Radioimmunoassay for Pancreatic Polypeptide, and Its Age-Related Changes in Concentration

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Pancreatic polypeptide (PP), a recently discovered pancreatic hormone, is potentially a marker for endocrine tumors. Consequently, we devised a radioimmunoassay for it, using antisera (raised in rabbits) to bovine PP, 125I-labeled bovine PP (purified by anion-exchange chromatography), and human PP standards. Concentrations circulating in fasting, normal subjects were measured. Statistical analysis of the results revealed a skewed distribution. An age-related increase was also observed. Evaluating PP concentrations in sera from 23 patients with endocrine tumors, we found increased values in a few cases of Zollinger–Ellison syndrome, medullary carcinoma of the thyroid, carcinoid syndrome, and one tumor producing vasoactive intestinal polypeptide (VIPoma). In contrast, values from five insulinomas and one glucagonoma were within the normal reference interval. Thus, an increased value for PP in a fasting individual may suggest the diagnosis of an endocrine tumor but is not a diagnostic prerequisite.

Additional Keyphrases: endocrine tumors • cancer • reference interval

Pancreatic polypeptide (PP),1 a 36-residue peptide, was discovered as a contaminant during the chemical purification of insulin (1), rather than by virtue of any distinct biological action. It has been localized predominantly to distinct endocrine cells of the pancreatic islets (2). Chemical characterization of PP derived from certain birds and mammals has revealed differences in primary structure, which may reflect differences in observed biological activity. In birds, the peptide has a metabolic role, primarily in hepatic glycogenolysis but possibly also in lipogenesis (3). In humans, PP inhibits gall-bladder contraction and, to a lesser extent, pancreatic exocrine secretion (4). Hyperplasia of PP-containing cells, in association with increases in its circulating concentrations, has been observed in patients with “mixed-type” pancreatic endocrine tumors (5) and in a proportion of patients with “VIPomas” (tumors that produce vasoactive intestinal polypeptide) or the “watery diarrhea hypokalemic achlorhydric” syndrome (6).

We developed a radioimmunoassay for this peptide and assessed the diagnostic value of circulating concentrations in a heterogeneous group of fasting patients with endocrine tumors.

Materials and Methods

Reagents

Bovine pancreatic polypeptide (615-D63-166-7) and human pancreatic polypeptide (615-1054B-200-8) were generously provided by Dr. R. E. Chance, Eli Lilly & Co., Indianapolis, IN 46268. The synthetic C-terminal hexapeptide of pancreatic polypeptide was obtained from Bioproducts, Peptide Department, Brussels, Belgium. Rabbit albumin was obtained from Sigma Chemical Co., St. Louis, MO 63178, and pure human albumin from Behringwerke AG, Marburg, F.R.G. Aprotinin (“Trasylol”) solution, containing 20 000 kallikrein inactivator units (K.I.U.) per milliliter of 150 mmol/L NaCl, was supplied by Bayer, Haywards Heath, U.K.

Antibody production. We used two methods of conjugating antigen to the carrier protein:

I. Carboxidiomide conjugation (7). This conjugate was prepared from 600 μg of bovine PP, 250 μg of rabbit albumin, and 0.5 mg of freshly weighed carboxidiomide (hygroscopic substance).

II. Glutaraldehyde conjugation (8). This conjugate was prepared by adding 600 μg of bovine PP and 250 μg of rabbit albumin to 2 mL of 40 mL/L glutaraldehyde solution. The efficiency of the conjugation methods was determined by estimating the amount of unconjugated PP present, by precipitating large-Mr (relative molecular mass) conjugated PP with absolute ethanol. The maximum percentage of unconjugated PP was 0.4%. We immunized three rabbits and one sheep with bovine PP conjugated by use of method I, and designated them PP204, PP205, PP206, and S98, respectively. Three rabbits—PP207, PP208, and PP209—were immunized by use of coupling method II. The primary immunization in each case consisted of approximately 100 μg of bovine PP in 1 mL of Freund’s complete adjuvant, injected subcutaneously at multiple sites. Secondary immunization, administered six weeks later, consisted of approximately 50 μg of antigen in 1 mL of Freund’s adjuvant (1/10, by vol, mixture of complete/incomplete), injected at a single site. We investigated the cross reactivity of the bovine PP antisera by testing them with human PP, bovine PP, the C-terminal hexapeptide of PP, insulin, gastrin, secretin, vasoactive intestinal polypeptide (VIP), gastric inhibitory polypeptide, and somatostatin. Two recently isolated peptides, neuropeptide Y and peptide YY (9, 10), which have considerable sequence homology with PP, were also tested for cross reactivity with the antisera.

Preparation of iodinated PP. We added 1 mCi of 125I to 10 μg of bovine PP in 100 μL of phosphate buffer (0.2 mol/L, pH 7.2) and stirred this at room temperature before adding 10 μL of a 2 g/L solution of Chloramine T. After 15 s, we added 100 μL of sodium metabisulphite (0.5 g/L solution) to terminate the reaction. After stirring the mixture for a further 40 s, we added 50 mL of a 10 g/L solution of potassium iodide. The resulting 123I-labeled bovine PP was promptly purified at 4 °C by anion-exchange chromatography, as follows. To a 30 × 0.9 cm column of QAE Sephadex A-25 equilibrated with Tris HCl buffer (80 mmol/L, pH 8.0) containing 2 g of human albumin per liter, we applied the labeled PP and eluted it with the same buffer solution at 7 mL/h. Fractions showing peaks corresponding to 123I-labeled bovine PP were pooled and stored in an equal volume of acidified ethanol.

1 Nonstandard abbreviations: PP, pancreatic polypeptide; VIP, vasoactive intestinal polypeptide; MEA, multiple endocrine adenomatosis.

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(ethanol/concentrated HCl, 99/1 by vol) at -20 °C. We determined the elution profile of nonlabeled peptide by eluting standard bovine PP under the same conditions as for \(^{125}\)I-labeled bovine PP.

Radioimmunoassay constituents. Human PP, the standard in our radioimmunoassay, was stored in aliquots at -20 °C in phosphate buffer (40 mmol/L, pH 7.4) containing 2 g of human albumin per liter. Before radioimmunoassay, the human PP was thawed and diluted to concentrations ranging from 19.5 to 1250 ng/L. The diluent was an ethanolic extract of horse serum, reconstituted in phosphate buffer, 40 mmol/L, pH 7.4. The horse serum had previously been stripped of endogenous peptides by treatment with activated charcoal (11).

Antiserum obtained from rabbit PP205 was used at a final assay dilution of 72,000-fold. The antiserum was stored at 1000-fold dilution at -20 °C, then thawed and diluted prior to assay in the phosphate buffer containing horse serum (20 mL/L).

\(^{125}\)I-labeled bovine PP was diluted in the phosphate buffer containing horse serum to give 100 counts/s per 100-μL aliquot.

Analytical samples. Blood samples for PP analysis were withdrawn, collected in heparinized tubes, and cooled to 4 °C. After centrifugation at 1000 × g for 20 min, 1-mL aliquots of plasma were extracted with 1.6 mL of absolute ethanol. The supernate obtained after further centrifugation (1500 × g, 20 min) was dried under a stream of air, stored at 4 °C, and reconstituted in phosphate buffer (40 mmol/L, pH 7.4) before assay. When necessary, analytical samples were diluted with standard diluent.

PP recovered from plasma. Stock standard used in the radioimmunoassay was diluted with charcoal-stripped plasma, then extracted with absolute ethanol in the same proportion as for the samples. Dried extracts were reconstituted in phosphate buffer (40 mmol/L, pH 7.4) and included in each assay as control recovery samples.

Procedures

All radioimmunoassay procedures were performed at 4 °C. Standards or analytical samples (100 μL), antiserum (100 μL), and standard diluent (100 μL) were incubated for 48 h before the addition of labeled peptide (100 μL). After a further 48 h, antibody-bound and free peptide were separated by using dextran-coated charcoal (12). To assess the percentage of free labeled peptide in the charcoal pellet, obtained by centrifugation at 1500 × g for 30 min, we used a gamma counter (NE 1600; Nuclear Enterprise Ltd., Sighthill, Edinburgh, U.K.).

Clinical studies

Stability of PP in whole blood. Blood samples were drawn from six normal volunteers and cooled to 4 °C. The plasma was separated and extracted for PP estimation immediately, and at various intervals thereafter, to assess the degradation of PP in whole blood. We determined the effect of Trasylol by adding it, 500 K.I.U./mL, to blood samples immediately after sampling (n = 5).

Circulating PP concentrations in fasting normal subjects. Circulating concentrations of PP were measured in normal volunteers with no history of gastrointestinal or endocrine disorders. The age, sex, and weight of each subject were recorded.

Basal PP concentrations. Fasting PP concentrations were estimated in 29 of the volunteers, at 15-min intervals over a 1-h period, to assess baseline fluctuation.

Circulating PP concentrations in fasting patients with endocrine tumors. We estimated pre-operatively the circulat-
tive peak provided a standard curve of greater sensitivity than the first-eluted peak and was therefore routinely used as tracer. Figure 2 demonstrates the superimposition of the dilution curve of labeled bovine PP and a standard curve for human PP standards.

Radioimmunoassay of PP. The efficiency of the extraction procedure used to measure PP was investigated by adding the peptide, in predetermined amounts, to charcoal-stripped plasma. The average analytical recovery was 89.6%. The precision of the assay was calculated by determining the standard deviation of replicate determinations. In the sensitive region of the calibration curve this was 5 ng/L, in the intermediate region, 22 ng/L, and in the insensitive region, 39 ng/L. The intra-assay coefficient of variation was 8.6%. The interassay coefficient of variation was 12.8%. The assay could distinguish 15 ng/L from zero with 95% confidence.

Clinical Studies

Degradation of PP in whole blood. We estimated PP concentrations in extracted blood samples. When processing of the samples was delayed for 3 h, the mean recovery of PP was 90.2 (SEM 5.6)% of that of samples that were processed immediately. After 7 h the recovery of PP decreased to 90 (7.7)%. When Trasylol was added to samples, the recovery after 3 h of delay was 94.8 (5.7)% and after 7 h 98.6 (0.8)%.

Circulating PP concentrations in fasting normal subjects. We estimated circulating concentrations of PP in 296 normal fasting volunteers, ages 13–84 years. Figure 3 demonstrates that the distribution curve for circulating PP concentrations in these subjects is skewed when age is not taken into account. When age is considered (Figure 4), the PP concentrations correlate positively with it (r = 0.41, p < 0.001). Approximately 90% of subjects had a circulating PP concentration of < 150 ng/L, and the three subjects with PP concentrations in excess of 400 ng/L were older than 80 years. We saw no significant sex-related differences in PP concentrations.

Basal PP concentrations. Figure 5 shows a decrease in circulating concentrations, followed by an increase, during continual sampling in the fasted state. Statistical analysis of results (paired t-test) showed significant differences, between 0 and 15 min (p < 0.05), 0 and 30 min (p < 0.025), and 30 and 45 min (p < 0.01).

Circulating PP concentrations in fasting patients with endocrine tumors. Figure 6 shows the circulating PP concentrations in fasting patients with endocrine tumors. None of the insulinoma patients nor the patient with the glucagonoma had an increased concentration of PP. One of the four
suitable for raising antisera to bovine PP, which cross react with the human peptide; however, these antisera are not C-terminal specific. Antiserum produced by the glutaraldehyde-conjugating method have inferior binding to both the bovine and human peptides but may be directed more specifically towards the C-terminal region.

Iodination of bovine PP by a modification of the Chloramine T method produced two distinct peaks of 125I-labeled bovine PP. The standard curves constructed with use of these labeled preparations were not identical, the material in the second peak producing a more sensitive curve than the first. PP contains four potential iodination sites at positions 7, 20, 27, and 36 (13). The presence of two peaks of 125I-labeled bovine PP suggests that there is more than one iodinated form of PP.

The assay has been used to determine normal circulating concentrations of PP in both sexes (ages 13–84 years), an investigation of the age and sex distribution of hormone concentrations being essential before those concentrations measured in disease states can be interpreted. We found no sex-related variations in concentrations of PP; therefore, we cannot confirm the previously reported observation of Track et al. (14), who measured greater PP concentrations in men than in women. However, we do confirm the observed increase in PP concentrations with age (15).

Fluctuations in basal values of human PP have not been previously reported. This may be related to changes in acid secretion, which, in patients with duodenal ulcers, have been shown to coincide with changes in circulating concentrations of PP (16). However, decreases in basal avian PP concentrations in turkeys have recently been attributed to handling and blood sampling (17). Therefore, further studies, involving longer periods of blood sampling during fasting, are required to assess the nature and significance of such basal fluctuations.

Tumor Studies

Given the circulating concentrations of PP we found in fasting patients with endocrine tumors—patients with Zollinger–Ellison syndrome, VIPoma, medullary carcinoma of thyroid, and carcinoid tumors—increased concentrations of PP may suggest the presence of an endocrine tumor. Patients with adenocarcinomas have values within the normal reference interval (18). However, PP concentrations were also normal in insulinoma patients and in one glucagonoma patient studied. Taylor et al. suggested (19) that increased PP concentrations in patients with Zollinger–Ellison syndrome reflect the presence of liver metastases. Indeed all of our four Zollinger–Ellison syndrome patients had metastatic tumors. Polak et al. (20) also proposed PP as a useful diagnostic marker for endocrine tumors and reported significant increases of PP values in serum of 18 of 28 patients. Floyd et al. (15) found three patients with increased PP had malignant insulinomas as a feature of the MIA Type I syndrome: one of six first-degree relatives had an increased concentration of PP associated with hyperparathyroidism, and one had the MIA Type I syndrome with a normal concentration of PP. In a study of 24 patients with Zollinger–Ellison syndrome (21), four patients had PP values in excess of 240 pmol/L, and 10 of 14 patients who also had the MIA Type I syndrome had PP values in excess of 100 pmol/L. Other groups (22, 23) have reported similar results for MIA Type I patients. In the group we studied, which is more heterogeneous than those previously reported, 10 of 23 patients had increased PP concentrations. However, neither of the two patients with insulinomas as part of their MIA Type I syndrome had abnormal PP values. Among our carcinoid patients, fewer than half had increased PP values,
and none of those had the classical clinical features of the carcinoid syndrome.

Using immunohistochemistry, Fiocca et al. (24) detected cells immunoreactive to bovine PP, and, to a lesser extent, human PP, in non-argentaffin rectal carcinoid tumors, which therefore have the potential to produce PP-like substances. The variation in circulating concentrations of PP may reflect the different characteristics of the tumor types in the carcinoid syndrome, or the abnormal circulating concentrations of PP may be a secondary phenomenon. Floyd et al. (25), however, postulated that PP may be derived from a non-tumorous site; they saw no decrease in high circulating concentrations of PP after the removal of an insulin-secreting islet cell tumor (assuming that all tumor tissue was removed). Increased concentrations of PP in the circulation may also reflect PP-cell hyperplasia in the pancreatic parenchyma outside of tumors (26). Thus, several possibilities have been suggested for the role of PP in endocrine tumors, but confirmation of a direct involvement of PP in the pathological process associated with these tumors has yet to be established.

In conclusion, these data confirm the previously reported age-related increase in PP concentrations. As regards diagnostic usefulness, the finding of an increased concentration of PP merits further investigation for an endocrine tumor, though a normal concentration—especially in the clinical context compatible with the diagnosis of an insulinoma—by no means excludes such a diagnostic possibility.

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References