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Short Communication

Establishment and clinical application of enzyme immunoassays for determination of luteinizing hormone releasing hormone and metastin

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Abstract: Metastin, a 54-residue peptide, was identified as the cognate ligand of human G-protein-coupled receptor GPR54. Since metastin is a gene product of the human metastasis suppressor gene '*KiSS-1*', early studies on metastin were focused on its activity as a tumor metastasis suppressor. Recently, there have been some reports that metastin is found in human plasma and is particularly abundant in the plasma of pregnant women. Dysfunction of the GPR54 receptor causes diseases that are characterized by an insufficient release of gonadotropin and lack or delay of pubertal maturation. This information strongly suggests that metastin is involved in the regulation of reproductive endocrine functions. In order to determine the plasma levels of metastin and luteinizing hormone releasing hormone (LHRH) in an isolated hypogonadotropic hypogonadism (IHH) patient, who received intermittent administrations of LHRH, we tried to establish a sensitive and specific enzyme immunoassay. The plasma LHRH levels of the patient were very high, while plasma metastin levels were at almost the same levels as circadian rhythms of healthy male humans. In the central nervous system, metastin stimulates the neuroendocrine reproductive axis. However, the effects of peripheral metastin are not known. Our result suggested that peripheral metastin had a genesis and activity different from central metastin. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: metastin; luteinizing hormone releasing hormone; enzyme immunoassay; clinical application; isolated hypogonadotropic hypogonadism

INTRODUCTION

Isolated hypogonadotropic hypogonadism (IHH) is characterized by complete or partial failure of pubertal development due to impaired secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). In molecular pathogenesis of IHH, the gonadotropin-releasing hormone receptor (GnRH-R) and associated proteins have evolved as a central element. GnRH-R germ line mutations were among the first genetic alterations identified in patients with IHH [1]. These mutations are associated with impaired GnRH binding, ligand-induced signal transduction, or both, leading to various degrees of LH and FSH deficiency. However, not all cases of IHH pathogenesis were explained by GnRH-R mutations [2]; therefore, a search for the possible pathogenetic origin has been continued by using the epidemiologic approach. In 2003, mutations of a previous orphan receptor GPR54 were identified in patients with IHH, opening a new pathway in the physiologic regulation of puberty and reproduction through the control of GnRH secretion [3,4].

LH releasing hormone (LHRH) (i.e. GnRH) is a peptide containing ten amino acids, and plays a central role in maintaining homeostasis of the hypothalamus–pituitary–gonads axis. More than a hundred LHRH analogs have been developed, and some of which are used to treat bladder and/or breast cancer (Figure 1).

The GPR ligands were identified as a 145-amino-acid protein that is enzymatically cleaved into a 54-amino-acid peptide, known as *kisspeptin or metastin*, as well as shortened peptides of 14 and 13 amino acids [5,6]. The essential bioactive sequence is the *C*-terminus ten amino acids of metastin (metastin-10) [7] (Figure 2). Our recent researches demonstrated how to synthesize downsized metastin-10 analogs with maintenance of high GPR54 agonist activity [7,8].

The expression of *KiSS-1* gene product was originally observed in tumor cells that were not apparently metastatic [9]. Thus, the gene was inferred to be a suppressor sequence because of its antimetastatic properties. On the other hand, exogenously administered kisspeptin exerts a profound stimulatory effect on gonadotropin secretion. It is now well documented that central and peripheral administration of kisspeptin stimulates a dose-dependent rise in serum levels of LH and FSH in adult male rats. Sheep, macaques, and humans

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also share this positive response to kisspeptin administration [10].

It is reported that continuous administration of LHRH inhibits temporary elevation of gonadotropin secretion, while intermittent administration maintains normal secretion of gonadotropin [11]. Therefore, general treatment of IHH is intermittent subcutaneous injections of LHRH (1–2 h intervals) [12–14].

In this study, we have tried to establish a sensitive and specific enzyme immunoassay (EIA) for detecting human LHRH and metastin-10-like immunoreactive substance (LI), and investigate the effect of peripheral administration of LHRH on plasma metastin levels.

MATERIALS AND METHODS

Materials

Gonadorelin acetate (Hypocrine injection; Tanabe Seiyaku Co., Ltd, Osaka, Japan) was used as LHRH.

Synthetic human metastin-10 and LHRH were purchased from the Peptide Institute (Osaka, Japan). Synthetic human and rat metastin was purchased from Merck (Darmstadt, Germany). Fragment LHRH (2–10), (4–10), and (7–10) were purchased from Sigma (St Louis, MO, USA). Fragment metastin (41–54) (metastin-14), and (42–54) (metastin-13) were synthetically prepared by standard Fmoc-based peptide synthesis. Antisera to LHRH (i400/001) were purchased from Biogenesis (Poole, UK), and metastin (G-048-56) from Phoenix Pharmaceuticals (Belmont, CA, USA). Goat affinity-purified antibody to rabbit IgGs (whole molecule) (55 641) was purchased from ICN Pharmaceuticals (Aurora, OH, USA). 4-Methylumbelliferyl-β-D-galactopyranoside (MUG) and *N*- (ε-maleimidocaproyloxy) (EMC)-succinimide were purchased from Sigma (St Louis, MO, USA). β-Gal and aprotinin (Trasylol) were purchased from Boerhinger Mannheim (Mannheim, Germany) and Bayer (Leverkusen, Germany), respectively. All other reagents were of analytical grade from commercial sources.

LHRH	5-oxoPro-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2
Buserelin	5-oxoPro-His-Trp-Ser-Tyr-D-Ser(*t*Bu)-Leu-Arg-Pro-NH-Et
Goserelin	5-oxoPro-His-Trp-Ser-Tyr-D-Ser(*t*Bu)-Leu-Arg-Pro-NHNHCONH_2
Leuprorelin	5-oxoPro-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-NH-Et

Figure 1 Structure of LHRH and its clinical derivatives.

Subjects

Six healthy male volunteers, aged between 24 and 31 years (median age: 29 years), weighing from 55 to 62 kg (median: 58 kg), participated in the study. Each subject received information on the scientific purposes of the study and gave written informed consent. The study was approved by the ethics committee of Oita Medical University. The subjects did not receive any medication for one month before and during the study. Before the study, their serum FSH, LH, and testosterone concentrations were within normal ranges.

Pregnant Women

Human milk samples were collected during late pregnancy and after delivery from seven healthy donors. Informed consent was obtained from each subject, and the protocol was approved by the ethics committee of Oita Medical University.

Patient

A male in his twenties, who suffered from IHH, was treated by intermittent subcutaneous injections of LHRH (20 μg at 90-min intervals, daily 320 μg) for 8 weeks. Before treatment, his serum FSH, LH, testosterone, and free testosterone concentrations were <0.5 mIU/ml, <0.5 mIU/ml, 12.7 ng/dl, and 1.5 ng/dl, respectively.

Study Schedule

Venous blood samples from a forearm vein were taken to measure plasma metastin-LI levels using the EIA to examine circadian rhythms of plasma metastin-LI levels. Blood samples were taken at 10:00, 10:15, 10:30, 10:45, 11:00, and 11:30 hours. All subjects were allowed to have breakfast until 8:00.

LHRH (20 μg) was administered subcutaneously at 90-min intervals. Venous blood samples were taken from a forearm vein before and 15, 30, 45, 60, 75, and 90 min after LHRH administration. The study was performed from 10:00 to 11:30 hours. Blood sampling was performed twice at four-week intervals. On the second occasion, the administration was not done just before the study.

Preparation of Enzyme-labeled Antigens

Human fragment LHRH (2–10) or metastin-10 was conjugated with β-Gal by EMC-succinimide according to the method of

Human metastin
GTSLSPPPESSGSRQQPGLSAPHSRQIPAPQGAVLVQREKDLPNYNWNSFGLRF
Metastin-14
Metastin-13
Metastin-10

Rat metastin
TSPCPPVENPTGHQRPGCATRSRLIPAPRGSVLVQREKDMSA YNWNSFGLRY

Figure 2 Structure of human and rat metastin.

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Kitagawa *et al.* [15]. In brief, fragment LHRH (2–10) (0.4 mg), or metastin-10 (1.0 mg) dissolved in 0.05 M phosphate buffer (pH 7.0, 0.20 ml), was mixed with EMC-succinimide (1.0 mg) in tetrahydrofuran (0.20 ml) at room temperature (20 °C) for 60 min. The EMC-peptides obtained were purified by separation through a Sephadex G-25 column (1.5 × 50 cm) pre-equilibrated with 0.05 M phosphate buffer (pH 7.0), which was also used to elute the column. Individual fractions (1.8 ml each) that showed an absorbance at 275 nm were collected. The purified EMC-peptide fractions were combined with β-Gal (4.0 mg) by mixing at room temperature for 60 min. The β-Gal conjugates were applied to a Sephacryl S-300 column (1.5 × 52 cm) and eluted with 0.05 M phosphate buffer (pH 7.0) containing 1 mM $MgCl_2$. Individual fractions (1.8 ml each) that showed an absorbance at 275 nm were collected. The fractions containing β-Gal activity were collected and stored at 4 °C after the addition of 0.2% bovine serum albumin (BSA) and 0.1% sodium azide.

EIA Procedure for LHRH- and Metastin-10-LI

We applied the EIA of these peptides to that of LHRH- and metastin-10-LI in plasma. The assay was performed by a delayed addition method. Separation of bound and free antigens was performed on an antirabbit IgG-coated immunoplate (Nunc-Immuno Module Maxisorp F8, InterMed, Denmark).

The assay buffer consisted of 0.05 M phosphate buffer (pH 7.0) containing 0.5% BSA, 1 mM $MgCl_2$, and 250 KIU/ml aprotinin. Diluted antiserum (100 µl) and the sample (100 µl of plasma extracts or standard) were mixed and incubated at 4 °C for 12 h. Diluted enzyme-labeled antigen (50 µl) was then added, and the solution was incubated at 4 °C for an additional 12 h. One hundred microliters of the antigen-antibody solution for each sample was added to the secondary antibody-coated immunoplate. The plate was incubated at 4 °C overnight, washed with 0.01 M phosphate buffer (pH 7.0) containing 0.15 M NaCl, and 0.05% Tween 20, and then 200 ml 0.1 mM MUG in 0.05 M phosphate buffer (pH 7.0) containing 1 mM $MgCl_2$ was added to each well. The plate was incubated at 37 °C for 180 min, and then the fluorescence intensity (λ_{Ex} 360 nm, λ_{Em} 450 nm) of the fluorescent product, 4-methylumbelliferon, was measured with an MTP-100F microplate reader (Corona Electric, Ibaraki, Japan).

Preparation of Plasma and Milk Extracts

Blood samples were placed in chilled tubes containing aprotinin (500 KIU units/ml) and ethylenediaminetetraacetic acid (EDTA) (1.2 mg/ml). Milk samples were defatted by centrifugation. After centrifugation, the plasma and milk samples were diluted fivefold with 4% acetic acid (pH 4.0), centrifuged, and the supernatants were loaded onto a C18 reversed-phase cartridge (Sep-Pak C18; Millipore Corp., Milford, MA, USA). After being washed with 4% acetic acid, the peptides in the plasma were eluted with 70% acetonitrile in 0.5% acetic acid (pH 4.0). The eluted samples were concentrated by spin-vacuum evaporation, lyophilized, and stored at −40 °C until assayed. The recovery and reproducibility of human plasma with LHRH and metastin EIA were examined by adding a standard solution to hormone-free plasma [16].

HPLC of Plasma and Milk Extracts

HPLC was performed using a reversed-phase C18 packed column (Cosmosil 5C18, Nacalai Tesque, Kyoto, Japan). The HPLC consisted of a model 600E pump system (Millipore Corp., Milford, MA, USA). The plasma (2.5 ml) and milk (1 ml) samples, purified by the Sep-Pak C18 cartridges as described above, were reconstituted to 100 µl aliquots with 0.1% trifluoroacetic acid (TFA) and passed through the column. LHRH- and metastin-10-LI were eluted with a linear gradient of acetonitrile (from 5 to 45% over 40 min) in 0.1% TFA. The flow rate was 1.0 ml/min and the fraction size was 1.0 ml. Eluted fractions were concentrated by spin-vacuum evaporation, lyophilized, and reconstituted to 100 µl with an assay buffer prior to undergoing EIA.

Statistical Analysis

The results are expressed as means ± S.D. Comparison of the mean values was made by a repeated one-way analysis of variance, followed by repeated paired *t*-test. A *P*-value of less than 0.05 indicated statistical significance.

RESULTS

Standard Curve

Typical calibration curves for the LHRH- and metastin-LI EIA are shown in Figures 3 and 4. When plotted as a semi-logarithmic function, the linear displacement of enzyme-linked fragment LHRH (2–10) or metastin-10 by LHRH or metastin was noted to be between 7.0 and 340 fmol/ml, and between 0.1 and 32.5 pmol/ml with each antiserum, respectively. The minimum amount of LHRH or metastin detectable by this EIA system was 7.0 fmol and 0.1 pmol, and the IC_{50} of the calibration curve was 53 fmol/ml and 1.9 pmol/ml, respectively.

Specificity of Antiserum i400/001

The immunospecificity of antiserum i400/001 was examined by EIA using fragment LHRH (2–10) conjugated with β-Gal. The displacement curves of human

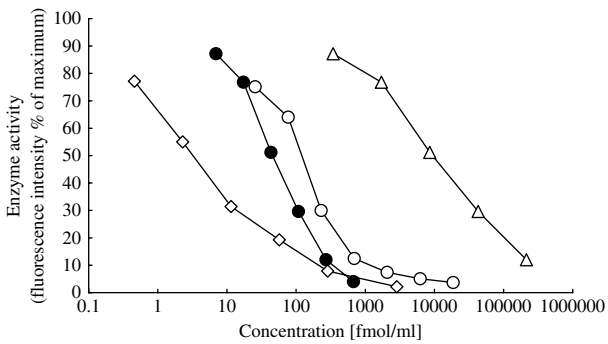

Figure 3 Inhibition curves of LHRH (●), LHRH (7–10) (◊), LHRH (4–10) (Δ), LHRH (2–10) (○), and other endogenous peptides (□) in the EIA by competition between LHRH (2–10) conjugated with β-Gal toward antiserum i400/001.

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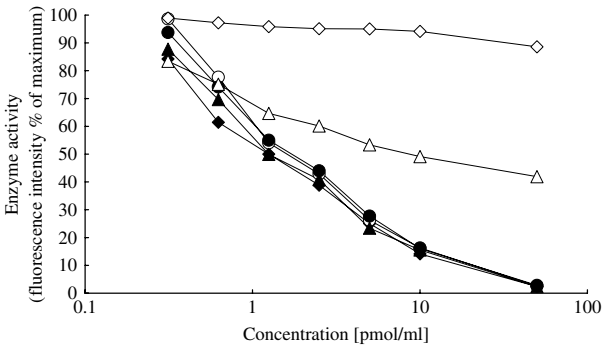

Figure 4 Inhibition curves of human metastin (●), metastin-14 (♦), metastin-13 (▲), metastin (1–12) (◊), metastin-10 (○), rat metastin (Δ), and other endogenous peptides (□) in the EIA by competition between metastin-10 conjugated with β-Gal toward antibody G-048-56.

LHRH, fragment LHRH (4–10), (7–10), and other endogenous peptides (human β- and γ-endorphin, vasoactive intestinal peptide (VIP), substance P, gastrin, gastrin-releasing hormone, thyrotropin releasing hormone, cholecystokinin (CCK), bradykinin, angiotensin II, Met-enkephalin) are shown in Figure 3. Fragments LHRH (2–10) and (7–10) exhibited cross-reactivity with synthetic LHRH. Other endogenous peptides showed minimal inhibition of the binding of β-Gal-conjugated LHRH (2–10) with LHRH antiserum i400/001.

Specificity of Antisera G-048-56

The immunospecificity of antiserum G-048-56 was examined by EIA using metastin-10 conjugated with β-Gal. The displacement curves of human metastin, metastin-14, metastin-13, fragment metastin (1–12), rat metastin and other endogenous peptides (human motilin, VIP, calcitonin gene-related peptide (CGRP), CCK, prolactin-releasing hormone (PrRH), and substance P) are shown in Figure 4. Metastin, metastin-14, and metastin-13 exhibited complete cross-reactivity with synthetic metastin-10. Although rat metastin exhibited a little cross-reactivity, other endogenous peptides, including fragment metastin (1–12), showed minimal inhibition of the binding of β-Gal-conjugated metastin-10 with metastin-10 antiserum G-048-56.

Measurement of LHRH-LI in Human Plasma and Milk by EIA

The recovery rates of human plasma LHRH in the proposed detectable range (13.5, 33.8, and 84.6 fmol/ml) with this EIA were 88, 87, and 73%, respectively. The reproducibility (expressed in % as the coefficient of variation (CV)) for human plasma (7.0 and 340 fmol/ml) with this LHRH EIA was 6.7 and 2.6%, respectively, for the inter-assay ($n = 8$), and 7.8 and 4.6%, respectively, for the intra-assay ($n = 10$) comparisons. Although the LHRH-LI in healthy humans was not detected (<0.7 fmol/ml), in milk, the levels of LHRH-LI was 3.5 ± 1.2 fmol/ml ($n = 6$, from 1.4 to 5.2 fmol/ml) by this EIA. Human milk extracts were subjected to reversed-phase HPLC to access the presence of LHRH-LI molecular variants in human milk. The elution profiles revealed the presence of a main immunoreactive peak (arrow) eluting at a position corresponding to standard LHRH (Figure 5).

Measurement of Metastin-10-LI in Human Plasma by EIA

Metastin-10-LI levels in a healthy male subject were 6.9 ± 1.9 fmol/ml ($n = 6$) by our EIA. The recovery rates of human plasma metastin-10 in the proposed detectable range (0.3, 1.3, and 10.0 pmol/ml) with

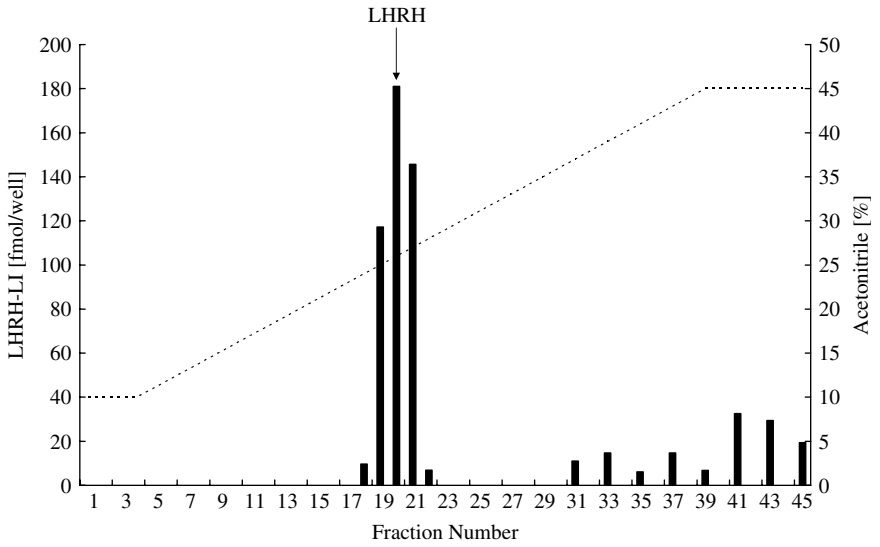

Figure 5 HPLC elution profiles of LHRH-LI in milk of pregnant women.

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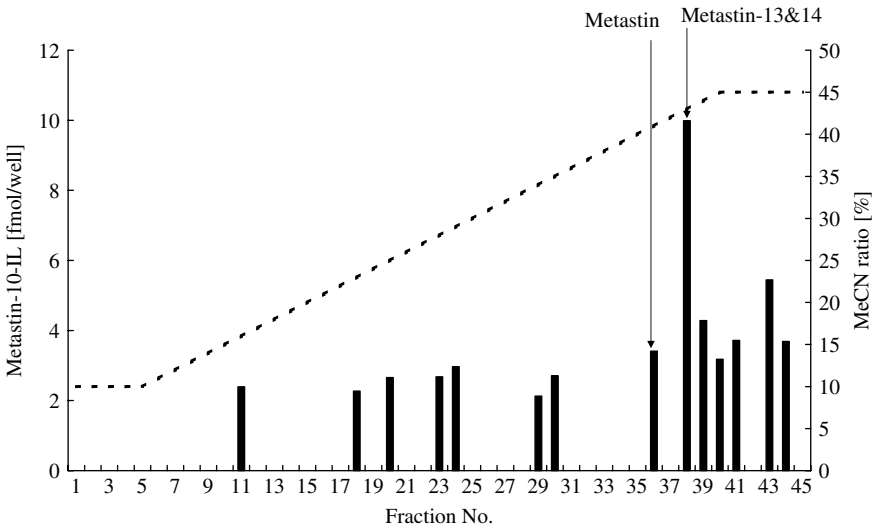

Figure 6 HPLC elution profiles of metastin-LI in human plasma. Synthetic human metastin and its bioactive fragments are run as separate chromatographs under the same condition (indicated by the arrows).

this EIA were 93, 92, and 101%, respectively. The reproducibility (expressed in % as the CV) for human plasma (0.1 pmol/ml and 32.5 pmol/ml) with this metastin-10 EIA was 13.4 and 8.6%, respectively, for the inter-assay ($n = 8$), and 15.0 and 8.8%, respectively, for the intra-assay ($n = 10$) comparisons. Human plasma extracts were subjected to reversed-phase HPLC to access the presence of metastin-10-LI molecular variants in human plasma. The elution profiles revealed the presence of a main immunoreactive peak (arrow) eluting at a position corresponding to standard metastin bioactive fragment (metastin-13 and -14) and several unknown peaks (Figure 6). There were no peaks at the positions corresponding to standard PrRH, which have the same *C*-terminus RF amide as metastin, CGRP, CCK, and gastrin, which have similar AF (CGRP) and DF (CCK and gastrin) amide in *C*-terminal sequence to metastin.

Plasma LHRH-LI Levels in Healthy Human Plasma and IHH Patient

Although all samples from the healthy human were below the limit of detection (<0.7 fmol/ml), all samples from the IHH patient had very high values (Figure 7). The plasma extracts of the IHH patient were subjected to reversed-phase HPLC to access the presence of LHRH-LI molecular variants in human plasma. The elution profiles were almost the same as the milk extract and revealed the presence of a main immunoreactive peak (arrow) eluting at a position corresponding to standard LHRH (Figure 8).

Plasma Metastin-10-LI Levels in Healthy Human Plasma and IHH Patient

Circadian rhythms of healthy human plasma metastin-10-LI levels in the morning and plasma metastin-10-LI

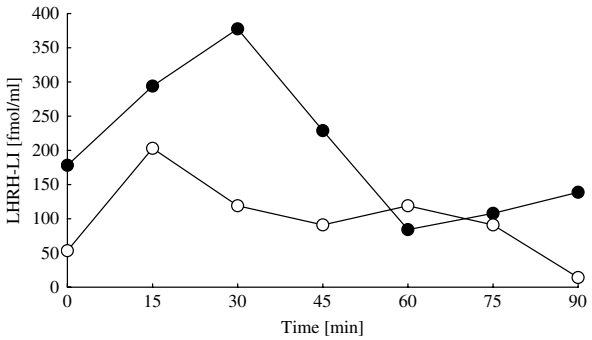

Figure 7 Plasma LHRH-LI levels after subcutaneous injections of 20 μg of LHRH. (●; first time, ○; second time).

levels before and after administration of LHRH are shown in Figure 9. All samples from healthy humans were in the range between 5.0 and 11.2 fmol/ml (no significant difference was observed), and those in the IHH patient were in the range between 10.7 and 14.5 fmol/ml.

DISCUSSION

Using β-Gal-labeled LHRH (2–10) and metastin-10 as a marker antigen, an antirabbit IgG-coated immunoplate as a bound/free separator, and MUG as a fluorogenic substrate, we developed a sensitive and specific EIA for the quantification of LHRH- and metastin-10-LI in human plasma. Our EIA for LHRH-LI was also sensitive (7.0 fmol/ml) and specific for LHRH, and the sharp inhibition curve obtained was linear between 7.0 and 340 fmol/ml. Furthermore, the EIA has no cross-reactivity with other endogenous peptides. From the cross-reactivities with LHRH fragments, antiserum i400/001

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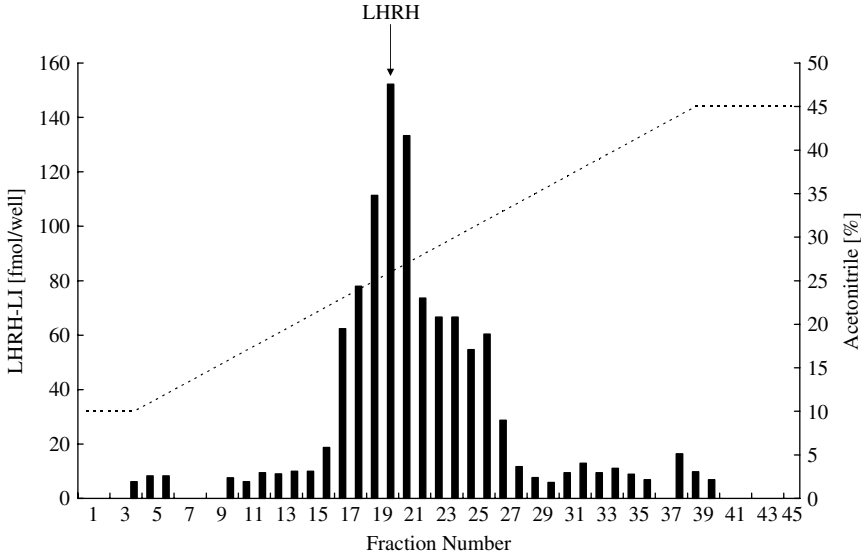

Figure 8 HPLC elution profiles of LHRH-LI in the plasma of IHH patient after administration of 20 μg LHRH.

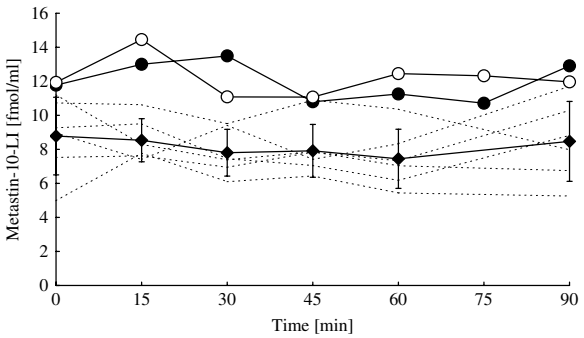

Figure 9 Circadian rhythms of plasma metastin-10-LI level profiles in five healthy human subjects (dotted line: each subject) in the morning (♦) (each value represents the mean ± SD, $n = 6$.) and in an IHH patient before and after LHRH injection (●; first time, ○; second time).

recognized the *C*-terminus of LHRH. Metastin has some molecular forms (metastin-13, -14, and -54) and the bioactive sequence is its *C*-terminal ten amino acids. Horikoshi *et al.* measured plasma metastin-54 levels using two-site EIA, which consists of two antibodies that recognize *N*-terminal or *C*-terminal [17]. The sensitivity of EIA has previously been reported as 0.3 fmol/well. Our EIA for metastin-10-LI was sensitive (0.1 pmol/ml) and specific for all bioactive metastin, and the sharp inhibition curve obtained was linear between 0.1 and 32.5 pmol/ml. The metastin-10 antibody G-048-56 was found to cross-react with metastin-13, -14, and -54. In endogenous peptides, there are some neuropeptides that have *C*-terminal RF (PrRH, etc.) and related XF amide (CGRP, etc.) sequence. However, the EIA has no cross-reactivity with these peptides. Therefore, we thought that the metastin-10 antibody G-048-56 recognized the *N*-terminus of metastin-10. With regard to practicability, our EIA enables the simultaneous measurement of many samples (96 wells) by using an antirabbit IgG-coated immunoplate as the bound/free separator. We applied the novel EIA to detect LHRH- and metastin-10-LI in human plasma. The recovery and reproducibility (CV % of inter-assay and intra-assay comparisons) of this EIA with plasma samples were satisfactory.

In a previous report, plasma metastin-10-LI levels in a healthy human (male or female) were about 1.3 fmol/ml [17]. Our results represented more than 5 times the former concentrations. Reported values are only metastin concentrations, while our values were bioactive metastin concentrations such as metastin-13 and -14, including metastin. In Figure 6, metastin-10-LI included metastin and metastin-13 and -14, which was about 3 times the concentration of metastin. Therefore, it is established that our EIA showed a higher value than in the reported method. In Figure 8, it is suggested that circulating metastin levels have no circadian rhythms. In the central nervous system, LHRH was secreted pulsatively in 90-min intervals in surges, which causes ovulation. Metastin is thought to be concerned with surge secretion [18]. It might be valid that plasma metastin-10-LI levels did not show circadian rhythms in males.

LHRH is secreted from the hypothalamus, and in humans the LHRH receptor exists in the pituitary gland at a limited level [19]. Since LHRH exists only in the brain and does not appear in circulating blood, the plasma LHRH-LI levels in a healthy male human were not detected. A single subcutaneous injection of LHRH (20 μg) causes the highest serum levels of LHRH to reach 216 fmol/ml and decline gently (half-life is about 30 min) [20]. Successive injections may

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cause accumulation of LHRH and show high plasma concentrations.

High dosage or continuous administration of LHRH causes decrease of plasma gonadotropins [21]. However, intermittent injections can promote gonadotropin secretion [11]. In this study, the serum FSH, LH, and testosterone concentration of the IHH patient is recovered at 4.1 and 4.5 mIU/ml, and 300 ng/dl, respectively. The plasma LHRH-LI levels were higher, and the half-life was longer than a single subcutaneous injection of a similar dose of LHRH [20]. Repeated administration could cause accumulation of LHRH, resulting in high concentration and long half-life. Considering its transferability to the brain, a high plasma concentration may be needed to treat IHH.

The plasma metastin-10-LI levels in the IHH patient were a little higher than in healthy humans, although LHRH-LI levels were much higher. Metastin is considered to be upstream of LHRH, and related surge secretion [18]. The surge LHRH secretion is quite another system, different from the pulse LHRH secretion. Metastin is secreted from the hypothalamus, placenta, and some types of tumors [22]. It is still unknown as to where the circulating metastin in healthy male humans comes from, how the peripheral metastin relates with central metastin, and how the circulating metastin is concerned with the regulation of hypothalamus–pituitary–gonads axis in male. If the peripheral metastin were closely related to central metastin, as sex steroid's negative feedback to the central metastin neurons regulating the pulse LHRH secretion [23], low sex steroid concentrations can cause high central and peripheral (plasma) metastin concentrations. To consider our result that showed low testosterone and a slightly high metastin-10-LI, the speculation is supported by our result. Furthermore, it is not denied that the sex steroid, or gonadotropin sensitive metastin secreting tissue, may exist in some peripheral areas, as our result showed high LHRH-LI concentration and a slightly high metastin-10-LI.

In summary, we established an EIA for the measurement of human metastin-10 and LHRH, and applied it for measurement of plasma metastin levels in an IHH patient treated with intermittent subcutaneous injections of LHRH. Although further study is needed, we experienced the possibility of peripheral metastin activity.

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