CCT GA-3′; LHRHR-II reverse 5′-GCC CCC AGA AGT TTC CTT AC-3′. 18s rRNA was used as an internal control: 18s rRNA forward 5′-TTC GGA ACT GAG GCC ATG AT-3′; 18s rRNA reverse 5′-TTT CGC TCT GGT CCG TCT TG-3′. Primers were designed using Primer3 (28), and the identity of their products was confirmed with sequencing. qPCR reactions were run for 40 cycles (10 secs at 95°C, 10 secs at 60°C, 30 secs at 72°C).

Initial amounts of cDNA in each reaction were quantified by comparison to a standard curve for the particular gene of interest. To create standard curves, flanking primers were designed to generate cDNA templates that included the products produced by the primers listed above. Template cDNA was produced using standard PCR. Flanking primer sequences are as follows: LHRH-I forward 5′-TCC TGT CCT TCA CTG TCC TTG-3′; LHRH-I reverse 5′-CCA TCT GTG GGA GAG AGG AC-3′; LHRH-II forward 5′-CCT GAA GGA GCC ATC TCA TC-3′; LHRH-II reverse 5′-CTC GCT TTG CTC CAG GGT A-3′; LHRHR-I forward 5′-GAA GGC AGA AAT AAA ACA TGG A-3′; LHRHR-I reverse 5′-AGG TCG CAG AGA GCA GAA AA-3′. For LHRHR-II and 18s rRNA, the same primers used for quantification were used to produce template. The amount of cDNA generated was determined by measuring the absorbance at 260 nm, and serial dilutions were prepared. These known amounts of template were then run with the samples during the PCR and used as a comparison in order to determine the initial amount of cDNA.

Peptide Synthesis. Peptides were synthesized with an Applied Biosystems 433A Peptide Synthesizer using Fmoc chemistry. The carboxyl terminal amino acid was attached to an amide resin (Applied Biosystems) using the instrument's normal 0.25-mmol synthesis program.

After loading the initial amino acid, the remaining reactive groups on the resin were capped using an acetic anhydride/HOBt/DIEA solution. Synthesis from that point continued with standard Fmoc protocols using HBTU/HOBT in DMF as the coupling reagent. Addition of the pyroglutamic acid (Pyr or pGlu) on the amino terminus was carried out using two different methods. Since the Pyr was a tBoc protected residue (Bachem, Torrance, CA) the initial addition to the peptide(s) was accomplished by synthesizing the majority of the peptide using Fmoc. The synthesis was paused at the point just prior to the addition of the Pyr, and the machine was flushed and converted to tBoc chemistry so as to add the Pyr as a symmetric anhydride. In later syntheses the Pyr was added using the standard Fmoc protocol, but the tBoc protective group on the Pyr amine end was removed during cleavage of the peptide from the amide resin using 95% TFA. MALDI-TOF of the peptides using both modes of Pyr addition revealed no mass differential.

All peptides were aliquoted into 10–20-mg aliquots and stored desiccated at −80°C. Prior to cell treatment, the peptides were diluted to 1 m*M*, aliquoted into 20-μl vials, and stored at −80°C. All diluted peptides were freeze-thawed no more than one time.

Immunocytochemistry. Ishikawa cells were plated and grown to ~70% confluence on an 8-well glass slide (Lab-Tek Chamber Slide with Cover, Nalge Nunc International, Naperville, IL). The medium was then removed and the cells fixed with 100% methanol (to visualize EP24.15) or buffered 4% paraformaldehyde (in 0.05 *M* phosphate buffer, pH 7.4; to visualize LHRH-I and LHRH-II) for 10 to 15 mins. After several washes in PBS, 5% normal goat serum (NGS) in 0.05 *M* PBS was added and allowed to block for 30 mins. The NGS solution was then poured off and the appropriate dilutions of the following well-characterized primary antibodies added: (i) a mouse monoclonal anti-LHRH-I antibody (1:1000; SMI-41; Sternberger, Inc., Baltimore, MD), (ii) the rabbit polyclonal antibodies that recognizes LHRH-II ([GF6; 1:1000; gift of

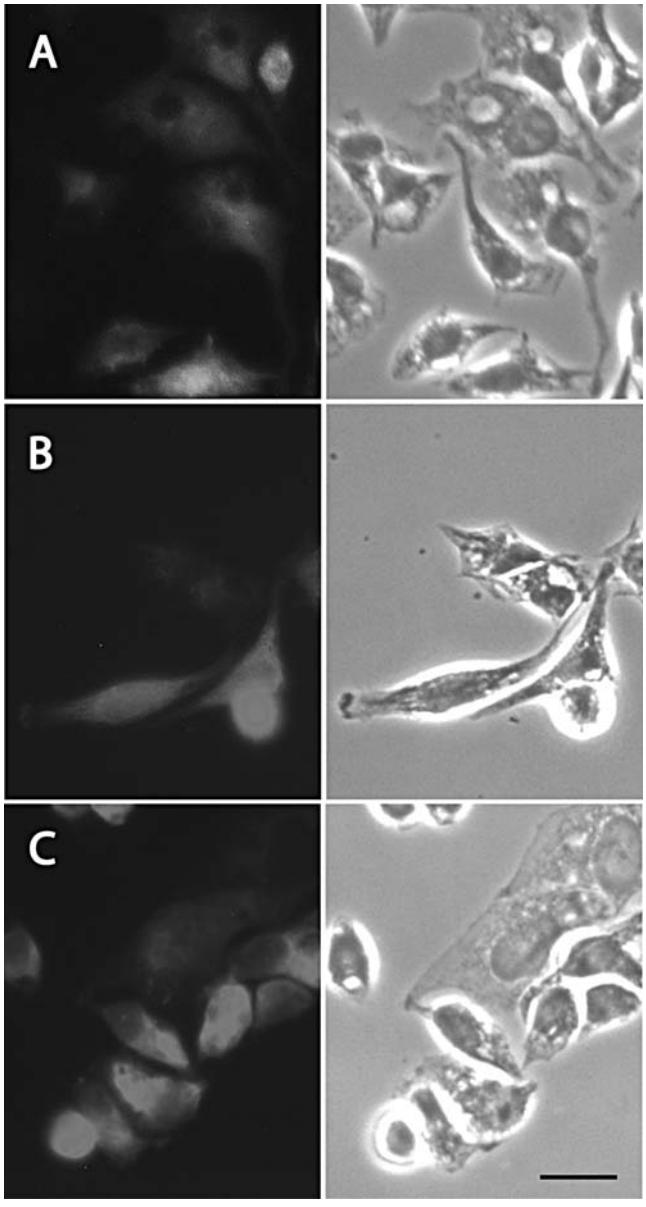

Figure 1. Immunocytochemical identification of (A) LHRH-I, (B) LHRH-II, and (C) EP24.15. LHRH-I was visualized using a mouse monoclonal antibody against LHRH-I (SMI-41) and a secondary anti-mouse antibody conjugated to Cy3. LHRH-II was visualized using a polyclonal antibody against LHRH-2 (gift of Dr. James Millam) and a secondary anti-rabbit antibody conjugated to Cy2. EP24.15 was visualized using a polyclonal antibody against EP24.15 (#48; gift of Dr. Marc Glucksman) and a secondary anti-rabbit antibody conjugated to Cy2. The left panels show the immunofluorescent expression of these peptides and protein. The right panel shows the photomicrograph of the same cells at the light level. Bar, 8 μm.

Dr. Nancy Sherwood; 29] and [#100; 1:1000; a gift of James L. Millam; 30]), and (iii) a rabbit polyclonal anti-EP24.15 antibody (1:2000; Ab#48; gift of Dr. M.J. Glucksman; 19, 31). Cells were incubated in primary antibody dissolved in PBS containing 0.05% Triton X-100 (EM Science, Gibbstown, NY) at 4°C overnight, after which they were washed several times with PBS. Subsequent to the primary antibody, the slides were treated with the appropriate secondary antibodies, diluted in PBS, and either Cy3 anti-mouse or Cy2 anti-rabbit secondary antibodies (1:500; Jackson Immunochemicals, West Grove, PA) were then added to the cells for 1 hr. A final round of washes in PBS was then conducted, after which the separating walls were removed and the slide coverslipped (24 × 50 mm Corning Cover Glass; Corning Corporation, Corning, NY) with an aqueous mounting medium (Dako Fluorescent Mounting Medium; DAKO Corporation, Carpinteria, CA, or Vectashield Mounting Medium with DAPI; Vector Laboratories, Burlingame, CA). Negative controls for the visualization of each epitope include preabsorptions of the antibodies with the appropriate peptide (50 μg/ml of the final antibody dilution) and omission of the primary antibody. None of these treatments show any staining pattern. For the analysis, cells were examined in triplicate from each passage number, and two passages were represented. The passage numbers used for the immunocytochemistry studies were representative of the passage numbers that were used for the gene expression and cAMP studies.

cAMP Assay. Intracellular cAMP levels were assayed using an enzyme fragment complementation technology (EFC) that is detectable with chemiluminescence (HitHunter XS Detection Kit, DiscoveRx Corporation, Fremont, CA; 32). The compound, 3-isobutyl-1-methylxanthine (IBMX), was added to inhibit phosphodiesterase activity and cAMP degradation. Standard curve of cAMP was obtained with a 1:3 serially diluted cAMP standards starting at 1.28×10^{-10} to 2.27×10^{-5} *M* in the same plate. All standards and samples were run in triplicates. Relative luminescence units (RLU) were measured with a luminometer (Wallac 1420 VICTOR Luminometer; PerkinElmer Life and Analytical Sciences, Inc., Wellesley, MA). The concentration of cAMP in samples was calculated based on the standard curve. The intra-assay coefficient was less than 5% for both high (1.8×10^{-5} *M*) and low (2.5×10^{-10} *M*) cAMP standards.

Data Analyses. All experiments were conducted in triplicate and each triplicate repeated at least three times. Only gene expression data using real-time PCR was transformed to percentage of controls. Differences in expression were determined by a one-way ANOVA followed by a *post hoc* comparison using Fisher's LSD (significance at $P < 0.05$).

Results

LHRH-I, LHRH-II, LHRHR-I, LHRHR-II, and EP24.15 Are Expressed in Ishikawa Cells. RT-PCR followed by DNA sequencing was conducted to confirm that LHRH-I, LHRH-II, LHRHR-I, and LHRHR-II are expressed in Ishikawa cells. All 4 genes were shown to be expressed using two sets of primer pairs, one set that was previously reported to be specific for these genes (26, 27) and the other based on the primer pair we designed for the

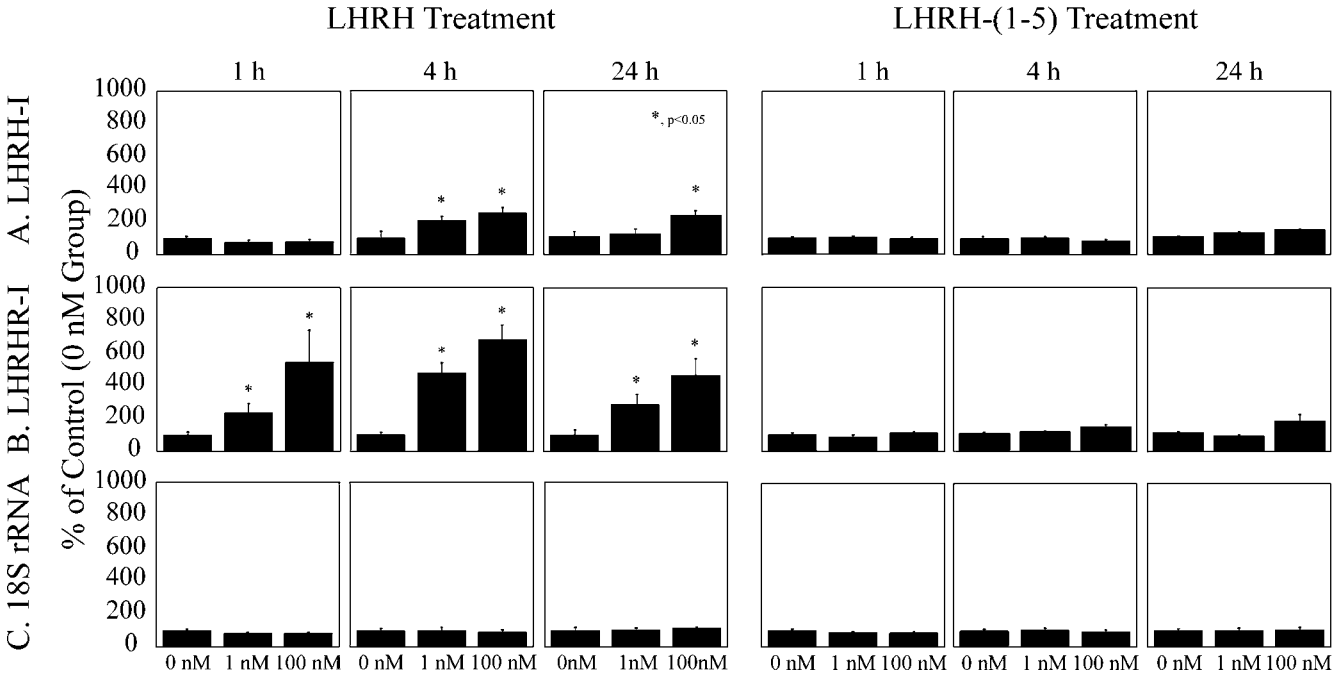

Figure 2. Effect of LHRH-I, LHRH-II, and LHRH-(1–5) treatment on (A) LHRH-I and (B) LHRHR-I mRNA expression in Ishikawa cells. Data are represented as the mean ± SEM of 6 experiments. Differences were considered statistically significant when $P < 0.05$.

real-time PCR quantification component of this paper (data not shown).

Immunocytochemistry was conducted in order to demonstrate that two of the peptides, LHRH-I and LHRH-II, and the enzyme involved in processing LHRH-I, EP24.15, are natively expressed in Ishikawa cells. Both peptides, LHRH-I and LHRH-II, and EP24.15 may be visualized by immunocytochemistry (Fig. 1). Preabsorption of each antibody with the appropriate peptide completely removed any staining; preabsorption of the LHRH-I antibody with LHRH-II and LHRH-(1–5) peptides and preabsorption of the LHRH-II antibody with LHRH-I and LHRH-(1–5) peptides did not affect its ability to immunostain. Likewise, preabsorption of the antibody to EP24.15 removed any staining (data not shown). Qualitative appraisal of each culture shows that EP24.15 is expressed in all cells, with rare exceptions. Likewise, LHRH-II is expressed in almost all cells, whereas LHRH-I is expressed in a majority significantly less than the expression for LHRH-II and EP24.15.

LHRH-I but Not LHRH-(1–5) Upregulates LHRH-I and LHRHR-I Expression. Figure 2 illustrates the result of treatment with LHRH-I and LHRH-(1–5) on LHRH-I (Fig. 2A), LHRHR-I (Fig. 2B), and 18S rRNA (Fig. 2C) mRNA expression. LHRH-I treatment resulted in increased expression of both itself and its cognate receptor in a time-dependent manner. At 1 hr, 1- or 100-n*M* LHRH-I treatment did not have a significant effect on LHRH-I mRNA levels (75 ± 12% and 79 ± 14%, respectively) when compared to those cells treated with 0 n*M* LHRH-I (100 ± 8%). At 4 hrs, LHRH-I treatment, both 1 n*M* and 100 n*M* (206 ± 27% and 253 ± 32%, respectively) doses resulted in significant upregulation ($P < 0.05$) of LHRH-I mRNA expression compared to controls (100 ± 23%). At 24 hrs, the 100 n*M* treatment (229 ± 16%) but not the 1 n*M* group (114 ± 9%) had a significant effect ($P < 0.05$) compared to controls (100 ± 9%).

The largest increases seen throughout these experiments were with LHRHR-I mRNA expression when treated with LHRH-I. Both 1- and 100-n*M* LHRH-I increased ($P < 0.05$) LHRHR-I mRNA levels at all three time points at 1 hr (293 ± 102% and 548 ± 155%, respectively), 4 hrs (320 ± 61% and 762 ± 135%, respectively), and 24 hrs (430 ± 53% and 666 ± 151%, respectively).

In contrast to the effect of LHRH-I, 1 n*M* LHRH-(1–5) did not change ($P > 0.10$) LHRH-I mRNA expression at 1 hr (103 ± 7%), 4 hrs (102 ± 8%), or 24 hrs (123 ± 11%) when compared to their corresponding controls (0 n*M*; 100 ± 6%, 100 ± 5%, and 100 ± 8%, respectively). At 100 n*M*, treatment of cells with LHRH-(1–5) did not affect ($P > 0.10$) LHRH-I mRNA levels at 1 hr (98 ± 5%) or 24 hrs (143 ± 29%) but decreased ($P < 0.05$) LHRH-I mRNA levels at 4 hrs (84 ± 6%). LHRHR-I mRNA levels were also not different ($P > 0.10$) in those cells treated with 1- or 100-n*M* LHRH-(1–5) at any of the treatment times when compared to controls (0 n*M*).

Neither LHRH-I nor LHRH-(1–5) affected 18S rRNA mRNA expression under any time or dose conditions (Fig. 2C).

LHRH-(1–5) but Not LHRH-I Increases LHRH-II and LHRHR-II Expression. Figure 3A illustrates the results of treatment with LHRH-I and LHRH-(1–5) on the

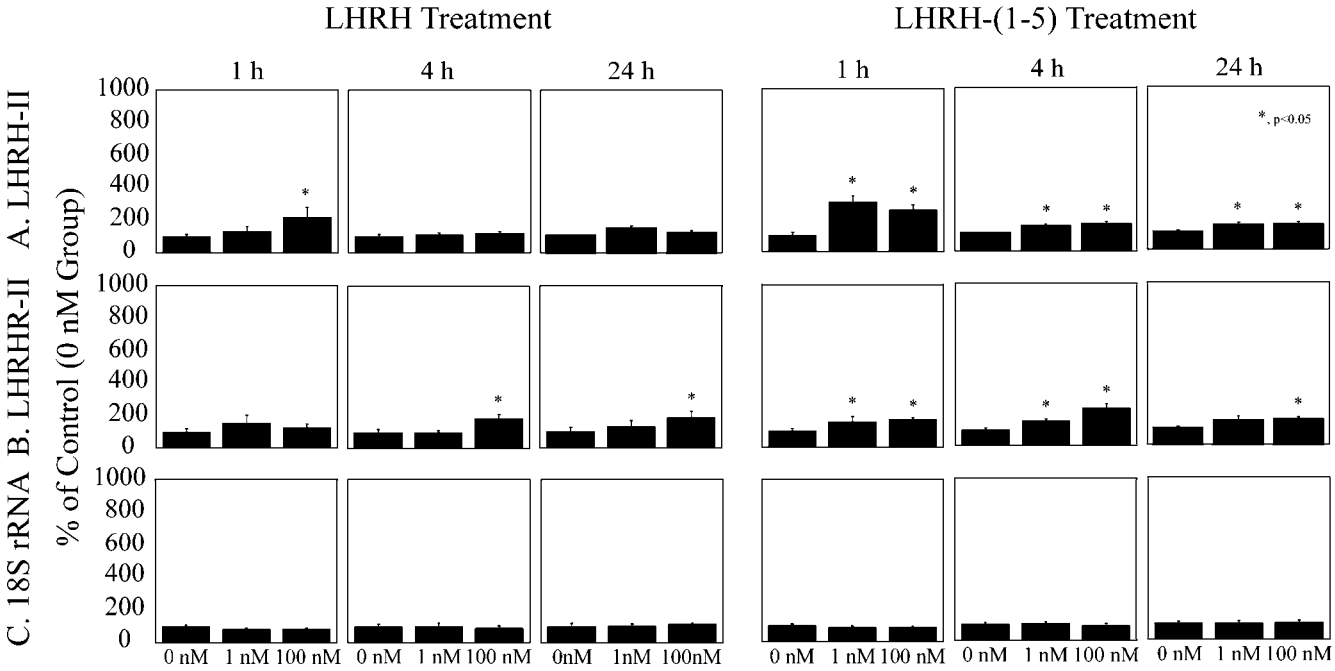

Figure 3. Effect of LHRH-I, LHRH-II, and LHRH-(1–5) treatment on (A) LHRH-II and (B) LHRHR-II mRNA expression in Ishikawa cells. Data are represented as the mean ± SEM of 6 experiments. Differences were considered statistically significant when $P < 0.05$.

expression of LHRH-II and LHRHR-II mRNA. Treatment of cells with LHRH-I did not change ($P > 0.10$) LHRH-II mRNA levels at any combination of dose and duration tested except when treated with 100 n*M* LHRH-I for 24 hrs ($P < 0.05$). While 1 n*M* LHRH-I also did not affect ($P > 0.10$) LHRHR-II mRNA levels at all three time points, 100 n*M* LHRH-I increased ($P < 0.05$) LHRHR-II mRNA at 4 hrs and 24 hrs (183 ± 27% and 212 ± 60%, respectively) when compared with their respective controls (100 ± 17% and 100 ± 24%, respectively) but not at 1 hr (158 ± 32%).

In contrast to the effect of LHRH, LHRH-(1–5) consistently affected both LHRH-II and LHRHR-II mRNA levels when compared to controls. Cells treated with 1- and 100-n*M* LHRH-(1–5) expressed greater ($P < 0.05$) LHRH-II mRNA levels at 1 hr (302 ± 38% and 255 ± 29%, respectively), 4 hrs (143 ± 14% and 153 ± 16%, respectively), and 24 hrs (135 ± 7% and 146 ± 16%, respectively) when compared with their controls (100 ± 15%, 100 ± 6%, and 100 ± 12%, respectively). Likewise, cells treated with 1- and 100-n*M* LHRH-(1–5) expressed greater ($P < 0.05$) LHRHR-II mRNA levels at 1 hr (158 ± 3% and 172 ± 9%, respectively) and 4 hrs (155 ± 11% and 233 ± 26%, respectively) when compared with their controls (100 ± 16%, 100 ± 10%, and 100 ± 11%, respectively). The cells treated with 100 n*M* LHRH-(1–5) (145 ± 27%) but not 1 n*M* LHRH-(1–5) (151 ± 16%) for 24 hrs had greater ($P < 0.05$) LHRHR-II mRNA levels than their respective controls.

Neither LHRH-I nor LHRH-(1–5) affected 18S rRNA mRNA expression under any time or dose conditions (Fig. 3C).

LHRH-1 but Not LHRH-(1–5) Enhances the Forskolin-Induced cAMP Accumulation in Cells. Treatment of Ishikawa cells with 0–100 μ*M* forskolin resulted in a dose-dependent increase ($P < 0.05$) in intracellular cAMP accumulation (Fig. 4A). By themselves, LHRH and LHRH-(1–5) did not affect intracellular cAMP levels ($P > 0.10$). When cotreated with a suboptimal dose of forskolin (1.0 μ*M*), cells treated with LHRH but not LHRH-(1–5) had greater cAMP levels ($P < 0.05$) when compared to those cells treated only with forskolin (Fig. 4B).

Discussion

To our knowledge, this is the first time that LHRH-(1–5) has been shown to regulate LHRH-II and LHRHR-II gene expression in endometrial cancer cells. This effect is in contrast to its parent molecule, LHRH-I, which affects its own expression as well as that of its cognate receptor but does not alter the expression of LHRH-II within this context. The effects of LHRH-(1–5) within this endometrial cell line are also in contrast with the effects previously observed in mouse neuronal GT_{1-7} cells, in which it upregulated LHRH-I mRNA (20).

It is well established that LHRH-I is distributed widely. While its primary effects have been attributed to have an endocrine function in its control of gonadotropin release form the pituitary, it has also been shown to have local effects (11, 33). For example, it has been shown that LHRH-

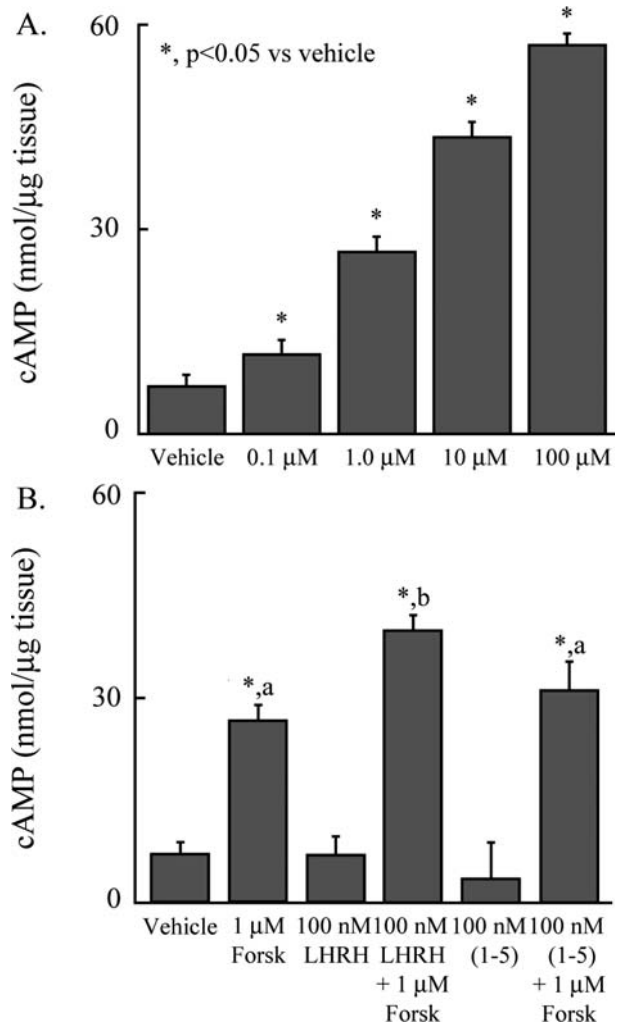

Figure 4. Effect of forskolin, LHRH-I and LHRH-(1–5) treatment on cAMP accumulation in Ishikawa cells. (A) Dose effect of 0.1-, 1.0-, 10-, and 100 µ*M* forskolin on cAMP accumulation in Ishikawa cells. (B) Effect of 1 µ*M* forskolin cotreated with vehicle, 100 n*M* LHRH-I, or 100 nM LHRH-(1–5) on cAMP accumulation. Data represented as the mean ± SEM of three separate experiments. Differences were considered statistically significant when $P < 0.05$ and indicated by (*) when compared to the appropriate controls or by different letters.

I treatment can inhibit its secretion and gene expression in GT_{1-7} cells, in hypothalamic explant tissues, and in the rat (20, 34–39). In peripheral tissues, LHRH-I has been shown to exert antiproliferative effects (8, 11, 26) in a number of tumors. Whereas the mechanism and circuitry is not completely understood, it appears that the antiproliferative effects reported are mediated by LHRH-I receptors because LHRH-I receptor antagonists block these LHRH-I effects (21, 36).

We initially hypothesized that LHRH-(1–5), a specific processed metabolite of LHRH-I composed of the first 5 amino acids, may be involved in regulating the LHRH-I and/or LHRH-II systems in endometrial cancer cells. The production of LHRH-(1–5) from LHRH-I has been shown to be cleaved by the zinc metalloendopeptidase EC3.4.24.15 (EP24.15). This enzyme was originally thought to be a degradation enzyme and LHRH-(1–5) to be a degradation intermediate. This enzyme prefers peptide substrates containing a hydrophobic amino-acid residue in the P1 and P2 positions in addition to a bulky hydrophobic residue in the P3′ position relative to the scissile bond (40). This means that EP24.15 is well-suited as a peptidase to cleave LHRH-I at the Tyr^5-Gly^6 bond to produce LHRH-(1–5) and LHRH-(6–10) (41). Previous studies in our laboratory showed that whereas LHRH-I treatment had a negative regulatory effect on its own expression in this cell line, its processed metabolite LHRH-(1–5) stimulates LHRH-I gene expression, indicating that LHRH-(1–5) is not only biologically active but likely operates through a distinct signaling mechanism when compared to its parent molecule (20, 21). Here, we extend those observations, showing that LHRH-(1–5), unlike its parent decapeptide, has a stimulatory effect on LHRH-II and LHRHR-II gene expression. The results suggest that in the endometrial Ishikawa cells, LHRH-I may have an effect on both the LHRH-I and LHRH-II systems *via* a direct effect of LHRH-I on the LHRH-I system and its processed metabolite exerting its effect on the LHRH-II system.

That LHRH-I and its processing metabolite LHRH-(1–5) have different effects on regulating gene expression for the LHRH-I and LHRH-II systems suggests the possibility that these two peptides act through different receptors. We recently showed in an *in vivo* rat model that the blockade of the LHRHR-I with a specific receptor antagonist, Antide, did not affect the ability of LHRH-(1–5) to facilitate lordosis behavior in rats (21). A similar finding suggests that not only is an LHRH-I antagonist unable to inhibit mating behavior facilitated by the LHRH fragment, Ac-LHRH-(5–10) (42), but the same LHRH fragment is also unable to interfere with the binding of an LHRH agonist to the receptor (43). On the other hand, Moss and co-workers (44, 45) found that even though Ac-LHRH-(5–10) may facilitate lordosis behavior in rats as well as stimulate CA1 pyramidal neuronal activity in an *in vitro* rat hippocampal slice preparation, LHRH-(1–6) had no activity in these same assays. Since the discovery of multiple forms of LHRH (over 20 in vertebrates) and some of their cognate receptors (2,9), it is possible that LHRH-(1–5) may act through one of the alternative LHRHRs. This is plausible, since LHRH-(1–5) shares the first 4 amino acids with at least 9 LHRH forms, including LHRH-II. Along with our findings on LHRH-(1–5), the collective studies suggest the importance of the tyrosine residue in LHRH-(1–5) in rendering biological activity.

Furthermore, that there may be two separate receptors mediating LHRH-1 and LHRH-(1–5) activity is supported in the present study; LHRH-I, but not its processed metabolite LHRH-(1–5), differentially enhanced the forskolin-induced cAMP. It is intriguing, however, that here, LHRH in the Ishikawa cells enhanced the forskolin-induced cAMP accumulation, whereas by itself it had no effect. In the pituitary, LHRHR has been shown to be coupled, *via* the

$G\alpha_q/G_{11}$ family of G proteins, to phospholipase C (46–49). In contrast, it has been thought that peripheral LHRHR is not only different from the pituitary signal transduction pathway ($G\alpha_q/G_{11}$-PLC system) but is linked to $G\alpha_i$ proteins and is involved in the inhibition of cAMP accumulation ($G\alpha_i$–cAMP pathway). This would suggest that for this endometrial cancer cell line, the LHRHR-I activation may lead to the activation of some regulatory proteins that indirectly enhance adenylate cyclase or may prevent the degradation of cAMP. This finding is the subject of current investigations in the laboratory.

In the human, only the truncated LHRHR-II has been isolated to date. The full-length transcript coding for a functional 7TM domain LHRHR-II receptor, however, has not yet been identified. The truncated LHRHR-II is due to a frame shift and premature stop-codon in the gene (3, 4). The LHRHR-II expressed in these Ishikawa cells was also found to be a truncated form in our hands.[2]

It is interesting that LHRH-II may promote luteinizing hormone secretion as well as facilitate sexual behavior in the rodent (50), a species where LHRHR-II is not known to be expressed (5–7, 51). It has been suggested that certain transcripts of the gene code for functional receptors or modulatory proteins. If translation were to begin at an alternate initiator codon at the end of the second transmembrane domain, for example, it could generate a five-transmembrane domain protein (52). This is an especially intriguing possibility given that a functional chemokine receptor containing a five-transmembrane domain has been reported (53). It has also been suggested that specific LHRHR-II transcripts could act as modulatory proteins (52, 54). Pawson *et al*., for instance, reported that a LHRHR-II gene fragment composed of transmembrane domains 6 and 7 inhibits the expression of LHRHR-I in COS-7 cells (54). Likewise, LHRHR-I fragments have also been shown to exert regulatory effects on full-length LHRHR-I (55). If this observation is correct, then a complex feedback loop linking the LHRH-I and LHRH-II systems exists. That LHRH-(1–5) in the present study upregulates LHRH-II and LHRHR-II suggests such a possible involvement. Alternatively, perhaps there exists another yet unidentified cognate receptor to LHRH-(1–5) that may be activated by LHRH-II or other structural homolog. In contrast to the possibility of a functional LHRHR-II or an unidentified cognate receptor, there exists another possibility that involves the LHRHR-I. It has been shown that binding of various ligands to the LHRHR-I may either stabilize or induce specific receptor active conformations, thereby causing different downstream effects potentially *via* its ability to couple to different G proteins (56, 57). There have been reports previously showing that LHRHR-I may bind LHRH-II (58).

The results from this study support the view that the LHRH-I system, while expressed widely, has different functions that are tissue-specific. Specifically, LHRH-(1–5) regulates LHRH-II mRNA expression in the endometrial cell line, whereas it regulates LHRH-I in GT_{1-7} cells (20). LHRH-I, on the other hand, induces its own expression as well as its cognate receptors in Ishikawa cells but inhibits LHRH-I expression in GT_{1-7} cells (20). This divergence between the cell lines is not at all surprising, considering the numerous reports of differences between LHRH effects and signaling pathways in different systems. In pituitary gonadotropes, for example, LHRHR-I is coupled to G protein q/11, thereby activating phospholipase C, which causes an IP3-mediated mobilization of intracellular Ca^{2+}, and protein kinase C, which activates various MAP kinases (1). LHRHR-I in reproductive cancers, on the other hand, couples to G_i proteins, subsequently activating a phosphotyrosine phosphatase (23, 59–63).

Summary

Our data indicate that the distinct actions of this processed peptide, LHRH-(1–5), and the parent molecule greatly expand the variety of effects of LHRH-I in the Ishikawa cell-line, leading to effects on both the LHRH-I and LHRH-II systems. Further studies elucidating the receptor and signaling pathway utilized by LHRH-(1–5) within these cells could lead to a novel target for treatment of reproductive system cancers.

[2] E.L. Baldwin, K. Oakley, H.N. Fugger and T.J. Wu, unpublished observation, 2005.

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