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1 **Fatty acid derivatised analogues of glucose-dependent insulinotropic polypeptide**
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4 **with improved antihyperglycaemic and insulinotropic properties**
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ABSTRACT

C-terminal acylation of Lys³⁷ with myristic (MYR; tetradecanoic acid), palmitic (PAL; hexadecanoic acid) and stearic (octadecanoic acid) fatty acids with or without N-terminal acetylation was employed to develop long-acting analogues of the glucoregulatory hormone, glucose-dependent insulinotropic polypeptide (GIP). All GIP analogues exhibited resistance to dipeptidylpeptidase-IV (DPP-IV) and significantly improved *in vitro* cAMP production and insulin secretion. Administration of GIP analogues to *ob/ob* mice significantly lowered plasma glucose - GIP(Lys³⁷MYR), *N*-AcGIP(Lys³⁷MYR) and GIP(Lys³⁷PAL) increased plasma insulin concentrations. GIP(Lys³⁷MYR) and *N*-AcGIP(Lys³⁷MYR) elicited protracted glucose-lowering effect when administered 24h prior to an intraperitoneal glucose load. Daily administration of GIP(Lys³⁷MYR) and *N*-AcGIP(Lys³⁷MYR) to *ob/ob* mice for 24 days decreased glucose and significantly improved plasma insulin, glucose tolerance and beta-cell glucose responsiveness. Insulin sensitivity, pancreatic insulin content and triglyceride levels were not changed. These data demonstrate that C-terminal acylation particularly with myristic acid provides a class of stable, longer-acting forms of GIP for further evaluation in diabetes therapy.

Keywords: dipeptidylpeptidase-IV (DPP-IV); glucose-dependent insulinotropic polypeptide (GIP); GIP agonist; glucose homeostasis; insulin secretion.

1. Introduction

Glucose-dependent insulinotropic polypeptide (GIP) is a gluco-regulatory hormone released from K-cells in response to feeding [1]. Despite initial characterisation based on its ability to inhibit gastric acid secretion, GIP, together with its sister hormone, glucagon-like peptide-1 (GLP-1), are now widely recognised as incretin hormones [2]. GIP and GLP-1 account for the total insulinotropic arm of the enteroinsular axis following meal ingestion, thereby reducing postprandial hyperglycaemia [3]. In contrast to currently used non-endogenous drugs, both GIP and GLP-1 elicit their potent insulinotropic actions in a glucose-dependent manner and as such avoid unwanted hypoglycaemic episodes. While GLP-1 based therapies, for example, exenatide, have now reached the market and are currently being prescribed to type 2 diabetic patients, the progression of GIP-based molecules has been somewhat hindered due to several fundamental limitations [4].

Firstly, insensitivity of the beta-cell to the insulin-releasing actions of GIP in type 2 diabetes has been noted [5,6]. However, it has emerged that rather than representing a GIP specific defect, this is a readily reversible phenomenon that can be rectified by improved diabetes control using other glucose-lowering drugs [7,8]. Thus combination therapy or use of modified GIP analogues can be expected to overcome any beta-cell insensitivity. Indeed the insulin-releasing and anti-diabetic potential of several amino-terminally modified GIP analogues have been demonstrated both in animal models and in preliminary studies in patients with type 2 diabetes [9]. Secondly, the pharmacokinetic profile of the native GIP hormone is severely compromised through rapid proteolytic degradation with the enzyme dipeptidylpeptidase-IV (DPP-IV; EC 3.4.14.5) generating the inactive major degradation product GIP(3-42) [10]. Thirdly, GIP and its associated metabolites are quickly eliminated and cleared from the body primarily via the kidney [11].

1 Furthermore, present appreciation of the role of circulating GIP in clinical diabetes and
2 particularly fat deposition is far from complete [4].
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8 These intrinsic limitations can be overcome through two different approaches including the
9 application of specific DPP-IV inhibitors or the development of structurally modified GIP
10 molecules which are resistant to the actions of DPP-IV [12]. Indeed, a range of structurally
11 modified analogues of GIP have been developed, several of which have improved
12 antihyperglycaemic and insulinotropic properties [4]. Recently, we have shown that GIP
13 conjugated at the C-terminus with a mini-polyethylene glycol residue exhibited prolonged and
14 enhanced biological actions *in vivo* [13]. However, another approach to avoid rapid renal
15 filtration and clearance from the body is fatty acid derivatisation. Studies in our laboratory have
16 shown that conjugating a C-16 fatty acid to the epsilon side-chain of Lys at position 37 resulted
17 in significant benefits in both glucose-lowering and insulin-releasing actions [14-18]. Fatty acid
18 derivatisation prolongs the biological half-life of GIP by masking the DPP-IV cleavage site, but
19 also facilitates binding to serum proteins thus reducing renal clearance [15,16]. Indeed, this
20 approach has been employed in the development of several GLP-1 based therapies, for example,
21 NN2211, which has been shown to elicit a prolonged pharmacodynamic profile (half-life >12h)
22 making it suitable for once-daily subcutaneous administration [19]. However, unlike GIP-based
23 mimetics, prolonged administration of NN2211 and GLP-1 mimetics in general leads to
24 gastrointestinal side-effects such as nausea due to a prolonged inhibitory effect on gastric
25 emptying [9].
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54 To date, only the biological efficacy of C-16 fatty acid derivatised GIP analogues have been
55 reported [14-18]. Therefore, in the present study we characterised the effects of a range of fatty
56 acid chain lengths (C-14, C-16 and C-18) with or without N-terminal acetylation on metabolic
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1 stability and biological actions. Stability of GIP analogues to DPP-IV degradation, as well as *in*
2
3 *vitro* cAMP production and insulin secretion were examined. Furthermore, acute and prolonged
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5 actions of selected analogues were assessed in *ob/ob* mice, a commonly employed animal model
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7 of type 2 diabetes displaying both beta-cell dysfunction and insulin resistance.
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10 11 12 13 14 **2. Materials and methods**

15 16 17 **2.1 Synthesis, purification and characterisation of GIP peptides**

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19 GIP peptides (see Table 1) were sequentially synthesised on an Applied BioSystems automated
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21 peptide synthesiser (ABI 432A Peptide Synthesiser; Warrington, Cheshire, UK) with a preloaded
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23 Fmoc-Gln(Trt)-Wang resin (typical loading 0.2-1.0 mmol/g; Sigma-Aldrich, Gillingham, Dorset,
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25 UK) using standard solid-phase Fmoc peptide chemistry [21]. Synthetic peptides were purified
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27 by semi-preparative HPLC followed by analytical HPLC and characterised using matrix-assisted
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29 laser desorption ionisation-time of flight (MALDI-TOF) mass spectrometry as described
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31 previously [16].
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38 39 **2.2. Degradation of GIP peptides by DPP-IV**

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41 GIP peptides were incubated at 37°C in 50 mM triethanolamine-HCl (pH 7.8) with purified
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43 porcine dipeptidylpeptidase-IV (5 mU; Sigma-Aldrich, UK) for 0, 2, 4, 8 and 24 h (no albumin
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45 was present in the incubation buffer). Enzymatic reactions were terminated by addition of 15 µl
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47 of 10% (v/v) TFA/water. Reaction products were applied to a Vydac C-4 column (4.6 x 250mm;
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49 The Separations Group, Hesperia, California, USA) and intact peptide separated from the major
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51 degradation fragment GIP(3-42). Absorption was monitored at 206 nm using a SpectraSystem
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53 UV2000 detector (Thermoquest Ltd, Manchester, UK) and HPLC peak area data were used to
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55 calculate percentage intact peptide remaining at time points indicated during the incubation.
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2.3. In vitro cAMP production and insulin secretion

The effects of GIP peptides on the stimulation of cAMP production and insulin secretion were measured using clonal pancreatic BRIN-BD11 cells as described previously [22]. For cAMP studies, BRIN-BD11 cells were seeded (100,000 cells per well) into 96-well plates (Nunc, Roskilde, Denmark) and washed with HBS buffer before being incubated with various concentrations of GIP peptides in the presence of 1 mM IBMX (Sigma-Aldrich, UK) for 20 min at 37°C. After incubation, medium was removed and the cells lysed prior to measurement of cAMP using an HTS Immunoassay Kit (Millipore, Watford, UK). For insulin-release studies, BRIN-BD11 cells were seeded (100,000 cells per well) into 24-well plates (Nunc, Roskilde, Denmark) and allowed to attach overnight at 37°C. Following a 40 min pre-incubation (1.1 mM glucose; 37°C), cells were incubated (20 min; 37°C) in the presence of 5.6 mM glucose with a range of peptide concentrations (estimated albumin concentration 76.5 µM). After 20 min incubation, buffer was removed from each well and aliquots (200 µl) stored at -20°C prior to measurement of insulin using radioimmunoassay.

2.4. Acute and persistent effects of GIP peptides on glucose-lowering and insulin release in vivo

Obese diabetic (*ob/ob*) mice and lean littermates from the Aston colony at 20-22 weeks of age were used to assess the *in vivo* biological properties of GIP peptides. The genetic background and characteristics of the colony used have been described in detail elsewhere [20]. Animals were age-matched, divided into groups and housed in an air-conditioned room at 22 ± 2°C with a 12 h light / 12 h dark cycle. Animals had free access to drinking water and normal laboratory chow (Trouw Nutrition, Cheshire, UK). All animal experiments were performed in accordance with the

1 UK Animals (Scientific Procedures) Act 1986. No adverse effects were observed following
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4 administration of any of the treatments.
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9 In a first series of experiments, overnight fasted normal mice received an intraperitoneal (ip)
10 injection of saline (0.9% (w/v) NaCl) or glucose (18 mmol/kg body weight) alone or in
11 combination with GIP or *N*-AcGIP(Lys³⁷MYR) (each at 25 nmol/kg body weight). In a second
12
13 set of experiments, overnight fasted *ob/ob* mice received an ip injection of glucose alone (18
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15 mmol/kg body weight) or in combination with GIP peptides (each at 25 nmol/kg body weight). In
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17 a third series of experiments, glucose (18 mmol/kg body weight; ip) was administered 24 and 48
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19 h after injection of GIP peptides (each at 25 nmol/kg body weight) to *ob/ob* mice. All test
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21 solutions were administered in a final volume of 8 ml/kg body weight.
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30 2.5. Sub-chronic effects of once daily administration of GIP peptides in *ob/ob* mice

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32 Once-daily ip injections of *N*-AcGIP, GIP(Lys³⁷MYR) or *N*-AcGIP(Lys³⁷MYR) (each at 25
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34 nmol/kg body weight) or saline vehicle (0.9% (w/v) NaCl) were administered at 16:00 h over a
35
36 24-day treatment period. Food intake, body weight, non-fasting plasma glucose and insulin levels
37
38 were monitored at intervals of 2-4 days. Glucose tolerance (18 mmol/kg body weight; ip) and
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40 insulin sensitivity (50 U/kg body weight; ip) tests were performed at the end of the study. At
41
42 termination, blood for measurement of circulating triglycerides was taken and pancreatic tissues
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44 excised and processed for measurement of insulin following extraction with 5 ml/g ice-cold acid
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46 ethanol.
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54 2.6. Biochemical analyses

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56 Blood samples were collected from the cut tip on the tail vein of conscious mice into chilled
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58 fluoride/heparin glucose micro-centrifuge tubes (Sarstedt, Numbrecht, Germany) at the
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1 timepoints indicated in the figures. Samples were immediately centrifuged using a Beckman
2 microcentrifuge (Beckman Instruments, Galway, Ireland) for 30 s at 13,000 x *g*. Plasma glucose
3 was assayed by an automated glucose oxidase procedure [23] using a Beckman Glucose Analyser
4 II (Beckman Instruments, Galway, Ireland). Plasma and pancreatic insulin was assayed by a
5 modified dextran-coated charcoal radioimmunoassay as described previously [24]. Plasma
6 triglyceride levels were measured using a Hitachi Automatic Analyser 912 (Boehringer
7 Mannheim, Germany) as per manufacturer instructions.
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21 2.7. Statistical analysis

22 Results are expressed as mean \pm SEM and data compared using the unpaired Student's *t*-test.
23 Where appropriate, data were compared using repeated measures ANOVA or one-way ANOVA,
24 followed by the Student-Newman-Keuls post-hoc test. Incremental area under the curve (AUC)
25 analyses for plasma glucose and insulin were calculated using GraphPad Prism version 3.02.
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3. Results

3.1. Structural characterisation and DPP-IV stability of GIP peptides.

Following solid-phase peptide synthesis and HPLC purification, the molecular mass of each
peptide was determined using MALDI-TOF MS (Table 1). The molecular masses resolved for
GIP peptides were: 5194.2 Da, GIP(Lys³⁷MYR); 5236.4 Da, *N*-AcGIP(Lys³⁷MYR); 5219.1 Da,
GIP(Lys³⁷PAL); 5263.3 Da, *N*-AcGIP(Lys³⁷PAL); 5249.8 Da, GIP(Lys³⁷STE); and 5292.8 Da,
N-AcGIP(Lys³⁷STE). Peptides were purified to >95% purity and molecular masses correlated
well with predicted theoretical molecular weights, thereby substantiating molecular identity. Also
shown in Table 1, native GIP was rapidly hydrolysed *in vitro* by DPP-IV with an estimated half-

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2 life of 2.2 h. In contrast, GIP analogues remained fully intact throughout the entire incubation
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4 period exhibiting half-lives of >24 h (Table 1).
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8 9 3.2. In vitro cAMP production and insulin secretion

10 Native GIP stimulated intracellular cAMP production in a concentration-dependent manner in
11 BRIN-BD11 cells with an EC₅₀ value of 0.70 nM. GIP analogues followed a similar pattern of
12 concentration-dependent stimulation of cAMP production but exhibited improved EC₅₀ values
13 (0.07 to 0.27 nM; Table 1). Consistent with effects on cAMP production, native GIP dose-
14 dependently stimulated insulin secretion (1.5 to 2.1-fold; $p < 0.05$) in BRIN-BD11 cells
15 compared to control (5.6 mM glucose alone) incubations (Fig. 1). Similarly, GIP analogues dose-
16 dependently stimulated insulin secretion but with enhanced potency compared to control (2.8 to
17 4.0-fold; $p < 0.001$) and to GIP (1.5 to 2.1-fold; $p < 0.01$ to $p < 0.001$). No significant differences
18 were observed in insulin-releasing potency between GIP analogues.
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35 3.3. Acute effects of GIP peptides on glucose-lowering and insulin release in vivo.

36 The acute effects of native GIP and a fatty acid modified GIP analogue, *N*-AcGIP(Lys³⁷MYR),
37 on glucose-lowering and insulin release in the absence and presence of a glucose load are shown
38 in Fig. 2. When native GIP and *N*-AcGIP(Lys³⁷MYR) were administered in combination with
39 saline vehicle to normal mice, no significant effects on plasma glucose and insulin concentrations
40 were observed (Fig. 2A). However, in the presence of a glucose load (18 mmol/kg body weight),
41 *N*-AcGIP(Lys³⁷MYR) significantly lowered plasma glucose (1.2 to 1.7-fold; $p < 0.05$ to $p < 0.01$)
42 and increased plasma insulin concentrations (1.3 to 1.8-fold; $p < 0.05$ to $p < 0.01$) compared to
43 GIP-treated animals (Fig. 2B).
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56 Fig. 3 depicts the plasma glucose responses to ip administration of glucose alone or in
57 combination with GIP peptides to *ob/ob* mice. Administration of glucose alone resulted in a rapid
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1 and protracted rise in plasma glucose concentrations. The plasma glucose excursion following
2 administration of native GIP was not significantly different to animals receiving glucose alone
3 (control). However, administration of GIP analogues resulted in significantly reduced plasma
4 glucose concentrations at all time points measured. This was corroborated by analysis of the area
5 under the curve AUC (0-60 min) where GIP analogues exhibited significantly lower plasma
6 glucose concentrations compared to glucose alone (1.2 to 1.4-fold; $p < 0.05$ to $p < 0.001$) and
7 animals treated with GIP (1.2 to 1.4-fold; $p < 0.05$ to $p < 0.001$). The improved glucose-lowering
8 action correlated with significantly elevated plasma insulin concentrations following
9 administration of GIP(Lys³⁷MYR), *N*-AcGIP(Lys³⁷MYR) and GIP(Lys³⁷PAL) compared to
10 control (1.2 to 1.8-fold; $p < 0.05$ to $p < 0.001$) and GIP-treated animals (1.2 to 1.8-fold; $p < 0.05$
11 to $p < 0.001$; Fig. 4). In contrast, *N*-AcGIP, *N*-AcGIP(Lys³⁷PAL), GIP(Lys³⁷STE) and *N*-
12 AcGIP(Lys³⁷STE) did not demonstrate significantly improved insulinotropic actions under
13 conditions used (Fig. 4).
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3.4. Persistent effects of GIP peptides on glucose-lowering in *ob/ob* mice

32 As illustrated in Fig. 5, the glucose-lowering action of GIP(Lys³⁷MYR) and *N*-
33 AcGIP(Lys³⁷MYR) is clearly evident when administered 24 h prior to an ip glucose load.
34 GIP(Lys³⁷MYR) and *N*-AcGIP(Lys³⁷MYR) significantly lowered plasma glucose at 15 and 60
35 min post injection compared to control (1.2 to 1.3 fold; $p < 0.01$ to $p < 0.001$) and *N*-AcGIP
36 treated mice (1.1 to 1.2 fold; $p < 0.05$ to $p < 0.01$). These results were supported by significantly
37 decreased plasma glucose AUC values compared to control (1.2 fold; $p < 0.05$) and *N*-AcGIP
38 treated mice (1.1 to 1.2 fold; $p < 0.05$ to $p < 0.01$). In contrast, GIP(Lys³⁷PAL), *N*-
39 AcGIP(Lys³⁷PAL), GIP(Lys³⁷STE) and *N*-AcGIP(Lys³⁷STE) demonstrated similar glycaemic
40 profiles to control animals (Fig. 5).
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1 3.5. Effects of daily administration of *N*-AcGIP, GIP(Lys³⁷MYR) and *N*-AcGIP(Lys³⁷MYR)
2 on bodyweight, food intake, non-fasting glucose and insulin concentrations in *ob/ob* mice
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4 Daily administration of *N*-AcGIP, GIP(Lys³⁷MYR) and *N*-AcGIP(Lys³⁷MYR) for 24 days had no
5 effect on body weight and food intake compared to saline-treated controls (Fig. 6A and 6B). Non-
6 fasting plasma glucose concentrations were significantly lower in GIP(Lys³⁷MYR) (1.4 to 1.5-
7 fold; $p < 0.05$ to $p < 0.01$) and *N*-AcGIP(Lys³⁷MYR) (1.5 to 1.7-fold; $p < 0.01$ to $p < 0.001$)
8 treated mice compared to saline-treated controls from Day 20 onwards (Fig. 6C). Furthermore, *N*-
9 AcGIP(Lys³⁷MYR) treated mice exhibited significantly reduced plasma glucose concentrations
10 (1.4-fold; $p < 0.05$) compared to *N*-AcGIP treated animals. This reduced plasma glucose was
11 correlated with significantly increased plasma insulin concentrations by Day 20 in
12 GIP(Lys³⁷MYR) (1.4 to 1.9-fold; $p < 0.01$ to $p < 0.001$) and *N*-AcGIP(Lys³⁷MYR) (1.6 to 2.2-
13 fold; $p < 0.01$ to $p < 0.001$) treated mice compared to saline controls and *N*-AcGIP treated
14 animals (Fig. 6D).
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34 3.6. Effects of daily administration of *N*-AcGIP, GIP(Lys³⁷MYR) and *N*-AcGIP(Lys³⁷MYR)
35 on glucose tolerance and plasma insulin response to glucose in *ob/ob* mice
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37 As shown in Fig. 7A, daily administration of GIP(Lys³⁷MYR) and *N*-AcGIP(Lys³⁷MYR) for 24
38 days resulted in an improved glycaemic response to an ip glucose load with significantly
39 decreased plasma glucose concentrations compared to saline control (1.4 to 1.5 fold; $p < 0.001$)
40 and *N*-AcGIP (1.3 to 1.4 fold; $p < 0.05$ to $p < 0.01$). The beneficial effects of GIP(Lys³⁷MYR)
41 and *N*-AcGIP(Lys³⁷MYR) were particularly evident from plasma glucose AUC values (0-60
42 min), which was significantly reduced compared to saline control (1.4 and 1.5 fold, respectively;
43 $p < 0.01$) (Fig. 7A). Glucose-mediated plasma insulin concentrations were significantly elevated
44 (1.5 to 4.4 fold; $p < 0.01$ to $p < 0.001$) for both GIP(Lys³⁷MYR) and *N*-AcGIP(Lys³⁷MYR)
45 compared to both saline and *N*-AcGIP treated animals (Fig. 7B).
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4 3.7. Effects of daily administration of *N*-AcGIP, GIP(Lys³⁷MYR) and *N*-AcGIP(Lys³⁷MYR)
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6 on insulin sensitivity, pancreatic insulin content and circulating plasma triglyceride
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8 concentrations in *ob/ob* mice
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11 As shown in Table 2, plasma glucose AUC values were not significantly changed in any of the
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13 treatment groups following administration of exogenous insulin. Similarly, pancreatic insulin
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15 content and circulating plasma triglyceride concentrations were not significantly different
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17 between treated and control groups (Table 2).
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25 **4. Discussion**

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27 Renewed interest into the potential use of incretin hormones as antidiabetic therapies has
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29 emerged in recent years [4]. The glucose-dependent insulin secretion induced by GLP-1 and GIP
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31 offers benefits over currently used single action, non-endogenous drugs that stimulate insulin
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33 secretion at any glucose concentration. Lack of effect of GIP on gastric emptying offers a
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35 potential advantage compared with therapies based on GLP-1 [9]. However, circulating GIP is
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37 rapidly hydrolysed by the ubiquitous enzyme DPP-IV and efficiently cleared from the body
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39 through renal filtration [11]. The potential of C-16 fatty acid (PAL) conjugated GIP analogues
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41 has been explored recently with *N*-AcGIP(Lys³⁷PAL) appearing to be the most promising
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43 analogue [15,16]. The present study examined further the effect of fatty acid chain length, with or
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45 without N-terminal acetylation, on biological efficacy through assessing in vitro and in vivo
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47 antihyperglycaemic and insulintropic properties.
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54 Following confirmation of successful Fmoc peptide synthesis by MALDI-TOF MS, the
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56 susceptibility of GIP peptides to DPP-IV was examined. Unlike the native hormone which
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58 displayed an in vitro biological half life of approximately 2.2 h, GIP analogues remained fully
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1 intact throughout the entire 24 h incubation (half-lives >24 h). While the conditions used for the
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3 DPP-IV assay in the present study were not able to discriminate $t_{1/2}$ between analogues, more
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5 detailed examination of SAR would be of interest in future studies. However, despite the
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7 probability of peptide adsorption during the longer incubation periods, this confirms in vitro
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9 DPP-IV resistance of each GIP analogue tested. It therefore appears that N-terminal acetylation
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11 and the addition of C14-C18 fatty acid residue close to the C-terminal region inhibits the action
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13 of DPP-IV, perhaps by disrupting the GIP hydro-affinity for the enzyme, as reported previously
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15 [16]. Interestingly, C14-C18 acylation without additional N-terminal acetylation also resulted in
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17 stable GIP analogues most likely due to steric hindrance.
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25 Broadly similar to previous studies, native GIP concentration-dependently stimulated cAMP
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27 production with an EC_{50} value of 0.70 nM [16]. Likewise, GIP analogues stimulated cAMP
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29 production in a concentration-dependent manner. However, analogues demonstrated up to a 10-
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31 fold reduction in EC_{50} value relative to GIP. This suggests enhanced potency at the GIP receptor,
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33 and is indicative of preserved receptor affinity with improved activation of the adenylate cyclase
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35 transduction pathway. This increased potency is corroborated by GIP analogues significantly
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37 inducing concentration-dependant stepwise increases in insulin secretion from BRIN-BD11 cells.
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39 At the highest concentrations, GIP analogues evoked a notable improvement in insulin secretion
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41 which quite likely reflects both increased potency and enhanced biological half-life [16]. Since
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43 no significant degradation of GIP occurred during these in vitro incubations, these data suggest
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45 that the combination of increased DPP-IV resistance and improved biological potency at the
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47 cellular level should provide analogues with greatly enhanced activity in vivo.
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56 To assess the antihyperglycaemic and insulinotropic effects of GIP analogues in vivo, we
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58 employed the *ob/ob* mouse model from the Aston colony [20]. On this genetic background, *ob/ob*
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1 mice present with hyperphagia, marked obesity, moderate hyperglycaemia and severe
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3 hyperinsulinaemia representing a very robust model of type 2 diabetes and obesity. As in
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5 previous studies, native GIP had no significant effect on plasma glucose or the insulin response
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7 following an intraperitoneal glucose load [16,17] due to its short biological half-life following
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9 degradation by DPP-IV and efficient renal clearance. In contrast, all GIP analogues demonstrated
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11 improved glucose-lowering actions. However, only GIP(Lys³⁷MYR), *N*-AcGIP(Lys³⁷MYR) and
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13 GIP(Lys³⁷PAL) appeared substantially more effective and longer-acting in stimulating insulin
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15 release. Reasons underlying these differences are unclear but likely reflect alterations in beta-cell
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17 stimulation possibly due to changes in effective concentrations as a result of protein binding and
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19 various non beta-cell and extrapancreatic actions [4,16]. Nevertheless, the magnitude of
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21 improvements in the glycaemic and insulinotropic responses of GIP(Lys³⁷MYR), *N*-
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23 AcGIP(Lys³⁷MYR) and GIP(Lys³⁷PAL) is notable given the severe insulin resistance of the
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25 *ob/ob* syndrome [20]. Importantly, when *N*-AcGIP(Lys³⁷MYR) was injected acutely together
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27 with saline in normal mice, no significant effects on glucose-lowering or insulin release were
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29 observed. However, when co-injected with a glucose load *N*-AcGIP(Lys³⁷MYR) significantly
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31 lowered plasma glucose and increased plasma insulin concentrations, thus demonstrating a potent
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33 glucose-dependent effect. From these acute studies it appears that DPP-IV resistant analogues of
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35 GIP can substantially overcome any possible defects in responsiveness of GIP in type 2 diabetes.
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46 The longer-acting glucose-lowering effects of GIP analogues were assessed through an
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48 intraperitoneal glucose tolerance in animals 24 h following peptide administration. Interestingly,
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50 only GIP(Lys³⁷MYR) and *N*-AcGIP(Lys³⁷MYR) elicited a persistent improvement in glycaemic
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52 control 24 h after administration. The reason why a persistent glucose-lowering effect was not
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54 observed with the palmitic acid GIP analogues is unclear [15,16], but could be due to differences
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56 in the conformation or activity of the analogue. The prolonged action of GIP(Lys³⁷MYR) and *N*-
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1 AcGIP(Lys³⁷MYR) suggests a much more extended plasma half-life, reflecting an enhanced
2 ability of this shorter C-14 chain to structurally orientate and bind more strongly to serum
3 proteins preventing kidney filtration [11]. The development of a specific assay to assess plasma
4 levels of these fatty acid GIP analogues would be necessary to provide more precise details of
5 these kinetics.
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16 Results of these acute in vivo studies provided a strong basis for the subsequent 24 day study.
17 Daily intraperitoneal injection of *N*-AcGIP, GIP(Lys³⁷MYR) and *N*-AcGIP(Lys³⁷MYR) over this
18 time had no apparent adverse or toxic effects. Bodyweight and food intake were similar to saline-
19 treated controls confirming previous findings [15]. Importantly, GIP(Lys³⁷MYR) and *N*-
20 AcGIP(Lys³⁷MYR), and to a lesser effect, *N*-AcGIP, significantly reduced plasma glucose levels
21 from day 20 of the study, accompanied by significantly elevated plasma insulin levels. Glucose
22 tolerance was markedly improved in animals treated with GIP(Lys³⁷MYR) and *N*-
23 AcGIP(Lys³⁷MYR) and this was accompanied by significantly increased plasma insulin
24 concentrations. This confirms the long-acting insulinotropic effects of these analogues and their
25 ability to overcome the severe beta-cell defect in this model. Improvement in glucose
26 homeostasis was independent of any change in insulin sensitivity which is in agreement with
27 previous findings [14-18]. There were no observed changes in pancreatic insulin content or
28 plasma triglyceride levels although a more prolonged treatment period may be necessary to see
29 improvements in these parameters.
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51 In conclusion, this study demonstrated that fatty acid derivatisation at position Lys³⁷, with or
52 without N-terminal acetylation confers DPP-IV resistance and improved biological efficacy
53 during in vitro and acute in vivo studies. Furthermore, derivatisation with a C-14 fatty acid
54 offered a more protracted and markedly improved biological function over C-16 and C-18 fatty
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1 acid counterparts. These data represent an important step in the progression of fatty acid
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3 derivatised GIP analogues as a potential therapy for type 2 diabetes. Further studies are required
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5 to answer whether or not these analogues will be useful in human diabetes treatment.
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10 11 12 **Acknowledgements**

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17
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19
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30 **References**

- 31
32 [1] Brown JC. Enteroinsular axis. In Gut Peptides: Biochemistry and Physiology; Dockray,
33
34 GJ, Walsh, JH, Eds; Raven Press: New York, 1994;pp 765-784.
35
36
37 [2] Holst JJ, Vilsbøll T, Deacon CF. The incretin system and its role in type 2 diabetes
38
39 mellitus. Mol Cell Endocrinol 2009;297:127-136.
40
41
42 [3] Creutzfeldt W. The entero-insular axis in type 2 diabetes – incretins as therapeutic agents.
43
44 Exp Clin Endocrinol Diabetes 2001;109:288-303.
45
46
47 [4] Irwin N, Flatt PR, Gault VA. GIP-based therapeutics for diabetes and obesity. Curr Chem
48
49 Bio 2008;2:61-68.
50
51 [5] Vilsbøll T, Knop FK, Krarup T, Johansen A, Madsbad S, Laren S, et al. The
52
53 pathophysiology of diabetes involves a defective amplification of late phase insulin
54
55 response to glucose by glucose-dependent insulintropic polypeptide-regardless of
56
57 etiology and phenotype. J Clin Endocrinol Metab 2003;88:4897-4903.
58
59
60
61
62
63
64
65

- 1
2 [6] Nauck MA, Heimesaat MM, Ørskov C, Holst JJ, Ebert R, Creutzfeldt W. Preserved
3
4 incretin activity of glucagon-like peptide 1 [7-36 amide] but not of synthetic human
5
6 gastric inhibitory polypeptide in patients with type 2 diabetes mellitus. *J Clin Invest*
7
8 1993;91:301-307.
9
- 10
11 [7] Piteau S, Oliver A, Kim SJ, Winter K, Pospisilik JA, Lynn F, et al. Reversal of islet GIP
12
13 receptor down-regulation and resistance to GIP by reducing hyperglycaemia in Zucker
14
15 rat. *Biochem Biophys Res Commun* 2007;362:1007-1012.
16
17
- 18 [8] Aaboe K, Knop FK, Vilsbøll T, Madsbad S, Deacon CF, Holst JJ, et al. Acute
19
20 administration of a sulfonylurea compound restores the insulinotropic effect of glucose-
21
22 dependent insulinotropic polypeptide in patients with type 2 diabetes. *Diabetologia*
23
24 2007;50(S1):S248 [Abstract].
25
26
- 27 [9] Green BD, Irwin N, Gault VA, O'Harte FPM, Flatt PR. Development and therapeutic
28
29 potential of incretin hormone analogues for type 2 diabetes. *Br J Diabetes Vas Dis*
30
31 2005;5:134-140.
32
33
- 34 [10] Kieffer TJ, McIntosh CH, Pederson RA. Degradation of glucose-dependant insulinotropic
35
36 polypeptide and truncated glucagon-like peptide 1 in vitro and in vivo by dipeptidyl
37
38 peptidase IV. *Endocrinology* 1995;136:3585-3596.
39
40
- 41 [11] Meier JJ, Nauck MA, Kranz D, Holst JJ, Deacon CF, Gaeckler D, et al. Secretion,
42
43 degradation, and elimination of glucagon-like peptide 1 and gastric inhibitory polypeptide
44
45 in patients with chronic renal insufficiency and healthy control subjects. *Diabetes*
46
47 2004;53:654-662.
48
49
- 50
51 [12] Flatt PR, Green BD, Bailey CJ. Dipeptidyl peptidase IV (DPP IV) and related molecules
52
53
54 in type 2 diabetes. *Front Biosci* 2008;13:3648-3660.
55
56
57
58
59
60
61
62
63
64
65

- 1
2 [13] Gault VA, Kerr BD, Irwin N, Flatt PR. C-terminal mini-PEGylation of glucose-dependent
3
4 insulinotropic polypeptide exhibits metabolic stability and improved glucose homeostasis
5
6 in dietary-induced diabetes. *Biochem Pharmacol* 2008;75:2325-2333.
7
- 8 [14] Irwin N, Green BD, Gault VA, Greer B, Harriott P, Bailey CJ, et al. Degradation, insulin
9
10 secretion and antihyperglycemic actions of two palmitate-derivitized N-terminal
11
12 proglutamyl analogues of glucose dependent insulinotropic polypeptide. *J Med Chem*
13
14 2005;48:1244-1250.
15
16
- 17 [15] Irwin N, Green BD, Mooney MH, Greer B, Harriott P, Bailey CJ, et al. A novel, long-
18
19 acting agonist of glucose-dependent insulinotropic polypeptide suitable for once-daily
20
21 administration in type 2 diabetes. *J Pharmacol Exp Ther* 2005;314:1187-1194.
22
23
- 24 [16] Irwin N, Gault VA, Green BD, Greer B, Harriot P, Bailey CJ, et al. Antidiabetic potential
25
26 of two novel fatty acid derivatised, N-terminally modified analogues of glucose-
27
28 dependent insulinotropic polypeptide (GIP): N-AcGIP(LysPAL¹⁶) and N-
29
30 AcGIP(LysPAL³⁷). *Biol Chem* 2005;386:679-687.
31
32
- 33 [17] Irwin N, O'Harte FPM, Gault VA, Green BD, Greer B, Harriott P, et al. GIP(Lys16PAL)
34
35 and GIP(Lys37PAL): novel long-acting acylated analogues of glucose-dependent
36
37 insulinotropic polypeptide with improved antidiabetic potential. *J Med Chem*
38
39 2006;49:1047-1054.
40
41
- 42 [18] Irwin N, Clarke GC, Green BD, Greer B, Harriott P, Gault VA, et al. Evaluation of the
43
44 antidiabetic activity of DPP IV resistant N-terminally modified versus mid-chain acylated
45
46 analogues of glucose-dependent insulinotropic polypeptide. *Biochem Pharmacol*
47
48 2006;72:719-728.
49
50
- 51 [19] Vilsbøll T, Brock B, Perrild H, Levin K, Lervang HH, Kølendorf K, et al. Liraglutide, a
52
53 once-daily human GLP-1 analogue, improves pancreatic B-cell function and arginine-
54
55
56
57
58
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- 1 stimulated insulin secretion during hyperglycaemia in patients with Type 2 diabetes
2
3
4 mellitus. *Diabet Med* 2008;25:152-156.
5
- 6 [20] Bailey CJ, Flatt PR. Influence of genetic background and age on the expression of the
7
8 obese hyperglycaemic syndrome in Aston *ob/ob* mice. *Int J Obes* 1982;6:11-21.
9
- 10 [21] Fields GB, Noble RL. Solid-phase peptide synthesis utilizing 9-florenylmethoxycarbonyl
11
12 amino acids. *Int J Pept Prot Res* 1990;35:161-214.
13
14
- 15 [22] McClenaghan NH, Barnett CR, Ah-Sing E, Abdel-Wahab YH, O'Harte FPM, Yoon TW,
16
17 et al. Characterization of a novel glucose-responsive insulin-secreting cell line, BRIN-
18
19 BD11, produced by electro-fusion. *Diabetes* 1996;45:1132-1140.
20
21
- 22 [23] Stevens JF. Determination of glucose by an automated analyser. *Clin Chem Acta*
23
24 1971;32:199-201.
25
26
- 27 [24] Flatt PR, Bailey CJ. Abnormal plasma glucose and insulin responses in heterozygous lean
28
29 (*ob/+*) mice. *Diabetologia* 1981;20:573-577.
30
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36
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Table 1 In vitro characteristics of GIP peptides with and without N-terminal acetylation

Peptide	MALDI-TOF MS		Estimated half-life (h)	cAMP EC ₅₀ (nM)
	Theoretical (Da)	Experimental (Da)		
GIP	4982.4	4982.2	2.2	0.70 ± 0.40
<i>N</i> -AcGIP	5023.0	5022.2	>24	0.17 ± 0.01
GIP(Lys ³⁷ MYR)	5192.8	5194.2	>24	0.27 ± 0.03
<i>N</i> -AcGIP(Lys ³⁷ MYR)	5235.8	5236.4	>24	0.07 ± 0.01
GIP(Lys ³⁷ PAL)	5218.0	5219.1	>24	0.07 ± 0.03
<i>N</i> -AcGIP(Lys ³⁷ PAL)	5264.0	5263.3	>24	0.11 ± 0.03
GIP(Lys ³⁷ STE)	5247.0	5249.8	>24	0.08 ± 0.02
<i>N</i> -AcGIP(Lys ³⁷ STE)	5290.0	5292.8	>24	0.09 ± 0.01

MALDI-TOF MS: purified peptide samples were mixed with α -cyano-4 hydroxycinnamic acid, applied to the sample plate of a Voyager-DE BioSpectrometry Workstation and mass-to-charge (m/z) ratio vs relative peak intensity recorded. **DPP-IV degradation:** resistance of GIP and GIP analogues to degradation by DPP-IV was measured (n=4) following 0, 2, 4, 8 and 24 h incubations. Reaction products were subsequently analysed by HPLC and degradation calculated as a percentage of intact peptide relative to the major degradation fragment, GIP(3-42). **cAMP production:** EC₅₀ values calculated are expressed as mean ± SEM of at least 3 independent experiments.

Table 2 Effects of daily administration of *N*-AcGIP, GIP(Lys³⁷MYR) and *N*-GIP(Lys³⁷MYR) on insulin sensitivity, insulin content and circulating triglycerides in *ob/ob* mice.

Peptide treatment	Insulin sensitivity (glucose AUC)	Insulin content (ng/g tissue)	Triglycerides (ng/dl)
Saline vehicle	898.7 ± 80.0	3.9 ± 0.4	2.2 ± 0.2
<i>N</i> -AcGIP	997.5 ± 72.6	4.0 ± 0.3	2.1 ± 0.3
GIP(Lys ³⁷ MYR)	912.0 ± 34.2	4.0 ± 0.4	2.4 ± 0.3
<i>N</i> -GIP(Lys ³⁷ MYR)	902.3 ± 95.9	4.5 ± 0.5	2.1 ± 0.2

Insulin sensitivity: exogenous insulin (50 U/kg body weight; ip) was administered after daily treatment with GIP peptides (25 nmol/kg body weight) for 24 days. Plasma glucose AUC values for 0-60 min post injection were calculated. **Pancreatic insulin content and circulating triglycerides:** parameters were measured after daily treatment with GIP peptides for 24 days. Data are expressed as mean ± SEM for 8 mice.

Legends to Figures

Fig. 1 - Effects of GIP peptides on insulin secretion. Various concentrations of GIP peptides were incubated with BRIN-BD11 cells in the presence of 5.6 mM glucose for 20 min (n=8) and insulin release measured using RIA. Data are expressed as mean \pm SEM. *p < 0.05, **p < 0.01, ***p < 0.001 compared to glucose alone. Δ p < 0.05, $\Delta\Delta$ p < 0.01, $\Delta\Delta\Delta$ p < 0.001 compared with GIP at the same concentration.

Fig. 2 - Acute effects of GIP and *N*-AcGIP(Lys³⁷MYR) on glucose-lowering and glucose-induced insulin release in normal mice. (A) Plasma glucose and insulin concentrations were measured prior to and after ip administration of saline alone (0.9% (w/v) NaCl) or in combination with GIP or *N*-AcGIP(Lys³⁷MYR) (each at 25 nmol/kg body weight). (B) Plasma glucose and insulin concentrations were measured prior to and after ip administration of glucose alone (18 mmol/kg bw) or in combination with GIP or *N*-AcGIP(Lys³⁷MYR) (each at 25 nmol/kg body weight). Plasma glucose and insulin AUC values for 0-60 min post injection are also included. Data are expressed as mean \pm SEM for 6 mice. *p < 0.05, **p < 0.01, ***p < 0.001 compared to glucose alone. Δ p < 0.05, $\Delta\Delta$ p < 0.01, $\Delta\Delta\Delta$ p < 0.001 compared with GIP treated mice. \square , control; \circ , GIP; ∇ , *N*-AcGIP(Lys³⁷MYR).

Fig. 3 - Acute effects of GIP peptides on glucose-lowering in *ob/ob* mice. Plasma glucose concentrations were measured prior to and after ip administration of glucose alone (18 mmol/kg body weight) or in combination with GIP peptides (25 nmol/kg body weight). Plasma glucose AUC values for 0-60 min post injection are also included. Data are expressed as mean \pm SEM for 6 mice. *p < 0.05, **p < 0.01, ***p < 0.001 compared to glucose alone. Δ p < 0.05, $\Delta\Delta$ p < 0.01, $\Delta\Delta\Delta$ p < 0.001 compared with GIP treated mice. \square , control; \circ , GIP; \blacktriangle , *N*-AcGIP; \blacksquare ,

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GIP(Lys³⁷MYR); ▽, *N*-AcGIP(Lys³⁷MYR); ▼, GIP(Lys³⁷PAL); ◇, *N*-AcGIP(Lys³⁷PAL); ●,
GIP(Lys³⁷STE); Δ, *N*-AcGIP(Lys³⁷STE).

Fig. 4 - Acute effects of GIP peptides on insulin release in *ob/ob* mice. Plasma insulin concentrations were measured prior to and after ip administration of glucose alone (18 mmol/kg body weight) or in combination with GIP peptides (25 nmol/kg body weight). Plasma insulin AUC values for 0-60 min post injection are also included. Data are expressed as mean ± SEM for 6 mice. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 compared to glucose alone. ^Δ*p* < 0.05, ^{ΔΔ}*p* < 0.01, ^{ΔΔΔ}*p* < 0.001 compared with GIP treated mice. □, control; ○, GIP; ▲, *N*-AcGIP; ■, GIP(Lys³⁷MYR); ▽, *N*-AcGIP(Lys³⁷MYR); ▼, GIP(Lys³⁷PAL); ◇, *N*-AcGIP(Lys³⁷PAL); ●, GIP(Lys³⁷STE); Δ, *N*-AcGIP(Lys³⁷STE).

Fig. 5 - Persistence of glucose-lowering effects of GIP peptides in *ob/ob* mice. Plasma glucose concentrations were measured prior to and after ip administration of glucose alone (18 mmol/kg body weight) in animals injected 24 h previously with GIP peptides (25 nmol/kg body weight). Plasma glucose AUC values for 0-60 min post injection are also included. Data are expressed as mean ± SEM for 6 mice. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 compared to glucose alone. ^Δ*p* < 0.05, ^{ΔΔ}*p* < 0.01 compared with *N*-AcGIP treated mice. □, control; ○, GIP; ▲, *N*-AcGIP; ■, GIP(Lys³⁷MYR); ▽, *N*-AcGIP(Lys³⁷MYR); ▼, GIP(Lys³⁷PAL); ◇, *N*-AcGIP(Lys³⁷PAL); ●, GIP(Lys³⁷STE); Δ, *N*-AcGIP(Lys³⁷STE).

Fig. 6 - Effects of daily administration of *N*-AcGIP, GIP(Lys³⁷MYR) and *N*-AcGIP(Lys³⁷MYR) on (A) body weight, (B) food intake, (C) non-fasting plasma glucose and (D) non-fasting plasma insulin in *ob/ob* mice. Parameters were measured prior to and 24 days during treatment with GIP

1 peptides (25 nmol/kg body weight) or saline vehicle (0.9% (w/v) NaCl). Data are expressed as
2 mean \pm SEM for 8 mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to saline-treated group.
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6 $\Delta p < 0.05$, $\Delta\Delta p < 0.01$, $\Delta\Delta\Delta p < 0.001$ compared with *N*-AcGIP-treated mice. \square , saline vehicle; \blacktriangle ,
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8 *N*-AcGIP; \blacksquare , GIP(Lys³⁷MYR); ∇ , *N*-AcGIP(Lys³⁷MYR).
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13 Fig. 7 - Effects of daily administration of *N*-AcGIP, GIP(Lys³⁷MYR) and *N*-AcGIP(Lys³⁷MYR)
14 on (A) glucose tolerance and (B) plasma insulin response to glucose in *ob/ob* mice. Tests were
15 conducted after daily treatment with GIP peptides (25 nmol/kg body weight) or saline vehicle
16 (0.9% (w/v) NaCl) for 24 days. Glucose (18 mmol/kg body weight; ip) was administered at time
17 $t=0$. Plasma glucose and insulin AUC values for 0-180 min post injection are also included. Data
18 are expressed as mean \pm SEM for 8 mice. ** $p < 0.01$, *** $p < 0.001$ compared to saline-treated
19 group. $\Delta p < 0.05$, $\Delta\Delta p < 0.01$, $\Delta\Delta\Delta p < 0.001$ compared with *N*-AcGIP-treated mice. \square , saline
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Figure 1

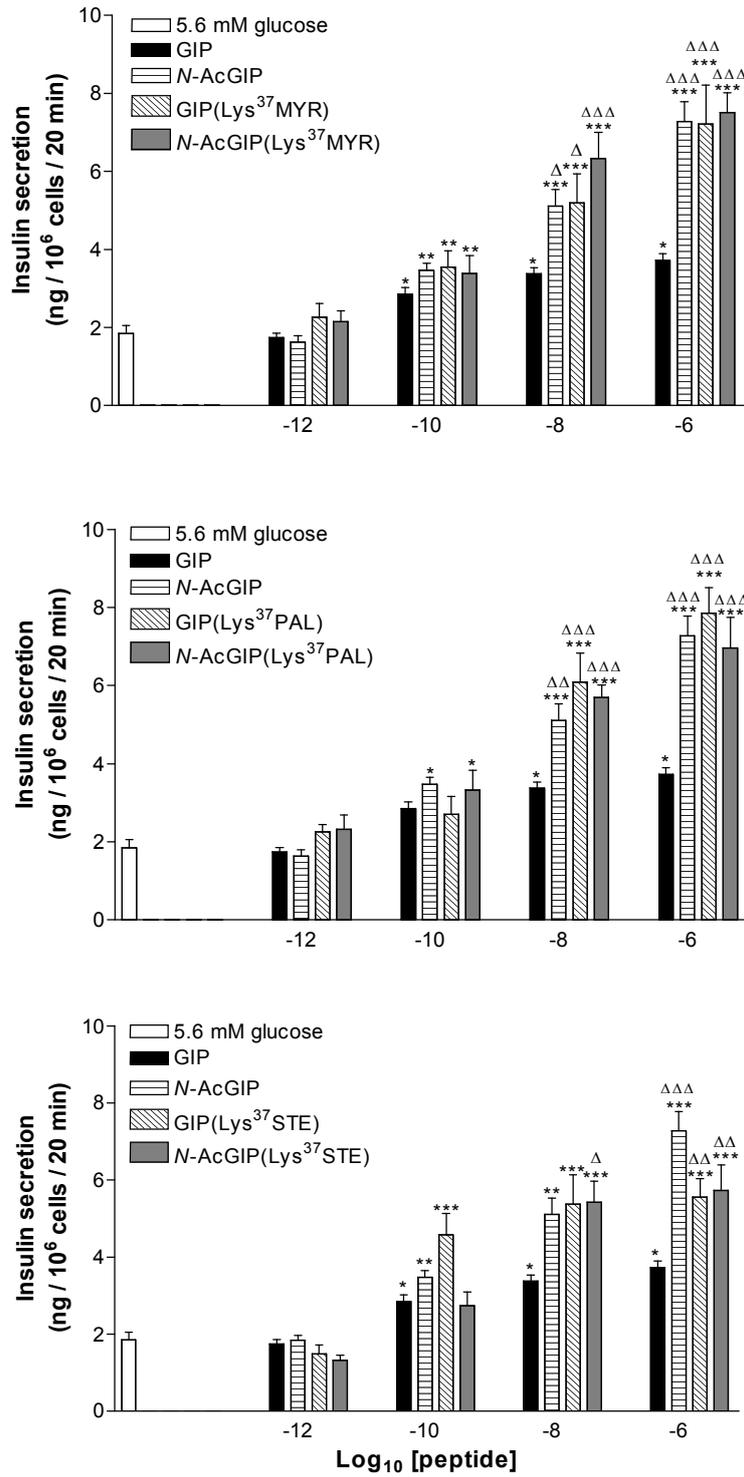


Figure 2

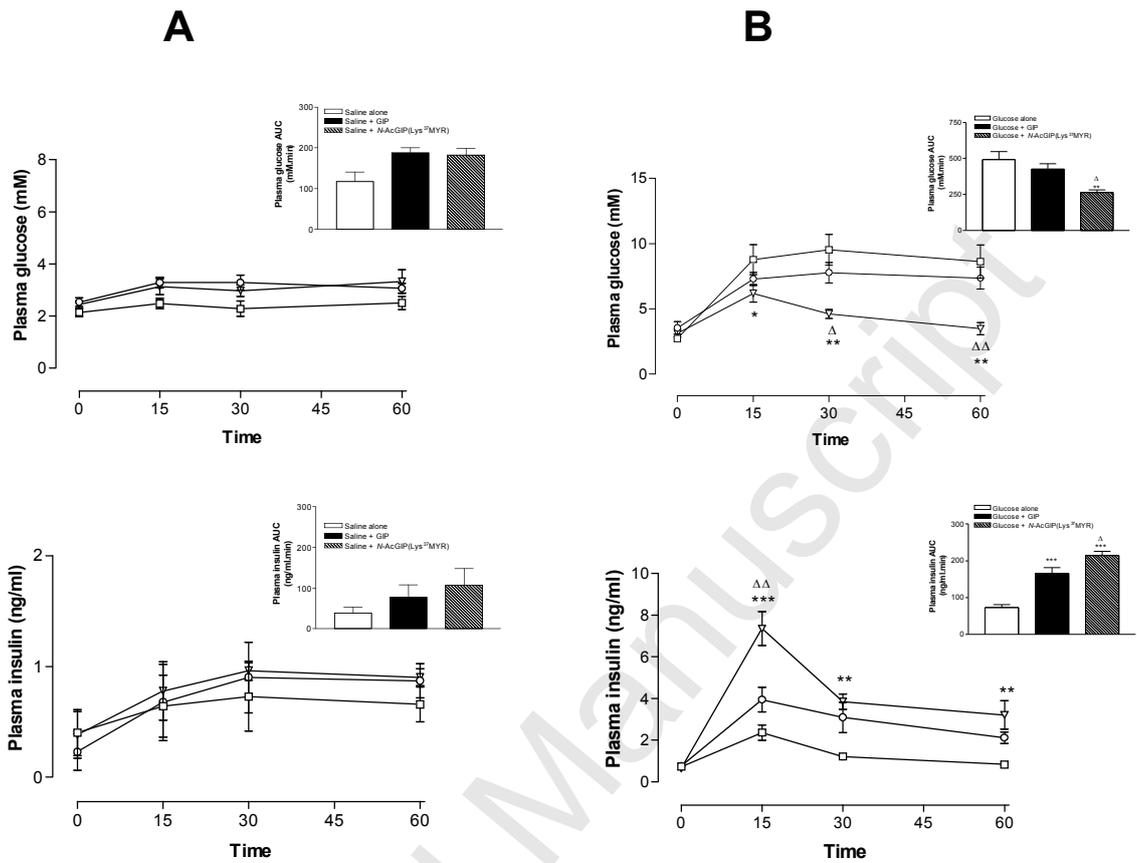


Figure 3

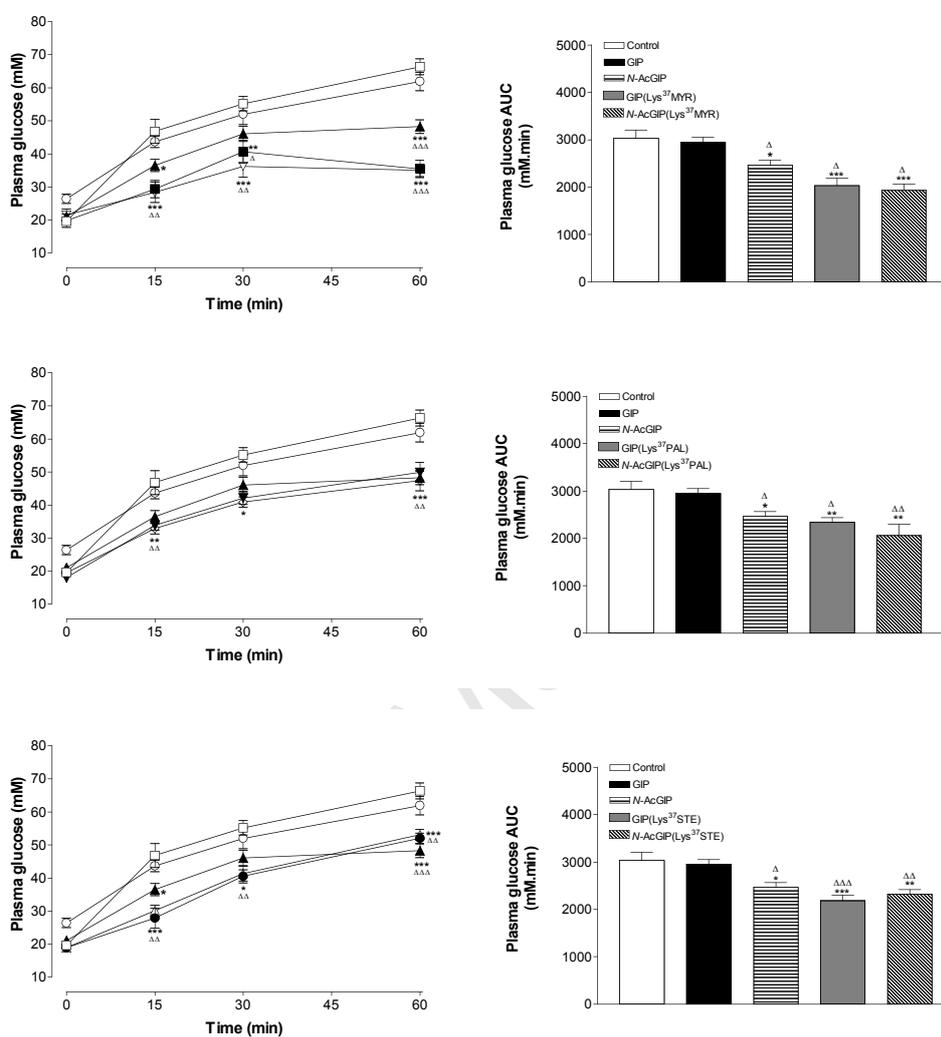


Figure 4

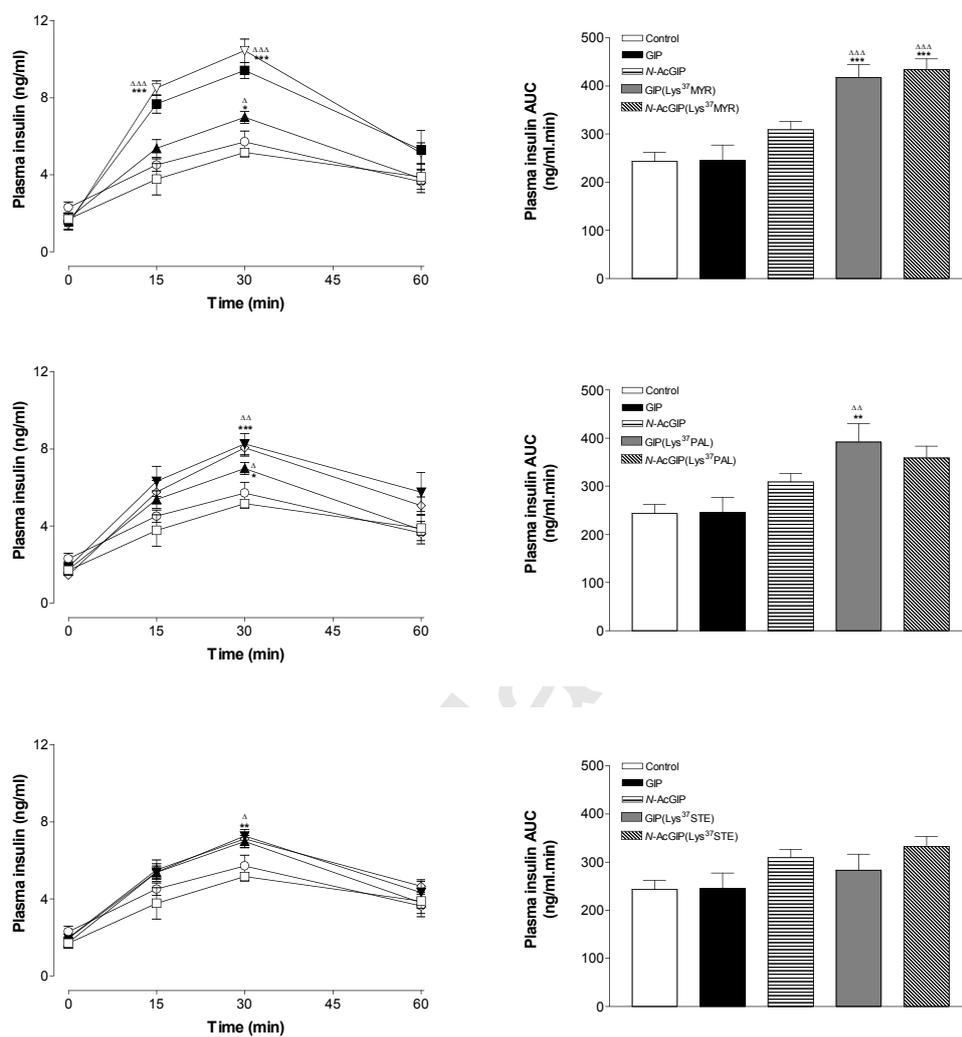


Figure 5

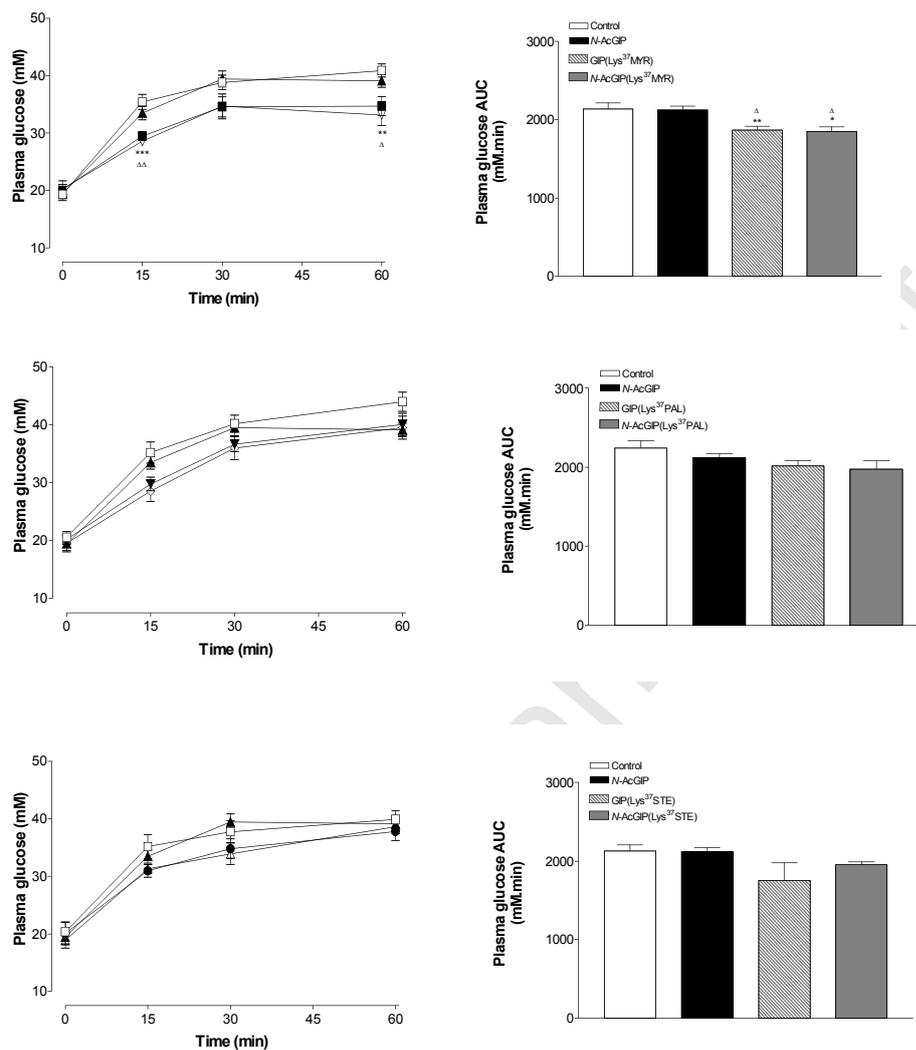


Figure 6

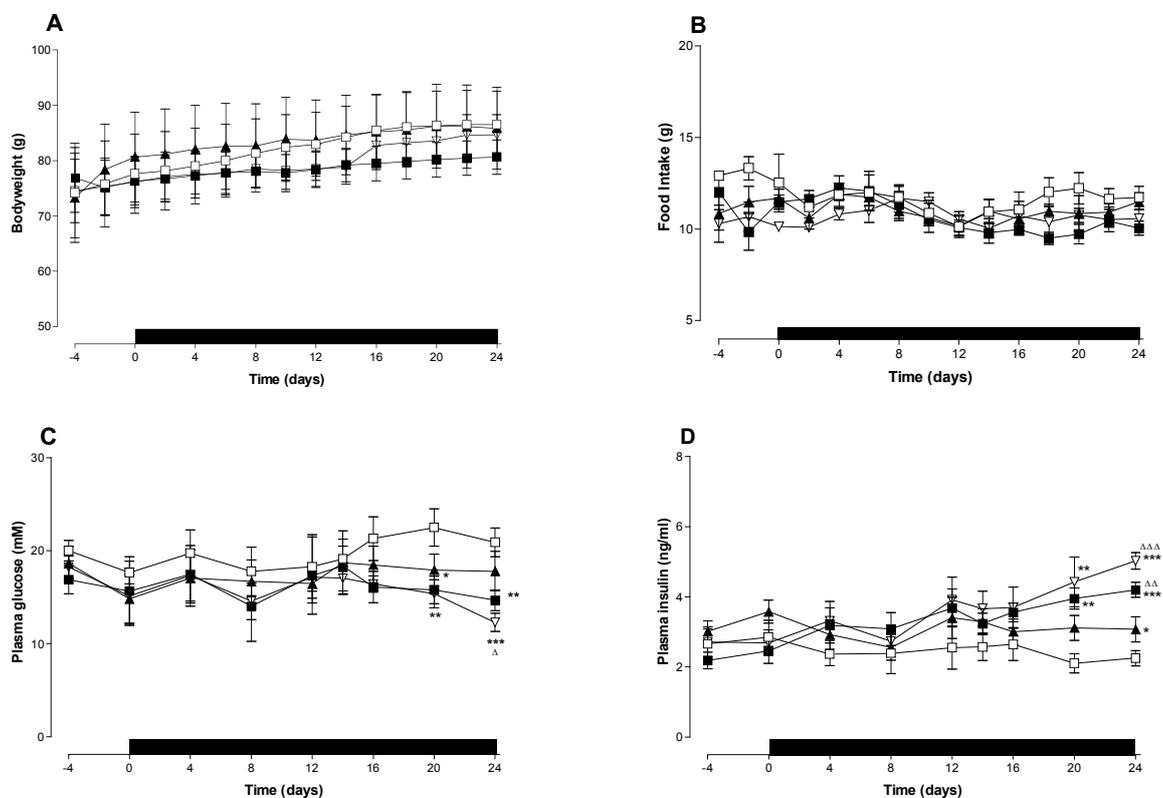


Figure 7

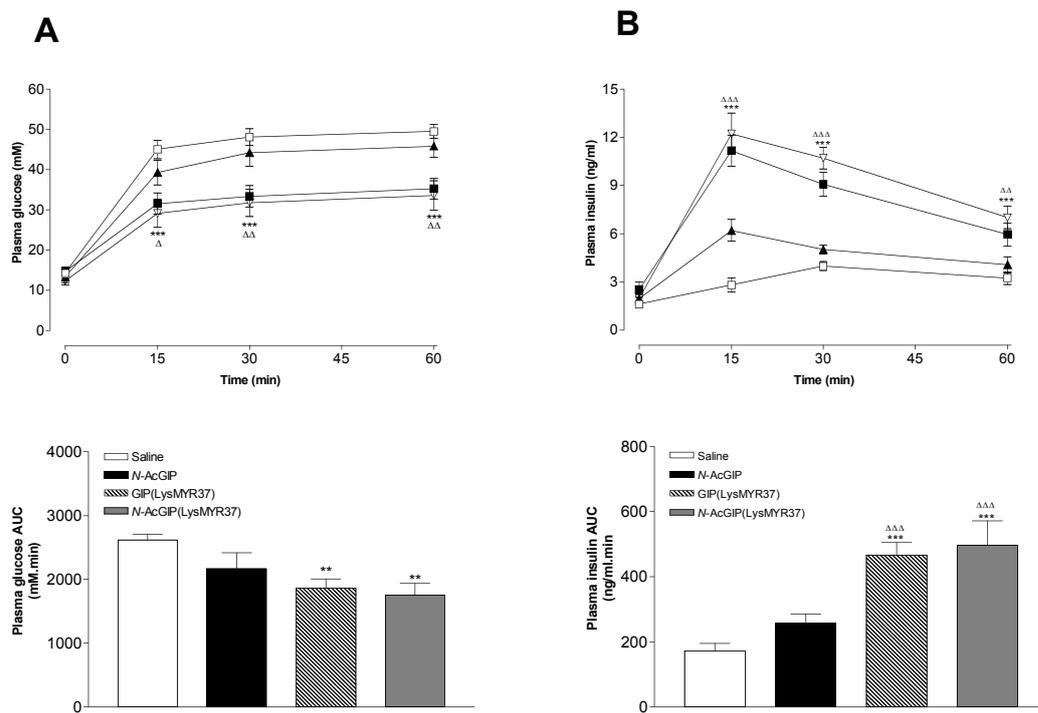


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GIP(Lys ³⁷ STE)	5247.0	5249.8	>24	0.08 ± 0.02
<i>N</i> -AcGIP(Lys ³⁷ STE)	5290.0	5292.8	>24	0.09 ± 0.01

MALDI-TOF MS: purified peptide samples were mixed with α -cyano-4 hydroxycinnamic acid, applied to the sample plate of a Voyager-DE BioSpectrometry Workstation and mass-to-charge (m/z) ratio vs relative peak intensity recorded. **DPP-IV degradation:** resistance of GIP and GIP analogues to degradation by DPP-IV was measured (n=4) following 0, 2, 4, 8 and 24 h incubations. Reaction products were subsequently analysed by HPLC and degradation calculated as a percentage of intact peptide relative to the major degradation fragment, GIP(3-42). **cAMP production:** EC₅₀ values calculated are expressed as mean ± SEM of at least 3 independent experiments.

Table 2 Effects of daily administration of *N*-AcGIP, GIP(Lys³⁷MYR) and *N*-GIP(Lys³⁷MYR) on insulin sensitivity, insulin content and circulating triglycerides in *ob/ob* mice.

Peptide treatment	Insulin sensitivity (glucose AUC)	Insulin content (ng/g tissue)	Triglycerides (ng/dl)
Saline vehicle	898.7 ± 80.0	3.9 ± 0.4	2.2 ± 0.2
<i>N</i> -AcGIP	997.5 ± 72.6	4.0 ± 0.3	2.1 ± 0.3
GIP(Lys ³⁷ MYR)	912.0 ± 34.2	4.0 ± 0.4	2.4 ± 0.3
<i>N</i> -GIP(Lys ³⁷ MYR)	902.3 ± 95.9	4.5 ± 0.5	2.1 ± 0.2

Insulin sensitivity: exogenous insulin (50 U/kg body weight; ip) was administered after daily treatment with GIP peptides (25 nmol/kg body weight) for 24 days. Plasma glucose AUC values for 0-60 min post injection were calculated. **Pancreatic insulin content and circulating triglycerides:** parameters were measured after daily treatment with GIP peptides for 24 days. Data are expressed as mean ± SEM for 8 mice.

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Figure 1

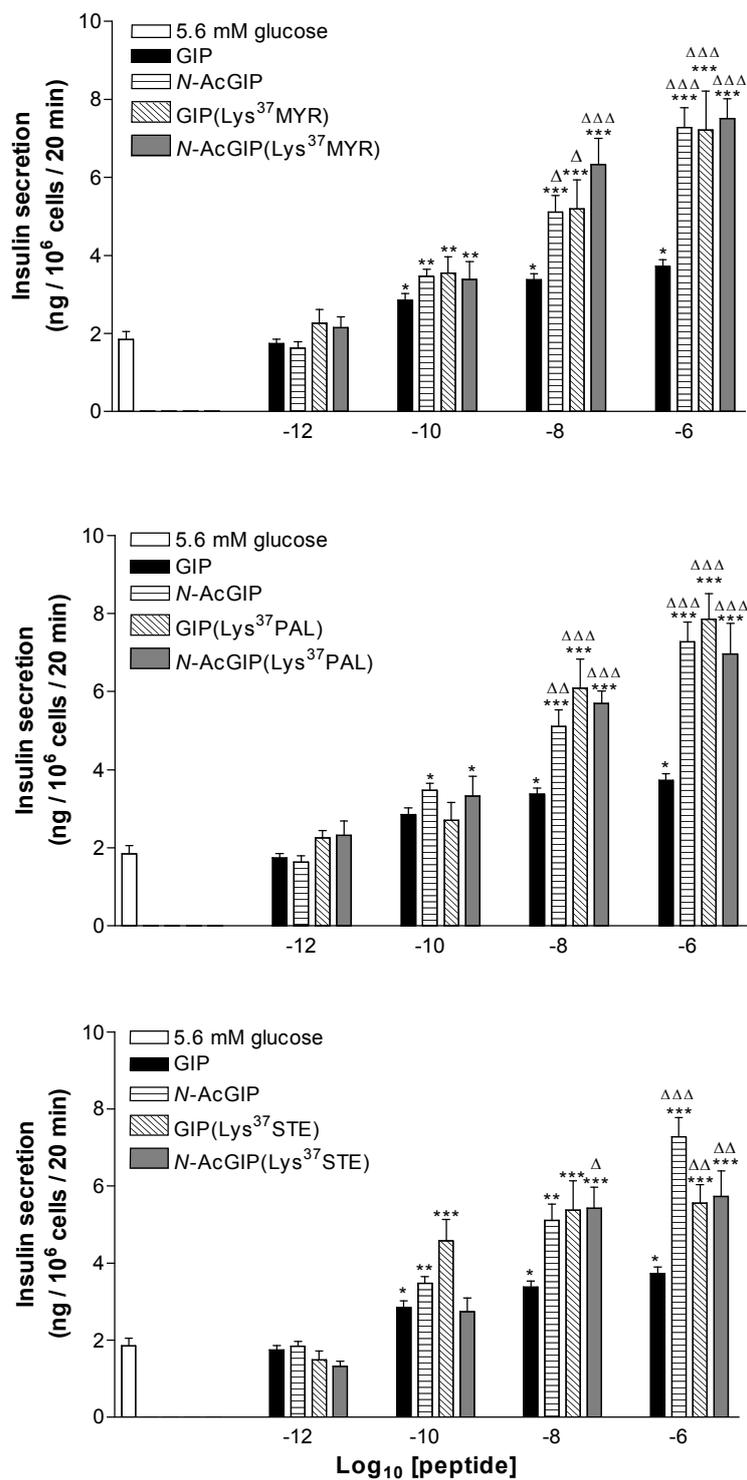


Figure 2

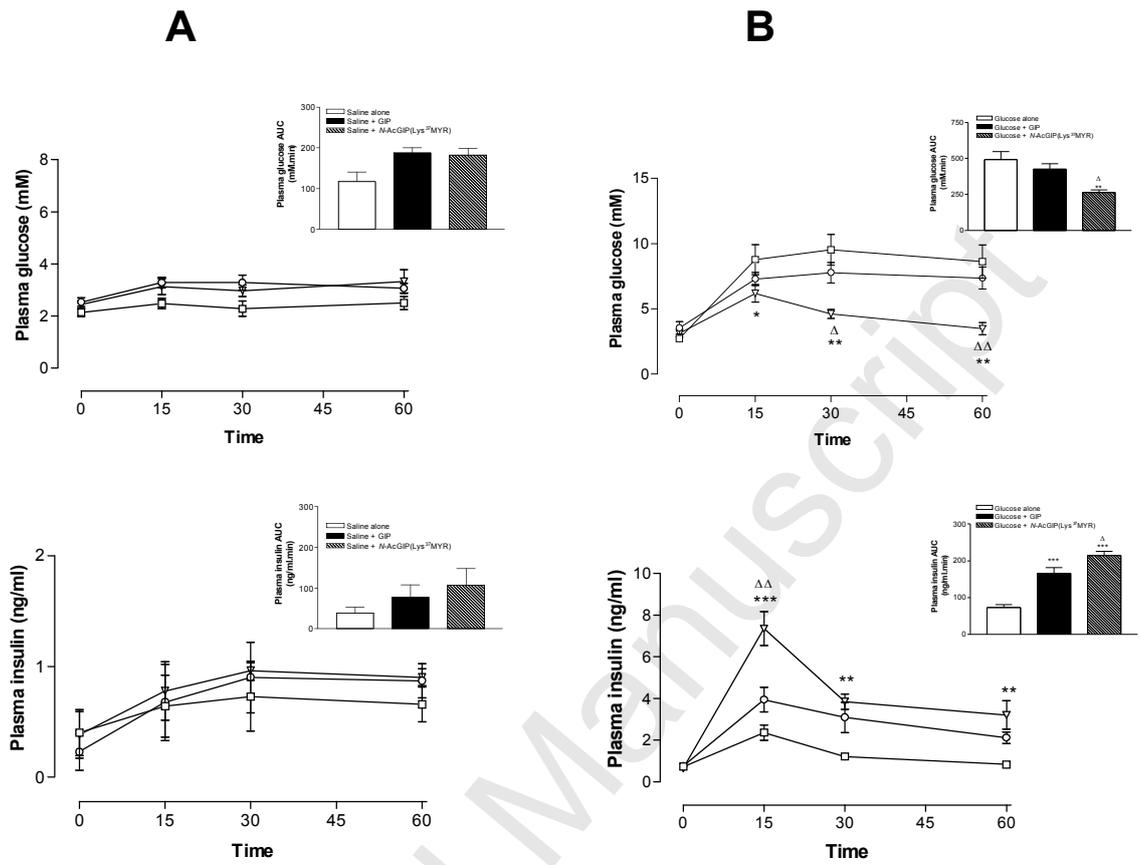


Figure 3

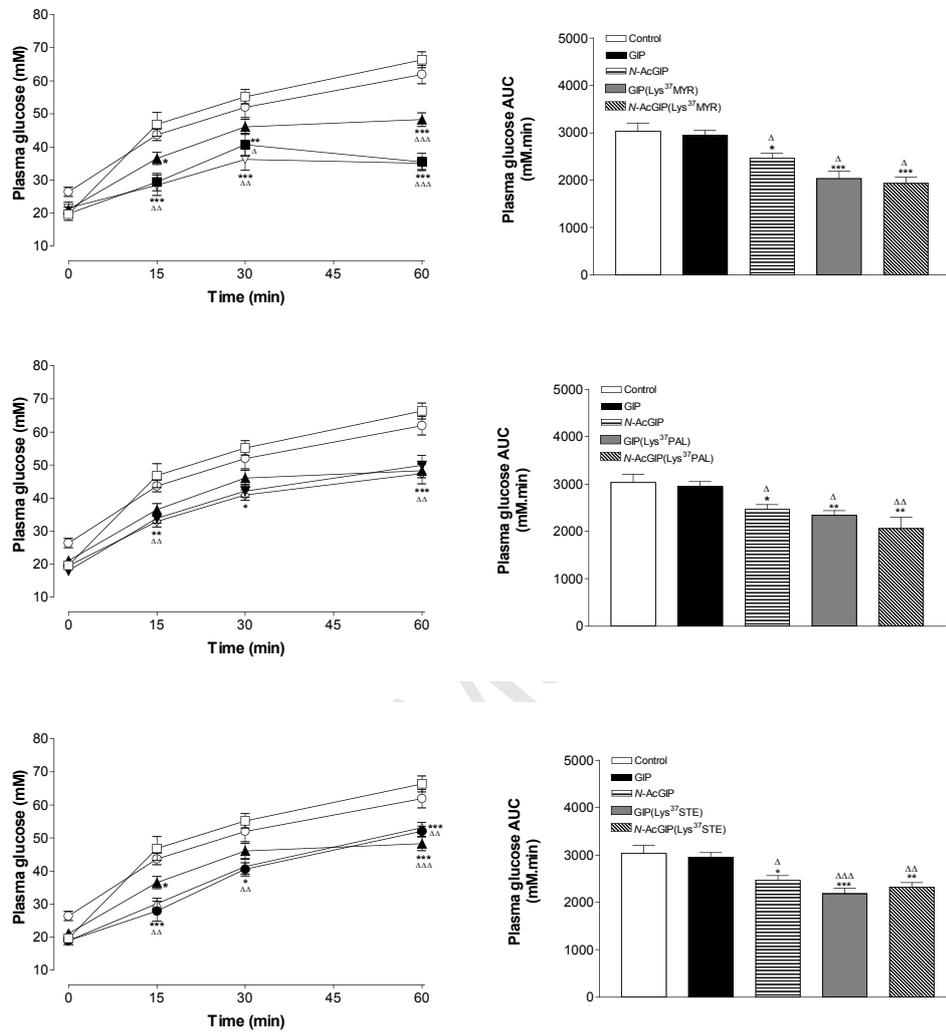


Figure 4

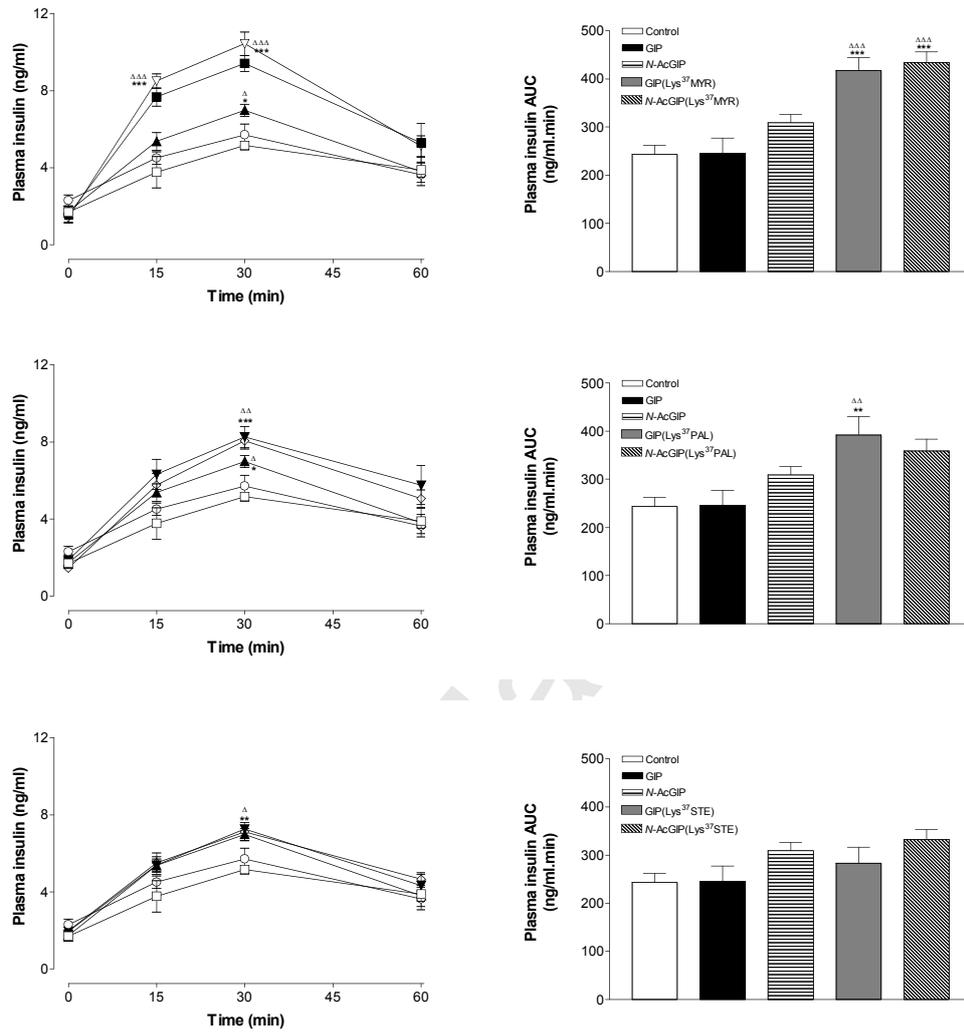


Figure 5

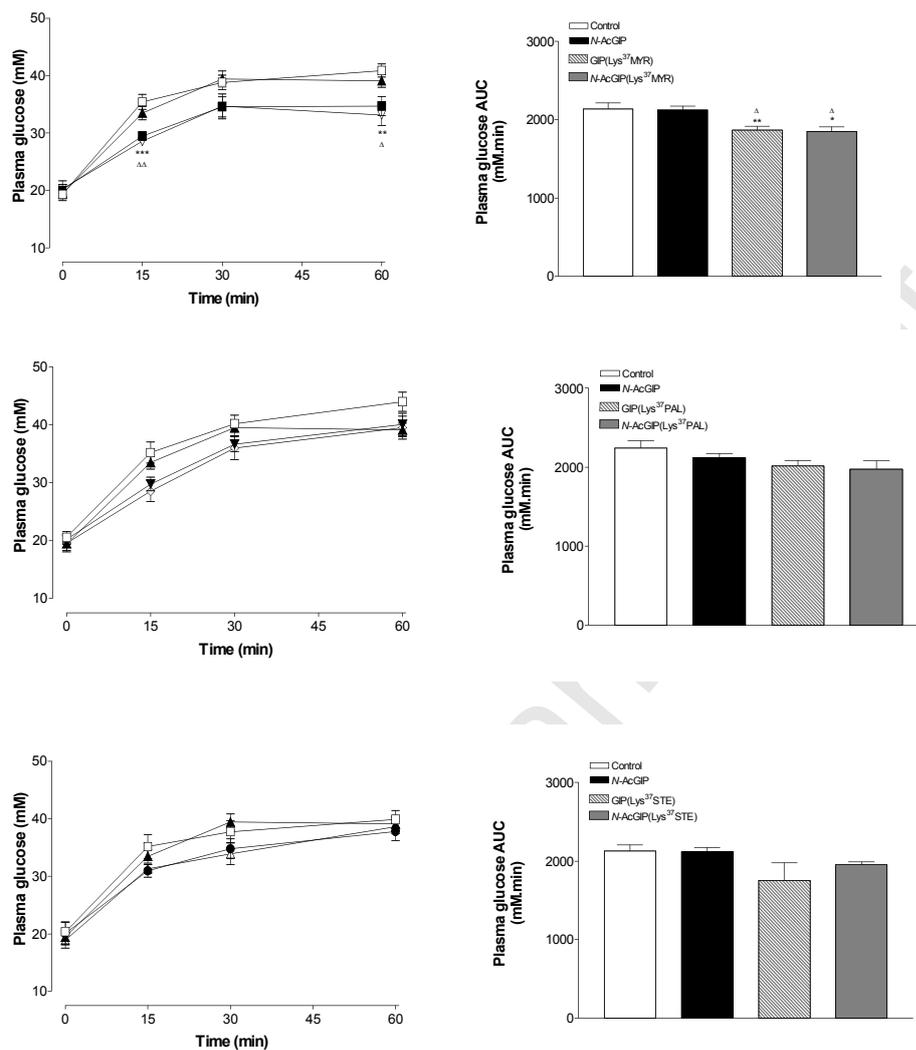


Figure 6

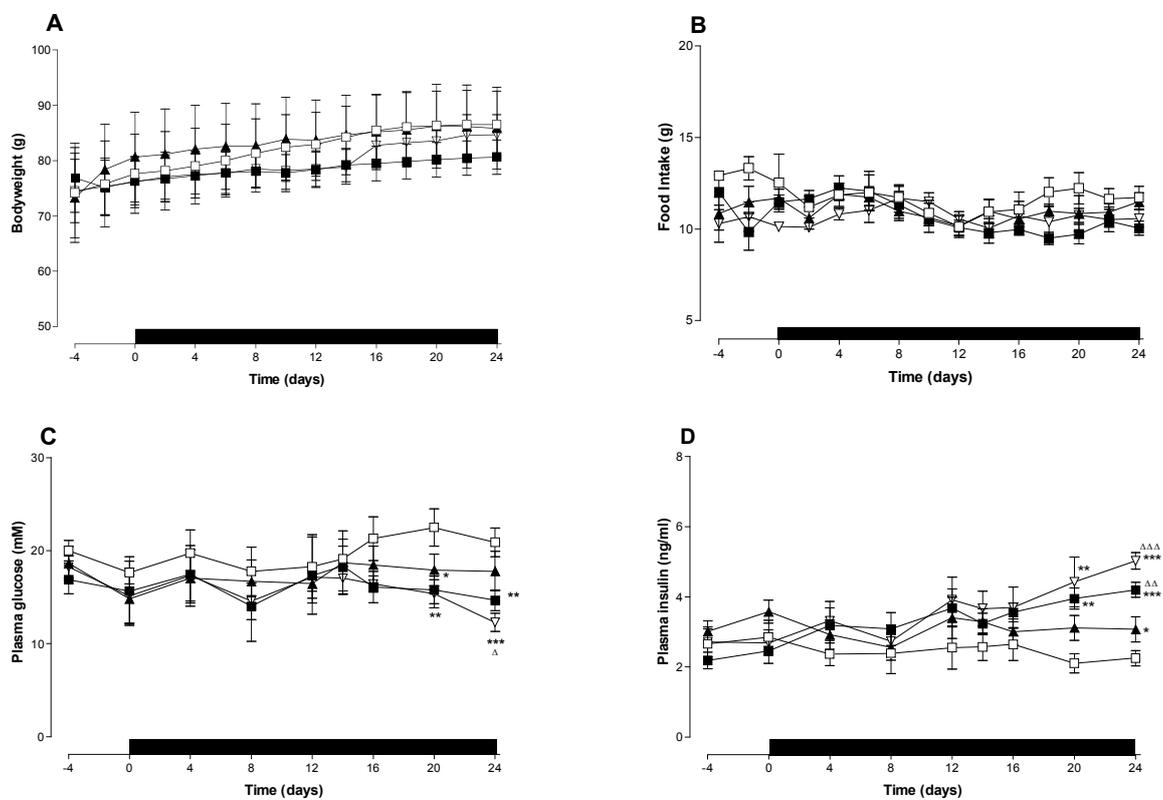
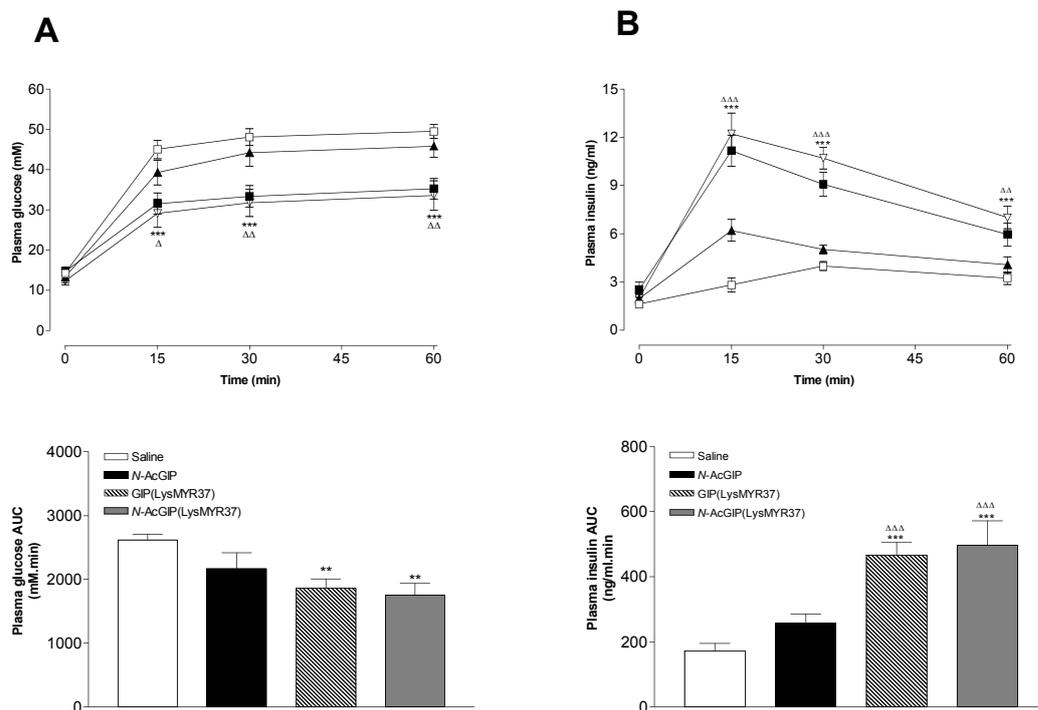


Figure 7



Graphical Abstract

C-terminal acylation particularly with myristic acid provides a class of stable, longer-acting forms of GIP for evaluation in diabetes therapy

1 10 20 30 40
NH₂-YAEGTFISDYSIAMDKIHQQDFVNWLLAQKGKKNDW**K**HNITQ-COOH

{ represents: myristic, palmitic or stearic acid

Albumin

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