Novel dipeptidyl peptidase IV resistant analogues of glucagon-like peptide-1(7–36)amide have preserved biological activities *in vitro* conferring improved glucose-lowering action *in vivo*

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

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

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

Abstract

Although the incretin hormone glucagon-like peptide-1 (GLP-1) is a potent stimulator of insulin release, its rapid degradation *in vivo* by the enzyme dipeptidyl peptidase IV (DPP IV) greatly limits its potential for treatment of type 2 diabetes. Here, we report two novel Ala⁸-substituted analogues of GLP-1, (Abu⁸)GLP-1 and (Val⁸)GLP-1 which were completely resistant to inactivation by DPP IV or human plasma. (Abu⁸)GLP-1 and (Val⁸)GLP-1 exhibited moderate affinities (IC₅₀: 4.76 and 81.1 nM, respectively) for the human GLP-1 receptor compared with native GLP-1 (IC₅₀: 0.37 nM). (Abu⁸)GLP-1 and (Val⁸)GLP-1 dose-dependently stimulated cAMP in insulin-secreting BRIN BD11 cells with reduced potency compared with native GLP-1 (1.5- and 3.5-fold, respectively). Consistent with other mechanisms of action, the analogues showed similar, or in the case of (Val⁸)GLP-1 slightly impaired insulin releasing activity in BRIN BD11 cells. Using adult obese (*ob/ob*) mice, (Abu⁸)GLP-1 had similar glucose-lowering potency to native GLP-1 whereas the action of (Val⁸)GLP-1 was enhanced by 37%. The *in vivo* insulin-releasing activities were similar. These data indicate that substitution of Ala⁸ in GLP-1 with Abu or Val confers resistance to DPP IV inactivation and that (Val⁸)GLP-1 is a particularly potent N-terminally modified GLP-1 analogue of possible use in type 2 diabetes.

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Introduction

Glucagon-like peptide-1(7–36)amide (GLP-1) is produced in the L cells of the small intestine by the tissue-specific post-translational processing of the product of the proglucagon gene (Bell *et al.* 1983). Upon ingestion of a meal, GLP-1 is released into the circulation (Fehmann *et al.* 1995) where it acts to stimulate insulin release from pancreatic β cells through interaction with specific receptors that are coupled to the stimulatory G protein (Thorens *et al.* 1993). Apart from its direct effect on insulin

secretion, GLP-1 has been shown to increase the rate of insulin biosynthesis (Fehmann & Habener 1992) and to restore the ability of the β cell to respond to glucose (Wang *et al.* 1997). Recent studies have highlighted mitogenic effects of GLP-1 on the pancreas and this has been associated with an ability to direct cell differentiation (Abraham *et al.* 2002) and increase β -cell mass (Tourrel *et al.* 2002). In addition to possessing potent insulinotrophic activity, GLP-1 also inhibits the release of glucagon (Ritzel *et al.* 1995) and both of these actions are glucose-dependent (Kreymann *et al.*

1987). Further metabolic properties of GLP-1 include peripheral effects such as inhibition of feeding (Turton *et al.* 1996) and reduction of gastrointestinal motility and secretion (Wettergren *et al.* 1993). Glycogenic effects of GLP-1 in liver, skeletal muscle and abdominal muscle (Valverde *et al.* 1994, Villanueva-Peñacarrillo *et al.* 1994, O'Harte *et al.* 1997) and lipogenic effects in adipose tissue (Oben *et al.* 1991, Perea *et al.* 1997) have also been reported, although there is no reproducible evidence that a GLP-1 receptor exists in these tissues (Bullock *et al.* 1996).

As most of the described properties of GLP-1 appear to be directly involved in lowering blood glucose, attention has focused on using GLP-1 as a therapeutic agent in the treatment of type 2 diabetes in man (Gutniak *et al.* 1992, Nathan *et al.* 1992, Nauck *et al.* 1996, Rachman 1996, Zander *et al.* 2002). However, a major limiting factor in such a use for GLP-1 is its susceptibility to degradation and inactivation *in vivo* by dipeptidyl peptidase IV (DPP IV; EC.3.4.14.5) – a member of the prolyl oligopeptidase family of serine proteases (Barrett & Rawlings 1992). DPP IV is ubiquitously found in mammalian organs and tissues including serum (Iwaki-Egawa *et al.* 1998) and cleaves peptides that contain penultimate proline, alanine or hydroxyproline residues (Mentlein 1999). In the case of GLP-1, DPP IV rapidly ($t_{1/2}$ 2–3 min) cleaves the His⁷-Ala⁸ dipeptide from the N-terminus generating GLP-1(9–36)amide (Mentlein *et al.* 1993). This truncated form of GLP-1 is inactive and may even behave as a receptor antagonist (Knudsen & Pridal 1996, Wettergren *et al.* 1998).

Various attempts have been made to prevent the degradation of GLP-1 by DPP IV through modification at the N-terminus (Deacon *et al.* 1998, Burcelin *et al.* 1999, O'Harte *et al.* 2001). In this study, the stability and activity of (Abu⁸)GLP-1 and (Val⁸)GLP-1 were examined. These novel GLP-1 analogues were prepared through substitution of the alanine at position 8 of GLP-1 with residues possessing a marginally larger side-chain. The *in vitro* stability, receptor binding affinity, cAMP production and insulinotropic activity of these analogues were investigated. In addition, we evaluated the effectiveness of these modified forms of GLP-1 following administration in obese diabetic (*ob/ob*) mice – a commonly used animal model of type 2 diabetes mellitus.

Materials and methods

Reagents

High performance liquid chromatography HPLC grade acetonitrile was obtained from Rathburn (Walkersburn, Scotland). Sequencing grade trifluoroacetic acid (TFA), dipeptidyl peptidase IV (DPP IV), forskolin (FSK), isobutylmethylxanthine (IBMX), adenosine 3',5'-cyclic monophosphate (cAMP) and adenosine 5'-triphosphate (ATP) were all purchased from Sigma (Poole, Dorset, UK). Fmoc-protected amino acids were obtained from Calbiochem Novabiochem (Beeston, Nottingham, UK). RPMI-1640 and DMEM tissue culture medium, fetal bovine serum (FBS), penicillin and streptomycin were all purchased from Gibco (Paisley, Strathclyde, Scotland). The chromatography columns used for cAMP assay, Dowex AG50 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, Bucks, UK. All water used in these experiments was purified using a Milli-Q, Water Purification System (Millipore, Milford, MA, USA). All other chemicals used were of the highest available purity.

Synthesis and purification of GLP-1, (Abu⁸)GLP-1 and (Val⁸)GLP-1

Peptide synthesis was carried out on an Applied Biosystems automated peptide synthesiser (model 432A) using standard solid-phase Fmoc (N-(9-fluorenyl)methoxycarbonyl) protocols (Fields & Noble 1990), starting with a rink amide MBHA resin. Synthetic peptides were cleaved from the resin and purified by reversed-phase HPLC on a Waters Millenium 2010 chromatography system (software version 2.1.5).

Electrospray ionisation-mass spectrometry (ESI-MS)

Intact and degradation fragments of GLP-1, (Abu⁸)GLP-1 and (Val⁸)GLP-1 were dissolved in water and eluted under isocratic conditions using an ion trap LCQ benchtop LC mass spectrometer (LC/MS; Finnigan MAT, Hemel Hempstead, UK). Mass spectra were collected using full ion scan mode over the mass-to-charge (m/z) range 150–2000. The molecular masses of each fragment

were determined using prominent multiple-charged ions and the following equation applied: $M_r = iM_i - iM_h$, where M_r is molecular mass, M_i is m/z ratio, i is the number of charges, and M_h is the mass of a proton.

Degradation of GLP-1, (Abu⁸)GLP-1 and (Val⁸)GLP-1 by DPP IV and human plasma

HPLC-purified peptides were incubated *in vitro* at 37 °C in 50 mM triethanolamine-HCl (pH 7.8, final peptide concentration 2 mM) with either DPP IV (1.25 mU) or pooled human plasma (7.5 µl) for 0, 6 and 12 h. The enzymatic reactions were terminated by the addition of 15 µl 10% (v/v) TFA/water. The reaction products were then applied to a Vydac C-18 column (4.6 × 250 mm) and the major degradation fragment GLP-1(9–36)amide was separated from intact GLP-1, (Abu⁸)GLP-1 and (Val⁸)GLP-1. The column was equilibrated with 0.12% (v/v) TFA/water at a flow rate of 1.0 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 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.

Cells and cell culture

Chinese hamster lung (CHL) fibroblasts stably transfected with the human GLP-1 receptor (Thorens *et al.* 1993) were cultured using DMEM tissue culture medium containing 10% (v/v) FBS, and 1% (v/v) antibiotics (100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.2 mg/ml gentimycin). BRIN-BD11 cells were cultured using RPMI-1640 tissue culture medium containing 10% (v/v) FBS, 1% (v/v) antibiotics (100 U/ml penicillin, 0.1 mg/ml streptomycin) and 11.1 mM glucose. The origin and insulin secretory characteristics of these cells have been described previously (McClenaghan *et al.* 1996). All cells were maintained in sterile tissue culture flasks (Corning, Glass Works, Sunderland, UK) at 37 °C in an atmosphere of 5% CO₂ and 95% air using a LEEC incubator (Laboratory Technical Engineering, Nottingham, UK).

Receptor binding studies

CHL fibroblasts stably transfected with the human GLP-1 receptor were seeded at a density of 1×10^5 cells per well into 24-multiwell plates (Nunc, Roskilde, Denmark). Following overnight culture at 37 °C, cells were washed twice with cold HBS buffer (130 mM NaCl, 20 mM HEPES, 0.9 mM NaHPO₄, 0.8 mM MgSO₄, 5.4 mM KCl, 1.8 mM CaCl₂, 25 mM glucose, 25 µM phenol red, pH 7.4). Test incubations were performed in HBS buffer (400 µl) with a range of concentrations (10^{-12} to 10^{-6} M) of GLP-1, (Abu⁸)GLP-1 or (Val⁸)GLP-1 plus ¹²⁵I-GLP-1 label (50 000 c.p.m./ml) and phenylmethylsulphonylfluoride (1 mM). ¹²⁵I-GLP-1 was prepared by the iodogen method (Salacinski *et al.* 1981). Following incubation for 24 h at 4 °C, cells were washed four times with cold saline solution (0.85% NaCl) and 500 µl lysis solution (5% trichloroacetic acid; 3% sodium dodecyl sulphate) were added. Plates were shaken for 10 min, 1 ml millipore water was added, 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 to calculate IC₅₀ values.

Effects of GLP-1, (Abu⁸)GLP-1 and (Val⁸)GLP-1 on cyclic AMP production

BRIN-BD11 cells were seeded into 24-multiwell plates at a density of 3.0×10^5 cells per well. The cells were then allowed to grow in culture for 48 h before being pre-incubated (16 h at 37 °C) in media supplemented with tritiated adenine (2 µCi). The cells were then washed twice with cold HBS buffer. The cells were then exposed for 20 min at 37 °C to varying concentrations (10^{-12} to 10^{-6} M) of GLP-1, (Abu⁸)GLP-1, (Val⁸)GLP-1 or forskolin (10 µM) in HBS buffer, in the presence of 1 mM IBMX. The medium was subsequently removed and 1 ml lysis solution added containing 0.3 mM unlabelled cAMP and 5 mM unlabelled ATP. The intracellular tritiated cAMP was then separated on Dowex and alumina exchange resins as previously described (Miguel *et al.* 2003).

In vitro insulin secretion

BRIN-BD11 cells were seeded into 24-multiwell plates at a density of 1.0×10^5 cells per well, and

allowed to attach overnight at 37 °C. Acute tests for 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, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 10 mM NaHCO₃, 0.5% (w/v) BSA, pH 7.4) supplemented with 1.1 mM glucose. Test incubations were performed in the presence of 5.6 mM glucose with a range of concentrations (10⁻¹² to 10⁻⁶ M) of GLP-1, (Abu⁸)GLP-1 or (Val⁸)GLP-1. After 20 min incubation, the buffer was removed from each well and aliquots (200 µl) were used in insulin RIA.

Effects of GLP-1, (Abu⁸)GLP-1 and (Val⁸)GLP-1 in (ob/ob) mice

Evaluation of the effects of GLP-1, (Abu⁸)GLP-1 or (Val⁸)GLP-1 on plasma glucose and insulin concentrations were examined using 12- to 16-week-old obese diabetic (*ob/ob*) mice. The genetic background and characteristics of the colony used have been outlined elsewhere (Bailey *et al.* 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 and a standard rodent maintenance diet (Trouw Nutrition Ltd, Cheshire, UK) were freely available. Food was withdrawn for an 18-h period prior to i.p. injection of saline (0.9% (w/v) NaCl) as control, glucose alone (18 mmol/kg body weight) or in combination with GLP-1, (Abu⁸)GLP-1 or (Val⁸)GLP-1 (each at 25 nmol/kg). All test solutions were administered in a final volume of 8 ml/kg body weight. Blood samples were collected from the cut tip of the tail vein of conscious mice into chilled fluoride/heparin microcentrifuge tubes (Sarstedt, Nümbrecht, Germany) immediately prior to injection and at 15, 30 and 60 min post injection. Plasma was aliquoted and stored at -20 °C for subsequent glucose and insulin determinations. All animal studies were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986.

Analyses

Plasma glucose was assayed by an automated glucose oxidase procedure using a Beckman Glucose Analyser II (Stevens 1971). Plasma insulin

was determined by dextran-charcoal RIA as described previously (Flatt & Bailey 1981). Incremental areas under the plasma glucose and insulin curves (AUC) were calculated using Graphpad PRISM version 3.0 (GraphPad Software, San Diego, CA, USA) which employs the trapezoidal rule (Burington 1973). Results are expressed as means ± S.E.M. and data were compared, as appropriate, using the 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 to be significantly different if *P* < 0.05.

Results

Synthesis and purification of peptides

Table 1 shows the monoisotopic masses obtained using ESI-MS for synthesised and purified GLP-1, (Val⁸)GLP-1 and (Abu⁸)GLP-1. Following spectral averaging, prominent multiple-charged species (M+2H)²⁺ and (M+3H)³⁺ were obtained for GLP-1, corresponding to an intact M_r of 3297.3 Da (theoretical mass 3297.5 Da); similarly, for (Abu⁸)GLP-1 corresponding to intact M_r of 3310.6 Da (theoretical mass 3311.7 Da), and finally for (Val⁸)GLP-1, corresponding to an M_r of 3324.4 Da (theoretical mass 3325.7 Da).

Degradation of GLP-1, (Abu⁸)GLP-1 and (Val⁸)GLP-1 by DPP IV and human plasma

GLP-1 was progressively metabolised by DPP IV over the 12-h period (47–82% degraded) giving rise to the appearance of a second peak corresponding to the degradation fragment GLP-1(9–36)amide. As shown in Table 1, similar incubation of GLP-1 with human plasma resulted in progressive metabolism with 78% degraded by 12 h. In contrast, when (Abu⁸)GLP-1 and (Val⁸)GLP-1 were incubated under similar conditions with DPP IV or human plasma, no formation of GLP-1(9–36)amide could be detected (Table 1).

Determination of GLP-1 receptor binding in CHL fibroblasts

The ability of GLP-1, (Abu⁸)GLP-1 or (Val⁸)GLP-1 to inhibit the binding of ¹²⁵I-GLP-1 to CHL

Table 1 Molecular characterisation and susceptibility of GLP-1 peptides to degradation by DPP IV and human plasma

Peptide	NH ₂ -terminal sequence	ESI-MS multiple-charged species		Molecular mass (Da)		Percentage degradation (12 h)	
		(M+2H) ²⁺	(M+3H) ³⁺	Measured	Theoretical	DPP IV	Human plasma
GLP-1	His-Ala-Glu-	1649.9	1099.9	3297.3	3297.5	82	78
(Abu ⁸)GLP-1	His-Abu-Glu-	1656.2	1104.6	3310.6	3311.7	0	0
(Val ⁸)GLP-1	His-Val-Glu-	1663.2	1109.1	3324.4	3325.7	0	0

The peptides were applied to 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 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.

fibroblast cells transfected with the human GLP-1 receptor is shown in Fig. 1. GLP-1 and GLP-1 analogues were all found to dose-dependently displace the radiolabelled tracer. Displacement by GLP-1 was complete at 10 nM and half-maximal inhibition of [¹²⁵I]-GLP-1 binding (IC₅₀) was observed at a GLP-1 concentration of 0.37 nM. (Abu⁸)GLP-1 and (Val⁸)GLP-1 were found to have slightly lower binding affinities

as defined by their ability to inhibit tracer binding with IC₅₀ values of 4.76 nM and 81.1 nM, respectively.

Stimulation of adenylate cyclase by GLP-1, (Abu⁸)GLP-1 and (Val⁸)GLP-1

The dose-dependent stimulatory effects of GLP-1, (Abu⁸)GLP-1 or (Val⁸)GLP-1 on intracellular cAMP production following incubation with BRIN-BD11 cells are shown in Fig. 2. At the highest concentrations, 10⁻⁶ and 10⁻⁵ M, both GLP-1 and its analogues induced the same maximal rise in cAMP levels. The concentrations of GLP-1, (Abu⁸)GLP-1 or (Val⁸)GLP-1 that produced 50% maximal formation of cAMP (EC₅₀) were approximately 4.7, 7.2 and 16.4 nM respectively. These values show good correlation with the relative affinity of GLP-1, (Abu⁸)GLP-1 and (Val⁸)GLP-1 for the GLP-1 receptor (Fig. 1).

Insulinotropic action of GLP-1, (Abu⁸)GLP-1 and (Val⁸)GLP-1

Figure 3 shows the effect of increasing concentrations of GLP-1, (Abu⁸)GLP-1 and (Val⁸)GLP-1 on insulin secretion from the glucose-responsive clonal pancreatic β-cell line, BRIN-BD11, in the presence of (A) 5.6 mM glucose or (B) a supraphysiological 16.7 mM glucose concentration. Figure 3A shows that all peptides stimulated insulin release (1.4- to 5.4-fold; *P* < 0.05 to *P* < 0.001) in a dose-dependent manner between 10⁻¹² and

Figure 1 Displacement of [¹²⁵I]-GLP-1 by unlabelled GLP-1, (Abu⁸)GLP-1 and (Val⁸)GLP-1 in CHL fibroblasts stably transfected with the human GLP-1 receptor. Values represent means ± S.E.M. for three different experiments.

Figure 2 Intracellular cAMP production in BRIN-BD11 cells exposed for 20 min to various concentrations of GLP-1, (Abu⁸)GLP-1 and (Val⁸)GLP-1. Each experiment was performed in triplicate ($n=3$) and the data expressed (means $\pm$ S.E.M.) as a percentage of the forskolin (10 μ M) response.

10⁻⁶ M compared with control (5.6 mM glucose). Compared with native GLP-1, (Abu⁸)GLP-1 was equipotent at stimulating insulin release over the entire concentration range. In contrast, (Val⁸)GLP-1 exhibited significantly reduced effects on insulin secretion at 10⁻⁸ and 10⁻⁷ M compared with GLP-1 but was equipotent at all other concentrations. At 16.7 mM glucose (Fig. 3B), the peptides similarly stimulated insulin secretion but the overall responses were increased, demonstrating the glucose-dependent nature of GLP-1 peptides. GLP-1, (Abu⁸)GLP-1 and (Val⁸)GLP-1 enhanced glucose-induced insulin secretion by 1.2- to 5.6-fold ($P<0.05$ to $P<0.001$) when compared with control. (Abu⁸)GLP-1 was again found to be equipotent to GLP-1 over the entire concentration range whilst (Val⁸)GLP-1 exhibited significantly reduced potency from 10⁻⁹ to 10⁻⁷ M. Interestingly, (Val⁸)GLP-1 and (Abu⁸)GLP-1 were found to have significantly enhanced potency at the lowest peptide concentrations (10⁻¹² to 10⁻¹¹ M) when compared with native GLP-1.

Effects of GLP-1, (Abu⁸)GLP-1 and (Val⁸)GLP-1 on glucose lowering and insulin secretion in obese diabetic (ob/ob) mice

Figures 4 and 5 show the plasma glucose and insulin responses to i.p. administration of saline control, glucose alone or in combination with GLP-1, (Abu⁸)GLP-1 or (Val⁸)GLP-1 in obese diabetic (ob/ob) mice. Saline had no effect on plasma glucose concentration (Fig. 4A). After injection of glucose alone, plasma glucose rose significantly at 15 min ($P<0.001$) and remained at elevated levels even after 60 min. Plasma glucose levels 15 min after native GLP-1 administration (28.2 ± 3.6 mM) were similar to those found with glucose alone. However, by 30 min plasma glucose had decreased dramatically after GLP-1 administration, to levels significantly lower ($P<0.01$) than those found with glucose alone, and glycaemic levels had virtually returned to basal by 60 min. Area under the curve (AUC, 0–60 min, Fig. 4B) analysis showed that administration of GLP-1 significantly ($P<0.001$) reduced the overall glycaemic excursion compared with glucose alone. (Abu⁸)GLP-1 acted with similar potency to GLP-1 also significantly reducing the AUC ($P<0.01$) and returning glucose levels to basal by 60 min. (Val⁸)GLP-1 was found to be significantly more effective than GLP-1 and (Abu⁸)GLP-1 at reducing glycaemic AUC ($P<0.01$) and at returning plasma glucose to a lower level at 60 min ($P<0.05$).

Figure 5 shows the corresponding plasma insulin response of obese diabetic (ob/ob) mice in this study. After injection of glucose alone, plasma insulin levels peaked at 15 min post administration to levels significantly higher ($P<0.001$) than pre-injection levels, returning to basal gradually over the remainder of the study. Although native GLP-1 induced a significantly greater insulin response (17.9 ± 0.6 mM; $P<0.01$) after 15 min compared with glucose, by 30 min the response to GLP-1 was not significantly different compared with glucose alone. Administration of (Abu⁸)GLP-1 resulted in a similar plasma insulin profile to that found with GLP-1; however (Val⁸)GLP-1 was found to evoke higher plasma insulin levels at 60 min (10.0 ± 0.4 ; $P<0.001$) compared with both GLP-1 and (Abu⁸)GLP-1. AUC analysis (Fig. 5B) confirmed the insulinotropic nature of GLP-1 with a significantly enhanced overall insulin response

Figure 3 Insulinotropic effects of GLP-1, (Abu⁸)GLP-1 and (Val⁸)GLP-1 during acute 20-min incubation of BRIN-BD11 cells in the presence of (A) 5.6 mM or (B) 16.7 mM glucose. Values represent the means $\pm$ S.E.M. for eight separate observations. * P <0.05, ** P <0.01, *** P <0.001 compared with glucose control. Δ P <0.05, $\Delta\Delta$ P <0.01 compared with native GLP-1 at the same concentration.

(P <0.01) compared with glucose alone. (Abu⁸)GLP-1 and (Val⁸)GLP-1 were also found to act with similar potency to GLP-1 significantly increasing the overall insulin response (P <0.01 to P <0.001).

Discussion

Classical insulinotropic secretagogues regularly used in the treatment of type 2 diabetes mellitus stimulate insulin secretion in an indiscriminate

Figure 4 Glucose lowering effects of GLP-1, (Abu⁸)GLP-1 and (Val⁸)GLP-1 in 18-h fasted (*ob/ob*) mice. (A) Plasma glucose concentrations were measured prior to and after i.p. administration of saline (0.9% (w/v) NaCl) as control, glucose alone (18 mmol/kg body weight), or in combination with native GLP-1, (Abu⁸)GLP-1 or (Val⁸)GLP-1 (25 nmol/kg body weight). The time of injection is indicated by the arrow (0 min). (B) Plasma glucose area under the curve (AUC) values for 0–60 min post injection. Data represent the means ± S.E.M. for eight mice. ** $P < 0.01$, *** $P < 0.001$ compared with glucose alone. $\Delta P < 0.05$, $\Delta\Delta P < 0.01$, $\Delta\Delta\Delta P < 0.001$ compared with native GLP-1.

manner, regardless of glucose concentrations, and therefore put diabetic patients at risk of developing hypoglycaemia. The glucose-dependent nature of incretin hormone action prevents hypoglycaemic episodes occurring by only triggering insulin secretion under hyperglycaemic conditions, and therefore these hormones have become a very attractive basis for the generation of potential novel diabetic therapies (Bailey & Flatt 1995).

Clinical studies using GLP-1 in human type 2 diabetic subjects have demonstrated that there is considerable therapeutic potential to be gained by the use of this hormone (Gutniak *et al.* 1992, Nauck *et al.* 1996, Zander *et al.* 2002). However, the rapid degradation of GLP-1 in the bloodstream by the enzyme DPP IV giving rise to the truncated and inactive GLP-1(9–36)amide is a major stumbling block to the efficient use of this hormone

Figure 5 Insulin releasing effects of GLP-1, (Abu⁸)GLP-1 and (Val⁸)GLP-1 in 18-h fasted (*ob/ob*) mice. (A) Plasma insulin concentrations were measured prior to and after i.p. administration of saline (0.9% (w/v) NaCl) as control, glucose alone (18 mmol/kg body weight), or in combination with native GLP-1, (Abu⁸)GLP-1 or (Val⁸)GLP-1 (25 nmol/kg body weight). The time of injection is indicated by the arrow (0 min). (B) Plasma insulin area under the curve (AUC) values for 0–60 min post injection. Data represent the means $\pm$ S.E.M. for eight mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with glucose alone. $\Delta P < 0.05$, $\Delta\Delta P < 0.01$, $\Delta\Delta\Delta P < 0.001$ compared with glucose+native GLP-1.

therapeutically (Mentlein *et al.* 1993, Kieffer *et al.* 1995). Administration of specific inhibitors of DPP IV have produced improved glucose tolerance in both animals (Sudre *et al.* 2002, Pospisilik *et al.* 2002) and humans (Ahren *et al.* 2002). However, as DPP IV is involved in diverse physiological processes including the inactivation of key regulatory hormones other than GLP-1 (Mentlein 1999), inhibition of DPP IV activity may not be a suitable

means of prolonging the action of either endogenous or exogenous GLP-1. As a consequence, efforts are now focused towards the development of GLP-1 analogues which display resistance to DPP IV whilst maintaining the biological potency of the native hormone.

In this study, the penultimate alanine residue from the N-terminus of the GLP-1 peptide was replaced with either a valine or a 2-aminobutyric

acid residue to generate two novel GLP-1 analogues - (Val⁸)GLP-1 and (Abu⁸)GLP-1. During *in vitro* incubation studies, native GLP-1 was progressively degraded over time by DPP IV and plasma. Both (Val⁸)GLP-1 and (Abu⁸)GLP-1 analogues proved to be completely resistant to proteolysis by DPP IV or plasma with no degradation products detected even after 12-h incubation. This suggests that increasing the size of the amino acid side chain at Ala⁸, achieved by substituting for either valine or 2-aminobutyric acid, drastically reduces the specificity of DPP IV for GLP-1. This finding is in accordance with previous studies, where Ala⁸ of GLP-1 was substituted with glycine (Deacon *et al.* 1998, Burcelin *et al.* 1999, Siegel *et al.* 1999, Doyle *et al.* 2001), serine (Deacon *et al.* 1998, Ritzel *et al.* 1998, Siegel *et al.* 1999), D-alanine (Siegel *et al.* 1999), threonine or α -aminoisobutyric acid (Deacon *et al.* 1998).

Although modified at the N-terminus, both (Val⁸)GLP-1 and (Abu⁸)GLP-1 retained biological activities normally associated with native GLP-1. Receptor binding studies demonstrated that (Val⁸)GLP-1 and (Abu⁸)GLP-1 bound with high affinity to the GLP-1 receptor, dose-dependently displacing ¹²⁵I-labelled GLP-1. However, these receptor affinities were reduced compared with native GLP-1. Additionally, although potent stimulators of intracellular cAMP, (Val⁸)GLP-1 and (Abu⁸)GLP-1 were, respectively, 1.5- and 3.5-fold less potent than native GLP-1. Taken together with previous data (Siegel *et al.* 1999), these observations indicate that a loss in receptor binding of Ala⁸-substituted analogues usually results in a loss in adenylate cyclase activity. However, in the present study losses in receptor affinity and cAMP production were not translated into losses in insulinotropic activity due to diverse mechanisms of GLP-1 action on β cells (MacDonald *et al.* 2002). (Val⁸)GLP-1 and (Abu⁸)GLP-1 maintained dose-dependent insulinotropic activity similar to GLP-1 at both basal and elevated glucose concentrations *in vitro*. The effects of (Abu⁸)GLP-1 were particularly impressive and at elevated glucose both analogues were found to be more potent than GLP-1 at the lowest concentration tested.

When administered to diabetic (*ob/ob*) mice, these novel GLP-1 analogues significantly lowered plasma glucose levels. Whilst (Abu⁸)GLP-1 had

similar *in vivo* glucose-lowering ability as native GLP-1, (Val⁸)GLP-1 was significantly more potent, reducing the overall glucose excursion by 37% more than native GLP-1. This glucose lowering activity was associated with increased insulin levels and GLP-1, (Abu⁸)GLP-1 and (Val⁸)GLP-1 appeared equipotent as insulin secretagogues *in vivo*. The more potent antihyperglycaemic activity of (Val⁸)GLP-1 may therefore relate to other beneficial actions such as inhibition of glucagon secretion or extrapancreatic effects (Fehmann *et al.* 1995). Other modifications of the GLP-1 molecule through substitution of Ala⁸ have also been reported. (Gly⁸)GLP-1 lowered blood glucose (Burcelin *et al.* 1999) and increased insulin secretion (Doyle *et al.* 2001) in diabetic mice and rats; however, this was less effective than native peptide. Also notable was (Ser⁸)GLP-1 which possessed enhanced insulinotropic and glucose-lowering activity in normal animals (Ritzel *et al.* 1998). Although comparison of the relative effectiveness of (Abu⁸)GLP-1 and (Val⁸)GLP-1 with these analogues is difficult due to differences in experimental design, it is clear that (Abu⁸)GLP-1 and particularly (Val⁸)GLP-1 rate favourably in terms of potency and spectrum of actions.

In conclusion, this study demonstrates that substitution of the Ala⁸ residue of GLP-1 by either valine or 2-aminobutyric acid confers resistance to DPP IV degradation without impairing insulin release or biological action *in vivo*. (Val⁸)GLP-1 exhibited increased antihyperglycaemic activity in *ob/ob* mice, also indicating important actions distinct from the stimulation of insulin secretion. This study lends support to the belief that GLP-1 analogues modified at the Ala⁸ position, such as (Val⁸)GLP-1, could be worthwhile therapeutic candidates for the treatment of type 2 diabetes mellitus.

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