N-terminal His⁷-modification of glucagon-like peptide-1(7–36) amide generates dipeptidyl peptidase IV-stable analogues with potent antihyperglycaemic activity

B D Green, M H Mooney, V A Gault, N Irwin, C J Bailey¹,
P Harriott², B Greer², F P M O'Harte and P R Flatt

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

¹Department of Pharmaceutical and Biological Sciences, Aston University, Birmingham, UK

²Centre for Peptide and Protein Engineering, School of Biology and Biochemistry, The Queen's University of Belfast, Belfast BT9 7BL, N. Ireland, UK

(Requests for offprints should be addressed to B D Green; Email: b.green@ulster.ac.uk)

Abstract

Glucagon-like peptide-1(7–36)amide (GLP-1) possesses several unique and beneficial effects for the potential treatment of type 2 diabetes. However, the rapid inactivation of GLP-1 by dipeptidyl peptidase IV (DPP IV) results in a short half-life *in vivo* (less than 2 min) hindering therapeutic development. In the present study, a novel His⁷-modified analogue of GLP-1, N-pyroglutamyl-GLP-1, as well as N-acetyl-GLP-1 were synthesised and tested for DPP IV stability and biological activity. Incubation of GLP-1 with either DPP IV or human plasma resulted in rapid degradation of native GLP-1 to GLP-1(9–36)amide, while N-acetyl-GLP-1 and N-pyroglutamyl-GLP-1 were completely resistant to degradation. N-acetyl-GLP-1 and N-pyroglutamyl-GLP-1 bound to the GLP-1 receptor but had reduced affinities (IC₅₀ values 32.9 and 6.7 nM, respectively) compared with native GLP-1 (IC₅₀ 0.37 nM). Similarly, both analogues stimulated cAMP production with EC₅₀ values

of 16.3 and 27 nM respectively compared with GLP-1 (EC₅₀ 4.7 nM). However, N-acetyl-GLP-1 and N-pyroglutamyl-GLP-1 exhibited potent insulinotropic activity *in vitro* at 5.6 mM glucose ($P < 0.05$ to $P < 0.001$) similar to native GLP-1. Both analogues (25 nM/kg body weight) lowered plasma glucose and increased plasma insulin levels when administered in conjunction with glucose (18 nM/kg body weight) to adult obese diabetic (*ob/ob*) mice. N-pyroglutamyl-GLP-1 was substantially better at lowering plasma glucose compared with the native peptide, while N-acetyl-GLP-1 was significantly more potent at stimulating insulin secretion. These studies indicate that N-terminal modification of GLP-1 results in DPP IV-resistant and biologically potent forms of GLP-1. The particularly powerful antihyperglycaemic action of N-pyroglutamyl-GLP-1 shows potential for the treatment of type 2 diabetes.

Journal of Endocrinology (2004) **180**, 379–388

Introduction

Glucagon-like peptide-1(7–36)amide (GLP-1) is a gastrointestinal hormone involved in the regulation of blood glucose, gastric function and food intake (Drucker 2002). After rapid release from the intestinal L-cells in response to ingested nutrients, GLP-1 mediates its actions at many sites, but the most widely recognised is its potent insulinotropic effects on the pancreatic beta cell (MacDonald *et al.* 2002). Pharmacological doses of GLP-1 administered to type 2 diabetic patients significantly raised circulating insulin and lowered plasma glucagon levels (Nathan *et al.* 1992). By such actions, GLP-1 acts to normalise blood glucose even in type 2 diabetic patients with secondary failure to sulphonylurea treatment (Nauck *et al.* 1993). Prolonged administration of GLP-1 to type 2 diabetic subjects showed that the hormone maintained its effects on

glycaemic control even after three weeks of treatment (Todd *et al.* 1997). Furthermore, unlike sulphonylureas, the insulinotropic action of GLP-1 is dependent on the prevailing plasma glucose concentration and this protects against hypoglycaemia (Holz *et al.* 1993).

The actions of GLP-1 are mediated by GLP-1 receptors in the pancreas, heart, kidney, central nervous system and gastrointestinal tract (Thorens 1992, Campos *et al.* 1994, Bullock *et al.* 1996). Following its discovery, much investigation has focused on GLP-1 as a gut-derived incretin that stimulates insulin secretion (Mojsov *et al.* 1987). The role of GLP-1 as a regulator of β -cell function has been demonstrated *in vivo* by studies using the truncated peptide exendin(9–39) derived from the salivary glands of the Gila monster (Eng *et al.* 1992). This peptide acts as a functional GLP-1 receptor antagonist, reducing the insulin response to feeding and increasing postprandial glycaemic excursion

(Kolligs *et al.* 1995, Gault *et al.* 2003). The biological importance of GLP-1 as an incretin is further underlined by disruption of the GLP-1 receptor in GLP-1R^{-/-} mice which causes impaired insulin secretion and glucose intolerance (Scrocchi *et al.* 1996).

Mounting evidence suggests GLP-1 is a regulator of islet neogenesis and β -cell proliferation. GLP-1 stimulates insulin gene transcription (Drucker *et al.* 1987), increases pancreatic β -cell mass (Perfetti *et al.* 2000) and promotes β -cell replication in mice (Edvell & Lindstrom 1999). GLP-1 causes the differentiation of pancreatic AR42J cells into glucagon- and insulin-producing cells (Zhou *et al.* 1999) and this differentiation to a β -cell phenotype occurs through a pancreatic duodenum homeobox-1 (PDX)-1-dependent pathway (Hui *et al.* 2001). Such an action could offset any β -cell depletion of insulin thus making GLP-1 a more useful diabetic therapy than those currently available.

Despite its many advantages as a potential therapeutic agent, GLP-1 is rapidly inactivated by degradation by the enzyme dipeptidyl peptidase IV (DPP IV) to a truncated GLP-1(9–36)amide metabolite, which serves as a GLP-1 receptor antagonist (Knudsen & Priddel 1996). DPP IV is widely distributed in the brush border epithelium and capillaries of the *lamina propria* as well as circulating in the bloodstream (Fukasawa *et al.* 1981, Duke-Cohan *et al.* 1995). This confers the peptide with a short circulating half-life of approximately 3 min (Hassan *et al.* 1999).

Whilst investigating the *in vitro* effects of glycation on GLP-1 action, it was noticed that N-glucitol-GLP-1 exhibited a modest reduction of insulinotropic potency (O'Harte *et al.* 1998) and a decreased ability to stimulate glucose uptake in muscle cells (O'Harte *et al.* 1997) when compared with native GLP-1. However, it was also noted that glycation, occurring at the N-terminal histidine was beneficial, conferring GLP-1 with a complete resistance to DPP IV and enhanced *in vivo* biological activity (O'Harte *et al.* 2000, 2001). Xiao *et al.* (2001) have synthesised analogues of GLP-1 with improved biological activity. One such analogue, N-acetyl-GLP-1, has improved insulinotropic action in normal mice. However, it is not known whether modifying GLP-1 by N-terminal acetylation can affect resistance to DPP IV or enhance insulin secretion either *in vitro* or *in vivo*. In this study we investigated the biological properties of an additional novel N-terminally His⁷-modified analogue of GLP-1, N-pyroglutamyl-GLP-1, as well as N-acetyl-GLP-1. In particular, their properties in terms of stability, receptor binding and *in vitro* and *in vivo* biological activities were compared with native GLP-1.

Materials and Methods

Reagents

HPLC grade acetonitrile, diethyl ether and dichloromethane were obtained from Rathburn (Walkersburn,

Scotland). Sequencing grade trifluoroacetic acid (TFA), dipeptidyl peptidase IV (DPP IV), forskolin, isobutylmethylxanthine IBMX, adenosine 3',5'-cyclic monophosphate (cAMP) and adenosine 5'-triphosphate (ATP) were purchased from Sigma (Poole, Dorset, UK). Fmoc protected amino acids were from Calbiochem Novabiochem (Beeston, Nottingham, Notts, UK). RPMI 1640 and DMEM tissue culture medium, foetal bovine serum, penicillin and streptomycin were all purchased from Gibco Life Technologies Ltd (Paisley, Strathclyde, UK). Chromatography columns used in the assay of cAMP, Dowex AG 50 WX and neutral alumina AG7 were obtained from Bio-Rad (Life Science Research, Alpha Analytical, Larne, N. Ireland). Tritiated adenine (TRK311) was obtained from Amersham Pharmacia Biotech, UK. All water used in experiments was purified using a Milli-Q Water Purification System (Millipore Corporation, Milford, MA, USA). All other chemicals used were of analytical grade.

Synthesis of GLP-1, N-acetyl-GLP-1 and N-pyroglutamyl-GLP-1

Utilising an automated 432 A peptide synthesiser (Applied Biosystems, Foster City, CA, USA) GLP-1, N-acetyl-GLP-1 and N-pyroglutamyl-GLP-1 were synthesised in a sequential fashion, starting with an Arg rink amide MBHA resin (Merrifield 1963). Completed peptides were cleaved from the resin and purified by reversed-phase HPLC on a Waters Millennium 2010 chromatography system (software version 2.1.5).

Electrospray ionisation mass spectrometry (ESI-MS)

Intact peptides and degradation fragments were dissolved in 0.12% (v/v) TFA/water and directly injected onto the electrospray ionisation source of an LCQ ion-trap mass spectrometer (Finnigan MAT, Hemel Hempstead, Herts, UK). Spectra were obtained from a quadrupole ion-trap mass analyser with the detector set to a mass-to-charge range of m/z 150–2000. The molecular masses of GLP-1, N-acetyl-GLP-1 and N-pyroglutamyl-GLP-1 were calculated from the prominent multiple charged ions using the equation, $M_r = iM_z - iM_h$ (where M_r is molecular mass, M_z is the m/z ratio, i is the number of charges and M_h is the mass of a proton).

Degradation of GLP-1, N-acetyl-GLP-1 and N-pyroglutamyl-GLP-1 following incubation with DPP IV and human plasma

GLP-1, N-acetyl-GLP-1 and N-pyroglutamyl-GLP-1 (final peptide concentration 2 mM) were incubated with either DPP IV (1.25 mU) or pooled human plasma (7.5 μ l) for 0, 2, 4, 6 and 12 h (37 °C; 50 mM triethanolamine-HCl buffer; pH 7.8). Concentrations of DPP IV and human plasma were selected in preliminary experiments

to provide degradation of approximately 50% of GLP-1 within 4–6 h, therefore allowing time-dependent degradation to be viewed over 12 h. Reactions were terminated by the addition of TFA/H₂O (15 µl, 10% (v/v)). The reaction products were then applied to a Vydac C-18 analytical column (4.6 × 250 mm) and the major degradation fragment GLP-1(9–36)amide separated from intact GLP-1. The column was equilibrated with TFA/H₂O (0.12% (v/v)) at a flow rate of 1.0 ml/min. Using 0.1% (v/v) TFA in 70% acetonitrile/H₂O, the concentration of acetonitrile in the eluting solvent was raised from 0% to 28% over 10 min, and from 28% to 42% over 30 min. The absorbance was monitored at 206 nm using a SpectraSystem UV 2000 detector (Thermoquest Limited, Manchester, UK) and peaks were collected manually prior to ESI-MS analysis.

Culture of BRIN-BD11 cells and Chinese hamster lung fibroblasts

Chinese hamster lung (CHL) fibroblasts stably transfected with the human GLP-1 receptor (Thorens 1992) were cultured using DMEM tissue culture medium (10% (v/v) foetal bovine serum, 1% (v/v) antibiotics (100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.2 mg/ml gentamycin)). BRIN-BD11 cells were cultured in RPMI-1640 tissue culture medium containing 10% (v/v) foetal calf serum, 1% (v/v) antibiotics (100 U/ml penicillin, 0.1 mg/ml streptomycin) and 11.1 mM glucose. BRIN-BD11 cells were produced by electrofusion of a New England Deaconess Hospital (NEDH) rat pancreatic beta-cell with RINm5F cell to produce an immortal, glucose sensitive cell line which is described in detail elsewhere (McClenaghan *et al.* 1996). All cells were maintained in sterile tissue culture flasks (Corning Glass Works, Corning, Sunderland, UK) at 37 °C in an atmosphere of 5% CO₂ and 95% air using a LEEC incubator (Laboratory Technical Engineering, Nottingham, Notts, UK).

Receptor binding studies

CHL fibroblasts transfected with the human GLP-1 receptor were seeded (1×10^5 per well) in 24-multiwell plates (Nunc, Roskilde, Denmark). Following overnight culture (37 °C; 5% CO₂) cells were washed twice with cold Hanks balanced saline (HBS) buffer. Test incubations (24 h; 4 °C) were performed in HBS buffer (400 µl) with a range of concentrations of GLP-1, N-acetyl-GLP-1 or N-pyroglutamyl-GLP-1 (10^{-6} M serially diluted threefold) plus ¹²⁵I-GLP-1 label (50 000 c.p.m./ml) and phenylmethylsulphonylfluoride (1 mM). ¹²⁵I-GLP-1 was prepared by the solid phase iodogen method (Salacinski *et al.* 1981) and purified by HPLC. Cells were washed four times with cold saline solution (0.85% NaCl₂) and 500 µl lysis solution (5% trichloroacetic acid, 3% sodium dodecyl sulphate) was added. Plates were shaken for 10 min, 1 ml

millipore water was added, and the content of the wells was removed and radioactivity was measured on a γ-counter (1261 Multigamma counter, LKB Wallac, Turku, Finland). Curves were analysed by non-linear regression using the sigmoidal dose-response equation ($Y = \min + (\max - \min) / (1 + 10^{\log EC_{50} - X})$) to calculate EC₅₀ values.

Determination of cAMP production by GLP-1, N-acetyl-GLP-1 and N-pyroglutamyl-GLP-1

BRIN-BD11 cells were seeded in 24-well plates (3×10^5 per well), cultured for 48 h before being pre-incubated in media supplemented with tritiated adenine (2 µCi) for 16 h. The cells were washed twice with cold HBS buffer and the test solution (400 µl; 37 °C) was added. The cells were then exposed to varying concentrations of GLP-1, N-acetyl-GLP-1 or N-pyroglutamyl-GLP-1 (10^{-5} M serially diluted threefold) in HBS buffer, in the presence of 1 mM IBMX and 5.6 mM glucose (20 min; 37 °C). Following incubation, test solutions were removed and 300 µl lysis solution (5% TFA, 3% SDS, 5 mM unlabelled ATP and 300 µM unlabelled cAMP) were added. Cell lysate was separated on Dowex and alumina exchange resins to isolate tritiated cAMP as described previously (Miguel *et al.* 2003).

Assessment of in vitro insulinotropic activity

BRIN-BD11 cells were seeded into 24-multiwell plates at a density of 1×10^5 per well, and allowed to attach during overnight culture. Acute studies of insulin release were preceded by 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 CaCl₂·2H₂O, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄·7H₂O, 10 mM NaHCO₃, 5 g/l bovine serum albumin, pH 7.4) supplemented with 1.1 mM glucose. Test incubations were performed at 37 °C in the presence of 5.6 mM glucose with a range of concentrations of GLP-1, N-acetyl-GLP-1 or N-pyroglutamyl-GLP-1 (10^{-12} – 10^{-6} M). After 20-min incubation, the buffer was removed from each well and aliquots were stored at –20 °C for measurement of insulin by radioimmunoassay.

Metabolic effects of GLP-1, N-acetyl-GLP-1 and N-pyroglutamyl-GLP-1 in obese diabetic (ob/ob) mice

The *in vivo* biological activity of GLP-1, N-acetyl-GLP-1 and N-pyroglutamyl-GLP-1 was assessed in 12- to 16-week-old obese diabetic (ob/ob) mice as described elsewhere (Bailey & Flatt 1982). The animals were housed individually in an air-conditioned room at 22 ± 2 °C with a 12 h light:12 h darkness cycle. Drinking water was available *ad libitum* and continuous access to standard rodent maintenance diet (Trouw Nutrition, Northwich, Cheshire, UK) was allowed. Mice were fasted for an 18-h

Table 1 Molecular characterisation and susceptibility of GLP-1 peptides to degradation by DPP IV and human plasma. The peptides were applied to an LC/MS equipped with a microbore C-18 HPLC column (150 mm × 2.0 mm) at a flow rate of 0.2 ml/min, under isocratic conditions in 35% (v/v) acetonitrile/water. Spectra were recorded using a quadrupole ion trap mass analyser and collected using full ion scan mode over the *m/z* range 150–2000. Data represent the percentage of the major degradation fragment, GLP-1(9–36)amide (following HPLC separation), relative to the intact peptide following incubation with purified DPP IV or human plasma. Linear regression 'best-fit' analysis was used to calculate the time at which half of the peptide was degraded

Peptide	NH ₂ -terminal sequence	ESI-MS multiple-charged species		Molecular mass (Da)		Percentage degradation (12 h)		Half-lives (t _{1/2}) (h)	
		(M+2H) ²⁺	(M+3H) ³⁺	Measured	Theoretical	DPP IV	Human plasma	DPP IV	Human plasma
GLP-1	His-Ala-Glu-	1649.9	1099.9	3297.3	3297.5	82	78	5.8	6.2
N-acetyl-GLP-1	Ac-His-Ala-Glu	1671.2	1114.2	3340.0	3339.6	0	0	>12	>12
N-pyroglutamyl-GLP-1	pGlu-His-Ala-Glu	1705.2	1137.1	3408.2	3408.7	0	0	>12	>12

period and intraperitoneally administered with saline (9 g/l NaCl), glucose alone (18 mM/kg body weight), or in combination with GLP-1, N-acetyl-GLP-1 or N-pyroglutamyl-GLP-1 (25 nM/kg body weight). All test solutions were administered in a final volume of 8 ml/kg body weight. Blood samples were collected into chilled fluoride/heparin microcentrifuge tubes (Sarstedt, Numbrecht, Germany) immediately prior to injection and at 15, 30 and 60 min post injection, and the plasma obtained was stored at –20 °C. All animal studies were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986.

Analyses

Plasma glucose levels were determined using an Analox glucose analyser (Hammersmith, London, UK), which employs the glucose oxidase method (Stevens 1971). Insulin levels were assayed by dextran-coated charcoal radioimmunoassay (Flatt & Bailey 1981). Incremental areas under plasma glucose and insulin curves (AUC) were calculated using GraphPad PRISM version 3.0 (Graphpad Software, San Diego, CA, USA). PRISM employs the trapezoidal rule (Burington 1973). Results were expressed as means ± S.E.M. and data were compared as appropriate using Student's *t*-test, repeated measures ANOVA or one-way ANOVA, followed by the Student-Newman-Keuls *post hoc* test. Groups of data were considered significantly different if *P* < 0.05.

Results

Confirmation of the structural identities of GLP-1 peptides

Table 1 shows the monoisotopic masses obtained for GLP-1, N-acetyl-GLP-1 and N-pyroglutamyl-GLP-1 using ESI-MS. Following spectral averaging, prominent multiple-charged species (M+2H)²⁺ and (M+3H)³⁺

were obtained for GLP-1 corresponding to *M_r* of 3297.3 Da (theoretical mass 3297.5 Da). Corresponding *M_r* values for N-acetyl-GLP-1 and N-pyroglutamyl-GLP-1 were 3340.0 Da (theoretical mass 3339.6 Da) and 3408.2 Da (theoretical mass 3408.7 Da), respectively.

Degradation of GLP-1, N-acetyl-GLP-1 and N-pyroglutamyl-GLP-1 by DPP IV and human plasma

GLP-1 was progressively metabolised by DPP IV and plasma (t_{1/2} = 5.8 and 6.2 h respectively) with 78–82% degradation by 12 h, giving rise to a major peak corresponding to GLP-1(9–36)amide upon ESI-MS analysis (Figs 1 and 2). In contrast, N-acetyl-GLP-1 and N-pyroglutamyl-GLP-1 did not yield a second peak on incubation with DPP IV or plasma (Figs 1 and 2) and were completely resistant to degradation (t_{1/2} > 12 h, Table 1).

Determination of GLP-1 receptor binding in CHL fibroblasts

Displacement of ¹²⁵I-GLP-1 by GLP-1, N-acetyl-GLP-1 and N-pyroglutamyl-GLP-1 in transfected CHL fibroblast cells is shown in Fig. 3. GLP-1 displaced the radiolabelled tracer reaching half-maximal inhibition of ¹²⁵I-GLP-1 binding (IC₅₀) at a concentration of 0.37 nM. N-acetyl-GLP-1 and N-pyroglutamyl-GLP-1 had reduced binding affinities presenting IC₅₀ values of 32.9 nM and 6.7 nM, respectively. Bonferri analysis of the sigmoidal dose-response curves showed that both synthetic analogues were statistically different (*P* < 0.001) from native GLP-1.

Stimulation of adenylate cyclase production by GLP-1, N-acetyl-GLP-1 and N-pyroglutamyl-GLP-1

The dose-dependent stimulatory effects of GLP-1 peptides on cAMP production are clearly observed in Fig. 4. N-acetyl-GLP-1 and N-pyroglutamyl-GLP-1 were potent at stimulating cAMP production with EC₅₀ values

Figure 1 Degradation profiles of (A and B) GLP-1, (C) N-acetyl-GLP-1 and (D) N-pyroglutamyl-GLP-1 after incubation with purified DPP IV. Representative HPLC profiles were obtained following the incubation of GLP-1 and analogues with DPP IV for 0 h (A) and 12 h (B-D).

of 16.3 nM and 27 nM respectively, compared with native GLP-1 (4.7 nM). Despite the maximal responses for cAMP production being equipotent, Bonferri analysis of the sigmoidal dose-response curves showed that N-acetyl-GLP-1 and N-pyroglutamyl-GLP-1 were statistically less potent ($P < 0.001$) than native GLP-1.

Insulinotropic action of GLP-1, N-acetyl-GLP-1 and N-pyroglutamyl-GLP-1

Figure 5 shows that at all concentrations tested (10^{-12} to 10^{-6} M) N-acetyl-GLP-1 caused a dose-dependent increase (1.2- to 2.7-fold) in insulin secretion *in vitro* compared with 5.6 mM glucose vehicle control ($P < 0.05$ to $P < 0.001$). However, native GLP-1 and N-pyroglutamyl-GLP-1 showed a dose-dependent increase in insulin secretion (1.4- to 2.7-fold) from 10^{-9} - 10^{-6} M compared with controls ($P < 0.05$ to $P < 0.01$). N-pyroglutamyl-GLP-1 was equipotent to native or N-acetyl-GLP-1 at all concentrations except at the higher peptide concentrations (10^{-7} and 10^{-6} M) where its effects on insulin secretion were reduced compared with GLP-1 ($P < 0.05$ and $P < 0.01$). Studies with GLP-1 analogues have also demonstrated that the effects of these peptides are glucose-dependent between concentrations of 3 and 16.7 mM glucose (data not shown).

Effects of GLP-1, N-acetyl-GLP-1 and N-pyroglutamyl-GLP-1 on glucose lowering and insulin secretion in obese diabetic (ob/ob) mice

Figures 6 and 7 show the plasma glucose and insulin responses to i.p. administration of saline, glucose alone or glucose in combination with GLP-1, N-acetyl-GLP-1 or N-pyroglutamyl-GLP-1. Injection of saline alone had no effect on either parameter (Figs 6A and 7A). Plasma glucose rose very significantly at 15 min after glucose alone and continued to rise even at 60 min ($P < 0.001$). The glucose excursion after administration of native GLP-1 was unchanged at 15 min but was reduced by 30 min ($P < 0.01$) compared with glucose alone. Basal values were almost attained by 60 min. Area under the curve analysis (AUC, 0–60 min; Fig. 6B) showed that administration of GLP-1 reduced the net glucose excursion significantly ($P < 0.001$) compared with glucose alone. N-acetyl-GLP-1 although significantly reducing the overall glucose excursion (AUC, $P < 0.05$), was not as potent as native GLP-1 (AUC, $P < 0.001$). N-pyroglutamyl-GLP-1 on the other hand, demonstrated an extremely significant antihyperglycaemic effect when compared with both glucose alone (AUC, $P < 0.001$), and native GLP-1 (AUC, $P < 0.05$).

Figure 2 Degradation profiles of (A and B) GLP-1, (C) N-acetyl-GLP-1 and (D) N-pyroglutamyl-GLP-1 after incubation with human plasma. Representative HPLC profiles were obtained following the incubation of GLP-1 and analogues with human plasma for 0 h (A) and 12 h (B-D).

Figure 7A shows the corresponding plasma insulin responses in this study. After glucose alone, plasma insulin rose significantly at 15 min, and then gradually returned to normal levels by 60 min. Insulin levels in response to native GLP-1 were higher at 15 min compared to glucose alone, but preinjection levels were attained by 60 min. Area under the curve (AUC, 0–60 min, Fig. 7B) analysis showed that administration of GLP-1 in combination with glucose increased the overall insulin response significantly ($P < 0.01$) compared with glucose alone. Administration of either N-acetyl-GLP-1 or N-pyroglutamyl-GLP-1 increased plasma insulin significantly during the test. The overall insulin responses of N-pyroglutamyl-GLP-1 as estimated by AUC revealed similar potencies to native GLP-1. N-acetyl-GLP-1 generated a significantly greater insulin response compared with native GLP-1 (Fig. 7B; $P < 0.05$).

Discussion

GLP-1 inhibits food intake, gastric emptying and glucagon secretion, and stimulates insulin secretion and β -cell proliferation (Nathan *et al.* 1992, Turton *et al.* 1996, Perfetti *et al.* 2000). As a result of these attributes, GLP-1 is

Figure 3 Receptor binding affinity of GLP-1, N-acetyl-GLP-1 and N-pyroglutamyl-GLP-1 in CHL cells stably transfected with the human GLP-1 receptor. Values represent means $\pm$ S.E.M. for three different experiments.

Figure 4 Intracellular cAMP production in BRIN BD11 cells exposed for 20 min to various concentrations of GLP-1, N-acetyl-GLP-1 and N-pyroglutamyl-GLP-1. Experiments were performed in triplicate ($n=4$) and the data expressed (means $\pm$ S.E.M.) as a percentage of the maximal response.

considered to be a promising candidate for therapeutic use in type 2 diabetes (Drucker 2002). The primary obstacle which hinders progression to the clinic is its rapid inactivation by the enzyme DPP IV (Deacon *et al.* 1995). Some effort has been devoted to the development and testing of DPP IV inhibitors to prolong GLP-1 action (Holst & Deacon 1998, Deacon & Holst 2002). However, given that DPP IV has at least 35 putative substrates within the body, this may prove to be a problematic strategy (Drucker 2003).

An alternative approach has been the development of DPP IV-resistant analogues of GLP-1 (Drucker 2002). For example, several studies have examined the naturally occurring GLP-1 agonist from the saliva of the *Heloderma suspectum* lizard found in the Arizona desert, exendin-4 (Eng *et al.* 1992). This peptide, closely related to GLP-1, does not contain an Ala residue at position 8 and is thus resistant to DPP IV action. Accordingly, it has been shown to exhibit a longer half-life and exert more potent biological activity than GLP-1 in healthy volunteers (Edwards *et al.* 2001) and both normal and diabetic animals (Szayna *et al.* 2000). We have similarly shown that N-terminal modification of GLP-1 by glycation (N-glucitol-GLP-1) conferred resistance to enzymatic degradation by DPP IV while maintaining antihyperglycaemic activity *in vivo*

Figure 5 Effects of GLP-1, N-acetyl-GLP-1 and N-pyroglutamyl-GLP-1 on insulin release from BRIN-BD11 cells at 5.6 mM glucose. Values are means $\pm$ S.E.M. for eight separate observations. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ compared with glucose control. $\Delta P<0.05$ and $\Delta\Delta P<0.01$ compared with GLP-1 at the same concentration.

Figure 6 Glucose-lowering effects of GLP-1, N-acetyl-GLP-1 and N-pyroglutamy-GLP-1 in obese diabetic (*ob/ob*) mice. (A) Plasma glucose concentrations were measured prior to and after i.p. administration of saline (0.9% NaCl (w/v)), glucose alone (18 mM/kg), or in combination with GLP-1, N-acetyl-GLP-1 or N-pyroglutamy-GLP-1 (25 nM/kg). The arrow indicates the time of injection ($t=0$). (B) Plasma glucose AUC values for 0–60 min post injection. Values are means $\pm$ S.E.M. for eight mice. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ compared with glucose alone. $\Delta P<0.05$, $\Delta\Delta P<0.01$, $\Delta\Delta\Delta P<0.001$ compared with native GLP-1.

(O'Harte *et al.* 2000). In this present study, the N-terminal of GLP-1 was extended by attaching either an acetyl or a pyroglutamy group to His⁷. Both analogues proved to be completely resistant to degradation by purified DPP IV and human plasma, with no visible signs of the major degradation metabolite even after 12 h. This is presumably due to structural protection of the DPP IV cleavage site.

In vitro studies revealed that N-acetyl-GLP-1 and N-pyroglutamy-GLP-1 dose-dependently displaced ¹²⁵I-labelled GLP-1 in GLP-1 receptor transfected fibroblasts with reduced affinity compared with native GLP-1 (89- and 18-fold respectively). Consistent with this observation, the two analogues were less potent stimulators of cAMP production than native GLP-1 (3.5- and 5.7-fold, respectively). Our findings with N-acetyl-GLP-1 are consistent with those of Xiao *et al.* (2001) who also found a loss of receptor binding and cAMP production in the related RINm5F cell line. However, no clear relationship existed between receptor binding affinity and ability to activate adenylate cyclase as the weaker analogue in terms of binding, N-acetyl-GLP-1, was the most potent stimulator of intracellular cAMP production. Additionally, these losses of potency compared with native GLP-1 were not mirrored by major differences in *in vitro* insulin-releasing

Figure 7 Insulin releasing effects of GLP-1, N-acetyl-GLP-1 and N-pyroglutamy-GLP-1 in obese diabetic (*ob/ob*) mice. (A) Plasma insulin concentrations were measured prior to and after i.p. administration of saline (0.9% NaCl (w/v)), glucose alone (18 mM/kg), or in combination with GLP-1, N-acetyl-GLP-1 or N-pyroglutamy-GLP-1 (25 nM/kg). The arrow indicates the time of injection ($t=0$). (B) Plasma insulin AUC values for 0–60 min post injection. Values are means $\pm$ S.E.M. for eight mice. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ compared with glucose alone. $\Delta P<0.05$, $\Delta\Delta P<0.01$, $\Delta\Delta\Delta P<0.001$ compared with native GLP-1.

activity. Thus with the exception of slightly reduced effects of N-pyroglutamy-GLP-1 at the highest concentrations (10^{-7} – 10^{-6} M), the insulinotropic actions of the two analogues were similar to GLP-1 at physiological glucose concentrations. This could be explained by GLP-1 also acting to release insulin by inhibition of β -cell K_{ATP} channels causing membrane depolarisation and influx of Ca^{2+} through voltage-dependent calcium channels (MacDonald *et al.* 2002).

Administration of native GLP-1, N-acetyl-GLP-1 or N-pyroglutamy-GLP-1 together with glucose to diabetic *ob/ob* mice significantly increased insulin secretion and decreased the glycaemic excursion compared with glucose alone. Although N-pyroglutamy-GLP-1 appeared to be no more potent than native GLP-1 in raising plasma insulin, this analogue exhibited substantially enhanced glucose-lowering ability in this commonly employed animal model of obesity, insulin resistance and diabetes (Bailey & Flatt 1982). This presumably reflects improved resistance to DPP IV and moderation of insulinotropic action of the analogue by the relatively low prevailing glucose concentrations in the latter part of the test. In contrast, N-acetyl-GLP-1 exerted a greatly enhanced plasma insulin response, but no greater improvement of

glucose tolerance than native GLP-1. Our studies extend the work of Xiao *et al.* (2001) who examined the glycaemic effects of acetyl-GLP-1 in normal mice. The present study confirms the antihyperglycaemic effects of N-acetyl-GLP-1 and in addition we have found for the first time that DPP IV resistance is associated with significant improvements in the insulinotropic activity of this analogue *in vitro* and *in vivo*. These differential effects of GLP-1 analogues suggest the possible importance of extrapancreatic effects of GLP-1 in tissues such as muscle, liver and adipose tissue which might contribute to glucose-lowering activity (Valverde *et al.* 2002). Additionally, the effects of analogues on the secretion of counter-regulatory hormones, such as glucagon, could be usefully explored.

Overall, these data concur with previous findings that N-terminal extension of GLP-1 at His⁷ prevents DPP IV action, providing a simple means of generating stable long-acting GLP-1 analogues (Gallwitz *et al.* 2000, O'Harte *et al.* 2000). The results support previous observations that such modifications to GLP-1 may result in some compromise of *in vitro* receptor binding affinity and cAMP production (Gallwitz *et al.* 2000). However, it is clear that these limitations do not necessarily compromise improved antihyperglycaemic potential *in vivo* due to prolonged circulating half-life and enhanced effects of some analogues on other target sites in addition to the stimulation of insulin secretion. Such findings add to a growing body of evidence that a diabetic drug therapy based on DPP IV resistant forms of GLP-1 is a feasible objective, and suggest that N-pyroglutamyl-GLP-1 is worthy of further study.

Acknowledgements

The authors wish to thank Professor Bernard Thorens (Institute of Pharmacology, University of Lausanne, Switzerland) for kindly providing the Chinese hamster lung (CHL) fibroblast cell transfected with the human GLP-1 receptor.

Funding

These studies were funded by the University of Ulster Strategy Funding and Research and Development Office of Health and Personal Social Services for N Ireland.

References

- Bailey CJ & Flatt PR 1982 Influence of genetic background and age on the expression of the obese hyperglycaemic syndrome in Aston *ob/ob* mice. *International Journal of Obesity* **6** 11–21.
- Bullock BP, Heller RS & Habener JF 1996 Tissue distribution of messenger ribonucleic acid encoding the rat glucagon-like peptide-1 receptor. *Endocrinology* **137** 2968–2978.
- Burington RS 1973 *Handbook of Mathematical Tables and Formulas*. New York: McGraw-Hill.
- Campos RV, Lee YC & Drucker DJ 1994 Divergent tissue-specific and developmental expression of receptors for glucagon and glucagon-like peptide-1 in the mouse. *Endocrinology* **134** 2156–2164.
- Deacon CF & Holst JJ 2002 Dipeptidyl peptidase IV inhibition as an approach to the treatment and prevention of type 2 diabetes: a historical perspective. *Biochemical and Biophysical Research Communications* **294** 1–4.
- Deacon CF, Nauck MA, Toft-Nielsen M, Pridal L, Willms B & Holst JJ 1995 Both subcutaneously and intravenously administered glucagon-like peptide I are rapidly degraded from the NH₂-terminus in type II diabetic patients and in healthy subjects. *Diabetes* **44** 1126–1131.
- Drucker DJ 2002 Biological actions and therapeutic potential of the glucagon-like peptides. *Gastroenterology* **122** 531–544.
- Drucker DJ 2003 Therapeutic potential of dipeptidyl peptidase IV inhibitors for the treatment of type 2 diabetes *Expert Opinion and Investigation in Drugs* **12** 87–100.
- Drucker DJ, Philippe J, Mojsov S, Chick WL & Habener JF 1987 Glucagon-like peptide I stimulates insulin gene expression and increases cyclic AMP levels in a rat islet cell line. *PNAS* **84** 3434–3438.
- Duke-Cohan JS, Morimoto C, Rocker JA & Schlossman SF 1995 A novel form of dipeptidyl peptidase IV found in human serum. Isolation, characterization, and comparison with T lymphocyte membrane dipeptidyl peptidase IV (CD26). *Journal of Biological Chemistry* **270** 14107–14114.
- Edvell A & Lindstrom P 1999 Initiation of increased pancreatic islet growth in young normoglycemic mice (Umea +/?). *Endocrinology* **140** 778–783.
- Edwards CM, Stanley SA, Davis R, Brynes AE, Frost GS, Seal LJ, Ghatei MA & Bloom SR 2001 Exendin-4 reduces fasting and postprandial glucose and decreases energy intake in healthy volunteers. *American Journal of Physiology Endocrinology and Metabolism* **281** E155–E161.
- Eng J, Kleinman WA, Singh L, Singh G & Raufman JP 1992 Isolation and characterization of exendin-4, an exendin-3 analogue, from *Heloderma suspectum* venom. Further evidence for an exendin receptor on dispersed acini from guinea pig pancreas. *Journal of Biological Chemistry* **267** 7402–7405.
- Flatt PR & Bailey CJ 1981 Abnormal plasma glucose and insulin responses in heterozygous lean (*ob/+*) mice. *Diabetologia* **20** 573–577.
- Fukasawa KM, Fukasawa K, Hiraoka BY & Harada M 1981 Comparison of dipeptidyl peptidase IV prepared from pig liver and kidney. *Biochimica et Biophysica Acta* **657** 179–189.
- Gallwitz B, Ropeter T, Morys-Wortmann C, Mentlein R, Siegel EG & Schmidt WE 2000 GLP-1-analogues resistant to degradation by dipeptidyl-peptidase IV *in vitro*. *Regulatory Peptides* **86** 103–111.
- Gault VA, O'Harte FPM, Harriott P, Mooney MH, Green BD & Flatt PR 2003 Effects of the novel (Pro3)GIP antagonist and exendin(9–39)amide on GIP- and GLP-1-inducing cyclic AMP generation, insulin secretion and postprandial insulin release in obese diabetic (*ob/ob*) mice: evidence that GIP is the major physiological incretin. *Diabetologia* **46** 222–230.
- Hassan M, Eskilsson A, Nilsson C, Jonsson C, Jacobsson H, Refai E, Larsson S & Efendic S 1999 *In vivo* dynamic distribution of ¹³¹I-glucagon-like peptide-1(7–36) amide in the rat studied by gamma camera. *Nuclear Medicine and Biology* **26** 413–420.
- Holst JJ & Deacon CF 1998 Inhibition of the activity of dipeptidyl-peptidase IV as a treatment for type 2 diabetes. *Diabetes* **47** 1663–1670.
- Holz GG, Kuhlreiber WM & Habener JF 1993 Pancreatic beta-cells are rendered glucose-competent by the insulinotropic hormone glucagon-like peptide-1(7–37). *Nature* **361** 362–365.

- Hui H, Wright C & Perfetti R 2001 Glucagon-like peptide 1 induces differentiation of islet duodenal homeobox-1-positive pancreatic ductal cells into insulin-secreting cells. *Diabetes* **50** 785–796.
- Knudsen LB & Pridal L 1996 Glucagon-like peptide-1(9–36) amide is a major metabolite of glucagon-like peptide-1(7–36) amide after *in vivo* administration to dogs, and it acts as an antagonist on the pancreatic receptor. *European Journal of Pharmacology* **318** 429–435.
- Kolligs F, Fehmann HC, Goke R & Goke B 1995 Reduction of the incretin effect in rats by the glucagon-like peptide 1 receptor antagonist exendin(9–39) amide. *Diabetes* **44** 16–19.
- McClenaghan NH, Barnett CR, Ah-Sing E, Abdel-Wahab YHA, O'Harte FPM, Yoon TW, Swanston-Flatt SK & Flatt PR 1996 Characterization of a novel glucose-responsive insulin-secreting cell line, BRIN-BD11, produced by electrofusion. *Diabetes* **45** 1132–1140.
- MacDonald PE, El-Kholy W, Riedel MJ, Salapatek AMF, Light PE & Wheeler MB 2002 The multiple actions of GLP-1 on the process of glucose-stimulated insulin secretion. *Diabetes* **51** S434–S442.
- Merrifield RB 1963 Solid phase peptide synthesis. *Journal of the American Chemical Society* **95** 2149–2159.
- Mojsov S, Weir GC & Habener JF 1987 Insulinotropin: glucagon-like peptide I(7–37) co-encoded in the glucagon gene is a potent stimulator of insulin release in the perfused rat pancreas. *Journal of Clinical Investigation* **79** 616–619.
- Miguel JC, Abdel-Wahab YH, Green BD, Mathias PC & Flatt PR 2003 Cooperative enhancement of insulinotropic action of GLP-1 by acetylcholine uncover paradoxical inhibition effect of beta cell muscarinic receptor on adenylate cyclase activity. *Biochemical Pharmacology* **65** 283–292.
- Nathan DM, Schreiber E, Fogel H, Mojsov S & Habener JF 1992 Insulinotropic action of glucagon-like peptide-I(7–37) in diabetic and nondiabetic subjects. *Diabetes Care* **15** 270–276.
- Nauck MA, Heimesaat MM, Orskov C, Holst JJ, Ebert R & Creutzfeldt W 1993 Preserved incretin activity of glucagon-like peptide 1 [7–36 amide] but not of synthetic human gastric inhibitory polypeptide in patients with type-2 diabetes mellitus. *Journal of Clinical Investigation* **91** 301–307.
- O'Harte FPM, Gray AM, Abdel-Wahab YH & Flatt PR 1997 Effects of non-glycated and glycated glucagon-like peptide-1(7–36) amide on glucose metabolism in isolated mouse abdominal muscle. *Peptides* **18** 1327–1333.
- O'Harte FPM, Abdel-Wahab YH, Conlon JM & Flatt PR 1998 Glycation of glucagon-like peptide-1(7–36)amide: characterization and impaired action on rat insulin secreting cells. *Diabetologia* **41** 1187–1193.
- O'Harte FPM, Mooney MH, Lawlor A & Flatt PR 2000 N-terminally modified glucagon-like peptide-1(7–36) amide exhibits resistance to enzymatic degradation while maintaining its antihyperglycaemic activity *in vivo*. *Biochimica et Biophysica Acta* **1474** 13–22.
- O'Harte FPM, Mooney MH, Kelly CM, McKillop AM & Flatt PR 2001 Degradation and glycemic effects of His(7)-glucitol glucagon-like peptide-1(7–36)amide in obese diabetic *ob/ob* mice. *Regulatory Peptides* **96** 95–104.
- Perfetti R, Zhou J, Doyle ME & Egan JM 2000 Glucagon-like peptide-1 induces cell proliferation and pancreatic-duodenum homeobox-1 expression and increases endocrine cell mass in the pancreas of old, glucose-intolerant rats. *Endocrinology* **141** 4600–4605.
- Salacinski PR, McLean C, Sykes JE, Clement-Jones VV & Lowry PJ 1981 Iodination of proteins, glycoproteins, and peptides using a solid-phase oxidizing agent, 1,3,4,6-tetrachloro-3 alpha,6 alpha-diphenyl glycoluril (Iodogen). *Biochemistry* **117** 136–146.
- Scrocchi LA, Brown TJ, McClusky N, Brubaker PL, Auerbach AB, Joyner AL & Drucker DJ 1996 Glucose intolerance but normal satiety in mice with a null mutation in the glucagon-like peptide 1 receptor gene. *Nature Medicine* **2** 1254–1258.
- Stevens JF 1971 Determination of glucose by an automatic analyser. *Clinica Chimica Acta* **32** 199–201.
- Szayna M, Doyle ME, Betkey JA, Holloway HW, Spencer RG, Greig NH & Egan JM 2000 Exendin-4 decelerates food intake, weight gain, and fat deposition in Zucker rats. *Endocrinology* **141** 1936–1941.
- Thorens B 1992 Expression cloning of the pancreatic beta cell receptor for the gluco-incretin hormone glucagon-like peptide 1. *PNAS* **89** 8641–8645.
- Todd JF, Wilding JP, Edwards CM, Khan FA, Ghatei MA & Bloom SR 1997 Glucagon-like peptide-1 (GLP-1): a trial of treatment in non-insulin-dependent diabetes mellitus. *European Journal of Clinical Investigation* **27** 533–536.
- Turton MD, O'Shea D, Gunn I, Beak SA, Edwards CM, Meeran K, Choi SJ, Taylor GM, Heath MM, Lambert PD, Wilding JP, Smith DM, Ghatei MA, Herbert J & Bloom SR 1996 A role for glucagon-like peptide-1 in the central regulation of feeding. *Nature* **379** 69–72.
- Valverde I, Villanueva-Pencarrillo ML & Malaisse WJ 2002 Pancreatic and extrapancreatic effects of GLP-1. *Diabetes and Metabolism* **28** 3S85–3S89.
- Xiao Q, Giguere J, Parisien M, Jeng W, St-Pierre SA, Brubaker PL & Wheeler MB 2001 Biological activities of glucagon-like peptide-1 analogues *in vitro* and *in vivo*. *Biochemistry* **40** 2860–2869.
- Zhou J, Wang X, Pineyro MA & Egan JM 1999 Glucagon-like peptide 1 and exendin-4 convert pancreatic AR42J cells into glucagon- and insulin-producing cells. *Diabetes* **48** 2358–2366.

Received in final form 3 September 2003

Accepted 14 November 2003