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IDENTIFICATION AND CHARACTERIZATION OF ADIPOKINETIC HORMONE (LOCUSTA MIGRATORIA)-LIKE IMMUNOREACTIVITY IN THE HUMAN CEREBROSPINAL FLUID+

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Summary: Using an antiserum raised against locust adipokinetic hormone I, considerable quantity of adipokinetic hormone-like immunoreactivity was demonstrated in the human cerebrospinal fluid. The immunoreactivity was characterized by gel permeation and high performance liquid chromatography.
The main immunoreactive component in the cerebrospinal fluid coeluted with adipokinetic hormone I. These results suggest that adipokinetic hormone may contribute to the neuronal function in the human central nervous system.

Adipokinetic hormone (AKH) was first isolated from extracts of locust corpora cardiaca (1). During the initial stages of flight, AKH is released from the secretory cells in the corpora cardiaca (2) in a calcium-dependent manner (3) and causes the release of specific diglycerides (4), used by the flight muscle as a source of energy (5). Recently, using an immunohistochemical technique, AKH has been demonstrated to be present not only in corpora cardiaca but also in the CNS of adult and all nymphal instars of the migratory locust (6). Furthermore, the immunoreactive nerve fibers and neurons have been present in the central nervous system of invertebrate species other than insects (7) and in the rat hypothalamic median eminence,

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periventricular nucleus and spinal cord (8). Therefore, AKH is thought to possess not only metabolic action but also neurotransmitter or neuromodulator function. In the present study we examined the possible occurrence of the AKH-like immunoreactivity (AKH-IR) in the human cerebrospinal fluid (CSF).

MATERIALS AND METHODS

Cerebrospinal Fluid (CSF). The CSF samples were obtained fresh via ventricular or cisternal drainage or by lumbar puncture from 9 patients with subarachnoid hemorrhage (2 males and 7 females; mean age 67±3.2 (mean±SEM) yr), 3 patients with subdural hematoma (2 males and 1 female; mean age 71.0±4.6 yr), and 5 patients with miscellaneous diseases including two with cerebral bleeding, one with cerebral infarction, one with metastatic brain tumor and one with olivo-ponto-cerebellar atrophy. Samples from cerebral bleeding and infarction were collected within two days after the onset. CSF samples from patients with subarachnoid hemorrhage were collected during 3-5 days after the onset. Other CSF samples were obtained at chronic stage. Five CSF samples were also collected by lumbar puncture from subjects (3 males and 2 females; mean age 68.2±4.5 yr), who had been suspected having cerebral or spinal tumors as non cerebrovascular accident controls. These samples were taken under overnight-fasted state and at lying position. These specimens were further processed for the determination of the immunoreactivity using Seppak C18 cartridges (Waters Associates Ltd., MA, USA). Usually 10-20 ml of CSF were passed through the cartridge and eluted 2 ml of 60 % acetonitrile (in water, vol/vol) with 0.1% trifluoroacetic acid(TFA, Nakarai Chemicals Ltd., Kyoto, Japan). These extracts were evaporated with Speed-Vac concentrator (Savant Instruments Inc., Farmingdale, NY, USA) and reconstituted with 1 ml of assay buffer.

Radioimmunoassay. The radioimmunoassay for AKH was performed with an antiserum raised against synthetic N-tyrosyl AKH (Tyr-AKH, Penninsula Lab. Inc., CA, USA) conjugated with bovine serum albumin with glutaraldehyde and emulsified in Freund's complete adjuvant for the primary immunization and in incomplete adjuvant for booster injections. The antisera were characterized by assessing their ability to bind labeled Tyr-AKH and their cross-reaction with other peptides (vasoactive intestinal polypeptide, pancreatic glucagon, glucose dependent insulinotropic peptide, somatostatin, neuropeptide Y, calcitonin gene-related peptide, human atrial natriuretic peptide, rat growth hormone, rat thyrotropin, rat luteinizing hormone, porcine insulin at concentration up to 1 nmol per tube). Among the red pigment concentrating or adipokinetic hormones (RPCH/AKH family), AKH-II(locust) and AKH(Manduca sexta) were also examined. Radiolabelled Tyr-AKH was prepared by radioiodination of the peptide using chloramine T. The purification was performed by Sephadex G-50 gel permeation chromatography. Standards were prepared gravimetrically from synthetic AKH-I(locust), lyophilized, and stored at -20°C. The assay was performed in

a total volume of 0.8 ml 0.06 M phosphate buffer, pH 7.4, containing 0.01 M ethylene diamine tetraacetate (EDTA; Wako Pure Chem. Ltd., Tokyo, Japan), 0.1% Triton X-100 (Sigma Chemical Co., St. Louis, MO, USA) and 150 μM bovine serum albumin (BSA, Sigma Chemical Co.). After 5 days incubation at 4°C, antibody-bound label was separated from free label by the addition of 8 mg charcoal(Wako Pure Chem. Ltd., Osaka, Japan) coated with clinical grade dextran.

Chromatography. The immunoreactivity assayed was characterized by gel permeation chromatography and reverse phase high performance liquid chromatography (HPLC) using μ Bondapak C18 column (Waters Associates Ltd.). A 1.4 x 90 cm of Sephadex G-50 superfine column was precalibrated with dextran blue, horse cytochrome C, and a trace amount of sodium [125-I] as molecular markers. Samples and synthetic standard were eluted at a flow rate of 3.2 ml/hour at 4°C with 0.06 M phosphate buffer containing 0.2M sodium chloride, 10 mM EDTA and 150 μM BSA. The HPLC system included pumps (LC-6A, Shimazu Co., Kyoto, Japan), a column oven(CTO-6A, Shimazu Co.), injector(Rhepdyne, Cotaro, CA, USA) and system controller(SCL-6B, Shimazu Co.). A combination of linear gradient and isocratic elution was used. The gradient from 10 to 30% of B (solvent A: 0.1% TFA/water, solvent B: 0.1% TFA/acetonitrile) for 10 min, 30 to 45% of B for the next 30 min and 45 to 60% for a further 10 min. Reaching 60% of B, the concentration was kept for 10 min. The flow rate was 1 ml/min and 1 ml fractions were collected for subsequent radioimmunoassay. Synthetic AKH-I(locust) was run as a standard under identical condition.

Calculations. Concentrations of the immunoreactivities examined were expressed as pmol per liter CSF and were given as the mean ± SEM. Statistical analysis was performed using Student's t test. Assay variation was calculated as a coefficient of variation (SD/X x 100) of 8 replicate samples.

RESULTS AND DISCUSSION

Rabbits immunized with glutaraldehyde conjugation revealed to produce antibody of sufficiently high affinity to be useful for the radioimmunoassay of tissue and CSF extract. Binding constant of the antibody (AKH-3) was 3.1×10^{10} M^{-1}. Following purification on gel chromatography, the specific activity of the label, as estimated by self displacement from the antibody, was 22.1 Bq/fmol (0.6 nCi/fmol). The lowest concentrations of AKH producing a fall in tracer binding greater than 2 SD from zero binding were 1.5 fmol/assay tube (ID_{50} value was 66 fmol/ tube), with intraassay variation of 8.5% and inter-assay variation of 12% at 25 fmol(n=8). Among the RPCH/AKH family 0.2% crossreaction to this system in a molar basis was

found in AKH-II(locust). No other known regulatory peptides tested cross-reacted in the assay. As shown in Fig.1, human CSF extract gave a parallel displacement of the label to the standard.

The mean concentration of AKH in CSF obtained from patients with subarachnoidal hemorrhage was 127.8±79.4 pmol/liter and seemed to be higher than that in controls (1.6±0.7 pmol/liter) though significant difference could not be observed ($p>0.05$). CSF AKH concentration from patients with subdural hematoma showed similar level to the control (2.5±1.4 pmol/liter). Mean CSF AKH concentration from patients with cerebral bleeding also seemed to be higher than that in controls (Fig.2).

As stated in the method section this immunoreactivity was further characterized with gel chromatography and reverse phase HPLC. Fig.3 showed representative gel permeation and HPLC profile for the immunoreactivity. In both systems the immunoreactive peak seemed to be single and the eluting position was exactly the same as the position where AKH-I (locust) emerged.

AKH-IR has been identified in the central nervous systems

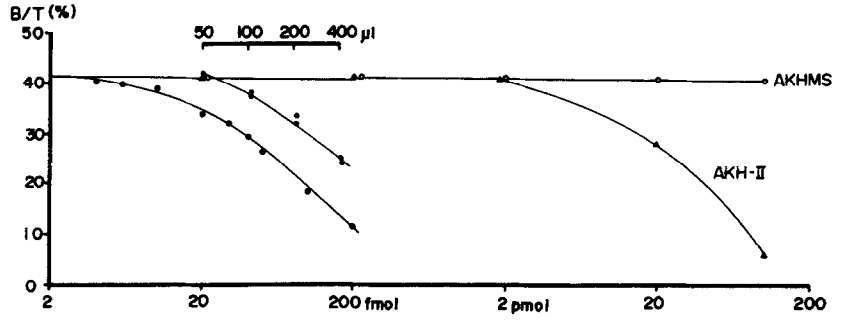

Figure 1. Standard curve of AKH (solid circle), mean dilution curve of CSF extract (duplicate solid circle) and the crossreaction to AKH II(triangle) or AKH MS (Manduca sexta; open circle).

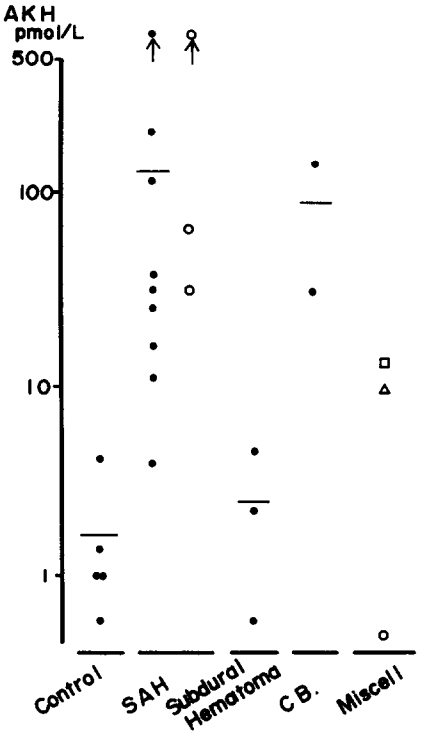

Figure 2. AKH-like immunoreactivity in cerebrospinal fluid in patients with cerebrovascular accidents and control subjects.
SAH:subarachnoid hemorrhage (closed circle: ventricular sample, open circle:cisternal sample), CB:cerebral bleeding, Miscell:miscellaneous disorders (cerebral infarction (open circle), olivo-ponto-cerebellar atrophy (open triangle), metastatic brain tumor (open square)).

of several invertebrate species including Locusta migratoria (6, 7) as well as the mammalian species (8,9). As the cerebral neurosecretory cell-corpus cardiacum complex in insects is thought to be analogous with the hypothalamic-neurohypophysial system in vertebrates (10), AKH action for a neuropeptide is supposed (8,9). As many peptides in the CSF are thought to be neurotransmitters or neuromodulators and exert their action partially via the CSF circulation (11), the result of the AKH-IR presence in the CSF might indicate its function for a neurotransmitter or neuromodulator in the human central nervous system. In our preliminary analysis AKH-IR was also found

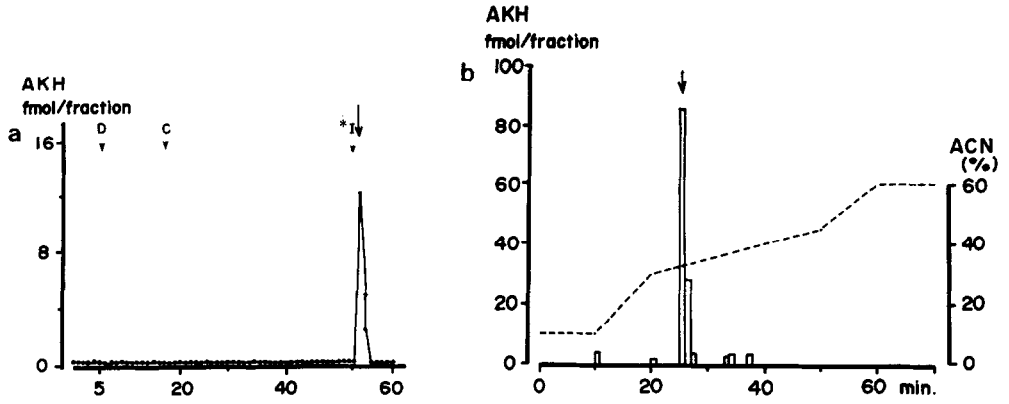

Figure 3. Representative gel chromatographic (3a) and reverse phase high performance liquid chromatographic (3b) profiles of AKH-like immunoreactivity in CSF extract. The gel column was calibrated with dextran blue (D), horse heart cytochrome C (C) and a trace amount of sodium [125-I]. The dotted line indicates the acetonitrile/water concentration. Arrow indicates the elution position of the synthetic AKH.

in the rat central nervous system, gastrointestinal tract and endocrine glands though the immunoreactivity was mainly present in more hydrophobic and larger molecular weight portion than the standard (Tsuchiya;unpublished), which was in accordance with Shueler et al (9). Furthermore, using rat pancreatic perfusion system pancreatic glucagon and insulin secretion induced with 10 mM arginine HCl was abolished by AKH infusion (Muramatsu;unpublished). These observation might support the presence of this insect hormone, AKH, in the mammalian tissues and play a role for a neurotransmitter or neuromodulator. Further study is obviously needed to elucidate the possibility.

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