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Effects of γ-glutamyl linker on DPP-IV resistance, duration of action and biological efficacy of acylated glucagon-like peptide-1

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ABSTRACT

Liraglutide, a GLP-1 mimetic has recently been approved for clinical use in obesity-diabetes. The purpose of this study was to assess if acylation of Liraglutide via its γ-glutamyl linker contributes to DPP-IV inhibition and efficacy of the molecule, given that such an approach could be useful in prolonging bioactivity of related peptides. Liraglutide lacking the γ-glutamyl linker (Lira-γGlu) and Liraglutide exhibited enhanced DPP-IV resistance with extension of $t_{1/2}$ plus effective cAMP production (EC_{50}: 0.15 ± 0.11 and 0.16 ± 0.11 nM, respectively) compared to GLP-1 (EC_{50} 3.81 ± 0.80 nM). GLP-1, Lira-γGlu and Liraglutide increased insulin secretion compared to glucose (1.5–3.0-fold; $p < 0.05$ to $p < 0.001$). *In vivo*, Lira-γGlu and Liraglutide significantly lowered plasma glucose when administered 4 and 8 h prior to a glucose load (1.3–1.9-fold; $p < 0.05$ to $p < 0.001$). Twice-daily administration of Lira-γGlu and Liraglutide for 14 days significantly decreased food intake (1.2-fold; $p < 0.05$) and plasma glucose (1.1–1.6-fold; $p < 0.05$ to $p < 0.01$) whilst increasing plasma insulin (1.4–1.6-fold; $p < 0.05$). At 14 days, Lira-γGlu and Liraglutide markedly improved glucose tolerance (1.4–3.4-fold; $p < 0.05$ to $p < 0.001$), insulin response to glucose (1.4–1.5-fold; $p < 0.05$), insulin sensitivity (1.3–1.4-fold; $p < 0.05$ to $p < 0.01$), as well as increasing pancreatic insulin content (1.4-fold; $p < 0.05$). Functional characteristics of Lira-γGlu and Liraglutide are almost indistinguishable, questioning necessity of γ-glutamyl linker in acylation for generation of long-acting incretin mimetics.

1. Introduction

The enteroinsular axis first coined by Unger and Eisentraut refers to all gut factors involved in stimulating insulin release following nutrient absorption [1]. This was further characterised to include hormonal gut factors defined as incretin hormones that stimulate insulin secretion in response to elevated blood glucose [2]. Whilst several gut factors (e.g. secretin and cholecystokinin) were initially considered to be incretin hormones, they were dismissed due to their inability to stimulate insulin release at physiological concentrations [3]. Glucose-dependent insulinotropic polypeptide (GIP) and sister hormone glucagon-like peptide-1 (GLP-1) are now acknowledged to account for the total hormonal post-prandial insulin response of the enteroinsular axis [4].

The primary action of incretin hormones is to stimulate glucose-dependent insulin secretion from pancreatic beta-cells in response to elevated blood glucose following nutrient absorption [4]. However, GLP-1 possesses a number of secondary actions that further enhance its therapeutic potential such as reducing bodyweight through altering feeding activity, inhibiting gastric emptying and glucagon secretion [5]. Furthermore, GLP-1 aids glucose homeostasis through up-regulation of insulin biosynthesis, pancreatic beta-cell growth, differentiation, proliferation and survival [5], as well as improving glucose uptake in peripheral tissues [6]. Despite this, progression of GLP-1-based therapies to the clinic has been severely hindered. Native GLP-1 has a short circulating half-life (~3–5 min) due to rapid degradation by the ubiquitous enzyme dipeptidylpeptidase-IV (DPP-IV) leaving truncated GLP-1(9–36)amide [7]. This enzymatic degradation is efficiently followed by rapid renal clearance of any remaining intact hormone and degradation products [8].

Several strategies have been employed to circumvent these limitations in order to exploit the pharmaceutical potential of GLP-1. Indeed, numerous N-terminally modified analogues of GLP-1 have shown DPP-IV resistance [9–11]. PEGylation and acylation appear to be the most commonly employed strategies to combat renal filtration of incretin analogues [12]. PEGylated forms of GLP-1 and GIP have been tested with varying degrees of success [13,14]. Acylation of GLP-1 has proved more successful, with the attachment of C_8 and C_{16} fatty acid moieties creating long-acting anti-hyperglycaemic agents including Liraglutide, LY315902, and Naliglutide [15]. The first incretin-based therapy to reach the market was exendin-4 (Byetta), a peptide isolated from the Gila monster salivary gland which shares 53% sequence homology to native GLP-1 [16]. Liraglutide has

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emerged as the second incretin mimetic in clinical use with acylation extending its half-life by binding to plasma proteins to permit once as opposed to twice-daily injection [15]. This GLP-1 receptor agonist shares 97% homology to human GLP-1 with structural variances restricted to amino acid substitution of K34R and addition of a C-16 acyl moiety at position 26 via a γ-glutamyl linker [17].

Despite numerous reports on the beneficial effects of Liraglutide in treatment of type 2 diabetes [18,19], necessity of the γ-glutamyl linker in augmenting biological function of this mimetic is debatable [17]. Clarification of this issue is particularly important given ongoing strategies to develop long-acting acylated analogues of other therapeutic gut peptides, including GIP and oxyntomodulin [20,21]. Thus, the present study explores effects of the GLP-1 mimetic, Liraglutide, and a structurally modified form lacking the γ-glutamyl linker (designated Lira-γGlu) on *in vitro* DPP-IV resistance, cellular cAMP and insulin secretion as well as acute and sub-chronic actions on glucose homeostasis.

2. Materials and methods

2.1. Peptide synthesis and characterisation

Human GLP-1, Lira-γGlu and Liraglutide (all >97% purity) were purchased from GL Biochem Ltd. (Shanghai, China) and structural identity confirmed using MALDI-Tof MS. Briefly, samples (1 μl) were mixed with matrix solution (1 μl of a 10 mg/ml solution of α-cyano-4-hydroxycinnamic acid (Sigma–Aldrich, UK) in acetonitrile/ethanol (1/1)) placed on one well of a 100-well stainless steel sample plate and allowed to dry at room temperature. Mass spectra were recorded using a Voyager-DE BioSpectrometry Workstation (PerSeptive BioSystems, Framingham, Massachusetts, USA). Masses were recorded as mass-to-charge (m/z) ratio *vs.* relative peak intensity and compared with theoretical values.

2.2. Degradation by DPP-IV

GLP-1, Lira-γGlu and Liraglutide were incubated at 37 °C in 50 mM triethanolamine–HCl (pH 7.8) with purified porcine DPP-IV (5 mU; Sigma–Aldrich, UK) for 0, 2, 4, 8 and 24 h. Enzymatic reactions were terminated through addition of trifluoroacetic acid (TFA) in water (15 μl of 10% (v/v)). Reaction products were applied to a Vydac C-4 column (4.6 mm × 250 mm; The Separations Group, Hesparia, California, USA) and intact peptide separated from the major degradation fragment GLP-1(9-36)amide. The column was equilibrated with 0.12% (v/v) TFA/water at varying flow rates of 1–2 ml/min using 0.1% (v/v) TFA in 70% acetonitrile/water. The concentration of acetonitrile in the eluting solvent was raised from 0% to 45% over 10 min and from 45% to 75% over 60 min. 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 remaining at the time points indicated during the incubation.

2.3. In vitro cAMP production and insulin secretion

The effects of GLP-1, Lira-γGlu and Liraglutide on the stimulation of cAMP production and insulin secretion were measured using clonal pancreatic BRIN-BD11 cells as described previously [20]. For cAMP studies, BRIN-BD11 cells were seeded (100,000 cells per well) into 96-well plates (Nunc, Roskilde, Denmark) and washed with Hank's Buffered Saline solution before being incubated with various concentrations of GLP-1 peptides in the presence of 1 mM IBMX for 20 min at 37 °C. After incubation, medium was removed and cells lysed prior to measurement of cAMP using an HTS Immunoassay Kit (Millipore, Watford, UK). For insulin-release studies, BRIN-BD11 cells were seeded (150,000 cells per well) into 24-well plates (Nunc, Roskilde, Denmark) and allowed to attach overnight at 37 °C. Following a 40 min pre-incubation with Krebs Ringer Buffer solution (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. After 20 min incubation, buffer was removed from each well and aliquots (200 μl) stored at −20 °C prior to measurement of insulin.

2.4. Animals

Male NIH *Swiss* mice (6–8 weeks and 26–30 g; Harlan Ltd., Blackthorne, UK) were age-matched, divided into groups ($n = 8$) 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 fed a high-fat diet composed of 45% fat, 20% protein and 35% carbohydrate (percent total energy 19.45 kJ/g; IPS product supplies Ltd., London, UK) for 140 days prior to commencement of *in vivo* studies. This diet resulted in progressive bodyweight gain (44.8 ± 3.0 g *vs.* 37.9 ± 2.9 g, $p < 0.05$) and hyperglycaemia (10.4 ± 1.1 mM/l *vs.* 6.9 ± 0.4 mM/l, $p < 0.05$) compared with age-matched controls on normal laboratory chow (data not shown). All 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 peptide administration.

2.5. In vivo studies

To assess duration of action, glucose (18 mmol/kg bodyweight) was administered intraperitoneally 4, 8 and 12 h following injection of saline (0.9% (w/v) NaCl) or in combination with GLP-1, Lira-γGlu or Liraglutide (each at 50 nmol/kg bodyweight). Plasma glucose was measured at 0, 15, 30 and 60 min post-glucose administration. To evaluate sub-chronic effects, twice-daily injections of Lira-γGlu and Liraglutide (50 nmol/kg bodyweight) or saline vehicle (0.9% (w/v) NaCl) were administered to high-fat fed mice at 10:00 and 16:00 h over a 14-day treatment period. The dosage used was chosen based on previous studies in rodents [27]. Food intake, bodyweight, plasma glucose and insulin were monitored at intervals of 2–4 days for the 14-day treatment period. Glucose tolerance (18 mmol/kg bodyweight; ip) and insulin sensitivity (50 U/kg bodyweight) tests were performed at day 14. At termination, pancreatic tissues were excised and processed for measurement of insulin following extraction with 5 ml/g ice-cold ethanol [20].

2.6. Biochemical analyses

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) at the time-points indicated in the figures. Whole blood samples were used to measure glucose with an Ascensia® CONTOUR® Microfill Blood Glucose Meter and Ascensia® MICROFILL® test strips (Bayer Healthcare, UK). Plasma samples were prepared immediately by centrifugation using a Beckman micro-centrifuge (Beckman Instruments, Galway, Ireland) for 30 s at 13,000 × *g*. Plasma and pancreatic insulin was assayed by a modified dextran-coated charcoal radioimmunoassay [22]. Plasma triglyceride levels were measured using a Hitachi Automatic Analyser 912 (Boehringer Mannheim, Germany). All analyses were carried out according to the manufacturer's instructions.

2.7. Statistical analysis

Results are expressed as mean ± SEM and data compared using the unpaired Student's *t*-test. Where appropriate, data were

compared using repeated measures ANOVA or one-way ANOVA, followed by the 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 $p < 0.05$.

3. Results

3.1. Confirmation of structural identities of peptides

HPLC retention times for GLP-1, Lira-γGlu and Liraglutide were 19.8, 24.7, and 26.2 min, respectively. Observed masses using MALDI-Tof MS were 3298.4, 3622.2, and 3752.2 Da, respectively which corresponded closely to theoretical values confirming their structural identities.

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

Native GLP-1 was progressively degraded over 24 h with an estimated half-life of 4.5 h (Fig. 1A). Contrastingly, Lira-γGlu and Liraglutide maintained a significantly higher percentage of intact peptide over the 24 h (up to 96-fold; $p < 0.001$), with biological half-lives >24 h. Significantly more Liraglutide was intact compared to Lira-γGlu (1.5-fold; $p < 0.001$) at 24 h. GLP-1, Lira-γGlu and Liraglutide stimulated cAMP production in a concentration-dependent manner with estimated EC_{50} values of 3.81 ± 0.80, 0.15 ± 0.11 and 0.16 ± 0.11 nM, respectively. GLP-1, Lira-γGlu and Liraglutide each stimulated insulin secretion in a concentration-dependent manner (1.5–3.0-fold; $p < 0.05$ to $p < 0.001$) compared to glucose alone (Fig. 1B).

3.3. Persistent anti-hyperglycaemic effects of Lira-γGlu and Liraglutide

As illustrated in Fig. 2, Lira-γGlu and Liraglutide significantly reduced glucose excursion at 15 and 60 min post-glucose load when administered 4 and 8 h previously, compared to saline-treated animals ($p < 0.05$ to $p < 0.001$). In contrast, GLP-1 had no significant effect on glucose excursion compared to saline-treated

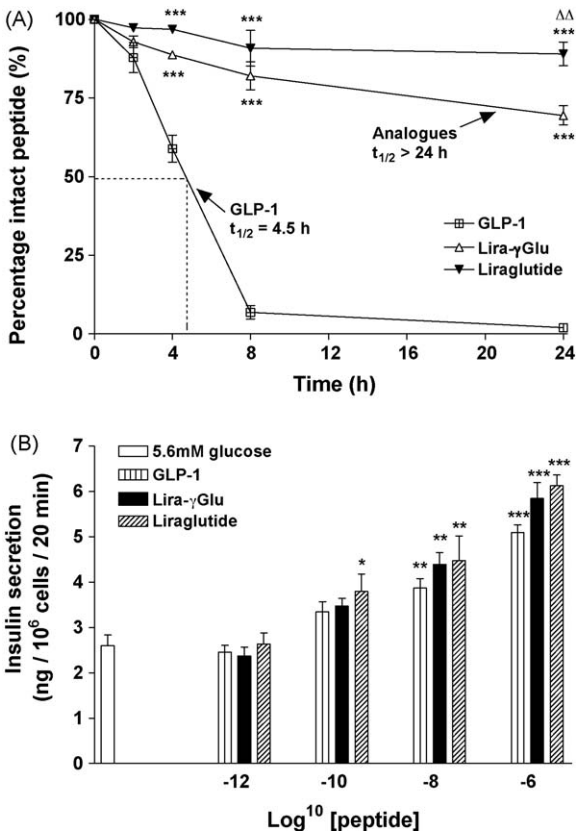

Fig. 1. Effects of Lira-γGlu and Liraglutide on (A) DPP-IV resistance, and (B) insulin secretion. (A) Resistance of native GLP-1, Lira-γGlu and Liraglutide to degradation by DPP-IV (5 mU) was measured ($n = 3$) following 0, 2, 4, 8 and 24 h incubations. Reaction products were subsequently analysed by HPLC and degradation expressed as a percentage of intact peptide. $^{***}p < 0.001$ compared with GLP-1, $^{\Delta\Delta}p < 0.01$ compared with Lira-γGlu. (B) 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 ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with 5.6 mM glucose control.

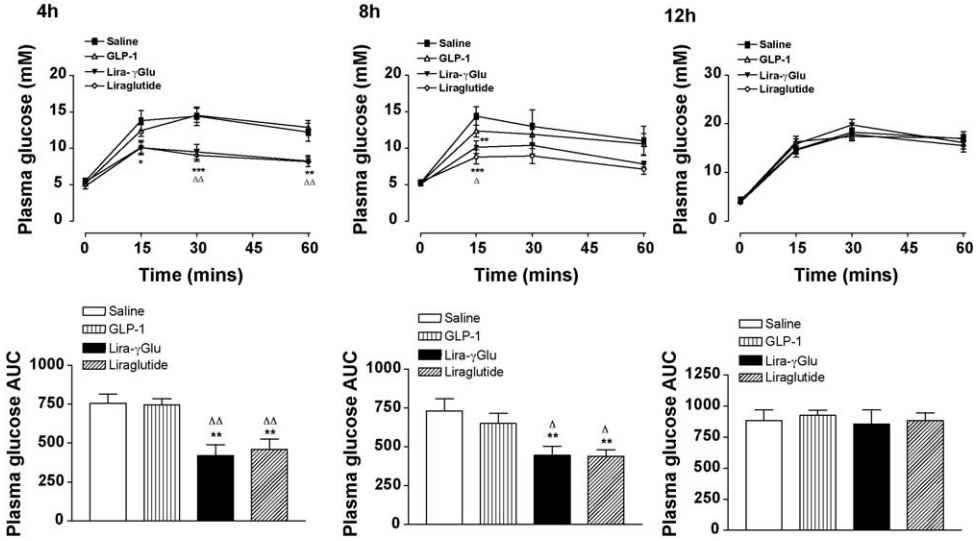

Fig. 2. Persistent glucose-lowering effects of Lira-γGlu and Liraglutide in high-fat fed mice. Plasma glucose concentrations were measured prior to and after intraperitoneal administration of glucose alone (18 mmol/kg bodyweight) in animals injected 4, 8 and 12 h previously with GLP-1, Lira-γGlu or Liraglutide (each at 50 nmol/kg bodyweight) or with saline vehicle (0.9% (w/v) NaCl). Plasma glucose area under the curve (AUC) values for 0–60 min post-injection are included. Values represent mean ± SEM for 8 mice. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to saline control. $^{\Delta}p < 0.05$, $^{\Delta\Delta}p < 0.01$ compared with GLP-1 treated mice.

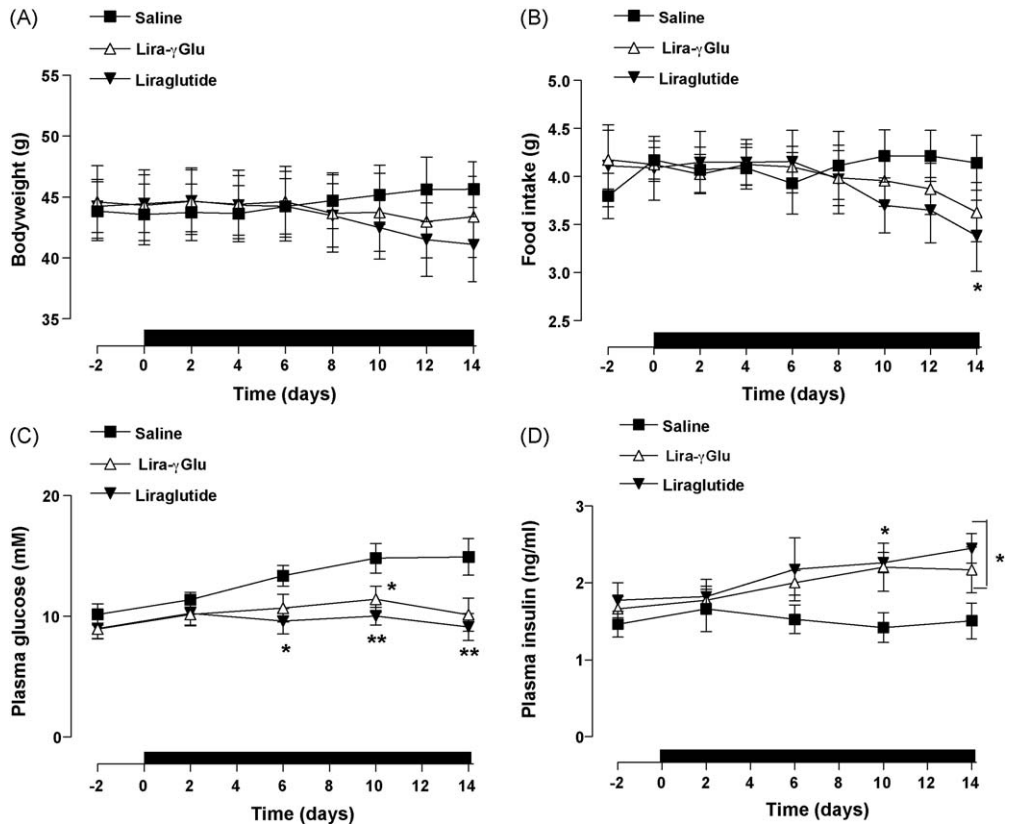

Fig. 3. Sub-chronic effects of twice-daily administration of Lira-γGlu and Liraglutide on (A) bodyweight, (B) food intake, (C) non-fasting plasma glucose and (D) non-fasting plasma insulin in high-fat fed mice. Parameters were measured prior to and during twice-daily treatment with Lira-γGlu or Liraglutide (each 50 nmol/kg bodyweight) or with saline vehicle (0.9% (w/v) NaCl) for 14 days. Values represent mean ± SEM for 8 mice. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to saline-treated animals.

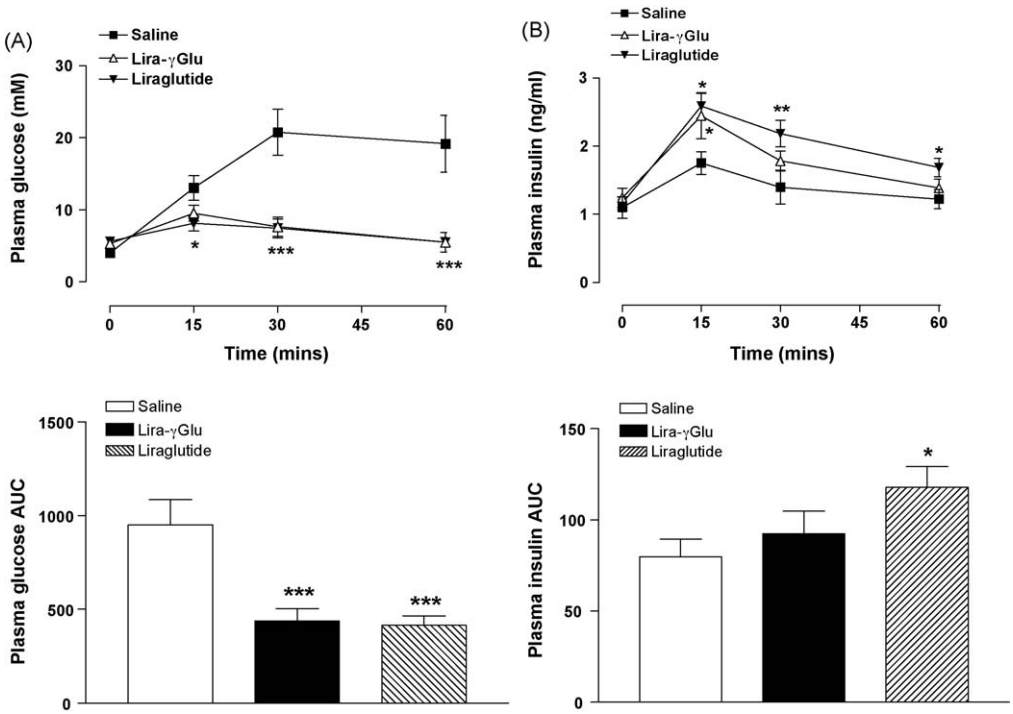

Fig. 4. Sub-chronic effects of twice-daily administration of Lira-γGlu and Liraglutide on (A) glucose tolerance and (B) insulin response to glucose in high-fat fed mice. Tests were conducted following twice-daily treatment with Lira-γGlu or Liraglutide (each at 50 nmol/kg bodyweight) or with saline vehicle (0.9% (w/v) NaCl) for 14 days. Data are expressed as mean ± SEM for 8 mice. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to saline-treated group.

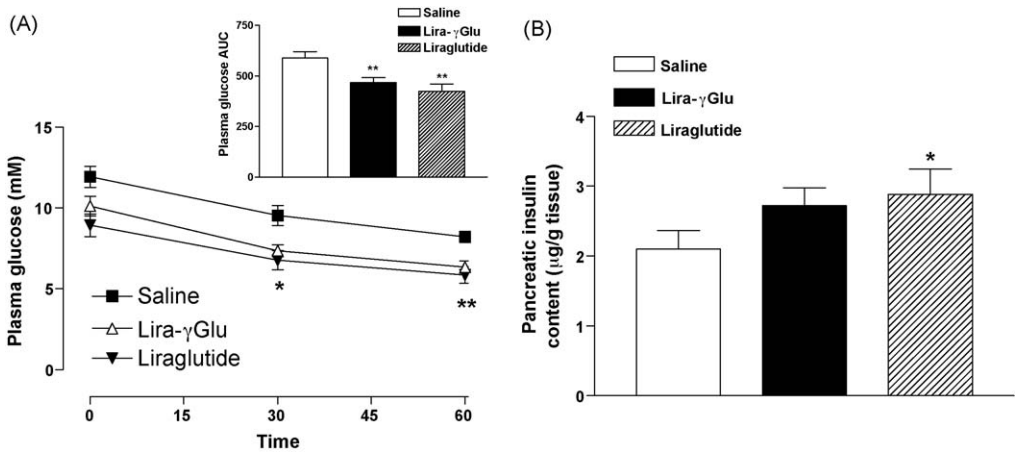

Fig. 5. Sub-chronic effects of twice-daily administration of Lira-γGlu and Liraglutide on (A) insulin sensitivity and (B) pancreatic insulin content in high-fat fed mice. Tests were conducted following twice-daily treatment with Lira-γGlu or Liraglutide (each at 50 nmol/kg bodyweight) or with saline vehicle (0.9% (w/v) NaCl) for 14 days. Data are expressed as mean ± SEM for 8 mice. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to saline-treated group.

animals. These results were supported by significantly decreased plasma glucose AUC values compared to saline-treated mice at 4 h (1.5–1.7-fold; $p < 0.01$) and 8 h (1.6-fold; $p < 0.05$ to $p < 0.01$) post peptide administration. No differences were noted 12 h post-injection nor between Lira-γGlu and Liraglutide.

3.4. Sub-chronic effects of Lira-γGlu and Liraglutide on bodyweight, food intake, non-fasting plasma glucose and insulin levels

Twice-daily administration of Lira-γGlu and Liraglutide for 14 days to high-fat fed mice had no significant effect on bodyweight (Fig. 3A) but did reduce food intake (Fig. 3B) by day 14 (1.2-fold; $p < 0.05$). Non-fasting plasma glucose (Fig. 3C) and insulin (Fig. 3D) levels were also improved with twice-daily administration of Lira-γGlu and Liraglutide. Non-fasting plasma glucose levels were significantly reduced by day 6 through day 14 (1.1–1.6-fold; $p < 0.05$ to $p < 0.01$). This was accompanied by significantly increased non-fasting plasma insulin levels on day 10 and day 14 (1.4–1.6-fold; $p < 0.05$). Lira-γGlu and Liraglutide did not differ in their effects.

3.5. Sub-chronic effects of Lira-γGlu and Liraglutide on glucose tolerance and insulin response to glucose

Twice-daily treatment of high-fat fed mice with Lira-γGlu and Liraglutide significantly improved glucose tolerance, with reduced plasma glucose levels at 15, 30 and 60 min (1.4–3.4-fold; $p < 0.05$ to $p < 0.001$; Fig. 4A). Similarly, AUC analysis demonstrated a powerful glucose-lowering effect of Lira-γGlu and Liraglutide relative to saline controls at day 14 (2.2–2.3-fold; $p < 0.001$). This action was accompanied by a significantly increased plasma insulin response to glucose at 15 min for Lira-γGlu and Liraglutide (1.4–1.5-fold; $p < 0.05$) and 30 and 60 min for Liraglutide (1.4–1.6-fold; $p < 0.01$) treated animals (Fig. 4B). This corresponded with increased AUC insulin levels for Liraglutide treated animals (1.5-fold; $p < 0.01$).

3.6. Sub-chronic effects of Lira-γGlu and Liraglutide on insulin sensitivity and pancreatic insulin content

Treatment with Lira-γGlu and Liraglutide significantly reduced plasma glucose concentrations 30 and 60 min after an insulin load (1.3–1.4-fold; $p < 0.05$ to $p < 0.01$) compared to saline-treated animals (Fig. 5A). This hypoglycaemic action was confirmed by AUC analysis, with Lira-γGlu and Liraglutide treatment significantly reducing plasma glucose levels relative to saline controls (1.3–1.4-fold; $p < 0.01$). As shown in Fig. 5B, Liraglutide treated mice demonstrated significantly greater pancreatic insulin content compared to saline controls (1.4-fold; $p < 0.05$).

4. Discussion

Several structurally modified mimetics or analogues of GLP-1 have demonstrated DPP-IV resistance with increased insulinotropic and anti-hyperglycaemic properties [9–11], with exendin-4 (Byetta) and Liraglutide emerging as the first incretin-based therapies established for clinical use. Liraglutide shares a 97% homology with human GLP-1 differing only at position K34 (R amino acid substitution) and position 26 (C-16 acylation through a γ-glutamyl linker) [23]. In a previous report, it was claimed that inclusion of a linker was crucial for bioactivity, with omission resulting in greater than 70-fold decrease in potency for the human GLP-1 receptor [17]. Since acylation is being increasingly explored in the development of therapeutic peptides for obesity-diabetes [20,21], we examined the functional characteristics of Lira-γGlu, a Liraglutide analogue without the γ-glutamyl linker at the acylation site.

Consistent with other studies [9–11], the GLP-1-based analogues examined demonstrated significantly improved DPP-IV resistance. Unlike GLP-1 which displayed an *in vitro* half-life of 4.5 h, Lira-γGlu and Liraglutide remained mostly intact throughout the entire incubation with significantly more intact peptide at 4, 8 and 24 h compared to the native peptide (half-lives > 24 h). Approximately 20% more intact Liraglutide was observed compared to Lira-γGlu at 24 h but chemical modifications on both analogues clearly infered a significantly prolonged DPP-IV resistance compared to the native peptide. Masking the enzyme cleavage site and alterations to charge or hydroaffinity could account for the positive effects of acylation on DPP-IV mediated N-terminal degradation [17,24]. Unlike other studies, we did not observe even small amounts of Liraglutide degradation products, suggesting that *in vivo* degradation of Liraglutide is likely to be of minor significance [25].

All peptides studied stimulated cAMP production with a concentration-dependent fashion in clonal beta-cells. Lira-γGlu and Liraglutide demonstrated substantially improved EC_{50} values compared with native GLP-1, suggesting that chemical modifications present in each analogue did not affect receptor affinity and offered improved activation of adenylate cyclase transduction pathways [26]. This view is supported by the concentration-dependent increase of insulin secretion from BRIN-BD11 cells. Interestingly, the omission of the γ-glutamyl linker in Lira-γGlu had no significant effect on biological function relative to Liraglutide. This contrasts sharply with the view that omitting

the linker destroys potency for the GLP-1 receptor in BHK cells expressing the cloned GLP-1 receptor [17]. The reason for this discrepancy between these two studies is unclear but could be due to the different approaches and outcomes measured, or activation of additional key signalling pathways in beta-cells that may contribute to the observed biological effects in the present paper. However, the stimulating effect of Liraglutide over basal values appeared to be achieved at lower concentrations.

To assess the persistent anti-hyperglycaemic effects of Lira-γGlu and Liraglutide a high-fat fed animal model of obesity and glucose intolerance was employed. Consistent with *in vitro* findings, administration of Lira-γGlu and Liraglutide significantly decreased plasma glucose excursion equi-potently when administered 4 and 8 h prior to a glucose load but not 12 h previously. This is indicative of a significantly reduced half-life in rodents ($t_{1/2} \sim 4$ h) compared with humans ($t_{1/2} \sim 13$ h) [27]. Native GLP-1 lacked anti-hyperglycaemic activity under these conditions, reflecting extensive degradation by DPP-IV. The persistent glucose-lowering actions of Lira-γGlu and Liraglutide suggest an extended plasma half-life as a direct result of the positional substitution with R34 and the C-16 acylation, independent of the γ-glutamyl linker, thereby offering both peptides *in vivo* DPP-IV resistance and protection from kidney filtration through binding to serum proteins such as albumin [8]. This is consistent with similar extension of plasma half-life in pigs recorded as 16 h for both peptides compared with 1.2 h for native GLP-1 [17].

In contrast to previous studies, twice-daily injection of Lira-γGlu and Liraglutide had no significant effect on bodyweight despite significantly reducing food intake by day 14 [27]. The independence from bodyweight change in this animal model may represent the progressive nature of weight loss together with counter-balancing effects on physical activity and energy expenditure [28]. However an extended treatment period may have resulted in significant bodyweight reductions. Interestingly, other studies in mice with various GLP-1 mimetics have also not shown weight loss as observed in humans [29,30]. However, the prominent reduction in non-fasting plasma glucose in the present study corresponded with significantly elevated insulin levels. Similarly, sub-chronic treatment with either peptide significantly augmented the glucose excursion and insulin response following a glucose load. Despite no significant difference in Lira-γGlu animals, Liraglutide treatment resulted in an enhanced plasma insulin excursion and improved pancreatic insulin content. Contrastingly, this was not the case for insulin sensitivity, as the inclusion of a γ-glutamyl linker had no beneficial effect, with both Lira-γGlu and Liraglutide treated animals demonstrating equal improvements of insulin action.

In conclusion, this study has demonstrated that Lira-γGlu, a Liraglutide analogue without the γ-glutamyl linker at the acylation site, possesses DPP-IV resistance, improved biological function *in vitro* and protracted anti-hyperglycaemic effects *in vivo* almost identical to Liraglutide. Furthermore, when administered sub-chronically, Lira-γGlu exerts strong anorexigenic, insulinotropic and anti-hyperglycaemic actions. The finding that the γ-glutamyl linker is unnecessary in the actions of the GLP-1 analogue suggests further examination of its efficacy in related therapeutic peptides such as GIP and oxyntomodulin.

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