Vol. 164, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 16, 1989 Pages 394-399

ESTABLISHMENT OF RADIOIMMUNOASSAY FOR HUMAN ISLET AMYLOID POLYPEPTIDE AND ITS TISSUE CONTENT AND PLASMA CONCENTRATION

Masamitsu NAKAZATO[1§], Junko ASAI[1], Kenji KANGAWA[2], Shigeru MATSUKURA[1] and Hisayuki MATSUO[2†]

[1]Third Department of Internal Medicine and [2]Department of Biochemistry, Miyazaki Medical College, Kiyotake, Miyazaki 889-16, Japan

Received September 4, 1989

SUMMARY: Using a synthetic C-terminal tetradecapeptide of human islet amyloid polypeptide (IAPP), we prepared an antiserum for human IAPP [24-37] and established a highly sensitive radioimmunoassay (RIA) for human IAPP. Analyses of human pancreatic extract using reverse-phase high performance liquid chromatography coupled with the RIA revealed that the antiserum specifically detects human IAPP. The content of IAPP in the pancreas of two non-diabetic patients was 604.0 and 1447.7 pg/mg wet weight, and a small amount of IAPP-immunoreactivity was detected in the stomach, duodenum, and jejunum. The mean plasma concentration of IAPP in 10 normal individuals was 13.5 ± 4.8 (SD) pg/ml. The RIA established in this study provides a useful tool to elucidate the physiological function of IAPP and its pathophysiological significance in non-insulin-dependent diabetes mellitus (NIDDM).

Amyloid deposition in the islets of Langerhans is a major pathological feature of NIDDM. Amyloid deposits are observed in 51% of NIDDM patients over the age of 40 (1), and small deposits are also noted in 14-18% of elderly non-diabetic individuals (1, 2). Islet amyloid is also quite common in insulinoma (3). Islet amyloid has been postulated to be derived from insulin or its precursor based on immunohistochemical studies using an anti-insulin antiserum. Recently a major protein constituent of the amyloid has been purified from human insulinoma (4) and from islet amyloid in NIDDM patients (5). This peptide, designated islet amyloid polypeptide (IAPP) (4), or diabetes-associated peptide (5) or amylin (6), consists of 37 amino acid residues and is 46% identical to the sequence of human calcitonin gene-related peptide (CGRP). IAPP has been observed immunohistochemically in normal B cells and in islet amyloid (7, 8). IAPP has been shown to inhibit both basal and insulin-stimulated rates of glycogen synthesis in stripped rat soleus muscle *in vitro* (6).

§To whom correspondence should be addressed.

† Present address: National Cardiovascular Center Research Institute, Suita, Osaka 565, Japan.

ABBREVIATIONS: CGRP, calcitonin gene-related peptide. HPLC, high performance liquid chromatography. IAPP, islet amyloid polypeptide. NIDDM, non-insulin-dependent diabetes mellitus. RIA, radioimmunoassay.

0006-291X/89 $1.50

In the present study, we prepared an antiserum against a synthetic C- terminal tetradecapeptide of human IAPP, and developed a sensitive and specific radioimmunoassay (RIA) for human IAPP. We identified immunoreactive species in human pancreas to be IAPP by using reverse- phase high performance liquid chromatography (HPLC) coupled with the RIA. The regional distribution and plasma concentration of human IAPP are also presented.

MATERIALS AND METHODS

Peptide synthesis: The tetradecapeptide with C- terminal tyrosine amide (Gly- Ala- Ile- Leu- Ser- Ser- Thr- Asn- Val- Gly- Ser- Asn- Thr- Tyr- NH_2) corresponding to the subsequence [24- 37] of human IAPP was synthesized by solid phase techniques, using peptide synthesizer Model 430A (Applied Biosystems). The peptide, human IAPP [24- 37], was purified by reverse - phase HPLC on a Chemcosorb 5ODS- H column. Correct synthesis was confirmed by amino acid analysis and sequencing.

Preparation of antiserum: Human IAPP [24- 37] (12 mg) was conjugated with bovine thyroglobulin (10 mg) by the glutaraldehyde method. The reaction mixture was dialyzed four times against one liter of 0.9% NaCl. Amino acid analysis of the conjugate showed that one thyroglobulin molecule was coupled with an average of 164 molecules of the peptide. The antigenic conjugate solution (1.5- 3 ml) was emulsified with an equal volume of Freund's complete adjuvant, and used for immunizing New Zealand white rabbits by intra- and subcutaneous injections. They were boostered every two weeks and bled 7 days after each injection. Two samples of the antisera, #203- 5 and #203- 9, were obtained after the fifth and ninth immunization, respectively.

Radioiodination of ligand: Human IAPP [24- 37] was radioiodinated by the chloramine- T method (9). The ^{125}I- labeled peptide was purified by reverse- phase HPLC on a TSK ODS 120A column.

Radioimmunoassay procedure: The incubation buffer for RIA was 0.05M sodium phosphate buffer (pH 7.4), containing 0.25% BSA, 0.08M NaCl, 0.025M EDTA•2Na, 0.05% NaN3, and 0.1% Triton X- 100. The sample (100 μ l) was incubated with antiserum diluent (100 μ l) for 24 hr. The tracer solution (16,000- 18,000 cpm in 100 μ l) was added and further incubated for 24 hr. Bound and free ligands were separated by the use of polyethyleneglycol. Radioactivity of the precipitate was measured in an Aloka ARC- 600 gamma counter. Samples were routinely assayed in duplicate. Assay was performed at 4 °C .

Characterization of the antiserum: Cross- reactivity of antiserum #203- 9 was examined with human IAPP [1- 37], rat IAPP [1- 37], human CGRPs, human insulin, human glucagon, human somatostatin, and human pancreatic polypeptide.

Identification of immunoreactive IAPP in the pancreas: The pancreas was obtained at autopsy from a 26- year- old male who died of epipharyngeal carcinoma. Amyloid deposits were not histochemically observed in the pancreas. The pancreas (3 mg wet weight) was boiled in 10 volumes of 1M acetic acid containing 20mM HCl for 10 min and then homogenized for 8 min. The homogenate was centrifuged at 20,000 x g for 30 min. The supernatant was applied to a Sep- Pak C- 18 cartridge (Waters), washed with 0.1M acetic acid, and eluted with 3 ml of 60% acetonitrile (CH_3CN) solution containing 0.1% trifluoroacetic acid (TFA). The eluate was subjected to reverse- phase HPLC on a TSK ODS 120A column. Chromatographic conditions are described in the legend of Fig. 2. All fractions were monitored by the RIA. The elution position of the immunoreactive fraction was compared with that of human IAPP [1- 37] purified from the pancreas.

Preparation of tissue and plasma samples: i) Tissue samples: Human tissues listed in Table I, except for the thymus, were resected at autopsy from two cases (aged 26, 64) expired from epipharyngeal carcinoma and cerebral hemorrhage, respectively. The thymus was obtained at thymectomy from two patients with thymoma (54- year- old male) and thymic hyperplasia (46- year- old female), respectively. Tissues were boiled in 10 volumes of 1M acetic acid containing 20mM HCl for 10 min and then homogenized for 8 min. The homogenate was centrifuged at 20,000 x g for 30 min. The supernatant was subjected to a Sep- Pak C- 18 cartridge as described above. The eluate was evaporated and reconstituted with the RIA buffer for submission to the RIA. ii) Plasma samples: Blood obtained from ten normal individuals (6 males and 4 females, aged 22- 37 yrs, mean 28.3 yrs) after overnight fasting was taken with EDTA•2Na (1 mg/ml blood) and aprotinin (500 units/ml blood) into a pre- chilled polypropylene tube. Samples were immediately centrifuged, and the plasma was applied to a Sep- Pak C- 18 cartridge which was pre- equilibrated with 0.9% saline. After washing the

column with saline and then 0.1% TFA solution, the adsorbed materials were eluted with 60% CH_3CN solution containing 0.1% TFA. The eluate was evaporated and submitted to the RIA.

RESULTS

RIA for IAPP: We synthesized human IAPP [24-37] with C-terminal tyrosine amide to use for immunization. Antiserum #203-9 recognized human IAPP [24-37] with high affinity at a final dilution of 30,000. As seen in Fig. 1, half-maximum inhibition by the peptide was observed at 26 fmol/tube, and the peptide was detectable at a low level of 3 fmol/tube. Human IAPP [1-37] showed 100% cross-reactivity with the antiserum. The dilution curve of human pancreatic extract was parallel to the standard curve. The antiserum did not exhibit cross-reactivity with human CGRPs, or other known peptides present in human pancreas: insulin, glucagon, somatostatin, and pancreatic polypeptide. Antiserum #203-9 showed only 0.20% cross-reactivity with rat IAPP, which we have recently isolated from the pancreata of normal rats and whose complete amino acid sequence we have determined (10). We prepared another antiserum, #203-5, obtained after the fifth immunization. This antiserum exhibited 0.56% cross-reactivity with rat IAPP (10), and was employed for the RIA in isolating rat IAPP.

Identification of immunoreactive species in the pancreas: Prior to the assay of IAPP, we identified immunoreactive species in human pancreas. The pancreatic extract was first applied to a Sep-Pak C-18 cartridge. Immunoreactivity was detected in the column eluate. The eluate was separated by reverse-phase HPLC to identify immunoreactive species. As shown in Fig. 2, IAPP-immunoreactivity emerged only at a position identical to that of human IAPP [1-37].

Tissue content and plasma concentration of IAPP: Tissue content and plasma concentration of IAPP are presented in Table I. When the tissues and plasma which were not extracted through

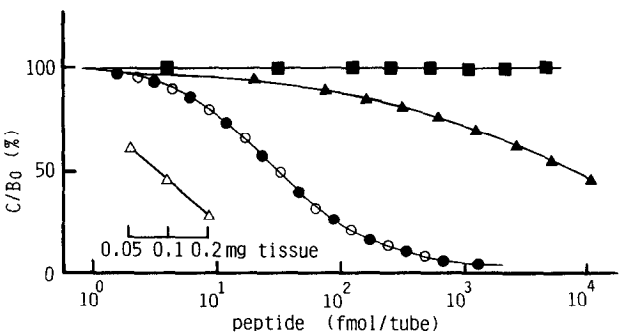

Fig. 1: Standard curve of RIA for human IAPP [24-37] and cross-reactivity of antiserum #203-9. Inhibition of ^{125}I-human [24-37] binding to the antiserum by serial dilutions of human IAPP [24-37] (●), human IAPP [1-37] (○), rat IAPP [1-37] (▲), human pancreatic extract (△), CGRPs (■), insulin (■), glucagon (■), somatostatin (■), and pancreatic polypeptide (■). The latter five were derived from human and none of them exhibited any cross-reactivity with the antiserum.

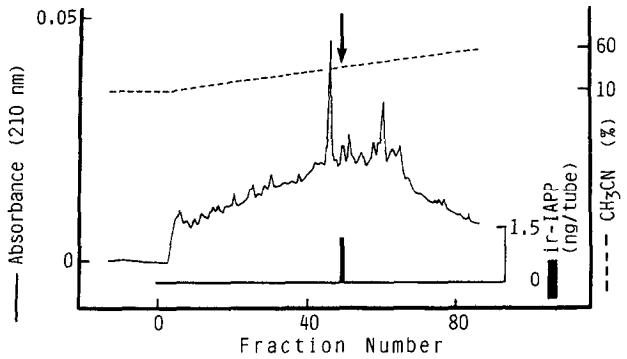

Fig. 2: Identification of human IAPP in pancreas by reverse-phase HPLC.
Sample: human pancreas (3 mg wet weight) extracted through a Sep-Pak C-18 cartridge.
Column: TSK ODS 120A (4.6 x 150 mm, Tosoh). Flow rate: 1.0 ml/min.
Solvent system: (A) H_2O : CH_3CN : 10% TFA = 90 : 10 : 1 (v/v).
(B) H_2O : CH_3CN : 10% TFA = 40 : 60 : 1 (v/v).
Linear gradient from (A) to (B) for 40 min.
All fractions were monitored by the RIA for IAPP and the immunoreactivity observed is shown by the black bar.
The arrow indicates the elution position of human IAPP [1-37].

the Sep-Pak C-18 cartridge were measured by the RIA, the results were two- to ten-fold higher than those presented in Table I, and the dilution curves of these samples were not parallel to the standard curve of the RIA. This suggested that admixed materials in the samples could interfere with valid determination of IAPP. To avoid this interference, the samples were extracted through the C-18 cartridge and then submitted to the RIA.

A large amount of IAPP was present in the pancreas and a small amount of IAPP-immunoreactivity was detected in the stomach, duodenum, and jejunum. No IAPP-

Table I. Tissue content and plasma concentration of IAPP in human

tissue	pg/mg wet weight		tissue	pg/mg wet weight	
	case 1	case 2		case 1	case 2
thyroid gland	<1	<1	pancreas	603.99	1447.72
lung	<1	<1	kidney	<1	<1
cardiac atrium	<1	<1	adrenal gland	<1	<1
ventricle	<1	<1	gall bladder	<1	<1
esophagus	<1	<1	spleen	<1	<1
stomach	5.33	8.19	liver	<1	<1
duodenum	4.51	12.57	thymus*	<1	<1
jejunum	4.14	7.72	spinal cord	<1	<1
ileum	<1	<1	plasma **	13.5 ± 4.8 pg/ml	
rectum	<1	<1			

case 1: A 26-year-old male expired from epipharyngeal carcinoma.
case 2: A 64-year-old male expired from cerebral hemorrhage.
Results are expressed as pg IAPP [1-37] equivalent/mg wet weight, except for plasma.
Thymus* obtained from two patients with thymoma and thymic hyperplasia, respectively.
Plasma** obtained from 10 normal individuals. Results are expressed as mean ± SD.

immunoreactivity was present in any of the other tissues examined. Plasma concentration of IAPP in 10 normal individuals ranged from 7.3 to 23.2 pg/ml with a mean of 13.5 ± 4.8 (SD) pg/ml.

DISCUSSION

Using a synthetic C- terminal fragment of human IAPP, we prepared antiserum #203- 9 and established a sensitive and specific RIA for human IAPP. C- terminal regions of human and rat IAPPs and human CGRPs are presented in Fig. 3. A whole molecule of human IAPP has 84% amino acid sequence homology with rat IAPP (10, 11) and 46% homology with human CGRPs; however, the C- terminal region of human IAPP is not homologous to rat IAPP, nor to human CGRPs. This could yield the very low cross- reactivity of antiserum #203- 9 with rat IAPP and the absence of cross- reactivity with human CGRPs as observed.

Figure 2 indicated that human IAPP was present in the pancreas as a 37- amino acid peptide, after being processed from a 67- amino acid precursor at Lys- Arg proteolytic cleavage sites (12, 13). The antiserum raised against human IAPP [24- 37] equally recognized human IAPP [1- 37] on a molar basis, indicating that a mature form of human IAPP was precisely measured by the RIA. For further confirmation of the specificity of the RIA, we identified immunoreactive species in human pancreas by using HPLC coupled with the RIA. A single immunoreactive peak was observed at an elution position identical to that of human IAPP [1- 37]. Thus, this RIA was confirmed to detect human IAPP specifically.

We have found that proteolysis of peptides takes place during extraction devoid of tissue heat treatment prior to homogenization (14). No fragment of IAPP was observed in the HPLC, indicating that the present procedure for tissue extraction was efficient in preventing nonspecific degradation of IAPP by intrinsic proteases.

The content of IAPP in human pancreas (604.0 and 1047.7 pg/mg wet weight) is equal to or greater than that of glucagon (160 pg/mg wet weight), somatostatin (556.9 pg/mg wet weight), and pancreatic polypeptide (514.4 pg/mg wet weight) (15). Abundance of IAPP in the pancreas suggests that IAPP must play an important role as a biologically active peptide. A

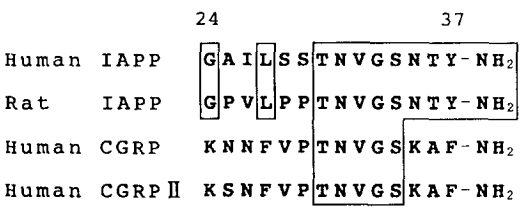

Fig. 3: Alignment of amino acid sequence from the 24th to 37th position of human and rat IAPPs and human CGRPs. Areas enclosed in rectangles indicate the same amino acid sequences.

small amount of IAPP-immunoreactivity was detected in the stomach, duodenum, and jejunum, as was true in the case of glucagon, somatostatin, and pancreatic polypeptide. The latter three peptides have some effects on secretory and motor functions of the gastrointestinal tract as well as on the metabolism of nutrients. Further investigation is needed to clarify whether IAPP exists in the gastrointestinal tract and whether it serves as a gastrointestinal hormone as well. IAPP has been shown to co-localize with insulin in the secretory granules (16). IAPP is probably co-secreted with insulin at a high rate in the early stage of NIDDM, which may be related to amyloid fibril formation in the islets. Measurement of the plasma concentration of IAPP could help to clarify the pathophysiological significance of IAPP in NIDDM. The RIA is a promising tool in elucidating the functional role of IAPP and in determining the various physiological factors involved in the secretion of IAPP.

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