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Evaluation of the antidiabetic activity of DPP IV resistant N-terminally modified versus mid-chain acylated analogues of glucose-dependent insulinotropic polypeptide

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Abbreviations:
GIP, glucose-dependent insulinotropic polypeptide
GLP-1, glucagon-like peptide-1
DPP IV, dipeptidylpeptidase IV
(*ob/ob*) mice, obese diabetic mice
HPLC, high performance liquid chromatography
MALDI-TOF, matrix-assisted laser desorption ionization-time of flight
AUC, area under curve
Lys, lysine
PAL, palmitate

ABSTRACT

Glucose dependent insulinotropic polypeptide (GIP) is a gastrointestinal hormone with therapeutic potential for type 2 diabetes due to its insulin-releasing and antihyperglycaemic actions. However, development of GIP-based therapies is limited by N-terminal degradation by DPP IV resulting in a very short circulating half-life. Numerous GIP analogues have now been generated exhibiting DPP IV resistance and extended bioactivity profiles. In this study, we report a direct comparison of the long-term antidiabetic actions of three such GIP molecules, *N*-AcGIP, GIP(Lys^{37}PAL) and *N*-AcGIP(Lys^{37}PAL) in obese diabetic (*ob/ob*) mice. An extended duration of action of each GIP analogue was demonstrated prior to examining the effects of once daily injections (25 nmol kg^{-1} body weight) over a 14-day period. Administration of either *N*-AcGIP, GIP(Lys^{37}PAL) or *N*-AcGIP(Lys^{37}PAL) significantly decreased non-fasting plasma glucose and improved glucose tolerance compared to saline treated controls. All three analogues significantly enhanced glucose and nutrient-induced insulin release, and improved insulin sensitivity. The metabolic and insulin secretory responses to native GIP were also enhanced in 14-day analogue treated mice, revealing no evidence of GIP-receptor desensitization. These effects were accompanied by significantly enhanced pancreatic insulin following *N*-AcGIP(Lys^{37}PAL) and increased islet number and islet size in all three groups. Body weight, food intake and circulating glucagon were unchanged. These data demonstrate the therapeutic potential of once daily injection of enzyme resistant GIP analogues and indicate that *N*-AcGIP is equally as effective as related palmitate derivatised analogues of GIP.

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1. Introduction

Recent research has elucidated that the two gut hormones, glucagon-like peptide-1 (GLP-1) and glucose dependent insulinotropic polypeptide (GIP), act as incretin hormones and account for the major part of meal-induced insulin release [1]. Studies with infusion of GIP and GLP-1 at postprandial levels under physiological conditions indicate that GIP is more important than GLP-1 [2]. In addition to enhancing insulin secretion, GIP has been shown to up-regulate proinsulin gene transcription and translation [3], increase pancreatic beta-cell growth and neogenesis [4], inhibit pancreatic beta-cell apoptosis [5], decrease hepatic insulin clearance and stimulate glucose uptake and metabolism [6,7]. Based on these glucose-lowering characteristics, GIP has received attention as a candidate for the treatment of type 2 diabetes [8].

Although the glucose-dependent insulinotropic action of GIP was discovered 30 years ago [9], antidiabetic therapeutic strategies based on GIP are not yet commercially available. The polypeptide structure of GIP necessitates parenteral administration, furthermore GIP is rapidly degraded by the ubiquitous enzyme dipeptidylpeptidase IV (DPP IV) yielding the major degradation fragment GIP(3–42), which lacks insulinotropic activity and may possibly act as a GIP receptor antagonist *in vivo* [10,11]. Therefore, the development of DPP IV resistant analogues of GIP would not only extend the half-life and increase biological potency of the peptide but also curtail production of GIP(3–42), thereby alleviating possible GIP receptor antagonism. However, it is important to note that the *in vivo* half-life of GIP not only depends on enzymatic degradation but also renal elimination [12]. One method to delay renal extraction of GIP is through covalent linkage of a free fatty acid chain, to promote plasma albumin binding [13].

Numerous N-terminally modified analogues of GIP have been developed, which exhibit profound resistance to DPP IV [14]. As a result of degradation resistance and enhanced *in vitro* activity, a number of these analogues displayed notable antidiabetic promise when administered acutely to obese diabetic (*ob/ob*) mice [14–17]. Modifications at Tyr^1 appeared to yield analogues with greater bioactivity than Ala^2 or Glu^3 modifications, with *N*-acetylated GIP (*N*-AcGIP) and *N*-pyroglutamyl GIP (*N*-pGluGIP) appearing most effective [18]. GIP analogues utilizing secondary modifications such as fatty acid derivatisation, which counters renal clearance by promoting

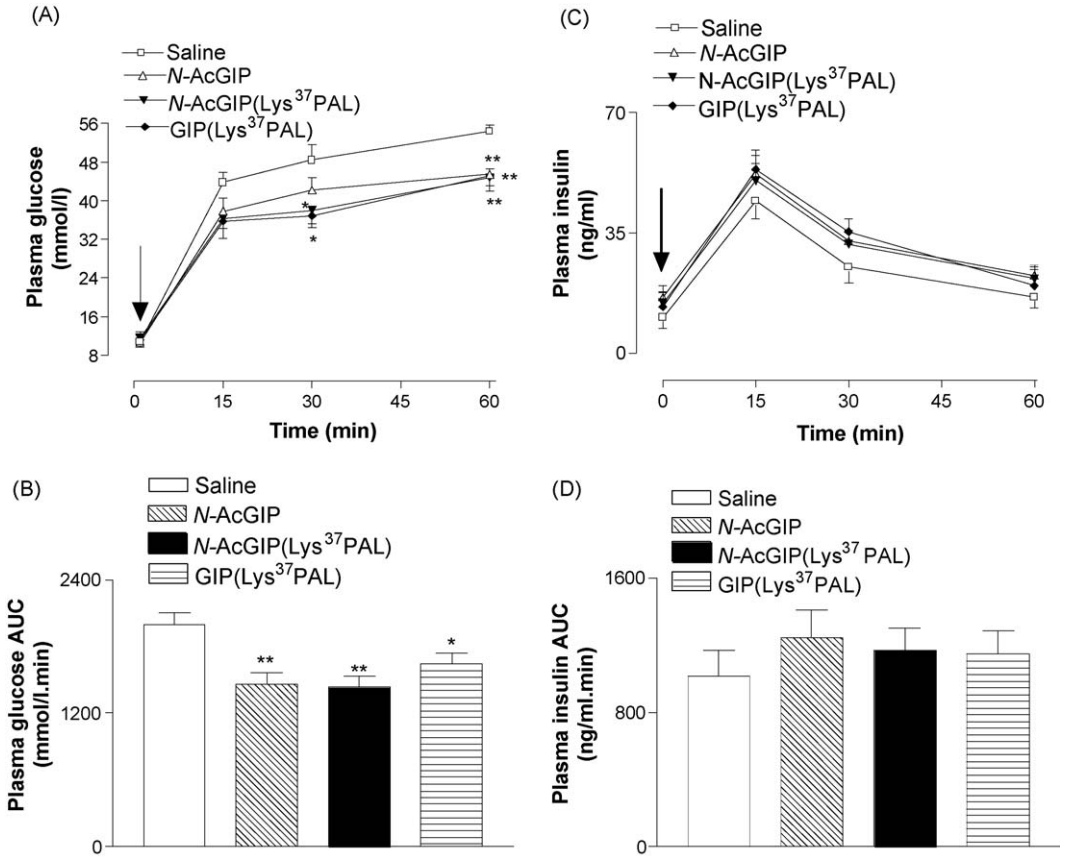

Fig. 1 – Persistence of effects of *N*-AcGIP, GIP(Lys^{37}PAL) and *N*-AcGIP(Lys^{37}PAL) on plasma glucose (A and B) and insulin (C and D) response 4 h after administration. This acute test was conducted 4 h after administration of *N*-AcGIP, GIP(Lys^{37}PAL), *N*-AcGIP(Lys^{37}PAL) (25 nmol kg^{-1}) or saline (0.9% NaCl) in 18 h-fasted *ob/ob* mice. Plasma glucose and insulin concentrations were measured prior to and at intervals after i.p. administration of glucose (18 mmol kg^{-1}) at the time indicated by the arrow. The incremental area under the glucose or insulin curves (AUC) between 0 and 60 min are shown in the right panels. Values represent mean ± S.E.M. for eight mice. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with saline alone group.

albumin binding of peptide, have also been studied [13,17]. Thus, native GIP engineered with palmitate at Lys^{16} or Lys^{37} (GIP(Lys^{16}PAL) and GIP(Lys^{37}PAL)) which show unexpected DPP IV resistance and the second generation analogue N-AcGIP(Lys^{37}PAL) displayed strong antidiabetic actions [17,19]. Interestingly, such a strategy has also been shown to prolong the duration of action of insulin and the sister incretin GLP-1 [20–23].

The present study was designed to directly compare the antidiabetic actions of extended treatment with three longer-acting GIP peptides namely, N-AcGIP, GIP(Lys^{37}PAL) and N-AcGIP(Lys^{37}PAL). This combination of GIP analogues permits ascertation of the metabolic and possible therapeutic significance of both N-terminal modification and mid-chain acylation of GIP. This is an important question in the selection of candidates for clinical development, as issues such as cost and freedom from adverse effects such as immunogenicity need to be addressed as well as efficacy. For this purpose, N-AcGIP, GIP(Lys^{37}PAL), N-AcGIP(Lys^{37}PAL) or saline as a control, were administered once daily by intraperitoneal injection to obese-diabetic (*ob/ob*) mice for 14 days. Effects on glucose homeostasis, pancreatic beta-cell function, insulin sensitivity and pancreatic morphology were examined. Furthermore, possible desensitization of GIP receptor action by prolonged exposure to elevated concentrations of these stable GIP analogues was examined. The results indicate significant antidiabetic potential of all three analogues, but suggest that mid-chain acylation of Tyr^1 modified N-AcGIP is unnecessary for long-term effectiveness.

2. Materials and methods

2.1. Animals and drug treatment protocols

Obese diabetic (*ob/ob*) mice derived from the colony maintained at Aston University, UK were used at 12–16 weeks of age [24]. Animals were housed in an air-conditioned room at 22 ± 2 °C with a 12 h light:12 h dark cycle (08:00–20:00 h) in groups of two. Drinking water and standard rodent maintenance diet (Trouw Nutrition, Cheshire, UK) were freely available. All animal experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986. GIP analogues were sequentially synthesized on an Applied Biosystems automated peptide synthesizer (Model 432 A, Foster City, CA, USA) using standard solid-phase Fmoc peptide chemistry as previously reported [18]. GIP(Lys^{37}PAL) were sequentially synthesized in the same way but with the exception that the lysine residue at position 37 was conjugated to an Fmoc protected C-16 palmitate fatty acid. In addition, an acetyl adduct was incorporated at the N-terminal Tyr^1 of the fatty acid derivatised GIP or native GIP as appropriate. The synthetic peptides were judged pure by reversed-phase HPLC on a Waters Millenium 2010 chromatography system (Software version 2.1.5) and structural identity was confirmed using matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry as described previously [25]. No adverse effects were observed following long-term administration of N-AcGIP, GIP(Lys^{37}PAL) or N-AcGIP(Lys^{37}PAL).

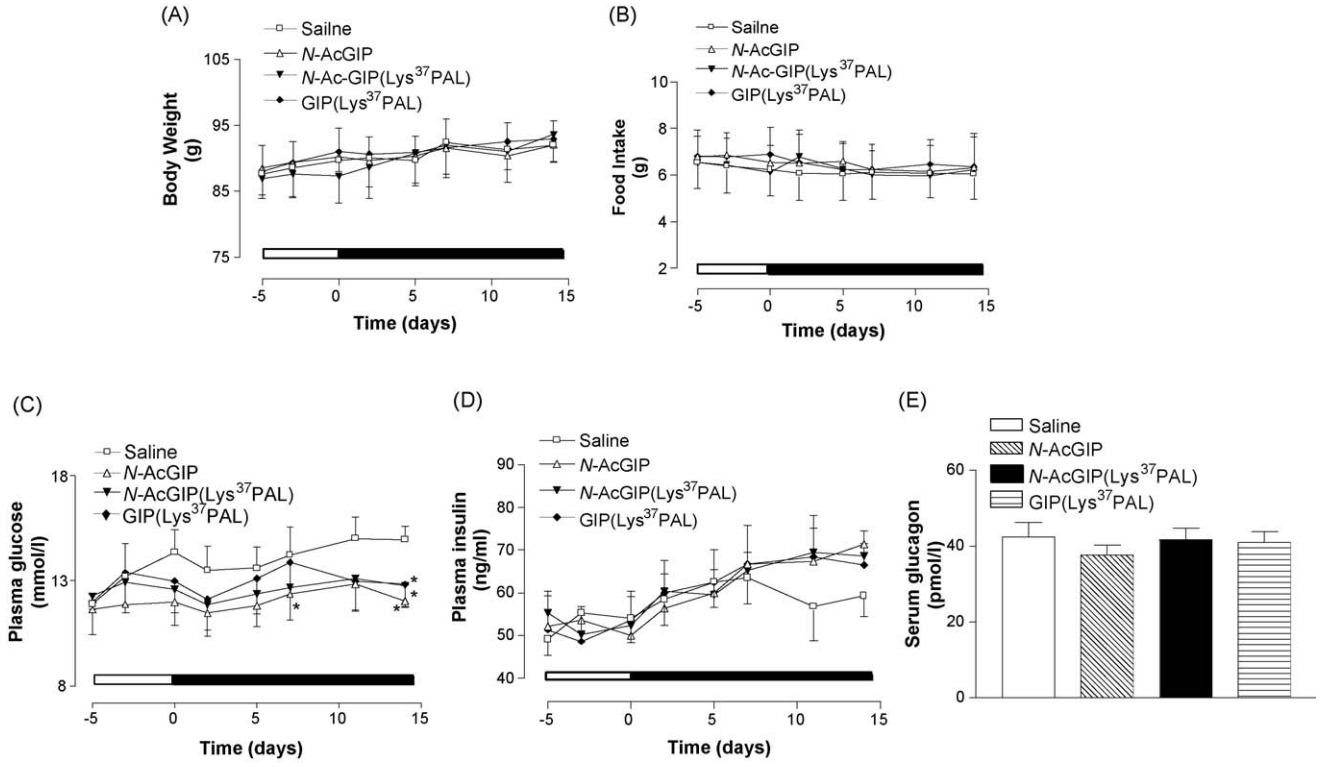

Fig. 2 – Subchronic effects of daily N-AcGIP, GIP(Lys^{37}PAL) and N-AcGIP(Lys^{37}PAL) administration on body weight (A), food intake (B) plasma glucose (C), insulin (D) and final glucagon concentrations (E) in non-fasted mice. N-AcGIP, GIP(Lys^{37}PAL), N-AcGIP(Lys^{37}PAL) (25 nmol kg^{-1} day^{-1}) or saline vehicle (control) were administered for the 14-day period indicated by the horizontal black bar. Values are mean ± S.E.M. for eight mice. $^{*}p < 0.05$ compared to control.

An initial *acute* experiment was preformed to compare extended biological activity of *N*-AcGIP, GIP(Lys^{37}PAL) and *N*-AcGIP(Lys^{37}PAL) by examining metabolic and endocrine responses to intraperitoneal injection of glucose alone (18 mmol kg^{-1} body weight) 4 h after GIP-related peptide or saline administration (25 nmol kg^{-1}) in 18 h fasted animals. Subsequently, a 14-day subchronic experiment was preformed whereupon groups of *ob/ob* mice ($n = 8$) received once daily intraperitoneal injections (17:00 h) of either saline vehicle (0.9%, w/v, NaCl), *N*-AcGIP, GIP(Lys^{37}PAL) or *N*-AcGIP(Lys^{37}PAL) (all at 25 nmol kg^{-1} day^{-1}). A dose of 25 nmol kg^{-1} was chosen based on previous findings demonstrating the biological effectiveness of *N*-AcGIP at this dose [18]. Food intake and body weight were recorded daily from 5 days before commencement of the subchronic treatment regime. Non-fasting plasma glucose and insulin concentrations (10:00 h) were monitored at 2–4 day intervals. At 14 days, non-fasted groups of animals were used to evaluate intraperitoneal glucose tolerance (18 mmol kg^{-1}), insulin sensitivity (50 U kg^{-1}) and metabolic responses to native GIP (25 nmol kg^{-1}). Mice fasted for 18 h were used to examine the metabolic response to 15 min feeding. All acute tests were commenced at 10:00 h. Circulating concentrations of GIP analogues given 17 h previously are likely to be quite low but require specific assay development for measurement.

At the end of the 14-day subchronic treatment period, pancreatic tissues were excised from non-fasted animals for immunohistochemistry or measurement of insulin following extraction with 5 ml/g ice-cold acid ethanol (75% ethanol, 2.35% H_2O, 1.5% HCl). Serum samples were taken for determination of glucagon. All plasma samples were collected from the cut tip of the tail vein of conscious mice into chilled fluoride/heparin coated glucose microcentrifuge tubes (Sarstedt, Nümbrecht, Germany) at the times indicated in the figures. Blood samples for glucagon were collected from the cut tip of the tail vein of conscious mice into chilled non-coated microcentrifuge tubes (Sarstedt, Nümbrecht, Germany) supplemented with aprotonin. All blood samples were immediately centrifuged using a Beckman microcentrifuge (Beckman Instruments, Galway, Ireland) for 30 s at 13,000 × *g*. The resulting plasma/serum was then aliquoted into fresh tubes and stored at −20 °C prior to glucose, insulin or glucagon determinations. In a separate acute experiment using untreated *ob/ob* mice, the effects of administering *N*-AcGIP (25 nmol kg^{-1}) in combination with insulin (50 U kg^{-1}) was determined.

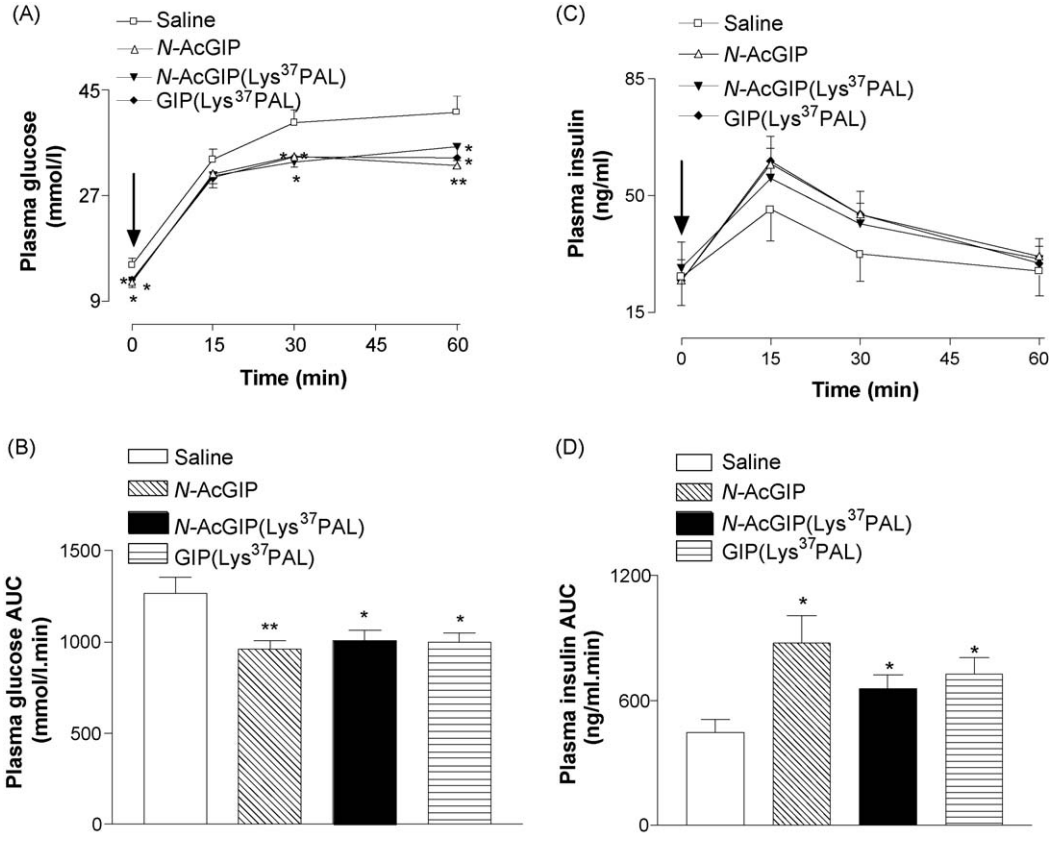

Fig. 3 – Subchronic effects of daily *N*-AcGIP, GIP(Lys^{37}PAL) and *N*-AcGIP(Lys^{37}PAL) administration on glucose tolerance (A and B) and plasma insulin (C and D) response to glucose. Tests were conducted after 14 daily injections of either *N*-AcGIP, GIP(Lys^{37}PAL), *N*-AcGIP(Lys^{37}PAL) (25 nmol kg^{-1} day^{-1}) or saline vehicle (control) in non-fasted mice. Glucose (18 mmol kg^{-1}) was administered by intraperitoneal injection at the time indicated by the arrow. Plasma glucose and insulin AUC values for 0–60 min post-injection are also shown in the bottom panels. Values are mean ± S.E.M. for eight mice. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to control.

2.2. Biochemical analyses

Plasma glucose was assayed by an automated glucose oxidase procedure using a Beckman Glucose Analyzer II [18]. Plasma and pancreatic insulin was assayed by dextran–charcoal RIA as described previously [18]. Serum glucagon concentrations were assayed by a commercially available glucagon RIA kit (Linco, St Charles, MI, USA).

2.3. Statistics

Results are expressed as mean ± S.E.M. Data were compared using repeated measures ANOVA or one-way ANOVA, followed by the Student–Newman–Keuls *post hoc* test. Incremental areas under plasma glucose and insulin curves (AUC) were calculated using a computer-generated program employing the trapezoidal rule with baseline subtraction. Groups of data were considered to be significantly different if $p < 0.05$ [18].

2.4. Immunocytochemistry

Pancreatic tissue from non-fasted animals was fixed in 4% paraformaldehyde/PBS and embedded in paraffin, sectioned at 8 μm and stained as described previously [26]. The average number and area of every islet in each examined section was estimated in a blinded manner using a microscope (Nikon Eclipse E2000, Diagnostic Instruments Incorporated, Michigan, USA) attached to a JVC camera Model KY-F55B (JVC, London, UK) and ImageJ 1.31 software (http://www.rsb.info.nih.gov/nih-image/). Approximately 60–70 random sections were examined from the pancreas of each mouse.

3. Results

3.1. Persistent effects of GIP peptide analogues on glucose tolerance and insulin response to glucose

As shown in Fig. 1, administration of *N*-AcGIP, GIP(Lys^{37}PAL) and *N*-AcGIP(Lys^{37}PAL) 4 h previously decreased plasma glucose levels following i.p. glucose injection (18 mmol kg^{-1}) corresponding to reductions of 27% ($p < 0.01$), 18% ($p < 0.01$) and 28% ($p < 0.05$), respectively (Fig. 1B). Enhancement of glucose-induced insulin release was less evident 4 h after administration (Fig. 1C and D). These data support a protracted and similar biological effect of *N*-AcGIP, GIP(Lys^{37}PAL) and *N*-AcGIP(Lys^{37}PAL).

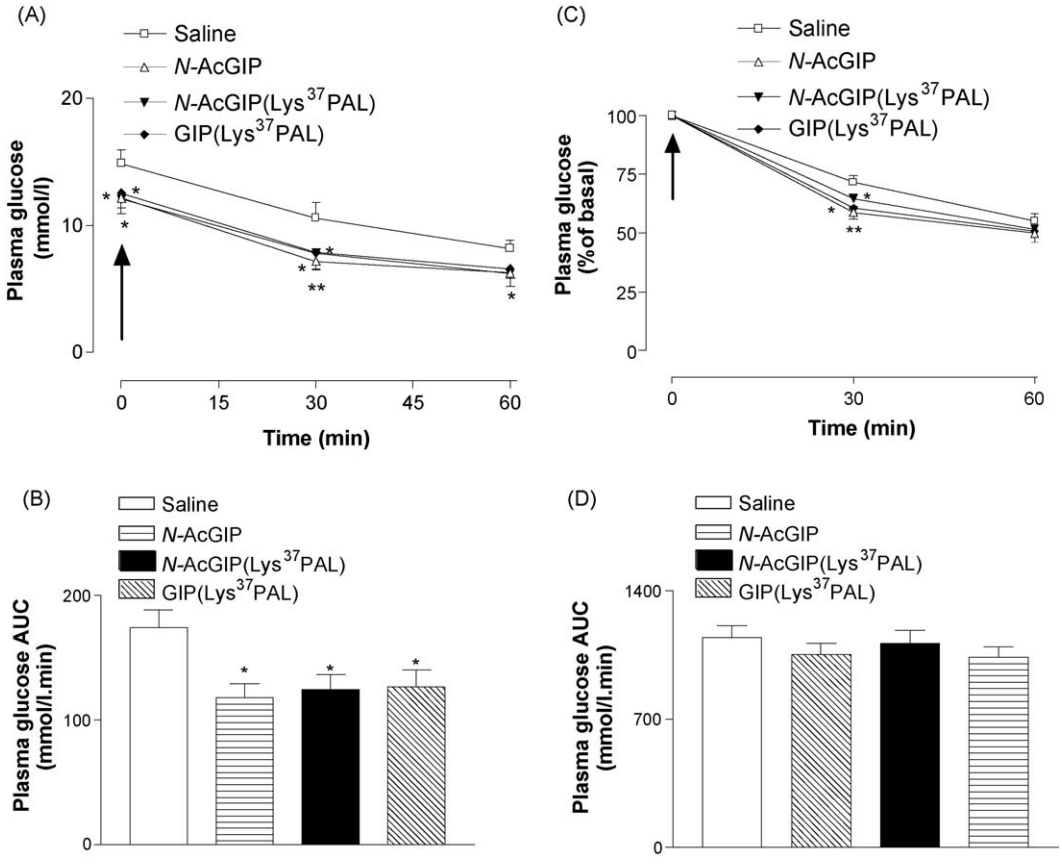

Fig. 4 – Subchronic effects of daily *N*-AcGIP, GIP(Lys^{37}PAL) and *N*-AcGIP(Lys^{37}PAL) administration on insulin sensitivity. Tests were conducted after 14 daily injections of either *N*-AcGIP GIP(Lys^{37}PAL), *N*-AcGIP(Lys^{37}PAL) (25 nmol kg^{-1} day^{-1}) or saline vehicle (control) in non-fasted mice. Insulin (50 U kg^{-1}) was administered by intraperitoneal injection at the time indicated by the arrow. Plasma glucose AUC values for 0–60 min post-injection are also shown in the bottom panels (B and D). Values are mean ± S.E.M. for eight mice. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to control.

3.2. *Effects of GIP peptide analogues on body weight, food intake and non-fasting concentrations of glucose, glucagon and insulin*

Administration of *N*-AcGIP, GIP(Lys^{37}PAL) or *N*-AcGIP(Lys^{37}PAL) had no effect on body weight or food intake (Fig. 2A and B). Plasma glucose concentrations were progressively reduced, resulting in significantly ($p < 0.05$) lowered glucose concentrations at 14 days in all three treatment groups (Fig. 2C). These changes were accompanied by a tendency towards elevated insulin concentrations, but these did not achieve statistical significance over the study period (Fig. 2D). No differences of serum glucagon were observed between control and treatment groups on day 14 (Fig. 2E).

3.3. *Effects of GIP peptide analogues on glucose tolerance*

Treatment with *N*-AcGIP, GIP(Lys^{37}PAL) or *N*-AcGIP(Lys^{37}PAL) for 14 days resulted in a significant improvement in glucose tolerance and glucose-mediated insulin release (Fig. 3). *N*-AcGIP produced a 24% reduction in the overall glycaemic excursion ($p < 0.01$) (Fig. 3B). This was accompanied by a 137% increase in insulin response ($p < 0.05$) (Fig. 3D). Both fatty acid derivatised GIP analogues produced similar effects ($p < 0.05$; in both cases) (Fig. 3).

3.4. *Effects GIP peptide analogues on insulin sensitivity*

As shown in Fig. 4, the hypoglycaemic action of insulin was significantly augmented in terms of post-injection values ($p < 0.05$–0.01) (Fig. 4A and C) and AUC measures ($p < 0.05$) (Fig. 4B and D) in *ob/ob* mice treated for 14 days with *N*-AcGIP, GIP(Lys^{37}PAL) or *N*-AcGIP(Lys^{37}PAL) compared to controls. The glucose-lowering action of insulin administered to untreated *ob/ob* mice simultaneously with *N*-AcGIP was not significantly different to insulin given alone (plasma glucose 0–60 min AUC values; 159.0 ± 28.60 mmol/l min versus 182.50 ± 24.360 mmol/l min, respectively; data not shown).

3.5. *Effects of GIP peptide analogues on metabolic response to feeding*

Plasma glucose responses to 15 min feeding were significantly lowered ($p < 0.05$) at 105 min in *ob/ob* mice treated for 14 days with *N*-AcGIP, GIP(Lys^{37}PAL) or *N*-AcGIP(Lys^{37}PAL) (Fig. 5A). Furthermore, the overall glycaemic response was significantly reduced in *N*-AcGIP and GIP(Lys^{37}PAL) treated mice ($p < 0.05$) (Fig. 5B). The corresponding plasma insulin responses to feeding were significantly increased ($p < 0.05$) at 15 min (Fig. 5C) and the overall insulinotropic response was significantly enhanced ($p < 0.05$) in all three treatment groups

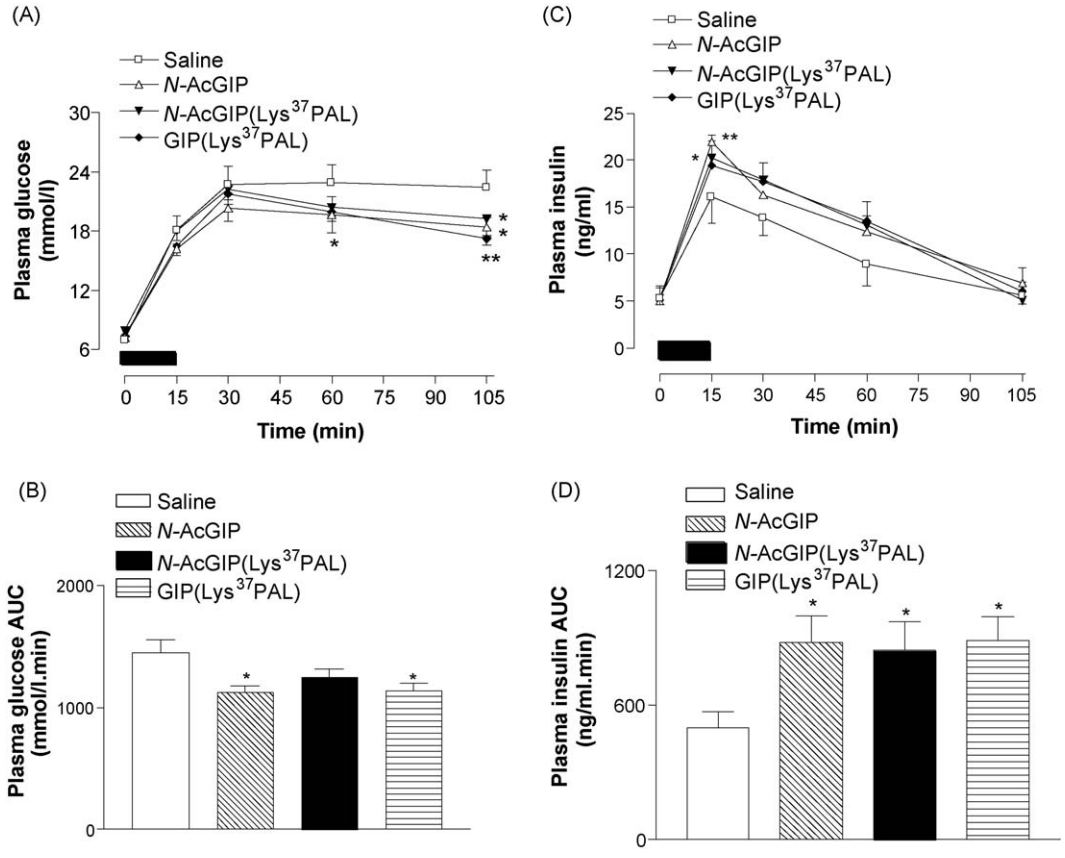

Fig. 5 – Subchronic effects of daily *N*-AcGIP, GIP(Lys^{37}PAL) and *N*-AcGIP(Lys^{37}PAL) administration on glucose (A and B) and insulin (C and D) responses to feeding in 18 h fasted *ob/ob* mice. Tests were conducted after 14 daily injections of either *N*-AcGIP, GIP(Lys^{37}PAL), *N*-AcGIP(Lys^{37}PAL) (25 nmol kg^{-1} day^{-1}) or saline vehicle (control). The horizontal black bar indicates the time of feeding (15 min). AUC values for 0–105 min post-feeding are shown in the bottom panels. Values are mean ± S.E.M. for eight mice. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to control.

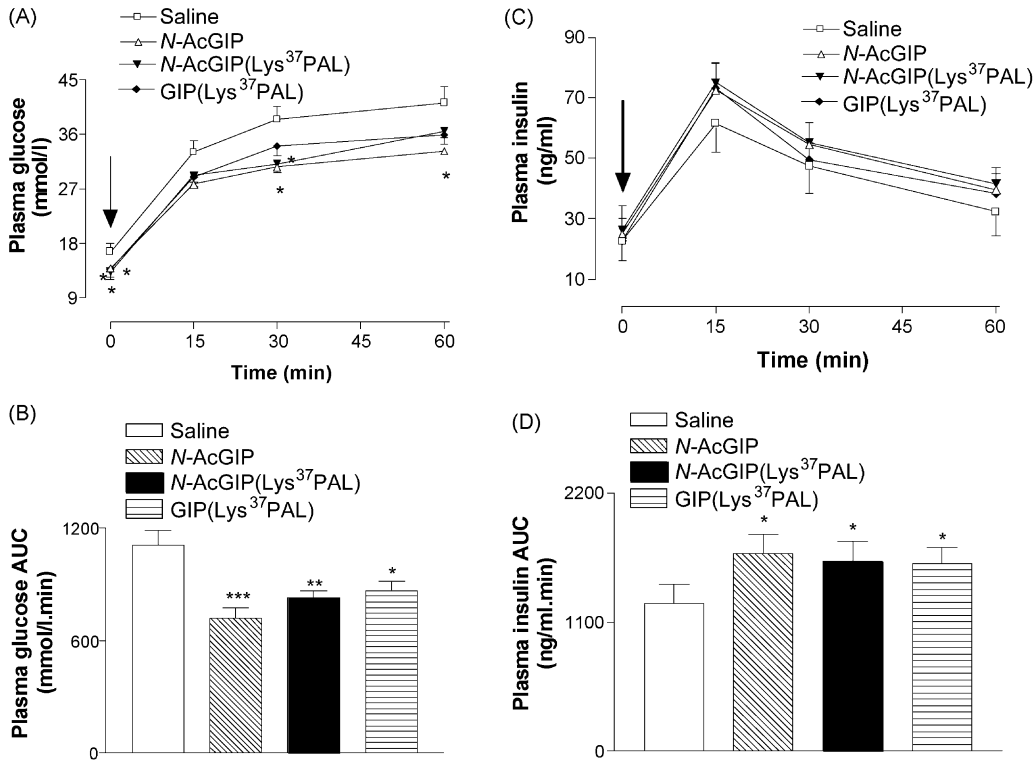

Fig. 6 – Subchronic effects of daily N-AcGIP, GIP(Lys^{37}PAL) and N-AcGIP(Lys^{37}PAL) administration on glucose tolerance (A and B) and plasma insulin (C and D) response to native GIP. Tests were conducted after 14 daily injections of either N-AcGIP, GIP(Lys^{37}PAL), N-AcGIP(Lys^{37}PAL) (25 nmol kg^{-1} day^{-1}) or saline vehicle (control) in non-fasted mice. Glucose (18 mmol kg^{-1}) in combination with native GIP (25 nmol kg^{-1}) was administered by intraperitoneal injection at the time indicated by the arrow. Plasma glucose and insulin AUC values for 0–60 min post-injection are shown in the bottom panels. Values are mean ± S.E.M. for eight mice. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to control.

(Fig. 5D). Food intake was similar (0.4–0.6 g/mouse/15 min) in all four groups of mice.

3.6. *Effects of GIP peptides analogues on response to native GIP*

Possible GIP receptor desensitization was assessed after 14-days treatment by evaluation of the responses to native GIP in combination with glucose. As shown in Fig. 6, treatment with N-AcGIP, GIP(Lys^{37}PAL) or N-AcGIP(Lys^{37}PAL) for 14 days significantly augmented the overall glucose-dependent insulinotropic response to native GIP compared to control ($p < 0.05$, in all cases) (Fig. 6D). In harmony, all three peptide treated groups displayed significantly decreased glycaemic excursions compared to control (Fig. 5B). Thus, 14-day treatment with N-AcGIP, GIP(Lys^{37}PAL) or N-AcGIP(Lys^{37}PAL) augmented the insulinotropic actions of native GIP.

3.7. *Effects of GIP peptide analogues on pancreatic insulin content and islet morphology*

Treatment N-AcGIP, GIP(Lys^{37}PAL) or N-AcGIP(Lys^{37}PAL) did not affect pancreatic weight (Fig. 7A) but insulin content was significantly increased ($p < 0.05$) by N-AcGIP(Lys^{37}PAL) compared with controls (Fig. 7C). All three GIP analogues significantly increased the number and size of pancreatic islets after 14-days treatment (Fig. 7B and D).

4. Discussion

Owing to its potent glucose-dependent insulinotropic actions, GIP has been considered alongside GLP-1 as a possible therapeutic option for the treatment of type 2 diabetes [14]. However, the peptidic nature, unfavorable pharmacokinetic profile and proposed inefficacy of GIP in type 2 diabetic patients has somewhat hindered the therapeutic progression of GIP in comparison to its sister incretin hormone, GLP-1 [30]. However, recent years have witnessed the advent of new designer, bioengineered longer-acting GIP molecules [8,13] and appreciation that the once postulated specific defect in GIP stimulation of insulin secretion [28] represents just one aspect of a generalized pancreatic beta-cell dysfunction in diabetes [29]. Thus, a clear rationale for future diabetes treatment strategies based on GIP now exists.

We have recently developed N-AcGIP, GIP(Lys^{37}PAL) and N-AcGIP(Lys^{37}PAL) as potent DPP IV resistant GIP receptor agonists [13,30,31]. Acute studies have highlighted the potential of these long-acting GIP analogues to enhance glucose-induced insulin release and moderate glycaemic excursions in *ob/ob* mice

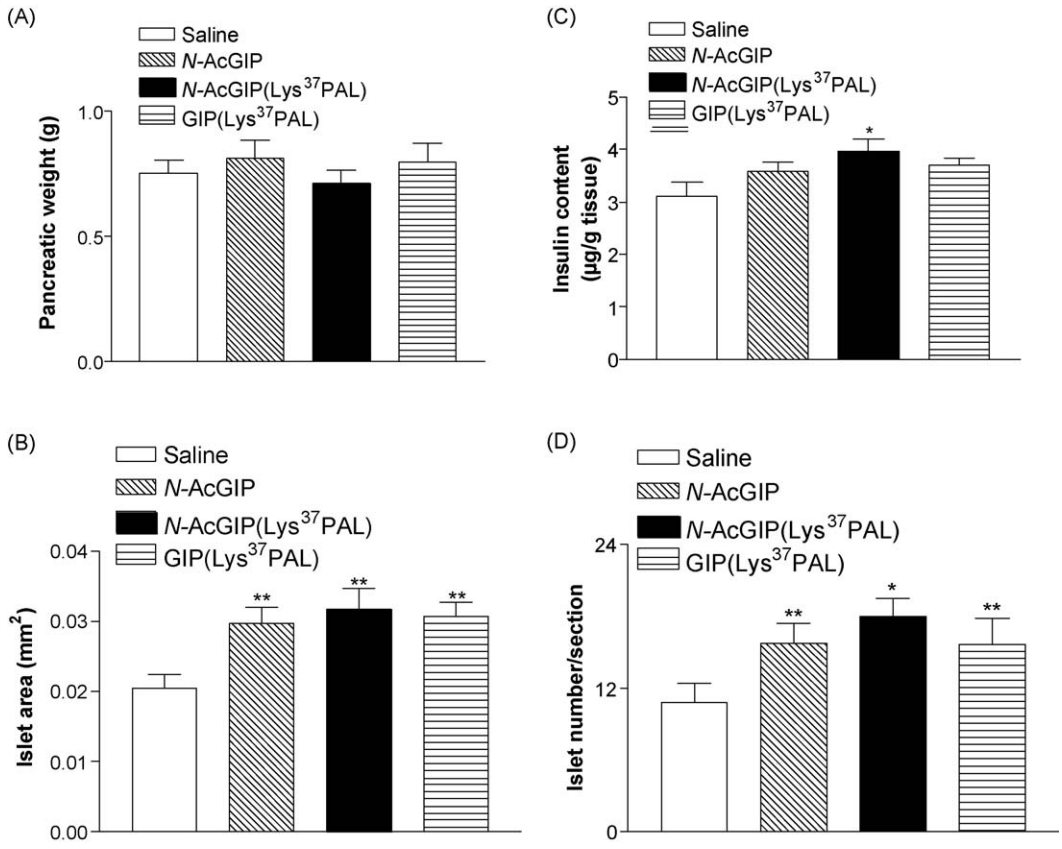

Fig. 7 – Subchronic effects of daily N-AcGIP, GIP(Lys^{37}PAL) and N-AcGIP(Lys^{37}PAL) administration on pancreatic weight (A), insulin content (C) and islet morphology (B and D). Parameters were determined after 14 daily injections of N-AcGIP, GIP(Lys^{37}PAL), N-AcGIP(Lys^{37}PAL) (25 nmol kg^{-1} day^{-1}) or saline vehicle (control) in non-fasted mice. Values are mean ± S.E.M. for eight mice. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to control.

[13,30,31]. It has also been shown that daily administration of N-AcGIP(Lys^{37}PAL), and surprisingly GIP(Lys^{37}PAL) which lacks N-terminal modification, resulted in significant amelioration of diabetes in *ob/ob* mice [13,19]. Exploitation of the effects of daily N-AcGIP treatment and direct comparison of the antidiabetic properties of these closely related analogues have not been carried out. The present study has thus examined extended daily treatment with N-AcGIP, GIP(Lys^{37}PAL) and N-GIP(Lys^{37}PAL). This involved once daily injections in *ob/ob* mice. Preliminary experiments determined that there was no significant difference in the bioactivity of N-terminally modified and/or acylated GIP on glucose tolerance 4 h after peptide administration (Fig. 1). This suggests that clearance of N-AcGIP may not be substantially different from its related palmitate analogues. However, development of specific assays are required to clarify this point. Notably, native GIP has previously been shown to have an *in vivo* half-life of 5.0 ± 1.2 min [12] and exerts weak effects in *ob/ob* mice even when administered conjointly with glucose [11].

Daily administration of N-AcGIP, GIP(Lys^{37}PAL) and N-AcGIP(Lys^{37}PAL) to *ob/ob* mice significantly reduced plasma glucose and improved glucose tolerance by 14 days. This effect was independent of any change in body weight or food intake (Fig. 2A and B). This accords with the view that GIP lacks effects on feeding activity and [27], unlike the sister incretin GLP-1, does not inhibit gastric emptying leading to unwanted side-effects such as nausea and vomiting [32]. Measurement of circulating glucagon in treated mice demonstrated no effect on endogenous glucagon secretion (Fig. 2E). Thus, opposition of the antihyperglycaemic actions of the GIP analogues by stimulation of glucagon secreting alpha-cells seems unlikely. Other studies have also shown that any stimulatory effects of GIP on glucagon secretion occur at physiological, and not elevated, glucose concentrations [33].

As would be expected from incretin hormone therapy, a significant proportion of the antihyperglycaemic effects stemmed from potent insulinotropic actions. Although native GIP is a weak stimulus to insulin secretion in *ob/ob* mice at the age studied [13], daily administration of all three GIP analogues resulted in enhanced beta-cell function and insulin secretion 14 days later (Figs. 3, 5 and 6). This is consistent with the action of GIP as a promoter of proinsulin gene expression [3]. N-AcGIP(Lys^{37}PAL) treated mice also displayed significantly elevated pancreatic insulin content (Fig. 7C). In harmony with this observation, the pancreas of all GIP peptide treated groups displayed more numerous and enlarged islets, consistent with the positive effect of GIP on islet neogenesis, beta-cell growth and survival [5,34] (Fig. 7B and D).

Daily treatment with N-AcGIP, GIP(Lys^{37}PAL) or N-AcGIP(Lys^{37}PAL) significantly enhanced the insulin response to

glucose, nutrient stimulation and native GIP (Figs. 3, 5 and 6). The magnitude of the effects were similar for the three analogues and it is important to note that glucose and GIP were administered as a bolus injection as opposed to continuous infusion, which has been reported to produce a weaker response in type 2 diabetes [35]. The enhanced insulin response resulted in reductions of glycaemic excursion both with intraperitoneal and alimentary administrations. This indicates that the enhancement of beta-cell function was more than adequate to overcome the severe insulin resistance of this mutant [24]. As observed previously with GIP(Lys^{37}PAL) or *N*-AcGIP(Lys^{37}PAL), a small enhancement of insulin sensitivity was also evident after 14 days treatment (Fig. 4). This glucose lowering action was not reproduced in untreated *ob*/*ob* mice by acute conjoint administration of *N*-AcGIP with insulin, indicating that the effect possibly represents an adaptive process to longer-term GIP analogue treatment. Such extrapancreatic effects might include reduction of hepatic insulin clearance [6] promotion of glucose uptake in muscle [7] and adipose tissue [36].

One major problem currently foreseen with extended GIP or GLP-1 treatment is desensitization of hormone receptor action [37]. There was no evidence that treatment with *N*-AcGIP, GIP(Lys^{37}PAL) or *N*-AcGIP(Lys^{37}PAL) for 14 days compromised the glucose lowering or insulin releasing actions of native GIP in any way (Fig. 6), in harmony with previous findings [17]. On the contrary, the insulinotropic effects of native GIP were amplified in the three treatment groups when compared to controls, consistent with amplification of pancreatic beta-cell responsiveness to GIP, similar to that observed with GLP-1 [26] (Fig. 6D).

Taken together the results of the present study indicate a marked improvement of diabetic state in *ob*/*ob* mice treated with N-terminally modified and acylated GIP molecules. All three analogues exhibit DPP IV resistance but it was not possible in the present study to show the benefit of fatty acid derivatisation aimed at retarding renal elimination [17–19]. Thus, in analogy with the stable GLP-1 mimetic, exendin(1–39)amide also termed exenatide or Byetta [38], further strategies to extend bioactivity by reduction of renal elimination by fatty acid derivatisation appear unnecessary for therapeutic usefulness. Such an approach has been adopted with insulin and the sister incretin hormone GLP-1 [22,23], although neither have achieved therapeutic application. Indeed given the difficulty, increased cost and possible immuongenicity of acylated peptides, the present study indicates that simple N-terminal acetylation represents an effective means of creating stable long-acting GIP analogue for potential treatment of type 2 diabetes.

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