Dipeptidyl peptidase IV resistant analogues of glucagon-like peptide-1 which have extended metabolic stability and improved biological activity

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Summary Glucagon-like peptide 1 (GLP-1) has great potential in diabetes therapy due to its glucose-dependent stimulation of insulin secretion, but this is limited by its rapid degradation, primarily by dipeptidyl peptidase IV. Four analogues, N-terminally substituted with threonine, glycine, serine or α-aminoisobutyric acid, were synthesised and tested for metabolic stability. All were more resistant to dipeptidyl peptidase IV in porcine plasma in vitro, ranging from a $t_{1/2}$ of 159 min (Gly^8 analogue) to undetectable degradation after 6 h (Aib^8 analogue; $t_{1/2}$ for GLP-1 (7–36) amide, 28 min). During i.v. infusion in anaesthetised pigs, over 50 % of each analogue remained undegraded compared to 22.7 % for GLP-1 (7–36) amide. In vivo, analogues had longer N-terminal $t_{1/2}$ (intact peptides: means, 3.3–3.9 min) than GLP-1 (7–36) amide (0.9 min; $p < 0.01$), but these did not exceed the C-terminal $t_{1/2}$ (intact plus metabolite: analogues, 3.5–4.4 min; GLP-1 (7–36) amide, 4.1 min). Analogues were assessed for receptor binding using a cell line expressing the cloned receptor, and for ability to stimulate insulin or inhibit glucagon secretion from the isolated perfused porcine pancreas. All bound to the receptor, but only the Aib^8 and Gly^8 analogues had similar affinities to GLP-1 (7–36) amide (IC_{50}; $Aib^8 = 0.45$ nmol/l; $Gly^8 = 2.8$ nmol/l; GLP-1 (7–36) amide = 0.78 nmol/l). All analogues were active in the isolated pancreas, with the potency order reflecting receptor affinities ($Aib^8 > Gly^8 > Ser^8 > Thr^8$). N-terminal modification of GLP-1 confers resistance to dipeptidyl peptidase IV degradation. Such analogues are biologically active and have prolonged metabolic stability in vivo, which, if associated with greater potency and duration of action, may help to realise the potential of GLP-1 in diabetes therapy. [Diabetologia (1998) 41: 271–278]

Keywords Glucagon-like peptide-1, analogue, dipeptidyl peptidase IV, non-insulin-dependent diabetes mellitus, therapy.

The insulinotropic hormone glucagon-like peptide-1 (GLP-1) is the product of tissue-specific post-translational processing of the glucagon precursor, proglucagon (PG) in the L-cells of the gastrointestinal mucosa [1, 2]. This results in the formation of GLP-1 (7–36) amide (corresponding to PG (78–107) amide), which is the predominant form in humans, although small amounts of non-amidated glycine-extended GLP-1 (7–37) are also produced [3]. GLP-1 is one of the most potent insulin secretagogues identified [4] and this, together with the glucose-dependency of its actions [5–7], has focussed interest on its role as a regulator of blood glucose and its potential as a therapeutic agent in the treatment of non-insulin-dependent diabetes mellitus (NIDDM) [6, 8–11].

Recent studies have shown that GLP-1 itself is the subject of further enzyme cleavage. In particular, dipeptidyl peptidase IV (DPP IV; EC 3.4.14.5) is im-

portant, resulting in a metabolite which is N-terminally truncated by 2 amino acids [12–14]. The resulting peptide, GLP-1 (9–36) amide, is an endogenous metabolite [13] which is an antagonist in vitro [15, 16]. Moreover, exogenously administered GLP-1 is also rapidly degraded in both diabetic and non-diabetic subjects, with GLP-1 (9–36) amide being the major metabolite [17]. In a strain of rats lacking DPP IV, this metabolite is not formed [14]. DPP IV is highly specific and has strict substrate requirements [18, 19], raising the possibility of developing analogues which the enzyme is unable to cleave. Studies with another peptide substrate of DPP IV, growth hormone-releasing factor (GRF), have shown that analogues with N-terminal amino acid substitutions have some resistance to the enzyme's action [20]. The present study was undertaken to examine whether small modifications of the N-terminus of GLP-1 would also confer resistance to degradation by DPP IV, while retaining the peptide's biological activity.

Materials and methods

Peptide synthesis. Peptides were synthesised on an Applied Biosystems 431A peptide synthesiser (Foster City, Calif., USA), according to the Fmoc strategy, using the following protected amino acid derivatives: Fmoc-Arg(Pmc), Fmoc-Trp(Boc), Fmoc-Glu(OBut), Fmoc-Lys(Boc), Fmoc-Gln(Trt), Fmoc-Tyr(But), Fmoc-Ser(But), Fmoc-Thr(But), Fmoc-His(Trt) and Fmoc-Asp(OBut) (Calbiochem-Novabiochem AG, Läufelingen, Switzerland). The peptides were deprotected and cleaved from the resin by treatment with trifluoroacetic acid (TFA)/phenol/thioanisole/water/ethanedithiol (83.25:6.25:4.25:2.00) for 180 min. After evaporation of the TFA, the crude peptide was precipitated with diethyl ether and purified by semipreparative HPLC on a C_{18} reversed-phase column eluted with a gradient of acetonitrile in 0.05 mol/l $(NH_4)_2SO_4$, pH 2.5. Peptide-containing fractions were applied to a Sep Pak C_{18} cartridge (Waters-Millipore, Milford, Mass., USA), eluted with 70 % acetonitrile/0.1 % TFA and lyophilised. The final products were characterised by amino acid analysis, analytical reversed-phase HPLC and by plasma desorption mass spectrometry. Purity was more than 95 % by HPLC with detection at 214 nm. Analogues, substituted at position 8 of GLP-1 with either threonine (Thr^8-GLP-1 (7–37)), glycine (Gly^8-GLP-1 (7–37)), serine (Ser^8-GLP-1 (7–36) amide) or α-aminoisobutyric acid (Aib^8-GLP-1 (7–36) amide and Aib^8-GLP-1 (7–37)) were prepared.

Peptide stability in porcine plasma in vitro. The stability of each peptide in porcine plasma was determined by incubation at 37 °C with 300 pmol/l of GLP-1 (7–36) amide or each analogue for up to 6 h. This was followed by reversed-phase HPLC and RIA according to a previously published method [13], using antiserum 2135 as described below.

Peptide pharmacokinetics in vivo. Danish LYY strain pigs (33–40 kg) were used. Food was withdrawn 24 h before surgery, but animals had free access to drinking water. After premedication with ketamine chloride (Ketaminol, 10 mg/kg; Veterinaria AG, Zurich, Switzerland), animals were anaesthetised with 1 % halothane (Halocarbon Laboratories, River Edge, NJ, USA), and

anaesthesia was maintained with intermittent positive pressure ventilation using an anaesthesia ventilator in a semi-open system. Catheters were placed in the right carotid artery for sampling of arterial blood, and into a vein of the left ear for peptide infusion. After surgical preparation, animals were heparinised, an infusion (0.9 % NaCl) was set up and given via the ear vein catheter (5 ml/min), and the animals were left undisturbed for 30 min.

Four groups of four animals were used. Each group received separate i.v. infusions of GLP-1 (7–36) amide and one analogue in a cross-over design with 80 min between each infusion. Synthetic GLP-1 (7–36) amide or analogues were dissolved in saline containing 1 % human serum albumin (HSA; Behringwerke, Marburg, Germany), and infused at a rate of 5 pmol · kg^{-1} · min^{-1} for 30 min using a syringe pump. Arterial blood samples (4 ml) were taken at 0, 5, 10, 15, 20, 22, 25, 27 and 30 min from the start of the infusion. After 30 min, the infusion was stopped, and further blood samples were taken at 1, 2, 4, 6, 10, 15, 20, 30, 40, 50 and 65 min. The amount of blood taken over the entire procedure was 160 ml, which, for a 40 kg pig, is equivalent to 4 % of the total blood volume.

Blood samples were collected into chilled tubes containing EDTA (7.4 mmol/l final concentration; Merck, Darmstadt, Germany), aprotinin (500 kallikrein inhibitory equivalents/ml blood; Novo Nordisk, Bagsværd, Denmark) and diprotin A (0.1 mmol/l final concentration; Bachem, Bubendorf, Switzerland), and kept on ice until centrifugation at 4 °C. Plasma was separated and stored at –20 °C until analysis with the RIAs described below. In addition, plasma collected during mins 22–30 of each infusion was separately pooled for each group of four animals, extracted on Sep Pak C_{18} cartridges and analysed by reversed-phase HPLC and RIA as before [13], using antiserum 2135, described below.

Expression of the cloned human GLP-1 receptor. The human GLP-1 receptor cDNA was obtained from Dr. B. Thorens. The cDNA was subcloned into the pcDNA 1 vector (Invitrogen Corporation, San Diego, Calif., USA) using the *Hind III – EcoRI* sites, and was then called pAH 260. Baby hamster kidney (BHK) cells were co-transfected with 20 μg pAH 260 and 0.6 μg pSV 2 neo vector [21] using the methods described by Chen and Okayama [22], and grown in Dulbecco's modified Eagle's medium, 10 % fetal calf serum, 100 IU penicillin, 100 μg/ml streptomycin and 1 mmol/l Na-pyruvate (all from Gibco, Life Technologies, Roskilde, Denmark). Stable clones were selected in medium containing 1 mg/ml Geneticin G-418 (Gibco) and maintained at 37 °C, in an atmosphere containing 5 % CO_2. Stable clones were screened in a receptor binding assay, and those expressing high levels of GLP-1 receptor were then recloned and screened in an adenylate cyclase assay to find clones with functional receptors. These clones were selected for further studies.

Receptor binding. Receptor binding was carried out as previously described [16], using BHK cells expressing the human pancreatic GLP-1 receptor. In brief, plasma membranes were prepared by homogenisation with two 10 s bursts using a Polytron PT 10–35 homogeniser (Kinematica, Lucerne, Switzerland), in a buffer consisting of 10 mmol/l Tris-HCl with 30 mmol/l NaCl, pH 7.4, containing in addition, 1 mmol/l dithiothreitol, 5 mg/l leupeptin, 5 mg/l pepstatin, 100 mg/l bacitracin (all from Sigma, St. Louis, Mo, USA) and 16 mg/l aprotinin, and centrifuged on top of a layer of 41 % (wt/vol) sucrose at 95 000 × *g* for 75 min. The white band between the two layers was diluted in buffer and centrifuged at 40 000 × *g* for 45 min. The precipitate containing the plasma membranes was suspended in buffer, and stored at –80 °C until use.

The binding assay was performed in 96-well filter microtitre plates. The buffer used was 50 mmol/l HEPES, pH 7.4, with the addition of 2.5 % (wt/vol) HSA grade V (Sigma). Peptide, tracer and plasma membranes were incubated for 30 min at 30 °C. The tracer was prepared by iodination of GLP-1 (7–36) amide using the lactoperoxidase method [23]. Purification by HPLC as previously described [24] yielded ^{125}I-Tyr^{19}-GLP-1 (7–36) amide with a specific activity of 80 kBq/pmol.

Isolated perfused pancreas. Danish LYY strain pigs (14–16 kg) were fasted overnight and anaesthetised with chloralose (100 mg/kg; Merck). The pancreases were isolated as previously described [25] and perfused in a single-pass system, using a gassed (5 % CO_2 in O_2) Krebs-Ringer-bicarbonate perfusion medium containing, in addition, 0.1 % HSA, 5 % dextran T 70 (Pharmacia, Uppsala, Sweden) and 5 mmol/l glucose. The venous effluent was collected for 1-min intervals, centrifuged at 4 °C, and stored at −20 °C until analysis. Synthetic GLP-1 (7–36) amide or peptide analogues were dissolved in 0.04 mol/l phosphate buffer, pH 7.4, containing 1 % HSA, and infused into the arterial line using a syringe pump, to give final concentrations of 1 nmol/l in the perfusate. Peptides were infused for 10 min periods, separated by 10–15 min rest periods, during which time endocrine secretion returned to basal levels. Insulin and glucagon concentrations in the venous effluent were measured by RIA as described below.

Hormonal analysis. HPLC fractions were analysed using antiserum 2135 [26, 27], which is "side-viewing", and which recognises all molecules containing the central sequence of GLP-1 regardless of C- or N-terminal truncations or extensions. It cross-reacts fully with GLP-1 (7–37), 79 % with GLP-1 (9–36) amide, and has a detection limit of 5 pmol/l. Plasma samples were assayed for GLP-1 immunoreactivity using RIAs which are specific for each terminus of the molecule. N-terminal immunoreactivity was measured using the newly described antiserum 93242 [28], which has a cross-reactivity of approximately 10 % with GLP-1 (1–36) amide, and less than 0.1 % with GLP-1 (8–36) amide and GLP-1 (9–36) amide, and has a detection limit of 5 pmol/l. C-terminal immunoreactivity was measured using antiserum 89390 or 92071 as appropriate. Antiserum 89390 [3, 29] has an absolute requirement for the intact amidated C-terminus of GLP-1 (7–36) amide, and cross-reacts less than 0.01 % with C-terminally truncated fragments, and 83 % with GLP-1 (9–36) amide. The detection limit is 1 pmol/l. Antiserum 92071 [3] is specific for the C-terminus of GLP-1 (7–37) and cross-reacts less than 0.1 % with GLP-1 (7–36) amide. It has a detection limit of 4 pmol/l. For all assays, the intra-assay coefficient of variation was less than 6 %. GLP-1 (7–36) amide, GLP-1 (7–37) or appropriate analogue were used as standard, and ^{125}I-labelled GLP-1 (7–36) amide or GLP-1 (7–37) were used as tracer. Separation of antibody-bound from free peptide was achieved using plasma-coated charcoal [26, 29]. Plasma samples were extracted with 70 % ethanol (vol/vol, final concentration) before assay, giving recoveries of 75 % [27]. The cross-reactivity of each analogue was determined for each antiserum.

Venous effluent from the perfused pancreas was assayed using antiserum 2004 for insulin [27], and antiserum 4305 for glucagon [30].

Calculations. During the peptide infusions, stable arterial peptide levels were achieved after 20 min, so the plateau concentration was defined as the mean of the last four measurements during the infusion. The plasma $t_{1/2}$ was calculated by ln-linear regression analysis of peptide concentrations in samples collected after termination of the infusion, after subtraction of endogenous arterial GLP-1 concentrations. Insulin and glucagon output from the perfused pancreas are expressed as percentages of basal secretion, which is defined as 100 %.

Statistical analysis. Data are expressed as means ± SEM, and were analysed using GraphPAD InStat software, version 1.13 (San Diego, Calif, USA). In vitro data were analysed using one-sample t-tests, analysis of variance (ANOVA) and 2-tailed t-tests with correction for multiple comparisons as appropriate. In vivo data were analysed using repeated measures ANOVA and 2-tailed t-tests for paired or unpaired data as appropriate. Values of p less than 0.05 were considered to be significant.

Results

Analogue cross-reactivity. All analogues cross-reacted more than 80 % with the appropriate C-terminal RIA; however, the cross-reactivity with the N-terminally directed 93242 assay varied considerably (Aib^8-GLP-1, < 5 %; Gly^8-GLP, 17 %; Ser^8-GLP-1, 21 %; Thr^8-GLP-1, 34 %). In practice, this meant that C-terminal assays (with appropriate standard) could be used for each analogue (detection limit, 5 pmol/l), but N-terminal immunoreactivity, using the appropriate analogue as standard, could be measured for only three analogues (Gly^8-GLP, Ser^8-GLP-1 and Thr^8-GLP-1; detection limit, 20 pmol/l).

Peptide stability in porcine plasma in vitro. GLP-1 (7–36) amide was degraded by porcine plasma in vitro at 37 °C, with a $t_{1/2}$ of 28.1 ± 1.2 min ($n = 12$). HPLC analysis revealed the time-dependent generation of a second peak corresponding to GLP-1 (9–36) amide, and no other immunoreactive peaks were detected. Incubation of the GLP-1 analogues revealed a significantly ($p < 0.0001$) prolonged $t_{1/2}$ compared to GLP-1 (7–36) amide itself (Gly^8-GLP-1, 159 ± 12 min, $n = 3$; Ser^8-GLP-1, 174 ± 12 min, $n = 9$; Thr^8-GLP-1, 197 ± 14 min, $n = 3$), and again, HPLC analysis showed the formation of only one other peak, in addition to the intact peptide. Degradation of Aib^8-GLP-1 ($n = 9$) was undetectable after 6 h.

Peptide pharmacokinetics in vivo. Stable arterial peptide concentrations were reached after 20 min of infusion. In all groups, concentrations determined by the C-terminal RIA (which measures both intact and N-terminally degraded peptide) exceeded those determined by the N-terminal assay (illustrated for Thr^8 analogue group in Figure 1), while the ratio of N-terminal to C-terminal immunoreactivity was greater for each analogue than for GLP-1 (7–36) amide (Table 1). HPLC analysis of plasma pooled from each group during each infusion revealed two immunoreactive peaks, corresponding to the intact peptide and the N-terminally truncated metabolite. In each
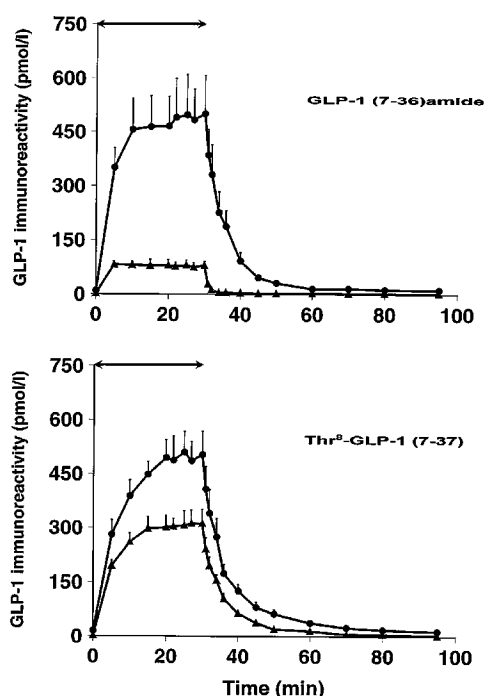
Fig.1. Plasma GLP-1 immunoreactivity in the carotid artery, measured with C-terminally (●) and N-terminally (▲) directed RIAs. Anaesthetised pigs received separate i.v. infusions (5 pmol · kg^{-1} · min^{-1}) of GLP-1 (7–36) amide and Thr^8-GLP-1 (7–37) in a cross-over design with 80 min between each infusion. Data are mean ± SEM; n = 4. The horizontal arrow indicates the period of the infusion

Fig.2. Binding affinity of GLP-1 analogues using the cloned human GLP-1 receptor. Individual curves are from one representative experiment where the data points are expressed as the mean of duplicate samples

group, a greater percentage of the analogue remained undegraded compared to GLP-1 (7–36) amide (Table 1).

For all four groups, there was no significant difference between the C-terminal $t_{1/2}$ for GLP-1 (7–36) amide or the analogues, but the N-terminal $t_{1/2}$ for each analogue was significantly prolonged (Table 2). For all four groups, the N-terminal $t_{1/2}$ was shorter than the C-terminal $t_{1/2}$ for GLP-1 (7–36) amide, but there was no significant difference between N- and C-terminal $t_{1/2}$ for the analogues (Table 2).
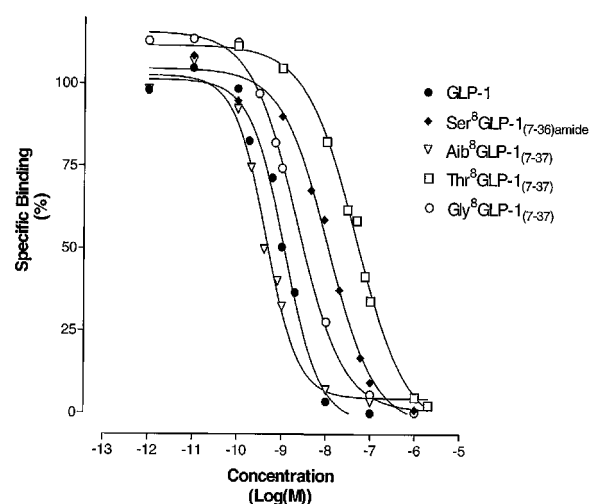
Receptor binding. All the analogues bound to the cloned human pancreatic GLP-1 receptor (Fig.2), but with widely differing binding affinities (IC$_{50}$; Table 3). The Aib^8 and Gly^8 analogues had similar affinities compared to GLP-1 (7–36) amide, while the other two analogues had lower receptor affinities than GLP-1 (7–36) amide.

Isolated perfused pancreas. Perfusion of the pancreas with 1 nmol/l GLP-1 (7–36) amide or the analogues increased insulin and decreased glucagon release relative to basal secretion (Fig.3). Of the analogues, Aib^8-GLP-1 (7–36) amide was at least as potent as GLP-1 (7–36) amide in stimulating insulin and inhibiting glucagon secretion, and was significantly ($p < 0.05$) more potent than the Ser^8 and Thr^8 ana-

Table 1. Plasma concentrations of GLP-1 peptides attained during infusion of GLP-1 (7–36) amide and N-terminally modified analogues calculated using N- and C-terminally directed RIAs

Infusion	Plateau concentrations (pmol/l)		N-terminal/C-terminal immunoreactivity	% intact peptide after HPLC
	N-terminal	C-terminal		
GLP-1 (7–36) amide	76.0 ± 12.2[a]	484.3 ± 103.1	16.4 ± 1.6[c]	24.9
Thr^8-GLP-1 (7–37)	437.8 ± 49.1[a]	689.3 ± 84.4	62.2 ± 0.6	62.5
GLP-1 (7–36) amide	108.0 ± 8.6[a]	500.3 ± 67.7	22.4 ± 2.1[c]	23.8
Gly^8-GLP-1 (7–37)	336.0 ± 10.4[a]	627.0 ± 24.0	53.8 ± 2.4	ND
GLP-1 (7–36) amide	104.7 ± 22.8[a]	387.0 ± 59.5	26.4 ± 2.1[c]	24.8
Ser^8-GLP-1 (7–36) amide	331.0 ± 59.7[b]	629.7 ± 137.1	55.7 ± 3.9	52.2
GLP-1 (7–36) amide	80.3 ± 7.4[a]	267.8 ± 22.6	31.7 ± 2.1	17.2
Aib^8-GLP-1 (7–36) amide	NDL	530.3 ± 16.2	NDL	67.1

Values are mean ± SEM; n = 4, except for the % intact peptide after HPLC, which was calculated after HPLC analysis of a single sample of plasma pooled from four animals.

[a] $p < 0.05$ vs C-terminal concentration; [b] NS, $p > 0.05$ vs C-terminal concentration; [c] $p < 0.005$ vs analogue; ND, not determined; NDL, not determined due to lack of cross-reactivity

Table 2. In vivo plasma $t_{1/2}$ for GLP-1 (7–36) amide and N-terminally modified analogues calculated using N- and C-terminally directed RIAs

Group	Analogue $t_{1/2}$ (min)		GLP-1 (7–36) amide $t_{1/2}$ (min)	
	N-terminal	C-terminal	N-terminal	C-terminal
Thr[8]-GLP-1 (7–37)	3.9 ± 0.2^{ab}	4.2 ± 0.4^{c}	0.7 ± 0.05^{d}	4.0 ± 0.1
Gly[8]-GLP-1 (7–37)	3.3 ± 0.4^{ab}	3.5 ± 0.7^{c}	0.9 ± 0.03^{d}	4.3 ± 0.5
Ser[8]-GLP-1 (7–36) amide	3.7 ± 0.4^{ab}	4.2 ± 0.2^{c}	0.9 ± 0.06^{d}	4.1 ± 0.2
Aib[8]-GLP-1 (7–36) amide	NDL	4.4 ± 0.2^{c}	1.1 ± 0.07^{d}	4.3 ± 0.3

Values are mean ± SEM; $n = 4$
[a] NS, $p > 0.05$ vs C-terminal $t_{1/2}$ for analogue; [b] $p < 0.01$ vs N-terminal $t_{1/2}$ for GLP-1 (7–36) amide; [c] NS, $p > 0.05$ vs C-terminal $t_{1/2}$ for GLP-1 (7–36) amide; [d] $p < 0.01$ vs C-terminal $t_{1/2}$ for GLP-1 (7–36) amide; NDL, not determined due to lack of cross-reactivity

Table 3. Receptor binding affinities of GLP-1 (7–36) amide and N-terminally modified analogues in baby hamster kidney cells expressing the human pancreatic GLP-1 receptor

Peptide	Binding affinity (IC_{50}, nmol/l)	n
GLP-1 (7–36) amide	0.78 ± 0.29	8
Thr[8]-GLP-1 (7–37)	49 ± 3.7^{a}	4
Gly[8]-GLP-1 (7–37)	2.8 ± 0.42^{b}	4
Ser[8]-GLP-1 (7–36) amide	9.0 ± 1.9^{a}	3
Aib[8]-GLP-1 (7–37)	0.45 ± 0.05^{b}	3

Values are mean ± SD of separate triplicate experiments.
[a] $p < 0.001$ vs GLP-1 (7–36) amide; [b] NS, $p > 0.05$ vs GLP-1 (7–36) amide

logues in raising insulin output. It was also the most potent ($p < 0.05$) of all the analogues in reducing glucagon output. The Gly[8] analogue was not significantly different to GLP-1 (7–36) amide in stimulating insulin or inhibiting glucagon secretion, but was more potent ($p < 0.05$) than the Ser[8] and Thr[8] analogues in inhibiting glucagon release, while the Thr[8] analogue was the least potent of the analogues tested.

Discussion

This study has demonstrated that small alterations in the N-terminus of GLP-1 confer resistance to the action of the enzyme DPP IV. Such analogues retain biological activity, and have an improved metabolic stability.

In incubations with human plasma in vitro, DPP IV is the main enzyme responsible for GLP-1 degradation [12, 13], and a thorough study by Pauly et al. [31] concluded that only minor secondary degradation could be attributed to other serum proteases. Accordingly, the in vitro $t_{1/2}$ of the GLP-1 analogues were considerably extended relative to GLP-1 (7–36) amide. There was no detectable degradation of the Aib[8] analogue, and the slow degradation of the other three analogues could reflect the substrate specificity of DPP IV, as was found for GRF; substitution of the alanine in position 2 of modified GRF analogues with either serine or threonine reduced the
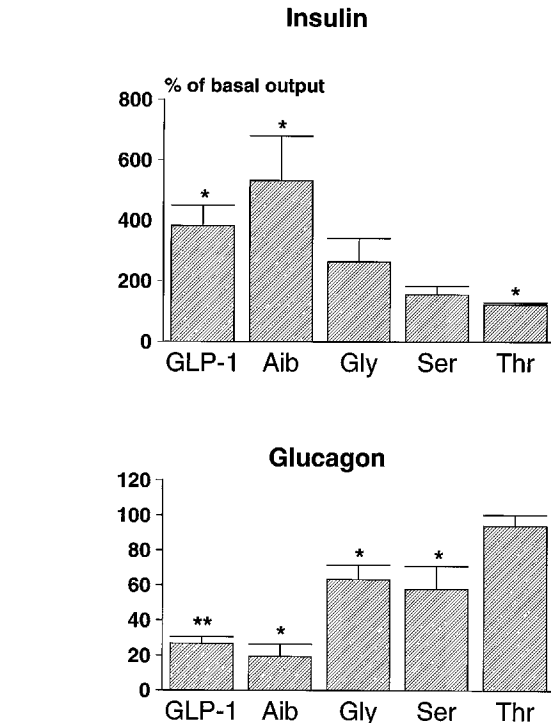
Fig. 3. Insulin and glucagon secretion by the isolated perfused porcine pancreas during infusion of 1 nmol/l GLP-1 (7–36) amide or N-terminally modified analogues. Data are expressed as a percentage of basal output (defined as 100 %), and are mean ± SEM of 4 experiments, except for the glucagon output during GLP-1 (7–36) amide infusion where $n = 3$.
$* p < 0.05$; $** p < 0.01$ compared to basal output

cleavage rates to less than 5 %, of that of the Ala[2] analogue [20]. That DPP IV is also the main enzyme responsible for in vivo N-terminal degradation of GLP-1 is illustrated by the increased proportions of N-terminal immunoreactivity (reflecting intact peptide) seen during i.v. infusion of each analogue relative to GLP-1 itself, and confirmed by HPLC analysis which revealed only two immunoreactive peaks corresponding to the intact peptide and the N-terminally truncated metabolite. In these in vivo studies, analogue N-terminal $t_{1/2}$ were prolonged and equalled the C-terminal $t_{1/2}$, but it is noteworthy that they could not be extended beyond those determined by

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