A novel DPP IV-resistant C-terminally extended glucagon analogue exhibits weight-lowering and diabetes-protective effects in high-fat-fed mice mediated through glucagon and GLP-1 receptor activation

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Abstract

Aims/hypothesis Modification of the structure of glucagon could provide useful compounds for the potential treatment of obesity-related diabetes.

Methods This study evaluated *N*-acetyl-glucagon, (D-Ser²)glucagon and an analogue of (D-Ser²)glucagon with the addition of nine amino acids from the C-terminal of exendin(1-39), namely (D-Ser²)glucagon-exe.

Results All analogues were resistant to dipeptidyl peptidase IV degradation. *N*-Acetyl-glucagon lacked acute insulinotropic effects in BRIN BD11 cells, whereas (D-Ser²)glucagon and (D-Ser²)glucagon-exe evoked significant ($p<0.001$) insulin release. (D-Ser²)glucagon-exe stimulated cAMP production ($p<0.001$) in glucagon- and GLP-1-receptor (GLP-1R)-transfected cells but not in glucose-dependent insulinotropic polypeptide-receptor-transfected cells. In normal mice, *N*-acetyl-glucagon and (D-Ser²)glucagon retained glucagon-like effects of increasing ($p<0.001$) plasma glucose and insulin levels. (D-Ser²)glucagon-exe was devoid of hyperglycaemic actions but substantially ($p<0.001$) increased plasma insulin levels. (D-Ser²)glucagon-exe reduced the glycaemic excursion ($p<0.01$) and increased the insulin secretory ($p<0.01$) response following a glucose challenge 12 h after administration. Studies in GLP-1R knockout mice

confirmed involvement of the GLP-1R pathway in the biological actions of (D-Ser²)glucagon-exe. Twice-daily administration of (D-Ser²)glucagon-exe to high-fat-fed mice for 28 days significantly ($p<0.05$ to $p<0.001$) reduced body weight, energy intake and non-fasting glucose levels, as well as increasing insulin concentrations. Glucose tolerance and insulin sensitivity were significantly ($p<0.01$) improved and energy expenditure, O₂ consumption and locomotor activity were ($p<0.05$ to $p<0.001$) augmented. The metabolic benefits were accompanied by increases in pancreatic islet number ($p<0.001$) and area ($p<0.05$), as well as beta cell area ($p<0.05$). Beneficial effects were largely retained for 14 days following cessation of treatment.

Conclusions/interpretation This study emphasises the potential of (D-Ser²)glucagon-exe for the treatment of obesity-related diabetes.

Keywords Diabetes · GLP-1 · Glucagon · Glucagon-like peptide-1 · Glucose tolerance · Obesity

Abbreviations

DPP IV	Dipeptidyl peptidase IV
GIP	Glucose-dependent insulinotropic polypeptide
GIP-R	GIP receptor
GLP-1	Glucagon-like peptide-1
GLP-2	Glucagon-like peptide-2
GLP-1R	GLP-1 receptor
GLP-2R	GLP-2 receptor
Glucagon-R	Glucagon receptor
OXM	Oxyntomodulin
PC	Prohormone convertase
RER	Respiratory exchange ratio
RP-HPLC	Reverse-phase HPLC

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Introduction

The proglucagon gene is the source of a family of peptides that play an important role in the function of the gut and regulation of insulin secretion, glucose homeostasis and energy balance [1]. Secretion of proglucagon peptides was originally considered to be confined to pancreatic alpha cells and gut L-cells [2]; however, more recent studies suggest that the brain also actively secretes proglucagon-derived peptides [3]. A single gene encodes proglucagon in mammalian species and identical mRNAs are produced in the pancreas and the intestine [4]. Thus, differences in the proglucagon products in these tissues are due to tissue-specific, post-translational processing [5]. In this regard, the action of prohormone convertase (PC) 2 results in formation of glucagon, whereas PC 1/3 activity yields glucagon-like peptide-1 (GLP-1), glucagon-like peptide-2 (GLP-2) and oxyntomodulin (OXM) [6].

Glucagon is a hormone with well-characterised effects on glycaemic control and energy expenditure [7]. GLP-1 is the classical incretin hormone, increasing insulin secretion from pancreatic beta cells in a glucose-dependent manner [8]. GLP-1 also possesses a number of other beneficial diabetes-protective actions that include decreasing glucagon secretion and increasing satiety and insulin sensitivity [9]. This favourable biological action profile has been successful for the progression of a number of GLP-1-based compounds to the diabetes clinic [10]. Liberation of the related 33-amino-acid product GLP-2, by PC 1/3, yields a peptide with important positive biological actions on intestinal growth, bone breakdown and neuroprotection [6]. Interestingly, OXM is a peptide hormone that can increase insulin secretion and reduce body weight by dual activation of GLP-1 receptors (GLP-1Rs) and glucagon receptors (glucagon-Rs), respectively [11–13]. The relative affinity of OXM for GLP-1- and glucagon-Rs has not been fully verified, although studies do confirm that OXM has a much weaker affinity than the native peptides for these receptors [14]. Nonetheless, a dual effect is beneficial since activation of glucagon-Rs will induce catabolic effects that

favour weight loss, while GLP-1R agonism will modulate glucose homeostasis [15]. Thus, it follows that modification of the structure of glucagon could yield useful therapeutic agents for obesity-related diabetes.

Therefore, in the present study, we have generated *N*-acetyl-glucagon and (D-Ser²)glucagon—N-terminally modified peptide analogues of glucagon. Furthermore, to promote potential GLP-1R interactions, the nine C-terminal amino acid residues of exendin(1-39) were added to (D-Ser²)glucagon to create the novel compound (D-Ser²)glucagon-exe (Table 1). Here we establish the metabolic stability and in vitro insulin-releasing activity of all three analogues compared with native glucagon. We also demonstrate beneficial diabetes-protective actions of (D-Ser²)glucagon-exe, and confirm activation of the glucagon- and GLP-1Rs to be key in this respect. Finally, the in vivo therapeutic potential of (D-Ser²)glucagon-exe was assessed using obese high-fat-fed mice with prominent beneficial metabolic effects noted on glycaemic status, body weight control, insulin sensitivity, pancreatic morphology and overall energy balance. The results provide experimental evidence that enzyme-resistant (D-Ser²)glucagon-exe may provide an effective novel means of treating obesity-related diabetes.

Methods

Peptides Glucagon, *N*-acetyl-glucagon, (D-Ser²)glucagon and (D-Ser²)glucagon-exe were synthesised by GL Biochem (Shanghai, China). Amino acid sequences and molecular mass data are provided in Table 1. Observed molecular masses for all peptides corresponded closely to theoretical values, confirming structural identity.

Dipeptidyl peptidase IV stability The stability of peptides to degradation by dipeptidyl peptidase IV (DPP IV) was assessed as described previously [13]. Briefly, peptides (40 µg) were incubated at 37°C in 50 mmol/l triethanolamine-HCl buffer

Table 1 Amino acid sequence of peptides together with RP-HPLC retention times, MALDI-ToF MS and DPP IV stability

Peptide	Sequence	Retention time (min)	Experimental mass (Da)	Theoretical mass (Da)	Intact peptide after 8 h (%)
Glucagon	HSQGTFTSDYSKYLDSSRAQDFVQWLMNT	23.5	3,483.7	3,482.82	44
<i>N</i> -Acetyl-glucagon	Ac -HSQGTFTSDYSKYLDSSRAQDFVQWLMNT	25.4	3,524.4	3,524.6	100
(D-Ser ²)glucagon	H[D S]QGTFTSDYSKYLDSSRAQDFVQWLMNT	23.2	3,482.7	3,482.82	100
(D-Ser ²)glucagon-exe	H[D S]QGTFTSDYSKYLDSSRAQDFVQWLMNT PSSGAPPPS	35.5	4,259.9	4,260.66	100

Values represent the % intact parent peptide remaining relative to parent peptide present at 0 h as determined from HPLC peak area data

Peptides were purified by RP-HPLC and retention times recorded. Purified samples were subsequently mixed with α-cyano-4-hydroxy-1-cinnamic acid, applied to a sample plate of a Voyager-DE Biospectrometry Workstation and experimental *m/z* ratio vs relative peak intensity determined

Chemical modifications are denoted in bold (D represents the D-isomer)

MALDI-ToF, matrix-assisted laser desorption/ionisation–time of flight

(pH 7.8) containing 5 mU DPP IV in a final volume of 500 μ l. Enzymatic reactions were terminated by addition of trifluoroacetic acid/H₂O (10 μ l, 10% [vol./vol.]) and stored at -20°C . Reactions products were applied to a Phenomenex C-8 analytical column (250 $\times$ 4.60 mm) (Phenomenex, Macclesfield, UK) and intact peptide, together with any degradation fragments, was separated using reverse-phase (RP) HPLC. HPLC peak area data were determined by UV2000 UV/Vis Detector (Thermo Separation Products, Hemel Hempstead, UK) and the percentage of intact peptide remaining at 8 h was expressed relative to HPLC peak area at 0 h.

In vitro insulin secretion Clonal BRIN-BD11 cells were used to determine peptide-induced insulin release [16]. Cells were seeded into 24-well plates (Orange Scientific, Braine-l'Alleud, Belgium) at a density of 1.0×10^5 cells per well and cultured overnight at 37°C to form cell monolayers. Culture medium was removed and 1.0 ml KRB (115 mmol/l NaCl, 4.7 mmol/l KCl, 1.28 mmol/l CaCl₂, 1.2 mmol/l MgSO₄, 1.2 mmol/l KH₂PO₄, 25 mmol/l HEPES and 10 mmol/l NaHCO₃; pH 7.4 with NaOH), supplemented with 0.1% (wt/vol.) BSA and 1.1 mmol/l glucose, was added to each well. Cells were incubated for 40 min at 37°C , after which the buffer was removed and replaced with fresh KRB (1.0 ml) supplemented with 5.6 mmol/l glucose, alanine, glucagon or a range of peptide concentrations (10^{-12} to 10^{-6} mol/l), as shown in Fig. 1. After 20 min incubation at 37°C , the buffer was removed and stored at -20°C for subsequent measurement of insulin by radioimmunoassay [17].

In vitro cAMP production Chinese hamster lung (CHL) cells transfected with either the human glucose-dependent insulinotropic polypeptide (GIP) receptor (GIP-R) or human GLP-1R, as well as human embryonic kidney (HEK293) cells transfected with the human glucagon-R, were used to assess effects on cAMP production [18]. Cells were seeded (200,000 cells per well) into 96-well plates (Nunc, Fisher Scientific, Loughborough, UK) and washed with Hanks' balanced salt solution buffer before incubation with test peptides (10^{-12} and 10^{-6} μ mol/l) in the presence of 200 μ mol/l 3-isobutyl-1-methylxanthine for 20 min at 37°C . After incubation, medium was removed and cells were lysed before measurement of cAMP using Parameter cAMP assay (R&D Systems, Abingdon, UK) according to the manufacturer's instructions.

Animals Male NIH Swiss mice (8 weeks old; Harlan, Blackthorn, UK) were housed in an air-conditioned room at $22 \pm 2^{\circ}\text{C}$ with a 12 h light:12 h dark cycle (08:00–20:00 hours). The mice had free access to drinking water and either a standard chow diet (10% fat, 30% protein, 60% carbohydrate; percentage of total energy 12.99 kJ/g; Trouw Nutrition, Cheshire, UK) or a high-fat diet (45% fat, 20% protein and 35% carbohydrate; percentage of total energy of 26.15 kJ/g;

Fig. 1 Acute effects of *N*-acetyl-glucagon (**a**), (*D*-Ser²)glucagon (**b**) and (*D*-Ser²)glucagon-exe (**c**) on insulin secretion from BRIN-BD11 cells. Grey bars, 5.6 mmol/l glucose control; white bars, 20 mmol/l alanine or 10^{-7} mol/l glucagon; black bars, *N*-acetyl-glucagon (**a**), (*D*-Ser²)glucagon (**b**) or (*D*-Ser²)glucagon-exe (**c**). Values represent means $\pm$ SEM, $n=8$. *** $p<0.001$ compared with 5.6 mmol/l glucose alone; †† $p<0.01$ and ††† $p<0.001$ compared with 10^{-7} mol/l glucagon alone

Special Diets Service, Essex, UK) for 3 months, before commencement of experiments. Other experiments used 14-week-old wild-type C57BL/6J and GLP-1R knockout (GLP-1R KO) mice, on the same genetic background, originally derived

from breeding pairs kindly supplied by D. Drucker (Banting & Best Diabetes Centre, University of Toronto, Canada). These mice were fed standard chow diet ad libitum. All animal studies were performed in accordance with the UK Animals (Scientific Procedures) Act 1986. No adverse effects were observed following administration of any of the peptides.

Acute and persistent effects on plasma glucose and insulin responses For acute studies, mice were administered an intraperitoneal injection of saline vehicle (0.9% [wt/vol.] NaCl), glucagon or (D-Ser²)glucagon-exe (each at 25 nmol/kg body weight). Blood samples were taken immediately before ($t=0$) and at 15, 30 and 60 min post injection. In another series of experiments, (D-Ser²)glucagon-exe (25 nmol/kg) was injected 4, 8 or 12 h before an intraperitoneal challenge with glucose (18 mmol/kg). Blood samples were taken immediately before ($t=0$) and at 15, 30 and 60 min post glucose injection. Blood glucose was measured using an Ascencia Microfill Blood Glucose Meter (Bayer, Newbury, UK). Blood samples for plasma insulin analysis were collected into fluoride-coated microcentrifuge tubes (Sarstedt, Numbrecht, Germany) and centrifuged at 12,000 g at 4°C for 3 min using a Beckman microcentrifuge (Beckman Instruments, High Wycombe, UK). Resulting plasma samples were collected and stored at –20°C until determination of insulin [17].

Effects of twice-daily administration of (D-Ser²)glucagon-exe on metabolic variables High-fat-fed mice ($n=8$) received twice-daily (09:30 and 16:00 hours) injections of saline vehicle (0.9% [wt/vol.] NaCl) or (D-Ser²)glucagon-exe (25 nmol/kg) for 28 days. Food intake, body weight and non-fasting plasma glucose and insulin were measured every 3–4 days. Glucose tolerance (18 mmol/kg) and insulin sensitivity (25 U/kg, intraperitoneal) tests were performed at the end of the study in non-fasted mice. In addition, on day 28, mice were placed in Complete Laboratory Animal Monitoring System (CLAMS) metabolism chambers (Columbus Instruments, Columbus, OH, USA) following the normal daily injection at 09:30 hours. Following an acclimatisation period of 24 h, the consumption of O₂,

production of CO₂, respiratory exchange ratio (RER), energy expenditure and locomotor activity of each mouse was determined as described previously [19]. Pancreatic tissues were also excised for the assessment of islet morphology as described elsewhere [20].

Statistical analysis Results are expressed as mean $\pm$ SEM and values were compared using repeated-measures ANOVA or one-way ANOVA (followed by the Student–Newman–Keuls post hoc test) or by Student's unpaired t test as appropriate. Incremental plasma insulin and glucose AUC was calculated using GraphPad Prism (version 5.0; San Diego, CA, USA). Groups of data were considered to be significantly different if $p<0.05$.

Results

Peptide characterisation and DPP IV stability Incubation with DPP IV rapidly degraded native glucagon (44% intact peptide remained at 8 h). However, none of the other analogues tested displayed any degradation when incubated with DPP IV for up to 8 h (Table 1).

In vitro insulin secretion and cAMP production Native glucagon, (D-Ser²)glucagon and (D-Ser²)glucagon-exe evoked significant insulin responses from BRIN BD11 cells compared with 5.6 mmol/l glucose control (Fig. 1b, c). *N*-acetylglucagon was ineffective (Fig. 1a) whereas (D-Ser²)glucagon-exe was the most significant secretagogue at 10^{–7} and 10^{–6} mol/l (Fig. 1c). The cAMP production capability of (D-Ser²)glucagon-exe was then examined in glucagon-R-, GIP-R- and GLP-1R-transfected cells (Fig. 2). (D-Ser²)glucagon-exe induced significant ($p<0.001$) cAMP generation in glucagon-R- and GLP-1R-transfected cells at concentrations of 10^{–8} mol/l and above when compared with control (Fig. 2a, c). Moreover, (D-Ser²)glucagon-exe was significantly ($p<0.01$ to $p<0.001$) more efficacious than native glucagon in glucagon-R-

Fig. 2 Effect of (D-Ser²)glucagon-exe on cAMP production in glucagon-R-, GIP-R- and GLP-1R-transfected cells. White bars, control; black bars, glucagon (a), GIP(1–42) (b) or GLP-1 (c); grey bars, (D-Ser²)glucagon-exe.

Values represent means $\pm$ SEM, $n=8$. * $p<0.05$ and *** $p<0.001$ compared with control; †† $p<0.01$ and ††† $p<0.001$ compared with respective native peptide at the same concentration

transfected cells (Fig. 2a). (D-Ser²)glucagon-exe had no effect on cAMP production in GIP-R-transfected cells (Fig. 2b).

Acute effects on plasma glucose and insulin Figure 3 displays the plasma glucose and insulin responses to administration of the various peptides in mice. Whereas *N*-acetyl-glucagon and (D-Ser²)glucagon were more hyperglycaemic than native glucagon (Fig. 3a, b), (D-Ser²)glucagon-exe was devoid of significant hyperglycaemic properties (Fig. 3c). All peptides increased plasma insulin concentrations, with particularly prominent effects observed with (D-Ser²)glucagon and (D-Ser²)glucagon-exe (Fig. 3e, f). Accordingly, (D-Ser²)glucagon-exe was taken forward for further evaluation.

Persistent effect of (D-Ser²)glucagon-exe on glucose lowering and insulin release As evident from electronic supplementary material (ESM) Fig. 1a–f, mice treated with (D-Ser²)glucagon-exe 4, 8 or 12 h previously displayed a significant ($p<0.01$ to $p<0.001$) decrease in plasma glucose with concomitant increases in plasma insulin levels ($p<0.05$ to $p<0.01$) in response to a glucose intraperitoneal load.

Acute effects on plasma glucose and insulin in GLP-1R KO mice Administration of native glucagon to wild-type C57BL/6J (Fig. 4a, c) or GLP-1R KO (Fig. 4b, d) mice induced characteristic glucose- and insulin-elevating effects, when compared with saline vehicle alone. In contrast, treatment with (D-Ser²)glucagon-exe did not significantly alter overall blood glucose concentrations in either mouse model when compared with saline control (Fig. 4a, b). Small but significant ($p<0.05$ to $p<0.01$) increases in plasma insulin levels were observed at 15, 30 and 60 min post (D-Ser²)glucagon-exe injection in GLP-1R KO mice (Fig. 4d). However, significant ($p<0.001$) increases in overall 0–60 min AUC insulin secretory response were noted in C57BL/6J (Fig. 4c) but not GLP-1R KO mice (Fig. 4d).

Effects of twice-daily administration of (D-Ser²)glucagon-exe in high-fat-fed mice Twice-daily administration of (D-Ser²)glucagon-exe significantly ($p<0.01$) reduced body weight compared with saline-treated controls (Fig. 5a). Overall energy intake was also reduced by (D-Ser²)glucagon-exe treatment (Fig. 5b). In addition, non-fasted plasma glucose concentrations were significantly ($p<0.05$ to $p<0.001$)

Fig. 3 Acute effects of *N*-Acetyl-glucagon, (D-Ser²)glucagon and (D-Ser²)glucagon-exe on glucose and insulin responses in normal mice. Insets show AUC values for 0–60 min post injection. Squares and black bars, saline; triangles and white bars, glucagon; circles and grey bars,

N-Acetyl-glucagon (a), (D-Ser²)glucagon (b) or (D-Ser²)glucagon-exe (c). Values are means $\pm$ SEM for eight mice. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ compared with respective saline controls; † $p<0.05$, †† $p<0.01$ and ††† $p<0.001$ compared with glucagon

Fig. 4 Acute effects of (D-Ser²)glucagon-exe on glucose and insulin responses in GLP-1R KO mice. Blood glucose (a, b) and plasma insulin (c, d) concentrations were measured before and after intraperitoneal administration of saline vehicle, glucagon or (D-Ser²)glucagon-exe (both at 25 nmol/kg) in C57BL/6J (a, c) and GLP-1R KO mice (b, d). Insets show AUC values for 0–60 min post injection. Squares and black bars, saline; triangles and white bars, glucagon; circles and grey bars, (D-Ser²)glucagon-exe. Values represent means $\pm$ SEM for eight mice. * p <0.05, ** p <0.01 and *** p <0.001 compared with saline alone; ††† p <0.001 compared with glucagon alone

decreased from day 9 onwards (Fig. 5c) while plasma insulin levels were significantly (p <0.05 to p <0.001) elevated by day 25 (Fig. 5d). Beneficial metabolic effects on body weight, plasma glucose and insulin persisted, to a lesser effect, after the cessation of treatment (Fig. 5a, c, d).

Effects of (D-Ser²)glucagon-exe on glucose tolerance and insulin sensitivity in high-fat-fed mice Administration of (D-Ser²)glucagon-exe twice daily for 28 days significantly (p <0.01) improved the overall glycaemic excursion after a glucose load (Fig. 6a, c). In addition, the glucose-mediated insulinitropic response was significantly elevated in terms of individual (p <0.01 to p <0.001) and overall AUC (p <0.001) values in the (D-Ser²)glucagon-exe treatment group (Fig. 6a, c). Two weeks after cessation of treatment with (D-Ser²)glucagon-exe, the glycaemic excursion and insulin response remained significantly (p <0.05 to p <0.001) improved (Fig. 6b, d). ESM Fig. 2 depicts the effect of (D-Ser²)glucagon-exe on insulin sensitivity on day 28 (ESM Fig. 2a, c) and two weeks after treatment cessation (ESM Fig. 2b, d); (D-Ser²)glucagon-exe treatment resulted in significantly (p <0.001) improved insulin sensitivity at both time points examined.

Effects of (D-Ser²)glucagon-exe on metabolic rate and energy expenditure in high-fat-fed mice (D-Ser²)glucagon-exe treatment significantly (p <0.05) increased O₂ consumption on day 28 but had no effect on CO₂ production (ESM Fig. 3a, b). RER was not changed but energy expenditure was significantly (p <0.01) increased in (D-Ser²)glucagon-exe-treated mice (ESM Fig. 3c, d). In addition, (D-Ser²)glucagon-exe-treated mice displayed significantly (p <0.01) increased ambulatory activity during the dark phase, but not the light phase, as assessed by X-beam breaks on day 28 (ESM Fig. 4a, b). These mice also exhibited significantly (p <0.001) increased rearing and jumping episodes during the dark phase, but not during the light phase (ESM Fig. 4c, d).

Effects of (D-Ser²)glucagon-exe on pancreatic islet morphology in high-fat-fed mice (D-Ser²)glucagon-exe significantly increased pancreatic islet number (p <0.001), as well as overall islet (p <0.05) and beta cell (p <0.05) areas (ESM Fig. 5a–c). These beneficial morphological changes persisted two weeks after cessation of treatment (ESM Fig. 5d–f).

Fig. 5 Effects of twice-daily injection, for 28 days, of (D-Ser²)glucagon-exe on body weight (**a**), energy intake (**b**), non-fasting plasma glucose (**c**) and plasma insulin (**d**) in high-fat-fed mice. Variables were measured at 3–4 day intervals for 5 days before, 4 weeks during (indicated by black horizontal bar) and 2 weeks following cessation of treatment (twice-daily

injection of (D-Ser²)glucagon-exe, 25 nmol/kg). Insets show % change from basal, with day 0 as baseline. Squares and black bars, saline; circles and white bars, (D-Ser²)glucagon-exe. Values are expressed as means $\pm$ SEM for eight mice. * p <0.05, ** p <0.01 and *** p <0.001 compared with saline-treated high-fat-fed mice

Discussion

The past ten years have witnessed major advances in the exploitation of peptide therapeutics for diabetes as demonstrated by the clinical use of GLP-1 and amylin mimetics [21, 22]. OXM and other dual- or triple-acting hybrid peptides have also been developed, incorporating the therapeutic potential of not only GLP-1R activity but also that of glucagon- and GIP-Rs [23]. Thus, it appears that stimulation of insulin secretion from pancreatic beta cells may overcome the hyperglycaemic actions of glucagon and allow exploitation of its beneficial effects on food intake, fat mobilisation and energy expenditure [24]. This study has therefore examined novel glucagon analogues in an attempt to reveal new molecular approaches to type 2 diabetes therapy.

Whereas native glucagon was susceptible to DPP IV-mediated degradation, the analogues that possessed either (D-Ser²) or *N*-acetyl modifications were stable. This is consistent with previous studies indicating that DPP IV cannot cleave N-terminally modified forms of glucagon

[25, 26]. (D-Ser²)glucagon and (D-Ser²)glucagon-exe also stimulated insulin secretion from clonal beta cells, whereas *N*-acetyl-glucagon was ineffective in this regard. These observations not only highlight the insulin-releasing properties of glucagon-like molecules [27] but also demonstrate that manipulation of the N-terminus of glucagon can hinder biological activity [28], as was the case for *N*-acetyl-glucagon. Intriguingly, this compound did possess insulinotropic actions when administered to mice, coupled with potent hyperglycaemic effects. Mechanisms responsible for the indirect insulinotropic effect are unclear but might simply reflect beta cell stimulation by raised glucose [24]. In contrast to the acetylated analogue, (D-Ser²)glucagon and (D-Ser²)glucagon-exe both possessed very pronounced insulin-releasing actions. However, unlike glucagon and the other two analogues, (D-Ser²)glucagon-exe did not induce elevations in blood glucose. This profile of (D-Ser²)glucagon-exe also differs from the biological actions of native OXM, which has previously been shown to increase glucose production

Fig. 6 Effects of twice-daily injection, for 28 days, of (D-Ser²)glucagon-exe on glucose and insulin responses to exogenous glucose immediately following (**a, c**) and 2 weeks after (**b, d**) cessation of treatment in high-fat-fed mice. Insets show overall plasma glucose and insulin AUC values for 0–105 min. Squares and black bars, saline; circles and white bars, (D-Ser²)glucagon-exe. Values are expressed as means $\pm$ SEM for eight mice. * p <0.05, ** p <0.01 and *** p <0.001 compared with saline-treated high-fat-fed mice

during a hyperinsulinaemic–euglycaemic clamp in mice [29]. Thus, (D-Ser²)glucagon-exe, which consists of the entire glucagon sequence, with a nine amino acid C-terminal extension, may have improved therapeutic use over OXM, similar to exendin(1-39). The C-terminal extension employed for (D-Ser²)glucagon-exe has previously been shown to stabilise peptides, reduce clearance and significantly enhance in vivo potency [30, 31]. Although we do not have an assay for (D-Ser²)glucagon-exe with which to determine pharmacokinetics, bioactivity studies revealed that the glucose-lowering actions of (D-Ser²)glucagon-exe were sustained for up to 12 h after administration. This is consistent with other regulatory peptides that possess similar amino acid substitutions [14, 31, 32].

Further studies were performed to determine which receptors were activated by (D-Ser²)glucagon-exe. Investigations using GLP-1R KO mice, revealed that the glucose-homeostatic and, particularly, insulin-releasing actions of (D-Ser²)glucagon-exe were largely dependent on GLP-1R activation. However, it is likely that glucagon-R activation would be important for beneficial effects on energy regulation [7, 12]. As such, (D-Ser²)glucagon-exe stimulated cAMP production in glucagon-R- and GLP-1-R-transfected cells with similar or enhanced efficacy when compared with the native peptide. This clearly shows that (D-Ser²)glucagon-exe is a potent dual agonist, with a C-terminal structure quite distinct from OXM [33]. The distinct specificity for incretin receptors [34] was confirmed by the lack of biological activity of (D-Ser²)glucagon-exe in GIP-R-transfected cells. However,

competitive receptor binding studies with labelled peptide would ultimately be required to unequivocally confirm which receptors are activated by (D-Ser²)glucagon-exe. Nonetheless, the results from in vitro and acute in vivo studies provide a strong basis for the subsequent 28 day study in high-fat-fed mice.

Chronic treatment, for 28 days, of mice with dietary-induced obesity with (D-Ser²)glucagon-exe resulted in a marked improvement in metabolic status. This included a decreased overall energy intake and a greater than 10% loss in body weight. Other hybrid peptides that interact with glucagon and GLP-1Rs have been reported previously [35, 36]. However, these peptides function as GLP-1R agonists and glucagon-R antagonists and as such lack the prominent effects on body weight loss noted in the current study. Circulating glucose levels and intraperitoneal glucose tolerance were significantly improved in (D-Ser²)glucagon-exe-treated high-fat-fed mice. These beneficial effects are symptomatic of the increased metabolic efficiency that typically accompanies weight loss [7]. In keeping with this, insulin sensitivity was significantly improved by day 28 and this was accompanied by a marked improvement in glucose-mediated insulin secretion. These data clearly support previous suggestions of improved beta cell function and glucose homeostasis following dual activation of glucagon- and GLP-1Rs [13]. Notably we observed a marked expansion of pancreatic islet and beta cell mass, with increased total numbers of islets in (D-Ser²)glucagon-exe-treated mice. This effect is consistent with the view that GLP-1 is a proliferative agent for beta cells

[37]. Thus, a major component of the beneficial biological actions of (D-Ser²)glucagon-exe appears to be mediated by direct effects on the endocrine pancreas.

In an attempt to further clarify the potential mechanisms behind the observed positive effects, we assessed aspects of metabolic rate and energy regulation following 28 days treatment with (D-Ser²)glucagon-exe. General locomotor activity and explorative episodes were elevated during the dark phase in (D-Ser²)glucagon-exe-treated mice, but unaltered during the light phase. This is a positive effect, given that locomotor activity of mice is already increased during the dark phase [19]. Energy expenditure and O₂ consumption were also increased by (D-Ser²)glucagon-exe, which may be due in part to increased activity [18]. Such effects, combined with significant reductions in food intake are clearly responsible for the substantial body weight loss [12]. Changes in feeding are likely modulated by both GLP-1- and glucagon-Rs, whereas beneficial effects on energy regulation most likely reflect activation of glucagon-Rs by (D-Ser²)glucagon-exe [24]. However, further studies, such as determining gene expression in metabolically active tissues, would help to elucidate the pathways and underlying beneficial mechanisms observed with (D-Ser²)glucagon-exe treatment.

An important observation in this study was the persistence of beneficial effects following discontinuation of (D-Ser²)glucagon-exe treatment in high-fat-fed mice. This contrasts with the findings of a previous study using a long-acting OXM molecule in *ob/ob* mice [13]. In the present study body weight, non-fasting circulating glucose, glucose tolerance, insulin sensitivity and pancreatic morphology were still significantly improved in (D-Ser²)glucagon-exe-treated mice 14 days after cessation of treatment. This suggests the possibility of change in metabolic programming [38], although persistence of the advantageous effects on blood glucose control could possibly reflect maintained reduction of body weight, which is known to impart positive metabolic effects [20]. The relative beneficial metabolic effects of (D-Ser²)glucagon-exe were comparable with, or indeed superior to, essentially similar studies conducted in our laboratory with the clinically approved GLP-1R agonist exendin-4 [39–41]; (D-Ser²)glucagon-exe had superior body-weight-lowering effects compared with exendin-4 and similar or superior metabolic benefits. Thus, (D-Ser²)glucagon-exe has significant potential to be further explored as a possible novel therapeutic compound for the treatment of obesity-related diabetes.

In conclusion, this study demonstrates for the first time that the stable glucagon analogue (D-Ser²)glucagon-exe causes sustained weight loss and improves glucose tolerance, insulin resistance, pancreatic morphology, metabolic rate and energy expenditure in high-fat-fed mice. Thus, (D-Ser²)glucagon-exe is a novel enzyme-resistant drug with potential application for the treatment of obesity-related diabetes.

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Duality of interest NI, VAG, FPMO'H and PRF hold shares with Diabetica Ltd, which has patents for exploitation of peptide therapeutics. All other authors declare that there is no duality of interest associated with their contribution to this manuscript.

Contribution statement NI, VAG, FPMO'H and PRF conceived the study, participated in the analysis and interpretation of data, drafted the manuscript and revised it critically for intellectual content. AML, NP and VP participated in the analysis and interpretation of data and drafted the manuscript and revised it critically for intellectual content. All authors approved the final version of the manuscript. VAG is the guarantor of this work.

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