Effects of sub-chronic exposure to naturally occurring N-terminally truncated metabolites of glucose-dependent insulintrophic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), GIP(3–42) and GLP-1(9–36)amide, on insulin secretion and glucose homeostasis in *ob/ob* mice

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Abstract

Glucose-dependent insulintrophic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are important enteroendocrine hormones that are rapidly degraded by an ubiquitous enzyme dipeptidyl peptidase IV to yield truncated metabolites GIP(3–42) and GLP-1(9–36)amide. In this study, we investigated the effects of sub-chronic exposure to these major circulating forms of GIP and GLP-1 on blood glucose control and endocrine pancreatic function in obese diabetic (*ob/ob*) mice. A once daily injection of either peptide for 14 days had no effect on body weight, food intake or pancreatic insulin content or islet morphology. GLP-1(9–36)amide also had no effect on plasma glucose homeostasis or

insulin secretion. Mice receiving GIP(3–42) exhibited small but significant improvements in non-fasting plasma glucose, glucose tolerance and glycaemic response to feeding. Accordingly, plasma insulin responses were unchanged suggesting that the observed enhancement of insulin sensitivity was responsible for the improvement in glycaemic control. These data indicate that sub-chronic exposure to GIP and GLP-1 metabolites does not result in physiological impairment of insulin secretion or blood glucose control. GIP(3–42) might exert an overall beneficial effect by improving insulin sensitivity through extrapancreatic action.

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Introduction

Glucose-dependent insulintrophic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are important gastrointestinal-releasing hormones involved in the regulation of postprandial nutrient homeostasis (Meier *et al.* 2003). Both the incretin hormones exert potent insulintrophic effects on pancreatic islets and glucose-lowering actions in peripheral tissues (Gault *et al.* 2003a, Meier *et al.* 2003, Green *et al.* 2004a). In addition, these incretin hormones have been reported to have other beneficial effects on β -cells, including stimulation of proliferation and neogenesis and inhibition of apoptosis (Buteau *et al.* 1999, Trumper *et al.* 2002, Ehses *et al.* 2003). Various extrapancreatic actions have been suggested, such as stimulation of glucose uptake and metabolism (Villanueva-Penacarrillo *et al.* 1994, O'Harte *et al.* 1998) and inhibition of hepatic insulin extraction (Trapote *et al.* 1996, Rudovich *et al.* 2004). In addition, GIP has been reported to affect the aspects of lipid metabolism, whereas a key effect of GLP-1 is inhibition of gastric emptying (Nauck 1999, Yip & Wolfe 2000).

One of the major factors limiting the biological activity of GIP and GLP-1 is their rapid degradation and subsequent elimination from the circulation. Kinetic studies investigating GIP and GLP-1 degradation in plasma have indicated that the major metabolites produced are GIP(3–42) and GLP-1(9–36)amide respectively (Pauly *et al.* 1996). This stepwise removal of an amino terminal dipeptide, Tyr¹–Ala² and His⁷–Ala⁸ respectively, was subsequently shown to be due to the enzyme dipeptidyl peptidase IV (DPP IV), a key regulator of incretin hormone metabolism (Mentlein *et al.* 1993). DPP IV is ubiquitously expressed in mammalian tissues and organs (Wrenger *et al.* 2000) and circulates at relatively high concentrations in the blood (Lojda *et al.* 1979). It has strict substrate specificity for removing dipeptides, where the penultimate amino acid residues are proline or alanine (Mentlein 1999). In addition to degradation, both hormones can be extracted in an organ-specific manner with the kidneys primarily responsible for the elimination and excretion of the metabolic products (Meier *et al.* 2004).

Early studies using GIP(3–42) originally isolated from porcine intestinal extracts demonstrated that this truncated

peptide lacked significant insulinotrophic activity (Brown *et al.* 1981). Subsequent studies have recently shown that the major degradation product of GIP metabolism, GIP(3–42), acts as a GIP receptor antagonist in acute studies *in vivo* (Gault *et al.* 2002). Parallel studies using the sister incretin hormone, GLP-1, have also shown that the truncated GLP-1(9–36) amide degradation product can act as a GLP-1 receptor antagonist (Knudsen & Pridal 1996, Green *et al.* 2004b). Interestingly, GLP-2(3–33), which also arises from proglucagon processing in the intestine, may act as a competitive GLP-2 receptor antagonist (Thulesen *et al.* 2002).

Recent RIA data indicate that approximately 70% of the circulating GIP may exist as GIP(3–42) in humans (Deacon *et al.* 2000). Similarly, 60% of the circulating GLP-1 exist as the major metabolite, GLP-1(9–36)amide (Deacon *et al.* 1995). Since the circulating levels of both incretin hormones exist predominantly as their major degradation products, this may have implications in the regulatory and physiological actions of the native hormones. Therefore, this study examined the effects of daily administration of GIP(3–42) and GLP-1(9–36)amide on metabolic parameters associated with glucose homeostasis and insulin secretion in obese diabetic *ob/ob* mice.

Materials and Methods

Reagents

HPLC grade acetonitrile was obtained from Rathburn (Walkersburn, Scotland). Sequencing grade trifluoroacetic acid (TFA) was obtained from Aldrich (Poole, Dorset, UK). Fmoc-protected amino acids were from Calbiochem Novabiochem (Beeston, Nottingham, UK). Dextran-T70-activated charcoal was obtained from Sigma. All water in these experiments was purified using a Milli-Q water purification system (Millipore, Milford, MA, USA). All other chemicals used were of the highest purity available.

Peptide synthesis and purification

GIP(3–42) and GLP-1(9–36)amide were sequentially synthesised on an Applied Biosystems automated peptide synthesiser (model 432A) using standard solid-phase Fmoc protocols as described previously (Gault *et al.* 2002). The peptides were purified by reversed-phase HPLC on a Waters Millennium 2010 chromatography system (Software version 2.1.5) and characterised using matrix-assisted laser desorption/ionisation time of flight (MALDI-TOF) mass spectrometry as described elsewhere (Gault *et al.* 2003b).

Animals

Obese diabetic (*ob/ob*) mice, derived from the colony maintained at Aston University, UK, were used at 12–16 weeks of age. The animals were housed individually in an

air-conditioned room at $22 \pm 2^\circ\text{C}$ with a 12 h light:12 h darkness cycle (0800–2000 h). Drinking water and a standard rodent maintenance diet (Trouw Nutrition Ltd, Cheshire, UK) were freely available. All animal experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986. No adverse effects were observed following administration of either GIP(3–42) or GLP-1(9–36)amide.

Sub-chronic biological effects of GIP(3–42) and GLP-1(9–36)amide

Ob/ob mice received once daily i.p. injections (1700 h) of either saline vehicle (0.9% (w/v) NaCl), GIP(3–42) or GLP-1(9–36) amide (both at 25 nmol/kg bw) over a 14-day period. This treatment regimen was based on that used in previous studies showing metabolic effects of N-terminally modified GIP (Gault *et al.* 2005). Food intake and body weights were recorded daily, while plasma glucose and insulin concentrations were monitored at 2–5-day intervals. Blood samples for determination of plasma glucose and insulin were taken at 2–4-day intervals from non-fasted animals at 1000 h. Intraperitoneal glucose tolerance (18 mmol/kg body weight (bw)) and insulin sensitivity (50 U/kg bw) were performed on days 14 and 28. In a separate series of experiments, *ob/ob* mice fasted for 18 h were used to examine metabolic response to feeding. At the end of the 14-day treatment period, pancreatic tissues were excised and processed for immunohistochemistry or measurement of insulin following extraction with 5 ml/g ice-cold acidic ethanol (750 ml ethanol, 235 ml H₂O and 15 ml concentrated HCl). Blood samples were collected from the cut tails of conscious mice into fluoride/heparin-coated microcentrifuge tubes (Sarstedt, Numbrecht, Germany) at the times indicated on the figures and immediately centrifuged (30 s at 13 000 g) using a Beckman microcentrifuge (Beckman Instruments, High Wycombe, Bucks, UK). The resulting plasma was then aliquoted into fresh Eppendorf tubes and stored at -20°C prior to glucose and insulin determinations.

Immunocytochemistry

For immunohistochemistry, tissue fixed in 4% paraformaldehyde/PBS and embedded in paraffin wax was sectioned at 8 μm . After de-waxing, the sections were stained with haematoxylin (BDH Chemicals, Poole, Dorset, UK) and then plunged into acid methanol (500 ml methanol, 500 ml H₂O and 2.5 ml concentrated HCl) prior to dehydration and mounting in Depex (BDH Chemicals). The stained slides were viewed under a microscope (Nikon Eclipse E2000, Diagnostic Instruments, Inc., San Diego, CA, USA) attached to an Olympus camera Model C-5060 (Olympus Corporation, Tokyo, Japan), analysed in a blinded manner using Image J software (National Institute of Health; Abramoff *et al.* 2004), and calibrated with a stage micrometer (Graticules Limited, Tonbridge, Kent, UK). Approximately, 60–70 random sections were examined from the pancreas of each mouse.

Statistical analysis

Plasma glucose was analysed using a Hitachi 912 Automatic Analyser (Boehringer Mannheim, Germany). Insulin was assayed using a modified dextran-coated charcoal RIA as described previously (Bailey *et al.* 1982). Results are expressed as mean $\pm$ S.E.M. Data were compared using ANOVA, followed by the Student–Newman–Keuls *post hoc* test. Area under the curve (AUC) analysis employed the trapezoidal rule with baseline subtraction (Burington 1973). Groups of data were considered to be significant if $P < 0.05$.

Results

Structural confirmation of GIP(3–42) and GLP-1(9–36)amide using MALDI-TOF mass spectrometry

Molecular mass of GIP(3–42) was detected at 4748.0 Da, which corresponds very closely to the theoretical mass of 4748.4 Da. Likewise, the molecular mass of GLP-1(9–36) amide was detected at 3088.1 Da, corresponding closely to the theoretical mass of 3088.4 Da.

Effects of GIP(3–42) and GLP-1(9–36)amide on body weight, food intake and non-fasting glucose and insulin

Administration of GIP(3–42) and GLP-1(9–36)amide had no significant effect on body weight or food intake (Fig. 1). Plasma glucose was significantly reduced ($P < 0.05$) in

GIP(3–42)-treated mice from day 9 onwards. Cessation of GIP(3–42) treatment returned plasma glucose concentrations towards control levels. No significant change was observed in plasma insulin levels during treatment with GIP(3–42). Plasma glucose and insulin were unchanged by daily GLP-1(9–36)amide treatment (Fig. 1).

Effects of GIP(3–42) and GLP-1(9–36)amide on glucose tolerance and insulin response to glucose

Daily administration of GIP(3–42) for 14 days significantly reduced ($P < 0.05$) plasma glucose concentrations at 60 min following an i.p. glucose challenge (Fig. 2). This was corroborated by a significantly decreased 0–60-min AUC value (1.5-fold; $P < 0.05$) when compared with saline control. There was no significant difference in plasma insulin levels following i.p. glucose. Cessation of GIP(3–42) treatment for 14 days returned the glycaemic excursion to control levels (629.0 ± 52.1 vs 702.1 ± 26.3 mmol/l per min respectively; data not shown). Daily administration of GLP-1(9–36)amide had no significant effect on the glucose or insulin responses to an i.p. glucose load (Fig. 2).

Effects of GIP(3–42) and GLP-1(9–36)amide on metabolic responses to feeding

Plasma glucose concentrations, in response to a 15-min feeding, were significantly lowered ($P < 0.05$) at 30-min post-feeding in GIP(3–42)-treated *ob/ob* mice (Fig. 3).

Figure 1 Effects of daily GIP(3–42) and GLP-1(9–36)amide administration on body weight, food intake, plasma glucose and insulin concentrations of *ob/ob* mice. The parameters were measured for 8 days prior to, 14 days during (indicated by black bar) and 14 days after treatment with saline, GIP(3–42) or GLP-1(9–36)amide (25 nmol/kg bw per day). Values are the mean $\pm$ S.E.M. for eight mice. * $P < 0.05$ compared with saline group.

Figure 2 Effects of daily GIP(3-42) and GLP-1(9-36)amide administration on glucose tolerance and plasma insulin response to glucose in *ob/ob* mice. Tests were conducted after daily treatment with GIP(3-42) or GLP-1(9-36)amide (25 nmol/kg bw per day) for 14 days. Glucose (18 mmol/kg bw) was administered at the time indicated by the arrow. Plasma glucose and insulin AUC values for 0–60-min post-injection are shown. Values are the mean $\pm$ S.E.M. for eight mice. * $P < 0.05$ compared with saline group.

Similarly, AUC values of 0–105-min post-feeding displayed a significant reduction (1.5-fold; $P < 0.05$) in the overall glycaemic excursion in GIP(3-42)-treated mice compared with saline controls. GIP(3-42) had no significant effect on plasma insulin concentrations. Cessation of treatment for 14 days returned the plasma glucose response to feed to control

levels (1228.0 ± 163.6 vs 1138.5 ± 167.2 mmol/l per min respectively; data not shown). Daily treatment with GLP-1(9-36)amide had no significant effects on the metabolic or insulin responses to feeding in 18-h fasted *ob/ob* mice (Fig. 3). Food intake of the various groups was similar during the 15-min feeding period ($0.3\text{--}0.5$ g/mouse per 15 min).

Figure 3 Effects of daily GIP(3-42) and GLP-1(9-36)amide administration on glucose and insulin responses to feeding in 18-h fasted *ob/ob* mice. Tests were conducted after daily treatment with GIP(3-42) or GLP-1(9-36)amide (25 nmol/kg bw per day) for 14 days. The arrow indicates the time of feeding (15 min). AUC values of 0–105 min post-feeding are also shown. Values are the mean $\pm$ S.E.M. for eight mice. * $P < 0.05$ compared with saline group.

Effects of GIP(3–42) and GLP-1(9–36) amide on insulin sensitivity

Following daily treatment with GIP(3–42), the hypoglycaemic action of insulin was significantly augmented ($P < 0.05$) at 60-min post-injection (Fig. 4). Cessation of treatment for 14 days reversed this effect (Fig. 4). Daily administration of GLP-1(9–36)amide had no significant effects on insulin sensitivity (Fig. 4).

Effects of GIP(3–42) and GLP-1(9–36)amide on pancreatic insulin and islet morphology

The pancreatic weights and insulin content were similar in the control, GIP(3–42) and GLP-1(9–36)amide groups after 14 days of treatment (Fig. 5). Similarly, no significant differences were observed in islet number per pancreatic section or average islet area during or after treatment (Fig. 5).

Discussion

The incretin hormones, GIP and GLP-1, are rapidly degraded in the circulation by the ubiquitous enzyme DPP IV to the N-terminally truncated metabolites, GIP(3–42) and GLP-1(9–36)amide respectively (Pauly *et al.* 1996). Both GIP(3–42) and GLP-1(9–36)amide constitute the major circulating forms of these native hormones (Deacon *et al.* 1995, 2000). While initial studies demonstrated that GIP(3–42) and GLP-1(9–36)amide were devoid of any biological activity, some studies have since demonstrated that they can act as antagonists at their respective receptors either *in vitro* or following acute injection (Knudsen & Pridal 1996, Gault *et al.* 2002, Green *et al.* 2004a).

In the present study, the effects of daily administration of the truncated metabolites GIP(3–42) and GLP-1(9–36)amide were examined in obese diabetic *ob/ob* mice. This is a commonly studied animal model displaying several abnormalities characteristic of type 2 diabetes, including obesity, insulin resistance, moderate hyperglycaemia and severe

hyperinsulinaemia (Bailey *et al.* 1982). These mice have been used previously to assess the acute actions of these truncated metabolites as well as synthetic antagonists of GIP and GLP-1 (Gault *et al.* 2002, Green *et al.* 2004b). Daily injection of *ob/ob* mice with GIP(3–42) and GLP-1(9–36) amide for 14 days had no adverse or toxic effects. In fact, food intake, body weight, islet structure and both pancreatic insulin content and morphology were identical to saline-treated control mice throughout the study. It was noted that basal plasma glucose levels were marginally lower and plasma insulin concentrations were higher 14 days after the cessation of treatment in all groups, probably reflecting the age-dependent progression of the *ob/ob* syndrome on the Aston background (Bailey *et al.* 1982). However, daily injection of GLP-1(9–36)amide had no significant effects on any of the measured metabolic parameters associated with glucose homeostasis and insulin secretion. This suggests that the naturally occurring fragment peptide of GLP-1 plays no role in the physiological modulation of glucose homeostasis *in vivo*, being consistent with the views of others (Deacon *et al.* 2002, Vahl *et al.* 2003). Thus, the antagonist actions of GLP-1(9–36) amide appear minor compared with exendin(9–39)amide (Kolligs *et al.* 1995, Edwards *et al.* 1999). Furthermore, recent studies suggest that GLP-1(9–36)amide does not regulate the insulinotropic and glucose homeostatic effects of GLP-1(7–36) amide (Vahl *et al.* 2003).

Interestingly, *ob/ob* mice treated with GIP(3–42) displayed a very mild improvement in their diabetic status. This included small but significant improvements of basal hyperglycaemia, glucose tolerance and glycaemic response to feeding. These effects were not associated with the inhibition of insulin secretion, which suggests that endogenously produced GIP(3–42) is not involved in the modulation of β -cell function through antagonism of the normal stimulatory effects of GIP. It should also be noted that recent results have shown GIP(3–42) not to antagonise the insulinotropic effects of GIP at physiological concentrations (Deacon *et al.* 2006), although dosing concentrations employed in the present study far exceeded the physiological concentration of GIP. Possible improvement of insulin

Figure 4 Effects of daily GIP(3–42) and GLP-1(9–36)amide administration on insulin sensitivity in *ob/ob* mice. Tests were conducted after daily treatment with GIP(3–42) or GLP-1(9–36)amide (25 nmol/kg bw per day) (A) for 14 days or (B) 14 days after cessation of treatment. Data are presented as percentage of basal value. Insulin (50 U/kg bw) was administered by i.p. injection at the time indicated by the arrow. Values are the mean $\pm$ S.E.M. for eight mice. * $P < 0.05$ compared with saline group.

Figure 5 Effects of daily GIP(3–42) and GLP-1(9–36)amide administration on pancreatic weight, insulin content, islet number and islet area in *ob/ob* mice. Parameters were measured after daily treatment with GIP(3–42) or GLP-1(9–36)amide (25 nmol/kg bw per day) for 14 days and 14 days after cessation of treatment (Day 28). Values are the mean $\pm$ S.E.M. for four mice.

sensitivity so as to moderate hyperglycaemia, therefore, seems likely. Indeed, insulin sensitivity tests conducted 14 days after GIP(3–42) treatment revealed a modest, but significant improvement in the glucose-lowering actions of exogenous insulin. The most plausible explanation for the present data stem from the appreciation of extrapancreatic effects of GIP(3–42) as depicted in Fig. 6. These actions include effects on hexose transport in the intestine (Tseng *et al.* 1999) or actions on liver, muscle or adipose tissue (O'Harte *et al.* 1998, Yip & Wolfe 2000, Rudovich *et al.* 2004). However, it may be possible that the reduction in basal plasma glucose concentrations by daily GIP(3–42) treatment led to reduced glucose toxicity in these animals and thus contributed to the improvement of their diabetic status.

Although modest, the actions of daily injection of GIP(3–42) in *ob/ob* mice are somewhat reminiscent of aspects of the effects of the well-characterised GIP-receptor antagonist, (Pro³)GIP (Gault *et al.* 2005). Such actions may have been more prominent, if it had been possible to use osmotic pumps in *ob/ob* mice to give more sustained exposure to the antagonist. However, single daily administration of (Pro³)GIP for just 11 days ameliorated insulin resistance and significantly improved glucose tolerance and abnormalities of islet structure and function in *ob/ob* mice. Although, it is clear from the present study that daily administration of GIP(3–42) did not effect hyperinsulinaemia, neither β -cell function nor islet morphology, the small enhancement of insulin sensitivity appears as a common theme. Thus, although considerably less potent than (Pro³)GIP as a receptor antagonist, these observations suggest that GIP(3–42) may also influence insulin sensitivity over the longer term without effecting body weight or the extent of hyperinsulinaemia (Gault *et al.* 2005).

In conclusion, previous cellular and acute *in vivo* studies have shown that antagonism of the GIP receptor by the truncated GIP(3–42) metabolite greatly reduces the biological effectiveness of this glucorecretin hormone (Gault *et al.* 2002). The present study shows that even when administering large exogenous doses of GIP(3–42), it is not possible to show long-term detrimental effects on blood glucose control. Similar observations were made using GLP-1(9–36)amide, but a small improvement in insulin sensitivity by GIP(3–42) suggests possible relevance of extrapancreatic effects as revealed previously using (Pro³)GIP (Gault *et al.* 2005). These alternative physiological and possibly therapeutic actions of GIP-like molecules merit further investigation.

Figure 6 Simplified scheme illustrating how GIP(3–42) might have beneficial effects on GIP-mediated extrapancreatic actions associated with insulin sensitivity and glucose tolerance.

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