Immunoreactive Corticotropin-Releasing Hormone (CRH) in Plasma and Hypothalamic Incubation Media as Assessed by a Novel and Highly Sensitive Immune Complex Transfer EIA

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Abstract. Previous methods of measuring CRH lack sensitivity for low concentrations of the peptide in peripheral blood or hypothalamic incubation media. We developed a novel immune complex transfer enzyme immunoassay (ICT-EIA) which utilizes two different antibodies, rabbit polyclonal antibody as a capture antibody and mouse monoclonal antibody, for β-D-galactosidase-conjugated Fab’ against different epitopes of the C-terminus portion of the CRH molecule. The least detectable value of the ICT-EIA was 10 amole/tube, which is 100 times more sensitive than a conventional RIA, and allowed us to measure CRH levels in plasma from pregnant women without any extraction procedures (the least detectable value, LDV, 6.8 pg/ml). Dilution curves of rat hypothalamic extracts or plasma samples from pregnant women were parallel with standard curves. The extent of cross-reaction (10,000 amole/tube of each peptide) by the ICT-EIA with ovine CRH, rat urocortin or any fragment of human GHRH(1-44)NH₂ was negligible, suggesting that EIA specificity was directed toward the C-terminus portion of the CRH molecule. Plasma CRH levels in pregnant women gradually increased from the first trimester (up to 14 weeks, n=4, below LDV, 6.8 pg/ml) to the third trimester of gestation (after 28 weeks, n=10, 503.4 ± 93.6 pg/ml). 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 complex, from 3-month-old control SD rats (CNT) were incubated every 30 min for 120 min in regular KRBG. Basal CRH release at 90–120 min was well measurable (mean ± SE: 3.8 ± 0.2 pg/hypothalamus/30 min, n=6). When hypothalami were incubated with KRBG containing 30 mM KCl, release was stimulated nearly 8-fold (30.0 ± 2.5 pg/hypothalamus/30 min, n=6, p<0.01 v.s. KRBG). These results suggest that the newly developed CRH ICT-EIA will be useful for the study of the physiology and pathophysiology of CRH release in both humans and rats.

Key words: corticotropin-releasing hormone (CRH), immune complex transfer-enzyme immunoassay (ICT-EIA), hypothalamic incubation, plasma levels

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Introduction

Corticotropin-releasing hormone (CRH) is a hypothalamic peptide that stimulates the release of ACTH (1). CRH in plasma or hypothalamic perfusates has been measured by radioimmunoassay after extraction with Sep-Pak cartridges (2). The extraction methods are not only time-consuming but also cause great variation in assay results. We recently developed a novel immune complex transfer EIA (3) for human GHRH (hGHRH) (4) to increase the sensitivity of conventional sandwich EIA (5) or RIA (6). In the present study we describe a highly sensitive immune complex transfer EIA (ICT-EIA) for CRH, and measured plasma CRH levels of pregnant women and CRH released in rat hypothalamic incubation media.

Materials and Methods

An antiserum against CRH (1-41)NH₂ was generated in the rabbit and has been well characterized (7, 8). Immunoglobulins have also been purified from mouse ascites, which contain monoclonal antibodies against CRH. The ICT-EIA for CRH was performed according to a method previously described (3) with minor modifications. Briefly, CRH in 20 μL of hypothalamic incubation medium or 10 μL of plasma mixed with 80 μL or 90 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 NaN₃), respectively, containing 0.4 mol/L NaCl was incubated at 4°C for 4 h with 50 μL of buffer A containing 0.4 mol/L NaCl, 2,4-dinitrophenyl (DNP)-biotinyl-bovine serum albumin (BSA)-anti-CRH Fab‘ conjugate (50 fmol), anti-CRH Fab‘-β-D-galactosidase conjugate (50 fmol) and nonspecific rabbit F(ab)‘, (0.1mg). Two colored polystyrene beads coated with affinity-purified (anti-2,4-DNP group) IgG were added to the reaction mixture, 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 0.1mol/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 × 10⁻⁶ mol/L 4-methylumbellifere. 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 CRH detection by the ICT-EIA were expressed as the minimal amount of CRH which gave a significant bound β-D-galactosidase activity in excess of that nonspecifically bound in the absence of CRH (background). The existence of a significant difference from the background was tested by Student’s t-test (p<0.01, n=5).

All subjects participating in this study gave their informed consent, and the project was approved by the Committee of Internal Medicine at Miyazaki Medical College. Nineteen pregnant women, aged 23–35 years, 6 non-pregnant women, aged 23–32 years, and 8 men, aged 25–35 years were studied between 9:00 a.m. and 10:00 p.m. Four pregnant women were in the first trimester of pregnancy (up to 14 weeks), 5 were in the second trimester (15–27 weeks), and 10 were in the third trimester (after 28 weeks). All were in good health and none were taking any medication. Blood, 2 ml, was collected in ice-chilled glass tubes containing EDTA-disodium and apotropane at 1 mg/ml and 1,000 KIU/ml blood, respectively, and immediately centrifuged
at 4°C for 15 min. Plasma was stored at -35°C until extraction.

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 a method previously described (9). 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 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% solvent A for 10 min, followed by a linear gradient from 20–60% solvent A over 40 min, 60–80% solvent A over a 5-min period, 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 EIA buffer, and measured for immunoreactive CRH by homologous CRH EIA.

Hypothalamic incubation was carried out according to a previous method (10). 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 detection limits of the ICT-EIA were 45 fg/tube (Fig. 1). This is 100-fold more sensitive than the conventional RIA. The extent of cross-reaction (10,000 amole/tube of each peptide) by the ICT-EIA with ovine CRH, rat urocrtin, any fragment of human GHRH (1-44)NH₂, other peptides of the glucagon-secretin family, somatostatin 14 or somatostatin 28, insulin, human ghrelin, GHRP-6, rat or human ACTH, and growth hormone was 0%, but 100% with [Met (O)²¹, ³⁸]CRH, suggesting that EIA specificity was directed toward the C-terminus portion of the CRH molecule. The dilution curves of pooled hypothalamic extracts or hypothalamic incubation media were parallel with the standard curves of the EIAs (Fig. 1). Intra-and inter-assay variation coefficients were 5 and 8%, respectively, at a level of 1000 fg/tube CRH.

The current EIA was, however, not sensitive enough to measure CRH levels in unextracted rat or nonpregnant human plasma from the peripheral circulation. Plasma CRH levels in pregnant women gradually increased from the first trimester (up to 14 weeks, n=4, below 6.8 pg/ml) to the third trimester of gestation (after 28 weeks, mean ± SE, n=10, 503.4 ± 93.6 pg/ml) (Fig. 2).

The HPLC of the rat hypothalamic extract showed a single major peak which corresponded to that of the synthetic peptide (Fig. 3).

Release of CRH from hypothalamic fragments incubated in KRBG decreased gradually from the initial preincubation period (0–30 min) to the last 30 min (90–120 min) (Fig. 4). The CRH release at 90–120 min occurred at a low but consistently detectable rate of 3.8 ± 0.2 pg/hypothalamus/30 min (KRBG, n=6). When hypothalami were incubated with KRBG containing 30 mM KCl, release was stimulated nearly 8-fold (30.0 ± 2.5 pg/hypothalamus/30 min, n=6, p<0.001 vs. KRBG).

These results suggest that the current ICT-EIA for CRH, providing precise evaluation of circulating CRH in pregnant women and CRH release from the rat hypothalami, could be useful for the study of the physiology and pathophysiology of CRH in both humans and rats.
Fig. 1 Standard curves of a highly sensitive immune complex transfer EIA (ICT-EIA, open squares) for CRH. Closed circles and squares indicate dilution curves for plasma samples from a pregnant woman at third trimester and rat hypothalamic (HT) extract, respectively. Vertical bars indicate standard deviations from several EIAs (n=3). Open triangles indicate cross-reactivity with rat urocrin and other related peptides described in the text.

Fig. 2 Plasma CRH concentration at different gestational stages. Plasma CRH levels increase toward the end of gestation.

Fig. 3 HPLC analysis of rat hypothalamic extract with the CRH EIA. The arrow indicates the elution position of synthetic rat CRH.
Fig. 4 Time course of CRH release from hypothalamic incubation. Marked stimulation of CRH release occurred in response to 30 mM K⁺ during the final 30-min period. Each column indicates the mean ± SE of 6 or 12 hypothalami, as indicated in parentheses. *indicates significant (P<0.01) increases in CRH release against regular KRBG solution.

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