



HAL
open science

Administration of an acylated GLP-1 and GIP preparation provides added beneficial glucose-lowering and insulinotropic actions over single incretins in mice with type 2 diabetes and obesity

Victor A Gault, Barry D Kerr, Patrick Harriott, Pr Flatt

► **To cite this version:**

Victor A Gault, Barry D Kerr, Patrick Harriott, Pr Flatt. Administration of an acylated GLP-1 and GIP preparation provides added beneficial glucose-lowering and insulinotropic actions over single incretins in mice with type 2 diabetes and obesity. *Clinical Science*, 2011, 121 (3), pp.107-117. 10.1042/CS20110006 . hal-00687792

HAL Id: hal-00687792

<https://hal.science/hal-00687792v1>

Submitted on 15 Apr 2012

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Administration of an acylated GLP-1 and GIP preparation provides added beneficial glucose-lowering and insulinotropic actions over single incretins in mice with type 2 diabetes and obesity

Victor A. Gault^{1*}, Barry D. Kerr¹, Patrick Harriott², and Peter R. Flatt¹

¹School of Biomedical Sciences, University of Ulster, Coleraine BT52 1SA, Northern Ireland, UK

²School of Biological Sciences, Queen's University of Belfast, Belfast BT9 7BL, Northern Ireland, UK

Correspondence: Dr. Victor A. Gault, The SAAD Centre for Pharmacy and Diabetes, School of Biomedical Sciences, University of Ulster, Cromore Road, Coleraine BT52 1SA, Northern Ireland, UK. E-mail address: va.gault@ulster.ac.uk; Tel.: +44 28 70323322; Fax: +44 28 70324965

Key words: Glucagon-like peptide-1 (GLP-1); glucose-dependent insulinotropic polypeptide (GIP); glucose-lowering; insulin secretion; *ob/ob* mice.

Short title: Acylated GLP-1 and GIP preparation

ABSTRACT

This study examined the glucose-lowering and insulinotropic properties of acylated GLP-1 and GIP peptides in type 2 diabetes and obesity. GLP-1, GIP, Liraglutide, *N*-AcGIP(Lys³⁷Myr), a simple combination of both peptides, and an overnight preparation of Liraglutide and *N*-AcGIP(Lys³⁷Myr) (Lira-AcGIP preparation) were incubated with DPP-IV to assess peptide stability and BRIN-BD11 cells were used to evaluate cAMP production and insulin secretion. Acute glucose-lowering and insulinotropic actions were evaluated in Swiss *TO* mice. Sub-chronic studies on glucose homeostasis, insulin secretion, food intake and bodyweight were evaluated in *ob/ob* mice. Liraglutide, *N*-AcGIP(Lys³⁷Myr), a simple combination of both peptides and Lira-AcGIP preparation demonstrated improved DPP-IV resistance ($P < 0.001$), while stimulating cAMP production and insulin secretion (1.4 to 2-fold; $P < 0.001$). Lira-AcGIP preparation was more potent at lowering plasma glucose (20 to 51% reduction; $P < 0.05 - P < 0.001$) and stimulating insulin secretion (1.5- to 1.8-fold; $P < 0.05 - P < 0.001$) compared to Liraglutide and *N*-AcGIP(Lys³⁷Myr) or a simple peptide combination. Daily administration of Lira-AcGIP preparation to *ob/ob* mice lowered bodyweight (7 to 9%; $P < 0.05$), food intake (23%; $P < 0.05$) and plasma glucose (46% reduction; $P < 0.001$) while increasing plasma insulin (1.5- to 1.6-fold; $P < 0.001$). Lira-AcGIP preparation enhanced glucose tolerance, insulin response to glucose and insulin content ($P < 0.05 - P < 0.001$). These data demonstrate that combined preparation of acylated GLP-1 and GIP peptides, Liraglutide and *N*-AcGIP(Lys³⁷Myr), markedly improved glucose-lowering and insulinotropic properties in diabetes-obesity compared with either incretin mimetic given individually.

INTRODUCTION

Type 2 diabetes mellitus is a disorder with major socioeconomic implications and a rapidly escalating global incidence [1]. Recent estimates suggest that approximately £710 million (~7% of the NHS budget) was spent on glucose-lowering drugs and monitoring of blood glucose control in 2008 [2]. As current therapies do not prevent complications there is a major unmet need for the development of more effective physiological drugs whose actions more tightly regulate the minute-to-minute variations of circulating blood glucose. The gut peptides glucagon-like-peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) are incretin hormones released by feeding which specifically target pancreatic beta-cells to enhance insulin secretion and aid reduction of postprandial hyperglycaemia [3]. Furthermore, incretin hormones only stimulate glucose-induced insulin-release under hyperglycaemic conditions and unlike other non-endogenous insulinotropic agents are unlikely to result in hypoglycaemia [4]. It is principally this unique attribute which has led to much interest in exploiting the incretin hormones as potential therapeutic agents [5, 6].

Despite their antidiabetic potential, both GLP-1 and GIP undergo enzymatic degradation by dipeptidylpeptidase-IV (DPP-IV) and rapid removal from the circulation [7-9]. Several strategies have now been successfully employed to circumvent these intrinsic limitations including development of DPP-IV inhibitors and so-called incretin mimetics [for review see 10]. The first GLP-1 mimetic, Exenatide (Byetta™), reached the market in 2005 closely followed by Liraglutide (Victoza®) [11]. Liraglutide shares a 97% sequence homology to human GLP-1 with structural variances restricted to amino acid substitution of Lys³⁴ Arg and addition of a C-16 acyl moiety at position 26 via a γ -glutamyl linker [12]. As a full agonist of the GLP-1 receptor, Liraglutide stimulates glucose-induced insulin secretion [13], inhibits glucagon secretion [14], enhances beta-cell mass [15], slows gastric emptying [16], restores beta-cell sensitivity to glucose [17] and promotes satiety resulting in decreased energy intake and bodyweight loss [18]. Whilst GIP mimetics have not entered clinical development, pre-clinical studies clearly demonstrate that N-terminally modified and acylated GIP peptides exhibit improved DPP-IV resistance, enhanced glucose-lowering and insulin-releasing actions in animal models of obesity-diabetes [19-29]. Furthermore, beta-cell insensitivity to GIP observed in humans with type 2 diabetes appears to be overcome by combination therapy using GIP with either sulphonylureas or insulin [30, 31].

The protracted duration of action of Liraglutide is understood to arise primarily from two mechanisms namely: (a) non-covalent reversible binding to serum albumin; and (b) the ability of the peptide to self-associate in solution, thus slowing absorption and facilitating resistance to degradation by DPP-IV [32]. Given this, it seems likely that acylated GIP peptides would behave in a similar manner to Liraglutide with regards to both of these mechanisms. Indeed, from a therapeutic perspective it would be attractive to develop an incretin-based preparation which would activate both arms of the enteroinsular axis normally triggered by feeding. We hypothesised that combining Liraglutide with the acylated GIP peptide *N*-AcGIP(Lys³⁷Myr) into a single preparation would facilitate peptide self-association, thereby providing a particularly effective means of exploiting both incretin pathways for the treatment of diabetes. Therefore, this study examined the effects of a combined preparation of the acylated GLP-1 and GIP peptides Liraglutide and *N*-AcGIP(Lys³⁷Myr) on *in vitro* DPP-IV resistance and biological activity as well as *in vivo* actions on glucose-lowering and insulin secretion. The effects were compared with the actions of either peptide alone.

MATERIALS AND METHODS

Peptide synthesis and characterization

GLP-1, GIP and Liraglutide were purchased from GL Biochem Ltd. (Shanghai, China). *N*-AcGIP(Lys³⁷Myr) was synthesised on an Applied BioSystems ABI 432A Peptide Synthesiser (Warrington, Cheshire, UK) with a Fmoc-Gln(Trt)-Wang resin (Bachem Ltd., Merseyside, UK) using Fmoc chemistry [29]. Briefly, the ϵ -amino group at Lys³⁷ of GIP was conjugated with C-14 (Myristic acid) and an acetyl adduct incorporated at Tyr¹. Peptides were purified using rp-HPLC on a Waters Millennium 2010 Chromatography System [29]. Identity of peptides was confirmed using Voyager-DE BioSpectrometry MALDI-TOF MS (PerSeptive BioSystems, Framingham, Massachusetts, USA) as described previously [29].

Preparation of peptide solutions

Prior to all experimentation (unless otherwise specified), GLP-1, GIP, Liraglutide and *N*-AcGIP(Lys³⁷Myr) and a preparation of both Liraglutide and *N*-AcGIP(Lys³⁷Myr) (equal concentrations and designated Lira-AcGIP) were aliquoted into sterile tubes (Sterlin Ltd., Hounslow, UK) containing experimental buffer (50 mM triethanolamine-HCl / Krebs Ringer Bicarbonate Buffer or saline vehicle (0.9% (w/v) NaCl)). Peptide solutions were then placed on an orbital shaker and agitated overnight at 4°C for 12 hours. A similar protocol has been used by others for self-association of acylated peptides, including GLP-1 [33]. HPLC analysis following overnight incubations revealed minimal loss of peptide (98.3 ± 0.8% intact peptide remaining) with no significant differences between Liraglutide, *N*-AcGIP(Lys³⁷Myr) or the Lira-AcGIP preparation. For simple combination of Liraglutide plus *N*-AcGIP(Lys³⁷Myr), the same protocol was followed with the exception that peptides were incubated separately overnight, then combined prior to experimentation.

Degradation by DPP-IV

GLP-1, GIP, Liraglutide, *N*-AcGIP(Lys³⁷Myr), a simple combination of Liraglutide plus *N*-AcGIP(Lys³⁷Myr), and the Lira-AcGIP preparation were incubated at 37°C in 50 mM triethanolamine-HCl (pH 7.8) with dipeptidylpeptidase-IV (DPP-IV, 5 mU; Sigma-Aldrich, UK) for 0, 2, 4, 8 and 24 hours. Enzymatic reactions were terminated with addition of 15 μ l of 10% (v/v) TFA / H₂O. Reaction products were separated on a Vydac C-4 column (4.6 x 250 mm; The Separations Group, Hesperia, California, USA) and intact peptide separated from associated major degradation products GIP(3-42) and GLP-1(9-36)amide. Absorption was monitored at 206 nm

using a SpectraSystem UV2000 detector (Thermoquest Ltd., Manchester, UK). HPLC peak area data was used to calculate percentage intact peptide and peptide purity.

***In vitro* cAMP production and insulin secretion**

Effects of GLP-1, GIP, Liraglutide, *N*-AcGIP(Lys³⁷Myr), a simple combination of Liraglutide plus *N*-AcGIP(Lys³⁷Myr), and the Lira-AcGIP preparation on stimulation of cAMP production and insulin secretion were measured in BRIN-BD11 cells. For cAMP studies, cells were seeded (100,000 cells / well) into 96-well plates (Nunc, Roskilde, Denmark) and washed with HBS buffer prior to incubation with peptides (20 min at 37°C) in presence of 200 mM IBMX. After incubation, medium was removed and cells lysed prior to measurement of cAMP using HTS Immunoassay Kit (Millipore, Watford, UK). For insulin-release studies, BRIN-BD11 cells were seeded (150,000 cells / well) into 24-well plates and allowed to attach overnight at 37°C. Following 40 min pre-incubation with 1.1 mM glucose, cells were incubated (20 min; 37°C) in the presence of 5.6 mM glucose with a range of peptide concentrations (10⁻¹² to 10⁻⁶ M). After 20 min incubation, buffer was removed from each well and insulin determined using radioimmunoassay.

Animals

Acute animal studies were carried out in normal male NIH Swiss *TO* mice (Harlan Ltd., Blackthorne, UK; 50-55 weeks old) while sub-chronic experiments were performed in obese diabetic (*ob/ob*) mice (20-22 weeks old) derived from the Aston colony [34]. Mice were age-matched, divided into groups (n = 8 to 9) and housed individually in an air-conditioned room (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 conducted according to UK Home Office Regulations (UK Animals Scientific Procedures Act 1986) and the "Principles of Laboratory Animal Care" (NIH Publication No. 86-23, revised 1985). No adverse effects were observed following administration of any of the peptides.

Acute glucose-lowering and insulin-releasing properties of GLP-1 and GIP peptides in Swiss *TO* mice

Plasma glucose and insulin concentrations were measured prior to and after intraperitoneal administration of glucose (18 mmol/kg bw) in animals injected 4 hours previously with saline vehicle (0.9% (w/v) NaCl), Liraglutide, *N*-AcGIP(Lys³⁷Myr) (each at 50 nmol/kg), a simple combination of Liraglutide plus *N*-AcGIP(Lys³⁷Myr) (each at 50 nmol/kg) or Lira-AcGIP preparation (100 nmol/kg bw). Blood samples were collected at the times shown in the Figures and glucose and insulin concentrations measured as indicated below. All peptides were administered by intraperitoneal injection.

Effects of once-daily administration of Liraglutide, *N*-AcGIP(Lys³⁷Myr) or Lira-AcGIP preparation in *ob/ob* mice

Once-daily injections of saline vehicle (0.9% (w/v) NaCl), Liraglutide, *N*-AcGIP(Lys³⁷Myr) (each at 50 nmol/kg bw), or Lira-AcGIP preparation (100 nmol/kg bw) were administered intraperitoneally at 16:00 h over 21 days to *ob/ob* mice. Food intake, bodyweight, plasma glucose and insulin concentrations were monitored at 2 to 4 day intervals. Glucose tolerance (18 mmol/kg bw; ip) and insulin sensitivity (50 U/kg bw; ip) tests were performed following 21 days treatment. At termination, blood for circulating triglycerides was taken and pancreatic tissues were excised and processed for measurement of insulin following extraction with 5 ml/g ice-cold ethanol as described previously [29].

Biochemical analysis

Blood samples were collected from the cut tip on the tail vein of conscious mice into chilled fluoride / heparin glucose micro-centrifuge tubes (Sarstedt, Numbrecht, Germany). Samples were immediately centrifuged using a Beckman micro-centrifuge (Beckman Instruments, Galway,

Ireland) for 30 s at 13,000 x g. Plasma glucose was measured from whole blood using the new 'plasma calibrated' Ascensia Contour® Blood Glucose Meter (Bayer AG, Leverkusen, Germany). Plasma and pancreatic insulin were assayed by a modified dextran-coated charcoal radioimmunoassay [35]. Plasma triglyceride levels were measured using a Hitachi Automatic Analyser 912 (Boehringer Mannheim, Germany).

Statistical analysis

Results are expressed as means \pm S.E.M. and data compared using the unpaired Student's *t*-test with Chi Squared analysis for correlations. Where appropriate, data were compared using repeated measures ANOVA or one-way ANOVA, followed by Student-Newman-Keuls post-hoc test. Incremental area under the curve (AUC) analyses for plasma glucose and insulin were calculated using GraphPad Prism version 3.02. Groups of data were considered to be significantly different if $P < 0.05$.

RESULTS

Structural characteristics of peptides

Table 1 displays the retention times and observed molecular masses for GLP-1, GIP, Liraglutide and *N*-AcGIP(Lys³⁷Myr) using rp-HPLC and MALDI-TOF MS, respectively. Peptide purity was calculated as being greater than 95% from HPLC area under the curve (AUC) analysis using appropriate standards. The molecular mass for each peptide corresponded closely to theoretical values therefore confirming correct peptide identity.

DPP-IV stability, in vitro cAMP production and insulin secretion

As shown in Figure 1A, native GLP-1 and GIP were progressively degraded over 24 hours with estimated half-lives of 4.5 and 2.2 hours, respectively. In contrast, Liraglutide, *N*-AcGIP(Lys³⁷Myr), a simple combination of Liraglutide plus *N*-AcGIP(Lys³⁷Myr) and Lira-AcGIP preparation maintained a significantly higher percentage of intact peptide over the 24 hours ($P < 0.05$ - $P < 0.001$). All test agents exhibited equipotent stimulation of cAMP production with EC₅₀ values ranging from 2.1 to 4.0 nM ($P > 0.05$; Figure 1B). Similarly, all peptides and combinations significantly stimulated insulin secretion from BRIN-BD11 cells in a concentration-dependent manner compared to control (1.4 to 2-fold; $P < 0.01$ - $P < 0.001$; Figure 1C).

Acute glucose-lowering and insulinotropic actions of GLP-1 and GIP peptides in Swiss TO mice

As shown in Figure 2A, Liraglutide, *N*-AcGIP(Lys³⁷Myr) and a simple combination of Liraglutide plus *N*-AcGIP(Lys³⁷Myr) significantly reduced the glycaemic response following intraperitoneal glucose compared to saline (25 to 35% reduction; $P < 0.05$ - $P < 0.001$). However, administration of the Lira-AcGIP preparation four hours previously resulted in a significant improvement in glycaemic control relative to all groups of mice (17 to 45% reduction; $P < 0.05$ - $P < 0.001$). This trend was confirmed by AUC analysis for Liraglutide, *N*-AcGIP(Lys³⁷Myr), simple combination of Liraglutide plus *N*-AcGIP(Lys³⁷Myr) and Lira-AcGIP preparation which revealed decreases of 26%, 25%, 30% and 47%, respectively compared to saline-treated mice ($P < 0.05$ - $P < 0.001$; Figure 2A). Importantly, the Lira-AcGIP preparation significantly lowered plasma glucose ($P < 0.05$) compared to simple combination of Liraglutide plus *N*-AcGIP(Lys³⁷Myr) (Figure 2A). As shown in Figure 2B, a simple combination of Liraglutide plus *N*-AcGIP(Lys³⁷Myr) significantly enhanced plasma insulin concentrations (1.6-fold; $P < 0.05$). Lira-AcGIP preparation was significantly more potent at stimulating insulin-release (1.5- to 1.8-fold; $P < 0.05$ - $P < 0.001$) compared to saline-treated animals. This was confirmed by a 1.6-fold increase in plasma insulin AUC ($P < 0.01$; Figure 2B). Administration of native GLP-1 and GIP four hours previously did not

result in significant modification of glycaemic or insulin responses compared to saline-treated controls (data not shown).

Effects of once-daily Liraglutide, N-AcGIP(Lys³⁷Myr) or Lira-AcGIP preparation on bodyweight, food intake, plasma glucose and insulin in *ob/ob* mice

Daily administration of Liraglutide and N-AcGIP(Lys³⁷Myr) did not alter bodyweight or food intake in *ob/ob* mice (Figure 3A and 3B). In contrast, Lira-AcGIP preparation significantly reduced bodyweight by day 18 to 21 (7 to 9% decrease; $P < 0.05$; Figure 3A) and decreased food intake by day 21 (23% decrease; $P < 0.05$; Figure 3B). As shown in Figure 3C, non-fasting plasma glucose concentrations were significantly improved following Liraglutide (29% reduction; $P < 0.01$), N-AcGIP(Lys³⁷Myr) (23% reduction; $P < 0.05$) and Lira-AcGIP preparation (46% reduction; $P < 0.001$). In addition, Lira-AcGIP preparation demonstrated significantly reduced plasma glucose concentrations relative to N-AcGIP(Lys³⁷Myr) treated animals (30% reduction; $P < 0.05$) by day 21. Improvements in glucose-lowering were accompanied by significantly increased non-fasting plasma insulin concentrations following administration of Liraglutide (1.3- to 1.4-fold increase; $P < 0.05$), N-AcGIP(Lys³⁷Myr) (1.2- to 1.3-fold increase; $P < 0.05$) and Lira-AcGIP preparation (1.5- to 1.6-fold increase; $P < 0.001$; Figure 3D).

Effects of once-daily Liraglutide, N-AcGIP(Lys³⁷Myr) or Lira-AcGIP preparation on glucose tolerance and insulin response to glucose in *ob/ob* mice

As shown in Figure 4A, Liraglutide, N-AcGIP(Lys³⁷Myr) and Lira-AcGIP preparation significantly reduced plasma glucose levels at 15, 30 and 60 min post-glucose injection (17 to 51% decrease; $P < 0.05 - P < 0.001$), which was corroborated by plasma glucose AUC analysis (21 to 40% reduction; $P < 0.01 - P < 0.001$). Importantly, Lira-AcGIP preparation exhibited a significantly improved glycaemic excursion compared to either Liraglutide and N-AcGIP(Lys³⁷Myr) alone (20 to 36% reduction; $P < 0.05 - P < 0.01$; Figure 4A). As shown in Figure 4B, glucose-lowering actions were mirrored by significantly increased plasma insulin response to glucose for Liraglutide and N-AcGIP(Lys³⁷Myr) (1.3- to 1.6-fold increase; $P < 0.05$) and Lira-AcGIP preparation (1.9 fold increase; $P < 0.05 - P < 0.001$) relative to saline-treated animals.

Effects of once-daily Liraglutide, N-AcGIP(Lys³⁷Myr) or Lira-AcGIP preparation on insulin sensitivity, pancreatic insulin and circulating triglycerides in *ob/ob* mice

No improvements on insulin sensitivity (Figure 5A) or circulating triglycerides (Figure 5B) were observed following daily treatment with Liraglutide, N-AcGIP(Lys³⁷Myr) or Lira-AcGIP preparation. All treatment groups exhibited significantly enhanced pancreatic insulin content (1.3- to 1.7-fold increase; $P < 0.05 - P < 0.01$; Figure 5C).

DISCUSSION

One successful strategy which has been employed to overcome the therapeutic barrier posed by very short half-life of native GLP-1 is the use of peptide acylation which promotes both stability to enzymatic degradation by DPP-IV and non-covalent binding to serum proteins thereby significantly prolonging biological half-life [6, 32]. Similarly, acylation of GIP has been shown to greatly improve bioactivity and therapeutic effects in diabetic animal models, especially when combined with N-terminal acetylation [23-26, 29]. Perhaps much less well recognised is the concept that acylation of peptides, including insulin and GLP-1, facilitates the process of self-association [32, 36]. Indeed, Liraglutide forms strongly self-associated heptamers in solution which appears key for protracted absorption after injection and the pharmacokinetic profile suitable for once-daily administration [37, 38]. As such, it is possible that a preparation of combined acylated GLP-1 and GIP peptides could provide additional non-covalent binding of peptide fatty acid chains thus promoting stability, potency, biological effectiveness and at the same time enabling activation of

both important physiological arms of the enteroinsular axis. In this study, we have examined the effects of a Liraglutide and *N*-AcGIP(Lys³⁷Myr) preparation (Lira-AcGIP), formed by simple self-association, on DPP-IV stability, *in vitro* cAMP production and insulin secretion, and *in vivo* glucose-lowering effects in normal and obese diabetic (*ob/ob*) mice. The actions of the Lira-AcGIP preparation were compared to either peptide given alone.

As observed in previous studies, both native GLP-1 and GIP were rapidly degraded by DPP-IV *in vitro* [29, 39]. Liraglutide, *N*-AcGIP(Lys³⁷Myr), a simple combination of both peptides and the Lira-AcGIP preparation remained intact throughout the entire 24 hours with significantly more intact peptide remaining at all time points compared to native GLP-1 and GIP. This enhanced DPP-IV resistance likely results from masking the enzyme cleavage site [40], alterations to peptide charge or possibly related to hydro-affinity [24, 40]. Importantly, the Lira-AcGIP preparation was completely stable over the incubation period, highlighting that combining Liraglutide with *N*-AcGIP(Lys³⁷Myr) did not have any detrimental effects on susceptibility to DPP-IV degradation.

Native GLP-1, GIP, Liraglutide, *N*-AcGIP(Lys³⁷Myr), a simple combination of both Liraglutide plus *N*-AcGIP(Lys³⁷Myr) and the Lira-AcGIP preparation concentration-dependently stimulated cAMP production in an equipotent manner in BRIN-BD11 cells. Estimated EC₅₀ values correlated well with previous observations and were not significantly different for any of the test groups [29, 39]. These observations demonstrate that peptide modifications and especially formation of the Lira-AcGIP preparation did not adversely affect cAMP second messenger production. Similarly, all test agents, including the Lira-AcGIP preparation stimulated insulin secretion equally, again with no apparent loss of potency relative to native ligands.

To assess the acute glucose-lowering and insulinotropic actions of the GLP-1 and GIP peptides normal Swiss *TO* mice were employed. The native incretins were inactive when administered four hours prior to an intraperitoneal glucose load, whereas Liraglutide and *N*-AcGIP(Lys³⁷Myr) both displayed notable effects on glycaemic control as previously described [29, 39]. Simple combination of Liraglutide plus *N*-AcGIP(Lys³⁷Myr) resulted in more prominent responses than saline controls but did not reveal significant differences compared to single doses of Liraglutide or *N*-AcGIP(Lys³⁷Myr) given alone. In contrast, the Lira-AcGIP preparation exhibited a significantly improved glycaemic profile compared to Liraglutide, *N*-AcGIP(Lys³⁷Myr) and a simple combination of both Liraglutide plus *N*-AcGIP(Lys³⁷Myr). In line with effects on glucose-lowering, the Lira-AcGIP preparation resulted in significantly greater stimulation of insulin release. The greater potency of the pharmacological preparations *in vivo* presumably reflects differences in the bioavailability and pharmacokinetics of injected preparations. These data demonstrate the beneficial effects of the Lira-AcGIP preparation over simple combination and use of either constituent incretin mimetic given alone.

Results from acute *in vivo* studies provided a strong basis for the subsequent 21-day sub-chronic study using genetically obese diabetic (*ob/ob*) mice [34]. Further, we chose to administer the Lira-AcGIP preparation once-daily and to compare this to either Liraglutide or *N*-AcGIP(Lys³⁷Myr) given alone. As expected, *N*-AcGIP(Lys³⁷Myr) treatment did not affect body weight or food intake corroborating other findings that GIP does not affect feeding activity [23-39, 41]. While both Liraglutide and the Lira-AcGIP preparation significantly reduced food intake towards the end of the study period, only the Lira-AcGIP preparation led to a significant reduction in body weight by day 18 onwards. This is most likely due to CNS actions as Liraglutide and other GLP-1 analogues have been shown to cross the blood-brain-barrier (42-45) and peripheral administration of Liraglutide activates several CNS nuclei (45-46). However, further studies examining the effects of Lira-AcGIP on weight reduction including gene and protein expression is necessary. Importantly, during routine physical examination of the mice over the 21-day study period, we did not observe any visible negative or toxic effects of drug treatment. Liraglutide, *N*-AcGIP(Lys³⁷Myr) and the Lira-AcGIP preparation significantly decreased non-fasting plasma glucose levels, accompanied by significantly elevated plasma insulin concentrations. These effects were particularly prominent in the Lira-AcGIP preparation-treated group, with significantly improved glucose-lowering compared with *N*-AcGIP(Lys³⁷Myr). Consistent with these actions, the

Lira-AcGIP preparation significantly improved glucose tolerance compared to either incretin mimetic given alone. This was accompanied by a significant enhancement of the plasma insulin response to glucose, demonstrating its ability to overcome the beta-cell defect in this animal model [47]. Thus, a large part of the glucose-lowering actions of these pharmacological preparations stems from their potent insulinotropic actions. However other receptor-mediated biochemical effects, such as inhibition of glucagon secretion, may be involved in improving metabolic control. Whilst all treatments improved pancreatic insulin content, changes in glucose homeostasis were not associated with any change of insulin sensitivity or circulating triglycerides highlighting a primary mode of action on the pancreatic beta-cell.

Defects in GLP-1 secretion and GIP action contribute to the pathogenesis of diabetes and the introduction of stable, long-acting GLP-1 mimetics represents one of the most significant therapeutic developments over recent years [1, 6, 48]. Reconstitution of normal physiological incretin action in diabetes using a combination of GLP-1 and GIP may enable further improvements in glycaemic control. Additional therapeutic strategies leading to improvement of beta-cell responsiveness to GIP are needed. However, this study demonstrates that an acylated GLP-1 and GIP preparation offers beneficial long-acting glucose-lowering and insulinotropic actions compared to single component peptide injections. These data point towards the possibility of developing novel formulations of acylated incretin peptides that will be effective in exploiting both arms of the enteroinsular axis for the treatment of diabetes.

AUTHOR CONTRIBUTIONS

Victor Gault and Peter Flatt conceived the experiments, co-wrote and proof-read the manuscript. Barry Kerr performed all experiments, analysed data and co-wrote the manuscript. Patrick Harriott synthesised *N*-AcGIP(Lys³⁷Myr) peptide.

FUNDING

These studies were supported by The SAAD Contracting and Trading Company and University of Ulster Strategic Research Funding.

ACKNOWLEDGEMENTS

The authors wish to thank Professor Clifford Bailey (Aston, UK) for provision of breeding pairs of *ob*/+ mice.

REFERENCES

1. Robertson, R.P., Kendall, D.M. and Seaquist, E.R. (2010) Diabetes: Strategies to prevent the type 2 diabetes mellitus epidemic. *Nat. Rev. Endocrinol.* **6**, 128-129
2. Currie, C.J., Peters, J.R. and Evans, M. (2010) Dispensing patterns and financial costs of glucose-lowering therapies in the UK from 2000 to 2008. *Diabet. Med.* **27**, 744-752
3. Baggio, L.L. and Drucker, D.J. (2007) Biology of incretins: GLP-1 and GIP. *Gastroenterology* **132**, 2131-2157
4. Creutzfeldt, W. (2001) The entero-insular axis in type 2 diabetes – incretins as therapeutic agents. *Exp. Clin. Endocrinol. Diabetes* **109**, S288-S303
5. Flatt, P.R., Bailey, C.J. and Green, B.D. (2009) Recent advances in antidiabetic drug therapies targeting the enteroinsular axis. *Curr. Drug Metab.* **10**, 125-137
6. Lovshin, J.A. and Drucker, D.J. (2009) Incretin-based therapies for type 2 diabetes mellitus. *Nat. Rev. Endocrinol.* **5**, 262-269

7. Deacon, C.F., Johnsen, A.H. and Holst, J.J. (1995) Degradation of glucagon-like peptide-1 by human plasma in vitro yields an N-terminally truncated peptide that is a major endogenous metabolite in vivo. *J. Clin. Endocrinol. Metab.* **80**, 952-957
8. Kieffer, T.J., McIntosh, C.H. and Pederson, R.A. (1995) Degradation of glucose-dependent insulinotropic polypeptide and truncated glucagon-like peptide 1 in vitro and in vivo by dipeptidyl peptidase IV. *Endocrinology* **136**, 3585-3896
9. Meier, J.J., Nauck, M.A., Kranz, D., Holst, J.J., Deacon, C.F., Gaeckler, D., Schmidt, W.E. and Gallwitz, B. (2004) Secretion, degradation and elimination of glucagon-like peptide 1 and gastric inhibitory polypeptide in patients with chronic renal insufficiency and healthy control subjects. *Diabetes* **53**, 654-662
10. Fonseca, V.A., Zinman, B., Nauck, M.A., Goldfine, A.B. and Plutzky, J. (2010) Confronting the type 2 diabetes epidemic: the emerging role of incretin-based therapies. *Am. J. Med.* **123**, S2-S10
11. Drab, S.R. (2010) Incretin-based therapies for type 2 diabetes mellitus: current status and future prospects. *Pharmacotherapy* **30**, 609-624
12. Knudsen, L.B., Nielsen, P.F., Huusfeldt, P.O., Johansen, N.L., Madsen, K., Pedersen, F.Z., Thøgersen, H., Wilken, M. and Agersø, H. (2000) Potent derivatives of glucagon-like peptide-1 with pharmacokinetic properties suitable for once-daily administration. *J. Med. Chem.* **43**, 1664-1669
13. Juhl, C.B., Hollingdal, M., Sturis, J., Jakobsen, G., Agersø, H., Veldhuis, J., Pørksen, N. and Schmitz, O. (2002) Bedtime administration of NN2211, a long-acting GLP-1 derivative, substantially reduces fasting and postprandial glycemia in type 2 diabetes. *Diabetes* **51**, 424-429
14. Nauck, M., Frid, A., Hermansen, K., Shah, N.S., Tankova, T., Mitha, I.H., Zdravkovic, M., Düring, M. and Matthews, D.R.; LEAD-2 Study Group (2009) Efficacy and safety comparison of liraglutide, glimepride, and placebo, all in combination with metformin, in type 2 diabetes: the LEAD (liraglutide effect and action in diabetes)-2 study. *Diabetes Care* **32**, 84-90
15. Bregenholt, S., Møldrup, A., Blume, N., Karlsen, A.E., Nissen, A.E., Friedrichsen, B., Tornhave, D., Knudsen, L.B. and Petersen, J.S. (2005) The long-acting glucagon-like peptide-1 analogue, liraglutide, inhibits beta-cell apoptosis in vitro. *Biochem. Biophys. Res. Commun.* **330**, 577-584
16. Ribel, U., Larsen, M.O., Rolin, B., Carr, R.D., Wilken, M., Sturis, J., Westergaard, L., Deacon, C.F. and Knudsen, L.B. (2002) NN2211: a long-acting glucagon-like peptide-1 derivative with anti-diabetic effects in glucose-intolerant pigs. *Eur. J. Pharmacol.* **451**, 217-225
17. Chang, A.M., Jakobsen, G., Sturis, J., Smith, M.J., Bloem, C.J., An, B., Galecki, A. and Halter, J.B. (2005) The GLP-1 derivative NN2211 restores beta-cell sensitivity to glucose in type 2 diabetic patients after a single dose. *Diabetes* **52**, 1786-1791
18. Feinglos, M.N., Saad, M.F., Pi-Sunyer, F.X., An, B. and Santiago, O; Liraglutide Dose-Response Study Group (2005) Effects of liraglutide (NN2211), a long-acting GLP-1 analogue, on glycaemic control and bodyweight in subjects with type 2 diabetes. *Diabet. Med.* **22**, 1016-1023
19. Gault, V.A., Flatt, P.R., Bailey, C.J., Harriott, P., Greer, B., Mooney, M.H. and O'Harte, F.P. (2002) Enhanced cAMP generation and insulin-releasing potency of two novel Tyr¹-modified enzyme-resistant forms of glucose-dependent insulinotropic polypeptide is associated with significant antihyperglycaemic activity in spontaneous obesity-diabetes. *Biochem. J.* **367**, 913-920
20. O'Harte, F.P., Gault, V.A., Parker, J.C., Harriott, P., Mooney, M.H., Bailey, C.J. and Flatt, P.R. (2002) Improved stability, insulin-releasing activity and antidiabetic potential of two novel N-terminal analogues of gastric inhibitory polypeptide: N-acetyl-GIP and pGlu-GIP. *Diabetologia* **45**, 1281-1291

21. Gault, V.A., Flatt, P.R., Harriott, P., Mooney, M.H., Bailey, C.J. and O'Harte, F.P. (2002) Improved biological activity of Gly²- and Ser²-substituted analogues of glucose-dependent insulinotropic polypeptide. *J. Endocrinol.* **176**, 133-141
22. Hinke, S.A., Gelling, R.W., Pederson, R.A., Manhart, S., Nian, C., Demuth, H.U. and McIntosh, C.H. (2002) Dipeptidyl peptidase IV-resistant [D-Ala(2)]glucose-dependent insulinotropic polypeptide (GIP) improves glucose tolerance in normal and obese diabetic rats. *Diabetes* **51**, 652-661
23. Irwin, N., Green, B.D., Gault, V.A., Greer, B., Harriott, P., Bailey, C.J., Flatt, P.R. and O'Harte, F.P. (2005) Degradation, insulin secretion, and antihyperglycaemic actions of two palmitate-derivatized, N-terminal pyroglutamyl analogues of glucose-dependent insulinotropic polypeptide. *J. Med. Chem.* **48**, 1244-1250
24. Irwin, N., Gault, V.A., Green, B.D., Greer, B., Harriott, P., Bailey, C.J., Flatt, P.R. and O'Harte, F.P. (2005) Antidiabetic potential of two novel fatty acid derivatised, N-terminally modified analogues of glucose-dependent insulinotropic polypeptide (GIP): N-AcGIP(LysPAL16) and N-AcGIP(LysPAL37). *Biol. Chem.* **386**, 679-687
25. Irwin, N., Green, B.D., Mooney, M.H., Greer, B., Harriott, P., Bailey, C.J., Gault, V.A., O'Harte, F.P. and Flatt, P.R. (2005) A novel, long-acting agonist of glucose-dependent insulinotropic polypeptide suitable for once-daily administration in type 2 diabetes. *J. Pharmacol. Exp. Ther.* **314**, 1187-1194
26. Irwin, N., O'Harte, F.P., Gault, V.A., Green, B.D., Greer, B., Harriott, P., Bailey, C.J. and Flatt, P.R. (2006) GIP(Lys16PAL) and GIP(Lys37PAL) : novel long-acting acylated analogues of glucose-dependent insulinotropic polypeptide with improved antidiabetic potential. *J. Med. Chem.* **49**, 1047-1054
27. Irwin, N., Clarke, G.C., Green, B.D., Greer, B., Harriott, P., Gault, V.A., O'Harte, F.P. and Flatt, P.R. (2006) Evaluation of the antidiabetic activity of DPP-IV resistant N-terminally modified versus mid-chain acylated analogues of glucose-dependent insulinotropic polypeptide. *Biochem. Pharmacol.* **72**, 719-728
28. Lamont, B.J. and Drucker, D.J. (2008) Differential antidiabetic efficacy of incretin agonists versus DPP-4 inhibition in high fat fed mice. *Diabetes* **57**, 190-198
29. Kerr, B.D., Irwin, N., O'Harte, F.P., Bailey, C.J., Flatt, P.R. and Gault, V.A. (2009) Fatty acid derivatised analogues of glucose-dependent insulinotropic polypeptide with improved antihyperglycaemic and insulinotropic properties. *Biochem. Pharmacol.* **78**, 1008-1016
30. Aaboe, K., Knop, F.K., Vilsboll, T., Vølund, A., Simonsen, U., Deacon, C.F., Madsbad, S., Holst, J.J. and Krarup, T. (2009) KATP channel closure ameliorates the impaired insulinotropic effect of glucose-dependent insulinotropic polypeptide in patients with type 2 diabetes. *J. Clin. Endocrinol. Metab.* **4**, 603-608
31. Hojberg, P.V., Vilsboll, T., Rabol, R., Knop, F.K., Bache, M., Krarup, T., Holst, J.J. and Madsbad, S. (2009) Four weeks of near-normalisation of blood glucose improves the insulin response to glucagon-like peptide-1 and glucose-dependent insulinotropic polypeptide in patients with type 2 diabetes. *Diabetologia* **52**, 199-207
32. Knudsen, L.B. (2004) Glucagon-like peptide-1: the basis of a new class of treatment for type 2 diabetes. *J. Med. Chem.* **47**, 4128-4134
33. Clodfelter, D.K., Pekar, A.H., Rebhun, D.M., Destrampe, K.A., Havel, H.A., Myers, S.R. and Brader, M.L. (1998) Effects of non-covalent self-association on the subcutaneous absorption of a therapeutic peptide. *Pharm. Res.* **15**, 254-262
34. Bailey, C.J., Flatt, P.R. and Atkins, T.W. (1982) Influence of genetic background and age on the expression of the obese hyperglycaemic syndrome in Aston ob/ob mice. *Int. J. Obes.* **6**, 11-21
35. Flatt, P.R. and Bailey, C.J. (1981) Abnormal plasma glucose and insulin responses in heterozygous lean (ob/+) mice. *Diabetologia* **20**, 573-577

36. Havelund, S., Plum, A., Ribel, U., Jonassen, I., Vølund, A., Markussen, J. and Kurtzhals P. (2004) The mechanism of protraction of insulin detemir, a long-acting, acylated analog of human insulin. *Pharm. Res.* **21**, 1498-1504
37. Knudsen LB, Nielsen PF, Steensgaard DB et al. Liraglutide, the once-daily human GLP-1 analogue, has a protracted profile based on both delayed absorption and a long plasma half-life. *Diabetologia* 2009; **52**[Suppl 1]: A775.
38. Steensgaard, D.B., Thomsen, J.K., Olsen, H.B. and Knudsen, L.B. (2008) The molecular basis for the delayed absorption of the once-daily human GLP-1 analog, Liraglutide. *Diabetes* **57**[Suppl 1]: A164
39. Kerr, B.D., Flatt, P.R. and Gault, V.A. (2010) Effects of gamma-glutamyl linker on DPP-IV resistance, duration of action and biological efficacy of acylated glucagon-like peptide-1. *Biochem. Pharmacol.* **80**, 396-401
40. Kuhn-Wache, K., Hoffman, T., Manhart, S., Brandt, W. and Demuth, H.U. (2003) The specificity of DPP IV for natural substances is peptide structure determined. *Adv. Exp. Med. Biol.* **524**, 57-63
41. Woods, S.C., West, D.B., Stein, L.J., McKay, L.D., Lotter, E.C., Porte, S.G., Kenney, N.J. and Porte, D. Jr. (1981) Peptides and the control of meal size. *Diabetologia* **20**, 305-313
42. Kastin, A.J., Akerstrom, V. and Pan, W. (2002) Interactions of glucagon-like peptide-1 (GLP-1) with the blood-brain-barrier. *J. Mol. Neurosci.* **18**, 7-14
43. Kastin, A.J. and Akerstrom, V. (2003) Entry of exendin-4 into brain is rapid but may be limited at high doses. *Int. J. Obes. Relat. Metab. Disord.* **27**, 313-318
44. McClean, P.L., Gault, V.A., Harriott, P. and Holscher, C. (2010) Glucagon-like peptide-1 analogues enhance synaptic plasticity in the brain: a link between diabetes and Alzheimer's disease. *Eur. J. Pharmacol.* **630**, 158-162
45. Holscher, C. (2010) The role of GLP-1 in neuronal activity and neurodegeneration. *Vitam. Horm.* **84**, 331-354
46. Holst, J.J., Burcelin, R. and Nathanson, E. (2011) Neuroprotective properties of GLP-1: theoretical and practical applications. *Curr. Med. Res. Opin.* **27**, 547-558
47. Flatt, P.R. and Bailey, C.J. (1981) Development of glucose intolerance and impaired plasma insulin response to glucose in obese hyperglycaemic (ob/ob) mice. *Horm. Metab. Res.* **13**, 556-560
48. Holst, J.J., Vilsbøll, T. and Deacon, C.F. (2009) The incretin system and its role in type 2 diabetes mellitus. *Mol. Cell. Endocrinol.* **297**, 127-136

Accepted Article

Table 1 Structural characteristics of GLP-1 and GIP peptides using rp-HPLC and MALDI-TOF MS

Peptide	rp-HPLC		MALDI-TOF MS	
	Retention (min)	time	Theoretical mass (Da)	Observed mass (Da)
GLP-1	19.8		3297.0	3298.4
GIP	18.6		4982.4	4982.9
Liraglutide	26.2		3751.0	3752.2
<i>N</i> -AcGIP(Lys ³⁷ Myr)	46.8		5235.8	5236.4

Peptides were purified by rp-HPLC [29] and retention times recorded. Purified samples were subsequently mixed with α -cyano-4-hydroxycinnamic acid, applied to sample plate of a Voyager-DE BioSpectrometry Workstation and *m/z* ratio vs relative peak intensity recorded. Theoretical mass was included for comparative purposes.

Accepted Manuscript

Figure Legends

Figure 1 DPP-IV resistance, cAMP production and insulin secretion of GLP-1 and GIP peptides. (A) Resistance of GIP, GLP-1, Liraglutide, *N*-AcGIP(Lys³⁷Myr), a simple combination of Liraglutide plus *N*-AcGIP(Lys³⁷Myr), and Lira-AcGIP preparation to degradation by DPP-IV (5 mU) was measured (n=3) following 0, 2, 4, 8 and 24 hour incubations. Reaction products were subsequently separated by rp-HPLC and degradation expressed as a percentage of intact peptide. ****P* < 0.001 compared with native GIP. ^Δ*P* < 0.05, ^{ΔΔ}*P* < 0.001 compared with native GLP-1. (B) BRIN-BD11 cells were exposed to various peptide concentrations for 20 min (n = 4) and cAMP production assayed using ELISA. (C) BRIN-BD11 cells were incubated with a range of peptide concentrations for 20 min (n = 8) in the presence of 5.6 mM glucose and insulin-release measured using RIA. Values represent mean ± S.E.M. ***P* < 0.01, ****P* < 0.001 compared with 5.6 mM glucose control.

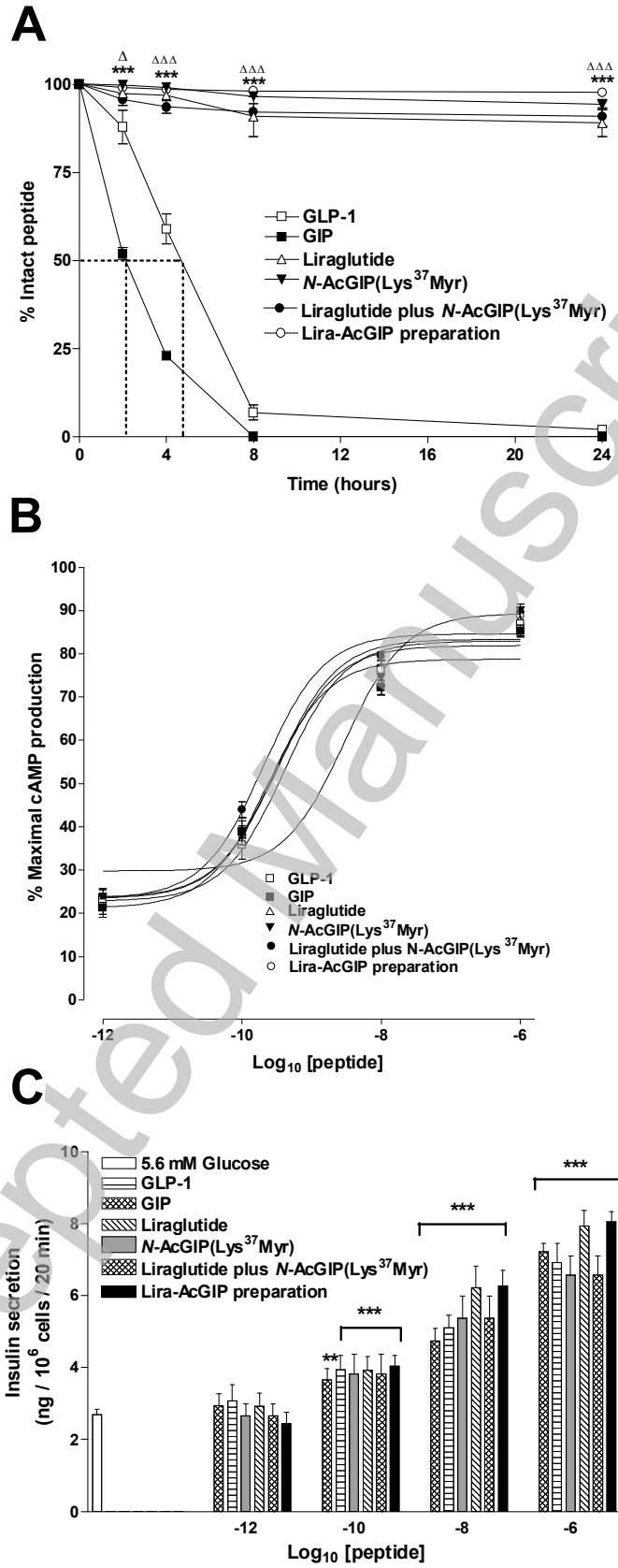
Figure 2 Comparative effects of acylated GLP-1 and GIP peptides plus peptide combinations on glucose tolerance and insulin response to glucose in Swiss *To* mice. (A) Plasma glucose and (B) insulin concentrations were measured prior to and after administration of glucose (18 mmol/kg bw) in animals injected previously (-240 min) with saline vehicle (0.9% (w/v) NaCl), Liraglutide, *N*-AcGIP(Lys³⁷Myr), simple combination of Liraglutide plus *N*-AcGIP(Lys³⁷Myr), or Lira-AcGIP preparation (100 nmol/kg bw). All peptides were administered ip at a dose of 50 nmol/kg unless otherwise stated. Time of injection is indicated by the arrow. Plasma glucose and insulin area under the curve (AUC) values for 0-60 min post-injection are included. Values represent mean ± S.E.M. for 8 mice. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared to saline control. ^Δ*P* < 0.05 compared with *N*-AcGIP(Lys³⁷Myr)-treated mice. ⁺*P* < 0.05 compared with Liraglutide-treated mice. [°]*P* 0.05 compared to a simple combination of Liraglutide plus *N*-AcGIP(Lys³⁷Myr)-treated mice.

Figure 3 Effects of daily administration of Liraglutide, *N*-AcGIP(Lys³⁷Myr) and Lira-AcGIP preparation on (A) bodyweight, (B) food intake, (C) plasma glucose and (D) plasma insulin in *ob/ob* mice. Parameters were measured prior to and 21 days during treatment with saline vehicle (0.9% (w/v) NaCl, ip), Liraglutide, *N*-AcGIP(Lys³⁷Myr) (each at 50 nmol/kg, ip), or Lira-AcGIP preparation (100 nmol/kg bw, ip). Values represent mean ± S.E.M. for 9 mice. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared to saline-treated group. ^Δ*P* < 0.05 compared with *N*-AcGIP(Lys³⁷Myr)-treated mice.

Figure 4 Effects of daily administration of Liraglutide, *N*-AcGIP(Lys³⁷Myr) and Lira-AcGIP preparation on (A) glucose tolerance and (B) plasma insulin response to glucose in *ob/ob* mice. Parameters were measured prior to and after administration of glucose (18 mmol/kg bw, ip) in animals receiving 21 days daily treatment with saline vehicle (0.9% (w/v) NaCl, ip), Liraglutide, *N*-AcGIP(Lys³⁷Myr) (each at 50 nmol/kg, ip), or Lira-AcGIP preparation (100 nmol/kg bw, ip). The time of injection is indicated by the arrow. Values represent mean ± S.E.M. for 9 mice. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared to saline-treated mice. ^Δ*P* < 0.05, ^{ΔΔ}*P* < 0.01 compared to *N*-AcGIP(Lys³⁷Myr)-treated mice. ⁺*P* < 0.05 compared to Liraglutide-treated mice.

Figure 5 Effects of daily administration of Liraglutide, *N*-AcGIP(Lys³⁷Myr) and Lira-AcGIP preparation on (A) insulin sensitivity, (B) circulating triglycerides and (C) pancreatic insulin content in *ob/ob* mice. Parameters were measured after 21 days daily treatment with saline vehicle (0.9% (w/v) NaCl, ip), Liraglutide, *N*-AcGIP(Lys³⁷Myr) (each at 50 nmol/kg, ip), or Lira-AcGIP preparation (100 nmol/kg bw, ip). Insulin (50 U/kg bw, ip) was injected at time indicated by the arrow. Values represent mean ± S.E.M. for 9 mice. **P* < 0.05, ***P* < 0.01 compared to saline-treated mice.

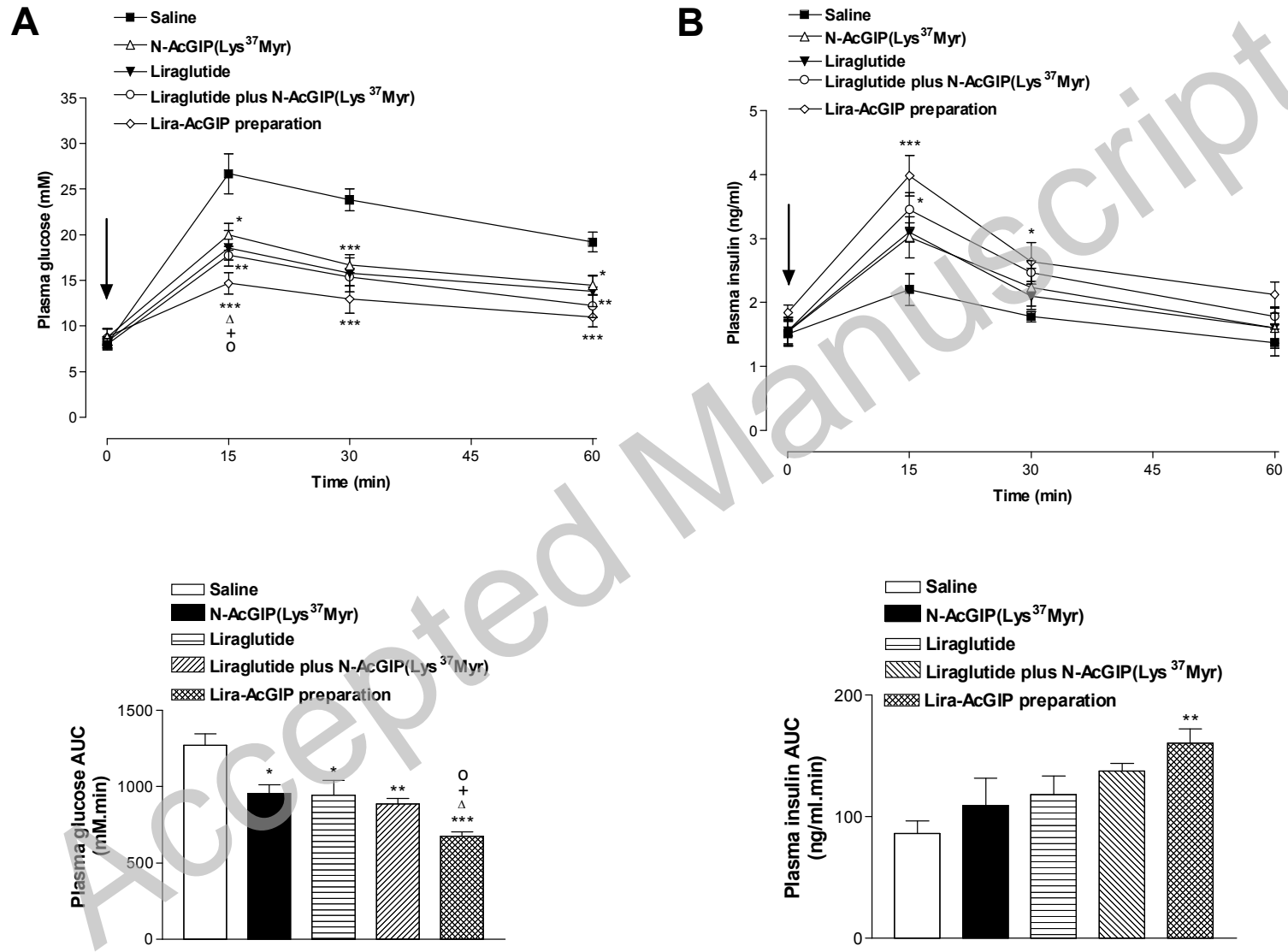
Figure 1



THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/CS20110006

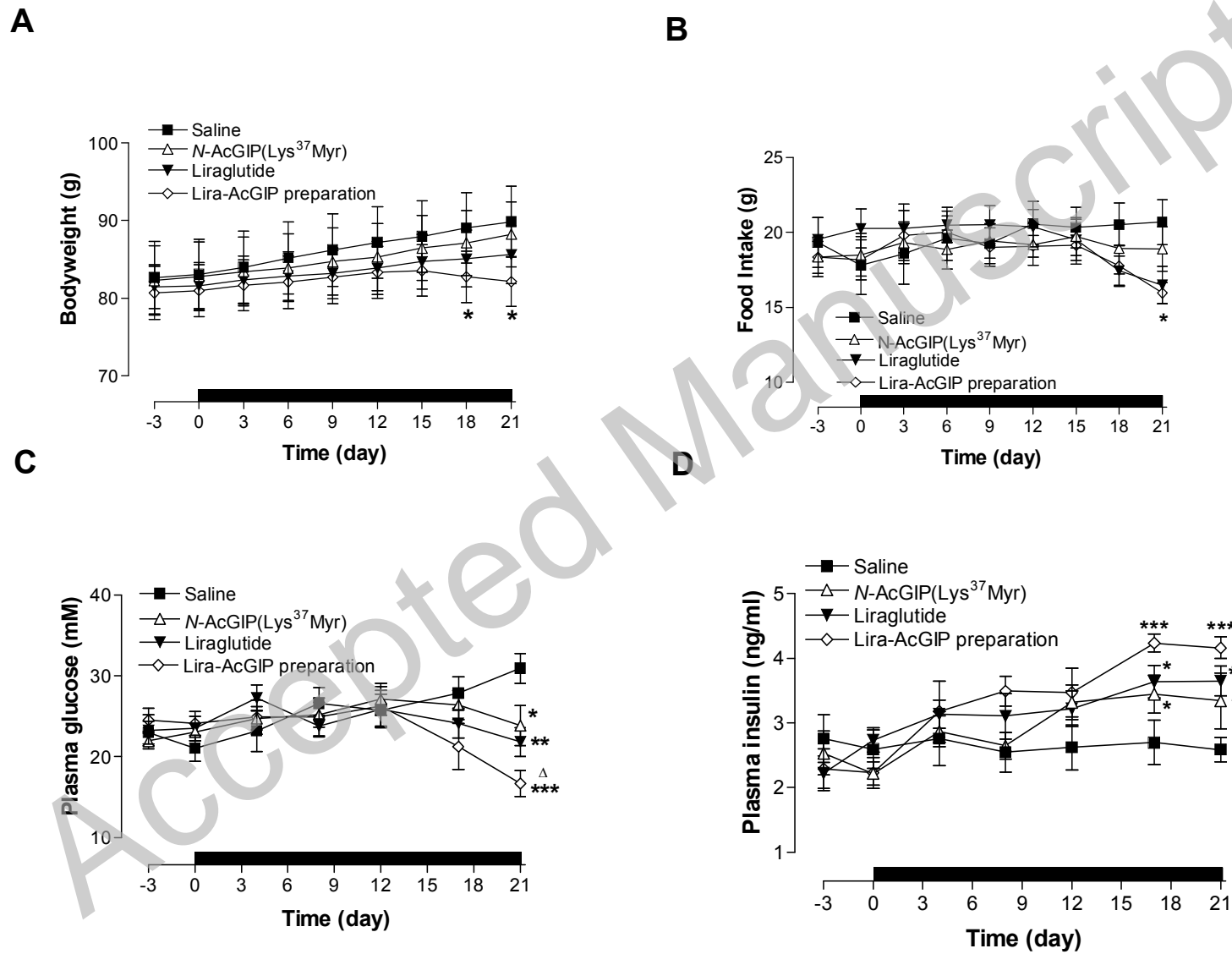
Accepted Manuscript

Figure 2



THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/CS20110006

Figure 3



THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/CS20110006

Figure 4

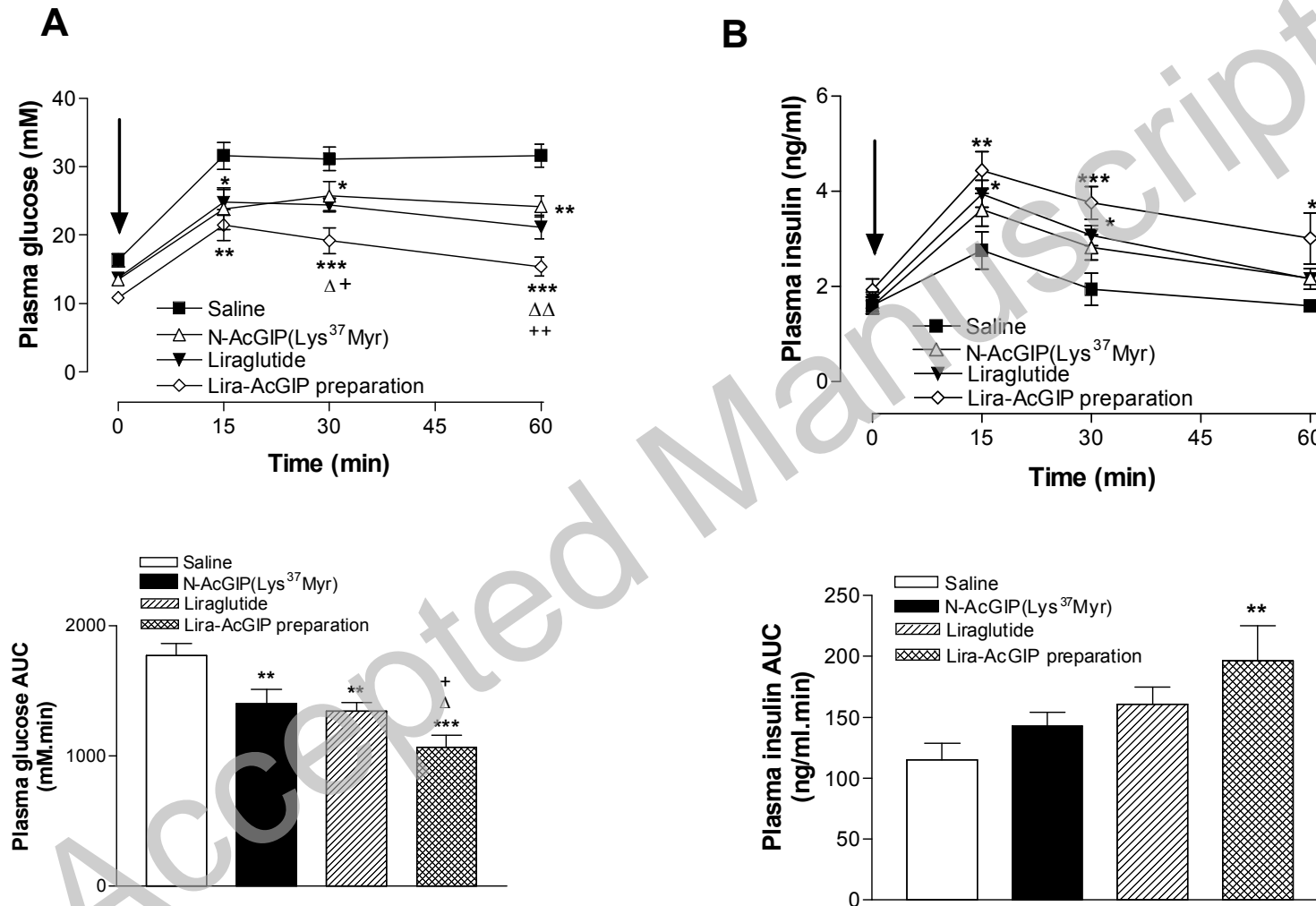
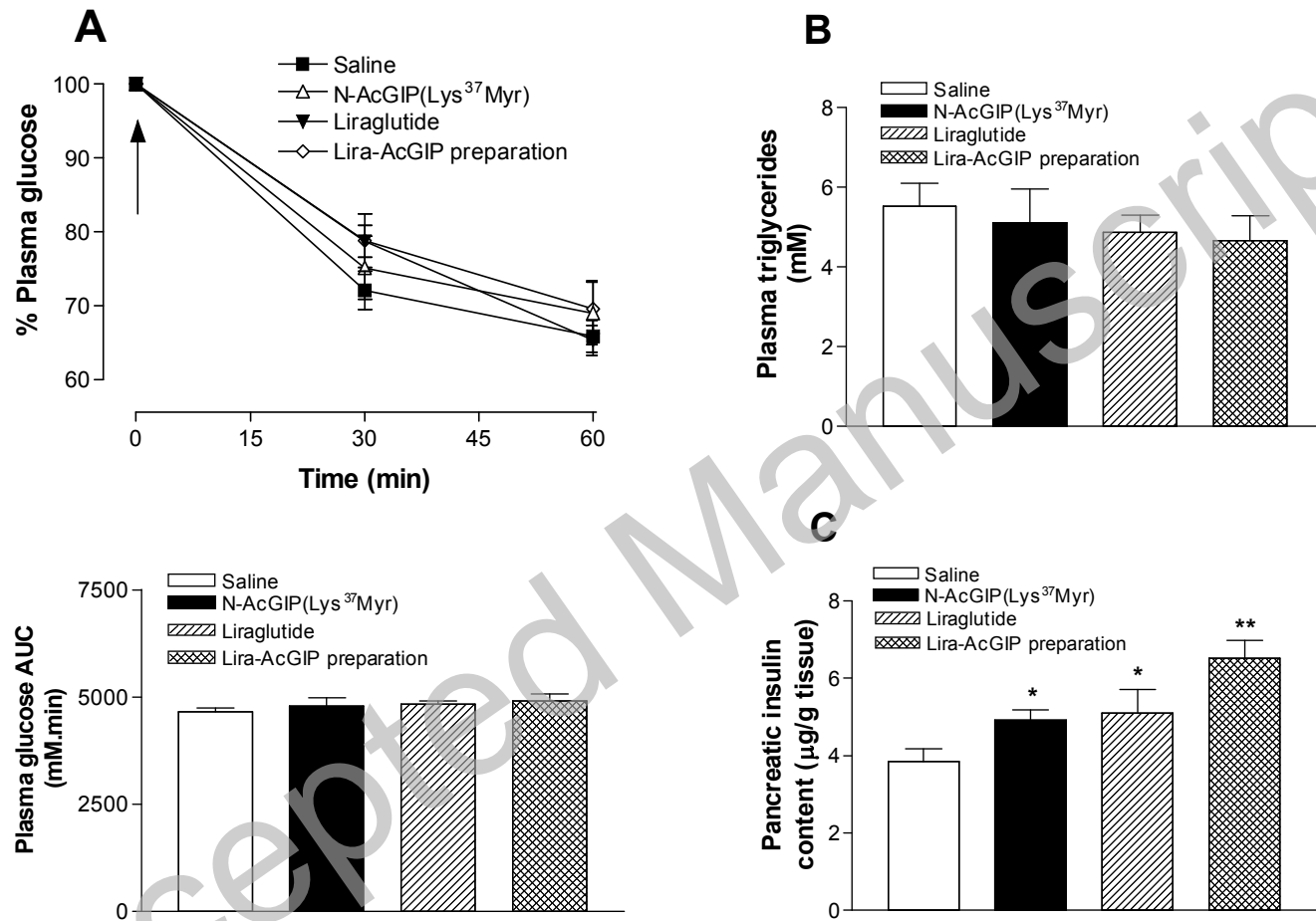


Figure 5



THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/CS20110006

Accepted Manuscript