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HISTIDYL-PROLINE DIKETOPIPERAZINE CYCLO (HIS-PRO): MEASUREMENT BY RADIOIMMUNOASSAY IN HUMAN BLOOD IN NORMAL SUBJECTS AND IN PATIENTS WITH HYPER- AND HYPOTHYROIDISM

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SUMMARY: An extraction procedure for cyclo (His-Pro) in human blood has been developed, employing sequential extraction with 90% methanol and 0.3 M perchloric acid. Chromatographic elution profiles of immunoreactive cyclo (His-Pro) in blood extracts measured by radioimmunoassay corresponded precisely to those of {^{3}H} cyclo (His-Pro) following separation with Sephadex G-25, DEAE-cellulose, and SP-Sephadex columns, and also HPLC. Concentrations of cyclo (His-Pro) in normal human subjects (5073 ± 730 fmoles/ml) were approximately 50 times greater than respective TRH concentrations measured in identical samples. Cyclo (His-Pro) concentrations in hypothyroid patients (12373 ± 802 fmoles/ml) were significantly higher than those in normal and those in hyperthyroid patients (5949 ± 566 fmoles/ml). TRH concentrations, by contrast, were similar in all 3 groups. Pyroglutamate aminopeptidase activity which catalyzes the conversion of TRH to cyclo (His-Pro), was identified also in peripheral plasma. The plasma enzyme activity in normal subjects was 76 ± 27 fmoles cyclo (His-Pro) formed/min/ml plasma. This activity was not statistically increased in hypothyroid patients, so that the increased concentrations of cyclo (His-Pro) in hypothyroidism could not be attributed to the enhanced conversion of TRH in the peripheral blood.

We have identified recently a novel neuropeptide, histidyl-proline diketopiperazine, cyclo (His-Pro), which can be derived from TRH by the limited proteolytic enzyme, pyroglutamate aminopeptidase (1). This dipeptide has been established by our specific RIA (2) to be present throughout the rat and monkey central nervous system like its precursor, TRH (3, 4). Moreover, elevated values of cyclo (His-Pro), but not TRH, have been found in the hypothalamus in hypothyroidism (5). On the basis of these observations and the

The abbreviations used are: cyclo (His-Pro), histidyl-proline diketopiperazine; TRH, thyrotropin-releasing hormone; RIA, radioimmunoassay; DEAE, diethylaminoethyl; SP-Sephadex, sulfopropyl-sephadex; HPLC, high performance liquid chromatography; PBS-BSA, phosphate buffered saline containing 1% bovine seruma albumin.

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recognition that other neuropeptides have been documented in peripheral blood (6-10), we wondered if cyclo (His-Pro) was present in human peripheral blood and if its concentrations could be influenced by thyroid status. Data that cyclo (His-Pro) is present in human blood and in increased concentrations in hypothyroidism form the substance of the present report.

MATERIALS AND METHODS

1. Peptides: Cyclo (His-Pro) was synthesized by us as described previously (11). {^{3}H} cyclo (His-Pro) was generated from {^{3}H} TRH (New England Nuclear, Boston, MA) as described previously (1). Synthetic TRH was purchased from Calbiochem-Behring, Corp., LaJolla, CA.

2. Extraction of cyclo (His-Pro) from blood: Five ml of blood was withdrawn from the antecubital vein of each human subject into a syringe containing 10 U/ml of heparin, and extracted immediately with 45 ml of chilled 99% methanol. The extract was centrifuged at 1200 xg for 30 minutes. Supernatants were then evaporated to dryness and suspended in 10 ml of 0.3 M perchloric acid (PCA). After freezing and thawing, the suspensions were centrifuged at 15,000 xg for 20 minutes. The resultant clear supernatants were neutralized with saturated $KHCO_3$ and centrifuged at 1200 xg for 30 minutes after freezing and thawing again. Supernatants were then lyophilized and resuspended in 1 ml of the assay buffer {0.01 M phosphate, 0.15 M NaCl and 1% bovine serum albumin, pH 7.5 (PBS-BSA)}. After centrifugation at 1200 xg for 30 minutes, the samples were used for RIA determinations.

3. Measurement of cyclo (His-Pro) and TRH by RIAs: RIAs for TRH and cyclo (His-Pro) were performed identically as described previously (2), using specific antisera for each peptide, diluted 1:10,000 for cyclo (His-Pro) and 1:52,000 for TRH. Assay sensitivities permitted detection of 43 fmole (10 pg) per tube of cyclo (His-Pro) and 2.8 mole (1 pg) per tube of TRH. The coefficient of intra-assay variation in both RIAs was less than 6%. Samples from human subjects were analyzed in the same RIA to minimize inter-assay variability.

4. Chromatographic characteristics of immunoreactive cyclo (His-Pro) in blood extracts: Chromatographic characterization of immunoreactive cyclo (His-Pro) from human blood was determined by applying extracted samples containing a trace amount of {^{3}H} cyclo (His-Pro) on 4 different chromatographic systems. A blood extract pool equivalent to 10 ml of unextracted blood was applied to a 1 x 55 cm Sephadex G-25 column and to a 1 x 15 cm DEAE-cellulose column. Columns were equilibrated respectively with PBS and distilled water, and eluted with the equilibrating buffers. High Performance Liquid Chromatography (HPLC) was carried out as described previously (5) by injecting 500 µl of the extracted pool into a µBondapak C18 reverse phase column (0.4 x 30 cm, Waters Association, Milford, MA), equilibrated with a solvent system of 22% ethanol, 0.01 M ammonium acetate, pH 4.0. One ml samples were collected, lyophilized, and resuspended in PBS-BSA for RIA determinations of cyclo (His-Pro). Sulfopropyl (SP)-Sephadex column chromatography was performed by applying 500 µl of blood extract dissolved in a 0.01 M ammonium acetate buffer, pH 3.6, to a 1 x 15 cm column as described previously (12). The sample was eluted using a discontinuous pH gradient (pHs 3.60, 5.55, 7.50) in an 0.01 M ammonium acetate buffer. One ml of each fraction was collected, lyophilized, and resuspended in PBS-BSA for ^{3}H-counting and determination of cyclo (His-Pro) by RIA.

5. Peripheral blood of pyroglutamate aminopeptidase activity: Enzyme activity was determined with slight modifications of our previously reported method (1, 5). In brief, 10 µl of plasma from each human subject was incubated for 10 minutes at 37^{0}C with 0.25 µCi (2.5 pmole) of {^{3}H-Pro} TRH in 50 µl of the incubation buffer (2.5 µM sodium phosphate, 50 nM EDTA and 50 nM dithiothreitol, pH 7.4).

Incubations were terminated by addition of 10 µl of 1 N acetic acid. Reaction mixtures were heated to 80°C for 10 minutes to facilitate cyclization of {^{3}H} His-ProNH_2 to {^{3}H-Pro} cyclo (His-Pro). Then, 143 nmol cyclo (His-Pro) was added to each tube as a marker peptide. Ten µl of each mixture was applied in duplicate on a Silicagel-60 TLC plate. The chromatogram was developed in a solvent system of $CHCl_3$ and CH_3OH (5:2; vol/vol), then sprayed with I_2-saturated $CHCl_3$, and dried. The Rf values for various peptide and amino acid standards in this system were as follows: cyclo (His-Pro) (0.38), TRH (0.08), acid TRH (0.01), His-Pro (0.01), prolineamide (0.01) and proline (0.01). The area corresponding to the carrier cyclo (His-Pro) was scraped out, and radioactivity was determined using a Packard liquid scintillation spectrometer.

6. Human subjects: Blood samples were collected from 10 normal patients, 6 patients with primary hypothyroidism, and 7 patients with hyperthyroidism as described above. Plasma TSH concentrations in hypothyroidism ranged from 18 to 52 µU/ml, and plasma T_4 concentrations ranged from 1.0 to 3.5 µg/dl (normal 5-13 µg/dl). In hyperthyroid patients, plasma T_4 concentrations ranged from 14.8 to 23 µg/dl.

RESULTS

The recoveries of tracer {^{3}H} cyclo (His-Pro) and {^{3}H} TRH added to blood samples extracted sequentially with 90% methanol and 0.3 M PCA were 79.6 ± 1.3 and 81 ± 1.5% respectively. Other extraction techniques, involving alternate solvents (e.g., 10 or 20% TCA, 1% charcoal, or 90% methanol) either gave excessively low yields or caused non-specific inhibition of the cyclo (His-Pro) RIA system and were therefore not used.

Cyclo (His-Pro) immunoreactivity was identified in all human blood extracts (Table 1). An inhibition curve of cyclo (His-Pro) immunoreactivity, derived from a human blood extract pool equivalent to 10 ml of whole blood, was identical over a 16-fold dilution assay to the curvilinear function generated with synthetic cyclo (His-Pro) (Fig. 1).

Chromatographic profiles of the human blood extract pool revealed that immunoreactivity (lower section) co-chromatographed precisely with {^{3}H} cyclo (His-Pro) elutions (upper section) in Sephadex G-25, DEAE-cellulose, HPLC (Fig. 2), and SP-Sephadex (Fig. 3) systems.

Concentrations of cyclo (His-Pro) and TRH in individual blood samples from euthyroid, hypothyroid, and hyperthyroid subjects are summarized in Table 1. Cyclo (His-Pro) concentrations were similar statistically in normal and hyperthyroid subjects, but were elevated significantly to 244% above euthyroid values in subjects with hypothyroidism. Cyclo (His-Pro) concentrations were approximately

Table 1. Concentrations of Cyclo (His-Pro) and TRH in Blood Extracts and Enzyme Activities of Pyroglutamate Aminopeptidse in Plasma

	Euthyroid(10)	Hyperthyroid(7)	Hypothyroid(6)
Cyclo (His-Pro) (fmoles/ml)	5073 ± 730	5949 ± 566	12373 ± 802*
TRH (fmoles/ml)	117.1 ± 7.4	110.4 ± 12.4	130.0 ± 10.2
Enzyme Activity (fmoles cyclo-(His-Pro) formed/ min/ml plasma	76.2 ± 27.3	60.9 ± 18.4	100.4 ± 14.1

Whole blood samples were extracted sequentially with 90% methanol and 0.3 M perchloric acid, and measured for each peptide by RIA. The enzyme activities were estimated by detecting {^{3}H-Pro} cyclo (His-Pro) formed from {^{3}H-Pro} TRH which was incubated in plasma. The data are expressed as mean ± SEM. The numbers of examined human subjects are parenthesized. The statistical significance was performed between normal and patient groups using Student's t-test.

50-fold higher than TRH on a molar basis in euthyroid and hyperthyroid subjects and were 95-fold higher than TRH in the hypothyroid group.

Low levels of pyroglutamate aminopeptidase activity was identified in all sera (lower section, Table 1). The levels found were statistically similar in all groups.

DISCUSSION

The data herein demonstrate that cyclo (His-Pro) immunoreactivity is easily detectable in human blood extracts and appears to be indistinguishable from synthetic cyclo (His-Pro) by immunoidentity (Fig. 1) and by chromatographic behavior on Sephadex G-25, DEAE-cellulose, HPLC, and SP-Sephadex systems (Figs. 2 & 3).

The mean concentration in normal subjects was 5073 fmoles/ml, approximately 50-fold higher than the corresponding concentration of TRH, whose average was 117.1 fmoles/ml, similar to values we have reported previously (13). It is of interest that cyclo (His-Pro) values in peripheral blood were considerably higher than those reported for other neuropeptides (6-10), raising intriguing questions about possible functions of this dipeptide in peripheral tissues.

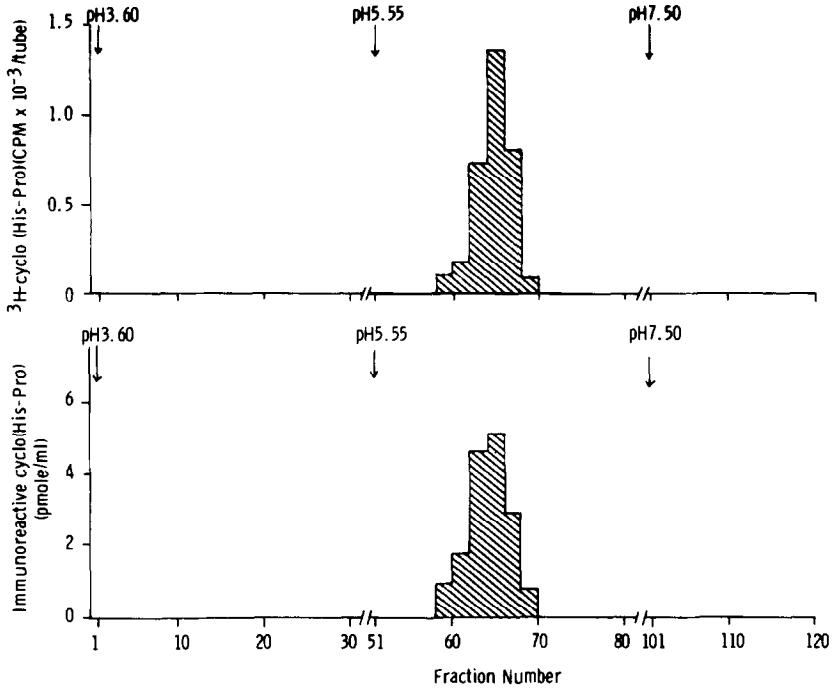

Figure 1. Inhibition curve of human blood extracts in the cyclo (His-Pro) radioimmunoassay (RIA).

Cyclo (His-Pro) concentrations were not altered in hyperthyroidism, and the slightly higher mean value of 5949 fmoles/ml was not statistically different from normal. TRH concentrations, similarly, were not different in hyperthyroid sera, as reported recently by us (13). However, cyclo (His-Pro) concentrations were substantially elevated in sera derived from hypothyroid subjects. The mean concentration of 12373 fmoles/ml, 240% above the euthyroid mean value, was significant statistically at $p < 0.01$. Such elevations in hypothyroidism could not be attributed to increased TRH concentrations nor to increased pyroglutamate aminopeptidase activity (Table 1). Presumably, the increased concentrations of cyclo (His-Pro) in hypothyroidism reflect enhanced extra-circulatory production and/or impaired clearance. Studies are in progress to elucidate what mechanisms may be responsible for these observed increments in cyclo (His-Pro) concentrations in hypothyroidism.

The precise source(s) of cyclo (His-Pro) in peripheral blood is not presently known. The high concentrations identified, however, suggest that non-CNS sources may be as important as the central nervous system itself. Potential

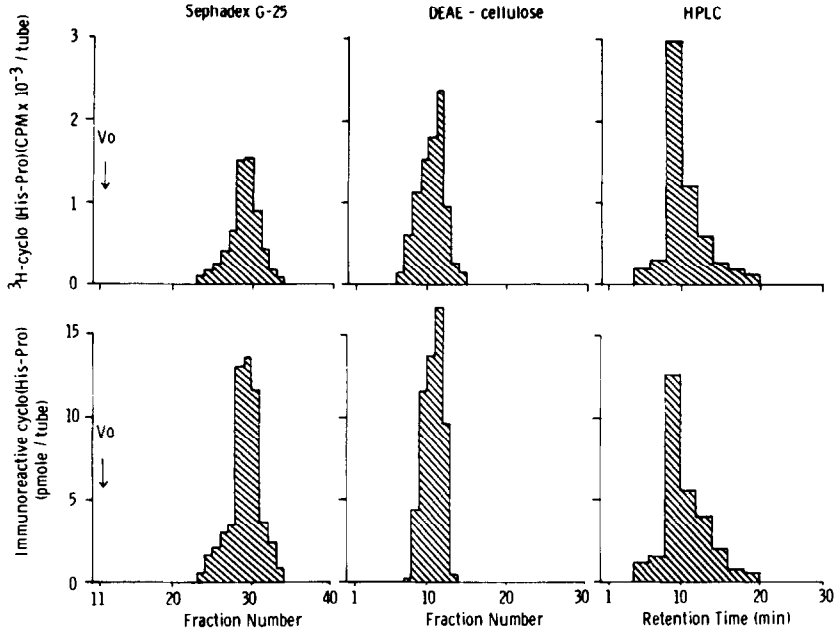

Figure 2. The elution profiles of immunoreactive and ^{3}H-labelled cyclo (His-Pro) on Sephadex G-25, DEAE-cellulose and HPLC columns. The concentrated human blood extracts were applied on each column and eluted as described in Materials and Methods. The activities of immunoreactive (lower column) and radioactive cyclo (His-Pro) (upper column) were determined respectively by RIA and liquid scintillation counting.

sources might include both pancreas and the gastrointestinal system, which have been demonstrated by us to be a rich source of cyclo (His-Pro) in the rat (14).

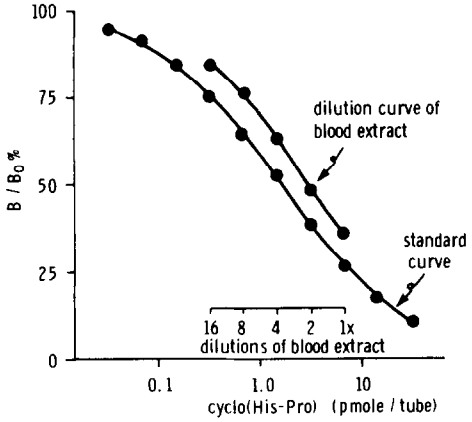

Figure 3. The elution profiles of immunoreactive and ^{3}H-labelled cyclo (His-Pro) on a SP-Sephadex column.

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