Enhanced cAMP generation and insulin-releasing potency of two novel Tyr¹-modified enzyme-resistant forms of glucose-dependent insulintropic polypeptide is associated with significant antihyperglycaemic activity in spontaneous obesity-diabetes

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Glucose-dependent insulintropic polypeptide (GIP) is an important incretin hormone, which potentiates glucose-induced insulin secretion. Antihyperglycaemic actions of GIP provide significant potential in Type II diabetes therapy. However, inactivation of GIP by the enzyme dipeptidyl peptidase IV (DPP IV) and its consequent short circulating half-life limit its therapeutic use. Therefore two novel Tyr¹-modified analogues of GIP, N-Fmoc-GIP (where Fmoc is 9-fluorenylmethoxycarbonyl) and N-palmitate-GIP, were synthesized and tested for metabolic stability and biological activity. Both GIP analogues were resistant to degradation by DPP IV and human plasma. In Chinese hamster lung (CHL) cells expressing the cloned human GIP receptor, both analogues exhibited a 2-fold increase in cAMP-generating potency compared with native GIP (EC₅₀ values of 9.4, 10.0 and 18.2 nM respectively). Using clonal BRIN-BD11 cells, both analogues demonstrated strong insulino-tropic activity compared with native GIP ($P < 0.01$ to $P < 0.001$). In obese diabetic (*ob/ob*) mice, administration of N-Fmoc-GIP or N-palmitate-GIP (25 nmol/kg) together with glucose

(18 mmol/kg) significantly reduced the peak 15 min glucose excursion (1.4- and 1.5-fold respectively; $P < 0.05$ to $P < 0.01$) compared with glucose alone. The area under the curve (AUC) for glucose was significantly lower after administration of either analogue compared with glucose administered alone or in combination with native GIP (1.5-fold; $P < 0.05$). This was associated with a significantly greater AUC for insulin (2.1-fold; $P < 0.001$) for both analogues compared with native GIP. A similar pattern of *in vivo* responsiveness was evident in lean control mice. These data indicate that novel N-terminal Tyr¹ modification of GIP with an Fmoc or palmitate group confers resistance to degradation by DPP IV in plasma, which is reflected by increased *in vitro* potency and greater insulintropic and antihyperglycaemic activities in an animal model of Type II diabetes mellitus.

Key words: dipeptidyl peptidase IV (DPP IV), GIP analogues, insulin secretion, obese diabetic (*ob/ob*) mice.

INTRODUCTION

Incretin hormones, which originate from the intestine, are released following absorption of nutrients, and under hyperglycaemic conditions they stimulate insulin release from pancreatic β -cells [1]. To date, only two known hormones are considered as physiological incretins, namely glucose-dependent insulintropic polypeptide (GIP) and glucagon-like peptide-1-(7–36)amide (GLP-1) [2]. Since the insulin-releasing actions of these hormones are strictly glucose-dependent, substantial interest has focused on their potential beneficial use in the treatment of Type II diabetes mellitus [3–8].

One of the key limitations hindering any possible use of GIP or GLP-1 in Type II diabetes therapy is their rapid clearance from the circulation (half-lives of approx. 3–5 min), following degradation by the ubiquitous enzyme dipeptidyl peptidase IV (DPP IV) [9–11]. DPP IV specifically removes the N-terminal

dipeptides Tyr¹-Ala² and His⁷-Ala⁸ from GIP and GLP-1 respectively, resulting in complete loss of insulintropic activity [12,13]. Any intact GIP and GLP-1 remaining in the circulation is rapidly filtered in the glomerulus and degraded by renal capillary peptidases, including DPP IV [14–16]. The kidney is involved in this removal of GIP, as demonstrated in patients suffering from chronic renal failure [17], as well as by reduced clearance of GIP in nephrectomized rats [18]. However, demonstration that DPP IV is the major route of degradation [19] indicates that strategies that prevent DPP IV action can potentially improve the therapeutic efficacy of GIP and GLP-1.

A number of key studies have clearly demonstrated the improved insulintropic potential of structurally modified stable analogues of GIP and, more particularly, GLP-1 [3,20,21]. Furthermore, we have demonstrated that a GIP analogue modified at the α -amino group by glycation possesses greatly improved resistance to DPP IV and insulin-releasing ability [6,8].

Abbreviations used: AUC, area under the curve; CHL, Chinese hamster lung; DPP IV, dipeptidyl peptidase IV; ESI-MS, electrospray ionization-MS; Fmoc, 9-fluorenylmethoxycarbonyl; GIP, glucose-dependent insulintropic polypeptide; GLP-1, glucagon-like peptide-1(7–36)amide; TFA, trifluoroacetic acid.

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Additional studies with GLP-1 have shown that ϵ -amino extension at internal Lys²⁶ and Lys³⁸ residues by covalent attachment of fatty acids greatly extends the plasma half-lives of GLP-1 (12–14 h) [22]. This principle of fatty acid derivatization has been used to prolong the action of insulin by facilitating binding to serum albumin [22–25]. It is thought that the fatty-acid-peptide complex binds to serum albumin, thus avoiding kidney filtration and clearance. Here the bioactive form of fatty acid-linked insulin exists in equilibrium with the albumin-bound inactive fraction and is slowly released from the protein-bound fraction.

The present study examines the metabolic stability and biological activity of a palmitate (C₁₆) α -amino-linked GIP analogue as well as of another N-terminally Tyr¹-modified GIP analogue (N-Fmoc-GIP). The base-labile 9-fluorenylmethoxycarbonyl (Fmoc) group is utilized as an α -amino-protecting group during peptide synthesis using standard Fmoc chemistry. The basic strategy underlying this approach is that the substitution of either palmitate or Fmoc groups at the Tyr¹ residue of GIP would prevent DPP IV-mediated degradation to GIP(3–42), thereby providing bioactive analogues with enhanced anti-diabetic properties. The stability of both N-Fmoc-GIP and N-palmitate-GIP to degradation by DPP IV, as well as their cAMP-stimulating abilities and insulin-releasing potencies *in vitro*, were examined and compared with those of the native peptide. Furthermore, the *in vivo* antihyperglycaemic efficacy of these analogues was compared with those of native GIP in obese diabetic (*ob/ob*) and lean control mice.

MATERIALS AND METHODS

Reagents

HPLC-grade acetonitrile was obtained from Rathburn (Walkersburn, Scotland, U.K.). Sequencing-grade trifluoroacetic acid (TFA) was obtained from Aldrich (Poole, Dorset, U.K.). DPP IV, forskolin, isobutylmethylxanthine, cAMP, ATP, dextran T-70 and activated charcoal were all purchased from Sigma (Poole, Dorset, U.K.). Fmoc-protected amino acids were from Calbiochem Novabiochem (Beeston, Nottingham, U.K.). RPMI 1640 and Dulbecco's modified Eagle's medium tissue culture media, foetal bovine serum, penicillin and streptomycin were all purchased from Gibco (Paisley, Strathclyde, U.K.). The chromatography columns used for cAMP assay, Dowex AG 50 WX and neutral alumina AG7 were obtained from Bio-Rad (Life Science Research, Alpha Analytical, Larne, N. Ireland, U.K.). All water used in these experiments was purified using a Milli-Q Water Purification System (Millipore, Milford, MA, U.S.A.).

Peptide synthesis

GIP, N-Fmoc-GIP and N-palmitate-GIP were synthesized sequentially on an Applied Biosystems automated peptide synthesizer (model 432A) using standard solid-phase Fmoc protocols [26], from a preloaded Fmoc-Gln-Wang resin. The following side-chain-protected amino acids were used: Fmoc-Gln(Trt)-OH, Fmoc-Thr(Trt)-OH, Fmoc-His(Trt)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Ser(Trt)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Asp(OtBu)-OH and Fmoc-Tyr(OtBu)-OH (where Trt is triphenylmethyl, Boc is *t*-butoxycarbonyl and OtBu is *t*-butoxy). In the synthesis of N-Fmoc-GIP, the labile Fmoc protecting group was left attached to the peptide chain, while palmitate-OH was added to the N-terminal Tyr¹ in the synthesis of N-palmitate-GIP. Deprotection and cleavage of the peptides from the resin was by use of TFA/water/thioanisole/ethanediol (90:2.5:5:2.5, by vol.; total volume of 20 ml/g of resin). The

resin was removed by filtration and the filtrate volume was decreased under reduced pressure. Dry diethyl ether was added slowly until a precipitate was observed. The precipitate was collected by low-speed centrifugation, resuspended in diethyl ether and centrifuged again. The procedure being repeated five times. The resulting pellets were then dried *in vacuo*, purified and judged pure by reverse-phase HPLC on a Waters Millennium 2010 chromatography system (Software version 2.1.5).

Electrospray ionization-MS (ESI-MS)

Samples for ESI-MS containing GIP, GIP degradation fragments, N-Fmoc-GIP or N-palmitate-GIP were dissolved (approx. 400 pM) in 100 μ l of water and applied to an LCQ benchtop LC mass spectrometer (Finnigan MAT, Hemel Hempstead, U.K.). Samples (20 μ l direct loop injection) were applied at a flow rate of 0.2 ml/min, under isocratic conditions in 35% (v/v) acetonitrile/water. Mass spectra were obtained from the quadrupole ion trap mass analyser and spectra were collected using full ion scan mode over the m/z range 150–2000. The molecular mass of each fragment was determined using prominent multiple charged ions, and the following equation was applied:

$$\text{Molecular mass} = iM_i - iM_h$$

where M_i is the m/z ratio, i is the number of charges and M_h is the mass of a proton.

Degradation of GIP and GIP analogues by DPP IV and human plasma

HPLC-purified GIP, N-Fmoc-GIP and N-palmitate-GIP were incubated *in vitro* at 37 °C in 50 mM triethanolamine/HCl (pH 7.8; final peptide concentration 2 mM) with either DPP IV (5 m-units) or pooled human plasma (10 μ l) for 0, 2, 8 and 24 h. The enzymic reactions were stopped by the addition of 10 μ l of 10% (v/v) TFA/water. The reaction products were then applied to a Vydac C-18 column (4.6 mm $\times$ 250 mm) and the major degradation fragment GIP(3–42) was separated from intact GIP. The column was equilibrated with 0.12% (v/v) TFA/water at a flow rate of 1.0 ml/min. Using 0.1% (v/v) TFA in 70% acetonitrile/water, the concentration of acetonitrile in the eluting solvent was raised from 0% to 28% over 10 min and then from 28% to 42% over 30 min for GIP. For the separation of both N-Fmoc-GIP and N-palmitate-GIP, the concentration of acetonitrile in the eluting solvent was raised from 0% to 28% over 10 min, and then from 28% to 50% over 50 min. The absorbance was monitored at 206 nm using a SpectraSystem UV 2000 detector (Thermoquest Limited, Manchester, U.K.), and peaks were collected manually prior to ESI-MS analysis. HPLC peak area data were used to calculate the percentage of the intact peptide remaining at each incubation time point.

cAMP production in transfected Chinese hamster lung (CHL) cells

CHL fibroblasts stably transfected with the human GIP receptor (A.T.C.C. CCL39) [27] were seeded into 12-multiwell plates (Nunc, Roskilde, Denmark) at a density of 1.0×10^5 cells per well. The cells were then allowed to grow for 48 h before incubation at 37 °C for 6 h with 2 μ Ci of [³H]adenine (TRK311; Amersham) in 1 ml of Dulbecco's modified Eagle's medium containing 0.5% (v/v) foetal bovine serum. The cells were then washed twice with HBS (130 mM NaCl, 20 mM Hepes, 0.9 mM NaHPO₄, 0.8 mM MgSO₄, 5.4 mM KCl, 1.8 mM CaCl₂, 25 mM glucose and 25 μ M Phenol Red, pH 7.4). The cells were then exposed ($n = 6$) to various concentrations (10^{-12} to 10^{-6} M) of

native GIP, N-Fmoc-GIP, N-palmitate-GIP or forskolin (10 μ M) in HBS, in the presence of 1 mM isobutylmethylxanthine, for 15 min at 37 °C. The medium was subsequently removed and the cells were lysed with 1 ml of 5% trichloroacetic acid containing 0.1 mM unlabelled cAMP and 0.1 mM unlabelled ATP. The intracellular [3 H]cAMP was then separated on Dowex and alumina exchange resins as previously described [28].

Acute tests for insulin secretion

Before experimentation, BRIN-BD11 cells were harvested with the aid of trypsin/EDTA (Gibco), seeded at 1.0×10^5 cells per well into 24-multiwell plates (Nunc) and allowed to attach overnight at 37 °C. The origin and characteristics of this clonal β -cell line have been described elsewhere [29]. Acute tests for insulin release were preceded by 40 min preincubation at 37 °C in 1.0 ml of Krebs–Ringer bicarbonate buffer [115 mM NaCl, 4.7 mM KCl, 1.28 mM CaCl_2 , 1.2 mM KH_2PO_4 , 1.2 mM MgSO_4 , 10 mM NaHCO_3 , 0.5% (w/v) BSA, pH 7.4] supplemented with 1.1 mM glucose. Test incubations were performed ($n = 8$) in the presence of 5.6 mM glucose with a range of concentrations (10^{-13} to 10^{-8} M) of native GIP, N-Fmoc-GIP or N-palmitate-GIP. After 20 min incubation, the buffer was removed from each well and aliquots (200 μ l) were used for measurement of insulin by RIA [30].

In vivo biological activities of GIP and GIP analogues in obese diabetic (ob/ob) and lean control mice

The effects of GIP, N-Fmoc-GIP and N-palmitate-GIP on plasma glucose and insulin concentrations were examined using 14–18-week-old obese diabetic (ob/ob) and lean control mice. The genetic background and characteristics of the colony used have been outlined in detail elsewhere [31]. The animals were housed individually in an air-conditioned room at 22 ± 2 °C with a 12 h light/12 h dark cycle. Drinking water and a standard rodent maintenance diet (Trouw Nutrition Ltd) were freely available. Food was withdrawn for an 18 h period prior to intraperitoneal injection of saline [0.9% (w/v) NaCl] as a control, or of glucose alone (18 mmol/kg body weight) or in combination with GIP or GIP analogues (25 nmol/kg body weight). All test solutions were administered in a final volume of 8 ml/kg body weight. Blood samples were collected from the cut tip of the tail of conscious mice into chilled fluoride/heparin microcentrifuge tubes (Sarstedt, Nümbrecht, Germany) immediately prior to injection and at 15, 30 and 60 min post-injection. Blood was immediately centrifuged using a Beckman microcentrifuge (Beckman Instruments) for 30 s at 13 000 *g*. Plasma glucose was assayed by an automated glucose oxidase procedure using a Beckman Glucose Analyzer II [32]. Plasma insulin was determined by dextran–charcoal RIA as described previously [30].

All animal studies were carried out in accordance with the U.K. Animals (Scientific Procedures) Act 1986. No adverse effects were observed following administration of any of the peptides.

Statistical analysis

Results are expressed as means $\pm$ S.E.M., and data for *in vitro* studies were compared using Student's *t* test. *In vivo* data were compared using ANOVA, followed by the Student–Newman–Keuls post hoc test. Incremental areas under the curve (AUCs) for plasma glucose and insulin were calculated using a computer-generated program (CAREA) employing the trapezoidal rule [33] with baseline subtraction. Groups of data from both were considered to be significantly different at $P < 0.05$.

RESULTS

Structural characterization of GIP and GIP analogues by ESI-MS

Following the synthesis and purification of GIP, N-Fmoc-GIP and N-palmitate-GIP, the monoisotopic molecular mass of each peptide was determined using ESI-MS (Figure 1). After spectral averaging was performed, the prominent multiply charged species ($M + 3\text{H}$) $^{3+}$ and ($M + 4\text{H}$) $^{4+}$ were detected from native GIP at m/z 1661.6 and 1246.8, corresponding to intact molecular masses of 4981.8 and 4983.2 Da respectively (Figure 1A). This corresponds very closely to the theoretical mass of 4984.2 Da. Similarly, for N-Fmoc-GIP, ($M + 3\text{H}$) $^{3+}$ and ($M + 4\text{H}$) $^{4+}$ were detected at m/z 1735.8 and 1302.7, corresponding to intact molecular masses of 5204.4 and 5206.8 Da respectively (theoretical mass 5202.5 Da) (Figure 1B). ($M + 3\text{H}$) $^{3+}$ and ($M + 4\text{H}$) $^{4+}$ were detected at m/z 1741.1 and 1306.3 for N-palmitate-GIP, corresponding to intact molecular masses of 5220.3 and 5221.2 Da respectively (Figure 1C). This correlates very closely with the theoretical mass of N-palmitate-GIP (5220.7 Da). The results from ESI-MS analysis confirmed that the correct primary structures for native GIP and related analogues had been synthesized successfully.

Degradation of GIP and GIP analogues by DPP IV and human plasma

The HPLC retention times of native GIP, N-Fmoc-GIP and N-palmitate-GIP incubated with DPP IV at $t = 0$ were 21.72, 45.22 and 49.25 min respectively. As shown in Table 1, degradation of native GIP by DPP IV was evident after just 2 h, as indicated by the appearance of a peak with an HPLC retention time of 20.81 min, which upon ESI-MS analysis corresponded to GIP(3–42). After 8 h, native GIP was completely degraded. In contrast, N-Fmoc-GIP and N-palmitate-GIP remained intact up to and including 24 h (Table 1), and no degradation fragment was evident. The half-lives of GIP, N-Fmoc-GIP and N-palmitate-GIP on incubation with DPP IV were 2.3, > 24 and > 24 h respectively.

In degradation studies using human plasma, the HPLC retention times for intact GIP, N-Fmoc-GIP and N-palmitate-GIP were 21.94, 45.52 and 50.29 min respectively. From Table 1 it can be seen that the degradation of native GIP by human plasma was in progress within 2 h, giving a major degradation peak of GIP(3–42) with a retention time of 20.21 min. GIP was completely degraded by 24 h, but in general the degradation with human plasma was not as rapid as with purified DPP IV (Table 1). In contrast with native GIP, N-Fmoc-GIP and N-palmitate-GIP remained intact up to and including 24 h (Table 1), and no degradation fragment was evident. In human plasma, the half-lives of native GIP, N-Fmoc-GIP and N-palmitate-GIP were 6.2, > 24 and > 24 h respectively.

Stimulation of intracellular cAMP production

Coupling of cloned human GIP receptors expressed on CHL cells to adenylate cyclase was assessed by measuring the accumulation of cAMP after exposure of the cells to various concentrations (10^{-12} to 10^{-6} M) of native GIP or GIP analogues. Figure 2 illustrates the dose-dependent production of cAMP upon binding of native GIP, N-Fmoc-GIP and N-palmitate-GIP to GIP receptors on CHL cells. Both N-Fmoc-GIP and N-palmitate-GIP were significantly more potent at stimulating cAMP production ($P < 0.05$ to $P < 0.01$) at concentrations from 10^{-8} to 10^{-6} M compared with native GIP. The calculated EC_{50} values for native GIP, N-Fmoc-GIP and N-palmitate-GIP were 18.2, 9.4 and 10.0 nM respectively. The maximal cAMP responses to N-Fmoc-GIP and N-palmitate-GIP were significantly higher

Figure 1 ESI-MS of (A) GIP, (B) N-Fmoc-GIP and (C) N-palmitate-GIP

The peptides were applied to LC/MS equipped with a microbore C-18 HPLC column (150 mm $\times$ 2.0 mm) at a flow rate of 0.2 ml/min, under isocratic conditions in 35% (v/v) acetonitrile/water. Spectra were recorded using a quadrupole ion trap mass analyser and collected using full ion scan mode over the m/z range 150–2000.

Table 1 Amount of intact peptide remaining after various incubation times with DPP IV or human plasma

Data represent the percentage of intact peptide remaining (following HPLC separation) relative to the major degradation fragment GIP-(3–42) after incubation with purified DPP IV or human plasma (shown in parentheses). The reactions were performed in triplicate, and results are means $\pm$ S.E.M.

Peptide	Intact peptide remaining (%)				
	0 h	2 h	4 h	8 h	24 h
GIP	100 (100)	52 $\pm$ 3 (78 $\pm$ 4)	23 $\pm$ 1 (62 $\pm$ 3)	0 (39 $\pm$ 1)	0 (0)
N-Fmoc-GIP	100 (100)	100 (100)	100 (100)	100 (100)	100 (100)
N-palmitate-GIP	100 (100)	100 (100)	100 (100)	100 (100)	100 (100)

(121.8 $\pm$ 2.2% and 129.5 $\pm$ 5.5% respectively; $P < 0.01$) than that to native GIP (100%). Exposure of either analogue to DPP IV or human plasma did not affect the ability of the re-purified peptide to increase cAMP levels (results not shown).

Dose-dependent effects of GIP and GIP analogues on insulin secretion

Figure 3 shows the effects of a range of concentrations of GIP, N-Fmoc-GIP and N-palmitate-GIP on insulin secretion from BRIN-BD11 cells at 5.6 mM glucose. Native GIP significantly stimulated insulin secretion (1.2–1.8-fold; $P < 0.01$ to $P < 0.001$) compared with control (5.6 mM glucose alone). N-Fmoc-GIP and N-palmitate-GIP were significantly more potent ($P < 0.05$ to $P < 0.001$) at stimulating insulin secretion at concentrations from 10^{-11} M to 10^{-8} M compared with native GIP. At 10^{-8} M, N-Fmoc-GIP and N-palmitate-GIP had a 1.2–1.3-fold greater stimulatory potency than native GIP.

Figure 2 Dose-dependent effects of native GIP, N-Fmoc-GIP and N-palmitate-GIP on intracellular cAMP production in GIP-receptor-transfected CHL cells

Data are expressed as a percentage of the maximal cAMP response induced by forskolin (FSK) for each peptide, and represent means $\pm$ S.E.M. of three independent experiments. * $P < 0.05$, ** $P < 0.01$ compared with native GIP at the same concentration.

Glucose-lowering effects of GIP and GIP analogues in obese diabetic (*ob/ob*) and lean control mice

Figure 4 shows the plasma glucose responses to intraperitoneal injection of saline [0.9% (w/v) NaCl] as a control, and of glucose

Figure 3 Dose-dependent effects of native GIP, N-Fmoc-GIP and N-palmitate-GIP on insulin secretion from BRIN-BD11 cells

After a preincubation of 40 min, the effects of various concentrations of the peptides (10^{-13} to 10^{-8} M) were tested during a 20 min incubation. Values are means $\pm$ S.E.M. for eight separate observations. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with 5.6 mM glucose control; $\Delta P < 0.05$, $\Delta\Delta P < 0.01$, $\Delta\Delta\Delta P < 0.001$ compared with native GIP at the same concentration.

Figure 4 Effects of GIP, N-Fmoc-GIP and N-palmitate-GIP on plasma glucose homeostasis in obese diabetic (*ob/ob*) mice

(A) Plasma glucose concentrations were measured prior to and at intervals after intraperitoneal administration of saline [0.9% (w/v) NaCl] as control, or of glucose alone (18 mmol/kg body weight) or in combination with GIP, N-Fmoc-GIP or N-palmitate-GIP (25 nmol/kg body weight). The time of injection is indicated by the arrow. (B) Plasma glucose AUC values for 0–60 min post-injection. Values are means $\pm$ S.E.M. for eight mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with glucose alone; $\Delta P < 0.05$, $\Delta\Delta P < 0.01$, $\Delta\Delta\Delta P < 0.001$ compared with native GIP.

alone (18 mmol/kg body weight) or in combination with GIP or GIP analogues (25 nmol/kg body weight) in obese diabetic (*ob/ob*) mice. When saline alone was injected, no effect was observed on plasma glucose (Figure 4A), while glucose given alone evoked a significant and prominent rise (34.4 ± 2.9 mM; $P < 0.001$) in plasma glucose after 15 min compared with basal

Figure 5 Effects of GIP, N-Fmoc-GIP and N-palmitate-GIP on plasma glucose homeostasis in lean control mice

(A) Plasma glucose concentrations were measured prior to and at intervals after intraperitoneal administration of saline [0.9% (w/v) NaCl] as control, or of glucose alone (18 mmol/kg body weight) or in combination with GIP, N-Fmoc-GIP or N-palmitate-GIP (25 nmol/kg body weight). The time of injection is indicated by the arrow. (B) Plasma glucose AUC values for 0–60 min post-injection. Values are means $\pm$ S.E.M. for eight mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with glucose alone; $\Delta P < 0.05$, $\Delta\Delta P < 0.001$ compared with native GIP.

(5.4 ± 0.5 mM) (Figure 4A). The peak glucose response to native GIP was reduced, but not significantly ($P > 0.05$), at 15–30 min compared with glucose alone. At 60 min, plasma glucose was significantly lower after administration of native GIP ($P < 0.05$), but this failed to reach significance in terms of overall glucose excursion, as identified by AUC (0–60 min; Figure 4B). In sharp contrast, both N-Fmoc-GIP and N-palmitate-GIP significantly reduced the peak 15–60 min glucose excursion (1.4- and 1.5-fold respectively; $P < 0.05$ to $P < 0.01$) compared with glucose alone. These GIP analogues also significantly reduced the AUC (1.5-fold; $P < 0.05$) compared with native GIP (Figure 4B). As shown in Figure 5, a very similar pattern of responsiveness to GIP and GIP analogues was evident in lean control mice.

Effects of GIP and GIP analogues on insulin secretion in obese diabetic (*ob/ob*) and lean control mice

Figure 6 shows the corresponding plasma insulin responses to the intraperitoneal injection of saline, or of glucose alone or in combination with GIP or GIP analogues (25 nmol/kg body weight). When saline alone was injected, no effect was observed on plasma insulin concentrations of obese diabetic (*ob/ob*) mice over the 60 min experimental period (Figure 6A). When glucose was given alone, a peak (9.0 ± 0.6 ng/ml) in plasma insulin was observed after 15 min compared with basal insulin (2.8 ± 0.5 ng/ml), and the plasma insulin concentration declined over the subsequent 45 min, returning to near basal values (Figure 6A). The overall insulin response to native GIP in the presence of glucose, as estimated by AUC, was significantly greater ($P < 0.05$) compared with that to glucose alone (Figure 6B). The

Figure 6 Effects of GIP, N-Fmoc-GIP and N-palmitate-GIP on plasma insulin responses in obese diabetic (*ob/ob*) mice

(A) Plasma insulin concentrations were measured prior to and at intervals after intraperitoneal administration of saline [0.9% (w/v) NaCl], or of glucose alone (18 mmol/kg body weight) or in combination with GIP, N-Fmoc-GIP or N-palmitate-GIP (25 nmol/kg body weight). The time of injection is indicated by the arrow. (B) Plasma insulin AUC values for 0–60 min post-injection. Values are means $\pm$ S.E.M. for eight mice. * $P < 0.05$, *** $P < 0.001$ compared with glucose alone; $\Delta P < 0.05$, $\Delta\Delta P < 0.01$, $\Delta\Delta\Delta P < 0.001$ compared with native GIP.

insulinotropic effects of both GIP analogues were significantly greater at 15 min (1.4-fold; $P < 0.01$) and at 60 min (2.4-fold; $P < 0.001$) post-injection compared with that of native GIP (Figure 6A). The overall insulinotropic effects of these analogues were significantly improved (2.1-fold; $P < 0.001$) compared with native GIP, as estimated by AUC values (Figure 6B). Lean control mice also exhibited an enhanced insulin secretory response to the two novel GIP analogues, as shown in Figure 7.

DISCUSSION

Studies using the specific GIP receptor antagonist GIP(7–30)-amide have bolstered the opinion that GIP is a potent insulin-releasing hormone of the enteroinsular axis and a key mediator in the physiological control of blood glucose homeostasis [34,35]. This insulinotropic action, together with several other potentially important extrapancreatic actions [36–40], has attracted much attention with regard to GIP having a possible therapeutic use in diabetes mellitus [6,8,41]. Although clinical trials have been restricted to GLP-1 [5], administration of peptide analogues of GIP with prolonged circulating half-lives, and inhibition of DPP IV, have been shown to greatly improve glucose tolerance in experimental animal models [6,8]. Currently, there is no consensus regarding the circulating levels of GIP in subjects with Type II diabetes; different studies have demonstrated increased, decreased and unaltered GIP levels [42–45].

Rapid inactivation in the circulation by the ubiquitous enzyme DPP IV and a biological half-life of less than 5 min [9,10] are major factors that hamper development towards the therapeutic use of GIP. This inactivation comes about through the rapid

Figure 7 Effects of GIP, N-Fmoc-GIP and N-palmitate-GIP on plasma insulin responses in lean control mice

(A) Plasma insulin concentrations were measured prior to and at intervals after intraperitoneal administration of saline [0.9% (w/v) NaCl], or of glucose alone (18 mmol/kg body weight) or in combination with GIP, N-Fmoc-GIP or N-palmitate-GIP (25 nmol/kg body weight). The time of injection is indicated by the arrow. (B) Plasma insulin AUC values for 0–60 min post-injection. Values are means $\pm$ S.E.M. for eight mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with glucose alone; $\Delta P < 0.05$, $\Delta\Delta P < 0.001$ compared with native GIP.

removal of the N-terminal dipeptide Tyr¹-Ala², giving rise to the major truncated metabolite GIP(3–42). One approach to counteract this drawback has been directed towards the inhibition of GIP degradation using specific DPP IV inhibitors [4]. A recent study in anaesthetized pigs with one such DPP IV inhibitor, valine-pyrrolidine, significantly extended the circulating half-life of GIP from 3.3 to 8.1 min, and enhanced the insulinotropic and antihyperglycaemic effects of the peptide following an intravenous glucose load in this species [19].

The alternative strategy to protect GIP from degradation is to structurally modify the N-terminal region of the peptide, which will not only disrupt enzymic hydrolysis but also potentially improve biological activity. The present study clearly demonstrates that structurally modifying GIP by attaching an Fmoc or a palmitate group at the α -amino group of Tyr¹ confers absolute protection against hydrolysis to GIP(3–42) when incubated for up to 24 h with purified DPP IV or human plasma. Thus, by masking the potential enzyme cleavage recognition sites, both the Fmoc and the palmitate adduct confer metabolic stability on the peptide, with a significant prolongation of the circulating half-life.

The *in vitro* biological efficacy of these DPP IV-resistant GIP analogues was assessed using stably transfected CHL fibroblasts and the clonal pancreatic β -cell line BRIN-BD11. In harmony with previous *in vitro* studies, GIP was shown to dose-dependently stimulate insulin release from BRIN-BD11 cells [46]. Both N-Fmoc-GIP and N-palmitate-GIP had an increased insulinotropic response (1.2- and 1.3-fold respectively; $P < 0.01$ to $P < 0.001$) compared with native GIP. Native GIP, N-Fmoc-GIP and N-palmitate-GIP had EC₅₀ values of 18.2, 9.4 and

10.0 nmol/l respectively for cAMP-stimulating ability. The reduced EC_{50} values for these GIP analogues signify an increased potency at the native GIP receptor. Such effects cannot be attributed merely to enzymic resistance, as GIP degradation in the *in vitro* systems employed was negligible. Taken together, the increased cAMP-stimulating potencies and the enhanced insulinotropic activities demonstrate that both analogues are biologically more potent than the native hormone.

Consistent with previous *in vivo* studies, administration of native GIP together with glucose to obese diabetic (*ob/ob*) mice resulted in an augmentation of insulin release and slight reduction of the glycaemic excursion compared with glucose alone [47]. The relatively modest effects on glucose excursion reflect the severe insulin insensitivity of these mutant mice [30]. In contrast, both N-Fmoc-GIP and N-palmitate-GIP significantly reduced the peak 15 min glycaemic rise (1.4- and 1.5-fold respectively; $P < 0.05$ to $P < 0.01$) compared with glucose alone (control). Indeed, the overall glycaemic excursion (estimated as AUC) was 45% lower following administration of either analogue when compared with native GIP. Associated with this greatly improved antihyperglycaemic activity was a corresponding increase in insulin-releasing activity. The plasma insulin responses following administration of either N-Fmoc-GIP or N-palmitate-GIP were significantly elevated (1.4-fold to 2.4-fold; $P < 0.01$ to $P < 0.001$) at all time points between 15 and 60 min when compared with native GIP. Further, the overall insulinotropic effects, as estimated by the insulin AUC values, were significantly improved (2.1-fold; $P < 0.001$) compared with native GIP. Interestingly, the insulin-releasing activity of these analogues was very much protracted compared with that of native GIP, even at 60 min post-injection, further emphasizing the resistance to degradation and potential of such analogues in the treatment of Type II diabetes mellitus. Responses to GIP and GIP analogues were similar in lean control mice as in obese mice, and notably no evidence was observed to suggest that the *ob/ob* mutant was refractory to the insulinotropic action of GIP.

The basis for the improved biological efficiency of these N-terminal Tyr¹-modified GIP analogues compared with the native GIP is likely to be increased potency at β -cells, and presumably other sites of GIP action [48,49], together with an extended circulating plasma half-life due to conferment of DPP IV resistance. The inhibition of N-Fmoc-GIP and N-palmitate-GIP catabolism will increase the effective concentrations of the biologically active peptides, while at the same time reducing the possible effect of feedback antagonism at the receptor level through decreased accumulation of the receptor antagonist GIP(3–42). It is perhaps surprising that the fatty acid-linked analogue N-palmitate-GIP was not more biologically potent *in vivo* than N-Fmoc-GIP, as studies with GLP-1 have shown fatty acid-derived analogues to be extremely potent due to considerable extension of half-life as a result of binding to albumin in blood [22]. Longer-term studies using N-palmitate-GIP in obese diabetic (*ob/ob*) mice would be interesting to assess possible greater beneficial effects of this analogue for the treatment of Type II diabetes.

In conclusion, we have demonstrated that two novel N-terminally Tyr¹-modified analogues of GIP, N-Fmoc-GIP and N-palmitate-GIP, exhibit DPP IV resistance, greatly reduced GIP(3–42) antagonist production and substantially enhanced cAMP-generating and insulin-secreting activity *in vitro* compared with native GIP. More importantly, these attributes are further exemplified *in vivo* by greatly enhanced antihyperglycaemic and insulin-releasing activities in a commonly employed animal model of Type II diabetes with β -cell dysfunction and severe insulin resistance.

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