Improved biological activity of Gly²- and Ser²-substituted analogues of glucose-dependent insulintrophic polypeptide

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Abstract

The therapeutic potential of glucagon-like peptide-1 (GLP-1) in improving glycaemic control in diabetes has been widely studied, but the potential beneficial effects of glucose-dependent insulintrophic polypeptide (GIP) have until recently been almost overlooked. One of the major problems, however, in exploiting either GIP or GLP-1 as potential therapeutic agents is their short duration of action, due to enzymatic degradation *in vivo* by dipeptidylpeptidase IV (DPP IV). Therefore, this study examined the plasma stability, biological activity and antidiabetic potential of two novel NH₂-terminal Ala²-substituted analogues of GIP, containing glycine (Gly) or serine (Ser). Following incubation in plasma, (Ser²)GIP had a reduced hydrolysis rate compared with native GIP, while (Gly²)GIP was completely stable. In Chinese hamster lung fibroblasts stably transfected with the human GIP receptor, GIP, (Gly²)GIP and (Ser²)GIP stimulated cAMP

production with EC₅₀ values of 18.2, 14.9 and 15.0 nM respectively. In the pancreatic BRIN-BD11 β-cell line, (Gly²)GIP and (Ser²)GIP (10⁻⁸ M) evoked significant increases (1.2- and 1.5-fold respectively; *P* < 0.01 to *P* < 0.001) in insulintrophic activity compared with GIP. In obese diabetic *ob/ob* mice, both analogues significantly lowered (*P* < 0.001) the glycaemic excursion in response to i.p. glucose. This enhanced glucose-lowering ability was coupled to a significantly raised (*P* < 0.01) and more protracted insulin response compared with GIP. These data indicate that substitution of the penultimate Ala² in GIP by Gly or Ser confers resistance to plasma DPP IV degradation, resulting in enhanced biological activity, therefore raising the possibility of their use in the treatment of type 2 diabetes.

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Introduction

Glucose-dependent insulintrophic polypeptide (GIP) is a 42 amino acid incretin hormone synthesised by enteroendocrine K-cells (Buchan *et al.* 1978), which upon secretion modulates pancreatic β-cell insulin release (Creutzfeldt 2001). GIP exerts its potentiating effects on glucose-induced insulin release via interaction with specific β-cell GIP receptors (Usdin *et al.* 1993). Binding of GIP is understood to activate a heterotrimeric G-protein, coupled to stimulation of adenyl cyclase and production of cAMP (Moens *et al.* 1996), activation of phospholipase A₂ (Ehse *et al.* 2001) and amplification of intracellular Ca²⁺ concentrations (Lu *et al.* 1993). In addition to actions on the pancreatic β-cell, GIP is also known to exert various extrapancreatic effects, which further promote glucose lowering. Thus, GIP has been shown to augment insulin-dependent inhibition of glycogenolysis in liver (Elahi *et al.* 1986) and to exert stimulatory effects on glucose

uptake and metabolism in muscle (O'Harte *et al.* 1998a). Furthermore, functional GIP receptors have been identified on adipocytes (Yip *et al.* 1998) and have been shown to stimulate glucose transport (Eckel *et al.* 1979), increase fatty acid synthesis (Oben *et al.* 1991) and stimulate lipoprotein lipase activity (Knapper *et al.* 1995). It is a combination of the above features that has aroused recent interest in the incretin hormones as novel potential therapeutic candidates for the treatment of type 2 diabetes (Bailey & Flatt 1995). To date, clinical trials have been limited to glucagon-like peptide-1 (GLP-1) (Holst 1999), although administration of an NH₂-terminally glycated form of GIP with a prolonged circulating half-life has been shown to produce improved glucose tolerance in experimental animal models of diabetes (O'Harte *et al.* 1999, 2000).

One of the crucial difficulties in attempting to utilise the insulintrophic activity of GIP as a potential therapeutic agent is its short biological half-life in plasma (Kieffer *et al.*

1995), due primarily to the action of the ectopeptidase, dipeptidylpeptidase IV (DPP IV; CD26; EC 3.4.14.5). DPP IV is expressed ubiquitously in mammalian tissues and organs (Mentlein 1999), being located on endothelial cells of blood vessels as well as in a soluble enzyme form in blood plasma (Lodja 1979). DPP IV belongs to the prolyl oligopeptidase family of serine proteases (Barrett & Rawlings 1992) and displays a strict specificity for hydrolysing peptides from the NH₂-terminus following a penultimate proline, alanine or hydroxyproline residue (Heins *et al.* 1988). Therefore, in the case of GIP, DPP IV hydrolyses the NH₂-terminal Tyr¹-Ala² dipeptide, producing the truncated peptide GIP(3–42). This truncated metabolite, initially isolated from porcine intestinal extracts, was originally shown to lack significant insulinotropic activity (Brown *et al.* 1981); however, more recent observations indicate that it behaves as a GIP receptor antagonist *in vivo* (Gault *et al.* 2002).

DPP IV-mediated inactivation of GIP and GLP-1 results in a considerable reduction in activity of the enteroinsular axis. This has led to the strategy of utilising DPP IV inhibitors to block this inactivation process and effectively upregulate the enteroinsular axis. Several studies investigating the efficacy of such agents in acute animal studies have revealed an improvement in glucose tolerance (Pederson *et al.* 1998, Deacon *et al.* 2001). More recently, a 12 week study using the DPP IV inhibitor P32/98 demonstrated sustained improvements in glucose tolerance, hyperinsulinaemia, β -cell glucose responsiveness and peripheral insulin sensitivity in fatty Zucker (*fa/fa*) rats (Pospisilik *et al.* 2002). While such inhibitors may well prolong the activity of GIP and GLP-1, they run the risk of conferring longer-term adverse effects through inhibition of numerous other DPP IV-mediated hormonal inactivation processes (Shipp & Look 1993). Rather than inhibiting DPP IV activity directly, an alternative means of prolonging therapeutic activity of GIP or GLP-1 would be to structurally modify domains within the molecule itself, thereby conferring on the peptide resistance to DPP IV activity. Taking into account the fact that the strict substrate specificity of DPP IV is well characterised, it was hypothesised that minor modifications at the NH₂-terminus may well render the peptide resistant to inactivation.

Recent studies in our laboratory have revealed that NH₂-terminal modification of GIP by glycation resulted in resistance to DPP IV and an extended plasma half-life (O'Harte *et al.* 1999). Furthermore, NH₂-terminal glucitol-GIP displayed markedly enhanced insulinotropic potency in clonal pancreatic β -cells and improved *in vivo* biological activity in animals with type 2 diabetes (O'Harte *et al.* 1998b, 2000). Based on these findings, and preliminary observations that substitution of the penultimate Ala² in GIP results in analogues with reduced DPP IV hydrolysis rates, we now report studies investigating the plasma stability, insulin-releasing activity and antihyperglycaemic

properties of two novel NH₂-terminal Ala²-substituted analogues of GIP, containing Gly² or Ser².

Materials and Methods

Reagents

HPLC-grade acetonitrile was obtained from Rathburn (Walkersburn, UK). Sequencing-grade trifluoroacetic acid (TFA) was obtained from Aldrich (Poole, Dorset, UK). DPP IV, forskolin (FSK), isobutylmethylxanthine (IBMX), cAMP and ATP were all purchased from Sigma (Poole, Dorset, UK). Fmoc-protected amino acids and diprotin A (DPA) were from Calbiochem Novabiochem (Nottingham, UK). RPMI 1640 and DMEM tissue culture medium, fetal bovine serum, penicillin and streptomycin were all purchased from Gibco (Paisley, Strathclyde, UK). 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, Larnie, UK). All water used 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.

Synthesis of GIP, (Gly²)GIP and (Ser²)GIP

Peptide synthesis was carried out on an Applied Biosystems (Warrington, Cheshire, UK) automated peptide synthesiser (model 432A) using standard solid-phase Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) protocols (Fields & Noble 1990), starting from a pre-loaded 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. The synthetic peptides were purified by reversed-phase HPLC on a Waters Millennium (Milford, MA, USA) 2010 chromatography system (Software version 2.1.5).

Electrospray ionisation-mass spectrometry (ESI-MS)

Intact and degradation fragments of GIP, (Gly²)GIP and (Ser²)GIP were dissolved in water and eluted under isocratic conditions using an ion trap LCQ benchtop LC mass spectrometer (Finnigan MAT, Hemel Hempstead, UK). Mass spectra were collected using full ion scan mode over the mass-to-charge (*m/z*) range 150–2000. The molecular masses of each fragment were determined using prominent multiple-charged ions and the following equation applied: $M_r = iM_i - iM_h$, where M_r is molecular mass, M_i is *m/z* ratio, *i* is the number of charges and M_h is the mass of a proton.

Degradation of GIP, (Gly²)GIP and (Ser²)GIP by human plasma

HPLC-purified peptides were incubated *in vitro* at 37 °C in 50 mM triethanolamine-HCl, (pH 7.8, final peptide concentration 2 mM) with pooled human plasma (10 µl) for 0, 2, 4 and 8 h. An 8 h plasma incubation was also performed for native GIP in the presence of the DPP IV specific inhibitor DPA (5 mU). The enzymatic reactions were stopped by the addition of 10 µl 10% (v/v) TFA/water. The reaction products were then applied to a Vydac C-18 column (4.6 × 250 mm) and the major degradation fragment GIP(3–42) separated from intact GIP, (Gly²)GIP or (Ser²)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 from 28 to 42% over 30 min. The absorbance was monitored at 206 nm using a SpectraSystem UV 2000 detector (Thermoquest Limited, Manchester, UK) and peaks were collected manually prior to ESI-MS analysis.

Cells and cell culture

Chinese hamster lung (CHL) fibroblast cells stably transfected with the human GIP receptor (Gremlich *et al.* 1995) were cultured in DMEM tissue culture medium containing 10% (v/v) fetal bovine serum, 1% (v/v) antibiotics (100 U/ml penicillin, 0.1 mg/ml streptomycin). BRIN-BD11 cells were cultured using RPMI-1640 tissue culture medium containing 10% (v/v) fetal bovine serum, 1% (v/v) antibiotics (100 U/ml penicillin, 0.1 mg/ml streptomycin) and 11.1 mM glucose. The origin and insulin secretory characteristics of these cells have been described previously (McClenaghan *et al.* 1996). The cells were maintained in sterile tissue culture flasks (Corning Glass Works, Sunderland, UK) at 37 °C in an atmosphere of 5% CO₂ and 95% air using an LEEC incubator (Laboratory Technical Engineering, Nottingham, UK).

Effects of GIP, Gly²(GIP) and Ser²(GIP) on cAMP production

GIP receptor-transfected CHL cells were seeded into 12-well plates (Nunc, Roskilde, Denmark) at a density of 1.0×10^5 cells/well. The cells were then allowed to grow for 48 h before being loaded with tritiated adenine (2 µCi; TRK311; Amersham Pharmacia Biotech UK Ltd, Little Chalfont, Bucks, UK) and incubated at 37 °C for 6 h in 1 ml DMEM, supplemented with 0.5% (w/v) fetal bovine serum. The cells were then washed twice with HBS buffer (130 mM NaCl, 20 mM Hepes, 0.9 mM NaH₂PO₄, 0.8 mM MgSO₄, 5.4 mM KCl, 1.8 mM CaCl₂, 25 mM glucose, 25 µM phenol red, pH 7.4). The cells were then exposed to varying concentrations (10^{-12} – 10^{-6} M) of

GIP, (Gly²)GIP, (Ser²)GIP or FSK (10 µM) in HBS buffer, in the presence of 1 mM IBMX, for 15 min at 37 °C. The medium was subsequently removed and the cells lysed with 1 ml 5% TCA containing 0.1 mM unlabelled cAMP and 0.1 mM unlabelled ATP. The intracellular tritiated cAMP was then separated on Dowex and alumina exchange resins as previously described (Widmann *et al.* 1993).

In vitro insulin secretion

BRIN-BD11 cells were seeded into 24-well plates (Nunc) at a density of 1.0×10^5 cells per well, and allowed to attach overnight at 37 °C. Acute tests for insulin release were preceded by 40 min pre-incubation at 37 °C in 1.0 ml Krebs–Ringer bicarbonate buffer (115 mM NaCl, 4.7 mM KCl, 1.28 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 10 mM NaHCO₃, 0.5% (w/v) BSA, pH 7.4) supplemented with 1.1 mM glucose. Test incubations were performed in the presence of 5.6 mM glucose with a range of concentrations (10^{-13} – 10^{-8} M) of GIP, (Gly²)GIP and (Ser²)GIP. After 20 min incubation, the buffer was removed from each well and aliquots (200 µl) were used for measurement of insulin by RIA (Flatt & Bailey 1981).

Effects of GIP, (Gly²)GIP and (Ser²)GIP in ob/ob mice

Evaluation of effects of GIP, (Gly²)GIP and (Ser²)GIP on plasma glucose and insulin concentrations were examined using 14- to 18-week-old obese diabetic *ob/ob* mice. The genetic background and characteristics of the colony used have been outlined elsewhere (Bailey & Flatt 1982). The animals were housed individually in an air-conditioned room at 22 ± 2 °C with a 12 h light:12 h darkness cycle. Drinking water and a standard rodent maintenance diet (Trouw Nutrition Ltd, Cheshire, UK) were freely available. Food was withdrawn for an 18 h period prior to i.p. injection of saline (0.9% (w/v) NaCl) as control, glucose alone (18 mmol/kg body weight) or in combination with GIP, (Gly²)GIP or (Ser²)GIP (each at 25 nmol/kg). 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 vein 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. Plasma was aliquoted and stored at –20 °C for subsequent glucose and insulin determinations. All animal studies were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986.

Analyses

Plasma glucose was assayed by an automated glucose oxidase procedure using a Beckman Glucose Analyser II

Table 1 Summary of the NH₂-terminal sequences and molecular masses of GIP and related analogues

Peptide	NH ₂ -terminal sequence	ESI-MS multiple-charged species		Molecular mass (Da)	
		(M + 3H) ³⁺	(M + 4H) ⁴⁺	Measured	Theoretical
GIP	Tyr-Ala-Glu-Gly...	1661·6	1246·8	4982·5	4980·5
(Gly ²)GIP	Tyr-Gly-Glu-Gly...	1656·8	1243·1	4967·9	4966·5
(Ser ²)GIP	Tyr-Ser-Glu-Gly...	1667·2	1250·6	4998·6	4996·5

(Beckman, Galway, Ireland) (Stevens 1971). Plasma insulin was determined by dextran-charcoal RIA as described previously (Flatt & Bailey 1981). Incremental areas under plasma glucose and insulin curves (AUCs) were calculated using a computer-generated program (CAREA) employing the trapezoidal rule (Burington 1973) with baseline subtraction. Results are expressed as means ± S.E.M. and data compared using Student's *t*-test. Where appropriate, data were compared using repeated measures ANOVA or one-way ANOVA, followed by the Student–Newman–Keuls post-hoc test. Groups of data were considered to be significantly different if *P* < 0·05.

Results

Structural characterisation of GIP, (Gly²)GIP and (Ser²)GIP by ESI-MS

Following solid-phase synthesis and HPLC purification of GIP, (Gly²)GIP and (Ser²)GIP, the monoisotopic molecular mass of each peptide was determined using ESI-MS (Table 1). The average molecular masses of GIP, (Gly²)GIP and (Ser²)GIP were 4982·5, 4967·9 and 4998·6 Da respectively. The differences between the observed average molecular masses of (Gly²)GIP (− 14·6 Da) and (Ser²)GIP (+ 16·1 Da) relative to the native peptide indicated that the penultimate Ala² in GIP had been substituted correctly with a Gly (~ 14 Da lighter) or a Ser (~ 16 Da heavier) residue respectively.

Degradation of GIP, (Gly²)GIP and (Ser²)GIP by human plasma

Incubating each peptide with human plasma followed by analysis of reaction products by HPLC was used to evaluate time-dependent degradation by DPP IV. Data were calculated as a percentage of intact peptide remaining relative to the major degradation fragment GIP(3–42) for each incubation time point. As shown in Fig. 1, degradation of native GIP was evident after just 2 h (78 ± 4% intact peptide), with only 39 ± 1% remaining intact at 8 h. (Ser²)GIP displayed a reduced hydrolysis rate with 95 ± 6 and 74 ± 6% remaining intact after 2 and 8 h respectively. In contrast, (Gly²)GIP remained completely intact up to

and including 8 h. From the degradation profiles, the half-life (*t*_{1/2}) of each peptide for the given experimental conditions was 6·2, >8 h and >8 h for GIP, (Gly²)GIP and (Ser²)GIP respectively. Addition of DPA (a specific DPP IV inhibitor) to the 8 h incubation with native GIP completely inhibited peptide degradation (data not shown), corroborating previous studies using human serum (O'Harte *et al.* 1999).

In vitro biological activity

A concentration-dependent (10^{−12}–10^{−6} M) increase in intracellular cAMP production was observed with GIP using GIP receptor-transfected CHL cells (Fig. 2). The half-maximal stimulatory concentration (EC₅₀ value) was 18·2 nM. Similarly, both (Gly²)GIP and (Ser²)GIP concentration-dependently stimulated cAMP production,

Figure 1 Degradation profiles of native GIP (Δ), (Gly²)GIP (□) and (Ser²)GIP (●) after incubation with human plasma. The resistance to degradation of each peptide was measured after incubation in the presence of human plasma for 0, 2, 4 and 8 h. The reaction products were subsequently analysed by HPLC and degradation expressed as a percentage of intact peptide remaining relative to the major degradation fragment GIP(3–42). Data represent means ± S.E.M. of three separate experiments performed in duplicate.

Figure 2 Intracellular cAMP production in stably transfected GIP-receptor CHL cells exposed for 15 min to various concentrations of GIP (Δ), (Gly²)GIP ($\square$) and (Ser²)GIP ($\bullet$). Each experiment was performed in triplicate and the data expressed (means $\pm$ S.E.M.) as a percentage of the FSK (10 μ M) response. * $P < 0.05$ compared with native GIP at the same concentration.

with EC₅₀ values of approximately 15.0 and 14.9 nM respectively (Fig. 2). The lower EC₅₀ values for both analogues suggest an increased cAMP-stimulating potency compared with native GIP. The observed maximal cAMP responses of (Gly²)GIP and (Ser²)GIP compared with GIP (100%) were 89.1 ± 3.0 and $113 \pm 4.5\%$ ($P < 0.05$) respectively.

In keeping with its role as a potent insulintropic hormone, GIP (10^{-11} – 10^{-8} M) stimulated insulin secretion (1.2- to 1.8-fold; $P < 0.01$) compared with control incubations (5.6 mM glucose alone) (Fig. 3). GIP and (Gly²)GIP were equipotent at stimulating insulin secretion from 10^{-11} – 10^{-9} M compared with control. However, at the highest concentration tested (10^{-8} M), (Gly²)GIP exhibited significantly greater (1.2-fold; $P < 0.01$) insulin-releasing activity compared with the native peptide (Fig. 3). GIP and (Ser²)GIP were equally potent over the concentration range 10^{-11} – 10^{-9} M, with (Ser²)GIP displaying a significantly enhanced insulintropic activity (1.4-fold; $P < 0.001$) compared with native GIP at 10^{-8} M (Fig. 3). The maximal insulin secretion induced by (Gly²)GIP and (Ser²)GIP compared with GIP (100%) was 117 ± 6.4 and $140 \pm 4.7\%$ respectively. (Ser²)GIP was significantly more potent (1.2-fold; $P < 0.01$) than

(Gly²)GIP. On the basis of combined cAMP and secretory data, it would appear that the rank order of potency is (Ser²)GIP > (Gly²)GIP > GIP.

In vivo biological activity in ob/ob mice

The glucose-lowering and insulin-releasing activities of (Gly²)GIP and (Ser²)GIP compared with the native peptide were evaluated in ob/ob mice. Administration of saline alone had no effect on plasma glucose concentrations (Fig. 4A). Injection of glucose (18 mmol/kg), resulted in a highly significant rise ($P < 0.001$) in plasma glucose concentration by 15 min, with the effect declining over the next 45 min (Fig. 4A). The peak glucose response to GIP was reduced at 15 min compared with glucose alone, but failed to reach significance in terms of overall glucose excursion as identified by the AUC (Fig. 4B). Both (Gly²)GIP and (Ser²)GIP reduced the glycaemic excursion after 15 min compared with the native peptide but failed to reach significance (Fig. 4A). At 60 min post-injection, both (Gly²)GIP and (Ser²)GIP reduced the glycaemic excursion (1.2-fold; $P < 0.01$) compared with GIP (Fig. 4A), indicating a longer-term beneficial glucose-lowering effect. In terms of overall glycaemic excursion, (Gly²)GIP and (Ser²)GIP displayed an increased glucose-lowering ability ($P < 0.01$) compared with control (Fig. 4B), with (Ser²)GIP decreasing the glycaemic response (1.3-fold; $P < 0.05$) compared with the native peptide (Fig. 4B).

The corresponding plasma insulin responses are illustrated in Fig. 5. Saline alone had no effect on plasma insulin concentrations over 60 min (Fig. 5A). When glucose was injected alone, a peak in plasma insulin was observed after 15 min, with concentrations returning to near basal values after 60 min (Fig. 5A). The insulin response to native GIP in the presence of glucose was significantly greater ($P < 0.05$) compared with glucose alone (Fig. 5B). The insulintropic effects of (Gly²)GIP and (Ser²)GIP were significantly greater ($P < 0.05$ to $P < 0.001$) than glucose alone at 15, 30 and 60 min (Fig. 5A). The AUC values demonstrated an improvement in insulin-releasing ability compared with glucose alone ($P < 0.001$), with (Gly²)GIP demonstrating a significantly improved insulintropic activity (1.5-fold; $P < 0.01$) compared with GIP (Fig. 5B).

Discussion

In blood, both GIP and GLP-1 are rapidly degraded by DPP IV, resulting in short biological half-lives *in vivo* (Kieffer *et al.* 1995) and greatly diminished therapeutic potential. Several studies have reported stable DPP IV-resistant analogues of GLP-1 with improved biological activity *in vivo* (Deacon *et al.* 1998, Burcelin *et al.* 1999). However, such structural modifications to the native

Figure 3 Insulinotropic effects of GIP and related analogues during an acute 20 min incubation period in the clonal pancreatic β -cell line, BRIN-BD11. Values represent the means $\pm$ S.E.M. ($n=8$). * $P<0.05$, ** $P<0.01$, *** $P<0.001$ compared with control (5.6 mM glucose alone). $\Delta P<0.05$, $\Delta\Delta P<0.01$, $\Delta\Delta\Delta P<0.001$ compared with native GIP at the same concentration.

GLP-1 molecule appear to impair receptor binding–activation characteristics and insulinotropic activity, thereby compromising the benefits of protection from intrinsic DPP IV degradation. Recent studies in our laboratory have shown that an NH₂-terminally glycosylated analogue of GIP (Tyr¹-glucitol GIP) exhibits profound resistance to proteolytic DPP IV degradation, and displays enhanced cellular insulin-releasing and antihyperglycaemic activity in a commonly employed animal model of obesity–hyperinsulinaemic type 2 diabetes (O’Harte *et al.* 1998b, 2000).

In the present study, two Ala²-substituted analogues of GIP, namely (Gly²)GIP and (Ser²)GIP, were synthesised and tested for DPP IV resistance and biological activity. Substituting the native Ala² with a Gly or a Ser residue resulted in more stable analogues with significantly prolonged plasma half-lives, which is in agreement with other studies, including observations on the sister incretin GLP-1 (Deacon *et al.* 1998, Burcelin *et al.* 1999, Kühn-Wache *et al.* 2000). The basis for this increased DPP IV stability resides in the strict substrate-binding and –cleavage sites of the enzyme. Substituting the alkyl side-chain methyl-group (CH₃–) of the native Ala² with a hydrogen group (H–), as is the case for (Gly²)GIP, makes this analogue less susceptible to degradation by DPP IV. Similarly, substitution of the side-chain CH₃– group with a hydroxy (OH–) group, as in (Ser²)GIP, renders this analogue more hydrophilic and a poor substrate for DPP IV (Ritzel *et al.* 1998). The resulting plasma stability, therefore, prevents removal of the NH₂-terminal dipeptide, preventing the formation of the GIP(3–42) antagonist (Gault *et al.* 2002).

Acute incubations with the native GIP hormone showed a concentration-dependent increase in cAMP production from GIP receptor-transfected CHL cells (EC₅₀ 18.2 nM), which is in agreement with previous results using this cell line (Gremlich *et al.* 1995). Both (Gly²)GIP and (Ser²)GIP exhibited concentration-dependent increases in cAMP stimulation, but with slightly lower EC₅₀ values than native GIP (15.0 and 14.9 nM respectively), indicating increased potency at the post-receptor level. Consistent with previous studies (O’Harte *et al.* 1998b), native GIP concentration-dependently stimulated glucose-induced insulin secretion at the physiological glucose concentration (5.6 mM) from BRIN-BD11 cells. (Gly²)GIP exhibited a moderately increased insulin response compared with the native peptide, while (Ser²)GIP produced a significantly greater insulin-releasing effect. The reason why (Ser²)GIP should elicit such a marked insulin response is unclear, especially when weighed against its cAMP-stimulating ability, but one explanation might be that its additional hydrophilicity may be linked to activation of phospholipase A₂ or other signal transduction pathways (Lu *et al.* 1993, Ehses *et al.* 2001). Unlike their DPP IV-resistant GLP-1 counterparts (Deacon *et al.* 1998, Ritzel *et al.* 1998, Burcelin *et al.* 1999), both GIP analogues displayed increased biological activity *in vitro*. Since no significant degradation of GIP occurred during these incubations, these data suggest that the combination of increased DPP IV resistance and improved biological potency at the cellular level should provide analogues with greatly enhanced activity *in vivo*.

To determine the insulin-releasing antidiabetic and antihyperglycaemic potential of the GIP analogues *in vivo*

Figure 4 Glucose-lowering effects of GIP and related analogues in 18 h-fasted *ob/ob* mice. (A) Plasma glucose concentrations were measured prior to and after i.p. administration of saline (0.9% (w/v) NaCl) as control (□), glucose alone (18 mmol/kg body weight) (Δ), or in combination with either native GIP (▲), (Gly²)GIP (○) or (Ser²)GIP (■) (25 nmol/kg body weight). The time of injection is indicated by the arrow (0 min). (B) Plasma glucose AUC values for 0–60 min post-injection. Data represent the means ± S.E.M. for eight mice. **P*<0.05, ***P*<0.01, ****P*<0.001 compared with glucose alone. ^Δ*P*<0.05, ^{ΔΔ}*P*<0.01, ^{ΔΔΔ}*P*<0.001 compared with native GIP.

we used *ob/ob* mice, as an extensively studied model of spontaneous obesity and diabetes (Bray & York 1979). Characteristically these mice exhibit hyperphagia, marked obesity, moderate hyperglycaemia and severe hyperinsulinaemia (Bailey & Flatt 1982). As reported previously (O'Harte *et al.* 2000), GIP reduced the glycaemic excursion induced by glucose in *ob/ob* mice, without evoking a particularly prominent insulinotropic response. This may be explained by defects in glucose sensitivity and β -cell secretory function in this mutant (Flatt & Bailey 1981). Both analogues of GIP further reduced the glycaemic

Figure 5 Insulin-releasing effects of GIP and related analogues in 18 h-fasted *ob/ob* mice. (A) Plasma insulin concentrations were measured prior to and after i.p. administration of saline (0.9% (w/v) NaCl) as control (□), glucose alone (18 mmol/kg body weight) (Δ), or in combination with either native GIP (▲), (Gly²)GIP (○) or (Ser²)GIP (■) (25 nmol/kg body weight). The time of injection is indicated by the arrow (0 min). (B) Plasma insulin AUC values for 0–60 min post-injection. Data represent the means ± S.E.M. for eight mice. **P*<0.05, ***P*<0.01, ****P*<0.001 compared with glucose alone. ^Δ*P*<0.05, ^{ΔΔ}*P*<0.01, ^{ΔΔΔ}*P*<0.001 compared with native GIP.

excursion at 60 min post-injection compared with the native peptide. (Ser²)GIP appeared to be the best analogue at reducing the glycaemic excursion as estimated by the overall plasma glucose response (AUC). This enhanced glucose-lowering effect was associated with moderately improved insulin-releasing activity. In contrast, (Gly²)GIP demonstrated a significantly improved insulin-releasing activity compared with the native peptide. These observations may also indicate that extrapancreatic actions (Elahi *et al.* 1986, O'Harte *et al.* 1998a) may be particularly important in mediating effects of (Ser²)GIP. Overall, these

in vivo results confirm the predictions that these DPP IV-resistant Ala²-substituted analogues of GIP exhibit increased biological potency and antihyperglycaemic activity in type 2 diabetes with β -cell dysfunction. The magnitude of improvement of glycaemic excursion is notable, given the severe insulin resistance of the *ob/ob* syndrome (Bailey & Flatt 1982). Furthermore, although other animal models and certain populations of type 2 diabetic patients may show decreased responsiveness to GIP (Nauck *et al.* 1993, Elahi *et al.* 1994), it is apparent that NH₂-terminally modified analogues of GIP can overcome a large part of any possible defect.

In conclusion, this study has revealed that the Ala²-substituted analogues of GIP, (Gly²)GIP and (Ser²)GIP, exhibit resistance to DPP IV and enhanced ability to elevate cAMP and stimulate insulin secretion *in vitro*. Any decrease in the degradation of the peptide will also limit the production of GIP(3–42), thereby relieving possible antagonism of the biological effects of GIP at its receptor (Gault *et al.* 2002). The observed net effect of these actions is improved *in vivo* biological activity evidenced by antihyperglycaemic activity and insulinotropic properties in *ob/ob* mice. The efficacy of these analogues appears to be similar to that of NH₂-terminally modified Tyr¹-glucitol-GIP, supporting the idea that DPP IV-resistant GIP analogues may prove to be useful agents in the treatment of diabetes and alleviation of its complications (O'Harte *et al.* 1999, 2000, Hinke *et al.* 2002).

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