Glucagon-like peptide-1(7–36)amide and glucose-dependent insulinotropic polypeptide secretion in response to nutrient ingestion in man: acute post-prandial and 24-h secretion patterns

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ABSTRACT

The acute effects of different macronutrients on the secretion of glucagon-like peptide-1(7-36)amide (GLP-1(7-36)amide) and glucose-dependent insulinotropic polypeptide (GIP) were compared in healthy human subjects. Circulating levels of the two hormones were measured over a 24-h period during which subjects consumed a mixed diet. In the first study, eight subjects consumed three equicaloric (375 kcal) test meals of carbohydrate, fat and protein. Small increases in plasma GLP-1(7-36)amide were found after all meals. Levels reached a maximum 30 min after the carbohydrate and 150 min after the fat load. Ingestion of both carbohydrate and fat induced substantial rises in GIP secretion, but the protein meal had no effect. In a second study, eight subjects consumed 75 g glucose or the equivalent portion of complex carbohydrate as boiled brown rice or barley. Plasma GIP, insulin and glucose levels increased after all three meals, the largest increase being observed following glucose and the smallest following the barley meal. Plasma GLP-1(7-36)amide levels rose only following the glucose meal. In the 24-h study, plasma GLP-1(7-36)amide and GIP concentrations were increased following every meal and remained elevated throughout the day, only falling to fasting levels at night. The increases in circulating GLP-1(7-36)amide and GIP levels following carbohydrate or a mixed meal are consistent with their role as incretins. The more sustained rises observed in the daytime during the 24-h study are consistent with an anabolic role in lipid metabolism.

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INTRODUCTION

The mammalian precursor gene proglucagon, which contains the glucagon sequence together with two structurally related glucagon-like peptides, glucagon-like peptides-1 and -2 (GLP-1 and GLP-2), is found in both gut and pancreas. In the pancreas, glucagon is the major biologically active hormone to be cleaved from proglucagon, but in the gut the cleavage site differs and glucagon-like peptide-1(7-36) amide (GLP-1(7-36)amide), a truncated form of GLP-1, is produced as a major active peptide (Bell et al. 1983; Mojsov et al. 1986). GLP-1(7-36)amide is also the predominant form found circulating post-prandially in man (Ørskov et al. 1989).

GLP-1(7-36)amide is a potent stimulator of insulin secretion (Holst et al. 1987; Kreymann et al. 1987). Together with the gastrointestinal hormone glucose-dependent insulinotropic polypeptide (GIP), it is capable of acting as an ‘incretin’ (Zunz & LaBarre, 1929), making an important contribution to insulin secretion via the entero–insular axis. In addition to stimulating insulin secretion, GLP-1(7-36)amide has, in common with GIP, a direct anabolic insulin-like action on adipose tissue, stimulating de novo lipogenesis (Oben et al. 1991a), and can also act as an entero gastrone, inhibiting pentagastrin-stimulated gastric acid secretion (Schjdager et al. 1989; O’Halloran et al. 1990).

The nutritional stimuli for GIP secretion have been well documented (Morgan et al. 1988). In contrast,
comparatively little is known about the circulating levels of GLP-1(7-36)amide and the effects of different nutrients on its secretion. There are discrepancies between the reported results from the small number of groups able to measure GLP-1(7-36)amide by immunoassay, with mean fasting levels of immunoreactive GLP-1 ranging between 15 pmol/l (Kreymann et al. 1987) and 236 pmol/l (Hirota et al. 1990). Several groups have found a small rise in circulating plasma immunoreactive GLP-1 following oral glucose (Takahashi et al. 1990; Ørskov et al. 1991) or a mixed meal (Ørskov & Holst, 1987). In contrast, Hirota et al. (1990) observed a reduction in total immunoreactive GLP-1 in normal subjects following an oral glucose load. These discrepancies are largely due to varying cross-reactivity with extended forms of GLP-1 secreted from the pancreas and problems associated with different plasma extraction techniques. It is therefore difficult to assign a physiological endocrine role to GLP-1(7-36)amide with any confidence.

The present study describes a radioimmunoassay to measure circulating levels of GLP-1(7-36)amide in man. It compares the acute effects of ingestion of different macronutrients on GIP and GLP-1(7-36)amide secretion, with particular emphasis on carbohydrate. In addition, circulating levels of GIP and GLP-1(7-36)amide have been investigated over a 24-h period in healthy subjects consuming a typical Western diet, to assess secretion of the hormone under more normal dietary circumstances.

**MATERIALS AND METHODS**

**Measurement of immunoreactive GLP-1**

Antisera against GLP-1(7-36)amide were raised in rabbits by immunizing with synthetic GLP-1(7-36)amide (Peninsula Laboratories, St Helens, Merseyside, U.K.) conjugated to bovine serum albumin (Fraction V; Sigma, Poole, Dorset, U.K.) using glutaraldehyde (Reichlin et al. 1968). Animals were primed with 50 μg GLP-1(7-36)amide as conjugate by the multisite intradermal method of Vaitukaitis et al. (1971), using a non-ulcerative Freund’s adjuvant. Antiserum of sufficient quality for radioimmunoassay was obtained after two intramuscular boosts of 25 μg GLP-1(7-36)amide, 9 and 13 months after the prime. Immunoreactive GLP-1 was measured in 200 μl samples of unextracted plasma by a double-antibody disequilibrium assay. Synthetic GLP-1(7-36)amide was used for standards and radiolabelled tracer and the standard curve was constructed with an equivalent amount of hormone-free plasma, as used in the radioimmunoassay for GIP (Morgan et al. 1978). The GLP-1(7-36)amide radiotracer was iodinated using a chloramine-T method (Kwasowski, 1986). The iodinated peptide was purified on a 10 × 0.4 cm Sephadex G-25 column and stored in aliquots at −20 °C. Immediately before use in the assay, an aliquot of tracer was further purified by affinity chromatography, using the gamma-globulin fraction of an early bleed of moderate titre from rabbits immunized with GLP-1(7-36)amide; this was conjugated to 100 μm porous amino-activated silica beads (Elliott, 1992). The assays were carried out in polyethylene Minisorp tubes (Life Technologies, Uxbridge, Middx. U.K.) to minimize peptide adsorption. Blood samples taken for immunoreactive GLP-1 measurement were collected into lithium-heparinized tubes containing 200 KIU aprotinin/ml blood (Novo Biolabs, Bagsvaerd, Denmark), and the plasma was separated immediately and stored at −20 °C until assay. The antiserum used in the assay exhibited no detectable cross-reactivity up to a concentration of 400 ng/ml with human GIP, GLP-2, glucagon, vasoactive intestinal peptide, secretin and motilin. It was specific for the C-terminal amidated form of GLP-1, cross-reacting 100% with GLP-1(1-36)amide but less than 0.2% with GLP-1(7-37) and GLP-1(1-37). No major proglucagon fragment (MPGF) could be detected in Sephadex G-50 chromatographs of rat or pig pancreatic extracts with the antiserum. The limit of sensitivity of the assay in plasma was 6 pmol/l, and the intra-assay coefficients of variation at 10, 19 and 25 pmol/l were 13, 10 and 9% respectively.

**Test meals**

**Isoenergetic carbohydrate, fat or protein meals**

Eight healthy volunteers aged between 21 and 24 years with body mass indices (BMIs) between 20 and 25 kg/m² participated in the study. On three separate occasions after an overnight fast, each consumed, in random order, meals of carbohydrate, fat or protein, each with an energy content of 375 kcal. The carbohydrate was given as 100 g glucose (Hycal; Beecham Products, Brentford, Middx, U.K.). The fat meal was served as 84 ml ‘double’ cream. A blackcurrant flavouring with no energy value (Scientific Hospital Supplies, Liverpool, U.K.) was added to improve palatability. The protein meal was in the form of 352 g grilled lean turkey. The meals were served with water so that the total liquid volume ingested (300 ml) was constant for all three meal types. Venous blood was collected from an indwelling cannula inserted into an antecubital forearm vein before and at frequent intervals for 3 h following the meal.

**Simple or complex carbohydrate test meals**

Eight healthy volunteers aged 19–23 years with BMIs ranging from 21 to 29 kg/m² participated in the study. On three separate occasions after an overnight fast,
each consumed, in random order, 75 g carbohydrate loads in the form of glucose (Hycal), boiled brown rice or boiled pearl barley. Both rice and barley meals were served together with 100 ml pureed canned tomatoes (containing less than 2 g carbohydrate) to improve palatability. The total volume of each meal was made up to 450 ml with water. Venous blood was collected as above.

24-h mixed meal study
Six healthy volunteers aged between 24 and 26 years with BMIs ranging from 21 to 31 kg/m² participated in the study. The subjects consumed a typical Western diet from 13.00 h on the first day of the study until 16.00 h on the second day. Lunch (600 kcal) was served at 13.00 h on both days. The evening meal (560 kcal) and breakfast (600 kcal) were served at 19.00 and 08.00 h respectively. In addition to the three main meals, the subjects were given mid-afternoon snacks (140 kcal, served at 15.30 h on both days), a mid-evening snack (260 kcal, served at 21.30 h) and a mid-morning snack (130 kcal, served at 10.30 h). Venous blood samples were taken hourly from 17.00 h on the first until 16.00 h on the second. Additional samples were taken 20 and 40 min after each main meal. The diet provided a total energy content of approximately 2370 kcal/24 h comprising 42% energy as carbohydrate, 45% as fat and 13% as protein.

All subjects participating in the studies gave their informed consent, and the studies were approved by the Ethical Committees of the University of Surrey and the Royal Surrey County Hospital.

Chemical analyses
Plasma immunoreactive GIP and insulin were measured by double-antibody radioimmunoassays (Morgan et al. 1978; Hampton, 1984). Plasma glucose was measured by an automated hexokinase method.

Statistical analyses
Repeated measures analysis of variance (ANOVA) was used to detect differences between the post-prandial circulating profiles of hormones and metabolites, followed by Duncan’s multiple range test to locate individual differences. Significant changes in the post-prandial concentrations of the different parameters were identified by comparison of the mean fasting concentrations with post-prandial concentrations using Student’s paired t-test. Hartley’s $F_{max}$ test was used to test for homoscedacity of data. Data analysis was carried out using the SPSS information system (SPSS Inc., Chicago, IL., U.S.A.). Values of $P<0.05$ were taken to be significant.

RESULTS

Isoenergetic carbohydrate, fat or protein meals
The plasma glucose, immunoreactive (IR)-insulin, -GIP and -GLP-1 responses to the test meals are shown in Fig. 1. Plasma IR-GIP levels underwent a sustained increase between 15 and 120 min after both the carbohydrate and fat meals ($P<0.01$), with mean peak levels 45 min following the carbohydrate and 90 min following the fat meal. Ingestion of protein had no effect on circulating GIP levels. Analysis of variance demonstrated differences between the post-prandial circulating IR-GLP-1 profiles. The IR-GLP-1 responses to different meals were, however, much smaller than those obtained for GIP. Mean fasting levels were in the range 14–17 pmol/l. Significant elevation of IR-GLP-1 was achieved following all three meals, but it was small and transient. GLP-1 levels peaked and reached statistical significance compared with basal levels at 30 min after carbohydrate and 150 min after the fat meals ($P<0.01$). After the protein meal, IR-GLP-1 levels showed a transient but statistically significant peak at 30 min ($P<0.01$), followed by a steady rise throughout the rest of the 3-h study period. Plasma glucose and IR-insulin levels rose following the carbohydrate test meal, but they were unaffected by the fat or protein test meals.

Simple or complex carbohydrate test meals
The plasma glucose, IR-insulin, -GIP and -GLP-1 responses to the carbohydrate test meals are shown in Fig. 2. Plasma IR-GIP levels rose post-prandially following all three meals; however, between 15 and 90 min the levels achieved following the glucose test meal were significantly ($P<0.05$) higher than those achieved after either the rice or the barley meals. Significant ($P<0.01$) increases in IR-GLP-1 were also seen 30–60 min following the glucose meal ($P<0.01$). However, neither the rice nor barley meals elicited any significant increase in circulating IR-GLP-1 levels; a transient increase in mean levels of IR-GLP-1 observed after ingestion of the rice meal, failed to achieve statistical significance. Plasma glucose and IR-insulin levels rose following all three test meals. Highest post-prandial levels for both glucose and IR-insulin were seen after the glucose test meal and the smallest increases after the barley meal.

24-h mixed meal study
The plasma glucose and IR-insulin levels over the study period are shown in Fig. 3, the IR-GIP and IR-GLP-1 levels in Fig. 4. Plasma glucose levels rose in a biphasic response following each main meal, but
were unaffected by the snacks, which were consumed 2 h after the main meals. There was some evidence that glucose tolerance tended to deteriorate towards the end of the day, with higher mean plasma glucose levels following supper compared with other main meals. Glucose levels declined almost to basal before consumption of the next main meal. Plasma IR-insulin levels followed a similar pattern to those for glucose. They were also unaffected by consumption of snacks and declined almost to basal between main meals. Plasma GIP and IR-GLP-1 patterns were similar to each other; they rose following consumption of each main meal, but unlike glucose and IR-insulin, remained elevated throughout the daytime, only reaching basal levels after an overnight fast, before the breakfast meal.

**DISCUSSION**

We have described a radioimmunoassay for GLP-1(7-36)amide of sufficient sensitivity to measure circulating levels of the peptide in unextracted plasma. The specificity of the antiserum to the C-terminal amidated form of GLP-1 enables measurement of the major biologically active circulating peptide, GLP-1(7-36)amide, without interference from other circulating gut glucagon-like peptides or pancreatic glucagon or the MPGF. The values for IR-GLP-1 in fasting plasma were in good agreement with those obtained by Kreymann et al. (1987), although the post-prandial increase following glucose or a mixed meal were somewhat lower than those reported by this group. The fall in IR-GLP-1 observed following oral glucose in the study reported by Hirota et al. (1990) has been attributed to the cross-reactivity of their antiserum with MPGF and the consequent masking of a post-prandial rise in GLP-1(7-36)amide by a concomitant fall in pancreatic MPGF levels. As we were unable to detect any MPGF in pancreatic extracts, it is unlikely that cross-reactivity with circulating MPGF interferes in our assay. Whilst GLP-1(7-37), not measured by our antiserum, has a similar spectrum of biological activity to that of GLP-1(7-36)amide, it does not
circulate in any appreciable quantities (Ørskov et al. 1989, 1992). The assay is therefore a reliable measure of circulating biologically active peptide.

Both GIP and GLP-1(7–36)amide were secreted in response to oral carbohydrate. The timing of the GLP-1(7–36)amide response to carbohydrate, although small, was consistent with the hormone acting as an incretin and stimulating glucose-mediated insulin secretion. The concomitant secretion of GIP and GLP-1(7–36)amide in response to carbohydrate ingestion supports the concept of several incretin factors in the entero-insular axis with functional interaction at pancreatic B-cell level; evidence for this in vitro comes from the finding that GIP and GLP-1(7–36)amide have a synergistic stimulatory effect on insulin secretion in isolated perfused rat pancreas (Fehmann et al. 1989).

The protein meal caused a small increase in GLP-1(7–36)amide secretion but had no effect on circulating GIP, insulin or glucose levels. GIP secretion is stimulated by some individual amino acids (Flatt et al. 1991), but ingested protein is absorbed largely in the form of short peptides rather than free amino acids (Matthews & Adibi, 1976). These peptides do not stimulate GIP secretion but they may act as a stimulus for GLP-1 secretion; however, the GLP-1(7–36)amide secreted following the protein meal did not stimulate insulin secretion.

A sustained increase in GIP levels was observed following the fat meal, with a small increase in circulating GLP-1(7–36)amide. Levels of both hormones were slower to rise following the fat meal, which may be related to the slower rate of gastric emptying and absorption of fat relative to carbohydrate.

The plasma glucose and insulin responses following ingestion of foods containing complex carbohydrates are dependent on a range of factors that affect the rate and extent of starch digestion (Thorne et al. 1983). The plasma glucose response following the rice and barley meals demonstrated a slower absorption of the complex carbohydrate than the glucose meals. This was likely to have been due to slow enzymic degradation of the starch as a result of interaction with other food components in the cereal grains.
FIGURE 3. Plasma glucose and immunoreactive insulin levels in healthy human subjects consuming a normal diet over a 24-h period. (Values are means ± S.E.M.; n = 6).

effect was more marked for the barley, which has a higher non-starch polysaccharide content, than for the rice. GIP secretion, which is dependent upon the rate of absorption of glucose (Morgan et al. 1979), was greater following the glucose meal than following either of the cereals. No significant increase in GLP-1(7–36)amide was observed following either cereal meal, in contrast to the rise in circulating levels seen following glucose ingestion. These results suggest that GLP-1(7–36)amide secretion is stimulated by glucose absorption and that a minimum rate of glucose absorption is necessary for an appreciable increase in plasma GLP-1(7–36)amide concentrations to be observed.

GIP-secreting cells are located mainly in the jejunum (Bloom & Polak, 1980), whereas most GLP-1(7–36)amide-secreting cells are situated in the distal small intestine and colon (Kreymann et al. 1988). It might be expected that, because of the distribution of GLP-1(7–36)amide in the gut, the more slowly digested complex carbohydrate absorbed further down the small intestine would provide a greater stimulus for GLP-1(7–36)amide than glucose, which is absorbed more proximally. This did not, however, occur; moreover, the timing of the increase in circulating GLP-1(7–36)amide was similar to that observed for GIP. The precise mechanism governing stimulation of GLP-1(7–36)amide by carbohydrate is not clear. One group, investigating the effect of a selection of sugars and sugar derivatives on GLP-1(7–36)amide secretion from canine ileal loops, have proposed that the release is under the influence of a glucose sensor on the enteroglucagon cells which has specific steric requirements for the binding of sugars. Any sugar that fulfills these requirements can bind to the sensor and stimulate GLP-1(7–36)amide release (Shima et al. 1990). However, the patterns of GLP-1(7–36)amide secretion observed in the present study, which are

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consistent with its role as an incretin, suggest that GLP-1(7–36)amide secretion may be linked to a signal, possibly neurocrine, originating in the upper small intestine.

Patterns of gastrointestinal hormone responses during the daytime in the 24-h study were very different from those observed following a single test meal after an overnight fast. The continuously elevated daytime levels reflect the fact that normal eating patterns are such that most people are absorbing nutrients, particularly fat, from the gut for approximately 18 out of 24 h. We have previously shown that GIP and GLP-1(7–36)amide have anabolic effects on lipid metabolism, stimulating fatty acid synthesis and increasing lipoprotein lipase activity (Oben et al. 1991a, b). The elevated post-prandial levels of GIP and GLP-1(7–36)amide remaining after post-prandial glucose and insulin levels have declined to basal may play a role in the metabolism of the fat component of a mixed meal.

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