Tissue-specific molecular heterogeneity of human growth hormone-releasing hormone receptor protein

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Abstract A site-directed anti-peptide antibody (anti-hGHRHRc18) was generated against the cytoplasmic tail of human GHRH receptor. The dissociation constant (K_d) and the antibody binding site (AbT) of anti-hGHRHRc18 were 2.5 nmol/l and 0.54 nmol/l, respectively. In an immunoblotting experiment, affinity-purified anti-hGHRHRc18 specifically recognized a single 50-kDa protein in human pituitary. In a screening of the expression of GHRH receptor protein in extra-pituitary tissues, only human kidney showed a single 52-kDa protein. Our results suggest that the GHRH receptor protein exhibits tissue-specific molecular heterogeneity.

Key words: GHRH; GHRH receptor; Anti-peptide antibody; Molecular heterogeneity; Immunoblotting; Kidney

1. Introduction

Growth hormone-releasing hormone (GHRH) is a hypothalamic releasing hormone which stimulates GH secretion from pituitary somatotrophs [1,2]. This activity was first isolated and characterized in human pancreatic islet tumors accompanying acromegaly [3,4] and was subsequently found to be identical to hypothalamic GHRH [5,6]. GHRH is produced mainly in the arcuate nucleus and is released into the hypophysial portal vein at the median eminence [7]. The released GHRH acts directly on pituitary somatotrophs through a G-protein-coupled receptor to stimulate adenylyl cyclase, resulting in the synthesis and release of growth hormone (GH) in the anterior pituitary. Since the discovery of GHRH, the presence of extra-hypothalamic GHRH has been reported in leukocytes, testis, ovary, placenta, endometrium, pancreas, and gastrointestinal tract [2,8–17]. However, it has not yet been determined whether these extra-hypothalamic GHRHs have any physiological significance. Additional evidence has suggested that these extra-hypothalamic GHRHs might use the VIP receptor, since the VIP receptor can bind GHRH [18–20]. To clarify the physiological significance of extra-hypothalamic GHRH, it is critical to determine whether these GHRH-positive extra-hypothalamic tissues coexpress GHRH receptor. Since GHRH binds with the VIP receptor and other secretin-family receptors, we cannot use a binding assay or chemical cross-linking analysis to address these questions.

A cDNA encoding GHRH receptor has recently been cloned from the pituitary. Furthermore, the tissue distribution of GHRH receptor was studied by measuring GHRH receptor mRNA, and an absence of GHRH receptor mRNA expression was found in extra-pituitary tissues [21–23]. However, these studies cannot exclude the possibility of technical problems, since the usual method for detecting mRNA expression is not sensitive enough to detect a minor population of mRNA.

In this study, we generated a site-specific anti-peptide antibody against the cytoplasmic tail of GHRH receptor protein and examined the presence of GHRH receptor protein in extra-pituitary tissues.

2. Materials and methods

2.1. Preparation of anti-hGHRHRc18

A synthetic oligopeptide (hGHRHRc18: TRAKWTTPSRSAAKVLTS) corresponding to the amino acid residue 404–421 of human GHRH receptor [21] was chemically synthesized by a solid-phase method. This hGHRHRc18 has 67% homology with the corresponding rat GHRH receptor protein (*T*CTE*W*TTP*P*R*S*RV*KVLT*S). 5 mg of hGHRHRc18 was conjugated with 20 mg of keyhole limpet hemocyanin (KLH) using glutaraldehyde. The conjugate was emulsified with complete Freund's adjuvant and injected subcutaneously into multiple sites over the backs of three male Japanese White rabbits (600 μg peptide/rabbit). All of the rabbits received booster injections of the conjugate emulsified with incomplete Freund's adjuvant at 14, 28, 42, 56, 70, 84, and 98 days after the initial immunization (450 μg/rabbit/boost). 10 ml of whole blood was obtained from the ear vein of each rabbit at each time point. Serum was separated from each blood sample and stored at −80°C until use.

2.2. RIA of hGHRHRc18

The specificity and titer of anti-hGHRHRc18 was assessed by radioimmunoassay (RIA). Since hGHRHRc18 does not contain a tyrosine residue, Tyr-hGHRHRc18 containing Tyr at the N-terminal of hGHRHRc18 was synthesized. Tyr-hGHRHRc18 (5 μg) was radioiodinated with 1 mCi of ^{125}I by the Iodogen method. A mixture of 0.2 ml of assay buffer (0.1 mol/l phosphate buffer, pH 7.4, containing 0.14 mol/l of NaCl, 0.05 mol/l of EDTA, 0.01% NaN_3, 0.1% BSA, and 0.1% Triton X-100), 0.05 ml of diluted anti-hGHRHRc18 serum (final dilution, 1:4200) and 0.05 ml of standard Tyr-hGHRHRc18 was incubated at 4°C for 24 h. Iodinated Tyr-hGHRHRc18 0.05 ml (about 5000 cpm) was then added, and the incubation was continued at 4°C for 24 h. Thereafter, 0.05 ml of anti-rabbit gamma-globulin (final dilution, 1:135) and 0.05 ml of normal rabbit serum (final dilution, 1:1350) were added. After incubation at 4°C for an additional 24 h, the mixture was centrifuged at 4°C for 30 min at 2000×*g* and the radioactivity of the precipitate was counted in a gamma counter (Aloka, Tokyo). All determinations were performed in duplicate. The dissociation constant (K_d) and concentration of the antibody binding site (AbT) values were calculated by Scatchard analysis.

2.3. Immunoaffinity purification of anti-hGHRHRc18

An hGHRHRc18-immobilized immunoaffinity column was prepared using 1 ml of Affi-Gel 10 (Bio-Rad Laboratories, Richmond,

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Abbreviations: GH, growth hormone; GHRH, growth hormone-releasing hormone

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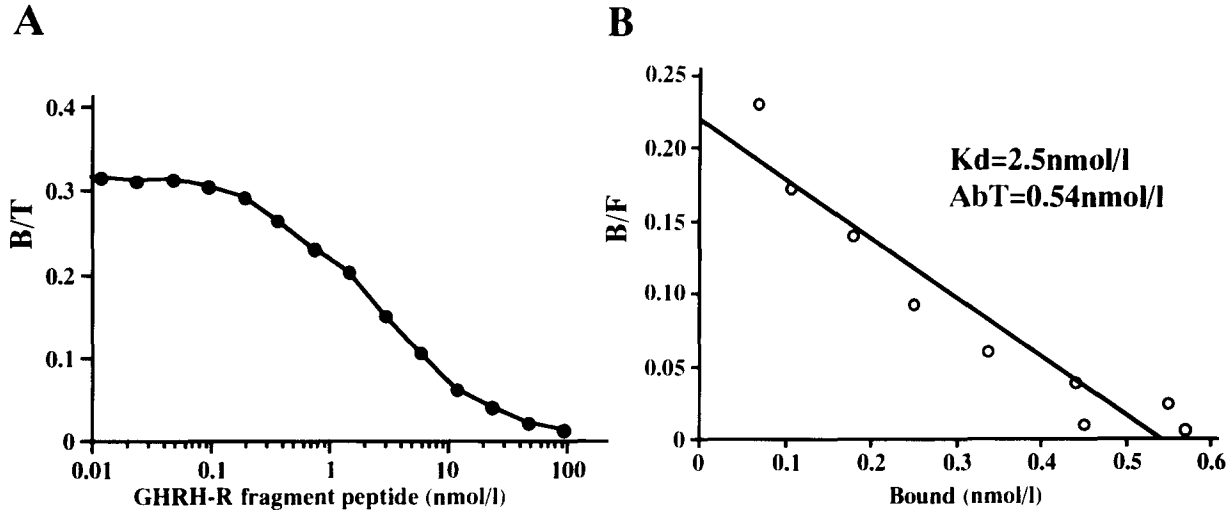

Fig. 1. Dissociation curves in radioimmunoassays for the synthetic Tyr-GHRH receptor fragment peptide. The specific binding of antiserum against the synthetic peptide was estimated by self-displacement (A). A Scatchard plot (B) revealed that the dissociation constant (K_d) and the antibody binding site concentration (AbT) were 2.5 and 0.54 nmol/l, respectively.

CA) and 1 mg of hGHRHRc18 following the protocol provided by the manufacturer. The IgG fraction of anti-hGHRHRc18 was prepared using protein A chromatography (Ampure PA kit: Amersham, Buckinghamshire, UK). The IgG fractions of anti-hGHRHRc18 were added to the hGHRHRc18-immobilized column and shaken for 12 h at 4°C. The column was washed with TBS (0.02 mol/l Tris-HCl, pH 7.5, 0.15 mol/l NaCl), followed by washing buffer (0.02 mol/l Tris-HCl, pH 7.5, 1 mol/l NaCl, 1% Triton X-100), TBS and 0.15 mol/l NaCl. Anti-hGHRHRc18 IgG fractions were eluted with elution buffer (0.1 mol/l glycine-HCl, pH 2.3). After neutralization with 1 mol/l Tris-HCl, pH 7.5, the protein concentrations in the fractions were measured with a BCA reagent kit (Amersham) and stored at −80°C.

2.4. Preparation of the membrane fraction

Human placenta was obtained from a woman at normal delivery and postmortem specimens of human pituitary, lung, liver, adrenal gland, kidney and ovary were obtained from two patients who had no endocrine disorders. Normal rat pituitary was also used in the study. Tissues were homogenized in ice-cold homogenate buffer (0.9% NaCl, 0.001 mol/l EDTA, 0.01 mol/l Tris-HCl, pH 7.4) in the presence of protease inhibitors (0.1 mg/l of leupeptin, 0.1 mg/l of chymostatin, 0.1 mg/l of pepstatin and 0.5 U/l of aprotinin) using a loose-fitting glass homogenizer. The homogenates were centrifuged at 750×g for 30 min at 4°C and the supernatants were centrifuged at 27000×g for 20 min. The pellets were washed three times with each homogenate buffer containing various concentrations of NaCl (0.5 mol/l, 1.0 mol/l and 2.0 mol/l). After resuspension with homogenate buffer containing 10% (v/v) glycerol and protease inhibitors, the membrane fractions were stored at −80°C until use.

2.5. Immunoblotting

Membrane fractions (100 μg protein) were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and electrophoretically transferred onto nitrocellulose membranes. The membrane was immersed in blocking buffer (5% nonfat dry milk in PBS) for 2 h at room temperature and incubated overnight at 4°C in a 1:600 dilution of purified anti-hGHRHRc18 in blocking buffer. The immunoblot was washed three times with blocking buffer containing 0.05% Tween-20 (10 min each wash), and then incubated in a 1:2000 dilution of horseradish peroxidase-labeled goat anti-rabbit IgG (Zymed Laboratories Inc., San Francisco, CA) for 2 h at room temperature. After washing, the membrane was visualized with an enhanced chemiluminescence kit (Amersham) and exposed to Kodak AR X-ray film (Eastman Kodak, Rochester, NY). In addition, an absorption test using hGHRHRc18 (100 μg/ml diluted antiserum) was also performed.

3. Results and discussion

All three of the rabbits that had been immunized with the synthetic oligopeptide hGHRHRc18 corresponding to the C-terminal intracellular tail of human GHRH receptor, produced antisera with high titers, which reached a maximum at 105 days after the initial immunization. The calculated maximal K_d and AbT values were 2.5 nmol/l and 0.54 nmol/l, respectively (Fig. 1).

Immunoblotting using affinity-purified anti-hGHRHRc18 showed a single specific band corresponding to a protein of about 50 kDa in human pituitary, and a 54-kDa protein in rat pituitary (Fig. 2). In spite of the fact that the molecular weight of human GHRH receptor protein predicted from the cDNA sequence was 45 kDa, previous studies using chemical cross-linking and GHRH analogs have provided conflicting results regarding the molecular weight of native GHRH receptor proteins: 75 kDa in human pituitary [24], 26 kDa in rat pituitary [25], and 42 and 27 kDa in rat thymocytes and splenocytes [26], respectively. There has also been a report that the 75-kDa band in human pituitary was in fact nonspecific binding of GHRH to bovine serum albumin which was present in the reaction buffer [25]. These discrepancies clearly demonstrate the methodological limitations of chemical cross-linking studies, and the possible tissue-specific modification of GHRH receptor protein. Recently, a photoaffinity cross-linking study showed a 55-kDa band of GHRH receptor proteins, which shifted to 45 kDa after deglycosylation by *N*-glycosidase, in the membrane fraction of HEK-293 cells transfected with human GHRH receptor [27], suggesting that the differences in molecular weight observed in chemical cross-linking are due to differences in the tissue- or species-specific glycosylation of GHRH receptor proteins. Our results also clearly showed a difference in the molecular weight of GHRH receptor proteins between human and rat pituitaries.

Immunoblotting of extra-pituitary tissues, including human lung, liver, adrenal gland, kidney, ovary, and placenta, showed no significant band except for human kidney (whole kidney was used for immunoblotting), which showed a single

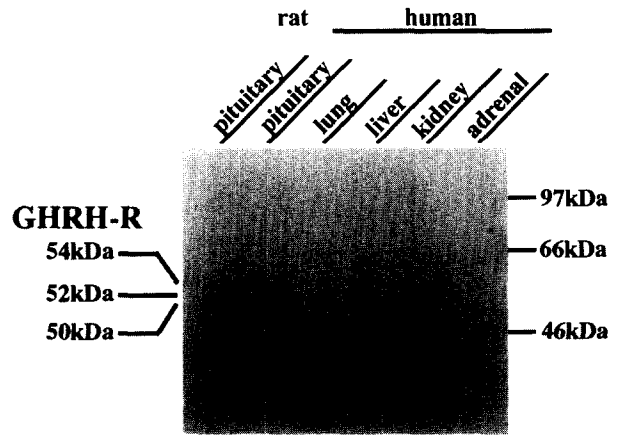

Fig. 2. Immunoblots of membrane fractions obtained from various tissues using anti-hGHRHRc18. GHRH receptor immunoreactivities corresponding to 54-kDa, 50-kDa, and 52-kDa protein were identified in the rat pituitary, human pituitary and human kidney, respectively.

band corresponding to a 52-kDa protein (Fig. 2). There has been no report on GHRH immunoreactivity in the kidney, and Northern blot analysis has failed to show the expression of GHRH receptor mRNA in any extra-pituitary tissues, including the kidney [21–23]. Thus, our results are the first to demonstrate the presence of GHRH receptor protein in human extra-pituitary tissues and the presence of a kidney-specific molecular form of GHRH receptor, suggesting that it may regulate the activity of nephrotic cells, which is in stark contrast to the classical role of GHRH, i.e. GH release from somatotrophs.

There has been a report that GHRH or GH transgenic mice develop glomerulosclerosis, while IGF-1 transgenic mice do not [28]. Originally, these results were thought to indicate a direct action of GH on nephrotic cells. However, the possibility of some direct action of GHRH on nephrotic cells should not be excluded. In addition, dwarf little (*lit*) mice with a missense mutation in the GHRH receptor gene showed no renal dysfunction [29,30]. Therefore, further analysis is required to clarify the biological significance of the GHRH/GHRH receptor system in the kidney.

The molecular heterogeneity of GHRH receptor proteins may result from tissue-specific alternative splicing or post-translational glycosylation of GHRH receptor protein, since several alternative forms of pituitary GHRH receptor cDNAs have been reported [21,31–33]. We should note the possibility of a truncated form of GHRH receptor which lacks the C-terminal cytoplasmic tail, which is necessary for recognition by anti-hGHRHRc18. If extra-pituitary tissues expressed such truncated GHRH receptor proteins, our anti-hGHRHRc18 would not be able to detect such subtypes of the GHRH receptor. Therefore, we cannot exclude the possibility that extra-pituitary tissues other than the kidney, which failed to show any band in our study, may express these truncated GHRH receptor proteins. Recently, Matsubara et al. reported the expression of GHRH and GHRH mRNA in various extra-pituitary tissues, including the kidney, using RT-PCR [34]. Their primer set used for detection of GHRH receptor mRNA covered the N-terminal region, so it could detect all of the alternative GHRH receptor mRNA. Other anti-peptide antibodies that recognize subtype-specific sequences must be developed to clarify these problems.

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