

# Antidiabetic potential of two novel fatty acid derivatised, N-terminally modified analogues of glucose-dependent insulinotropic polypeptide (GIP): *N*-AcGIP(LysPAL<sup>16</sup>) and *N*-AcGIP(LysPAL<sup>37</sup>)

Nigel Irwin<sup>1,\*</sup>, Victor A. Gault<sup>1</sup>, Brian D. Green<sup>1</sup>, Brett Greer<sup>2</sup>, Patrick Harriott<sup>3</sup>, Clifford J. Bailey<sup>4</sup>, Peter R. Flatt<sup>1</sup> and Finbarr P.M. O'Harte<sup>1</sup>

<sup>1</sup>School of Biomedical Sciences, University of Ulster, Coleraine, BT52 1SA, N. Ireland, UK

<sup>2</sup>School of Biology and Biochemistry, Queen's University of Belfast, Medical Biology Centre, Belfast, BT9 7BL, N. Ireland, UK

<sup>3</sup>Centre for Synthesis and Chemical Biology, Department of Pharmaceutical and Medicinal Chemistry, Royal College of Surgeons in Ireland, St. Stephen's Green, Dublin 2, Ireland

<sup>4</sup>School of Life and Health Sciences, Aston University, Aston Triangle, Birmingham, B4 7ET, UK

\*Corresponding author  
e-mail: n.irwin@ulster.ac.uk

## Abstract

Fatty acid derivatisation was used to develop two novel, long-acting, N-terminally modified, glucose-dependent insulinotropic polypeptide (GIP) analogues, *N*-AcGIP(LysPAL<sup>16</sup>) and *N*-AcGIP(LysPAL<sup>37</sup>). In contrast to GIP, which was rapidly degraded by *in vitro* incubation with dipeptidylpeptidase IV (DPP IV) (52% intact after 2 h), the analogues remained fully intact for up to 24 h. Both fatty acid-derivatised analogues stimulated cAMP production in GIP receptor Chinese hamster lung (CHL) fibroblasts (EC<sub>50</sub> 12.1–13.0 nM) and significantly improved *in vitro* insulin secretion from BRIN-BD11 cells (1.1- to 2.4-fold;  $p < 0.05$  to  $p < 0.001$ ) compared to control (5.6 mM glucose). Administration of *N*-AcGIP(LysPAL<sup>16</sup>) and *N*-AcGIP(LysPAL<sup>37</sup>) together with glucose in obese diabetic (*ob/ob*) mice significantly reduced the glycaemic excursion (1.4- and 1.5-fold, respectively;  $p < 0.05$  to  $p < 0.01$ ) and improved the insulinotropic response (1.5- and 2.3-fold, respectively;  $p < 0.01$  to  $p < 0.001$ ) compared to native peptide. Dose-response studies with *N*-AcGIP(LysPAL<sup>37</sup>) revealed that even the lowest concentration (6.25 nmol/kg) induced a highly significant decrease (1.4-fold;  $p < 0.001$ ) in the overall glycaemic excursion, coupled with a significant increase (2.0-fold;  $p < 0.01$ ) in circulating insulin. Furthermore, basal glucose values remained significantly reduced ( $p < 0.05$ ) and insulin values increased 24 h following a single injection of *N*-AcGIP(LysPAL<sup>37</sup>). The glucose-lowering action of the fatty acid-derivatised peptide was greater than that of *N*-AcGIP. These data demonstrate that novel fatty acid-derivatised analogues of N-terminally modified AcGIP

function as long-acting GIP-receptor agonists, with significant antidiabetic potential.

**Keywords:** dipeptidylpeptidase IV (DPP IV); fatty acid derivatisation; GIP analogues; glucose-dependent insulinotropic polypeptide (GIP); insulin secretion; obese diabetic *ob/ob* mice.

## Introduction

Glucose-dependent insulinotropic polypeptide (GIP) is a 42-amino acid peptide secreted from enteroendocrine K-cells following nutrient ingestion (Brown, 1994). Although initially characterised for its ability to inhibit gastric acid secretion, GIP is now widely acknowledged as an incretin hormone promoting glucose-dependent insulin release from pancreatic  $\beta$ -cells (Creutzfeldt, 2001). In addition, GIP stimulates proinsulin gene transcription and translation (Wang et al., 1996) and acts synergistically with glucose as a  $\beta$ -cell growth factor (Trumper et al., 2001; Pospisilik et al., 2003) and anti-apoptotic factor (Ehnes et al., 2001; Trumper et al., 2002). However, the potent antidiabetic effects of GIP cannot only be attributed to effects at the pancreatic  $\beta$ -cell, as antihyperglycaemic effects of the hormone have been observed at several extrapancreatic sites (Morgan, 1996; Yip and Wolfe, 2000; Gault et al., 2003a). Therefore, due to this broad range of biological activities there has been renewed interest in GIP as a novel therapeutic candidate for the treatment of type 2 diabetes (Holst, 2002; Meier et al., 2002; Gault et al., 2003a,b).

Despite its potent antihyperglycaemic properties, the ubiquitous enzyme dipeptidylpeptidase IV (DPP IV; EC 3.4.15.5) rapidly hydrolyses the native hormone to the truncated metabolite GIP(3–42) (Kieffer et al., 1995). Recent studies in *ob/ob* mice demonstrate that GIP(3–42) acts as a GIP receptor antagonist (Gault et al., 2002a). In addition to enzyme degradation, recent studies in patients with chronic renal insufficiency underline the importance of the kidneys in the final elimination of GIP from the circulation (Meier et al., 2004a). As a result, native GIP has a very short half-life and pharmacokinetic profile, which significantly limits its therapeutic potential.

One approach to counter both renal clearance and enzyme degradation could be the utilisation of fatty acid derivatisation together with N-terminal modification. Fatty acid derivatisation has previously been shown to prolong the half-life of insulin (Kurtzhals et al., 1995) and the sister incretin glucagon-like peptide-1 (GLP-1) (Knudsen et al., 2000; Kim et al., 2003; Green et al., 2004). In addition, several N-terminally modified GIP analogues have

**Table 1** Structural characterisation of GIP and GIP analogues by MALDI-ToF MS.

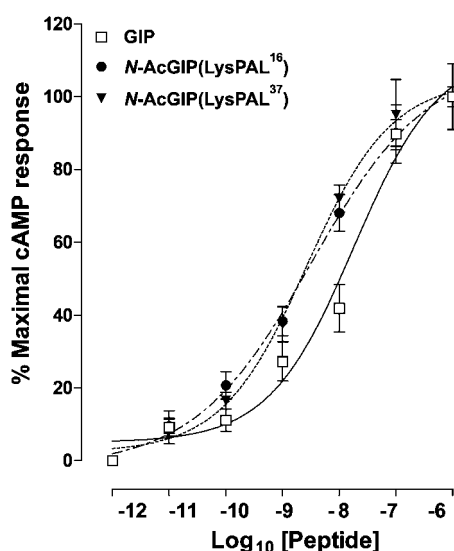
Peptide	$M_r$		
	Experimental	Theoretical	Difference
GIP	4983.7	4982.4	1.3
<i>N</i> -AcGIP(LysPAL <sup>16</sup> )	5268.9	5266.1	2.8
<i>N</i> -AcGIP(LysPAL <sup>37</sup> )	5267.7	5266.1	1.6

Peptide samples were mixed with matrix ( $\alpha$ -cyano-4-hydroxycinnamic acid) and the  $m/z$  ratio versus relative peak intensity was recorded using a Voyager-DE BioSpectrometry Workstation.

**Table 2** Percentage intact peptide remaining after incubation with DPP IV.

Peptide	Intact peptide remaining (%)			
	0 h	2 h	8 h	24 h
Native GIP	100	52±3	0	0
<i>N</i> -AcGIP(LysPAL <sup>16</sup> )	100	100	100	100
<i>N</i> -AcGIP(LysPAL <sup>37</sup> )	100	100	100	100

Values represent the percentage intact peptide remaining relative to the major degradation product GIP(3–42) following incubation with DPP IV as determined from HPLC peak area data. The reactions were performed in triplicate and the mean values ±SEM were calculated.

**Figure 1** Intracellular cAMP production.

Intracellular cyclic AMP production by GIP and fatty acid-derivatised GIP analogues, as determined by column chromatography, in CHL cells stably expressing the human GIP receptor. Each experiment was performed in triplicate ( $n=3$ ) and the results are expressed (mean±SEM) as a percentage of the maximum GIP response.

been developed with enhanced DPP IV stability and biological activity (O'Harte et al., 1999, 2002; Gault et al., 2002b, 2003c; Hinke et al., 2002). Of these, *N*-AcGIP has emerged as the most effective DPP IV-resistant analogue, substantially augmenting the plasma insulin response and curtailing the glycaemic excursion following conjoint administration with glucose to *ob/ob* mice (O'Harte et al., 2002).

The current study was designed to evaluate the metabolic stability and biological activity of two novel, second-generation, fatty acid-derivatised, N-terminally

modified *N*-AcGIP analogues, *N*-AcGIP(LysPAL<sup>16</sup>) and *N*-AcGIP(LysPAL<sup>37</sup>). Both GIP analogues contain a C-16 palmitate group linked to the  $\epsilon$ -amino group of Lys at positions 16 or 37, in combination with an N-terminal (Tyr<sup>1</sup>) acetyl group (O'Harte et al., 2002). The relative stability to DPP IV degradation, insulin secretion and cyclic AMP properties of both analogues was examined. Furthermore, acute and dose-response studies in obese diabetic *ob/ob* mice were designed to further elucidate the antihyperglycaemic and insulinotropic properties of these two novel GIP analogues. The results indicate that novel fatty acid derivatised, N-terminally modified AcGIP analogues add value to the exploitation of GIP as a potential therapeutic agent for the treatment of type 2 diabetes.

## Results

### Structural characterisation by MALDI-ToF MS

Following synthesis and HPLC purification, the molecular masses were obtained for GIP, *N*-AcGIP(LysPAL<sup>16</sup>) and *N*-AcGIP(LysPAL<sup>37</sup>) using MALDI-ToF MS (Table 1). The mass/charge ( $m/z$ ) ratio for native GIP was calculated to be 4983.7 Da, corresponding very closely to the theoretical mass of 4982.4 Da. Similarly, the  $m/z$  ratios for *N*-AcGIP(LysPAL<sup>16</sup>) and *N*-AcGIP(LysPAL<sup>37</sup>) were 5268.9 and 5267.7 Da, respectively. These values correlate very closely to the theoretical mass (5266.1 Da), therefore confirming the correct structures for each of the synthetic peptides.

### Degradation by DPP IV

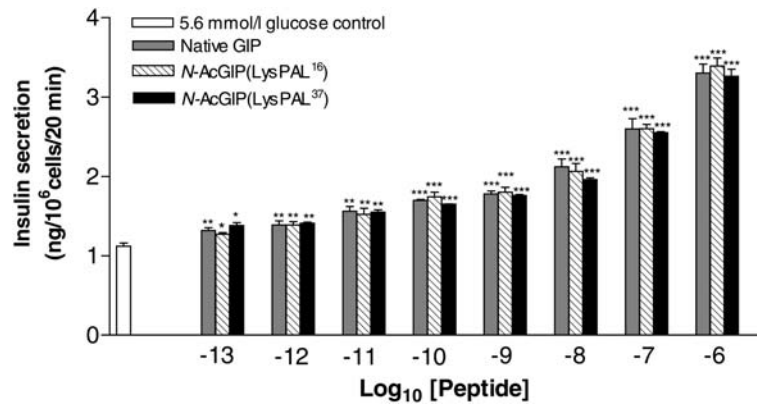
Table 2 illustrates the percentage intact peptide remaining after incubation with DPP IV. Degradation of native GIP was evident after just 2 h, with only 52±3% of the peptide remaining intact. After 8 h of incubation the native peptide was entirely degraded to GIP(3–42). In contrast, both *N*-AcGIP(LysPAL<sup>16</sup>) and *N*-AcGIP(LysPAL<sup>37</sup>) remained completely intact (no degradation fragment evident) even after 24-h incubation with DPP IV.

### Cyclic AMP production

A concentration-dependent ( $10^{-13}$ – $10^{-6}$  M) increase in cyclic AMP production was observed with native GIP ( $EC_{50}$  value 18.2 nM) using CHL cells transfected with the human GIP receptor (Figure 1). Likewise, both *N*-AcGIP(LysPAL<sup>16</sup>) and *N*-AcGIP(LysPAL<sup>37</sup>) followed a similar pattern of stimulation to that of native GIP, with calculated  $EC_{50}$  values of 12.1 and 13.0 nM, respectively. The lower  $EC_{50}$  values for both analogues suggest enhanced cAMP-stimulating potency.

### *In vitro* insulin-releasing activity

Consistent with its role as a potent insulinotropic hormone, native GIP concentration-dependently stimulated insulin secretion (1.1- to 2.3-fold;  $p<0.01$  to  $p<0.001$ ) compared to control (5.6 mM glucose alone; see Figure 2). Likewise, both *N*-AcGIP(LysPAL<sup>16</sup>) and *N*-AcGIP(LysPAL<sup>37</sup>) significantly stimulated glucose-



**Figure 2** Insulin-releasing activity of GIP and fatty acid-derivatised GIP analogues in the clonal pancreatic  $\beta$ -cell line BRIN-BD11. After pre-incubation (40 min), the effects of various concentrations of peptide were tested on insulin release during a 20-min incubation. Values are mean  $\pm$  SEM for eight separate observations. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  compared to control (5.6 mM glucose alone).

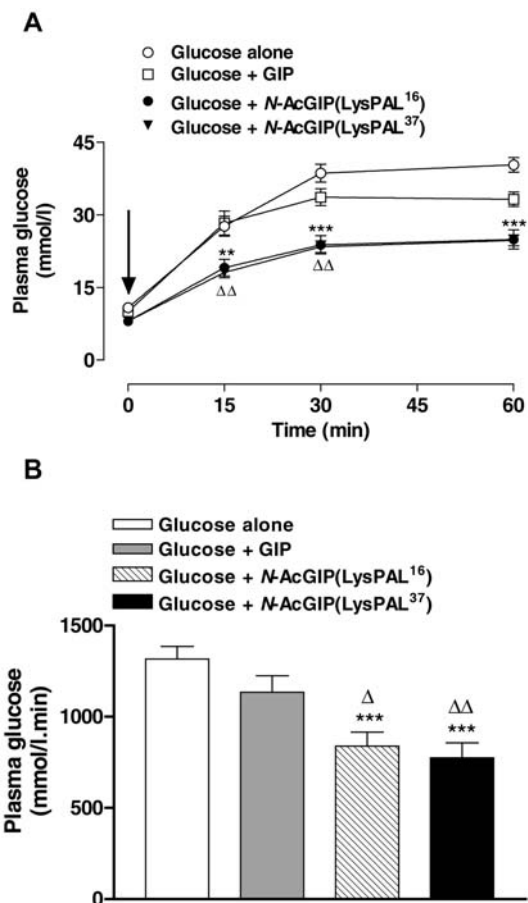
induced insulin secretion (1.1- to 2.4-fold and 1.1- to 2.3-fold, respectively;  $p < 0.05$  to  $p < 0.001$ ). On the basis of cyclic AMP and insulin secretory data, both GIP analogues appear to be at least equipotent to the native peptide.

#### Metabolic effects in *ob/ob* mice

Figure 3 illustrates the antihyperglycaemic activities of native GIP and GIP analogues in *ob/ob* mice. Basal blood glucose levels of the experimental groups were not significantly different at the start of the study ( $p > 0.05$ ). After injection of glucose alone, plasma glucose levels increased from basal  $10.0 \pm 1.1$  to  $27.7 \pm 2.1$  mM at 15 min and continued to rise during the remaining 45 min, peaking at  $40.3 \pm 1.5$  mM (Figure 3A). Native GIP reduced plasma glucose at each of the time points monitored; however, this failed to reach significance in terms of overall glucose excursion, as revealed by the area under the curve (AUC) values (Figure 3B). Administration of *N*-AcGIP(LysPAL<sup>16</sup>) and *N*-AcGIP(LysPAL<sup>37</sup>) produced a significant reduction in plasma glucose at each time point ( $p < 0.01$  to  $p < 0.001$ ; Figure 3A) and significantly lowered glucose AUC (1.6- and 1.7-fold, respectively;  $p < 0.001$  to  $p < 0.001$ ; Figure 3B) when compared to glucose alone. In addition, *N*-AcGIP(LysPAL<sup>16</sup>) and *N*-AcGIP(LysPAL<sup>37</sup>) decreased the overall glucose excursion (1.4- and 1.5-fold, respectively;  $p < 0.05$  to  $p < 0.001$ ) when compared to native GIP (Figure 3B).

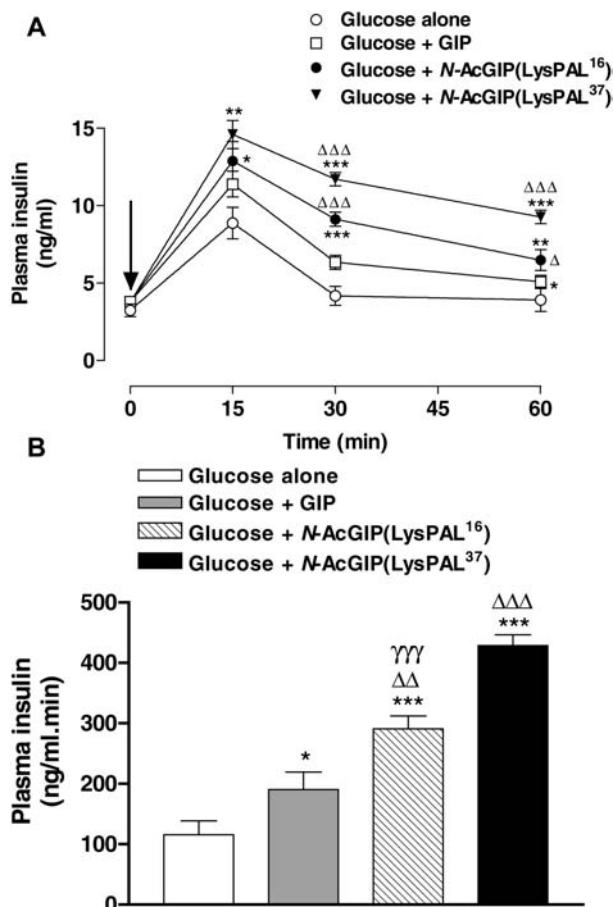
The corresponding plasma insulin responses are illustrated in Figure 4. After administration of glucose alone (control), the maximal rise in plasma insulin was observed at 15 min ( $8.8 \pm 1.0$  ng/ml), which then fell towards basal levels over the remaining 45 min (Figure 4A). Administration of native GIP significantly elevated the overall insulinotropic response (1.7-fold;  $p < 0.05$ ) compared with glucose alone (Figure 4B). When *N*-AcGIP(LysPAL<sup>16</sup>) or *N*-AcGIP(LysPAL<sup>37</sup>) was administered together with glucose, a maximum plasma insulin concentration was observed at 15 min ( $14.6 \pm 0.9$  and  $12.9 \pm 1.3$  ng/ml, respectively). Furthermore, protracted biological activity for both analogues was clearly evident from 30 to 60 min. Glucose-mediated plasma insulin concentrations were significantly higher compared to both control ( $p < 0.01$  to

$p < 0.001$ ) and GIP-treated animals ( $p < 0.05$  to  $p < 0.001$ ; Figure 4A). The corresponding AUC values for *N*-AcGIP(LysPAL<sup>16</sup>) and *N*-AcGIP(LysPAL<sup>37</sup>) revealed significant enhancements in overall glucose-mediated insulin



**Figure 3** Glucose-lowering effects of GIP and fatty acid-derivatised GIP analogues in 18-h-fasted *ob/ob* mice.

(A) Plasma glucose concentrations were measured prior to and after i.p. administration of glucose alone (18 mmol/kg bw) as a control, or in combination with GIP or GIP analogues (25 nmol/kg bw). (B) The incremental area under the glucose curve (AUC) between 0 and 60 min. Values represent mean  $\pm$  SEM for eight mice. \*\* $p < 0.01$  and \*\*\* $p < 0.001$  compared to glucose alone.  $\Delta p < 0.05$ ,  $\Delta\Delta p < 0.01$  compared to native GIP.



**Figure 4** Insulin-releasing activity of GIP and fatty acid-derivatised GIP analogues in 18-h-fasted *ob/ob* mice. (A) Plasma insulin concentrations were measured prior to and after i.p. administration of glucose alone (18 mmol/kg bw) as a control, or in combination with GIP or GIP analogues (25 nmol/kg bw). (B) The incremental area under the insulin curve (AUC) between 0 and 60 min. Values represent mean  $\pm$  SEM for eight mice. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  compared to glucose alone.  $\Delta p < 0.05$ ,  $\Delta\Delta p < 0.01$  and  $\Delta\Delta\Delta p < 0.001$  compared to native GIP.  $\gamma\gamma\gamma p < 0.001$  compared with *N-AcGIP(LysPAL<sup>37</sup>)*.

release compared to native GIP (1.5- and 2.3-fold, respectively;  $p < 0.01$  to  $p < 0.001$ ; Figure 4B). Furthermore, *N-AcGIP(LysPAL<sup>37</sup>)* was significantly more potent (1.5-fold;  $p < 0.001$ ) than *N-AcGIP(LysPAL<sup>16</sup>)* at stimulating insulin secretion (Figure 4B).

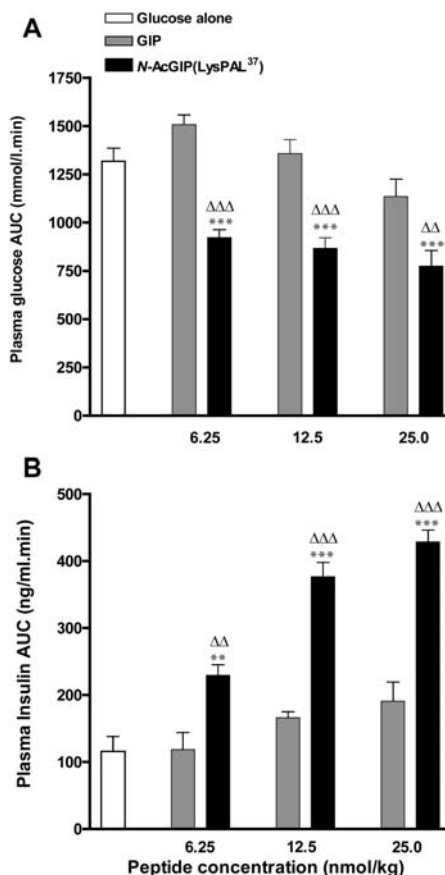
#### Dose-dependent metabolic effects in *ob/ob* mice

Figure 5 illustrates the dose-dependent antihyperglycaemic and insulinotropic effects of GIP and the more potent analogue *N-AcGIP(LysPAL<sup>37</sup>)* when administered with glucose to *ob/ob* mice. Data are presented as overall AUC responses for convenience. Expressed in this manner, native GIP did not significantly affect AUC glucose and insulin at any of the doses tested. *N-AcGIP(LysPAL<sup>37</sup>)* was substantially more potent than native GIP ( $p < 0.01$  to  $p < 0.001$ ) and exhibited prominent dose-dependent antihyperglycaemic and insulinotropic actions at all doses administered (Figure 5). Remarkably, even the lowest concentration of *N-AcGIP(LysPAL<sup>37</sup>)* tested (6.25 nmol/kg) had highly significant antihyperglycaemic

properties compared to glucose alone (1.4-fold;  $p < 0.001$ ) (Figure 5A). Consistent with this observation, 6.25 nmol/kg *N-AcGIP(LysPAL<sup>37</sup>)* elicited a prominent insulin response (2.0-fold;  $p < 0.01$ ) compared to glucose alone (Figure 5B).

#### Long-acting effects in *ob/ob* mice

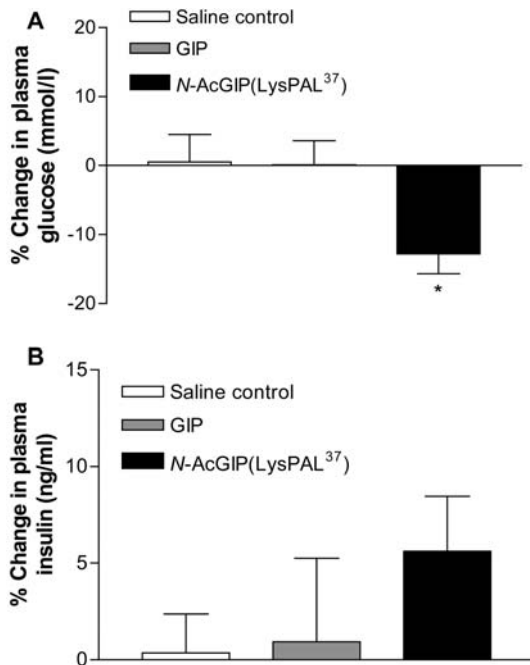
Plasma glucose and insulin concentrations of *ob/ob* mice 24 h after i.p. injection of native GIP were not significantly different to saline-treated controls (Figure 6). However, 24 h after administration of a single injection of 12.5 nmol/kg *N-AcGIP(LysPAL<sup>37</sup>)*, plasma glucose concentrations were significantly reduced by 13% ( $p < 0.05$ ) (Figure 6A). Plasma insulin concentrations were higher in animals treated with *N-AcGIP(LysPAL<sup>37</sup>)* compared to controls; however, these did not reach significance ( $p > 0.05$ ) (Figure 6B). As shown in Figure 7, the glucose-lowering action of *N-AcGIP(LysPAL<sup>37</sup>)* over 1–24 h was significantly greater ( $p < 0.05$ ) and more protracted than GIP(*LysPAL<sup>37</sup>*), which was, in turn, more active than *N-AcGIP* ( $p < 0.05$ ). Administration of native GIP did not affect plasma glucose levels at any time point.



**Figure 5** Dose-dependent effects of GIP and *N-AcGIP(LysPAL<sup>37</sup>)* in 18-h-fasted *ob/ob* mice. The incremental area under the curve (AUC) for glucose (A) and insulin (B) between 0 and 60 min after i.p. administration of glucose alone (18 mmol/kg bw) or in combination with GIP or *N-AcGIP(LysPAL<sup>37</sup>)* (each at 6.25, 12.5 and 25 nmol/kg bw). Values represent mean  $\pm$  SEM for eight mice. \* $p < 0.01$  and \*\*\* $p < 0.001$  compared to glucose alone.  $\Delta p < 0.01$  and  $\Delta\Delta p < 0.001$  compared to native GIP at the same dose.

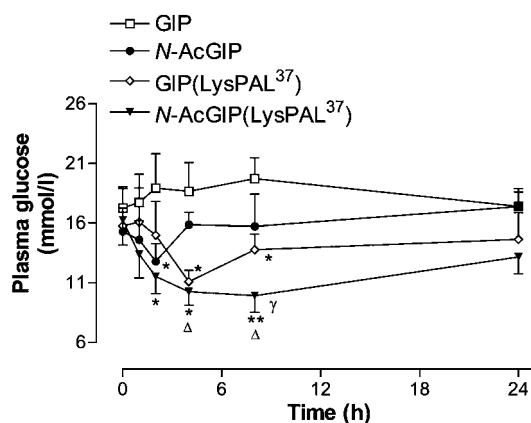
## Discussion

New strategies to counter the rapid degradation of circulating GIP and GLP-1 have generated much current interest in the exploitation of these incretin hormones for the treatment of type 2 diabetes (Holst, 2002; Meier et al., 2002; Gault et al., 2003a,b).



**Figure 6** Long-lasting effects of *N*-AcGIP(LysPAL<sup>37</sup>) in *ob/ob* mice.

Changes in plasma glucose (A) and insulin (B) concentrations in *ob/ob* mice 24 h after i.p. injection of saline vehicle as control, GIP or *N*-AcGIP(LysPAL<sup>37</sup>) (12.5 nmol/kg bw). Values are the mean  $\pm$  SEM for eight mice. \* $p < 0.05$  compared to saline-treated group.



**Figure 7** Comparison of glucose-lowering effects of *N*-AcGIP(LysPAL<sup>37</sup>), related analogues and native GIP.

Plasma glucose concentrations were measured prior to and after i.p. injection of fed *ob/ob* mice with 25 nmol/kg bw *N*-AcGIP(LysPAL<sup>16</sup>), *N*-AcGIP, GIP(LysPAL<sup>37</sup>) or native GIP. The arrow indicates the time of injection. Animals had free access to food throughout. Values represent the mean  $\pm$  SEM for six mice. \* $p < 0.05$ , \*\* $p < 0.05$  compared with native GIP;  $\Delta p < 0.05$  compared with *N*-AcGIP;  $\gamma p < 0.05$  compared with GIP(LysPAL<sup>37</sup>).

After secretion or external administration, GIP and GLP-1 are rapidly degraded by the enzyme dipeptidyl peptidase IV (DPP IV) (Kieffer et al., 1995) and cleared from the circulation by renal filtration (Meier et al., 2004a). To date, research has focused primarily on strategies preventing DPP IV degradation through the use of specific DPP IV inhibitors (Drucker, 2003) or the generation of DPP IV-resistant analogues of GIP and GLP-1 (Meier et al., 2002; Gault et al., 2003a). Although several positive effects on glycaemic control have been reported with DPP IV inhibitors (Ahren et al., 2002; Drucker, 2003), the long-term efficacy and possible side-effects of interference in DPP IV-mediated metabolism of numerous additional peptide substrates require elucidation (Gault et al., 2003a). An alternative approach has been the successful synthesis of specific GIP and GLP-1 analogues modified in the region around the enzyme cleavage site to impart DPP IV resistance (O'Harte et al., 1999; Meier et al., 2002).

Despite their many attributes, DPP IV-resistant analogues of GIP and GLP-1, like their native counterparts, are still subject to renal filtration. To circumvent this problem, fatty acid derivatisation has already been used successfully to improve the duration of action of GLP-1 (Knudsen et al., 2000; Kim et al., 2003; Green et al., 2004). The most promising analogue, NN2211 (Liraglutide), appears effective in improving blood glucose control in type 2 diabetic subjects, despite a tendency towards promotion of nausea, possibly due to inhibition of gastric emptying (Agersø et al., 2002).

The present study describes the results of introducing two specific modifications to the native GIP hormone, namely N-terminal acetylation and C-terminal fatty acid derivatisation. N-terminal acetylation was employed, as previously described (O'Harte et al., 2002), to significantly enhance stability to DPP IV. In contrast, conjugation of a C-16 palmitate residue at the  $\epsilon$ -amino group of Lys<sup>16</sup> or Lys<sup>37</sup> was introduced to extend the biological half-life through binding to circulating proteins (Kurtzhals et al., 1995). Unlike the native peptide, both GIP analogues appeared to be completely resistant to enzymatic breakdown by DPP IV, which corroborates previous observations with *N*-AcGIP (O'Harte et al., 2002). Furthermore, both analogues displayed similar or slightly better insulin-releasing and cyclic AMP production properties to native GIP and *N*-AcGIP when tested in *in vitro* cellular systems (O'Harte et al., 2002).

To assess the antihyperglycaemic and insulinotropic potential of the fatty acid-derivatised GIP analogues *in vivo*, we employed obese diabetic (*ob/ob*) mice. The *ob/ob* syndrome is an extensively studied model of spontaneous obesity and diabetes, exhibiting hyperphagia, marked obesity, moderate hyperglycaemia and severe hyperinsulinaemia (Bailey and Flatt, 1982). As described in previous studies (Gault et al., 2002b, 2003c), native GIP only modestly reduced the glycaemic excursion in *ob/ob* mice, reflecting the severe insulin resistance of this mutant animal model (Bailey and Flatt, 1982). In sharp contrast, both *N*-acetylated GIP analogues additionally substituted with a palmitate molecule at Lys<sup>16</sup> or Lys<sup>37</sup> [*N*-AcGIP(LysPAL<sup>16</sup>) and *N*-AcGIP(LysPAL<sup>37</sup>)] significantly

lowered plasma glucose levels compared to the native peptide. This was accompanied by significantly enhanced insulin-releasing activity, especially in the case of *N*-AcGIP(LysPAL<sup>37</sup>). The significantly protracted insulinotropic response to both fatty acid-derivatised GIP analogues at 60 min, despite substantially lower plasma glucose, is indicative of a much more extended plasma half-life. This is presumably due to extensive binding of both palmitate-derivatised GIP analogues to serum albumin, therefore significantly impairing their clearance via the kidneys (Meier et al., 2004a). However, further studies, including the establishment of sensitive and specific immunoassays for the novel GIP analogues, would be needed to confirm such actions.

*N*-AcGIP(LysPAL<sup>37</sup>) appeared to be the best fatty acid-derivatised analogue *in vivo*, displaying a more protracted, significantly enhanced insulin-releasing potency over *N*-AcGIP(LysPAL<sup>16</sup>). Reasons for the increased potency of *N*-AcGIP(LysPAL<sup>37</sup>) remain unclear, but possible explanations include differences in half-life or extrapancreatic actions such as glucagon secretion. A further possibility may be that a fatty acid chain linked to the Lys closer to the C-terminus of the peptide may have less of a detrimental effect on the bioactive region of the molecule known to be located within the N-terminus (Hinke et al., 2001; Gault et al., 2002d; Manhart et al., 2003). However, similarities between the *in vitro* biological activities of the two palmitate-derivatised analogues make this less likely.

Given that *N*-AcGIP(LysPAL<sup>37</sup>) was the more potent of the two analogues *in vivo*, it was further utilised in dose-response studies. Considering that native GIP itself has only very modest effects in *ob/ob* mice, as observed with continuous infusion but not bolus injection in type 2 diabetic subjects (Jones et al., 1987; Nauck et al., 1993; Meier et al., 2004b), it is remarkable that *N*-AcGIP(LysPAL<sup>37</sup>), even at the lowest dose of 6.25 nmol/kg, exhibited significant glucose-lowering and insulinotropic activity when administered with glucose. Considering that *N*-AcGIP(LysPAL<sup>37</sup>) is subject to albumin binding, the fact that it is still highly biologically active at lower concentrations indicates striking potency, even compared with similar fatty acid derivatives of N-terminal pyroglutamyl GIP (Irwin et al., 2004). Furthermore, the observation that a one-off injection of *N*-AcGIP(LysPAL<sup>37</sup>) decreased hyperglycaemia and sustained a 13% ( $p < 0.05$ ) decrease in plasma glucose at 24 h provides direct functional evidence for a prolonged duration of action on glucose-lowering *in vivo*. By this time, plasma insulin had subsided, suggesting possible involvement of actions of GIP other than on the  $\beta$ -cell in its glucose-lowering activity (Gault et al., 2003b). Direct comparison of glucose-lowering activity indicated that *N*-AcGIP(LysPAL<sup>37</sup>) was more potent than GIP(LysPAL<sup>37</sup>), which was, in turn, more active than *N*-AcGIP. This indicates a clear benefit of fatty acid derivatisation when the peptide was given as a single injection to *ob/ob* mice.

In conclusion, this study demonstrates that N-terminally acetylated GIP analogues carrying an additional palmitate group display resistance to DPP IV and an impressive profile of bioactivity, manifested by potent and long-acting glucose-lowering activity in *ob/ob* mice.

Unlike its sister incretin GLP-1, GIP lacks significant effects on gastric emptying in man (Meier et al., 2003). Accordingly, the ability of N-terminally modified fatty acid-derivatised 'super GIP' molecules to overcome severe insulin resistance and  $\beta$ -cell dysfunction (including poor response to the native hormone) indicates that they may offer notable potential as future therapeutic agents for the treatment of type 2 diabetes.

## Materials and methods

### Materials

High-performance liquid chromatography (HPLC) grade acetonitrile was obtained from Rathburn (Walkersburn, UK). Trifluoroacetic acid (TFA) and trichloroacetic acid (TCA) were obtained from Aldrich (Poole, UK). DPP IV, isobutylmethylxanthine (IBMX),  $\alpha$ -cyano-4-hydroxycinnamic acid, cyclic AMP and ATP were all purchased from Sigma (Poole, UK). Fmoc-protected amino acids were from Calbiochem Novabiochem (Nottingham, UK). RPMI-1640 and DMEM tissue culture medium, foetal bovine serum (FBS), penicillin and streptomycin were all purchased from Gibco (Paisley, UK). The chromatography columns used for cyclic AMP assay, Dowex AG50 WX and neutral alumina AG7, were obtained from Bio-Rad (Life Science Research, Alpha Analytical, Larne, 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, purification and characterisation of GIP and related analogues

Native GIP was sequentially synthesised using standard solid-phase Fmoc peptide chemistry (ABI 432A Peptide Synthesiser) as described previously (Gault et al., 2002b). *N*-AcGIP(LysPAL<sup>16</sup>), *N*-AcGIP(LysPAL<sup>37</sup>) and GIP(LysPAL<sup>37</sup>) were synthesised in the same way as native GIP, with the exception that the  $\epsilon$ -amino groups of Lys at positions 16 or 37 were conjugated with a C-16 palmitate fatty acid. In addition, an acetyl adduct was incorporated at the N-terminal Tyr<sup>1</sup> of the fatty acid-derivatised or native GIP molecules. The synthetic peptides were judged pure by reversed-phase HPLC on a Waters Millennium 2010 chromatography system (software version 2.1.5) and subsequently characterised using matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-ToF MS) as described previously (Gault et al., 2002c).

### DPP IV degradation studies

GIP and fatty acid-derivatised GIP analogues were incubated at 37°C with purified porcine dipeptidylpeptidase IV (5 mU in 50 mM triethanolamine-HCl, pH 7.8) for 0, 2, 4, 8 and 24 h (final peptide concentration 2 mM). The reactions were subsequently terminated by addition of 10% (v/v) TFA/water and the reaction products separated using HPLC. Reaction products were applied to a Vydac C-4 column (4.6 × 250 mm; The Separations Group, Hesperia, CA, USA) and the major degradation product GIP(3–42) 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, with the concentration of acetonitrile in the eluting solvent increased from 0% to 40% over 10 min, and then from 40% to 75% over 35 min. The absorbance was monitored at 206 nm using a SpectraSystem UV 2000 detector (Thermoquest Limited, Manchester, UK); the fractions corresponding to the peaks were col-

lected manually prior to MALDI-ToF MS analysis. HPLC peak-area data were used to calculate the percentage intact peptide remaining throughout the incubation.

### Cells and cell culture

Chinese hamster lung (CHL) fibroblasts stably transfected with the human GIP receptor (Gremlich et al., 1995) were cultured in DMEM tissue culture medium containing 10% (v/v) FBS and 1% (v/v) antibiotics (100 U/ml penicillin, 0.1 mg/ml streptomycin). Clonal pancreatic BRIN-BD11 cells (McClenaghan et al., 1996) were cultured using RPMI-1640 culture medium containing 10% (v/v) FBS, 1% (v/v) antibiotics (100 U/ml penicillin, 0.1 mg/ml streptomycin) and 11.1 mM glucose. Cells were maintained at 37°C in an atmosphere of 5% CO<sub>2</sub> and 95% air using an LEEC incubator (Laboratory Technical Engineering, Nottingham, UK).

### In vitro biological activity

Intracellular cyclic AMP production was measured using GIP receptor-transfected CHL fibroblasts (Gault et al., 2003c). In brief, CHL cells were seeded into 12-well plates (Nunc, Roskilde, Denmark) at a density of 10<sup>5</sup> cells/well and allowed to grow for 48 h before being loaded with tritiated adenine (2 µCi; TRK311; Amersham, Buckinghamshire, UK). The cells were then incubated at 37°C for 6 h in 1 ml of DMEM supplemented with 0.5% (w/v) BSA and subsequently washed twice with HBS buffer (pH 7.4). Cells were then exposed to GIP/GIP analogues (10<sup>-13</sup>–10<sup>-6</sup> M) 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 of 5% TCA containing 0.1 mM unlabelled cyclic AMP and 0.1 mM unlabelled ATP. The intracellular cyclic AMP was then separated on Dowex and alumina exchange resins as described previously (Gault et al., 2003c).

Insulin-release studies were carried out using clonal pancreatic BRIN-BD11 cells as described previously (Gault et al., 2003c). Briefly, BRIN-BD11 cells were seeded into 24-well plates at a density of 10<sup>5</sup> cells/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 of Krebs Ringer bicarbonate buffer supplemented with 1.1 mM glucose. Test incubations were performed in the presence of 5.6 mM glucose with a range of concentrations (10<sup>-13</sup>–10<sup>-6</sup> mol/l) of GIP and GIP analogues. After 20 min of incubation, the buffer was removed from each well and aliquots (200 µl) were used for the measurement of insulin.

### In vivo biological activity

Effects of GIP and GIP analogues on plasma glucose and insulin concentrations were examined in 12–15-week-old obese diabetic *ob/ob* mice. The genetic background and characteristics of the colony used have been outlined in detail elsewhere (Bailey and Flatt, 1982). Animals were housed 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, Cheshire, UK) were freely available. Metabolic and dose-response effects of GIP and *N*-AcGIP(LysPAL) analogues [at 6.25–25 nmol/kg body weight (bw)] following glucose administration (18 mmol/kg bw) were examined in 18-h-fasted mice. In a separate experiment, non-fasted *ob/ob* mice received a one-off injection of either saline vehicle (0.9% w/v NaCl), native GIP or *N*-AcGIP(LysPAL<sup>37</sup>) (12.5 nmol/kg bw). A similar approach, with multiple blood sampling for glucose, was used to compare bioactivity of *N*-AcGIP(LysPAL<sup>37</sup>), *N*-AcGIP, GIP(LysPAL<sup>37</sup>) and native GIP. Animals had free access to food throughout these tests. All test solutions were administered by i.p. injection in a final volume of 5 ml/kg bw. Blood was collected from the cut tip of the tail vein of conscious mice into chilled fluoride/heparin

glucose microcentrifuge tubes immediately prior to injection and at the times indicated in the figures. Plasma was separated using a Beckman microcentrifuge (Beckman Instruments, High Wycombe, UK) at 13 000 *g* for 30 s and stored at -20°C prior to glucose and insulin determinations. All animal experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986. No adverse effects were observed following administration of any of the peptides.

### Analyses

Plasma glucose was assayed by an automated glucose oxidase procedure (Stevens, 1971) using a Beckman Glucose Analyser II (Beckman, Galway, Ireland). Plasma insulin was determined by dextran-charcoal RIA as described previously (Flatt and Bailey, 1981). Incremental areas under plasma glucose and insulin curves were calculated employing the trapezoidal rule (Burlington, 1973) with baseline subtraction. Results are expressed as mean values ±SEM and data compared using the unpaired Student *t*-test. Where appropriate, data were compared using repeated-measures or one-way ANOVA, followed by the Student-Newman-Keuls *post hoc* test. Groups of data were considered to be significantly different for *p*<0.05.

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