

Comparison of the anti-diabetic effects of GIP- and GLP-1-receptor activation in obese diabetic (*ob/ob*) mice: studies with DPP IV resistant *N*-AcGIP and exendin(1–39)amide

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Abstract

Background The two major incretin hormones, glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are being actively explored as anti-diabetic agents because they lower blood glucose through multiple mechanisms. The rapid inactivation of GIP and GLP-1 by the ubiquitous enzyme, dipeptidyl peptidase IV (DPP IV) makes their biological actions short-lived, but stable agonists such as *N*-acetylated GIP (*N*-AcGIP) and exendin(1-39)amide have been advocated as stable and specific GIP and GLP-1 analogues.

Methods The present study examined the sub-chronic (14 days) anti-diabetic actions of single daily doses of *N*-AcGIP and exendin(1-39)amide given alone or in combination to obese diabetic (*ob/ob*) mice over a 14-day period.

Results Initial experiments confirmed the potent anti-hyperglycaemic and insulinotropic properties of *N*-AcGIP and exendin(1-39)amide. Sub-chronic administration of *N*-AcGIP alone or in combination with exendin(1-39)amide significantly decreased non-fasting plasma glucose and improved glucose tolerance compared to control *ob/ob* mice. This was associated with a significant enhancement of the insulin response to glucose and a notable improvement of insulin sensitivity. Combined treatment with *N*-AcGIP and exendin(1-39)amide also significantly decreased glycated haemoglobin. Exendin(1-39)amide alone had no significant effect on any of the metabolic parameters monitored. In addition, no significant effects were observed on body weight and food intake in any of the treatment groups.

Conclusions The results illustrate significant anti-diabetic potential of *N*-AcGIP alone and in combination with exendin(1-39)amide. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords glucose-dependent insulinotropic polypeptide (GIP); dipeptidylpeptidase IV (DPP IV); glucagon-like peptide-1 (GLP-1); analogue; exendin; glucose homeostasis



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Introduction

Glucose-dependent insulintropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are gastrointestinal hormones that regulate post-prandial glucose homeostasis [1]. The primary function of these two incretin hormones is the glucose-dependent stimulation of insulin release from the pancreatic beta-cells following nutrient absorption [2]. However, recent significant advances in the understanding of GIP and GLP-1 function have shown that they possess a number of secondary activities important for blood glucose regulation [3]. These actions include up-regulation of insulin biosynthesis, stimulation of glucose uptake and metabolism as well as beta-cell proliferation, survival and growth [4,5]. Despite their similarities, GIP and GLP-1 have several distinguishing features. GLP-1 is known to decrease the feeding activity and body weight and to inhibit gastric emptying and glucagon secretion, whereas GIP has no such effects [3,6]. GIP and GLP-1 are transcribed on separate genes [7,8] and each possess its own specific G-protein coupled receptor [9]. Recent evidence has shown that the signalling pathways of GIP and GLP-1 are mechanistically different [10].

These anti-diabetic features of GIP and GLP-1 provide the basis for a recent upsurge of interest in the pharmaceutical industry for exploitation as new therapies [4,5]. Furthermore, since the insulin-releasing actions of GIP and GLP-1 are glucose-dependent, unwanted hypoglycaemic episodes are rare, providing a distinct advantage over more traditional anti-diabetic drugs such as the sulphonylureas [11]. However, the therapeutic potential of GIP and GLP-1 is severely hindered through rapid *N*-terminal degradation in the circulation by the ubiquitous enzyme dipeptidyl peptidase IV (DPP IV). DPP IV cleaves the *N*-terminal dipeptide of GIP and GLP-1 (Tyr¹-Ala² in GIP and His¹-Ala² in GLP-1), causing a complete loss of insulintropic action of these incretin hormones [12].

Nonetheless, various strategies to overcome the rapid degradation of GIP and GLP-1 are proving successful. Several *N*-terminally modified analogues of GIP and GLP-1 have been tested that are profoundly resistant to DPP IV. For example, in GIP, modifications at Tyr¹ appear to yield DPP IV resistant analogues with greater bioactivity than Ala² or Glu³ modifications, with *N*-acetylated GIP (*N*-AcGIP) being most effective [13]. Interestingly, exendin(1-39)amide is a 39-amino acid peptide isolated from the Gila monster salivary gland that shares 53% sequence homology with GLP-1 and acts as a specific DPP IV resistant agonist of the GLP-1 receptor [14]. This peptide also termed exenatide has been approved for the clinical treatment of type 2 diabetes in United States under the trade name Byetta [15]. Thus, *N*-AcGIP and exendin(1-39)amide represent DPP IV resistant GIP and GLP-1 agonists with enhanced therapeutic potential.

Although many studies have demonstrated the enhanced anti-diabetic potential of various DPP IV resistant analogues of GIP and GLP-1 [4-6], none have evaluated the therapeutic potential of their combined administration. The present study has directly compared the anti-diabetic actions of a 14-day treatment of *ob/ob* mice with *N*-AcGIP, exendin(1-39)amide or a combination of both peptides. Effects on basal plasma glucose and insulin, glucose homeostasis, insulin sensitivity, glycated haemoglobin and pancreatic insulin content were examined. The results illustrate significant anti-diabetic potential of *N*-AcGIP alone and in combination with exendin(1-39)amide.

Methods

Animals

Obese diabetic (*ob/ob*) mice derived from the colony maintained at Aston University, UK, [16] were used at 15-19 weeks of age. 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). 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. No adverse effects were observed following a long-term administration of *N*-AcGIP, exendin(1-39)amide or a combination of both peptides.

Synthesis, purification and characterization of GIP, *N*-AcGIP and GLP-1

GIP, *N*-AcGIP and GLP-1 were sequentially synthesized on an Applied Biosystems automated peptide synthesizer (Model 432 A, Foster City, CA, USA) using a standard solid-phase Fmoc peptide chemistry as previously reported [13]. For *N*-AcGIP, an acetyl adduct was incorporated at the *N*-terminal Tyr¹ of native GIP. Peptides were judged pure by reversed-phase HPLC on a Waters Millennium 2010 chromatography system (Software version 2.1.5) and subsequently characterized using matrix-assisted laser desorption ionization-time of flight mass spectrometry as described previously [17]. Exendin(1-39)amide was purchased from the American Peptide Company (Sunnyvale, CA, USA) and characterized using matrix-assisted laser desorption ionization-time of flight mass spectrometry.

In vitro insulin secretion

BRIN-BD11 cells were seeded into 24-multi-well plates at a density of 1.0 × 10⁵ cells per well, and allowed to attach overnight at 37 °C. Acute tests for insulin release were

preceded by a 40-min pre-incubation at 37°C in 1.0-mL Krebs Ringer bicarbonate buffer (115 mM NaCl, 4.7 mM KCl, 1.28 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 10 mM NaHCO₃, 0.5% (w/v) BSA, pH 7.4) supplemented with 1.1-mM glucose. Test incubations were performed in the presence of 5.6-mM glucose with a range of concentrations (10⁻¹² – 10⁻⁸ M) of GIP, GLP-1, N-AcGIP and exendin(1–39)amide. After a 20-min incubation, the buffer was removed from each well and aliquots (200 µL) were used for the measurement of insulin. The origin and functional characteristics of BRIN BD11 cells are detailed elsewhere [18].

Acute effects of GIP, GLP-1, N-AcGIP and exendin(1–39)amide on glucose-lowering and insulin release in (*ob/ob*) mice

The effects of GIP, GLP-1, N-AcGIP and exendin(1–39)amide on plasma glucose and insulin concentrations were examined in an 18-h fasted obese diabetic (*ob/ob*) mice. *Ob/ob* mice received intra-peritoneal injection of glucose alone (18-mmol/kg body weight) or in combination with GIP, GLP-1, N-AcGIP or exendin(1–39)amide (each at 25-nmol/kg body weight). Blood samples were collected from the cut tip of the tail vein of a conscious mice into the chilled fluoride/heparin coated glucose micro-centrifuge tubes (Sarstedt, Nümbrecht, Germany) at the times indicated in the Figures. The resulting plasma was then aliquoted into fresh tubes and stored at –20°C prior to glucose and insulin determinations.

Sub-chronic effects of N-AcGIP, exendin(1–39)amide and combined peptide administration in *ob/ob* mice

Groups of *ob/ob* mice received once daily intra-peritoneal injections (17:00 h) of either saline (0.9%, w/v, NaCl), N-AcGIP, exendin(1–39)amide or a combination of both peptides (all at 12.5 nmol/kg body weight/day). This dose was chosen such that the combined peptide administration corresponded to a net incretin dose of 25-nmol/kg body weight as used in the acute tests. Previous studies using GIP and GLP-1 analogues have shown to exert *in vivo* effects within this dose range [19,20]. Food intake and body weight were recorded daily from 4 days before commencement of the treatment regimes. Plasma glucose and insulin concentrations were monitored at 2–4-day interval (10:00 h). On Day 14, groups of animals were used to evaluate intra-peritoneal glucose tolerance (18 mmol/kg) and insulin sensitivity (50 U/kg). Glycated haemoglobin was also determined on Day 14. All acute tests were commenced at 10:00 h. All blood samples were collected from the cut tip of the tail vein of a conscious mice into the chilled fluoride/heparin coated glucose micro-centrifuge tubes (Sarstedt, Nümbrecht, Germany) at the times indicated in the Figures. Blood samples

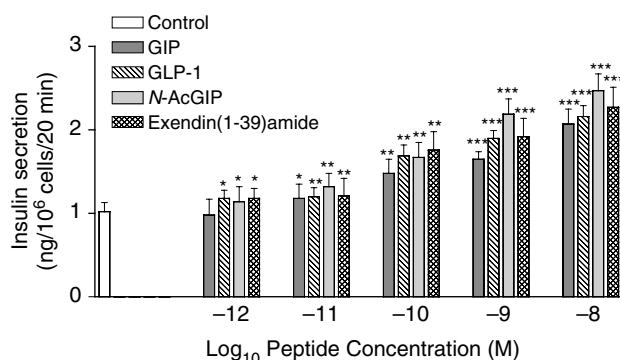


Figure 1. Insulin-releasing activity of GIP, GLP-1, N-AcGIP and exendin(1–39)amide in the clonal pancreatic beta-cell line, BRIN-BD11. After a pre-incubation (40 min), the effects of various concentrations of peptide were tested on insulin release during a 20-min incubation. Values are means ± SEM for eight separate observations. **p* < 0.05, ***p* < 0.01, ****p* < 0.01 compared to 5.6-mM glucose control

were immediately centrifuged using a Beckman micro-centrifuge (Beckman Instruments, Galway, Ireland) for 30 s at 13 000 g. The resulting plasma was then aliquoted into fresh tubes and stored at –20°C prior to glucose and insulin determinations.

Biochemical analyses

Plasma glucose was assayed by an automated glucose oxidase procedure [13] using a Beckman Glucose Analyser II. Glycated haemoglobin was measured using a commercially available kit purchased from Chirus Ltd. (Watford, UK) Insulin was assayed by a modified dextran-charcoal RIA as described previously [13].

Statistics

Results are expressed as mean ± SEM. 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 were calculated using a computer-generated program employing the trapezoidal rule [13] with baseline subtraction. Groups of data were considered to be significantly different if *p* < 0.05.

Results

Stimulation of *in vitro* insulin secretion

All four peptides; GIP, GLP-1, N-ACGIP and exendin(1–39)amide, significantly (*p* < 0.05 – *p* < 0.001) enhanced insulin release in a concentration-dependent manner compared to 5.6-mM glucose control (Figure 1). There was no significant difference in potency between the four peptides. At the highest concentration tested (10⁻⁸ M), the peptides stimulated insulin secretion by 2.1–2.4-fold compared to 5.6-mM glucose control.

Acute anti-hyperglycaemic and insulin releasing activity in *ob/ob* mice

The relative glucose-lowering abilities of GIP, GLP-1, *N*-AcGIP and exendin(1–39)amide (25-nmol/kg body weight) in *ob/ob* mice are shown in Figure 2(A). Injection of glucose alone resulted in a rapid and marked increase in plasma glucose that continued to rise until 60 min. Native GIP had a tendency to reduce glucose concentrations, and by 60 min, levels were significantly ($p < 0.05$) decreased compared to control (Figure 2(A)). Similarly, *N*-AcGIP, GLP-1 and exendin(1–39)amide reduced plasma glucose concentrations compared to control with significantly ($p < 0.01 - p < 0.001$) reduced glucose levels between 30 and 60 min. The overall glucose area under the curve values were significantly ($p < 0.05 - p < 0.001$) reduced in all four peptide treated groups compared to control, with *N*-AcGIP being significantly ($p < 0.05$) more effective than native GIP. As shown in Figure 2(B), these anti-hyperglycaemic effects were linked to corresponding changes in plasma insulin. All four peptides significantly increased glucose-stimulated insulin concentrations between 15 and 60 min. Native GIP caused a significantly greater ($p < 0.05$) overall insulin response compared to glucose alone. *N*-AcGIP was significantly more potent than native GIP ($p < 0.01$). GLP-1 and exendin(1–39)amide had similar enhanced

insulinotropic effects, evoking significantly ($p < 0.01$ and $p < 0.001$; respectively) greater overall insulin release compared to control (Figure 2(B)).

Sub-chronic effects of *N*-AcGIP, exendin(1–39)amide and a combination of both peptides on non-fasting plasma glucose and insulin levels and glycated haemoglobin concentrations

Administration of *N*-AcGIP, exendin(1–39)amide or a combination of both peptides had no effect on food intake or body weight (data not shown). Plasma glucose concentrations were progressively reduced, resulting in significantly ($p < 0.05$) lowered glucose concentrations at 14 days in the groups treated with *N*-AcGIP alone or in combination with exendin(1–39)amide (Figure 3). Glycated haemoglobin concentrations were significantly ($p < 0.05$) reduced in the combined *N*-AcGIP and exendin(1–39)amide treated group (Figure 3). These changes were accompanied by a tendency towards elevated insulin concentrations, but these did not achieve a statistical significance over the study period (Figure 3). No differences on plasma glucose or insulin were observed in exendin(1–39)amide treated mice alone (Figure 3).

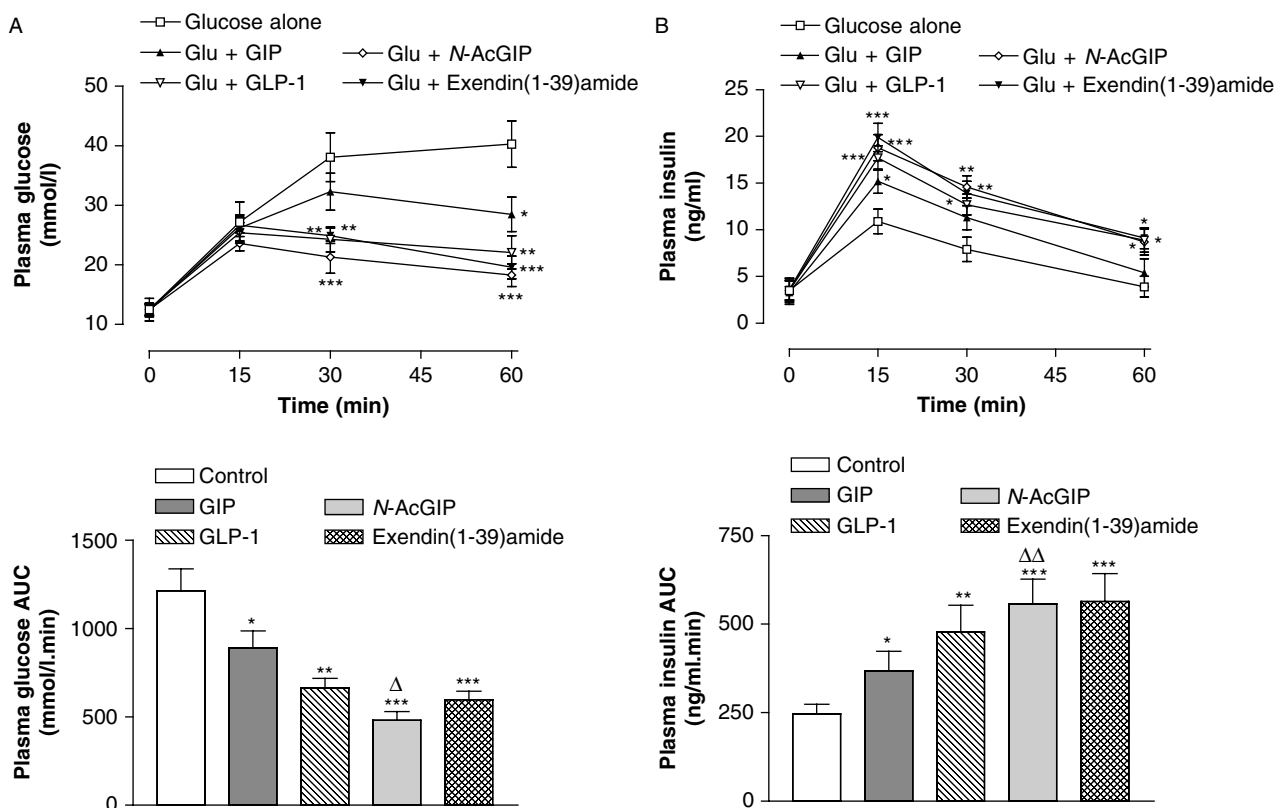


Figure 2. Acute glucose lowering and insulin releasing activity of GIP, GLP-1, *N*-AcGIP and exendin(1–39)amide in an 18-h fasted (*ob/ob*) mice. Plasma glucose and insulin concentrations were measured prior to and after intra-peritoneal administration of glucose alone (18 mmol/kg) as a control, or in combination with peptide (25 nmol/kg). The glucose and insulin area under the curve (AUC) between 0 and 60 min are shown in bottom panels. Values represent means \pm SEM for eight mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.01$ compared to glucose alone. $\Delta p < 0.05$, $\Delta\Delta p < 0.01$ compared to native GIP

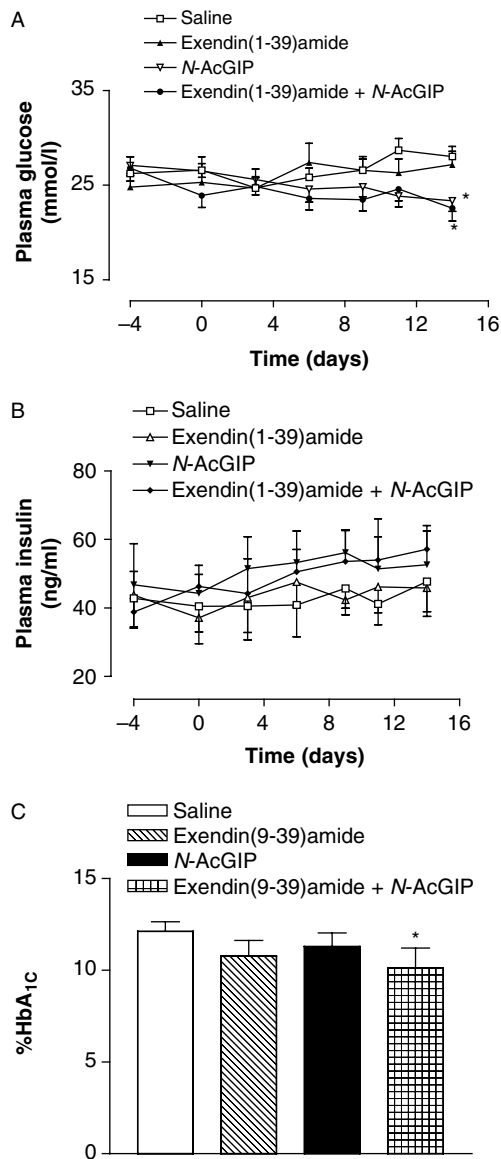


Figure 3. Effects of daily *N*-AcGIP, exendin(1–39)amide or combined peptide administration on plasma glucose (A), insulin (B) and glycated haemoglobin concentrations (C). *N*-AcGIP, exendin(1–39)amide, a combination of both peptides (each at 12.5 nmol/kg/day) or saline (control) were administered for 14 days as indicated by the horizontal black bar. Values are mean \pm SEM for eight mice. * $p < 0.05$ compared to control

Sub-chronic effects of *N*-AcGIP, exendin(1–39)amide and a combination of both peptides on glucose tolerance

Treatment with *N*-AcGIP alone or in combination with exendin(1–39)amide for 14 days resulted in a significant improvement in glucose tolerance and glucose-mediated insulin release. *N*-AcGIP produced a 27% reduction in the overall glycaemic excursion ($p < 0.05$). This was accompanied by a 194% increase in the overall insulin release ($p < 0.05$) (Figure 4). When *N*-AcGIP administration was combined with exendin(1–39)amide, a similar effect was noted ($p < 0.05$; in both cases)

(Figure 4). However, exendin(1–39)amide treatment alone had no significant effects on glucose stimulated insulin release or glycaemic excursion (Figure 4).

Sub-chronic effects of *N*-AcGIP, exendin(1–39)amide and a combination of both peptides on insulin sensitivity

As shown in Figure 5, the hypoglycaemic action of insulin was significantly augmented in terms and area under the curve measures in *ob/ob* mice treated for 14 days with *N*-AcGIP (1.3-fold; $p < 0.05$) alone or combined with exendin(1–39)amide (1.5-fold; $p < 0.01$). Treatment for 14 days with exendin(1–39)amide alone had no significant effects on the glucose lowering effects of exogenous insulin in *ob/ob* mice (Figure 5).

Discussion

Previous studies have shown that daily administration of exendin(1–39)amide or stable GIP analogues, including *N*-AcGIP result in a significant amelioration of diabetes in type 2 animal models [19,21–23]. Direct comparison of the anti-diabetic properties of these stable incretin peptide analogues, and evaluation of the possible effects of combined treatment, have not been carried out. The present study examined the effects of sub-chronic administration of *N*-AcGIP, exendin(1–39)amide or a combination of both peptides in adult *ob/ob* mice. In harmony with previous findings, acute studies in *ob/ob* mice confirmed the potent anti-hyperglycaemic and insulinotropic properties of *N*-AcGIP and exendin(1–39)amide [13,24].

These observations of unchanged body weight, food intake and non-fasting plasma insulin concentrations are broadly similar to the previously reported long-term effects of stable GIP agonists in this animal model, thereby confirming a spectrum of useful anti-diabetic effects [20,23]. Combination of *N*-AcGIP with an equal dose of exendin(1–39)amide did not result in appreciably greater effects than observed with *N*-AcGIP alone. However, a small decrease of glycated haemoglobin was observed over a short 14-day study period in this group, suggesting a potential synergistic action. This effect may have been magnified, if study was carried out over a longer time course.

Perhaps, the greatest surprise from the present study was the lack of effect of exendin(1–39)amide when given alone by daily injection over 14 days to *ob/ob* mice. This contrasts sharply with similar studies in *db/db* mice, *fa/fa* rats and Zucker rats, which showed that long-term administration of exendin(1–39)amide lowered blood glucose and HbA_{1c}, significantly reduced food intake causing weight loss and improved insulin sensitivity, while insulin concentrations were also reported to be higher [21,22,25]. Importantly, the batch of exendin(1–39)amide used in the present study was of

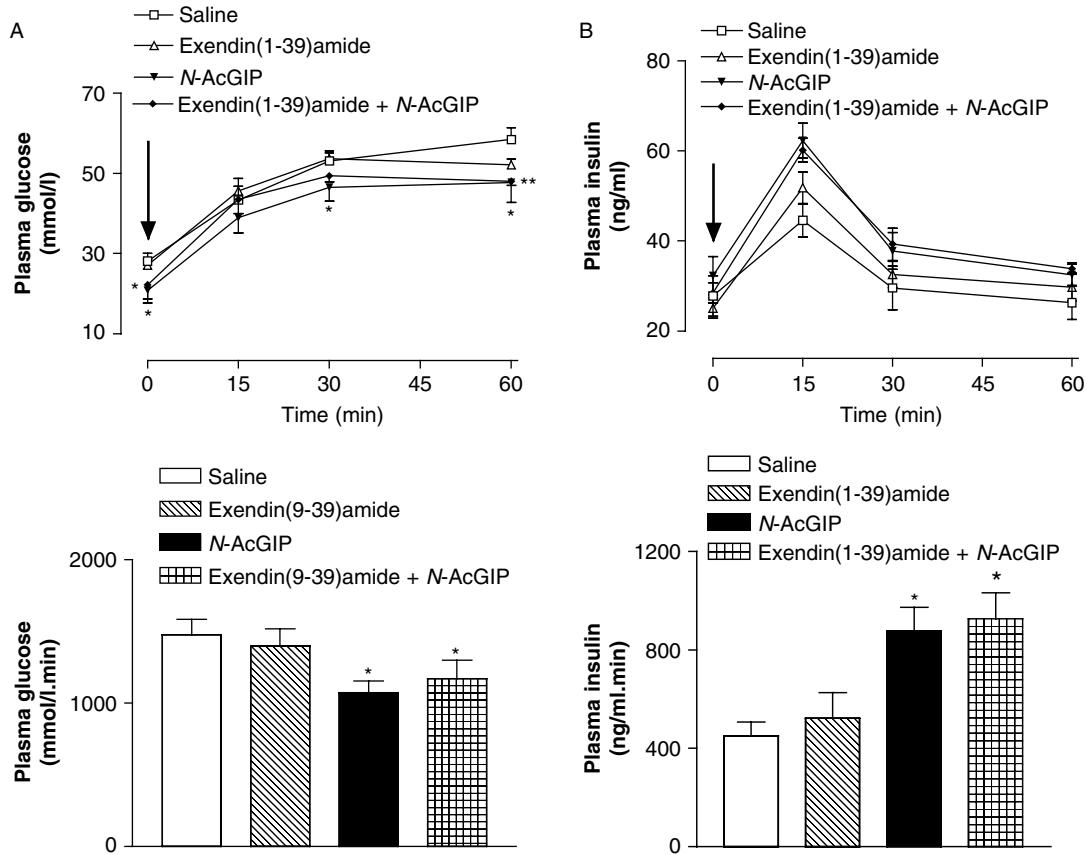


Figure 4. Effects of daily *N*-AcGIP, exendin(1-39)amide and combined peptide administration on glucose tolerance (A) and plasma insulin response to glucose (B). Tests were conducted after 14 daily injections of either *N*-AcGIP, exendin(1-39)amide, a combination of both peptides (each at 12.5 nmol/kg/day) or saline (control). Glucose (18 mmol/kg) was administered by intra-peritoneal injection at the time indicated by the arrow. Plasma glucose and insulin AUC values for a 0–60 min post-injection, are shown in the bottom panels. Values are mean ± SEM for eight mice. **p* < 0.05 and ***p* < 0.01 compared to control

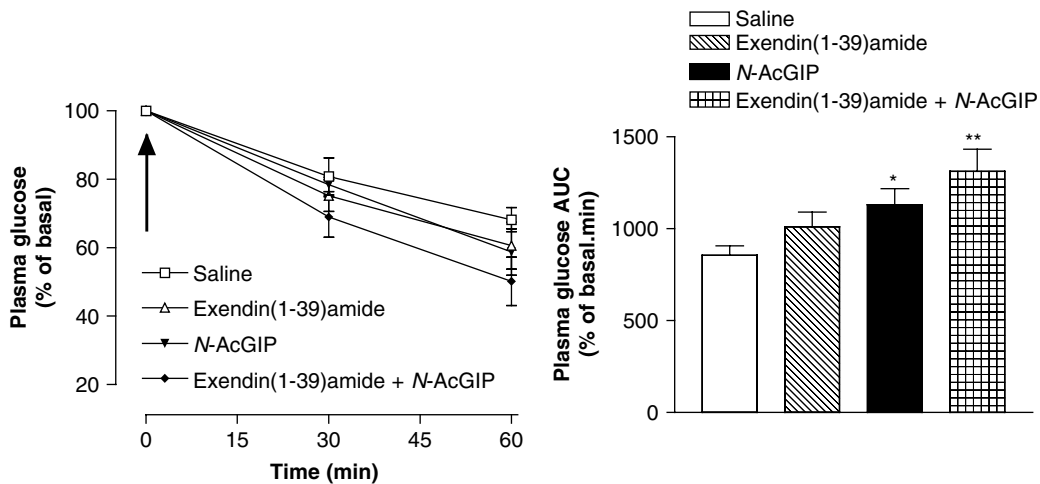


Figure 5. Effects of daily *N*-AcGIP, exendin(1-39)amide or combined peptide administration on insulin sensitivity. Tests were conducted after 14 daily injections of either *N*-AcGIP, exendin(1-39)amide, a combination of both peptides (each at 12.5 nmol/kg/day) or saline (control). Insulin (50 U/kg) was administered by intra-peritoneal injection at the time indicated by the arrow. Plasma glucose AUC values for a 0–60 min post-injection, are shown in the bottom panel. Values are mean ± SEM for eight mice. **p* < 0.05 and ***p* < 0.01 compared to control

confirmed molecular identity and proven to be active in both *in vitro* insulin secretion tests and acute studies in *ob/ob* mice. Indeed, results of the latter correspond

with similarly published acute studies in this animal model [21]. The most plausible explanation for the lack of such chronic effects of exendin(1-39)amide,

therefore, lies with either the dose or possible GLP-1 receptor de-sensitization. It seems unlikely to us that a reduced dose of 12.5 nmol/kg would remove an effect of exendin(1–39)amide, whereas potency of *N*-AcGIP is retained. Furthermore, other studies testing exendin(1–39)amide in animal models have employed doses of 1 [26], 3 [27], 24 [22] or 50 nmol/kg [21]. The alternative that GLP-1 receptor de-sensitization lies behind the present poor long-term responses in *ob/ob* mice is perhaps more plausible because such a phenomenon has been observed previously with GLP-1 *in vitro* [28], although not to any appreciable extent *in vivo* [29]. However, this effect would appear to be species and animal model specific. Also, the disappointing sub-chronic effects of exendin(1–39)amide observed here contrast sharply with the prominent anti-diabetic actions of the stable agonist (Val⁸)GLP-1 administered at 25 nmol/kg to *ob/ob* mice [19]. Thus, further studies are required to resolve this issue, which seems to stem from issues surrounding chemical nature of the GLP-1 mimetic, dose and animal model employed. Determination of the pharmacokinetics of exendin(1–39)amide in *ob/ob* mice would also be useful, especially in relation to the time of administering glucose load.

In conclusion, the present study indicates that once daily injection of *N*-AcGIP is an effective means of improving diabetes control in *ob/ob* mice. Some evidence for benefit of combined treatment with exendin(1–39)amide was obtained, but this GLP-1 mimetic is considerably less effective than *N*-AcGIP or (Val⁸)GLP-1 [19] in this commonly employed diabetic animal model.

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