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

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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 His\(^7\)-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 (IC\(_{50}\) values 32.9 and 6.7 nM, respectively) compared with native GLP-1 (IC\(_{50}\) 0.37 nM). Similarly, both analogues stimulated cAMP production with EC\(_{50}\) values of 16.3 and 27 nM respectively compared with GLP-1 (EC\(_{50}\) 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.


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 beta-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 AR-42J 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 & Pridal 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). Fnoc 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, Lerne, 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 ring 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, \( M_i = \frac{iM_i - iM_0}{i} \) (where \( M_i \) is molecular mass, \( M_i \) is the m/z ratio, i is the number of charges and \( M_0 \) 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 µl) 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/H$_2$O (15 µl, 10% (v/v)). The reaction products were then applied to a Vydac C-18 analytical column (4/6 x 250 mm) and the major degradation fragment GLP-1(9–36)amide separated from intact GLP-1. The column was equilibrated with TFA/H$_2$O (0:12% (v/v)) at a flow rate of 1·0 ml/min. Using 0·1% (v/v) TFA in 70% acetonitrile/H$_2$O, 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 electrophorosis 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% CO$_2$ 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% CO$_2$) 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) 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 lystate 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 CaCl$_2$.2H$_2$O, 1·2 mM KH$_2$PO$_4$, 1·2 mM MgSO$_4$.7H$_2$O, 10 mM NaHCO$_3$, 5 g/l bovine serum albumin, pH 7·4) 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 M NaOH, 5 mM unlabeled ATP and 300 µM unlabeled cAMP) were added. Cell lystate was separated on Dowex and alumina exchange resins to isolate tritiated cAMP as described previously (Miguel et al. 2003).

**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
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 mM/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 Mᵣ of 3297.3 Da (theoretical mass 3297.5 Da). Corresponding Mᵣ 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.

<table>
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<th>Peptide</th>
<th>NH₂-terminal sequence</th>
<th>ESI-MS multiple-charged species</th>
<th>Molecular mass (Da)</th>
<th>Percentage degradation (12 h)</th>
<th>Half-lives (h)</th>
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<td>His-Ala-Glu</td>
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<td>5.8</td>
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<tr>
<td>N-acetyl-GLP-1</td>
<td>Ac-His-Ala-Glu</td>
<td>1671.2</td>
<td>3340.0</td>
<td>0</td>
<td>&gt;12</td>
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<tr>
<td>N-pyroglutamyl-GLP-1</td>
<td>pGlu-His-Ala-Glu</td>
<td>1705.2</td>
<td>3408.2</td>
<td>0</td>
<td>&gt;12</td>
</tr>
</tbody>
</table>

**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 (t₁/₂=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 (t₁/₂ >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. Bonferroni 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
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 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
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 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 (mean ± S.E.M.) as a percentage of the maximal response.

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 ± S.E.M. for eight separate observations. *P<0.05, **P<0.01 and ***P<0.001 compared with glucose control. ΔP<0.05 and ΔΔP<0.01 compared with GLP-1 at the same concentration.
In this present study, the N-terminal of GLP-1 was extended by attaching either an acetyl or a pyroglutamyl 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-pyroglutamyl-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 adenylyl 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 activity. Thus with the exception of slightly reduced effects of N-pyroglutamyl-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-pyroglutamyl-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-pyroglutamyl-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 glycemic effects of acetyl-GLP-1 in normal mice. The present study confirms the antihyperglycemic 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 His prevents DPP IV action, providing a simple means of generating stable analogues that such modifications to GLP-1 may result in 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.

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Received in final form 3 September 2003
Accepted 14 November 2003