RADIOIMMUNOASSAY FOR HYPOCRETIN-2

T. Mitsuma, [1]Y. Hirooka, M. Kayama, Y. Mori, Y. Yokoi, M. Izumi, N. Rhue, J. Ping, K. Adachi, R. Ikai, N. Kawai, A. Nakayashiki, [2]T. Nogimori

Fourth Department of Internal Medicine, [1]Department of Laboratory Medicine, Aichi Medical University, Nagakute, Aichi, Aichi 480-1195, Japan
[2]Department of Internal Medicine, Konanshowa Hospital, Konan, Aichi, Aichi, Japan

Objective. To develop radioimmunoassay for hypocretin-2(Hcrt-2) and search for its presence in certain rat tissues.

Methods. Anti-Hcrt-2 antiserum has been raised in New Zealand white rabbits immunized with a conjugate of synthetic Hcrt-2 with bovine serum albumin. Radioiodination of Hcrt-2 was performed by chloramine T method followed by purification of radioiodinated material on Sephadex G-25 column.

Results. The obtained antibody did not crossreact with hypocretin-1, hypothalamic hormones, pituitary hormones, neuropeptides or gut hormones. The assay was performed with a double antibody system. Hcrt-2 was extracted from the tissues with acid acetone. The dilution curve of acid acetoneextracts of rat hypothalamus in the radioimmunoassay system was parallel to the standard curve. The recovery of tissue Hcrt-2 was about 85 %, and the intra-assay and inter-assay variations were 5.6 % and 8.0 %, respectively. Hcrt-2 was found in the hypothalamus, cerebrum, brain stem and testis.

Conclusions. These data suggest that this assay system is suitable for the estimation of Hct-2 in the tissues and that Hcrt-2 is mainly found in the hypothalamus.

Key words: Hypocretin-2 – Radioimmunoassay – Immunohistochemistry – Rat

Recently De Lecea et al. (1998) identified two peptides called hypocretins (Hcrt) which showed neuroexcitory activity. They are found in the hypothalamus and may be related to the food comsuption (Kalra et al. 1999). These peptides are identical to orexins which were isolated by Sakurai et al. (1998). The hypothalamus is known to be the main control center of food intake and energy expenditure (Kalra et al. 1999).

To study the physiological and pathophysiological significance of Hcrt, it is necessary to measure Hcrt in tissues. We previously reported a radioimmunoassay for orexin-A (Hcrt-1) (Mitsuma et al. 1999). The present study designed to establish a radioimmunoassay system for measuring Hcrt-2 in the tissues.

Materials and Methods

Animals. Male Wistar rats weighing 250 g were housed in a temperature (22 °C) and humidity (60 %) controlled room with 12 h illumination cycle. They were fed with laboratory chow and water ad libitum. After twelve hours fasting, the rat was sacrificed under sodium pentobarbital anesthesia (60 mg/kg) and the tissues were obtained. The brain was removed and the hypothalamus, cerebrum, brain stem, thalamus, hippoampus, striatum and cerebellum were separated according to the method previously described (Mitsuma et al. 1991).

Drugs. Synthetic Hcrt-1, Hcrt-2, hypothalamic hormones, pituitary hormones, neuroepeptides and gut hormones were obtained from the Protein Research

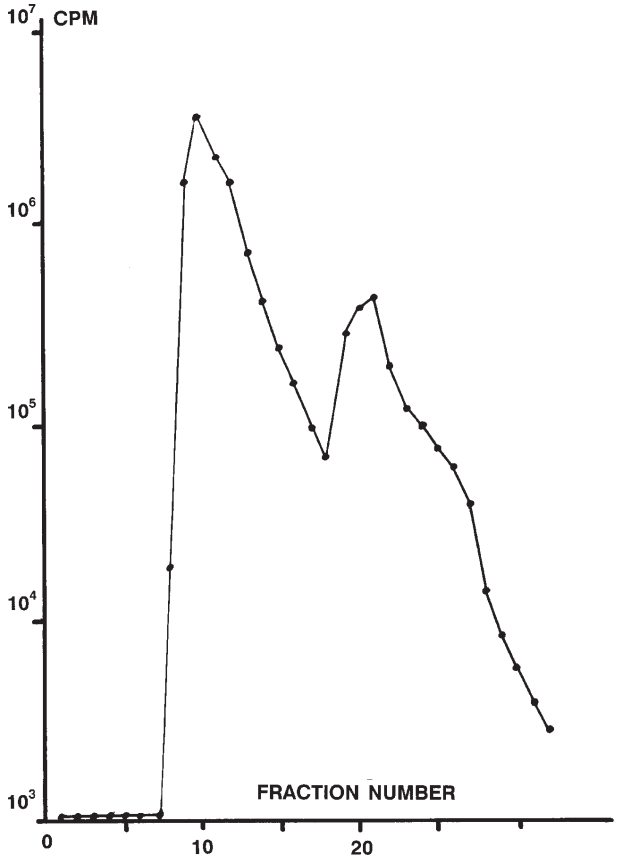

Fig 1 The elution profile of radioiodinated hypocretin-2 on Sephadex G-25

Foundation (Osaka, Japan). Bovine serum albumin (BSA) and glutaraldehyde were purchased from the Wako Chem. Co, Ltd. (Tokyo, Japan). Sephadex G-25 was purchased from Pharmacia (Sweden).

Preparation of Hcrt-2 conjugate to BSA. Synthetic Hcrt-2 was conjugated on an equal weight basis to BSA by the method previously described for anti-GHRH antibody, using glutaraldehyde (MITSUMA et al. 1983).

Immunization. New Zealand white rabbits were immunized with the emulsion of one mg of this Hcrt-2 conjugate in one ml water and complete Freund's adjuvant (1:2, v/v) which was injected into the foot pad at intervals of three weeks. Blood was withdrawn one week after each injection and the presence of anti-Hcrt-2 was checked by radioimmunoassay.

Radioiodination of Hcrt-2 and purification. Radioiodination of Hcrt-2 was performed with the chloramin T method according to the GREENWOOD et al. (1963). The radioiodinated materials were chromatographed on a Sephadex G-25 (1x20cm), eluted with 0.01 M phosphate buffer (pH 7.4) and collected in one ml fractions. The first peak was Hcrt-2-I-125 and the second peak was free ^{125}I (Fig. 1). Specific activity was calculated to be approximately 200 μCi/μg.

Assay buffer. The assay buffer consisted of 0.01 M phosphate buffer (pH 7.4) with 0.1 % BSA, 0.1 % mercaptoethanol and 0.1 % triton X-100.

Assay procedure. Double antibody radioimmunoassay system was used, the schematic diagram of the which is shown in Tab. 1.

Extraction of Hcrt-2 from the tissues. The extraction of Hcrt-2 was performed by the method previously described for Hcrt-1 (MITSUMA et al. 1999). The freshly obtained tissues were weighed and placed in 5.0 ml acid acetone, homogenized and centrifuged. The supernatants were dried under the air stream in a water bath (56 °C). The recovery of this extraction method was evaluated by adding a known amount of synthetic Hcrt-2 to the tissues. The recovery was found to be approximately 85 %.

Results

Generation of antibodies to Hcrt-2. All three rabbits used responded to the immunization and developed the antibodies at a final dilution 1:1000 or higher. The antiserum used in this study was obtained one week after the third injection and showed a specific binding at a final dilution of 1:8000.

Specificity of antiserum. The specificity of anti-Hcrt-2 is shown in Tab. 2. No crossreactivity was observed with Hcrt-1, hypothalamic hormones, pituitary hormones, neuropeptides and gut hormones.

Standard curve and dilution of acid-acetone-extracted hypothalamus. Typical standard curve is shown

Table 1
Time scheme of the assay procedure

Standard or samples	0.1 m
Antibody(1:1000)	0.1 ml
Hcrt-2-I-125	0.1 ml
Buffer	0.5 ml
Incubated for 24 hours at 4 °C	
Added second antibody solution at 4 °C	
Incubated 24 hours at 4 °C	
Centrifuged	
Decanted supernatant	
Counted (precipitates)	
Calculated bound/total count (B/T)	

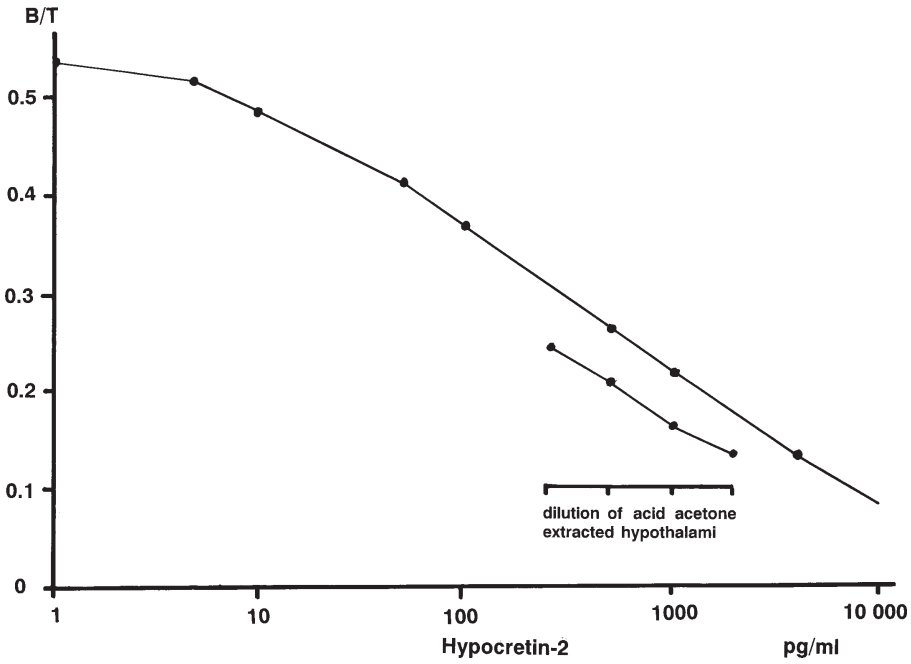

Fig 2 Standard curve an dilution of acid acetone extracted hypothalami

in Fig. 2. The detection limit in this system was calculated as 10 pg/ml. Parallel curves were obtained for the dilution of acid acetone extracted hypothalamus (Fig. 2).

Recovery experiment. Recovery of Hcrt-2 was evaluated with adding known amount of Hcrt-2 to the hypothalamus and measured with radioimmunoassay. The recovery was approximately 100 %.

Intra-assay and inter-assay variation. The intra-assay variation was 5.6 %, and the inter-assay variation was 8.0 %.

Hcrt-2 in the tissues. Hcrt-2 was found in the central nervous system and testis (Tab. 3).

Discussion

A specific method for measurement of Hcrt-2 is essential for the investigations of physiological and pathophysiological significance of this peptide. The present study tried to develop a Hcr-2 radioimmunoassay for measuring Hcrt-2. The antibody used in this study did not cross-react with Hcrt-1, hypothalamic hormones, pituitary hormones, neuropeptides or gut hormones. The data indicate that this antibody is specific to Hcrt-2 and suitable for the estimation of Hcrt-2 in a radioimmunoassay system. Radioiodination was performed with chloramin T method and the elution pro-

Table 2

Relative reactivity of hypothalamic hormones, pituitary hormones, neuropeptides or gut hormones in Hcrt-2 radioimunassay system

Hert-2	100	Adrenomedullin	0.001
Hcrt-1	0.001	Endothelin-1	0.001
TRH	0.001	Endothelin-2	0.001
GHRH	0.001	Endothelin-3	0.00l
Somatostatin	0.001	ACTH	0.001
CRH	0.001	TSH	0.001
LHRH	0.001	LH	0.001
Neurotensin	0.001	FSH	0.001
Leucine-enkephalin	0.001	Prolactin	0.001
Beta-endorphin	0.001	GH	0.001
Dynorphin	0.001	Alpha-MSH	0.001
Alpha-neoendorphin	0.001	Vasopression	0.001
Beta-neoendorphin	0.001	Oxytocin	0.001
Substance P	0.001	Secretin	0.001
VIP	0.001	Glucagon	0.001
CGRP	0.001	Gastrin	0.001
ANP	0.001	Insulin	0.001
BNP	0.001	GLP-1	0.001
CNP	0.001		

Arbitary values of Hcrt-2 = 100

Table 3
Immunoreactive Hcrt-2 in various rat organs (ng/g wet weight)

Hypothalamus	330±22 ng/g
Cerebral cortex	4.0±0.3 ng/g
Striatum	1.2±0.2 ng/g
Hippocampal formation	20.2±1.8 ng/g
Thalamus	30.2±2.2 ng/g
Brain stem	50.4±4.2 ng/g
Testis	0.2±0.02 ng/g

Not detected by this assay system in:
Cerebellum, Anterior pituitary, Posterior pituitary, Retina, Stomach, Duodenum, Jejunum and ileum, Colon, Pancreas, Liver, Lung, Heart, Spleen, Kidney, Thyroid, Adrenals, Fat, Muscle
The vales are shown by mean±S.E. in each group of seven rats

file showed two peaks: the first peak was Hcrt-2-I-125 and the second peak was free I-125. First peak was used as the radiactive ligand. The dilution curve of acid acetone extracted hypothalamus was found parallel to the standard curve, and the results of recovery experiment, intra-assay and inter-assay variation were satisfactory. These data suggest that this assay system is suitable to measure Hcrt-2 in the tissues.

The present study demonstrated that Hcrt-2 was mainly found in the central nervous system, especially in the hypothalamus. The hypothalamus is known to regulate food consumption and body weight (Levine et al. 1997; Rosenbaum et al. 1997; Flier et al. 1998; Kalra et al. 1999). It was also reported that neuropeptides and neurotransmitter in the hypothalamus regulate food intake and energy expenditure (Kalra et al. 1999). The hypocretins are known to induce hyperphagia and their concentrations in the hypothalamus were increased by fasting (Sakurai et al. 1998). Thus, it is of interest to measure Hcrt-2 in the hypothalamus in various physiological and pathophysiological conditions. We also found Hcrt-2 in the testis, although its physiological significance in this organ remained unclear.

The present data and previous reports taken together suggest that this assay system is suitable to measure Hcrt-2 in the tissues and Hcrt-2 may play an important role in food consumption. However, the physiological role of Hcrt-2 in tissues is unknown at present. Further investigations are needed to clarify this point.

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Corresponding author: T. Mitsuma, M.D.
Fourth Department of Internal Medicine
Aichi Medical University
Nagakute,
Aichi 480-1195
Japan
Phone: 0561-62-3311
Fax: 0561-62-1570

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