Development of a Highly Sensitive Immune Complex Transfer Enzyme Immunoassay (EIA) for Rat Growth Hormone-Releasing Hormone

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Abstract. The previous methods of measuring rGHRH lack sensitivity at the low levels of endogenous rGHRH in the peripheral blood or hypothalamic incubation media. In the present study we developed a novel immune complex transfer enzyme immunoassay (ICT-EIA) which utilized the same polyclonal antibody for both capture antibody and β-D-galactosidase-conjugated Fab against different epitopes of the rGHRH molecule. The least detectable EIA value for rGHRH was 10 amole/tube, which was 50 times more sensitive than a conventional RIA. Dilution curves of rat hypothalamic extracts paralleled standard curves. The extent of cross-reaction (10,000 amole/tube of each peptide) by EIA with any fragments of human GHRH(1-44)NH₂, ovine GHRH were 0%, but 100% with rGHRH(1-29)NH₂, suggesting that antibody specificity was directed toward the segment between the N-terminus and middle portion of the rGHRH molecule. The HPLC of the rat hypothalamic extract showed a single major peak which corresponded to that of the synthetic peptide. Hypothalamic fragments, which contained the arcuate nucleus-median eminence complexes from control animals, were incubated every 30 min for 120 min in regular KRBB. Basal GHRH release at 90-120 min was easily measured (3.3 ± 0.1 pg/hypothalamus/30 min, n=6). When hypothalami were incubated with KRBB containing 30 mM KCl, release was stimulated nearly 7-fold (22.2 ± 2.0 pg/hypothalamus/30 min, n=6, p<0.001 v.s. KRBB). These results suggest that the newly developed EIA is useful for the study of the physiology and pathophysiology of hypothalamic GHRH release.

Key words: rat growth hormone-releasing hormone (rGHRH), immune complex transfer enzyme immunoassay (EIA), radioimmunoassay, hypothalamic incubation

Introduction

Growth hormone-releasing hormone (GHRH) is a hypothalamic peptide that stimulates the release of growth hormone (1-3). GHRH in plasma or hypothalamic perfusates has been measured by radioimmunoassay after extraction with Sep-Pak cartridges (4-6). The extraction methods are not only time-consuming but also cause great variation in assay results. We have recently developed a novel immune complex transfer EIA (7) for human GHRH (hGHRH) (8) to increase the sensitivity of conventional sandwich EIA (9) or RIA (4). In the
present study we describe a highly sensitive immune complex transfer EIA (ICT-EIA) for rat GHRH(1-43)OH, and measured rGHRH released in hypothalamic incubation media.

**Materials and Methods**

Anti-hGHRH serum (RS #51) was prepared according to the previous method with modifications (6). rGHRH(1-43)OH (Peninsula Laboratories, Inc., Belmont, CA) was conjugated to bovine serum albumin (fraction V, Sigma Chemical Co., St. Louis, MO, USA) in the presence of 0.22% 1-ethyl 3-(3-dimethylaminopropyl) carbodiimide hydrochloride (Sigma Chemical Co.). For the initial immunization, the conjugate dissolved in saline (100 μg/ml of rGHRH) was emulsified with an equal volume of Freund’s complete adjuvant. Female New Zealand albino rabbits (2.5-3.0 kg) were injected subcutaneously at multiple sites with 2.0 mL of the emulsion. For booster injections, the conjugate dissolved in saline (50 μg/mL of rGHRH) was emulsified with an equal volume of Freund’s incomplete adjuvant, and 2 mL of the emulsion was injected at monthly intervals as described for the initial immunization. Blood was collected 7-14 days after the last booster injection, and anti-rGHRH serum was stored at -30°C.

RIA of rGHRH was performed according to the method previously described (6). rGHRH(1-43)OH was iodinated with 125I by the choline-T method, and the iodinated rGHRH was purified by HPLC. The anti-rGHRH serum (#51) described above was used at a final dilution of 1:40,000. There was no significant cross-reaction with 2 μg/mL of hGHRH(1-44)NH₂, hGHRH(1-37)OH, hGHRH(1-29)NH₂ (Peninsula Laboratories, Inc., Belmont, CA), pancreatic polypeptide (Peninsula), human peptid histidine isoleucine (1-27) (hPHI) (Peninsula), vasoactive intestinal polypeptide (VIP) (Peninsula), glucagon (Peninsula), somatostatin 14 (Peninsula), somatostatin 28 (Peninsula), insulin (porcine) (Peninsula), human ghrelin (10) (Peptide Institute, Inc., Osaka, Japan), GHRP-6 (Bachem California Inc., Torrance, CA) or rat growth hormone (provided by the NIDDK, NIH, Bethesda, Washington DC). The extent of cross-reaction by RIA with rGHRH(1-43)OH and rGHRH(1-29) NH₂ (peninsula) was 100%, suggesting that antibody specificity was directed toward the segment between the N-terminus and middle portion of the rGHRH molecule. The least detectable values of the current rGHRH RIA were 2.5 pg/tube.

The ICT-EIA for rGHRH was performed according to the method previously described (7) with modifications (8) (Fig. 1). rGHRH in 20 μL of hypothalamic incubation medium mixed with 80 μL of buffer A (10 mmol/L sodium phosphate buffer, pH 7.0, containing 0.1 g/L bovine serum albumin, 1.0 mmol/L MgCl₂ and 1.0 g/L NaCl) containing 0.4 mol/L NaCl was incubated at 4°C for 4 h with 50 μL of bufferA containing 0.4 mol/L NaCl, 2,4-dinitrophenyl (DNP)-biotinyl-bovine serum albumin (BSA)-anti-rGHRH Fab’ conjugate (50 fmol), anti-rGHRH Fab’-β-D-galactosidase conjugate (50 fmol) and nonspecific rabbit F(αb’). (0.1 mg). To the reaction mixture two colored polystyrene beads coated with affinity purified (anti-2,4-DNP group) IgG were added, and the incubation was continued at 4°C overnight. The next day the colored polystyrene beads were washed twice by the addition and aspiration of 2 mL of buffer A containing 0.1 mol/L NaCl and then incubated at room temperature for 3 h with 150 μL of buffer A containing 0.1 mol/L NaCl and 1.0 mmol/L ε-N-2,4-dinitrophenyl-L-lysine and two white polystyrene beads coated with streptavidin. All the following processes were performed at room temperature. After removing the colored polystyrene beads, the white polystyrene beads were washed as described above, and bound β-D-galactosidase activity was assayed at 30°C for 20 h by fluorometry with 4-methylumbelliferyl-β-D-galactoside as the substrate. The fluorescence intensity was measured relative to 1 x 10⁻⁸ mol/L 4-methylumbelliferone. In some experiments, β-D-galactosidase activity bound to the colored
polystyrene beads coated with affinity-purified (2,4-dinitrophenyl group) IgG was assayed as described above. The limits of rGHRR detection by the ICT-EIA were expressed as the minimal amount of rGHRR which gave a significant bound β-D-galactosidase activity in excess of that nonspecifically bound in the absence of rGHRR (background). The existence of a significant difference from the background was confirmed by Student's t-test (p<0.01, n=5).

The HPLC analysis of the hypothalamic extract was carried out with a Gilson Model 305 (Gilson Inc., Middleton, WI), and the elution profile was continuously monitored at 2 wavelengths, 210 nm and 280 nm. The hypothalamic extract (6) was subjected to analytical HPLC on a Cosmosil C-18 reverse phase silica column (5C18 AR-300, 0.46 × 25 cm; particle size 5 μm; pore size 300 Å) (Nakarai Tesque, Kyoto, Japan) at ambient temperature and a solvent flow rate of 1.0 ml/min. The mobile phase was acetonitrile (CH₃CN) containing 0.1% TFA (solvent A) and 0.1% TFA in organic pure water (solvent B), under the following elution conditions: an isocratic initial elution of 20% solution A for 10 min, followed by a linear gradient from 20–60% solution A over 40 min, 60–80% solution A over a 5 min period, and an isocratic final elution of 80% solution A for 5 min. The HPLC fractions (1.0 mL) were collected over a period of 60 min, lyophilized, reconstituted in EIA buffer, and measured for immunoreactive rGHRR by homologous GHRH EIA.

Hypothalamic incubation was carried out according to the previous method [11] with minor modifications. Male Sprague–Dawley rats, weighing 220–250 g (Kiwa Laboratory Animals Co., Ltd, Wakayama, Japan), were kept for at least one week in an environmentally controlled room (lights on 0600–1800 h, temperature, 23 ± 1°C), and provided tap water and food ad libitum. The rats were killed by decapitation and the hypothalami were rapidly dissected. The tissue block, weighing 2–4 mg, and containing the median eminence, arcuate nucleus and medial
portion of the ventromedial nucleus, was placed in ice-cold Krebs-Ringer bicarbonate buffer, pH 7.4, containing 14mM glucose, 0.2% BSA (KRBG). After a 30-min preincubation, two hypothalamic fragments were incubated in a single polypropylene tube containing 0.5 mL KRBG for three successive 30 min (basal) periods. During a final 30-min period, tissues were exposed to 0.5 mL KRBG containing 30 mM KCl.

**Results and Discussion**

The least detectable value for the rGHRH RIA was 2.5 pg/tube with half-maximal displacement at 12 pg/tube. The detection limits of the ICT-EIA were 50 fg/tube (Fig. 2). The detection limits with the current ICT-EIA were 50-fold lower than that with the RIA. The current EIA was not sufficiently enough to measure GHRH levels in unextracted rat plasma from the peripheral circulation. The extent of cross-reaction (10,000 amole/tube of each peptide) by the EIA with any fragments of human GHRH(1-44)NH₂, other peptides of the glucagon-secretin family, somatostatin 14 or somatostatin 28, insulin, human ghrelin, GHRP-6, rat growth hormone, or ovine GHRH was 0%, but 100% with rGHRH(1-43)OH or rGHRH(1-29)NH₂, suggesting that antibody specificity was directed toward the segment between the N-terminus and middle portion of the rGHRH molecule. The dilution curves of pooled hypothalamic extracts or hypothalamic incubation media were parallel with the standard curves of the ELAs (Fig. 2). Within-assay and between-assay variation coefficients were 4 and 7%, respectively, at a level of 1000 fg/tube rGHRH. The HPLC of the rat hypothalamic extract showed a single major peak which corresponded to that of the synthetic peptide (Fig. 3).

**Fig. 2** Standard curves of a highly sensitive immune complex transfer EIA (B, open squares) for the rGHRH(1-43)OH. Closed symbols indicate dilution curve for hypothalamic (HT) extract from an adult male Sprague-Dawley rat. Cross reaction with rat GHRH(1-29)NH₂ is 100%, but negligible with any fragments of human GHRHs, glucagon-secretin family peptides, GHRP-6 and human ghrelin. Vertical bars indicate standard deviations from several ELAs (n=3).

**Release of rGHRH from KRBG containing 30 mM KCl.** Release was stimulated nearly 7-fold (22.2±2.0 pg/hypothalamus/30 min, n=6, p<0.001 v.s. KRBG). These results are in good accord with those by RIA coupled with extraction methods (11).

These results suggest that the current immune
complex transfer EIA for rGHRH, providing precise evaluation of GHRH release from the rat hypothalami, could be useful for the study of the physiology and pathophysiology of hypothalamic GHRH release.

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References

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