Lys^9 for Glu^9 Substitution in Glucagon-Like Peptide-1(7-36)Amide Confers Dipeptidylpeptidase IV Resistance With Cellular and Metabolic Actions Similar to Those of Established Antagonists Glucagon-Like Peptide-1(9-36)Amide and Exendin (9-39)

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The incretin hormone glucagon-like peptide-1(7-36)amide (GLP-1) has been deemed of considerable importance in the regulation of blood glucose. Its effects, mediated through the regulation of insulin, glucagon, and somatostatin, are glucose-dependent and contribute to the tight control of glucose levels. Much enthusiasm has been assigned to a possible role of GLP-1 in the treatment of type 2 diabetes. GLP-1's action unfortunately is limited through enzymatic inactivation caused by dipeptidylpeptidase IV (DPP IV). It is now well established that modifying GLP-1 at the N-terminal amino acids, His^7 and Ala^8, can greatly improve resistance to this enzyme. Little research has assessed what effect Glu^9-substitution has on GLP-1 activity and its degradation by DPP IV. Here, we report that the replacement of Glu^9 of GLP-1 with Lys dramatically increased resistance to DPP IV. This analogue, (Lys^9)GLP-1, exhibited a preserved GLP-1 receptor affinity, but the usual stimulatory effects of GLP-1 were completely eliminated, a trait duplicated by the other established GLP-1-antagonists, exendin (9-39) and GLP-1(9-36)amide. We investigated the in vivo antagonistic actions of (Lys^9)GLP-1 in comparison with GLP-1(9-36)amide and exendin (9-39) and revealed that this novel analogue may serve as a functional antagonist of the GLP-1 receptor.

THE INCRETIN HORMONE, glucagon-like peptide-1(7-36)amide (GLP-1), is secreted from the L-cells of the distal ileum and colon in response to meal ingestion and nutrient absorption.[1] It has been demonstrated that GLP-1 has a catalogue of beneficial effects on the endocrine pancreas and several other tissues. In the islets of Langherhans, GLP-1 stimulates insulin and somatostatin secretion and inhibits the release of glucagon.[2] GLP-1 has been shown to stimulate β-cell growth and regeneration[3] in addition to causing the transdifferentiation of ductal pancreatic cells into insulin-secreting cells.[4,5] In addition, GLP-1 delays gastric emptying and so acts to retard absorption of nutrients, thereby helping to minimize the glucose-excursion after a meal.[6] In skeletal muscle, GLP-1 promotes glycogen formation and lactate production and encourages glucose uptake.[7] This evidence suggests that GLP-1 may not only have therapeutic potential in regulating acute glycemic excursions, but may also have long-lasting effects[8] reversing the progression of type 2 diabetes.

The potential of GLP-1 as a treatment for type 2 diabetes is hindered by its short circulating half-life, as the N-terminal dipeptide, His-Ala, is rapidly removed by dipeptidylpeptidase IV (DPP IV).[9] The resulting truncated form, GLP-1(9-36)amide, lacks biologic activity and may even function as a GLP-1 receptor antagonist.[10] To circumvent this problem, several groups have sought to minimize the degradation of GLP-1 using novel DPP IV inhibitors.[11,12] Although such inhibitors prolong GLP-1 action, there is concern over interference with other regulatory processes, because DPP IV inactivates a wide range of hormones, including enterostatin, gastrin-releasing peptide, GLP-2, and neuropeptide Y.[13]

As an alternative to nonspecific enzyme inhibition, attention has focused on the generation of DPP IV-resistant analogues of GLP-1. Deacon et al[14] generated four N-terminally modified GLP-1 analogues with resistance to DPP IV, improved biologic half-life and strong receptor affinities by replacing Ala^8 with either threonine, glycine, serine, and α-aminoisobutyric acid. Other substitutions at position 8 have also produced analogues with substantially improved plasma stability compared with native GLP-1. These showed similar or reduced potencies in terms of insulin secretion and cyclic adenosine monophosphate (cAMP) production when compared with native GLP-1.[15-18] N-terminal extension of GLP-1 at His^7 by addition of glucitol or other groups also confers DPP IV resistance, but such analogues also tend to have reduced receptor affinity and cellular actions.[19,20] It is known that the substitution of Glu^9 with either Ala or Asp can have dramatic effects on receptor binding activity of GLP-1,[21] but nothing is known in terms of how amino acid substitution at this position affects GLP-1 stability to DPP IV and in vivo biologic activity.

In the present study, a novel Glu^9-substituted analogue of GLP-1, (Lys^9)GLP-1, was assessed in terms of DPP IV stability, receptor affinity, cAMP production, cellular insulin secretion, and in vivo glucose lowering and insulinotropic activities. In addition, we have compared the actions of this novel GLP-1 analogue with GLP-1(9-36)amide, exendin (9-39), and exendin 4 (1-39).

MATERIALS AND METHODS

Reagents

High-performance liquid chromatography (HPLC) grade acetonitrile, diethyl ether, and dichloromethane (DCM) were obtained from Rathburn (Walkersburn, Scotland). Sequencing grade trifluoroacetic acid (TFA), DPP IV, forskolin (FSK), isobutylmethylxanthine (IBMX),

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cAMP, and adenosine 5′-triphosphate (ATP) were purchased from Sigma (Poole, Dorset, UK). Fmoc protected amino acids were purchased from Calbiochem Novabiochem (Beeston, Nottingham, UK). RPMI 1640 and Dulbecco's modified Eagles's medium (DMEM) tissue culture medium, fetal bovine serum, penicillin, and streptomycin were all purchased from Gibco Life Technologies (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 Analyical, Larne, N. Ireland). Tritiated adenine (TRK311) was obtained from Amersham Pharmacia Biotech, Buckinghamshire, UK. Exendin 4 (1-39) and exendin (9-39) were gifts from Amylin (San Diego, CA). All water used in experiments was purified using a Milli-Q Water Purification System (Millipore, Milford, MA). All other chemicals used were of analytical grade.

Synthesis of GLP-1 and (Lys^9)GLP-1

GLP-1, GLP-1(9-36)amide and (Lys^9)GLP-1 were synthesized in a sequential fashion, starting with an Arg rink amide MBHA resin[22] using an automated 432 A peptide synthesizer (Applied Biosystems, Foster City, CA). Synthesis 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 Ionization Mass Spectrometry

Intact peptides and degradation fragments were dissolved in 0.12% (vol/vol) TFA/water and directly injected onto the electrospray ionization source of an LCQ ion-trap mass spectrometer (Finnigan MAT, Hemel Hempstead, Hertfordshire, UK). Spectra were obtained from a quadrupole ion-trap mass analyzer with the detector set to a mass-to charge range of *m/z* 150 to 2,000. The molecular masses of GLP-1 and related peptides were calculated from the prominent multiply charged ions using the equation, $M_r = iM_i - iM_h$ (where M_r is molecular mass, M_i is the *m/z* ratio, i is the number of charges, and M_h is the mass of a proton).

Degradation of GLP-1 and (Lys^9)GLP-1 After DPP IV and Plasma Incubation

GLP-1 and (Lys^9)GLP-1 (final peptide concentration 2 mmol/L) were incubated with either DPP IV (1.25 mU) or pooled human plasma (7.5μL) for 0, 2, 4, 6, and 12 hours (37°C; 50 mmol/L triethanolamine-HCl buffer; pH 7.8). Reactions were terminated by the addition of TFA/H_2O (15 μL, 10% [vol/vol]). The reaction products were then applied to a Vydac C-18 analytical column (4.6 $\times$ 250 mm) and the major degradation fragment GLP-1(9-36)amide separated from intact GLP-1 or (Lys^9)GLP-1. The column was equilibrated with TFA/H_2O (0.12% [vol/vol]) at a flow rate of 1.0mL/min. Using 0.1% (vol/vol) TFA in 70% acetonitrile/H_2O, the concentration of acetonitrile in the eluting solvent was increased from 0% to 28% over 10 minutes and from 28% to 42% over 30 minutes. The absorbance was monitored at 206 nm using a SpectraSystem UV 2000 detector (Thermoquest, Manchester, UK) and peaks were collected manually before electrospray ionization-mass spectrometry (ESI-MS) analysis.

Culture of Chinese Hamster Lung Fibroblasts and BRIN-BD11 Cells

Chinese hamster lung (CHL) fibroblasts transfected with the human GLP-1 receptor[23] were cultured using DMEM tissue culture medium (10% (vol/vol) fetal bovine serum, 1% (vol/vol) antibiotics (100 U/mL penicillin, 0.1 mg/mL streptomycin, and 0.2 mg/mL genetimycin). BRIN-BD11 cells were cultured in RPMI-1640 tissue culture medium at 11.1 mmol/L glucose with similar additions. BRIN BD11 cells are a glucose-responsive pancreatic β-cell line generated by the electrofusion of New England Deaconess Hospital (NEDH) rat pancreatic β cells and rat insulinoma-derived RINm5F cells. Full details of the origin and characteristics of BRIN BD11 cells are given elsewhere.[24] All cells were maintained in sterile tissue culture flasks (Corning Glass Works, Corning, UK) at 37°C in an atmosphere of 5% CO_2 and 95% air using a LEEC incubator (Laboratory Technical Engineering, Nottingham, UK).

Receptor Binding Studies

CHL fibroblasts were seeded (1×10^5 per well) in 24-multiwell plates (Nunc, Roskilde, Denmark). After overnight incubation (37°C; 5% CO_2) cells were washed twice with cold Hanks' Balanced Salt Solution (HBSS). Each peptide was tested by a single experiment (n = 3), which incorporated an internal control incubation of GLP-1 (60 nmol/L) to ensure consistency and accuracy. Test incubations (24 hours; 4°C)) were performed in HBS buffer (400 μL) with a range of concentrations of GLP-1 and related peptides (10^{-6} mol/L serially diluted 3-fold) plus ^{125}I-GLP-1 label (50,000 cpm/mL) and phenylmethylsuphonylfluoride (1 mmol/L). ^{125}I-GLP-1 was prepared from GLP-1(7-36)amide by the solid phase iodogen method[25] and purified by HPLC. Cells were washed 4 times with cold saline solution (0.85% $NaCl_2$) and 500 μL of lysis solution (5% trichloroacetic acid; 3% sodium dodecyl sulfate [SDS]) was added. Plates were shaken for 10 minutes and 1 mL millipore water was added before measurement of radioactivity on a γ-counter (1261 Multigamma counter, LKB Wallac, Turku, Finland). Curves were analyzed by nonlinear regression using the sigmoidal dose-response equation ($Y = \min + (\max - \min/1 + 10^{\text{LogEC50-X}})$ to calculate EC_{50} values.

Assessment of the Adenylate Cyclase Activity

BRIN-BD11 cells were seeded in 24-well plates (3×10^5 per well), cultured for 48 hours before being preincubated in media supplemented with tritiated adenine (2 μCi) for 16 hours. The cells were washed twice with cold HBSS buffer and test solution (400 μL; 37°C) was added. The cells were then exposed to varying concentrations of GLP-1 and related peptides (10^{-5} mol/L serially diluted 3-fold) in HBS buffer in the presence of 1 mmol/L IBMX and 5.6 mmol/L glucose (20 minutes; 37°C). After incubation, test solutions were removed and 300 μL of lysis solution (5% TFA, 3% SDS, 5 mmol/L of unlabelled ATP and 300 μmol/L of unlabelled cAMP) was added. Cell lysate was separated on Dowex and alumina exchange resins to isolate tritiated cAMP as described elsewhere.[26]

Assessment of In Vitro Insulinotropic Action

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 a 40-minute preincubation at 37°C in 1.0 mL Krebs Ringer bicarbonate buffer (115 mmol/L NaCl, 4.7 mmol/L KCl, 1.28 mmol/L $CaCl_2 \cdot 2H_2O$, 1.2 mmol/L KH_2PO_4, 1.2 mmol/L $MgSO_4 \cdot 7H_2O$, 10 mmol/L $NaHCO_3$, 5 g/L bovine serum albumin, pH 7.4) supplemented with 1.1 mmol/L glucose. Test incubations were performed at 37°C in the presence of 5.6 or 16.7 mmol/L glucose with a range of concentrations of GLP-1 and related peptides (10^{-6} to 10^{-12} mol/L). After a 20-minute incubation, the buffer was removed from each well and aliquots were stored at −20°C for measurement of insulin.

Metabolic and Insulin-Releasing Effects of GLP-1 and Related Peptides in Obese Diabetic (ob/ob) Mice

The actions of GLP-1 and related peptides were assessed in 12- to 16-week-old obese diabetic (*ob/ob*) mice, described elsewhere.[27] The animals were housed individually in an air-conditioned room at 22°C ± 2°C with a 12-hour light:12-hour dark cycle and access to drinking water and standard rodent maintenance diet (Trouw Nutrition,

Table 1. ESI-MS Identification of Peptides

Analogue	Theoretical Molecular Mass (d)	Monoisotopic Masses $(M+2H)^2$ (d)	Monoisotopic Masses $(M+3H)^3$ (d)	Monoisotopic Masses $(M+4H)^{4+}$	ESI-MS Molecular mass (d)	Molecular Mass Difference (d)
GLP-1	3,297.5	1,649.9	1,099.9	—	3,297.3	0.2
GLP-1(9-36)amide	3,089.4	1,545.1	1,030.5	—	3,088.4	1.0
(Lys^9)GLP-1	3,296.8	1,649.6	1,100.0	—	3,297.1	0.3
Exendin 4 (1-39)	4,186.7	—	1,396.4	1,047.7	4,186.6	0.1
Exendin (9-39)	3,370.8	1,685.6	1,124.2	—	3,369.4	1.4

NOTE. Peptide samples were applied to a LC/MS equipped with a microbore C-18 HPLC column (150 mm × 2.0 mm). Spectra were recorded using a quadruple ion trap mass analyzer and collected using ion scan mode over the mass-to-charge (m/z) range 150 to 2,000.

Cheshire, UK) ad libitum. Mice were fasted for 18 hours before intraperitoneal (IP) administration of saline (9 g/L NaCl), glucose alone (18 mmol/L/kg body weight) or in combination with either GLP-1, GLP-1(9-36), (Lys^9)GLP-1, exendin (9-39), or exendin 4 (1-39) (25 nmol/L/kg). In a second experimental series to assess possible GLP-1 receptor antagonism, GLP-1, (Lys^9)GLP-1, exendin (9-39), or GLP-1(9-36)amide (25 nmol/L/kg body weight) were given immediately after injection of GLP-1 (25 nmol/L/kg body weight), combined with glucose (18 mmol/L/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 before injection and at 15, 30, and 60 minutes postinjection, and the plasma obtained was stored at −20°C. All animal studies were performed in accordance with the UK Animals (Scientific Procedures) Act of 1986.

Measurement of Glucose and Insulin

Plasma glucose levels were determined using an Analox glucose analyzer (Hammersmith, London, UK), which uses the glucose oxidase method. Insulin was measured by dextran-coated charcoal radioimmunoassay.[28] Incremental areas under plasma glucose and insulin curves (AUC) were calculated using the trapezoidal rule using GraphPad PRISM version 3.0 (Graphpad Software, San Diego, CA). Results were expressed as mean ± SEM and data compared using the Student's *t* test and, where appropriate, repeated measures analysis of variance (ANOVA) or 1-way ANOVA, followed by the Student-Newman-Keuls post hoc test. Groups of data were considered significant if $P < .05$.

RESULTS

Confirmation of the Identities of Peptides by ESI-MS

Table 1 shows the monoisotopic masses obtained for GLP-1 and related peptides using ESI-MS. Prominent multiply-charged species $(M+2H)^{2+}$ and $(M+3H)^{3+}$ were obtained for GLP-1, corresponding to M_r of 3,297.3 Da; for GLP-1(9-36)amide of 3,088.4 Da; for (Lys^9)GLP-1 of 3,297.1 Da, and for exendin 4 (1-39) of 4,186.6 dalton (d). Finally, for exendin (9-39), $(M+3H)^{3+}$ and $(M+4H)^{4+}$ charged species were observed corresponding to of 3,369.4 d. Values measured compared closely to the theoretical molecular mass in each case (Table 1).

Degradation of GLP-1 and (Lys^9)GLP-1 by DPP IV and Human Plasma

Figure 1 shows a set of typical HPLC profiles of the products obtained from the incubation of GLP-1 or (Lys^9)GLP-1 with purified DPP IV (Fig 1A and B) or human plasma (Fig 1C and D) for 12 hours. GLP-1 was progressively metabolized by DPP IV and plasma over 4 to 12 hours (Table 2) giving rise to the appearance of a major degradation peak corresponding to GLP-1(9-36)amide. In contrast, when (Lys^9)GLP-1 was incubated under identical conditions, the truncated metabolite was not detected until the 12-hour time point (Fig 1B and D; Table 2). The estimated half-lives of DPP IV and plasma-mediated degradation of GLP-1 and (Lys^9)GLP-1 were 5.5 to 6.2 hours and >12 hours, respectively (Table 2).

Determination of GLP-1 Receptor Binding

Displacement of ^{125}I-GLP-1 by GLP-1, (Lys^9)GLP-1 and related peptides in transfected CHL fibroblast cells is shown in Fig 2. The extent of ^{125}I-GLP-1 binding was 94.6%. GLP-1 displaced the radiolabelled tracer reaching half-maximal inhibition of ^{125}I-GLP-1 binding (IC_{50}) at a concentration of 0.37 nmol/L. GLP-1(9-36)amide, exendin 4 (1-39), exendin (9-39), and (Lys^9)GLP-1 had binding affinities presenting IC_{50} values of 53.2, 0.42, 2.35, and 6.3 nmol/L, respectively. Bonferri analysis of the sigmoidal dose-response curves showed that all peptides, with the exception of exendin 4 (1-39), were significantly different ($P<0.05$) from native GLP-1.

Stimulation of Adenylate Cyclase Production

The effects of GLP-1 and related peptides on cAMP production in BRIN-BD11 cells are illustrated in Fig 3. Only GLP-1, exendin 4 (1-39) exerted dose-dependent stimulatory effects on cAMP production with EC_{50} values of 4.7 and 6.0 nmol/L, respectively. GLP-1(9-36)amide, exendin (9-39), and (Lys^9)GLP-1 did not increase cellular cAMP even at the highest peptide concentrations.

Insulinotropic Action on Clonal β Cells

As shown in Fig 4, GLP-1 and exendin 4 (1-39) stimulated insulin secretion in a dose-dependent manner between 10^{-12} and 10^{-6} mmol/L. GLP-1(9-36)amide, exendin (9-39), and (Lys^9)GLP-1 did not demonstrate any insulinotropic activity even at the highest peptide concentrations tested.

Metabolic Effects of GLP-1, (Lys9)GLP-1, GLP-1(9-36)Amide, Exendin 4 (1-39), and Exendin (9-39) in Obese (ob/ob) Mice

Figure 5 shows the plasma glucose and insulin responses to IP administration of saline, glucose alone, or in combination with either GLP-1, (Lys^9)GLP-1, GLP-1(9-36)amide, exendin 4 (1-39), or exendin (9-39). Whereas injection of saline had no effect, administration of glucose alone resulted in a rapid and sustained increase in plasma glucose. The glucose response

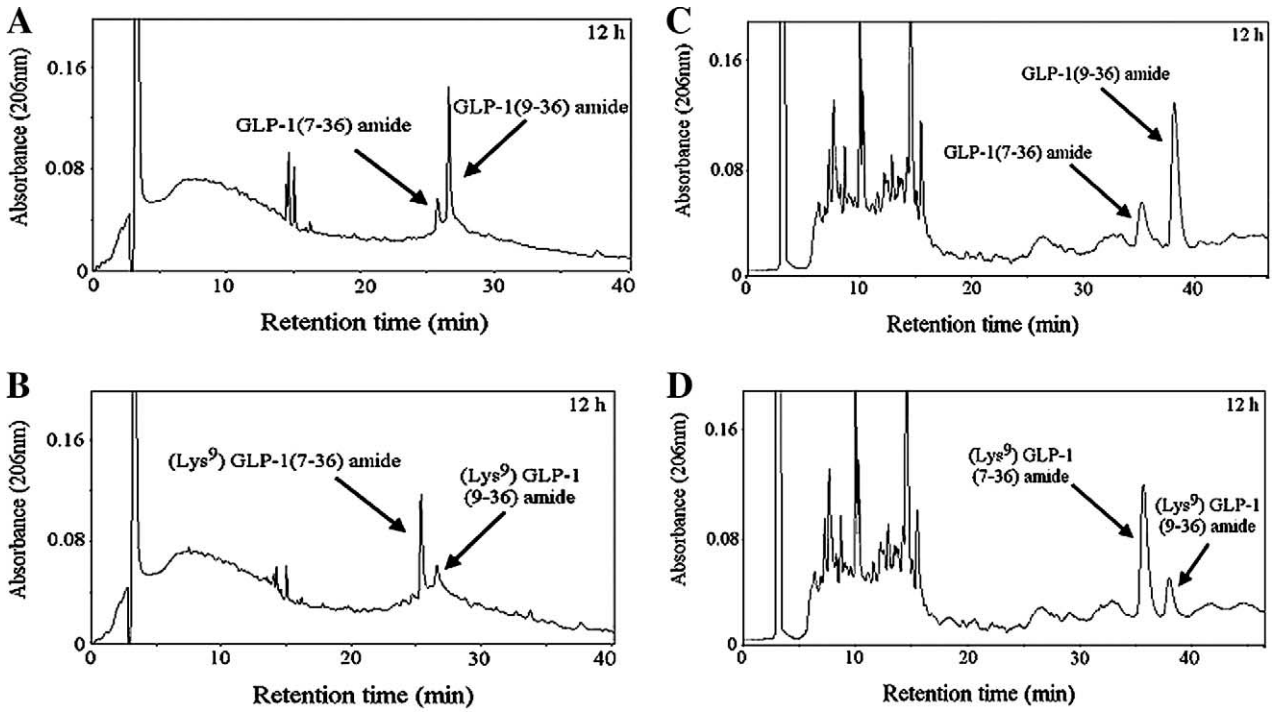

Fig 1. (A and C) HPLC degradation profiles of GLP-1 and (B and D) (Lys^9) GLP-1 after incubation with purified DPP IV or human plasma, respectively, for 12 hours. Degradation can be assessed as a percentage of intact peptide remaining relative to the major degradation fragments GLP-1(9-36)amide or (Lys^9)GLP-1(9-36)amide.

after native GLP-1 was similar at 15 minutes, but glucose concentrations were reduced to near normal levels by 60 minutes ($P < .001$). Exendin 4 (1-39) had similar potency to GLP-1 (Fig 5, AUC, 729 ± 40 *v* 606 ± 84 mmol/L · min, respectively), but (Lys^9)GLP-1, GLP-1(9-36)amide and exendin (9-39) were devoid of glucose-lowering ability (AUC, 1,301 ± 72, 1,146 ± 102, and 1,100 ± 62 mmol/L · min, respectively). These actions of GLP-1 and exendin 4 (1-39) were associated with markedly enhanced insulin responses (AUC, 509 ± 37 and 496 ± 21 ng/mL · l min, respectively). In contrast, (Lys^9)GLP-1, GLP-1(9-36)amide, and exendin (9-39)

Table 2. Degradation of GLP-1 and (Lys^9)GLP-1 by Incubation With Purified DPP IV or Human Plasma

	Percentage of Peptide Degraded			
	DPP IV		Human Plasma	
Time (h)	GLP-1	(Lys^9) GLP-1	GLP-1	(Lys^9) GLP-1
0	0	0	0	0
4	37 ± 0.4	0	54 ± 0.5	0
6	59 ± 0.9	0	67 ± 0.8	0
12	84 ± 0.6	17 ± 1.1	78 ± 1	21 ± 2
$T_{1/2}$(h)	6.2	>12	5.5	>12

NOTE. DPP IV and plasma half-lives were calculated by constructing a graph of percentage degradation against time. Linear regression "best-fit" analysis was used to calculate the time at which half of the peptide was degraded. Values are mean ± SEM for 2 separate experiments.

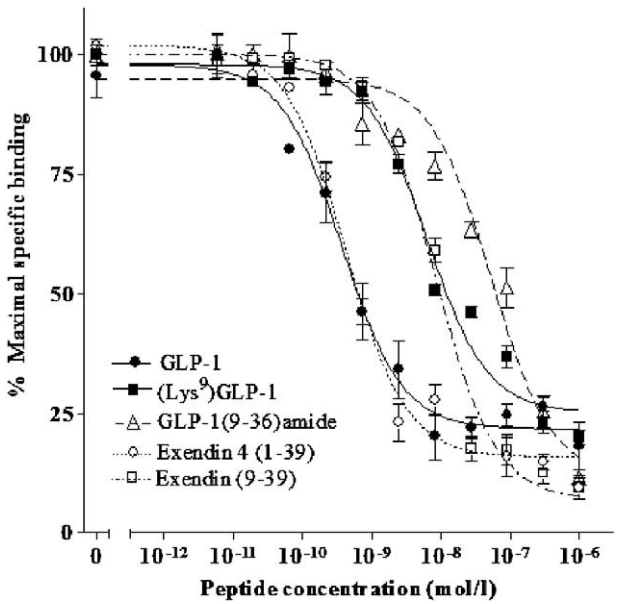

Fig 2. Receptor binding of GLP-1, (Lys^9)GLP-1 and related peptides in CHL cells stably transfected with the human GLP-1 receptor. Values are mean ± SEM (n = 3). Curves were analyzed by nonlinear regression using the sigmoidal dose-response equation to calculate EC_{50} values: GLP-1, 0.37 nmol/L; (Lys^9)GLP-1, 6.3 nmol/L; GLP-1(9-36)amide, 53.2 nmol/L; exendin 4 (1-39), 0.42, exendin (9-39), 2.35 nmol/L.

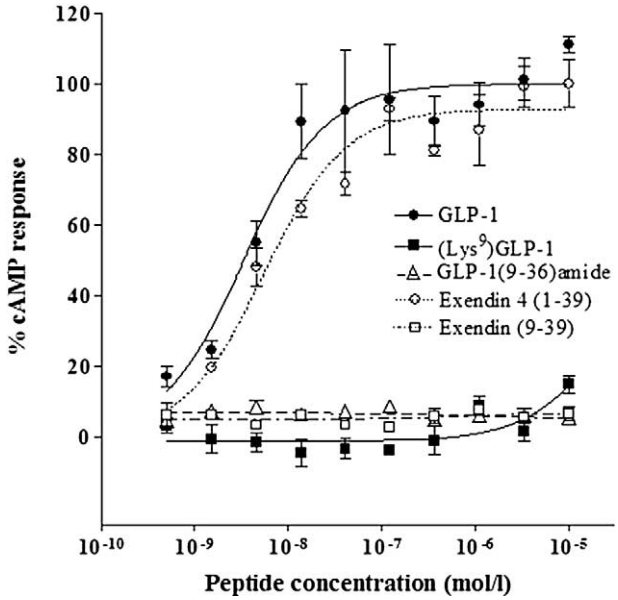

Fig 3. cAMP production in BRIN-BD11 cells exposed for 20 minutes to various concentrations of GLP-1, (Lys^9)GLP-1 and related peptides. Values are mean ± SEM (n = 4). Curves were analyzed by nonlinear regression using the sigmoidal dose-response equation to calculate EC_{50} values: GLP-1, 4.7 nmol/L and exendin 4 (1-39), 6.0 nmol/L.

did not stimulate plasma insulin, and the overall (AUC) insulin responses (AUC, 229 ± 35, 289 ± 31, and 266 ± 30 ng/mL · min, respectively) were similar to glucose alone (AUC, 260 ± 21 ng/mL · min).

Antagonism of GLP-1 Metabolic Actions by (Lys^9)GLP-1, GLP-1(9-36)Amide, and Exendin (9-39) in Obese (ob/ob) Mice

Figure 6 shows the plasma glucose and insulin responses to saline, glucose alone, or in combination with either GLP-1 plus either equipotent GLP-1, (Lys^9)GLP-1, GLP-1(9-36)amide, or exendin (9-39). As expected, administration of GLP-1 markedly decreased the glycemic excursion (AUC, 523 ± 57 mmol/L · min, $P < .001$) and enhanced plasma insulin concentrations (AUC, 509 ± 37 ng/mL · min, $P < .001$) compared with glucose alone (AUC, 1,156 ± 36 mmol/L · min and 261 ± 21 ng/mL · min, respectively). These beneficial actions of GLP-1 were abolished by conjoint injection of (Lys^9)GLP-1 (AUC, 1,057 ± 31 mmol/L · min and 278 ± 31 ng/mL · min) and substantially blocked by the established GLP-1 receptor antagonists, GLP-1(9-36)amide (AUC, 935 ± 62 mmol/L · min and 402 ± 30 mmol/L · min), and exendin (9-39) (AUC, 980 ± 58 mmol/L · min and 382 ± 21 mmol/L · min).

DISCUSSION

Much interest has been generated by attempts to develop DPP IV-resistant analogues of GLP-1 using N-terminal modifications, such as Ala^8-substitution[14-18,29] and His^7-modification.[19,20] These studies have met with varying degrees of success with many analogues suffering from reduced receptor affinity and diminished insulinotropic potency.[14,20,29] Other analogues have displayed similar biologic activity to native GLP-1.[14,16-19] As yet, no study has investigated how modification at the Glu^9 position affects the actions and susceptibility of GLP-1 to DPP IV.

In the present study, we examined a novel synthetic Glu^9-substituted GLP-1 analogue, (Lys^9)GLP-1. Degradation studies indicated that this analogue exhibited markedly enhanced stability

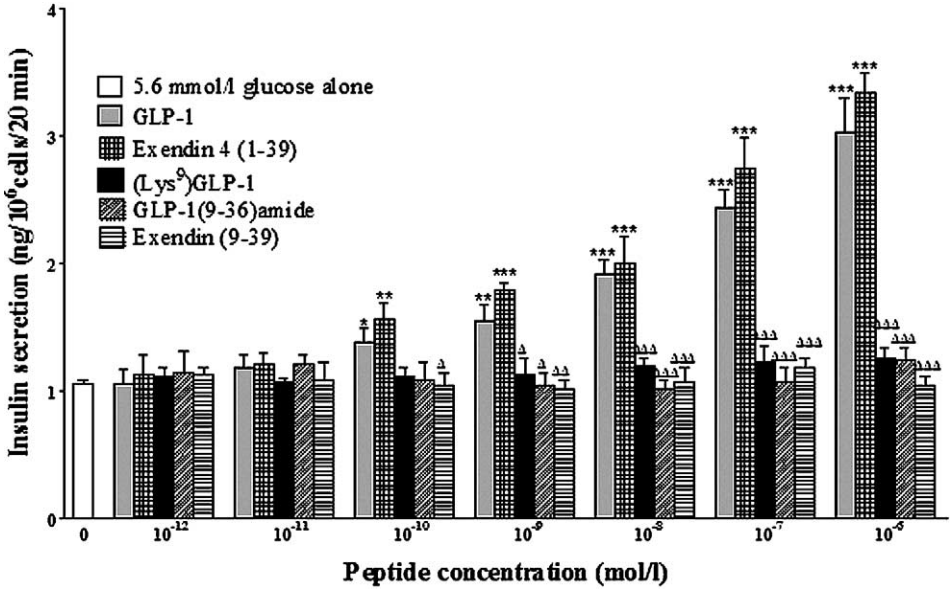

Fig 4. Effects of GLP-1, (Lys^9)GLP-1 and related peptides on insulin release from BRIN-BD11 cells. Values are mean ± SEM (n = 8) *$P < .05$, **$P < .01$, and ***$P < .001$ compared with 5.6 mmol/L glucose control. $^{\Delta}P < .05$, $^{\Delta\Delta}P < .01$, and $^{\Delta\Delta\Delta}P < .001$ compared with GLP-1 at the same concentration.

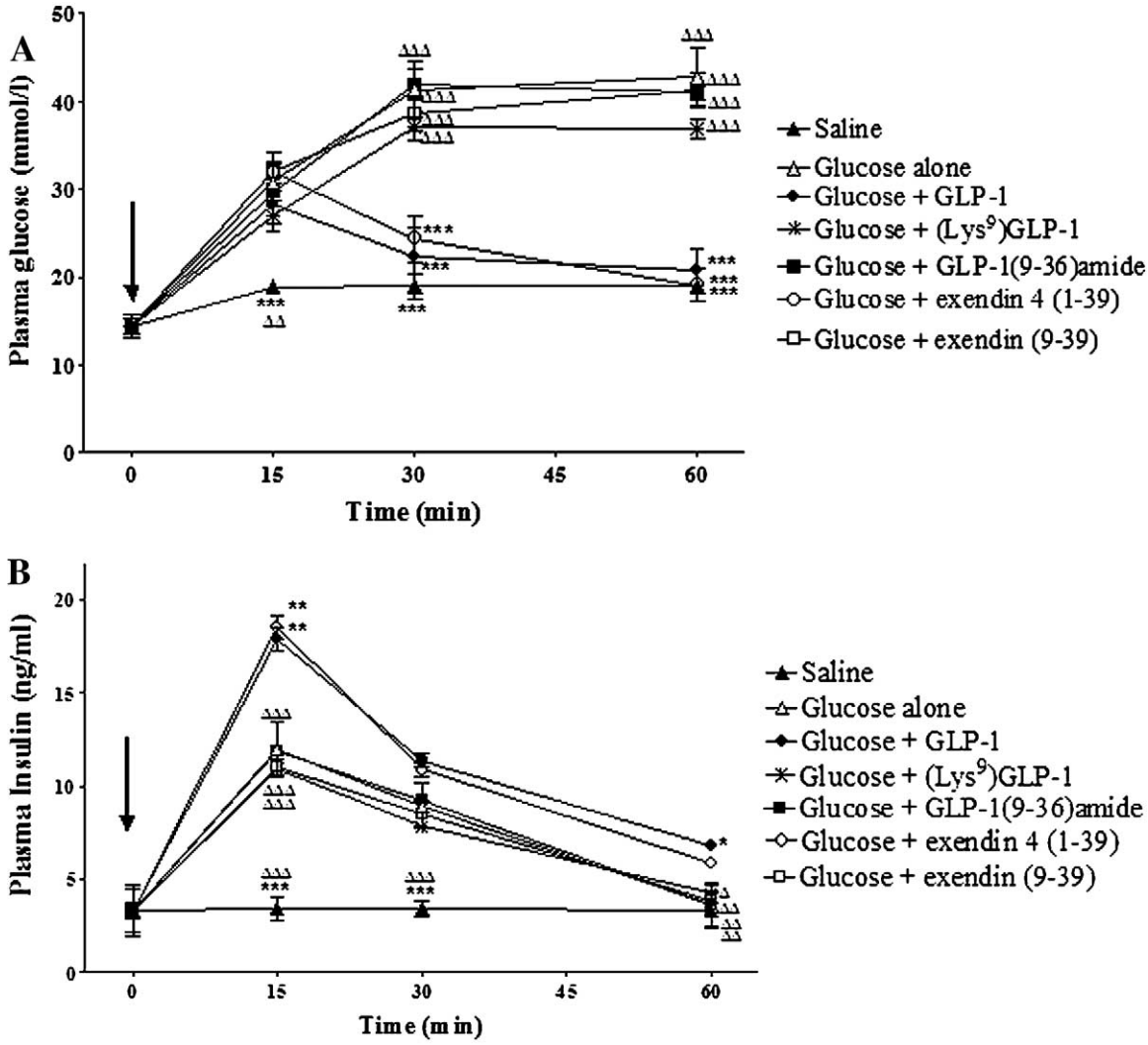

Fig 5. Effects of GLP-1, (Lys[9])GLP-1 and related peptides on glycemic excursion and circulating insulin in obese diabetic (*ob/ob*) mice. Concentrations of (A) plasma glucose and (B) insulin before (t = 0) and up to 60 minutes after IP administration of saline (0.9% [wt/vol]), glucose alone (18 mmol/L/kg) or in combination with GLP-1, (Lys[9])GLP-1, exendin 4 (1-39), exendin (9-39), or GLP-1(9-36)amide (25 nmol/L/kg) at 0 minute. Values are mean ± SEM for 8 mice. *$P < .05$, **$P < .01$, ***$P < .001$ compared with glucose alone. $^{\Delta}P < .05$, $^{\Delta\Delta}P < .01$, $^{\Delta\Delta\Delta}P < .001$ compared with native GLP-1.

to degradation by DPP IV or human plasma in contrast to GLP-1, which was rapidly degraded. (Lys[9])GLP-1 exhibited strong binding to CHL fibroblasts transfected with the human GLP-1 receptor, although affinity was reduced approximately 17-fold compared with native GLP-1 or exendin 4 (1-39). This indicates that Glu[9] is important for GLP-1 receptor activation and binding and is in agreement with observations of a substantial reduction in receptor affinity when Glu[9] was substituted for Ala.[21,30] However, Xaio et al[21] noted very little loss in receptor binding when Glu[9] was replaced with Asp. Comparison of receptor binding characteristics of (Lys[9])GLP-1 with GLP-1(9-36)amide and exendin (9-39) revealed close similarities. These 2 established GLP-1 receptor antagonists exhibited weaker binding affinities than GLP-1 as described previously.[10,31,32]

Consistent with previous observations,[31] exendin 4 (1-39) and GLP-1 were approximately equipotent in stimulating cAMP generation in GLP-1 receptor transfected fibroblasts. This property was mirrored by the ability of the 2 peptides to stimulate insulin secretion from clonal BRIN-BD11 cells. As expected, the established GLP-1 antagonists, GLP-1(9-36)amide and exendin (9-39) lacked stimulatory effects of both cAMP production and insulin secretion in vitro. A very similar profile was observed with the novel (Lys[9])GLP-1, which did not stimulate cAMP or insulin secretion.

In addition to being a potent insulinotropic peptide in vitro, GLP-1 has been shown exert antihyperglycemic and insulin-releasing activity in obese (*ob/ob*) mice, an animal model of type 2 diabetes. The results presented here concur with previous findings[27] and additionally indicate that exendin 4 (1-39) also has potent activity in this animal model. No significant difference in potency was observed between the 2 peptides, which was somewhat surprising given that exendin 4 (1-39) is

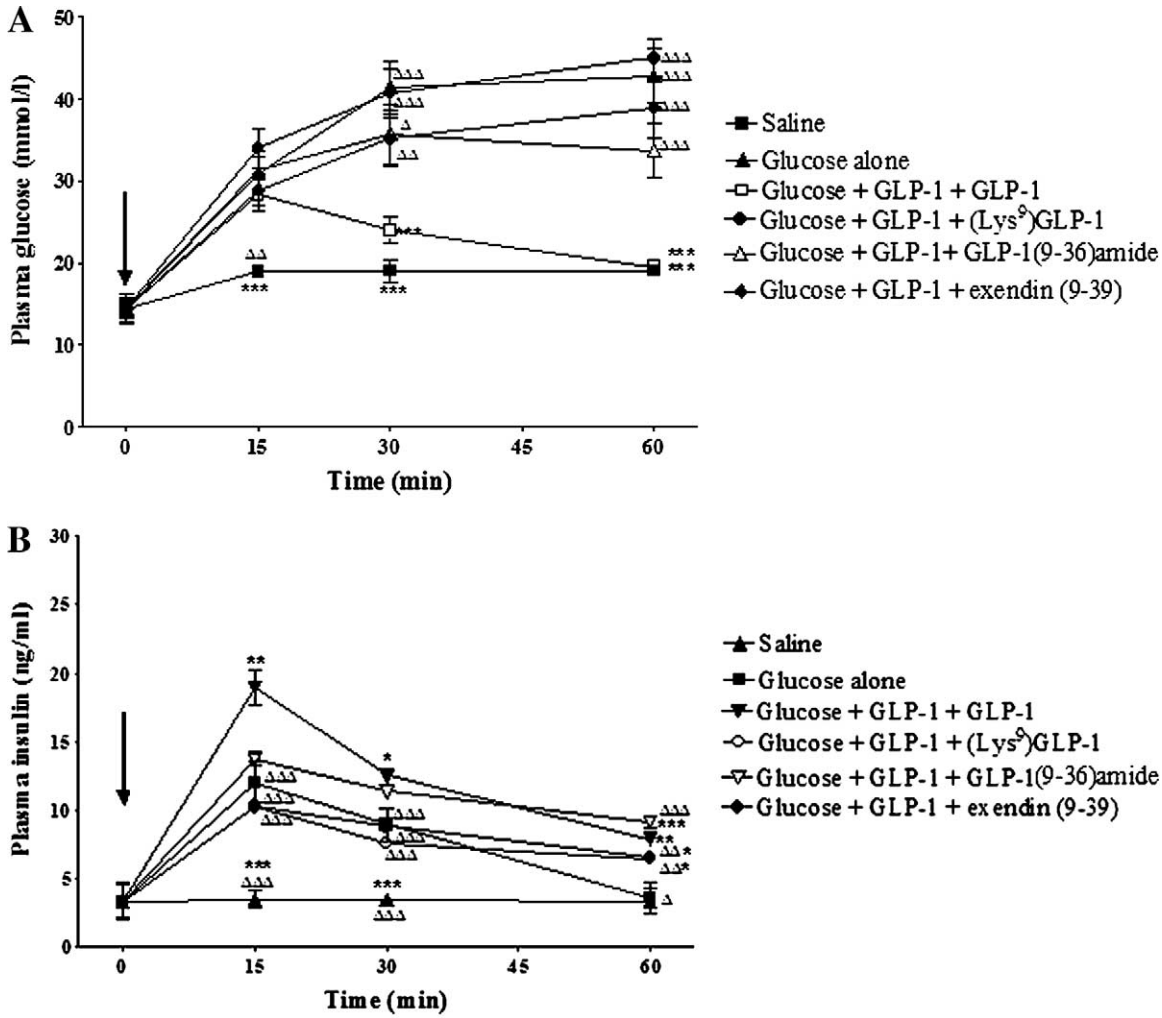

Fig 6. Antagonistic effects of (Lys^9)GLP-1, exendin (9-39), and GLP-1(9-36)amide on glucose-lowering and insulinotropic actions of GLP-1 in obese diabetic *(ob/ob)* mice. Concentrations of (A) plasma glucose and (B) insulin were measured before (t = 0) and up to 60 minutes after IP administration of saline (0.9% [wt/vol]), glucose alone (18 mmol/L/kg) or in combination with GLP-1 + GLP-1, GLP-1 + (Lys^9)GLP-1, GLP-1 + exendin (9-39), or GLP-1 + GLP-1(9-36)amide (25 nmol/L/kg + 25 nmol/L/kg) at 0 minute. *P < .05, **P < .01, ***P < .001 compared with glucose alone. $^{\Delta}P$ < .05, $^{\Delta\Delta}P$ < .01, $^{\Delta\Delta\Delta}P$ < .001 compared with native GLP-1.

reported to be less susceptible than native GLP-1 to DPP IV-mediated degradation.[33] In line with in vitro observations, no significant glucose-lowering or insulinotropic actions were observed when (Lys^9)GLP-1 was injected into *ob/ob* mice. In fact, the responses were almost identical to those observed with GLP-1(9-36)amide and exendin (9-39).

In view of these results, a further series of experiments were conducted in *ob/ob* mice to evaluate whether (Lys^9)GLP-1 functions as a GLP-1 antagonist in vivo. When (Lys^9)GLP-1 was administered in combination with GLP-1, there was total inhibition of the glucose-lowering effect of the native peptide and a complete inhibition of its insulinotropic activity. Similar observations were made with the established antagonists GLP-1(9-36)amide and exendin (9-39). Indeed, the actions of (Lys^9)GLP-1 were somewhat more pronounced, as some residual beneficial effect of GLP-1 was observed in the presence of GLP-1(9-36)amide and exendin (9-39).

In conclusion, these studies have explored the cellular and metabolic actions of the stable GLP-1 analogue, (Lys^9)GLP-1. Substitution of Glu^9 for Lys^9 in the GLP-1 molecule provided a novel DPP IV-resistant analogue, which served as a potent antagonist of the actions of GLP-1 in vivo. These observations reveal a central role of Glu^9 in mediating the biologic actions of GLP-1. In addition, the data suggests that (Lys^9)GLP-1 might serve as a useful tool to evaluate the physiologic and possible pathophysiologic roles of GLP-1 after molecular ablation of endogenous GLP-1 action in vivo.

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