

Plasma enteroglucagon related to malabsorption in coeliac disease

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SUMMARY Plasma enteroglucagon was measured before and during three hours after a standard meal in 21 untreated adult patients with suspected coeliac disease who all had villous atrophy of the small intestinal mucosa and malabsorption, and in nine control subjects. In 11 of these patients the diagnosis of coeliac disease was confirmed and 10 were again investigated on a gluten free diet. The coeliac patients had higher basal (37 ± 9 pmol/l, mean \pm SE, $p < 0.05$) and postprandial (70 ± 9 pmol/l, $p < 0.005$) mean plasma enteroglucagon concentrations than the control subjects (basal 14 ± 4 pmol/l, postprandial 25 ± 5 pmol/l). The 10 coeliac patients on gluten free diet for five to 20 months had a basal mean plasma enteroglucagon concentration not significantly lower than before treatment (25 ± 5 pmol/l) but significantly lower postprandial enteroglucagon concentrations than before treatment (40 ± 7 pmol/l, $p < 0.025$). Postprandial plasma enteroglucagon concentration after 90 minutes in untreated patients correlated positively to the faecal fat excretion ($r = 0.58$, $p < 0.02$). It correlated negatively to the urinary five hour D-xylose excretion after an oral load of 165 mmol D-xylose ($r = -0.71$, $p < 0.01$). Thus, the postprandial plasma enteroglucagon concentrations in untreated coeliac disease were related to the degree of malabsorption and they normalised during treatment with a gluten free diet.

Biological activity similar to pancreatic glucagon has long been known to occur in the intestine. The presence of glucagon like immunoreactants also became evident after the development of glucagon radioimmunoassay. Purified preparations of 'enteroglucagon' from porcine intestine have been reported to be composed of the pancreatic glucagon amino acid sequence of 29 amino acids and a C-terminal octapeptide extension.¹ Antibodies against the N-terminal part of the glucagon molecule may cross react completely with enteroglucagon, but those against the C-terminal part may have negligible such cross reactivity.² Enteroglucagon is located within the mucosal L-cells with a maximal concentration in the distal small intestine.³ It is released into the circulation after an oral glucose load or a mixed meal.⁴ Plasma enteroglucagon concentrations are increased greatly by intrajejunal glucose infusion and moderately by triglycerides whereas they are unchanged after infusion of hyperosmolar sodium chloride.⁵ The physiological role of enteroglucagon in man is so far unknown but a trophic effect on the

small intestinal mucosa has been suggested⁶ as well as an inhibition of the gastrin effect on the gastric acid secretion.⁷

High basal and postprandial plasma enteroglucagon concentrations have been found in patients with untreated coeliac disease.⁸ Coeliac patients treated with a gluten free diet have, however, normal enteroglucagon concentrations. It has therefore been suggested that determination of plasma enteroglucagon might be of value for detection and follow up of patients with coeliac disease.^{8,9} No studies of the same coeliac patients before and after gluten withdrawal, however, have to our knowledge been reported.

The purpose of this investigation was to determine fasting and postprandial plasma enteroglucagon concentrations in coeliac patients before and after treatment with a gluten free diet and to see if enteroglucagon assay may be useful in the follow up of the dietary treatment.

Methods

PATIENTS

Twenty one patients with villous atrophy and nine

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healthy volunteers were investigated. The study was approved by the ethical committee of the Medical Faculty of the University of Göteborg.

Twelve patients were studied before gluten withdrawal, nine women and three men aged between 24 and 78 years (mean age 49 years). Nine of these patients had a subtotal villous atrophy and three a partial villous atrophy of the small intestinal mucosa. The diagnosis of coeliac disease was confirmed by complete normalisation or obvious improvement of the mucosal morphology by light microscopy of a second biopsy specimen after treatment with a gluten free diet. Eleven of these patients were also studied after five to 20 months (mean 11 months) on a gluten free diet. One patient will be described separately; before the investigation for coeliac disease an intestinal resection was carried out because of a jejunal adenocarcinoma.

Nine patients, aged between 19 and 85 years (mean age 58 years), with probable coeliac disease and malabsorption, were also studied before any treatment with a gluten free diet. One patient refused any further investigations and one patient, 85 years old, died three months after the initial investigation. In two cases a second biopsy showed no improvement of the intestinal morphology; this investigation had been made three and six months, respectively, after they were prescribed a gluten free diet. Five patients have been treated for a short time and a second biopsy has not yet been performed.

The renal function, judged by the concentration of serum creatinine, was normal in all patients.

Nine healthy volunteers took part in the study; there were five women and four men, aged between 18 and 46 years (mean age 35 years).

D-XYLOSE ABSORPTION TEST

D-xylose, 165 mmol in 500 ml water, was given to the patient in the morning after an overnight fast. Urine was collected for five hours and analysed for xylose.^{10, 11} An excretion of 33 mmol or more is considered to be normal. The test was carried out once as part of the routine clinical evaluation before therapy.

FAT ABSORPTION TEST

The patient received a diet containing about 150 g fat daily (ordinary hospital diet plus 200 ml milk cream) for five days. Faeces was collected during the final three days and the fatty acid content determined after alkaline hydrolysis.¹² An excretion of less than 60 mmol fatty acids per 72 h has been taken as normal. The test was carried out once as part of the routine clinical evaluation before therapy.

TEST MEAL

After an overnight fast the patients and the control

subjects received a breakfast meal consisting of two boiled eggs, 60 g protein free bread, 15 g butter, 40 g orange marmalade, and 150 ml orange juice with a total energy content of about 2500 kJ. The amount of carbohydrate was estimated to be about 65 g.

BLOOD SAMPLING

Blood was collected with 30 minute interval from 30 minutes before to three hours after the breakfast. Blood was drawn into heparinised, ice cooled glass tubes (Vacutainer®, Becton Dickinson) containing 500 IU aprotinin (Trasylo®l®, Bayer AG) per ml blood. The tubes were placed in a water bath with ice. Plasma was separated by centrifugation at 4°C within 15 minutes and stored at -20°C until analysis.

ASSAY OF PLASMA ENTEROGLUCAGON

Plasma enteroglucagon concentrations were determined by radioimmunoassay,¹³ using reagents from Novo Research Institute (Bagsvaerd, Denmark). Antibody K 4023 was used for determination of the total plasma glucagon immunoreactivity and antibody K 5563 for the pancreatic glucagon immunoreactivity. The antibody K 4023 has been shown to give linear dilution curves with crude pork gut glucagon like immunoreactants (GLI).¹⁴ When measured with K 5563, the reaction with gut GLI has been found to be lower than 1%.¹⁵ The difference between the dose estimates with these antibodies was taken as a measure of the plasma enteroglucagon concentration.¹³ Porcine glucagon in phosphate buffer solution (0.04 M phosphate buffer, pH 7.4, containing 1 g human albumin (Behringwerke), 6 g NaCl, and 240 kIU aprotinin/l) was used as standard and ¹²⁵I-labelled pork glucagon as radioligand. Calibrators were assayed in triplicate and ethanol extracted plasma samples in duplicates. Plasma extracts were prepared by adding 1.8 ml 96% ethanol to 0.5 ml plasma diluted with 0.5 ml 0.04 M phosphate buffer (pH 7.4) followed by centrifugation. The supernatants were decanted to small bottles and evaporated to dryness in a desiccator. The residues were dissolved in 0.5 ml of the phosphate buffer. Calibrators and plasma samples were incubated at 4°C for 20–24 hours before addition of [¹²⁵I] glucagon solution. After another 20–24 hours 96% ethanol was added to a final concentration of 85% to precipitate the antibody bound glucagon. The within assay imprecision of the glucagon assays was calculated from differences between the duplicate assays of single plasma sample extracts using values from pool samples and patient samples (Table 1). The within assay imprecision and bias (assay drift) for the enteroglucagon assays was calculated from

Table 1 Within assay imprecision of glucagon analysis of extracted plasma samples calculated from the differences between duplicate assays of single plasma extracts. 'Pancreatic' glucagon was assayed with antibody K 5563 and 'total' glucagon was assayed with antibody K 4023

Antibody preparation	Concentration interval (pmol/l)	Plasma samples (no)	Mean concentration (pmol/l)	SD (pmol/l)	CV (%)
K 5563	0-29	321	22	1.3	5.8
	30-57	142	34	1.3	3.9
	58	1	58	1.9	3.3
K 4023	0-29	8	19	2.7	14
	30-86	309	56	2.9	5.2
	≥87	141	130	4.7	3.6

the differences in values obtained from two plasma pools analysed at the start and end of each assay run. The between assay imprecision of enteroglucagon was calculated from the differences between runs for three to five different plasma pool samples analysed at start of each assay run (Table 2).

STATISTICAL ANALYSIS

Student's *t* test was used for statistical analysis of significance. The regression line was determined by the method of least squares.¹⁶

Results

PLASMA ENTEROGLUCAGON CONCENTRATIONS

A significant increase of the mean plasma enteroglucagon concentration was found in the first samples 30 minutes after initiation of the meal in both control subjects and in patients (Fig. 1). After

Table 2 Within assay imprecision and total between assay imprecision for assay of plasma enteroglucagon. Within assay imprecision was calculated from differences between samples from two plasma pools analysed at start and at end of each assay run. The between assay imprecision was calculated from the differences between runs for 3-5 plasma pool samples analysed at start of each assay run

Mean concentration of plasma pool sample (pmol/l)	Assay runs (no)	Coefficient of variation (%)	
		Within assay	Between assay
19	16	14	19
28	12	—	13
40	13	3.4	8.6
46	12	—	9.7
50	16	—	17

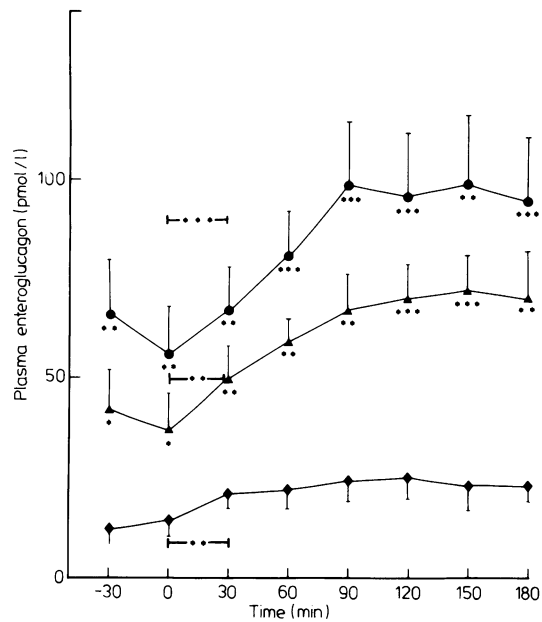


Fig. 1 Plasma enteroglucagon concentration (mean ± SE) before and after a test meal in control subjects (◆, n=9), patients with probable coeliac disease (●, n=9), and patients with untreated coeliac disease (▲, n=11). Patients and control subjects had meal at 0 minutes and had usually finished within 15 minutes. Stars indicate the level of significance in relation to control subjects and stars within solid lines that between 0 samples and 30 minute samples for each group. Levels of significance: **p*<0.05, ***p*<0.01, ****p*<0.001.

90 minutes no further concentration increase was observed in the patients or in controls.

The mean basal plasma enteroglucagon concentration (mean ± SE) was at all sampling times significantly higher in untreated coeliac disease (37±9 pmol/l) and in probable coeliac disease (56±12 pmol/l) than in control subjects (14±4 pmol/l). During the period 90-180 minutes the patients with coeliac disease and those with probable coeliac disease had significantly higher plasma enteroglucagon concentrations than control subjects (mean value for the four samples being 70±9 pmol/l and 100±16 pmol/l, respectively, compared with 25±5 pmol/l, *p*<0.005).

The treated patients (n=10) had significantly lower concentrations (40±7 pmol/l) during the period 90-180 minutes (*p*<0.025) than before treatment, which was not significantly higher than in control subjects (Fig. 2). The basal mean plasma enteroglucagon concentration (25±5 pmol/l) was not significantly lower than the pretreatment

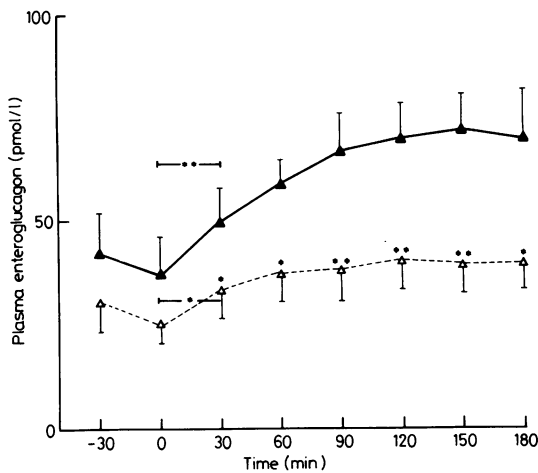


Fig. 2 Plasma enteroglucagon concentration (mean \pm SE) before and after a test meal in patients with coeliac disease before (\blacktriangle , $n=11$) and after (\triangle , $n=10$) treatment with a gluten free diet. Time for the meal as in Fig. 1. Symbols for degree of statistical significance of differences as in Fig. 1.

concentration. The patient with jejunal resection because of adenocarcinoma had the highest postprandial plasma enteroglucagon concentration, highest value being 210 pmol/l before treatment and 70 pmol/l after treatment.

An overlap of the postprandial concentrations in patients with coeliac disease and in control subjects was observed (Fig. 3).

RELATION BETWEEN PLASMA ENTEROGLUCAGON AND MALABSORPTION

Plasma enteroglucagon concentration either at 90, 120, or 150 minutes was found to be correlated to the faecal fat excretion (Fig. 4). A negative correlation to the D-xylose excretion during an absorption test was found at 90, 120, 150, as well as at 180 minutes (Fig. 5). Table 3 shows the frequency of abnormal malabsorption tests and postprandial plasma enteroglucagon concentrations above the range of the control subjects.

Discussion

This study confirms results from a previous study⁸ that postprandial plasma enteroglucagon concentrations are higher in untreated coeliac disease than in control subjects. It also shows that dietary treatment of these patients is followed by lower plasma enteroglucagon concentrations. Furthermore, we found that the postprandial plasma enteroglucagon concentrations were related to the

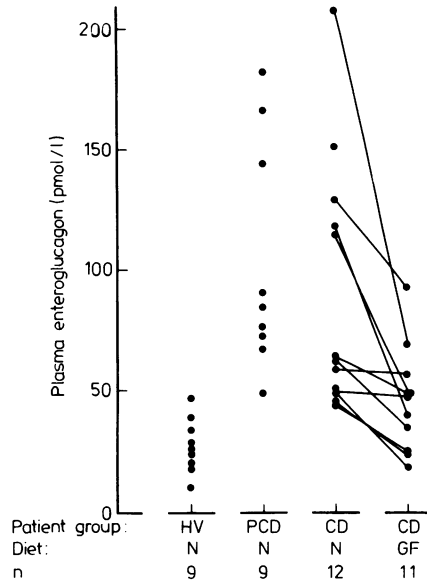


Fig. 3 Maximal postprandial plasma enteroglucagon concentrations in healthy volunteers (HV), in patients with probable coeliac disease (PCD), and in patients with coeliac disease (CD) on normal diet (N) and after treatment with a gluten free diet (GF).

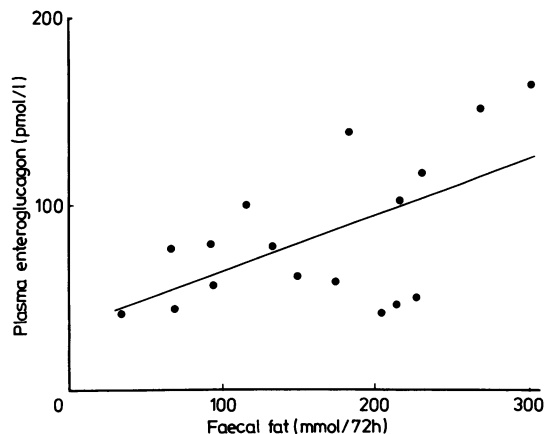


Fig. 4 Correlation between plasma enteroglucagon concentrations at 90 minutes after the test meal and the faecal fat excretion (expressed as fatty acids) in patients with coeliac disease and probable coeliac disease ($n=17$, $r=0.58$, $p<0.02$).

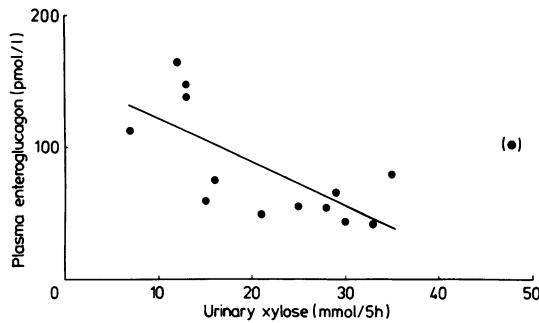


Fig. 5 Correlation between plasma enteroglucagon concentrations at 90 minutes after test meal and urinary D-xylose excretion after oral loading test in untreated patients with coeliac disease and probable coeliac disease ($n=13$, $r=-0.71$, $p<0.01$). One value, a D-xylose excretion of 48 mmol/5 h, was regarded as an outlier and has been excluded from the calculations.

degree of malabsorption in untreated coeliac disease. This finding may partly explain the differences found in comparison between our values and those reported by others (Table 4) as well as the large inter-individual differences in our study as well as in reference 8 (judged by the reported variations). Methodological differences conceivably are present as well.

That the release of enteroglucagon is related to the faecal fat excretion and negatively to the urinary D-xylose excretion in coeliac disease agrees with the findings reported by Carson *et al.*,⁹ who studied children during gluten challenge. They reported negative correlations between the total N-terminal glucagon like immunoreactivity (enteroglucagon plus pancreatic glucagon) and the blood D-xylose concentration 60 minutes after oral D-xylose ingestion as well as to fasting cholesterol.

Table 3 Frequency of positive test results in untreated coeliac disease. Postprandial plasma enteroglucagon concentrations above the range in control subjects (>48 pmol/l) are regarded as positive results for coeliac disease

Test	Patients (no)	Positive test results (no) (frequency, %)
Faecal fat excretion	17	16 (94)
D-xylose excretion	14	11 (78)
Postprandial enteroglucagon	21	19 (90)

The small increase of the plasma enteroglucagon concentrations in the control subjects after the meal in our study accords with the results reported by Besterman *et al.*⁸ A more pronounced increase has been observed after intrajejunal infusion of glucose or triglycerides.⁵ In several gastrointestinal disorders, such as chronic pancreatitis,¹⁷ Crohn's disease,¹⁸ dumping syndrome,¹⁹ after small intestinal resections²⁰ and jejunioleal bypass²¹ the postprandial plasma enteroglucagon concentrations are significantly higher than in control subjects. After jejunioleal bypass operations Holst *et al.*²¹ found higher plasma enteroglucagon concentrations in the patients with a short remaining jejunal part and a longer ileal part than in those with the reversed relation with a long jejunal segment and a short ileal part. These findings support the hypothesis²¹ that enteroglucagon is released when unabsorbed intestinal content reaches the distal part of the small intestine and the colon, where the tissue concentration of enteroglucagon is highest. Enteroglucagon is located within the L-cells, which reach the intestinal lumen, terminating in microvilli.³ In coeliac disease the proximal part of the small intestine is more severely affected than the distal part.^{22, 23} Thus, that part of the intestine containing

Table 4 Reported values (mean \pm SE) for plasma enteroglucagon concentration in coeliac disease. Data from ref 9 were recalculated to SI units assuming a molecular mass of 3485 dalton

Type of sample	Clinical group	Enteroglucagon (pmol/l)		Total GLI* (pmol/l) Carson <i>et al.</i> ⁹
		Besterman <i>et al.</i> ⁸	Present study	
Fasting	Controls	28 \pm 7	14 \pm 4	—
	Coeliac disease	97 \pm 31	37 \pm 9	—
	Treated coeliac disease [†]	34 \pm 7	25 \pm 5	32 \pm 12
	After gluten challenge	—	—	53 \pm 14
Postprandial [‡]	Controls	45 \pm 11	25 \pm 5	—
	Coeliac disease	263 \pm 62	70 \pm 9	—
	Treated coeliac disease [†]	59 \pm 8	40 \pm 7	28 \pm 9
	After gluten challenge	—	—	60 \pm 15

* Total glucagon like immunoreactivity. [†] Gluten free diet. [‡] After a mixed meal in ref 8 and in present study; after an oral glucose load in ref 9.

the enteroglucagon cells is preserved and it is exposed to an increased amount of unabsorbed luminal content after a meal. Our findings of a correlation between the postprandial plasma enteroglucagon concentrations and the degree of malabsorption support the hypothesis mentioned above and indicate that the amount of unabsorbed intestinal content is important for the release of enteroglucagon.

The number of patients and control subjects in our study is too low to allow a full evaluation of the possibility that determination of plasma enteroglucagon concentrations could be used as a test for detection of coeliac disease in adults. Postprandial values seem to separate the groups more clearly than fasting values. The fact that the postprandial plasma enteroglucagon concentrations were lower after treatment with a gluten free diet implies that it is possible to follow the improvement of the malabsorption and the adherence to the diet with repeated determinations. The plasma enteroglucagon assay still presents some problems as regards assay performance and interpretation of values: (a) the methodological variations are high even if the CVs of both the 'glucagon' and 'total glucagon' assays are satisfactory by present standards for polypeptide assays; (b) extraction efficiency and degree of heterogeneity of plasma 'enteroglucagon' in different clinical states are still less well defined. The present results, our results for children,²⁴ as well as the results reported by others do indicate a clinical value of the enteroglucagon assay in coeliac patients which will encourage work in development of more satisfactory assays.

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