Novel GLP-1 analogue (Val⁸)GLP-1 results in significant improvements of glucose tolerance and pancreatic beta cell function after 3 weeks daily administration in obese diabetic (ob/ob) mice.

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a) **Running Title:** Long-term effects of (Val\textsuperscript{8})GLP-1 in *ob/ob* mice.

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ABSTRACT

Aims/hypothesis: This study evaluates the antidiabetic potential of an enzyme resistant analogue, (Val^8)GLP-1. Methods: The effects of daily administration of a novel DPP IV resistant GLP-1 analogue, (Val^8)GLP-1, on glucose tolerance and pancreatic beta cell function were examined in obese-diabetic ob/ob mice. Results: Acute intraperitoneal administration of (Val^8)GLP-1 (6.25-25 nmoles/kg) with glucose increased the insulin response and reduced the glycaemic excursion in a dose-dependent manner. The effects of (Val^8)GLP-1 were greater and longer-lasting than native GLP-1. Once-daily subcutaneous administration of (Val^8)GLP-1 (25 nmoles/kg) for 21 days reduced plasma glucose concentrations, increased plasma insulin and reduced body weight more than native GLP-1 without a significant change in daily food intake. Furthermore, (Val^8)GLP-1 improved glucose tolerance, reduced the glycaemic excursion after feeding, increased the plasma insulin response to glucose and feeding, and improved insulin sensitivity. These effects were consistently greater with (Val^8)GLP-1 than native GLP-1, and both peptides retained or increased their acute efficacy compared with initial administration. (Val^8)GLP-1 treatment increased average islet area 1.2-fold without changing the number of islets, resulting in an increased number of larger islets. Conclusions/interpretation: These data demonstrate that (Val^8)GLP-1 is more effective and longer-acting than native GLP-1 in obese-diabetic ob/ob mice.
INTRODUCTION

Glucagon-like peptide-1 (GLP-1) is a gut-derived incretin hormone which lowers circulating glucose levels post-prandially. Importantly the action of GLP-1 is glucose-dependent avoiding the occurrence of hypoglycaemia (Drucker 2003; Nauck et al., 2003; Green et al. 2004a; Vilsboll and Holst, 2004; Deacon, 2004). Glucose-lowering induced by GLP-1 appears to be mediated by a potent insulin-releasing action, as well as, a range of other effects including inhibition of glucagon secretion and gastric emptying, increased satiety, stimulation of glucose-uptake and glyconeogenesis, (Villanueva-Penacarillo et al. 1994; Turton et al. 1996; Zander et al. 2002; Green et al. 2004a; Vilsboll and Holst, 2004). GLP-1 also appears to exert trophic effects on the beta-cell, stimulating growth and differentiation and inhibiting cytokine- and FFA- and STZ-mediated apoptosis (Buteau et al. 1999; Zhou et al. 1999; Liu et al. 2004; Bregenholt et al. 2005).

In view of these attributes GLP-1 is now the focus of pharmaceutical industry’s attention. The duration of action of GLP-1 (t½ 2-3min) is limited by inactivation due to N-terminal degradation by the enzyme dipeptidyl peptidase IV (DPP IV) (Deacon et al. 1995). DPP IV is a ubiquitous cell surface and circulating enzyme found in large amounts at the brush-border of kidney epithelium. Two main intervention strategies are under development to prevent degradation of GLP-1: specific inhibitors of DPP IV, and subtle modifications of the GLP-1 molecule to generate analogues that are resistant to DPP IV. We have modified the N-terminus of GLP-1 to generate a family of novel DPP IV-resistant analogues (Green et al. 2003, 2004a). (Val8)GLP-1 is a GLP-1 analogue with profound resistance to DPP IV, and greater biological activity than other N-terminally modified analogues and native GLP-1 (Green et al. 2003).
Acute administration of (Val\textsuperscript{8})GLP-1, in combination with glucose, showed similar insulin-releasing activity to native GLP-1 and greater glucose-lowering than GLP-1. The more potent antihyperglycaemic activity of (Val\textsuperscript{8})GLP-1 may therefore relate to other beneficial actions such as inhibition of glucagon secretion or extrapancreatic effects (Fehmann et al. 1995).

While structural modification of GLP-1 may overcome degradation by DPP IV, this does not address the loss of GLP-1 by renal filtration (Meier et al. 2004). Ourselves and others have attempted to prevent renal filtration of GLP-1 by acylation (attaching long-chain fatty acid molecules) (Green et al. 2004b). Acylating peptides facilitates binding to plasma proteins, such as albumin, thereby minimising their elimination by the kidney. LY315902 (Eli Lilly), for example, is an acylated GLP-1 analogue with an octanoyl fatty acid chain (Holz and Chepurney, 2003). NN2211 (Liraglutide, NovoNordisk) contains a hexanoyl fatty acid group attached to the \(\varepsilon\)-amino group of Lys\textsuperscript{26} (Holz and Chepurney, 2003) and CJC-1131 (Conjuchem) contains a reactive chemical linker attached to the \(\varepsilon\)-amino group of Lys\textsuperscript{34} (Holz and Chepurney, 2003). NN2211 and CJC-1131 show sustained activities and half-lives greatly in excess of 8 hours. Other attempts to acylate GLP-1 with palmitate (18 carbon fatty acid) produce analogues with moderately prolonged activities, but with greatly reduced bioavailability (Green et al. 2004b). Albugon, is a recombinant GLP-1-albumin protein which decreases the glycaemic excursion in mice, but has a reduced ability to activate the GLP-1 receptor (Baggio et al. 2004).
Since (Val\(^8\))GLP-1 differs by one amino acid from physiological form of mammalian
GLP-1 and exhibits increased stability and acute biological activity, it offers particular
promise for therapeutic use. However, whether this translates to improved duration of
action and tangible metabolic long-term benefits in type 2 diabetes remains to be
evaluated. In this study, we assessed the magnitude of (Val\(^8\))GLP-1’s glucose-
lowering and insulin-releasing actions compared with GLP-1. Furthermore, we also
describe the effects of 21 days long-term daily administration of (Val\(^8\))GLP-1 on
feeding activity, body weight, basal glucose and insulin concentrations, glucose
tolerance, pancreatic beta-cell function, insulin sensitivity and islet morphology of
obese diabetic (\(ob/ob\)) mice, a commonly employed animal model of non-insulin
dependent diabetes.

**METHODS**

**Reagents**

Na\(^{125}\)I for iodination of insulin was obtained from Amersham (Buckinghamshire,
UK). Bovine insulin, dextran-T70 and activated charcoal, were obtained from Sigma
(Poole, Dorset, UK). All other chemicals were of the highest purity available.
(Val\(^8\))GLP-1 and GLP-1 were synthesised, purified and characterised as described
previously (Green et al. 2004b). Briefly, (Val\(^8\))GLP-1 and GLP-1 were sequentially
synthesised on an Applied Biosystems automated peptide synthesiser (Model 432A)
using standard solid-phase Fmoc peptide chemistry. They were judged to be >99%
pure by reversed-phase high-performance liquid chromatography (HPLC) using a
Waters Millenium (Milford, MA) 2010 chromatography system. Peptide structures
were confirmed using electrospray ionisation-mass spectrometry (ESI-MS), as
described previously (Green et al. 2004b).
Animals

The genetic background and characteristics of the ob/ob colony have been described elsewhere (Bailey and Flatt, 1995). Males, aged 15-19 weeks were housed individually in an air-conditioned room at 22 ± 2 °C with a 12 h light (06:00-18:00): 12 h dark cycle (18:00-06:00). Drinking water and a standard rodent maintenance diet (Trouw Nutrition Ltd., Belfast, UK) were freely available. All animal experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986. No adverse effects were observed following administration of any of the peptides.

Evaluation of acute and long-term effects of (Val^{8})GLP-1 and native GLP-1 in ob/ob mice.

Initial experiments were performed to evaluate the acute dose-dependent glucose-lowering and insulin-releasing effects of (Val^{8})GLP-1 and GLP-1 when administered by intraperitoneal injection to 18h fasted ob/ob mice together with glucose (18mmoles/kg). On the basis of results obtained subsequent studies were all conducted with a peptide dose of 25 nmoles/kg. To demonstrate the longer term duration of action of (Val^{8})GLP-1, glucose tolerance (18 mmoles/kg) and insulin release were assessed 4 h after administration of (Val^{8})GLP-1, GLP-1(25nmol/kg) or saline.

For long-term studies separate groups of ob/ob mice received once daily subcutaneous injections (17:00h) of either (Val^{8})GLP-1, GLP-1 (25 nmol/kg in saline) or saline (0.9%, w/v, NaCl) over a 21-day period. Prior to the 21-day treatment period animals were stratified so that all groups were of similar age, body weight and diabetes status.
as judged by non-fasting plasma glucose concentration. Food intake and body weight were recorded daily. Blood samples were collected on days 0, 1, 3, 7, 11, 14 and 20 (09:00h) from the cut tail tip of conscious fed mice. Glucose tolerance (18 mmol/kg, intraperitoneally), meal tolerance (15 min refeeding after 18 h fast), peptide response (25 nmol/kg peptide with 18 mmol/kg glucose, intraperitoneally) and insulin sensitivity (50 U/kg insulin, intraperitoneally) tests were conducted on day 21. Procedures were commenced between 09:00-10:00h. For all experiments, blood samples were collected at the times indicated in the figures into chilled fluoride/heparin coated microcentrifuge tubes (Sarstedt, Numbrecht, Germany) and centrifuged (30 s at 13,000 g) using a Beckman microcentrifuge (Beckman Instruments, UK). The resulting plasma was then aliquoted into fresh eppendorf tubes and stored at -20°C prior to glucose and insulin analysis.

Immunohistochemistry

At the end of the experimental period, islet morphology was evaluated in 4 mice from each group. Tissue fixed in 4% paraformaldehyde/PBS (phosphate buffered saline) and embedded in paraffin was sectioned at 8 μm. After de-waxing, sections were incubated with blocking serum (Vector Laboratories, CA, USA) prior to exposure to insulin antibody. Tissue samples were then incubated consecutively with secondary biotinylated universal, pan-specific antibody (Vector Laboratories, CA, USA) and streptavidin/peroxidase preformed complex (Vector Laboratories, CA, USA). Following washing, the stained pancreatic tissue was counterstained with hematoxylin (BDH Chemicals, Dorset, UK) and then washed in acid methanol (500 ml methanol, 500 ml H2O and 2.5 ml concentrated HCl) prior to dehydration and mounting in Depex (BDH Chemicals, Dorset, UK). The stained slides were viewed under a
microscope (Nikon Eclipse E2000, Diagnostic Instruments Incorporated, Michigan, USA) attached to a JVC camera Model KY-F55B (JVC, London, UK) and analyzed using Kromoscan imaging software (Kinetic Imaging Limited, Faversham, Kent, UK). The average number and area of islets in each section were estimated in a blinded manner using ImageJ software (NIH) (Abramoff et al. 2004) calibrated with a stage micrometer (Graticules Limited, Tonbridge, Kent, UK). Approximately 60-70 random sections were examined from the pancreas of each mouse.

Analyses

Plasma glucose was assayed by an automated glucose oxidase procedure using a Beckman Glucose Analyser II. Plasma insulin was assayed by a modified dextran charcoal radioimmunoassay (Flatt and Bailey, 1981). Results were expressed as means ± SEM. Plasma glucose, plasma insulin values and islet area data were compared using the unpaired Student’s t-test. ∆AUC0-60 values data were compared using repeated measures one-way Analysis of Variation (ANOVA) followed by the Student-Newman-Keuls post hoc test. Incremental areas under plasma glucose and insulin curves (∆AUC0-60) were calculated using a computer-generated program employing the trapezoidal rule (Burington, 1973) with baseline subtraction. Groups of data were considered to be significantly different if P<0.05. It should be noted that in most cases these parameters did not regain baseline values by the end of the test period. Indeed, in separate glucose tolerance tests plasma glucose concentrations of untreated ob/ob mice only returned to baseline values at approximately 240 min (Figure 1). Routine, extension of the observation period in other tests was not possible.
because there is a limit on the number of sequential blood samples that could be taken for glucose and insulin analyses in each mouse.

RESULTS

Acute dose-dependent glucose-lowering and insulin-releasing effects of (Val$^8$)GLP-1 in ob/ob mice

(Val$^8$)GLP-1 or native GLP-1 (both at doses of 6.25, 12.5 or 25 nmoles/kg) were administered intraperitoneally with glucose (18 mmoles/kg) and metabolic responses were monitored (Figure 2). At all doses tested (Val$^8$)GLP-1 the glycaemic excursions 30-60 min post-injection were lowered significantly more by (Val$^8$)GLP-1 than native GLP-1 (P<0.05-P<0.001; Figure 2A). Plasma glucose levels Analysis by area under the curve (\(\Delta\text{AUC}_{0-60}\)) confirmed this and revealed that a 6.25 nmoles/kg (Val$^8$)GLP-1 dose was more potent than 25 nmoles/kg native GLP-1 (P<0.001; Figure 2). At doses of 12.5 and 25 nmoles/kg (Val$^8$)GLP-1 was more effective than native GLP-1 in augmenting insulin release (P<0.05-0.01; Figure 2) despite lower glucose concentrations.

Long-lasting biological actions of (Val$^8$)GLP-1 in ob/ob mice

(Val$^8$)GLP-1 was evaluated for long-lasting metabolic actions after single dose injection. As shown in Figure 3, the glucose-lowering and insulin-releasing effects of (Val$^8$)GLP-1 were clearly evident when given 4h before administration of an intraperitoneal glucose load. Glucose values were decreased by 21% and 22% at 30 min and 60 min, respectively compared with saline-treated controls (P<0.05; Figure 3). Corresponding insulin concentrations were increased by 22% at 60min (P<0.05).
In contrast, native GLP-1 lacked significant effects on glucose and insulin values when given 4h before the glucose load (Figure 3).

Long-term effects of (Val$^8$)GLP-1 in ob/ob mice

Figure 4 shows the effects of daily administration of (Val$^8$)GLP-1, GLP-1 or saline on body weight, food intake and non-fasting plasma concentrations of glucose and insulin in ob/ob mice. GLP-1 had no significant effect on body weight or food intake. However, mice treated with (Val$^8$)GLP-1 displayed significantly reduced body weights by day 16 (P<0.05) without a significant change in food intake. Over the 21 day period, plasma glucose levels of ob/ob mice treated with saline ranged from 21 ± 3 mmol/l to 25 ± 1 mmol/l. Mice chronically treated with (Val$^8$)GLP-1 had significantly lower plasma glucose levels after 18 days (P<0.05) and elevated insulin levels after 9 days of treatment (P<0.01). Glucose and insulin levels in GLP-1-treated mice did not differ from saline-treated mice on any of the days tested.

Long-term effects of (Val$^8$)GLP-1 on glucose tolerance in ob/ob mice

Figure 5 shows the effects of intraperitoneal glucose (18 mmol/kg) on glucose and insulin concentrations of ob/ob mice treated for 21 days with either (Val$^8$)GLP-1, GLP-1 or saline. Treatment with (Val$^8$)GLP-1 significantly lowered plasma glucose levels from 15 min onwards compared with saline-treated controls (P<0.01), mice treated with GLP-1 showed lower plasma glucose levels from 30 min (P<0.05). Treatment with (Val$^8$)GLP-1 also significantly increased insulin responses to glucose at 15 and 30 min after injection (P<0.01). Insulin responses in GLP-1-treated mice did not differ significantly from those of saline-treated mice.
Long-term effects of (Val\textsuperscript{8})GLP-1 on insulin sensitivity in ob/ob mice

Figure 6 shows the effect of intraperitoneal insulin (50 U/kg) on glucose concentrations in 21 day treated mice. (Val\textsuperscript{8})GLP-1 treatment resulted in significantly lower plasma glucose levels 30 min after insulin injection (P<0.01). The glucose ∆AUC\textsubscript{0-60} value was also significantly decreased (437 ± 111 versus 238 ± 51 mmol/l x min; P<0.001). GLP-1 treatment had no significant effect on insulin-induced glucose-lowering.

Long-term effects of (Val\textsuperscript{8})GLP-1 on metabolic response to feeding in ob/ob mice

Figure 7 shows the glucose and insulin responses of ob/ob mice to refeeding following 21 days treatment with (Val\textsuperscript{8})GLP-1 and GLP-1. Glucose responses in (Val\textsuperscript{8})GLP-1-treated mice were significantly lower than saline-treated mice 15 and 30 min post-feeding (P<0.05). Insulin concentrations were not significantly different at individual time points but the ∆AUC\textsubscript{0-60} value (Val\textsuperscript{8})GLP-1-treated mice was 36% greater (P<0.05) than in saline-treated controls (765 ± 60 versus 564 ± 60 ng/ml x min). The glycaemic and insulin responses were not changed by GLP-1 treatment. Food intake of (Val\textsuperscript{8})GLP-1-treated (0.8 ± 0.1g/mouse/15 min), GLP-1-treated (0.8 ± 0.1g/mouse/15 min) and saline-treated (0.7 ± 0.1g/mouse/15 min) mice were not significantly different.

Long-term effects of (Val\textsuperscript{8})GLP-1 on peptide response in ob/ob mice

Figure 8 shows the effects of intraperitoneal administration of glucose alone or in combination with peptide (GLP-1 or (Val\textsuperscript{8})GLP-1) in mice treated with (Val\textsuperscript{8})GLP-1, GLP-1 or saline for 21 days. Significant glucose-lowering and insulin-releasing actions of both GLP-1 peptides were preserved following treatment with (Val\textsuperscript{8})GLP-1.
or GLP-1 (Figure 8). However, mice treated with (Val\textsuperscript{8})GLP-1 exhibited lower glucose and elevated insulin concentrations compared with saline-treated mice (P<0.01). Overall glucose and insulin responses estimated from ∆\text{AUC}_{0-60} values were also significantly different (P<0.05) between (Val\textsuperscript{8})GLP-1 (65 ± 15 mmol/l x min, 1692 ± 104 ng/ml x min) and GLP-1 (286 ± 50 mmol/l x min, 1306 ± 80 ng/ml x min) treated groups.

**Long-term effects of (Val\textsuperscript{8})GLP-1 on islet morphology**

Figure 9 shows the effects 21 days treatment with (Val\textsuperscript{8})GLP-1, GLP-1 or saline on the area, number and morphology of pancreatic islets. The average islet area of mice treated with (Val\textsuperscript{8})GLP-1 increased 1.2-fold (P<0.01) compared with saline. Whilst (Val\textsuperscript{8})GLP-1 treatment did not change the number of islets per pancreas section, the proportion of small islets (<0.075 mm\textsuperscript{2}) was reduced (P<0.01) and the proportion of medium (0.075-0.15 mm\textsuperscript{2}) and large (>0.15 mm\textsuperscript{2}) islets was increased (Figure 9; P<0.01). No changes in islet area or number were observed in mice treated with GLP-1 but the proportion of large islets (>0.15 mm\textsuperscript{2}) was decreased (P<0.01).

**DISCUSSION**

Although GLP-1 is inactivated rapidly by dipeptidyl peptidase IV (DPP IV), synthetic analogues of GLP-1 have been designed which reproduce the biological actions of GLP-1 and are resistant to DPP IV degradation. As reviewed recently, numerous GLP-1 analogues have been produced with varying degrees of resistance to DPP IV and varying biological activities (Green et al. 2004a; Holz & Chepurny, 2003). (Val\textsuperscript{8})GLP-1 is a novel N-terminally modified GLP-1 analogue displaying profound
resistance to DPP IV degradation (Green et al. 2003). No degradation fragments were detected when (Val$^8$)GLP-1 was incubated for up to 12 h in purified DPP IV or pooled human plasma (Green et al. 2003). The replacement of alanine with the larger valine residue appears to cause sufficient steric hinderance to diminish the susceptibility of GLP-1 to DPP IV-mediated degradation, but this change is sufficiently subtle retain the biological activities of GLP-1 at the cellular level (Green et al. 2003). In vitro (Val$^8$)GLP-1 has similar cAMP stimulating and insulin releasing activity to another position 8 substituted analogue, (Abu$^8$)GLP-1, but in vivo demonstrated a significantly greater ability to lower glucose (Green et al. 2003). Analogues of GLP-1 developed by modification of His$^7$ are stable to DPP IV but suffer losses in biological potency (Green et al. 2004a).

Acute in vivo effects of (Val$^8$)GLP-1 were compared with native GLP-1 using obese diabetic (ob/ob) mice. This spontaneous model of obesity and diabetes is devoid of biologically active leptin and characterised by hyperphagia, obesity, hyperglycaemia, defective beta cell function and severe insulin resistance (Bailey et al. 1982). Consistent with previous observations (Green et al. 2003), acute administration native GLP-1 together with glucose augmented insulin release but had minimal effects on the glucose excursion of ob/ob mice. (Val$^8$)GLP-1 produced a greater increase in glucose mediated insulin release and lowered the glucose values up to 70% more than GLP-1. In contrast to an earlier report (Green et al. 2004b), these more detailed observations also demonstrated a greater in vivo insulin-releasing activity of (Val$^8$)GLP-1. Most notably in the present study, the glucose-lowering potency of 6.25 nmoles/kg (Val$^8$)GLP-1 given acutely was superior to a 4 times greater dose of native GLP-1. Further, the effect persisted for more than 4 hours after injection,
indicating an extended duration of action of (Val⁸)GLP-1. Since (Val⁸)GLP-1 and GLP-1 can be expected to be equally susceptible to renal filtration this suggests that it is degradation by DPP IV and not renal filtration which immediately curtails hormone action. Interestingly, the glucose-lowering activity of (Val⁸)GLP-1 was accompanied by relatively modest increases in insulin concentrations. This could indicate that (Val⁸)GLP-1 may also possess prolonged/enhanced effects on glucose-lowering mechanisms, in particular inhibition of glucagon secretion and stimulation of glucose-uptake and glyconeogenesis.

The major interest of the present study concerns evaluation of how the stability of (Val⁸)GLP-1 translates to improved diabetes control following long-term once daily injection. Administration of (Val⁸)GLP-1 to adult ob/ob mice (25 nmoles/kg/day) for 21 days resulted in progressive elevation of plasma insulin and a lowering of basal glucose concentrations. Body weight also declined but this was not matched with any measurable change in food intake. Whilst there is evidence that GLP-1 can reduce feeding (Turton et al. 1996; Flint et al. 2000) this is not a consistent finding (Thiele et al 1997). It is possible that small differences in meal pattern, physical activity or enhanced metabolic efficiency may have been missed by the current study. Interestingly, studies in db/db mice showed that the GLP-1 analogue, exendin-4 (exenatide) also decreased body weight after 10 weeks and lowered both fasting glucose and insulin concentrations (Greig et al. 1999). In clinical studies, in type 2 diabetic patients, chronic treatment with exendin-4 improved glycaemic control with a reduction in body weight (DeFronzo et al. 2005).
Evaluation of the spectrum of antidiabetic effects of 21 days treatment of ob/ob mice with (Val8)GLP-1 revealed substantial improvements of glucose tolerance and glycaemic responses to feeding. This can be attributed in part to considerable improvements of beta cell responsiveness. Islet number was not affected, arguing against stimulation of neogenesis over the time period studied. However, administration of (Val8)GLP-1 modestly enhanced islet area by increasing the proportion of larger islets in the pancreas suggesting increased beta-cell numbers. This accords to observations with exendin-4 in db/db mice (Greig et al. 1999; Young et al. 1999) and the reported ability of GLP-1 to stimulate beta cell replication and inhibit apoptosis (Zander et al. 2002; Buteau et al. 1999). However, insulin sensitivity was also significantly improved by (Val8)GLP-1 administration. This may be due in part to reduced glucotoxicity indicated by the consistently lower glucose concentrations, but also may be due to the reduction in bodyweight imparted by (Val8)GLP-1 treatment. However, in contrast to (Val8)GLP-1, administration of native GLP-1 resulted in little change to insulin sensitivity, although improvement of glucose tolerance was significant.

The observation of significant improvements in glucose homeostasis and beta-cell function of ob/ob mice treated with (Val8)GLP-1 for 21 days suggests that the GLP-1 receptor is not down-regulated by prolonged exposure to the peptide. Consistent with this view, acute administration of (Val8)GLP-1 together with glucose retained ability to moderate the glycaemic excursion and enhance insulin secretion. Indeed, these attributes of (Val8)GLP-1 given acutely were slightly enhanced after 21 days of treatment. This appears to reflect enhanced insulin sensitivity and improved beta cell responsiveness. Similarly long-term treatment with GLP-1 improved the glycaemic
excursion when the peptide was administered acutely with glucose, although there was no further improvement to the insulin response. The extent to which this reflects possible long-term exposure to the truncated metabolite GLP-1(9-36)amide that acts as a weak antagonist at the GLP-1 receptor (Green et al. 2004c) is unknown. However, daily administration of the GLP-1 receptor antagonist, exendin(9-39)amide mildly impaired glucose homeostasis in normal mice due to changes of insulin secretion (Green et al. 2005). It remains to be seen whether DPP IV processing of GLP-1 to this degradation fragment results in any alterations of metabolism in obese-diabetic ob/ob mice.

It is possible that small differences in meal pattern, physical activity or enhanced metabolic efficiency may have been missed by the current study. Long-acting GLP-1 analogues and exendin(1-39) can produce nausea and taste aversion (Thiele et al. 1997; Mark, 2003; Green et al. 2004). It is not known whether (Val^8)GLP-1 has such effects, but no adverse effects were noted during the 21 day treatment period.

In conclusion, this study shows that long-term treatment with (Val^8)GLP-1 was associated with significant acute and long-term antidiabetic actions in ob/ob mice. Further development of stable long-acting GLP-1 analogues, such as (Val^8)GLP-1, promises to provide new effective agents for diabetes therapy.
REFERENCES


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). *Metabolism* **53**:252-259


FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. Plasma glucose concentrations over 4h period following administration of glucose alone or together with (Val^8)GLP-1 or GLP-1 in 18h fasted ob/ob mice. Plasma glucose concentrations were measured prior to and at intervals after intraperitoneal injection of glucose (18 mmoles/kg) alone or in combination with 25 nmoles/kg (Val^8)GLP-1 or native GLP-1. The time of injection is indicated by the arrow. Values are mean ± SEM for groups of 6 mice. *P<0.05 **P<0.01 and ***P<0.001 compared to mice receiving glucose alone.

Figure 2. Acute glucose-lowering and insulin-releasing effects of (Val^8)GLP-1 and GLP-1 in 18h fasted ob/ob mice. (A) Plasma glucose and (B) plasma insulin concentrations were measured prior to and at intervals after intraperitoneal injection of glucose (18 mmoles/kg) alone or in combination with 6.25, 12.5 or 25 nmoles/kg (Val^8)GLP-1 or native GLP-1. The time of injection is indicated by the arrows. Glucose and insulin ∆AUC_{0-60} values for 0-60 min post injection are shown in the lower panels. Values are mean ± SEM for groups of 8 mice. *P<0.05 **P<0.01 and ***P<0.001 compared to mice receiving a similar dose of native GLP-1. ∆P<0.05, ∆∆P<0.01 and ∆∆∆P<0.001 compared to GLP-1 (25 nmoles/kg).

Figure 3. Persistence of glucose-lowering and insulin-releasing effects of (Val^8)GLP-1 in 18h fasted ob/ob mice. (A) Plasma glucose and (B) plasma insulin were measured prior to and after ip administration of glucose (18mmol/kg body weight) in mice injected 4h previously with (Val^8)GLP-1, GLP-1 (25nmol/kg body weight, ip) or saline. Injection times are indicated by the arrows. Values are means ± SEM for groups of 6 mice. *P<0.05 compared mice injected 4 h earlier with saline.
Figure 4. Food intake, body weight, plasma glucose and insulin concentrations of \(ob/ob\) mice receiving 21 daily injections of either (Val\(^8\))GLP-1, GLP-1 or saline. (A) Body weight (B) food intake, (C) non-fasting plasma glucose and (D) insulin concentrations were measured on days 0, 1, 3, 7, 11, 14 and 20 (09:00h) during (indicated by black box) treatment with (Val\(^8\))GLP-1, GLP-1 or saline (25nmol/kg body weight). Values are mean ± SEM for groups of 8 mice. *\(P<0.05\) and **\(P<0.01\) compared with saline.

Figure 5. Effects of long-term treatment with (Val\(^8\))GLP-1 or GLP-1 on glucose and insulin responses to intraperitoneal glucose in \(ob/ob\) mice. (A) Plasma glucose and (B) plasma insulin concentrations were measured prior to and at intervals after intraperitoneal administration of glucose (18mmol/kg body weight) following 21 days treatment with (Val\(^8\))GLP-1 or GLP-1. Time of injection is indicated by arrow. Values are mean ± SEM for groups of 8 mice. *\(P<0.05\) and **\(P<0.01\), ***\(P<0.001\) compared with saline. \(\Delta\Delta P<0.01\) compared with GLP-1.

Figure 6. Insulin sensitivity of \(ob/ob\) mice following 21 days treatment with (Val\(^8\))GLP-1 or GLP-1. Plasma glucose concentrations were measured prior to and at intervals after intraperitoneal administration of insulin (50U/kg body weight). Tests were conducted following 21 day treatment with (Val\(^8\))GLP-1, GLP-1 or saline. Values are mean ± SEM for groups of 8 mice. **\(P<0.01\) compared with saline.

Figure 7. Effects of long-term treatment with (Val\(^8\))GLP-1 or GLP-1 on glucose and insulin responses to feeding in \(ob/ob\) mice. Following the 21-day treatment period mice were fasted (18 h) overnight. At 09:00 free access to food was allowed.
for 15 min (indicated by the black bar) and (A) plasma glucose and (B) plasma insulin concentrations were measured. Values are mean ± SEM for groups of 8 mice. *P<0.05 compared with saline.

Figure 8. Glucose lowering and insulin releasing effects of (Val^8)GLP-1 and GLP-1 following daily peptide treatment of ob/ob mice for 21 days. (A) Plasma glucose and (B) plasma insulin concentrations were measured prior to and at intervals after intraperitoneal injection of glucose alone, or glucose in combination with either (Val^8)GLP-1 or GLP-1 after 21 days of treatment with (Val^8)GLP-1, GLP-1 or saline. Time of injection is indicated by arrow. Values are mean ± SEM for groups of 8 mice. *P<0.05 **P<0.01 compared with saline.

Figure 9. Effects of daily administration of (Val^8)GLP-1, GLP-1 or saline on islet size, number and morphology in ob/ob mice. Parameters were measured after daily treatment with (Val^8)GLP-1, GLP-1 (25 nmoles/kg body weight/day) or saline for 21 days. Graph are: (A) a scatter plot of the individual areas of islets and (B) mean islet area in mm^2. (C) Graph shows the mean number of islets observed per slide section. (D) Percentage of islets with areas classified as > 0.15 mm^2, 0.075 – 0.15 mm^2 and < 0.075 mm^2 are shown. Values are mean ± SEM for groups of 8 mice. *P<0.05 and **P<0.01 compared with saline-treated mice.
Figure 3A

Plasma glucose (mmol/l)

- SALINE
- SALINE + GLP-1
- SALINE + (VAL^8)GLP-1

GLUCOSE

Time (h) 0 1 2 3 4
Time (min) 0 15 30 45 60

*
Figure 3B

Plasma insulin (ng/ml)

- SALINE
- SALINE + GLP-1
- SALINE + (VAL₈)GLP-1

GLUCOSE

Time (h)

0 1 2 3 4

Time (min)

0 15 30 45 60

0 10 20 30 40 50
Figure 4A

- ■ SALINE
- □ GLP-1
- ○ (VAL^8)GLP-1

Body weight (g)

Time (days)

-5 0 5 10 15 20

70 80 90 100
Figure 4B

- SALINE
- GLP-1
- (VAL²)GLP-1

Food intake (g/day)

Time (days)
Figure 4C

- **SALINE**
- **GLP-1**
- **(VAL\(^8\))GLP-1**

Plasma glucose (mmol/l)

Time (days)
Figure 5A

Plasma glucose (mmol/l)

Time (min)

- SALINE
- GLP-1
- (Val^8)GLP-1

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Figure 6

Plasma glucose (mmol/l)

Time (min)

SALINE

GLP-1

(Val^8)GLP-1
Figure 7A

- **SALINE**
- **GLP-1**
- **(Val^8)GLP-1**

Plasma glucose (mmol/l)

Time (min)

0  15  30  45  60  75  90  105
Figure 7B

Plasma insulin (ng/ml)

SALINE
GLP-1
(Val^8)GLP-1

Time (min)
Figure 8A

Plasma glucose (mmol/l)

- **SALINE:** Glucose alone
- **GLP-1:** Glucose + GLP-1
- **(Val^8)GLP-1:** Glucose + (Val^8)GLP-1

Time (min)
Figure 8B

Plasma insulin (ng/ml)

- **SALINE:** Glucose alone
- **GLP-1:** Glucose + GLP-1
- **(Val^8)GLP-1:** Glucose + (Val^8)GLP-1

Time (min)
Figure 9B

The graph shows the islet area in mm² for different treatments: Saline, GLP-1, and (Val⁸)GLP-1. The data indicates that (Val⁸)GLP-1 has a significantly higher islet area compared to Saline and GLP-1, as indicated by the asterisks above the (Val⁸)GLP-1 bar.
Figure 9D

Percentage of islets

Saline  GLP-1  (Val^8)GLP-1

<0.075 mm^2
0.075-0.15 mm^2
>0.15 mm^2

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