Glucagon from the phylogenetically ancient paddlefish provides a template for the design of a long-acting peptide with effective anti-diabetic and anti-obesity activities

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Highlights

- [D-Ser^2]palmitoyl-paddlefish glucagon is resistant to degradation by plasma peptidases and DPP-IV
- The peptide enhanced *in vitro* insulin secretion from BRIN-BD11 clonal β-cells and mouse islets
- The peptide augmented β-cell proliferation and reduced cytokine-mediated apoptosis
- The peptide improved glucose tolerance in lean mice when injected up to 4 h before a glucose load.
- Twice daily administration to high-fat fed mice over 21 days reduced food intake and body weight
- The peptide improved glucose tolerance, insulin sensitivity and functional islet gene expression.

Abstract

This study has examined the *in vitro* and *in vivo* anti-diabetic properties of the peptidase-resistant analogues [D-Ser^2]palmitoyl-paddlefish glucagon and [D-Ser^2]palmitoyl-lamprey glucagon. The peptides stimulated insulin release from BRIN-BD11 clonal β-cells and isolated mouse pancreatic islets and also enhanced cAMP production in cells transfected with the human GLP-1 receptor and with the human glucagon receptor. The insulinotropic actions of the peptides were attenuated in INS-1 cells lacking GLP-1 and glucagon receptors. [D-Ser^2]palmitoyl-paddlefish glucagon stimulated proliferation of BRIN-BD11 cells and protected against cytokine-mediated apoptosis as effectively as GLP-1. The analogue was more effective than the native peptide or the lamprey glucagon analogue in acutely lowering blood glucose and elevating plasma insulin in lean mice even when administered up to 4 h before a glucose load. Twice daily administration of [D-Ser^2]palmitoyl-paddlefish glucagon to high-fat fed mice over 21 days reduced food intake, body weight, non-fasting blood glucose and plasma insulin concentrations, as well as significantly improving glucose tolerance and insulin resistance and decreasing α-cell area and pancreatic insulin content. Islet expression of the Gcgr, Glp1r, Gipr and Slc2a2 (GLUT-2) genes significantly increased. These data demonstrate that long-acting peptide [D-Ser^2]palmitoyl-paddlefish glucagon exerts beneficial metabolic properties in diabetic mice via Ggcr- and Glp1r-activated pathways and so shows potential as a template for further development into an agent for treatment of patients with obesity-related Type 2 diabetes.

Key words: Glucagon, lamprey, paddlefish, dual agonist, obesity, Type 2 diabetes.

1. Introduction

Glucagon-like peptide-1 (GLP-1) stimulates insulin release as well as supressing both glucagon release and appetite and promoting proliferation of ß-cells (Hare et al., 2010; Dailey and Moran, 2013; Drucker, 2018). Consequently, the use of GLP-1 receptor (GLP1R) agonists in the treatment of patients with Type 2 diabetes mellitus (T2DM) is well established. Several long-acting chemically modified analogues, such as liraglutide, albiglutide, and lixisenatide, have been developed and are in use in clinical practice (Bailey, 2018). The idea that glucagon, a potent stimulator of glycogenolysis and gluconeogenesis, or its derivatives may find application in T2DM therapy appears at first sight to be paradoxical. However, administration of glucagon promotes weight loss, an important factor in reversing insulin resistance, by increasing energy expenditure and lipid catabolism suggesting a possible therapeutic role in the management of obesity-related T2DM (Scott and Bloom, 2018; Al-Massadi et al., 2019).

The last decade has seen the development of a new approach to incretin-based therapy by design of a range of unimolecular co-agonists of GLP1R and the glucagon receptor (GCGR) that are capable of activating different signalling pathways (Pocai et al., 2009; Day et al., 2009; Henderson et al., 2016; Evers et al., 2017; Zhou et al., 2017). Such agents combine the insulinotropic and anorectic effects of GLP-1 with the lipolytic and thermogenic actions of glucagon and are effective in reducing body weight and improving glucose tolerance, lipid metabolism, and hepatic steatosis (Brandt et al., 2018). In addition, the hyperglycaemic action of glucagon restrains the insulin-mediated glucose lowering action of GLP-1 preventing episodes of hypoglycaemia. The naturally occurring GCCR/GLP1R dual-agonist peptide, oxyntomodulin stimulates insulin release and improves glucose tolerance

when administered to mice and, at pharmacological doses, will inhibit appetite and food intake (Lynch et al., 2014; Holst et al., 2018). In the past, clinical application of glucagon has largely been confined to the emergency management of insulin-induced hypoglycaemia (Hawkes et al., 2019) but, with the availability of long-acting, dual-agonist peptides based upon the primary structure of glucagon and either GLP-1 or glucose-dependent insulinotropic peptide (GIP), this situation is likely to change.

Glucagon isolated from the dogfish *Scyliorhinus canicula* (Elasmobranchii) targets both the GLP-1 and glucagon receptors *in vitro* (O'Harte et al., 2016a) and shows strong anti-hyperglycaemic and insulinotropic effects in mice (O'Harte et al., 2016b). A more recent study has shown that the potent glucose-lowering and insulinotropic actions of glucagons from the phylogenetically ancient sea lamprey *Petromyzon marinus* (Petromyzontiformes) and the North American paddlefish *Polyodon spathula* (Acipenseriformes) in mice are also mediated by activation of both Glp1r and Gcgr (Graham et al., 2018a). Lamprey and paddlefish glucagons are rapidly cleaved by plasma peptidases and dipeptidyl peptidase IV (DPP-IV, EC 3.4.14.5) producing inactive fragments (Graham et al., 2018a) thereby limiting their therapeutic potential. A successful strategy for producing long-acting, peptidase-resistant anti-hyperglycaemic agents derived from dogfish glucagon (O'Harte et al., 2016b) and lamprey GLP-1 (Graham et al., 2020) involved modification of the naturally occurring peptides at position 2 by substitution of L-Ser by D-Ser and attachment of a C-16 fatty acid to a lysine residue in the molecule via a γ-glutamyl linker. The present study has adopted this strategy to produce analogues of paddlefish and lamprey glucagons with increased resistance to proteolytic degradation and their anti-diabetic properties are investigated *in vitro* and in both lean and high-fat fed mice.

2. Materials & methods

2.1. Peptides

[D-Ser^2,Lys^{30}-γ-glutamyl-palmitate]-lamprey glucagon ([D-Ser^2]palmitoyl-lamprey glucagon) and [D-Ser^2,Lys^{30}-γ-glutamyl-palmitate]-paddlefish glucagon ([D-Ser^2]palmitoyl-paddlefish glucagon) were supplied in crude form by Synpeptide Co. Ltd. (Shanghai, China). The peptides were purified to > 98% homogeneity by reversed-phase HPLC on a (2.2 cm x 25 cm) Vydac 218TP1022 (C-18) column. The concentration of acetonitrile in the eluting solvent was raised from 38.5% to 70 % over 60 min using a linear gradient. Absorbance was measured at 214 nm and the flow rate was 6 ml/min. The molecular masses of the peptides were confirmed by matrix-assisted laser desorption ionization time-of-flight mass (MALDI-TOF) spectrometry using a Voyager DE PRO instrument (Applied Biosystems, Foster City, USA) as previously described (Conlon et al., 2018). The primary structures and molecular masses of the peptides used in this study are shown in Table 1.

2.2. Degradation assays

Lamprey glucagon, paddlefish glucagon, human glucagon, [D-Ser^2]palmitoyl-lamprey glucagon and [D-Ser^2]palmitoyl-paddlefish glucagon (30 μg and 100 μg) were separately incubated in 50 mM triethanolamine buffer, pH 7.8 (390 μl) with either (a) purified porcine DPP-IV (5 mU; Merck Millipore) or (b) pooled plasma (10 μl) from National Institutes of Health (NIH) Swiss mice for 4 h at 37 °C. Each reaction mixture was chromatographed at a flow rate of 1.0 ml/min on a (250 x 4.6 mm) Luna 5μ C-8 column (Phenomenex, Macclesfield, U.K.) using linear gradients of acetonitrile (0% to 35% from 0 min to 10 min,

35% to 42% from 10 min to 30 min, and 42 to 70% from 30 min to 35 min) for elution. Peptides were detected by measurement of absorbance at 214 nm and analysed by MALDI-TOF mass spectrometry. % intact peptide was calculated from the peak area.

2.3. In vitro insulin release studies using BRIN-BD11 cells

BRIN-BD11 clonal β-cells (McClenaghan et al., 1996) were used to assess *in vitro* insulin secretory activity of peptides as previously described (Graham et al., 2018b; 2019). Briefly, the cells were seeded (150,000 cells/well) into 24-well plates (Nunc, Roskilde, Denmark) and allowed to attach overnight at 37 °C. Cells were incubated for 20 min at 37 °C in Krebs-Ringer Bicarbonate (KRB) buffer, pH 7.4 supplemented with 5.6 mM glucose in the presence of the peptides in the concentration range (1 pM - 3 μM; n = 8). After 20 min incubation, the supernatant was removed from each well and stored at −20 °C prior to measurement of insulin concentrations by radioimmunoassay (Flatt and Bailey, 1981).

2.4. Insulin release studies using isolated mouse islets

Effects of the fish glucagon analogues on *ex-vivo* insulin secretion were assessed using isolated pancreatic islets from adult, male NIH Swiss mice (Harlan Ltd, Bicester, UK) as previously described (Ojo et al., 2016). The islets were cultured for 48 h at 37 °C in an atmosphere of 5% CO_2 and 95% air prior to incubation with peptides (10 nM and 1μM; n = 4) and alanine (10 mM; n = 4) for 1 h at 37˚C in KRB buffer supplemented with 16.7 mM glucose. Insulin release was measured by radioimmunoassay and the insulin content was measured by extraction of the islets with acid-ethanol as previously described (Ojo et al., 2016).

2.5. Effects on cAMP production

The effects of the peptides to stimulate cAMP production were determined using Chinese hamster lung (CHL) cells transfected with GLP1R (Thorens et al., 1993) and human embryonic kidney (HEK293) cells transfected with GCGR (Ikegami et al., 2001) as previously described (Graham et al., 2018a). The cells were pre-incubated for 40 min with KRB buffer supplemented with 0.1% (w/v) bovine serum albumin (BSA) and 1.1 mmol/l glucose. A test solution containing KRB buffer supplemented with 0.1% (w/v) BSA, 5.6 mM glucose, 200 μM 3-isobutyl-1-methylxanthine and peptides (10^{-8} and 10^{-6} M) were added to the cells. After incubating for 60 min, the medium was removed and cells were lysed before measurement of cAMP using a Parameter cAMP assay kit (R&D Systems, Abingdon, UK) according to the manufacturer's recommended protocol.

2.6. Insulin release studies using CRISPR/Cas9-engineered INS-1 cells

The effects of the peptides to stimulate insulin release were determined using wild-type INS-1 832/3 rat clonal pancreatic β-cells and CRISPR/Cas9-engineered cells with knock-out of the glucagon receptor (Gcgr KO) (Graham et al., 2020), GLP-1 receptor (Glp1r KO) (Naylor et al., 2016) or GIP receptor (Gipr KO) (Naylor et al., 2016). The cells were cultured and incubated as described for BRIN-BD11 cells with the addition of 1 mM sodium pyruvate and 50 mM 2-mercaptoethanol to the medium (Graham et al., 2018b; 2019) The cells were incubated with 10^{-8} and 10^{-6} M concentrations of GLP-1, glucagon, GIP, and the fish peptide analogues for 20 min at 37 °C in KRB buffer supplemented with 5.6 mM glucose and insulin release was measured by radioimmunoassay.

2.7. Effects on proliferation of BRIN-BD11 cells

To assess the effects of [D-Ser^2]palmitoyl-lamprey glucagon, [D-Ser^2]palmitoyl-paddlefish glucagon, and human GLP-1 (10^{-8} and 10^{-6} M) on proliferation, BRIN-BD11 cells were incubated with the peptides for 18 h at 37 °C and fixed as previously described (Khan et al., 2016; Graham et al., 2018a). After fixation, cells were stained with rabbit anti-Ki-67 primary antibody and subsequently with Alexa Fluor 594 secondary antibody (Abcam. Cambridge, UK). Proliferation frequency was determined in a blinded fashion and expressed as % of total cells analyzed. Approximately 150 cells per replicate were analyzed.

2.8. Effects on cytokine-induced apoptosis in BRIN-BD11 cells

BRIN-BD11cells were incubated with peptides (10^{-8} and 10^{-6} M) and a cytokine mixture (200 U/ml tumour-necrosis factor-α, 20 U/ml interferon-γ, and 100 U/ml interleukin-1β) for 16 h at 37 °C as previously described (Khan et al., 2016). Effects on apoptosis were measured using an *in situ* Cell Death Detection Kit (Roche Diagnostics, Burgess Hill, UK) for 1 h at 37 °C following the manufacturer's recommended procedure. Slides were viewed using a fluorescent microscope with 488 nm filter (Olympus System Microscope, model BX51; Southend-On-Sea, UK) and photographed by a DP70 camera adapter system.

2.9. Animals

All animal experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and EU Directive 2010/63EU for animal experiments and approved by Ulster University Animal Ethics Review Committee (ref. UOA3/B/115). All

necessary steps were taken to prevent any potential animal suffering. Mice were maintained in environmentally controlled rooms (12h:12 h light/darkness cycle, 22 ± 2 ºC) with free access to food and water. Acute *in vivo* insulin-release studies were carried out using 8 - 10 week-old male NIH Swiss mice maintained on a standard rodent diet (10% fat, 30% protein, 60% carbohydrate: total energy 12.99 kJ/g; Trouw Nutrition, Northwich, UK) and water. Food intake studies were carried out in 8 - 10 week-old male NIH Swiss mice as previously described (O'Harte et al., 2017).

Longer term effects of peptide treatment were investigated using 8-12 week-old male NIH Swiss mice maintained on high-fat chow (45% fat, 20% protein, and 35% carbohydrate (total energy, 26.15 KJ/g; Special Diets Service, Essex, UK) with free access to water for 3 months prior to the start of experiments in order to produce obesity, glucose intolerance, and insulin resistance. Body weight, blood glucose, and plasma insulin concentrations were monitored at regular intervals. The animals were allocated into groups with matching average body weights and blood glucose levels prior to starting the experiment. Mice remained on a high-fat diet for the duration of the study.

2.10. Effects of peptide administration on glucose tolerance, insulin release and food intake in lean mice

Blood glucose and plasma insulin concentrations were measured after intraperitoneal (i.p.) injection of glucose (18 mmol/kg body weight) either alone or together with peptides (25 nmol/kg) in overnight-fasted lean mice as previously described (Owolabi et al., 2016a). In a separate series of experiments, blood glucose and plasma insulin were measured after i.p. injection of glucose (18 mmol/kg) either 2 h or 4 h after ip administration of saline, lamprey glucagon, paddlefish glucagon, [D-Ser^2]palmitoyl-lamprey glucagon or [D-Ser^2]palmitoyl-

paddlefish glucagon (25 nmol/kg). For food intake studies, mice (21 h fasted) received ip injections of either 0.9% (w/v) NaCl solution or peptides (25nmol/kg) and were allowed free access to food pellets. The weight of food consumed was recorded at 30 min intervals up to 180 min. Consumption of water was not restricted.

2.11. Effects of twice daily peptide administration on insulin resistance in high-fat-fed mice

High-fat fed mice received twice-daily ip injections of 0.9 % saline solution for three days before administration of either 0.9% saline (control) or peptides (25 nmol/kg) twice daily (09:30 and 17:00 h) over 21 days. Non-fasting blood glucose, cumulative food and water intake, body weight, and plasma insulin concentrations were monitored at 3-day intervals before daily injection of test peptides. The peptide treatment groups (n = 8 for all groups) were as follows: Group 1: 0.9% saline; Group 2: liraglutide, (25 nmol/kg); Group 3: [D-Ser^2]palmitoyl-paddlefish glucagon (25 nmol/kg). Oral and i.p. glucose tolerance (18 mmol/kg) and insulin sensitivity (25 U/kg; i.p.) tests were performed after 21 days of treatment as previously described (Owolabi et al., 2016b). HOMA-β and HOMA-IR were calculated using standard formula as described (Wallace et al., 2004). Mice were killed at the end of the treatment period to obtain pancreata and terminal blood. Bone mineral density and body tissue composition were measured using DEXA scanning (Owolabi et al., 2016b).

2.12. Biochemical analyses

Blood glucose concentration was measured using an Ascenacia Counter Blood Glucose Meter (Bayer, Newbury, UK). Blood samples were collected in fluoride/heparin microcentrifuge tubes (Sarstedt, Numbrecht, Germany) and centrifuged for 30 s at 13,000 × g. Terminal non-fasted blood was collected for measurement of plasma glucagon

concentrations (Glucagon Chemiluminescent Assay, Millipore Corporation, Germany). Plasma amylase (Abcam, ab102523) and alanine aminotransferase (ALT Reagent, Randox, UK) activities were measured following the manufacturer's instructions. Plasma cholesterol levels were measured using an automated analyser (I-Lab 650 Integrated Chemistry System, Instrumentation Laboratory, Warrington, UK). LDL-cholesterol concentrations were calculated using the Friedewald equation as described (Johnson et al., 1997). Pancreatic tissues were homogenised and extracted using buffer containing 20 mM Tris HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA and 0.5% Triton X 100 and stored at -80^0 C. Protein concentrations were determined using Bradford reagent (Sigma, Dorset, UK).

2.13. Insulin release studies using islets from 21-day treated high-fat fed animals

Pancreatic islets were isolated as previously described (Ojo et al., 2016). Following a 60-min pre-incubation with 1.4 mM glucose, the islets were incubated with KRB buffer (500 µl) supplemented with 0.5% BSA and 16.7 mM glucose and the test agents (10 mM alanine, 20 mM KCl, 10 mM arginine, 1 µM human GLP-1 and 1µM human GIP). Tubes were incubated for 60 min at 37˚C followed by centrifugation at 800 x g for 5 min. The supernatants were stored at -20 ˚C for determination of insulin release by radioimmunoassay. The remaining islets were stored at -70˚C for the gene expression studies.

2.14. Tissue processing for histological analyses

Pancreatic tissues from each group of mice were fixed and processed as previously described (Vasu et al 2014). The following primary antibodies were used as appropriate: mouse monoclonal anti-insulin antibody (ab6995, 1:400; Abcam, Cambridge, UK) and

guinea-pig anti-glucagon antibody (PCA2/4, 1:50; raised in-house). The following secondary antibodies were used as appropriate: Alexa Fluor 488 goat anti-guinea pig IgG (1:400) and Alexa Fluor 594 goat anti-mouse IgG (1:400). The slides were viewed under a fluorescent microscope (Olympus model BX51) with the DP70 camera adapter system. Islet parameters (islet number, islet area, β-cell area, α-cell area and islet size distribution) were determined using Cell^F image analysis software (Olympus Soft Imaging Solutions GmbH, Münster, Germany).

2.15. Islet gene expression

mRNA was extracted from collagenase-digested islets using TriPure Isolation Reagent (Roche, Burgess Hill , UK) (1ml/0.5 g of cells) following the manufacturer's instructions and converted to cDNA using SuperScript™ II Reverse Transcriptase (Invitrogen Life Technologies, Carlsbad, CA, USA) (Vasu et al. 2013). Real-time polymerase chain reaction (PCR) was performed using a MJ Mini Personal Thermal Cycler (Bio-Rad Laboratories Ltd., Watford, U.K.). PCR reaction mixture (containing 4.5 µl of QuantiFast SYBR green PCR mix (Qiagen, Venlo, Netherlands), 1 µl of forward and 1 µl reverse primers, 3 µl of cDNA and 1 µl of nuclease-free water) was added to the PCR tube strips. DNA denaturation was performed at 95°C for 5 min followed by a 40-cycle cDNA amplification with final denaturation at 95°C for 30 s, annealing at 58 °C for 30 s and elongation at 72°C for 30 s. SYBR Green fluorescence was recorded after each cycle. Data were normalized to β-actin (Actb) gene expression and analysed using the ΔΔCt method as previously described (Owolabi et al., 2016b).

2.16. Statistical analysis

Data were compared using one-way ANOVA with Bonferroni post-hoc test and unpaired Student's t-test (non-parametric, with two-tailed P values and 95% confidence interval) as appropriate (GraphPad PRISM Version 5.0 San Diego, California). Area under the curve (AUC) analysis was performed using the trapezoidal rule with baseline correction. Data are presented as mean ± S.E.M.

3. Results

3.1. Degradation studies

Both [D-Ser^2]palmitoyl-lamprey glucagon and [D-Ser^2]palmitoyl-paddlefish glucagon were stable to degradation after 4 h incubation with either DPP-IV or mouse plasma (Fig. 1A and Table 2). In contrast, under the same conditions the underivatized lamprey and paddlefish glucagons and human glucagon were susceptible to rapid proteolytic cleavage by DPP-IV at the Ser^2 residue site to generate inactive metabolites.

3.2. In vitro insulin-releasing activities of the fish glucagon analogues

Incubation of [D-Ser^2]palmitoyl-lamprey glucagon and [D-Ser^2]palmitoyl-paddlefish glucagon with BRIN-BD11 cells increased the rate of insulin release in a concentration-dependent manner (Figs.1B and 1C). The rate of insulin release stimulated by the lamprey glucagon analogue at 3 μM concentration was significantly ($P < 0.05$) less than that the parent peptide but the threshold concentration was lower (0.3 nM vs 3 nM) (Table 3). The effect of the paddlefish glucagon analogue at 3 μM concentration was comparable to that of

the underivatized peptide and human GLP-1 and the threshold concentration was 10-fold lower (0.01 nM vs 0.1 nM). Incubation with [D-Ser^2]palmitoyl-paddlefish glucagon (10 nM and 1 μM) increased the rate of insulin release from isolated mouse islets by amounts that were comparable to those produced by human GLP-1 (Fig. 1D). The small increases in rate produced by incubation of islets with the lamprey analogue (10 nM and 1 μM) were not significant.

3.3. Effects of the fish glucagon analogues on cAMP production

Incubation of CHL cells expressing GLP1R with [D-Ser^2]palmitoyl-lamprey glucagon resulted in significantly increased cAMP production at both 10 nM and 1 μM concentration compared with control. However, the effect at 10 nM was significantly less than that produced by human GLP-1 (Fig. 1E). The stimulatory effect of [D-Ser^2]palmitoyl-paddlefish glucagon was comparable to that of human GLP-1 at both 10 nM and 1 μM concentrations (Fig. 1E). Both analogues at concentrations of 10 nM and 1 μM stimulated cAMP production in HEK293 cells transfected with the human glucagon receptor but the magnitude of responses produced by [D-Ser^2]palmitoyl-lamprey glucagon were appreciably less than those produced by human GLP-1 (Fig. 1F).

3.4. Insulin release studies using CRISPR/Cas9-engineered INS-1 cells

Incubations with [D-Ser^2]palmitoyl-lamprey glucagon and [D-Ser^2]palmitoyl-paddlefish glucagon at both 10 nM and 1 μM concentrations significantly increased the rate of insulin release from wild-type INS-1 cells (Fig. 2A). Incubation of the analogues with Glp1r KO cells and Gcgr KO cells attenuated the stimulatory effects on insulin secretion compared to wild-type INS-1 cells (Fig. 2B and 2C). In contrast, incubation of the analogues

with Gipr KO cells produced a stimulation in the rate of insulin release that was not significantly different from the response in wild-type cells (Fig. 2D) indicating the actions of the analogues are not mediated through activation of the GIP receptor.

3.5. Effects on proliferation and survival of BRIN-BD11 cells

Incubations with human GLP-1 and the fish glucagon analogues (10 nM and 10 μM) significantly ($P < 0.001$) augmented the proliferation of BRIN-BD11cells compared with incubations with media alone (Fig. 2E). In the second series of experiments, incubation, of the cells with a mixture of proinflammatory cytokines significantly ($P < 0.001$) increased the number of cells undergoing apoptosis. [D-Ser^2]palmitoyl-lamprey glucagon (10 nM and 10 μM) was equally as effective as GLP-1 in protecting BRIN-BD11cells against cytokine-induced DNA damage but the lamprey analogue was inactive (Fig. 2F).

3.6. Effects on glucose tolerance in overnight fasted lean mice

Administration of [D-Ser^2]palmitoyl-paddlefish glucagon along with a 18 mmol/kg i.p. glucose load significantly ($P < 0.01$ - $P < 0.001$) reduced blood glucose concentrations at 15, 30 and 60 min (Fig. 3A) and also the integrated glucose response (Fig. 3C) compared with the effect of administration of glucose alone. The effects of the paddlefish glucagon analogue were not significantly different from those of human GLP-1 and exendin-4 at each time point. These observations were consistent with a significantly ($P < 0.01$ - $P < 0.001$) increased insulin secretory response to the paddlefish glucagon analogue at 15, 30 and 60 min (Fig. 3B) and a greater integrated response (Fig. 3D) compared with i.p. injection of glucose alone. Plasma insulin concentrations after administration of the paddlefish analogue and

exendin-4 were significantly ($P < 0.001$) greater at 60 min post-injection compared with those following GLP-1 but the integrated responses were similar. In marked contrast, [D-Ser^2]-palmitoyl lamprey glucagon (25 nmol/kg) had lost the beneficial glucose-lowering properties of the parent peptide and administration failed to elicit any significant effects on glucose (Fig. 3A) or insulin (Fig. 3B) concentrations.

When administrated 2 h (Supplementary Fig, 1) and 4 h (Supplementary Fig. 2) prior to the glucose load, [D-Ser^2]palmitoyl-paddlefish glucagon and exendin-4 significantly reduced circulating glucose concentrations and elevated insulin concentrations at each time point compared with glucose injection only whereas, as expected, the glucose-lowering and insulin-releasing actions of GLP-1 had disappeared. The integrated glucose responses to the paddlefish glucose analogue and exendin-4, administered 2 h (Supplementary Fig, 1) and 4 h (Figs. 3E and 3F) before the glucose load were significantly ($P < 0.001$) less and corresponding integrated insulin responses significantly ($P < 0.001$) greater than those following GLP-1 injection.

3.7. Effects of fish glucagon peptides on food intake in lean mice

Administration of [D-Ser^2]palmitoyl-paddlefish glucagon at a dose of 25 nmol/kg body weight to overnight fasted animals significantly ($P < 0.05$) suppressed feeding at all time points up to 180 min (Supplementary Fig. 3B). The appetite suppressive effects of paddlefish glucagon and its analogue were comparable to the effects of exendin-4. In contrast, ip administration of the lamprey glucagon analogue resulted in a small reduction of cumulative food intake only after 180 min (Supplementary Fig. 3A).

3.8 Effects of 21-day administration [D-Ser^2]-palmitoyl paddlefish glucagon on food intake, body weight, and circulating glucose and insulin concentrations in high-fat fed mice

Based on the data obtained *in vitro* and in lean mice, [D-Ser^2]palmitoyl-paddlefish was selected for longer-term studies. Twice-daily administration of the peptide to high-fat fed mice resulted in a significant ($P < 0.05$ - $P < 0.001$) reduction of food intake starting from day 13 onwards when compared with high-fat control animals (Fig. 4A). Peptide treatment also led to a reduction in body weight from day 18 onwards when compared with control mice (Fig. 4B). Administration of the paddlefish glucagon analogue and liraglutide resulted in a significant ($P < 0.001$) decrease in body weight and in the integrated blood glucose concentration (Fig. 4C and E) compared with high-fat fed control mice. A reduction of non-fasting insulin concentrations ($P < 0.05$) on days 10 and 16 was observed for liraglutide and [D-Ser^2]palmitoyl-paddlefish glucagon respectably (Fig. 4F).

3.9. Effects of 21-day administration [D-Ser^2]palmitoyl paddlefish glucagon on glucose tolerance, insulin sensitivity and insulin resistance in high-fat fed mice

Following 21-day treatment with [D-Ser^2]palmitoyl-paddlefish glucagon, a significant ($P < 0.05$ - $P < 0.001$) reduction in blood glucose concentration was observed at the 15 - 120 min time points after i.p. injection of glucose. This translated to a significant reduction ($P < 0.001$) in integrated blood glucose concentration (Fig. 5A). Glucose-stimulated insulin concentrations were significantly ($P < 0.05$ - $P < 0.001$) increased at 15 - 60 min for the group treated with the analogue and the integrated response of insulin release was also significantly ($P < 0.001$) increased (Fig. 5B). [D-Ser^2]-palmitoyl paddlefish glucagon treatment resulted in significantly ($P < 0.05$) greater stimulation of insulin release compared with liraglutide treatment. In a second series of experiments involving oral glucose

administration, there was a significant ($P < 0.05$) reduction in glucose concentrations at 60, 90 and 120 min together with significantly ($P < 0.05$) lowered integrated response the group receiving [D-Ser^2]palmitoyl-paddlefish glucagon in compared with the control group (Fig. 5C). Concomitant with the lower blood glucose levels, the concentration of plasma insulin was significantly ($P < 0.05$) greater at 15 min in the group treated with the analogue and the integrated insulin responses were significantly ($P < 0.05$) greater compared with high-fat fed control mice (Fig. 5D).

No significant differences in blood glucose concentrations were observed in the three groups after administration of exogenous insulin (Fig. 5E). However, HOMA-IR values were significantly greater and HOMA-β values significantly less in the mice receiving either liraglutide or the paddlefish glucagon analogue demonstrating that long-term administration of the peptides decreased insulin resistance and increased β-cell function compared with high-fat fed control mice (Fig. 5F).

3.10 Effects of 21-day administration [D-Ser^2]palmitoyl-paddlefish glucagon on plasma glucagon, amylase and alanine aminotransferase activities, blood lipid profile and body mass in high-fat fed mice.

Analysis of plasma after 21-day treatment showed that amylase activity was significantly ($P < 0.05$) elevated (Fig. 6B), and plasma alanine aminotransferase activity was significantly ($P < 0.05$ and $P < 0.001$) reduced (Fig. 6C) in the peptide-treated groups compared with control mice. Plasma glucagon concentrations were not significantly different between the groups (Fig. 6A).

The plasma lipid profile revealed significant ($P < 0.05$) reductions in total cholesterol and triglyceride concentrations in the group treated with [D-Ser^2]palmitoyl-paddlefish glucagon when compared with control mice (Figs. 6D and 6E). LDL-cholesterol and HDL-

cholesterol concentrations were not significantly different between the groups (Figs. 6F and 6G). A significant ($P < 0.05$ - $P < 0.01$) reduction in fat mass and % body fat was also observed in the peptide treatment groups (Figs. 6H and 6I).

3.11. Effects of 21-day administration [D-Ser^2]palmitoyl-paddlefish glucagon on pancreatic insulin and glucagon content and islet histology in high-fat fed mice

Pancreatic glucagon content in [D-Ser^2]palmitoyl-paddlefish glucagon- and liraglutide-treated mice was not significantly different compared with high-fat fed control animals (Fig. 7B). In contrast, pancreatic insulin content was significantly reduced in both treated groups compared to control (Fig. 7A). No appreciable change in islet morphology was noted between the liraglutide-treated and control groups (Figs. 7C - 7G). In the group treated with [D-Ser^2]palmitoyl-paddlefish glucagon there was a significant reduction in α-cell area ($P < 0.05$) (Fig. 7F), as well as a significant ($P < 0.05$) increase in the number of small (< 10,000 μm^2) islets compared to the control group (Fig. 7G).

3.12. Effects of 21-day administration of [D-Ser^2]palmitoyl-paddlefish glucagon on ex-vivo insulin secretion from isolated mouse islets and islet gene expression in high-fat fed mice

Islets isolated from high-fat fed mice showed weak insulin secretory responses to stimulatory concentrations of glucose, alanine, arginine, potassium chloride, human GLP-1 and GIP that were improved by 21-day treatment with liraglutide and paddlefish glucagon analogue (Fig. 8A). Treatment with both the paddlefish glucagon analogue and liraglutide significantly ($P < 0.05$) reduced the islet expression of *Ins1* (mouse insulin 1) (Fig. 8B) and increased the expression of *Slc2a2* (glucose transporter 2; GLUT2) (Fig. 8D), Glp1r (Fig. 8E)

and Gcgr (Fig. 8F) compared with control mice but only the paddlefish peptide upregulated Gipr expression (Fig. 8G).

4. Discussion

This study has extended previous investigations for the design of long-acting anti-diabetic agents exploiting the structures of proglucagon-derived peptides from phylogenetically ancient fish (O'Harte et al., 2016b; Graham et al., 2020) by development of a peptidase-resistant analogues of paddlefish and lamprey glucagons. Both [D-Ser^2]palmitoyl-paddlefish glucagon and [D-Ser^2]palmitoyl-lamprey glucagon displayed increased resistance to degradation by DPP-IV compared with the native peptide (Table 2) DPP-IV converts human glucagon into inactive fragments (Pospisilik et al., 2001) and is important in terminating the action of the peptides *in vivo* (Deacon 2018). The additional modification involving the attachment of a fatty acid to the Lys^{30} residue of the peptide promotes binding to albumin and delays renal clearance (Son et al., 2009; Kerr et al., 2010). This modification has been used in the design of the long-acting incretin, liraglutide which has a half-life of 11-15 hours (Wajcberg and Amarah, 2010). Both analogues stimulated cAMP production in CHL cells transfected with GLP1R as well as in HEK293 cells transfected with GCGR (Figs. 1E and 1F) and their insulinotropic properties were attenuated in INS-1 cells lacking Glp1r and Gcgr (Figs. 2B and 2C) demonstrating that the modifications to the naturally occurring peptides have not abolished their dual-agonist properties.

Paddlefish glucagon contains the segments Glu^{15}-Glu^{16} and Glu^{24}-Trp^{25}-Leu^{26}-Lys^{27}-Asn^{28}-Gly^{29} in common with long-acting GLP1R agonist, exendin-4 leading to the suggestion that it represents a naturally occurring hybrid glucagon/exendin-4 molecule

(Graham et al., 2018a). The modifications to the peptide resulted in an analogue that was equally effective in stimulating insulin release from BRIN-BD11 clonal β-cells but was 10-fold more potent (Table 2) and also increased the rate of insulin release from isolated mouse islets at a concentration of 10 nM (Fig 1D). In contrast, the modified lamprey glucagon showed reduced effectiveness in stimulating insulin release from BRIN-BD11 cells (Table 2) and did not produce a significant response in isolated mouse islets at a concentration of 1 μM (Fig. 1C). This marked difference in properties may be a consequence of the fact that the palmitate residue is attached to the C-terminal amino acid in the lamprey peptide. The site of attachment is important in determining the activities of acylated analogues of lamprey and paddlefish GLP-1 (Graham et al., 2020) and structure-activity studies involving human GLP-1 showed that the addition of a fatty acid and γ-glutamyl linker at certain sites in the molecule resulted in up to a 70-fold reduction in potency (Knudsen et al. 2000; Madsen et al. 2007). However, both analogues at a concentration of 10 nM stimulated proliferation of BRIN-BD11 cells (Fig. 2E) but only the paddlefish analogue protected the cells against cytokine-induced apoptosis (Fig. 2F).

In studies with overnight-fasted lean mice, administration of the paddlefish glucagon analogue significantly lowered blood glucose concentrations and increased circulating insulin concentrations even when injected 4 h before an ip glucose load (Figs. 3E and 3F; Supplementary Figure 2). The peptide was as effective as exendin-4 whereas human GLP-1 and underivatized paddlefish glucagon were ineffective under these conditions. In studies with high-fat fed mice injected twice-daily for 21 days with either the paddlefish glucagon analogue or liraglutide, animals treated with the paddlefish analogue were associated with a significant decrease in non-fasting blood glucose, plasma insulin and pancreatic insulin content (Fig. 4). Moreover, glycaemic responses to intraperitoneal and oral glucose loads were improved which correlated with beneficial effects upon insulin resistance and β-cell

function (Fig. 4). These improvements in glycaemic control and insulin resistance will decrease the functional demand for insulin and so permit beneficial β-cell rest (Pathak et al., 2015).

In addition to positive effects on glucose homeostasis, the paddlefish glucagon analogue significantly suppressed food intake and body weight in these animals. This finding is consistent with the DEXA scan results showing a reduction in adipose tissue which would also be expected to contribute to the improvements of insulin sensitivity and metabolic control. Consumption of a diet high in fact induces dyslipidemia with increased concentrations of plasma cholesterol, low levels of HDL cholesterol and increased levels of triglycerides (Ipsen et al., 2016). Treatment with the paddlefish glucagon analogue reduced total cholesterol and decreased circulating triglycerides in high-fat fed mice (Fig. 6). In the present study, liraglutide failed to improve the lipid profile. However, other investigations have shown a decrease in triglyceride levels in mice treated once daily with liraglutide (Millar et al., 2017; O'Harte et al., 2018). Feeding a high-fat diet also results in steatohepatitis and inflammasome activation (Ganz et al., 2014; Stephenson et al., 2018). Treatment with the analogue reduced plasma aminotransferase activity (Fig. 6), which suggests that the peptide, like liraglutide, may exercise a protective action against liver damage. On the other hand, administration of liraglutide and the paddlefish glucagon analogue modestly increased levels of circulating amylase which may suggest a possible risk of pancreatitis (Gier et al., 2012). Indeed, there has been concern regarding possible adverse effects of GLP1R agonist drugs on pancreatic and thyroid tissue since animal studies and analyses of drug databases suggest a possible link between these medications and pancreatitis, pancreatic cancer, and thyroid cancer (Filippatos et al., 2014). However, meta-analyses (Alves et al., 2012) and LEADER randomised trials (Steinberg et al., 2017) failed to

confirm that the increase of serum amylase caused by GLP-1 mimetics is implicated in these conditions.

A high-fat diet leads to development of insulin resistance with a concomitant elevation in blood glucose levels arising from reduced insulin-mediated glucose uptake by the peripheral tissues and increased hepatic glucose production. In response, β-cells compensate by releasing more insulin and there is an increase in β-cell mass via proliferation (Golson et al., 2010; Sachdeva and Stoffers, 2009; Vasu et al., 2014). The histological findings reveal that treatment with the paddlefish glucagon analogue significantly reduced α-cell area compared to high-fat fed controls. Although not significant, the β-cell area was also decreased in this group which correlated with a higher number of smaller-sized islets. Consistent with this observation, although no change in plasma glucagon concentrations were detected, islet insulin and glucagon content were decreased in this group. This finding may indicate a restoration of α-cell function and ability to respond to glucose similar to the observed beneficial action of GLP-1 (Vilsboll, 2009; Hare et al., 2010). In contrast, there were no significant changes in any histological parameters in the liraglutide treatment group.

A major challenge in diabetes research is understanding the multiple effects of glucose on β-cells in molecular terms by revealing the sites of dysregulation in gene expression caused by chronically elevated glucose concentrations (Schuit et al., 2002). Effective drugs that will reverse these molecular abnormalities are clearly required. Insulin gene (*Ins1*) transcription, which is up-regulated by an increase in blood glucose levels (Mosley et al., 2004), was decreased in mice treated with liraglutide and the paddlefish glucagon analogue compared to high-fat fed controls (Fig 8). This may be a consequence of the decreased circulating glucose levels in the treatment groups. Moreover, expression of *Slc2a2* gene encoding GLUT2, which regulates the entry of glucose into the β-cell, was significantly higher in the treatment groups. Decreased expression of *Slc2a2* is a hallmark of

β-cell glucose unresponsiveness (Thorens, 2015) so that the increased expression observed in this study may be responsible for the enhanced glucose sensitivity which in turn is beneficial for the preservation of insulin secretion by the pancreatic β- cells (Laukkanen et al., 2005). Stimulation of insulin release by GLP-1 and GIP is impaired in T2DM which correlates with the decreased expression GLP1R and GIPR receptors in diabetic subjects (Xu et al., 2007). Both liraglutide and the paddlefish glucagon analogue had a positive impact on the expression of Glp1r in high-fat fed mice compared with controls but only treatment with the paddlefish glucagon analogue resulted in a higher expression of Gipr despite the fact that treatment with both peptides enhanced the insulinotropic effects of GLP-1 and GIP in isolated islets (Fig. 8A). A higher expression of the Gcgr gene was observed in both peptide treatment groups. Previous studies suggest that glucagon may play a crucial role in pancreatic β-cell growth and differentiation. $Gcgr^{-/-}$ mice were shown to have a decreased percentage of β-cells per islet compared to control animals and less severe hyperglycaemia was observed in RIP-Gcgr mice overexpressing the glucagon receptor in the pancreas (Gelling et al., 2009).

To conclude, [D-Ser^2]palmitoyl-paddlefish glucagon is a potent dual-acting agonist that activates both GLP-1 and glucagon receptors and exhibits anti-hyperglycaemic, insulinotropic and proliferative effects as well as enhanced stability in plasma. Administration of the analogue had beneficial effects on food intake, body weight, glucose tolerance, insulin resistance, lipid profile, islet morphology, islet gene expression and β-cell function in high-fat fed mice with actions similar or superior to those of liraglutide. Exploitation of the structures of proglucagon-derived peptides from phylogenetically ancient fish, such as the paddlefish, may lead to the development new improved agents for treatment of patients with obesity-related T2DM.

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Legends to Figures

Fig. 1. (A) Stability of the fish glucagon analogues after 4 h incubation with plasma. Effects of (B) [D-Ser^2]palmitoyl-lamprey glucagon and (C) [D-Ser^2]palmitoyl-paddlefish glucagon on insulin release from BRIN-BD11 cells. (D) Effects of fish glucagon peptides (10^{-8} and 10^{-6} M) on insulin release from isolated mouse pancreatic islets. Effects of fish glucagon peptides (10^{-8} and 10^{-6} M) on cAMP production in (E) GLP1R-transfected CHL cells and (F) GCGR-transfected HEK293 cells. Values are mean ± S.E.M. In panels B and C, n = 8. *P < 0.05, **P < 0.01, ***P < 0.001 compared to 5.6 mM glucose alone. In panel D, n = 4; *P < 0.05, **P < 0.01, ***P < 0.001 compared to 16.7mM glucose alone. In panels E and F, n = 3. *P < 0.05, ** P < 0.01 and ***p < 0.001 compared with 5.6 glucose alone. $^{\Delta}$P< 0.05, $^{\Delta\Delta}$P < 0.01 and $^{\Delta\Delta\Delta}$P < 0.001 compared with the corresponding effects of GLP-1 or glucagon.

Fig. 2. Effects of [D-Ser^2]palmitoyl-lamprey glucagon and [D-Ser^2]palmitoyl-paddlefish glucagon (10^{-8} and 10^{-6} M) on the rate of insulin release from (A) wild-type INS-1 cells, (B) Glp1r KO INS-1 cells, (C) Gcgr KO INS-1 cells and (D) Gipr KO cells. (E) Effects of the fish glucagon analogues and human GLP-1 (10^{-8} and 10^{-6} M) on proliferation of BRIN-BD11 cells. (F) Effects of the fish glucagon analogues and human GLP-1 (10^{-8} and 10^{-6} M) on the protection of BRIN-BD11 cells against cytokine-induced apoptosis. Values are mean ± S.E.M. In panels A-D, n = 8; *P < 0.05, **P < 0.01 and ***P < 0.001 compared with 5.6 mM glucose alone; $^{\Delta}$P < 0.05, $^{\Delta\Delta}$P < 0.01, $^{\Delta\Delta\Delta}$P < 0.001 compared with effects in wild-type INS-1 cells. In panel E, n = 3 ***P < 0.001 compared with control cultures. In panel F, n = 3; ***P < 0.001 compared with cytokine-treated cells.

Fig. 3. Effects of administration of lamprey glucagon, paddlefish glucagon, [D-Ser^2]palmitoyl-lamprey glucagon and [D-Ser^2]palmitoyl-paddlefish glucagon (25 nmol/kg) on (A) blood glucose and (B) plasma insulin concentrations in lean mice after simultaneous ip injection of glucose (18 mmol/kg). The integrated responses (area under the curve, AUC) are shown in panels C and D. The integrated responses when administered 4 h before intraperitoneal injection of glucose (18 mmol/kg) are shown in panels E and F. The values are mean ± S.E.M. for n = 6. *P < 0.05, **P < 0.01 and ***P < 0.001 compared with glucose alone; $^{\Delta}$P < 0.05, $^{\Delta\Delta}$P < 0.01, $^{\Delta\Delta\Delta}$P < 0.01 compared with human GLP-1.

Fig. 4. Effects of 21-day administration of [D-Ser^2]palmitoyl-paddlefish glucagon on (A) food intake, (B) body weight, (C) % change in body weight, (D) non-fasting blood glucose concentrations, (E), % change in blood glucose concentrations and (F) plasma insulin concentrations in high-fat fed mice. The black horizontal bar represents the treatment period. Values represent mean ± S.E.M. for 8 mice. *P < 0.05, **P < 0.01 and ***P < 0.001 compared with high-fat fed control mice; $^{\Delta}$P < 0.05, $^{\Delta\Delta}$P < 0.01 compared with liraglutide.

Fig. 5. Effects of [D-Ser^2]palmitoyl-paddlefish glucagon an (A) blood glucose concentration and (B) plasma insulin concentration after ip glucose administration, (C) blood glucose concentration and (D) plasma insulin concentration after oral glucose administration (E) insulin sensitivity and (F) HOMA-β and HOMA-IR in high-fat fed mice. The parameters were measured on day 21 following twice-daily treatment with saline vehicle, liraglutide (25 nmol/kg) or [D-Ser^2]palmitoyl-paddlefish glucagon (25 nmol/kg). Values represent mean ± S.E.M., n = 8. *P<0.05, **P<0.01 and ***P<0.001 compared with high-fat fed control animals.

Fig. 6. Effects of 21-day intraperitoneal administration of D-Ser^2]palmitoyl-paddlefish glucagon on (A) plasma glucagon concentrations, (B) amylase and (C) alanine aminotransferase activities (D), cholesterol (E) triglyceride, (F) HDL, (G) LDL concentrations, (H) whole body fat mass and (I) % fat mass in high-fat fed mice. Parameters were measured on day 21 following twice-daily treatment with 0.9% NaCl, liraglutide (25 nmol/kg) or the paddlefish glucagon analogue (25 nmol/kg). Data are expressed as mean ± S.E.M.; n = 4. *P < 0.05, ** P < 0.01, *** P < 0.001 compared with saline control.

Fig. 7. Effects of 21-day administration [D-Ser^2]-palmitoyl paddlefish glucagon and liraglutide on (A) pancreatic insulin content, (B) pancreatic glucagon content (C) number of islets, (D) islet area, (E) β-cell area, (F) α-cell area, and (G) islet size distribution (% of total area) in high-fat fed mice. Parameters were measured on day 21 following twice-daily treatment with peptides (25 nmol/kg). ***P < 0.001 compared to high-fat fed control animals. Approximately 50 islets per mouse were analysed using Cell^F analysis software.

Fig. 8. (A) Effects of established insulin secretogogues on insulin secretion from isolated mouse islets from high-fat fed mice after treatment with D-Ser^2]-palmitoyl paddlefish glucagon or liraglutide. Effects of 21-day treatment with peptides on islet gene expression of (B) Ins1, (C) Gck, (D) Slc2α2, (E) Gcgr, (F) Glp1r, and (G) Gipr. Data were normalised to β-actin (Actb) gene expression. Values are means ± S.E.M. for n = 3. *P < 0.05, ** P< 0.01 and *** P< 0.001 compared with high-fat fed control animals.

Table 1. Primary structures and molecular masses of the glucagon-related peptides investigated in this study.

Peptide	**Amino acid sequence**	**Calculated molecular mass (Da)**	**Observed molecular mass (Da)**
Human glucagon	HSQGTFTSDYSKYLDSRRAQDFVQWLMNT	3482.8	3484.4
Lamprey glucagon	HSEGTFTSDYSKYLENKQAKDFVRWLMNA	3466.9	3466.5
Paddlefish glucagon	HSQGMFTNDYSKYLEEKRAKEFVEWLKNGKS	3751.1	3750.8
[D-Ser^2]palmitoyl-lamprey glucagon	HsEGTFTSDYSKYLENKQAKDFVRWLMNA[K-γ-glutamyl-palmitate]	3962.6	3964.1
[D-Ser^2]palmitoyl-paddlefish glucagon	HsQGMFTNDYSKYLEEKRAKEFVEWLKNG[K-γ -glutamyl-palmitate]S	4118.7	4120.0

Table 2. Degradation of fish and human glucagon-related peptides after 4 h incubation with dipeptidyl peptidase IV (DPP-IV) and plasma

Peptide	**% degraded by DPP-IV**	**% degraded in plasma**	**M_r of DPP-IV-degraded product, Da**	**Proposed cleavage site**
Lamprey glucagon	42	ND	3243.1	Ser^2-Glu^3
Paddlefish glucagon	14	ND	3429.7	Ser^2-Gln^3
[D-Ser^2]palmitoyl-lamprey glucagon	0	0	NA	NA
[D-Ser^2]palmitoyl-paddlefish glucagon	0	0	NA	NA
Human glucagon	24	35	3259.5	Ser^2-Gln^3

ND not determined. NA not applicable

Table 3. Effects of fish and human glucagon-related peptides on the rate of insulin release from BRIN-BD11 cells.

Peptide	**Threshold concentration (nM)**	**Effect at 3µM (ng/10^6 cells/20min)**
5.6 mM glucose	-	1.0 ± 0.02
Lamprey glucagon^{+}	3^{b}	3.3 ± 0.2^{c}
Paddlefish glucagon^{+}	0.1^{a}	3.6 ± 0.2^{c}
[D-Ser^{2}]palmitoyl-lamprey glucagon	0.3^{a}	2.4 ± 0.1^{c}
[D-Ser^{2}]palmitoyl-paddlefish glucagon	0.01^{a}	3.3 ± 0.2^{c}
Human glucagon	100^{a}	2.7 ± 0.2^{c}

The threshold concentration is the minimum concentration of the peptide producing a significant ($p < 0.05$) increase in the rate of insulin release. The values are mean ± S.E.M. for n = 8. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ compared to 5.6 mM glucose alone. $^{+}$Data taken from Graham et al., 2018a.

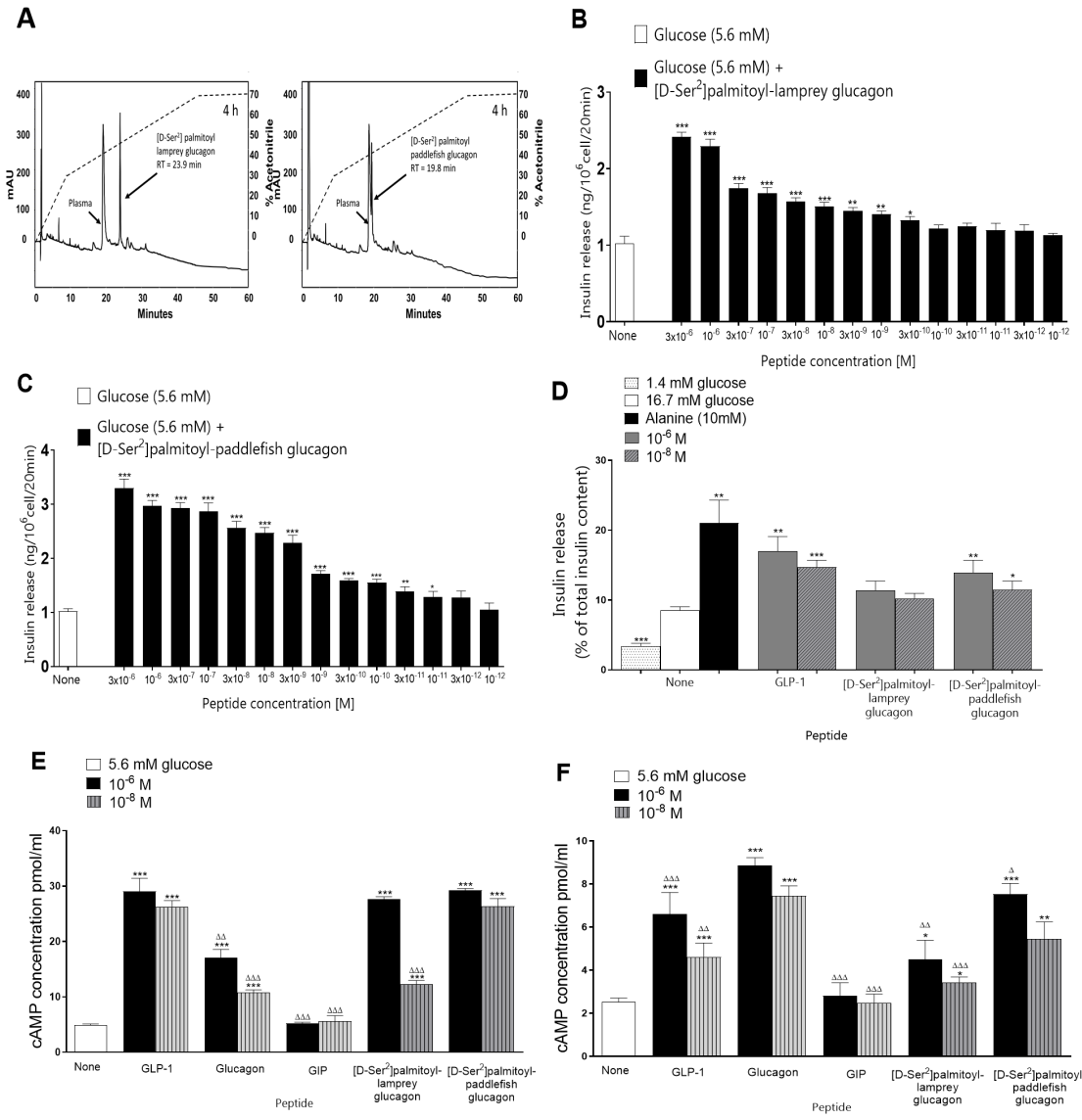

A
4 h
[D-Ser2] palmitoyl lamprey glucagon RT = 23.9 min
Plasma
mAU
% Acetonitrile
Minutes
4 h
[D-Ser2] palmitoyl paddlefish glucagon RT = 19.8 min
Plasma
B
Glucose (5.6 mM)
Glucose (5.6 mM) + [D-Ser2]palmitoyl-lamprey glucagon
Insulin release (ng/10^6 cell/20min)
Peptide concentration [M]
C
Glucose (5.6 mM)
Glucose (5.6 mM) + [D-Ser2]palmitoyl-paddlefish glucagon
Insulin release (ng/10^6 cell/20min)
Peptide concentration [M]
D
1.4 mM glucose
16.7 mM glucose
Alanine (10mM)
10-6 M
10-8 M
Insulin release (% of total insulin content)
None
GLP-1
[D-Ser2]palmitoyl-lamprey glucagon
[D-Ser2]palmitoyl-paddlefish glucagon
Peptide
E
5.6 mM glucose
10-6 M
10-8 M
cAMP concentration pmol/ml
None
GLP-1
Glucagon
GIP
[D-Ser2]palmitoyl-lamprey glucagon
[D-Ser2]palmitoyl-paddlefish glucagon
Peptide
F
5.6 mM glucose
10-6 M
10-8 M
cAMP concentration pmol/ml
None
GLP-1
Glucagon
GIP
[D-Ser2]palmitoyl-lamprey glucagon
[D-Ser2]palmitoyl-paddlefish glucagon
Peptide

Figure 1

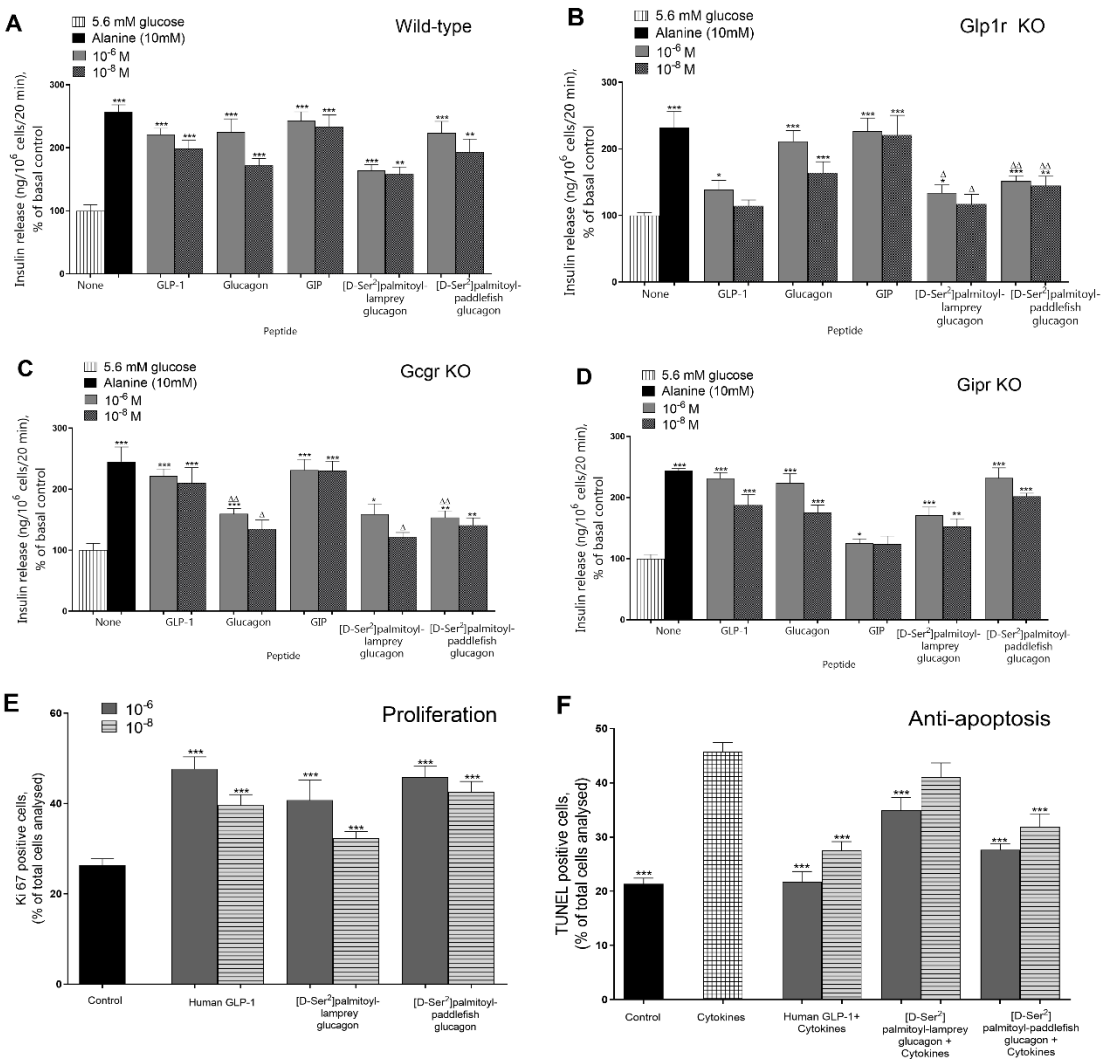

A
5.6 mM glucose
Alanine (10mM)
10-6 M
10-8 M
Wild-type
Insulin release (ng/10^6 cells/20 min), % of basal control
None
GLP-1
Glucagon
GIP
[D-Ser2]palmitoyl-lamprey glucagon
[D-Ser2]palmitoyl-paddlefish glucagon
Peptide
B
Glp1r KO
C
Gcgr KO
D
Gipr KO
E
Proliferation
Ki 67 positive cells, (% of total cells analysed)
Control
Human GLP-1
F
Anti-apoptosis
TUNEL positive cells, (% of total cells analysed)
Cytokines
Human GLP-1+ Cytokines
[D-Ser2] palmitoyl-lamprey glucagon + Cytokines
[D-Ser2] palmitoyl-paddlefish glucagon + Cytokines

Figure 2

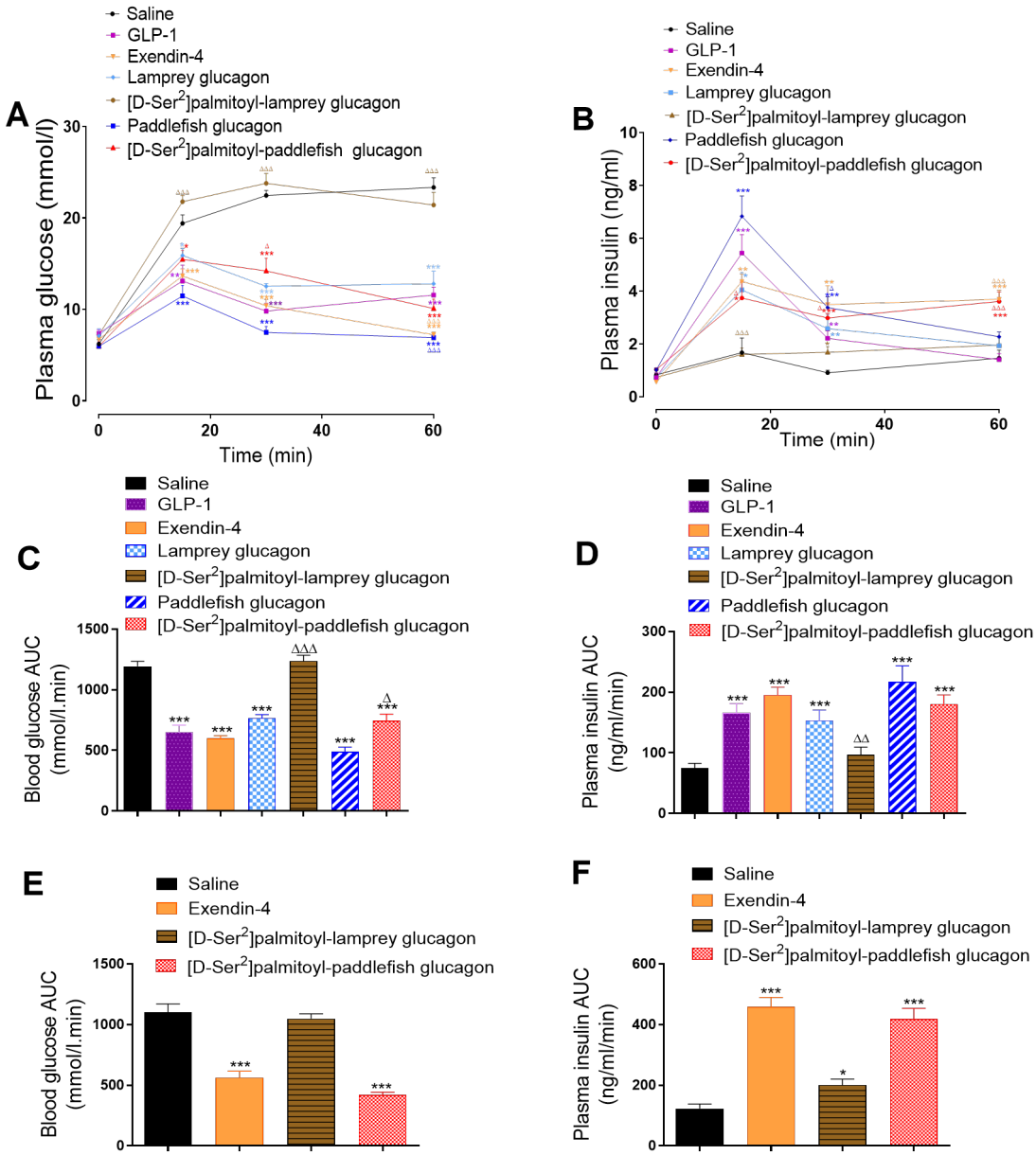

A
Saline
GLP-1
Exendin-4
Lamprey glucagon
[D-Ser2]palmitoyl-lamprey glucagon
Paddlefish glucagon
[D-Ser2]palmitoyl-paddlefish glucagon
Plasma glucose (mmol/l)
Time (min)
B
Plasma insulin (ng/ml)
Time (min)
C
Blood glucose AUC (mmol/l.min)
D
Plasma insulin AUC (ng/ml/min)
E
Blood glucose AUC (mmol/l.min)
F
Plasma insulin AUC (ng/ml/min)

Figure 3

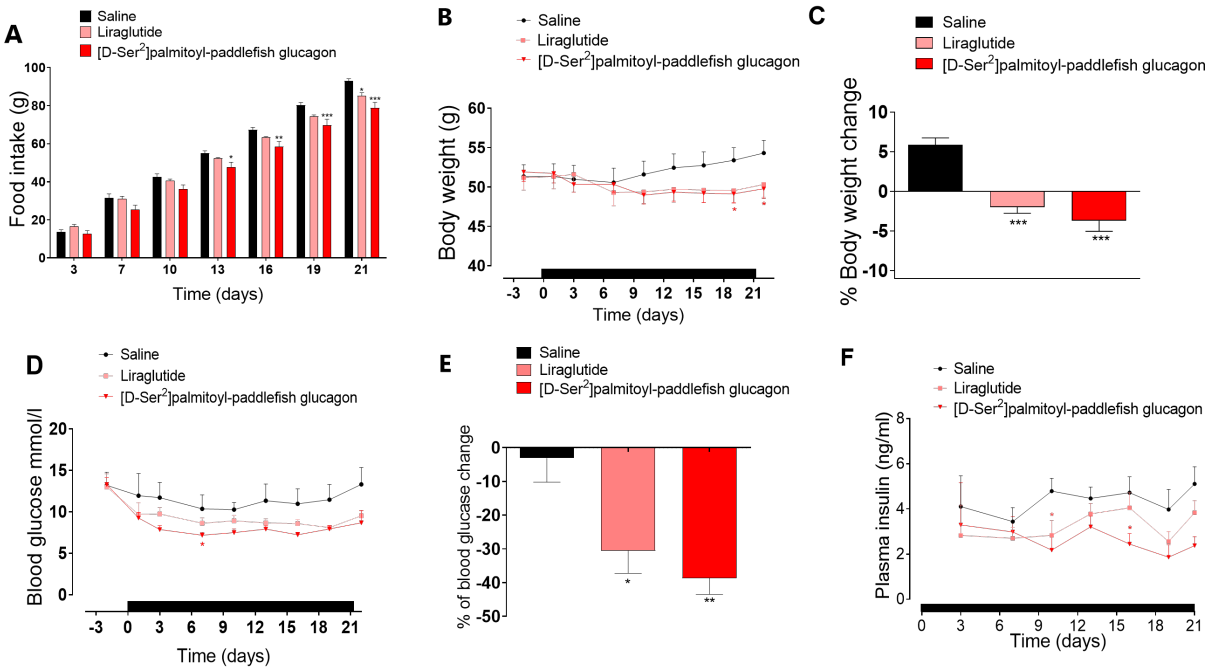

A
Saline
Liraglutide
[D-Ser2]palmitoyl-paddlefish glucagon
Food intake (g)
Time (days)
B
Body weight (g)
Time (days)
C
% Body weight change
D
Blood glucose mmol/l
Time (days)
E
% of blood glucase change
F
Plasma insulin (ng/ml)
Time (days)

Figure 4

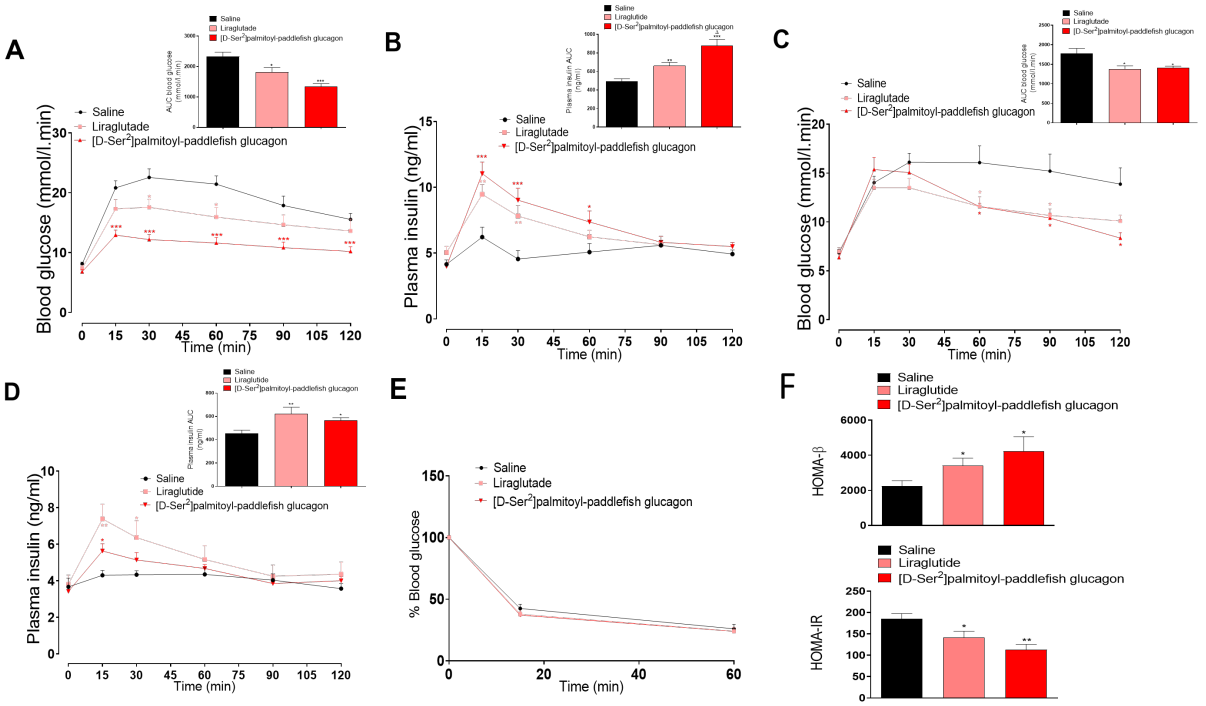

A
B
C
D
E
F
Saline
Liraglutide
[D-Ser2]palmitoyl-paddlefish glucagon
Blood glucose (mmol/l.min)
Plasma insulin (ng/ml)
% Blood glucose
HOMA-β
HOMA-IR
Time (min)

Figure 5

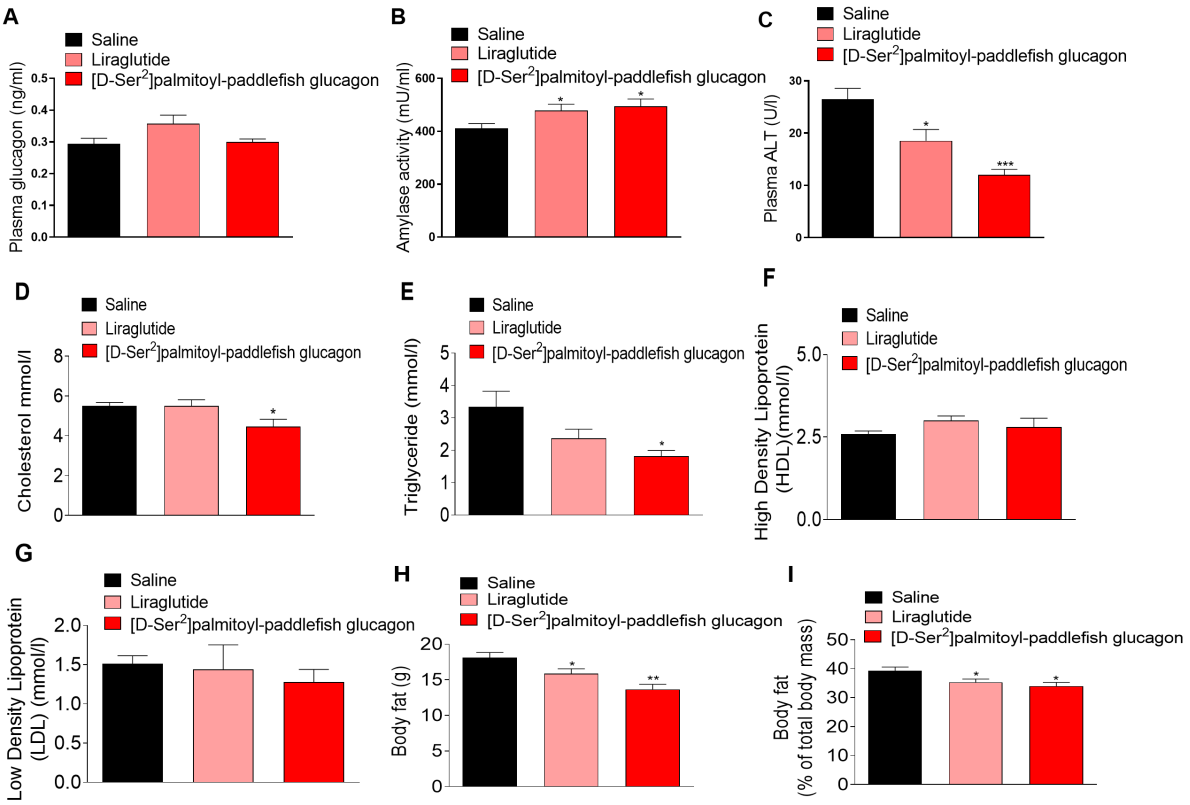

A
Saline
Liraglutide
[D-Ser2]palmitoyl-paddlefish glucagon
Plasma glucagon (ng/ml)
B
Amylase activity (mU/ml)
C
Plasma ALT (U/l)
D
Cholesterol mmol/l
E
Triglyceride (mmol/l)
F
High Density Lipoprotein (HDL)(mmol/l)
G
Low Density Lipoprotein (LDL) (mmol/l)
H
Body fat (g)
I
Body fat (% of total body mass)

Figure 6

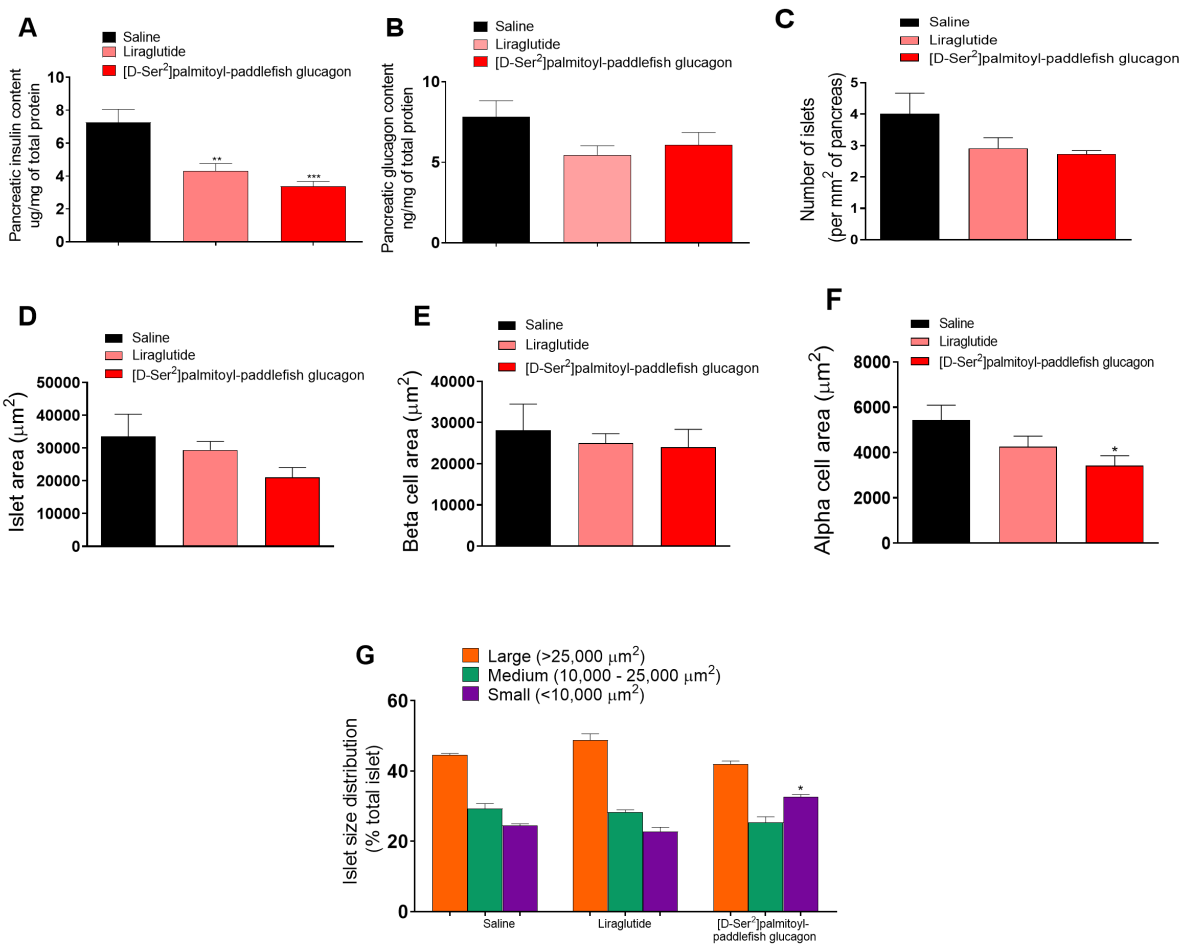

A
Saline
Liraglutide
[D-Ser2]palmitoyl-paddlefish glucagon
Pancreatic insulin content ug/mg of total protein
B
Pancreatic glucagon content ng/mg of total protien
C
Number of islets (per mm2 of pancreas)
D
Islet area (μm2)
E
Beta cell area (μm2)
F
Alpha cell area (μm2)
G
Large (>25,000 μm2)
Medium (10,000 - 25,000 μm2)
Small (<10,000 μm2)
Islet size distribution (% total islet)
Saline
Liraglutide
[D-Ser2]palmitoyl-paddlefish glucagon

Figure 7

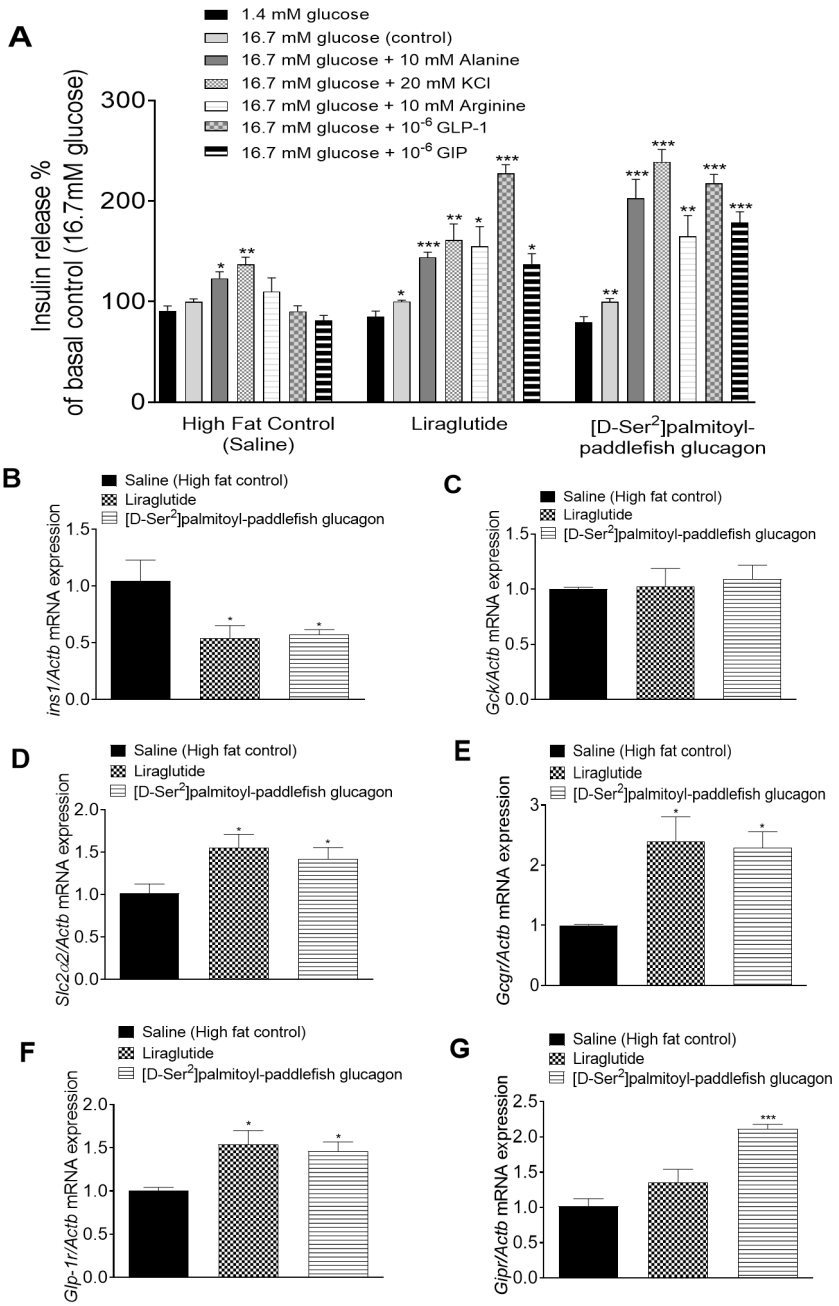
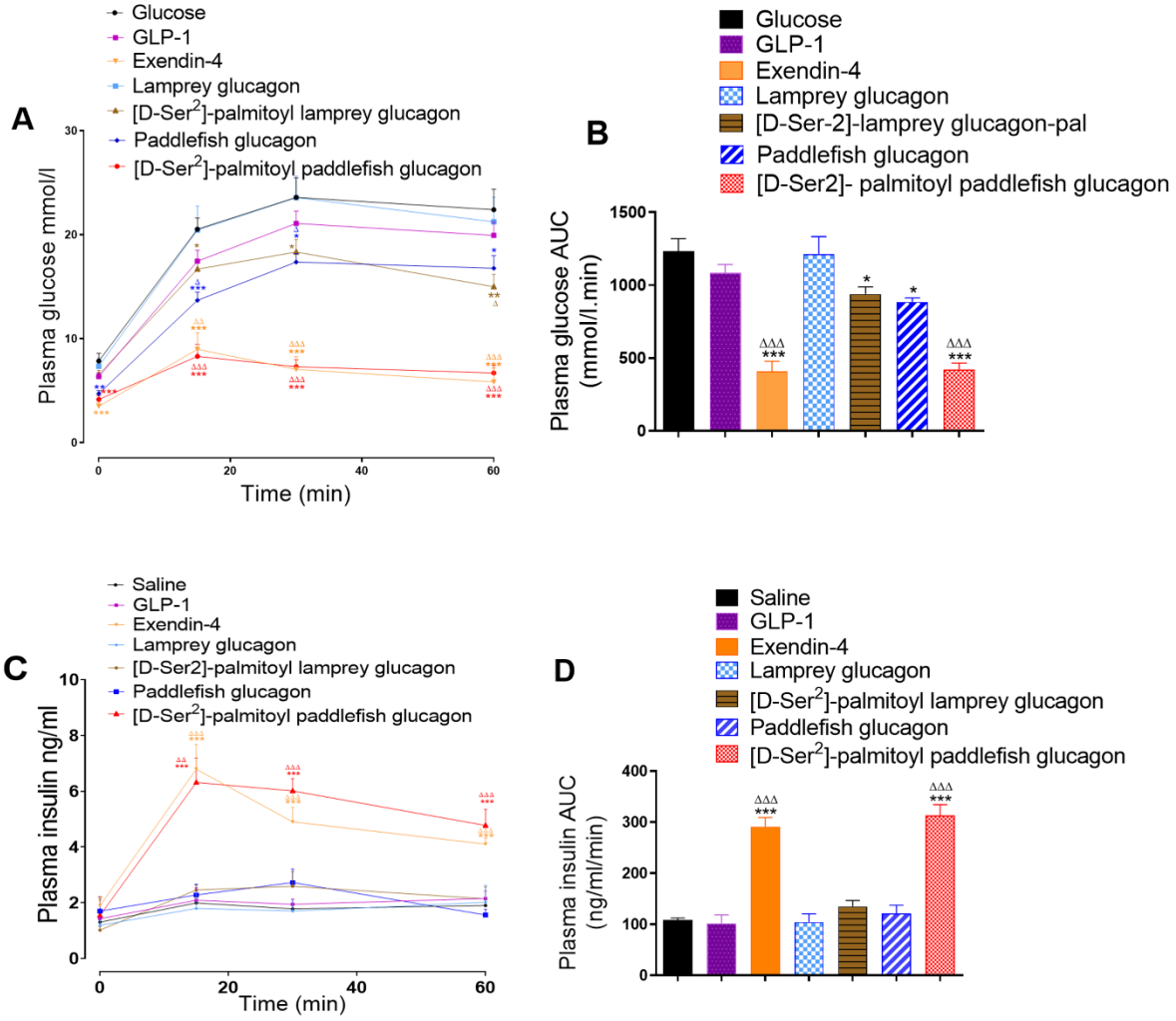
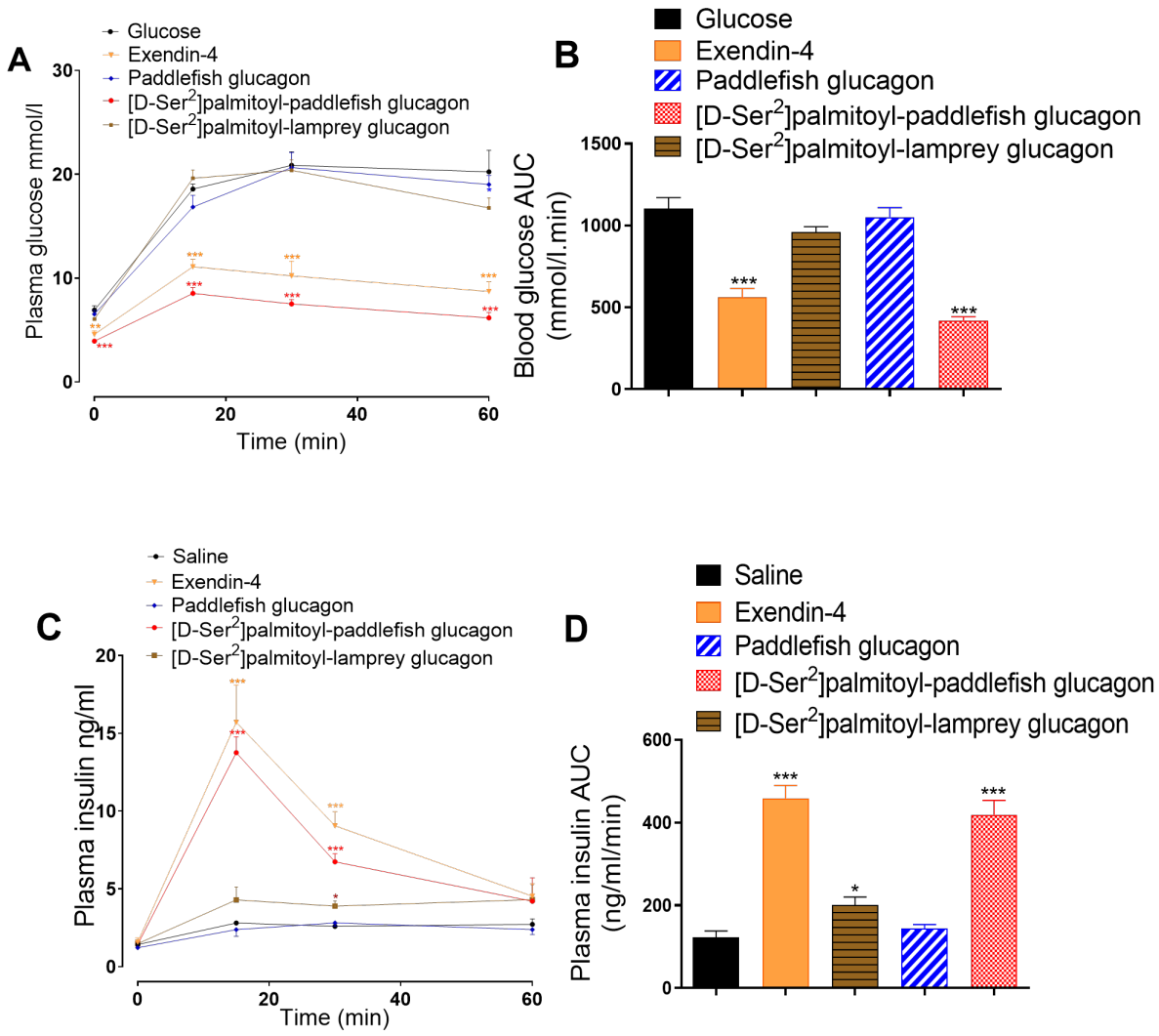

A
1.4 mM glucose
16.7 mM glucose (control)
16.7 mM glucose + 10 mM Alanine
16.7 mM glucose + 20 mM KCl
16.7 mM glucose + 10 mM Arginine
16.7 mM glucose + 10-6 GLP-1
16.7 mM glucose + 10-6 GIP
Insulin release % of basal control (16.7mM glucose)
300
200
100
0
High Fat Control (Saline)
Liraglutide
[D-Ser2]palmitoyl-paddlefish glucagon
B
Saline (High fat control)
Liraglutide
[D-Ser2]palmitoyl-paddlefish glucagon
ins1/Actb mRNA expression
C
Gck/Actb mRNA expression
D
Slc2a2/Actb mRNA expression
E
Gcgr/Actb mRNA expression
F
Glp-1r/Actb mRNA expression
G
Gipr/Actb mRNA expression

Figure 8

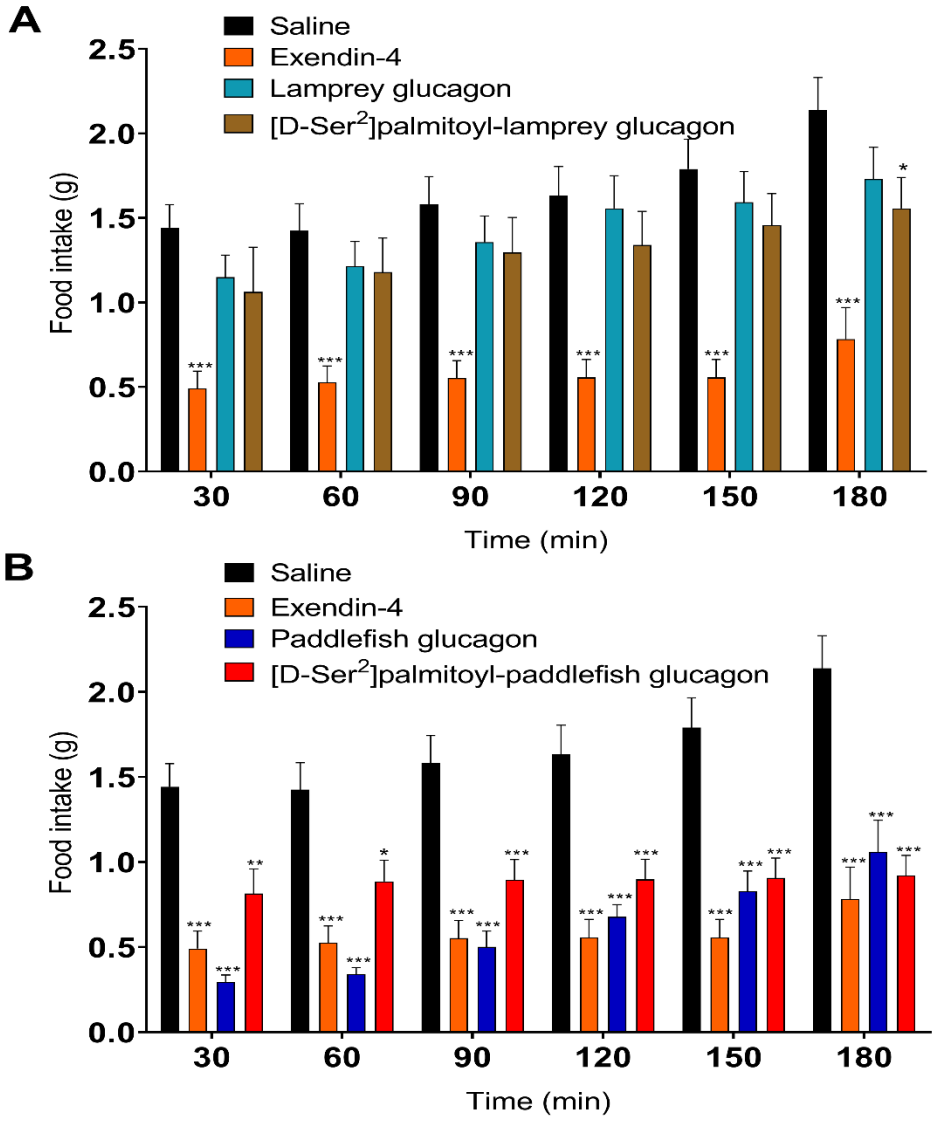
Supplementary material



Supplementary Fig. 1. Effects of lamprey glucagon, [D-Ser^2]palmitoyl-lamprey glucagon, paddlefish glucagon, [D-Ser^2]palmitoyl-paddlefish glucagon and exendin-4 (25 nmol/kg) on circulating (A) glucose and (C) insulin concentrations in lean mice administered 2 h before i.p. injection of a glucose load (18 mmol/kg). The respective integrated responses (area under the curve, AUC) are shown in panels B and D. The values are mean ± SEM for n = 6. *P<0.05 **P<0.01 and ***P<0.001 compared to glucose alone; $^{\Delta}$ P<0.05, $^{\Delta\Delta}$ P<0.01 and $^{\Delta\Delta\Delta}$ P<0.001 compared to human GLP-1.



Supplementary Fig. 2. Effects of [D-Ser^2]palmitoyl-lamprey glucagon, paddlefish glucagon, [D-Ser^2]palmitoyl-paddlefish glucagon and exendin-4 (25 nmol/kg) on circulating (A) glucose and (C) insulin concentrations in lean mice administered 4 h before i.p. injection of a glucose load (18 mmol/kg). The respective integrated responses (area under the curve, AUC) are shown in panels B and D. The values are mean ± SEM for n = 6. *P<0.05 **P<0.01 and ***P<0.001 compared to glucose alone.



Supplementary Fig. 3. Effects of (A) lamprey glucagon and [D-Ser^2]palmitoyl-lamprey glucagon and (B) paddlefish glucagon and [D-Ser^2]palmitoyl-paddlefish glucagon on cumulative food intake over 3 h feeding in 12 h fasted lean mice. Cumulative food intake was measured after intraperitoneal administration of fish peptides and exendin-4 (25 nmol/kg). The values are mean ± SEM for n = 8. *P < 0.05, **P < 0.01 and ***P < 0.001 compared with control mice treated with saline only.