Development of Highly Sensitive RIA for Somatostatin: Direct Measurement of Plasma Somatostatin in Humans, Monkeys and Rats

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Abstract. Somatostatin (SRIF) is originally isolated from the hypothalamus and inhibits GH secretion from the pituitary. SRIF in the peripheral circulation is believed to originate mainly in the gastrointestinal tract. Previous RIAs for plasma SRIF lack sensitivity and need extraction procedures. Here we developed a highly sensitive RIA for SRIF (1-14), which allowed us to directly measure plasma SRIF levels without any extraction procedures. The antiserum to SRIF (1-14) was generated in a rabbit (873), and used at a final dilution of 1.60,000 with [¹²⁵I]-Tyr⁴-SRIF (1-14) as a radiolabeled ligand. The least detectable RIA value was 9.8 fg/tube (IC50: 50.0 fg/tube), showing the 65.7% cross-reactivity with SRIF (1-28) but not with any other pituitary or gut peptides, including GH, insulin and ghrelin. Intra- and inter-assay coefficients of variation were 6% and 9%, respectively. The dilution curves of hypothalamic extracts from rats and monkeys or peripheral plasma (from 1.25 µl to 10.0 µl) from humans, monkeys and rats paralleled, respectively, the standard curve of SRIF. The recovery rate of SRIF (1-14) added to plasma was 82%. The HPLC analysis of rat hypothalamic extracts showed two peaks, one corresponding to SRIF 1-28 and the other to SRIF 1-14. Hypothalamic fragments, which contained the arcuate nucleus-median eminence complex from 1.5-month-old control SD rats (CNT), were incubated every 30 min for 120 min in regular KRKB. Basal SRIF release at 90–120 min was easily measured (mean ± SE: 0.7 ± 0.1 pg/hypothalamus/30 min, n=8). When hypothalami were incubated with KRKB containing 30 mM KCl, release was stimulated nearly 5-fold (3.8 ± 0.5 pg/hypothalamus/30 min, n=6, p<0.01 v.s. KRKB). When plasma SRIF levels were measured in healthy adults (45 males and 41 females) and children (6 boys and 7 girls), there was no significant difference among four groups (mean ± SE, adult males: 9.7 ± 0.7 pg/ml, adult females: 10.3 ± 0.6 pg/ml, boys: 10.1 ± 1.3 pg/ml and girls: 10.6 ± 0.7 pg/ml). Plasma SRIF levels in a patient with a SRIF-producing tumor were high (20–36.0 pg/ml). Plasma SRIF levels in conscious adult monkeys (m. fuscata, n=10 each, males: 11.9 ± 0.9 pg/ml, females: 11.0 ± 0.6 pg/ml) and adult rats were measurable (n=12 each, males: 12.5 ± 1.3 pg/ml, females: 13.3 ± 1.6 pg/ml). The present SRIF RIA is useful for the study of the physiology and pathophysiology of GH secretion in both humans and laboratory animals.

Key words: somatostatin, highly sensitive RIA, direct measurement of plasma SRIF levels, humans and monkeys, rats

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Introduction

Somatostatin (SRIF) is a hypothalamic peptide that inhibits the release of GH (1) along with other gastrointestinal hormones. SRIF in plasma and hypothalamic perfusates has been measured by radioimmunoassay after extraction with Sep-Pak cartridges or affinity chromatography (2, 3) due to nonspecific interference by serum components and lack of sensitivity (4). The extraction methods are not only time-consuming but also cause great variation in assay results. In the present study we describe a highly sensitive RIA for SRIF, allowing us to use a tiny amount of plasma samples with negligible nonspecific interference by plasma, and to measure plasma SRIF levels in normal human subjects as well as monkeys and rats without any extraction procedures.

Materials and Methods

Chemicals and peptides

All synthetic brain-gut peptides, motilin, glucagon, secretin, NPY, human GHRH (1-44)NH₂, SRIF (1-14), SRIF (1-28), [Tyr⁶]-SRIF (1-14), [Tyr¹⁴]-SRIF (1-14) and octreotide (5) were purchased (Peninsula Laboratories, Inc., Belmont, CA), with the exception of ghrelin (6). Pituitary hormone, including rat and human GH, prolactin, TSH, ACTH, LH, FSH and AVP, were provided by NIDDK, NIH (Washington, DC). Synthetic human ghrelin (1-28) and ghrelin (13-28) were kindly provided by Dr. Kojima M (Research Institute, Cardiovascular Center, Osaka, Japan).

Antiserum

An antiserum to SRIF (1-14) was generated in rabbit according to the previous method with modifications (7). SRIF (1-14) 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 SRIF) was emulsified with an equal volume of Freund’s complete adjuvant. Female Japanese white 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 SRIF) 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-SRIF serum was stored at −30°C.

RIA for SRIF

The RIA for SRIF was performed according to the method previously described (7) with minor modifications. Tyr⁶-SRIF (1-14) was iodinated with¹²⁵I by the chlomamine-T method, and the iodinated SRIF was purified by HPLC (8). An anti-SRIF serum (#73) was used at a final dilution of 1:60,000. Briefly, SRIF standards, hypothalamic incubation media, hypothalamic extracts, or 1.25–10.0 μL of plasma were mixed with RIA buffer (50 mM phosphate saline buffer with pH 7.4, containing 0.01% TritonX-100, 25 mM EDTA-Na and 0.1% BSA). SRIF RIA was carried out by the delayed addition of tracer¹²⁵I-SRIF (1-14) and the double antibody technique with goat anti-rabbit serum.

Plasma samples and hypothalamic extracts

All subjects participating in this study gave informed consent, and the project was approved by the Committee of Internal Medicine at Miyazaki Medical College. Fourteen healthy adults, 8 men aged 23–35 years, 6 non-pregnant women, aged 23–32 years, and healthy children of constitutional short stature (six boys and seven girls) (9) were studied between 9:00 a.m. and 10:00 a.m. after overnight fasting. All were in good health and none were taking any medication. Plasma samples from a patient with
SRIF-producing pancreatic tumor (10) were obtained for the SRIF RIA.

Ten each of adult male and female Japanese macaques (Macaca fuscata), aged from 7 to 17 years for male and from 8 to 15 years for female, were used for blood sampling. All monkeys, healthy at the time of the experiment, were bred at the Primate Research Institute of Kyoto University. The living animals were cared for and treated in accordance with The Guide for the Care and Use of Laboratory Animals established by NIH (1985) and The Guide for the Care and Use of Laboratory Primates (1986) established by the Primate Research Institute of Kyoto University. After overnight fasting, two milliliters of blood was collected in ice-chilled glass tubes containing EDTA-disodium and aprotinin at 1 mg/ml and 1,000 KIU/ml blood, respectively, and immediately centrifuged at 4°C for 15 min. Plasma was stored at −30°C.

Blood samples (0.2 ml) were collected from conscious male and female Sprague Dawley (SD) rats two months old after overnight fasting through chronic indwelling intra-atrial cannulae (11) into EDTA-aprotinin-containing test tubes, and the plasma was immediately separated and stored at −30°C for subsequent RIA.

Hypothalami from 40-day-old male SD rats were collected at the time of decapitation. Tissues were rapidly dissected, frozen on dry ice, and stored at −80°C until subsequent extraction (7). Individual frozen tissues, c.a. 10 mg, were weighed, and immediately put into test tubes containing a ten-fold volume of 1 N acetic acid, and the tubes were boiled for 20 min. The tissues were individually homogenized with a glass-Teflon homogenizer. The homogenates were centrifuged at 10,000 g for 20 min at 4°C and the supernatants were lyophilized and stored at −30°C for subsequent SRIF measurement. Recovery of SRIF added prior to extraction was 96%. All animal experiments were conducted in accordance with mandated standards for humane care and were approved by the Institutional Animal Care and Use Committee of Miyazaki Medical College.

HPLC

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, according to the method previously described (12). The hypothalamic extract 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 A) (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% solvent A for 10 min, followed by a linear gradient from 20 to 60% solvent A for 40 min, 60–80% solvent A for 5 min, and an isocratic final elution of 80% solvent A for 5 min. The HPLC fractions (1.0 mL) were collected over a period of 60 min, lyophilized, reconstituted in RIA buffer, and measured for immunoreactive SRIF by SRIF RIA.

Hypothalamic incubation

Hypothalamic incubation was carried out according to the previous method (13) with minor modifications. Male SD 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 with tap water and food ad libitum. Rats were killed by decapitation and hypothalami were rapidly dissected. The tissue block, weighing 2–4 mg, contained 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 14 mM glucose, 0.2% BSA (KRBG).
After a 30-min preincubation, two hypothalamic fragments were incubated in a single polypropylene tube containing 0.5 mL KRPG for three successive 30-min (basal) periods. During a final 30-min period, tissues were exposed to 0.5 mL KRPG containing 30 mM KCl. The significance of differences between groups was determined with Student's t-test as performed on a computer with a StatView 4.5J program for Macintosh computers. A probability of p<0.05 was considered significant.

Results and Discussion

RIA standard curves

Anti-SRIF serum (RS #73) used in the present study at a final dilution of 1:60,000 exhibited no significant cross-reactivity (<1 pmol/tube) with ghrelin (1-28), des-acyl rat ghrelin (1-28), rat ghrelin (13-28), motilin, rat growth hormone-releasing hormone (1-43)OH, rat corticotropin-releasing factor, peptide histidyl isoleucine, vasoactive intestinal polypeptide, glucagon, secretin, gonadotropin-releasing hormone, thyrotropin-releasing hormone, gastric inhibitory polypeptide, gastrin releasing peptide, cholecystokinin 8, neurotensin, substance P, angiotensin 1, rat GH, TSH, or prolactin. As shown in Fig.1, the sensitivity of the SRIF assay was 9.8 fg/tube with half-maximal displacement of 50.0 fg/tube (IC50). The cross-reactivity of the antisera with SRIF (1-28), [Tyr1]-SRIF (1-14), [Tyr11]-SRIF (1-14) and octreotide was 65.7%, 100%, 30% and less than 0.01%, respectively. The intra- and inter-assay coefficients of variation were 5 and 7%, respectively, at a concentration of 50.0 fg/tube SRIF (1-14). The recovery of SRIF (1-14) added to plasma was 90%. The dilution curves of rat hypothalamic extract and plasma samples (1.25–10.0 μL) from humans, monkeys and rats were parallel to those of the synthetic SRIF (1-14) (Fig. 1). Since larger volumes of plasma samples from humans, monkeys or rats, 15.0 μL and 30.0 μL, did not show parallelism with standard SRIF, 7.5 μL of plasma samples from humans, monkeys and rats were used for the SRIF RIA. The current RIA is sensitive enough to directly measure SRIF levels in small plasma samples.

Plasma SRIF levels in humans, monkeys and rats

Direct measurement of plasma SRIF levels in healthy adults (45 males and 41 females) and children (6 boys and 7 girls) showed no significant difference among the four groups (mean ± SE, adult males: 9.7 ± 0.7 pg/mL, adult females: 10.3 ± 0.6 pg/mL, boys: 10.1 ± 1.3 pg/mL and girls: 10.6 ± 0.7 pg/mL). As expected (14), SRIF levels in patients with an SRIF-producing islet cell tumor were high (20.0–36.0 pg/mL). The current values are in good agreement with previous results obtained by direct measurement (15), affinity chromatography (3) or extraction with a Sep-Pak cartridge for SRIF (10).

Plasma SRIF levels in conscious adult monkeys (n=10 each, males: 11.9 ± 0.9 pg/mL, females: 11.0 ± 0.6 pg/mL) and rats (n=12 each, males: 12.5 ± 1.3 pg/mL, females: 13.3 ± 1.6 pg/mL) were measurable. Values obtained by the present RIA are also comparable to those reported both in monkeys (16) and rats (17). SRIF in plasma is believed to originate mainly in the gastrointestinal tract since circulating SRIF levels are not affected by GH-excess or deficiency (16, 18–20). Although the number of children was limited in the present study, there seems to be no difference in plasma SRIF levels according to age or gender. These results suggest that the highly sensitive RIA provides an accurate method of measuring SRIF-like immunoreactivity in the plasma of humans and laboratory animals.

HPLC analysis

The HPLC analysis of rat hypothalamic extracts showed two peaks, one corresponding to SRIF (1-28) and the other to SRIF (1-14) (Fig. 2). Since the cross reactivity of the RIA
with SRIF 1-28 is 65.7%. SRIF (1-14) appears to be the dominant form in the rat hypothalamus.

Hypothalamic incubation
Basal SRIF release at 90–120 min was easily measured (mean ± SE: 0.7 ± 0.1 pg/hypothalamus/30 min, n=8) (Fig. 3). When hypothalami were incubated with KRBG containing 30 mM KCl, release was stimulated nearly 5-fold (3.8 ± 0.5 pg/hypothalamus/30 min, n=6, p<0.01 v.s. KRBG). The magnitude of SRIF stimulation by high potassium is comparable to that in previous reports (21, 22).

The present SRIF RIA is useful for the study of the physiology and pathophysiology of GH secretion in both humans and laboratory animals.

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Fig. 2  HPLC analysis of rat hypothalamic extract with the SRIF RIA. The arrows indicate the elution position of the synthetic SRIF (1-14) and SRIF (1-28).

Fig. 3  Time course of SRIF release from hypothalamic incubation. Stimulation of SRIF release occurs in response to 30 mM K+ during a final 30-min period. Each column indicates the mean ± SE for 6 or 8 hypothalami, as indicated in parentheses. a indicates significant (P<0.001) increases in SRIF release against regular KRBG solution.

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References


