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Title

Novel dual agonist peptide analogues derived from dogfish glucagon show promising *in vitro* insulin releasing actions and antihyperglycaemic activity in mice.

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Keywords

Dogfish glucagon, glucagon-like peptide-1 (GLP-1), glucose dependent insulinotropic polypeptide (GIP), glucagon, dual agonist, diabetes, acute effects, antidiabetic and antihyperglycaemic.

Abstract

The antidiabetic potential of thirteen novel dogfish glucagon derived analogues were assessed *in vitro* and in acute *in vivo* studies. Stable peptide analogues enhanced insulin secretion from BRIN-BD11 β -cells ($p < 0.001$) and reduced acute glycaemic responses following intraperitoneal glucose (25 nmol/kg) in healthy NIH Swiss mice ($p < 0.05$ - $p < 0.001$). The *in vitro* insulinotropic actions of [S2a]dogfish glucagon, [S2a]dogfish glucagon-exendin-4(31-39) and [S2a]dogfish glucagon-Lys³⁰- γ -glutamyl-PAL, were blocked ($p < 0.05$ - $p < 0.001$) by the specific GLP-1 and glucagon receptor antagonists, exendin-4(9-39) and (desHis¹Pro⁴Glu⁹)glucagon amide but not by (Pro³)GIP, indicating lack of GIP receptor involvement. These analogues dose-dependently stimulated cAMP production in GLP-1 and glucagon ($p < 0.05$ - $p < 0.001$) but not GIP-receptor transfected cells. They improved acute glycaemic and insulinotropic responses in high-fat fed diabetic mice and in wild-type C57BL/6J and GIPR-KO mice ($p < 0.05$ - $p < 0.001$), but not GLP-1R-KO mice, confirming action on GLP-1 but not GIP receptors. Overall, dogfish glucagon analogues have potential for diabetes therapy, exerting beneficial metabolic effects via GLP-1 and glucagon receptors.

Introduction

Since the start of the twenty first century there has been an explosion of interest in the use of stable incretin peptides (mimetics) for type 2 diabetes (T2DM) therapy (Holst 2004; Campbell & Drucker 2013; Irwin & Flatt 2015; Nauck 2015). These injectable agents based upon the structure of human GLP-1 have multiple antidiabetic actions, including promotion of postprandial glucose-induced insulin secretion, suppression of glucagon secretion, reduction in gastric emptying, augmentation of glucose uptake in tissues and, at least in animal models, potential benefits on pancreatic β -cell growth and regeneration (Drucker 2013; Campbell & Drucker 2013). Incretin mimetics such as exendin-4 (Byetta) and the acylated GLP-1 analogue, liraglutide (Victoza) showed promising clinical efficacy in early human trials (Madsbad 2009; Nikfar *et al.* 2012; Kela & Davies 2012; Wysham *et al.* 2013; McCormack 2014; Scott 2014) and are now widely used in clinical practice. Nevertheless, the use of single therapeutic agents to overcome the many challenges of posed by obesity and T2DM have been quite disappointing (Sadry & Drucker 2013). As a result, there is growing interest in the use of dual or co-agonist peptides which could enhance multiple metabolic pathways and provide better treatments options (Claus *et al.* 2007; Tom *et al.* 2007; Franklin *et al.* 2011; Bhat *et al.* 2013a, 2013b; Fosgerau *et al.* 2013; Trevaskis *et al.* 2013; Finan *et al.* 2013; Skarbaliene *et al.* 2015; Irwin & Flatt 2015; Irwin *et al.* 2015).

In addition to the widely accepted classical counter regulatory role for glucagon in combatting hypoglycaemia through promoting hepatic glucose output (Ramnanan *et al.* 2011), this hormone has many extrahepatic actions, including stimulation of insulin secretion, lipolysis and energy expenditure (Gelling *et al.*, 2003; Sadry & Drucker 2013; Charron & Vuguin 2015; Ye *et al.* 2015). Interestingly, both the glucagon receptor (GCGR) and GLP-1 receptor (GLP-1R) are members of the class B G-protein-coupled receptor superfamily but have opposing mechanisms of action in glucose homeostasis. However, because activation of both receptors induces satiety, co-agonist peptide analogues with optimal ratios of GLP-1 to glucagon agonism

may exert synergistically superior effects on body weight, glycaemic control and lipid metabolism (Sadry & Drucker 2013). Furthermore, a study by Pocai and colleagues (2009) testing GLP-1/glucagon dual-agonists found that normalisation of glycaemic control, as well as significant weight loss, was more impressive in diet-induced obese (DIO) mice treated with the dual agonist than treatment with a GLP-1R selective agonist alone. In another study (Tan *et al.*, 2013), GLP-1/glucagon co-agonism improved resting energy expenditure in non-diabetic, DIO mice, and it was inferred that the dual activation of these receptors would have beneficial effects in terms of glycaemic control and lipid metabolism in humans with T2DM. Day *et al.* (2009) reported that full agonism of the GLP-1 receptor in combination with a certain degree of glucagon agonism normalises glycaemic levels and enhances body fat reduction in obese rodents. Design of the best GLP-1R to glucagon ratio is difficult to predict and research in this respect is currently lacking.

Over the past 15 years, we have developed a strong interest in examining the potential of synthetic and naturally occurring peptides which have structural similarities with incretin hormones and glucagon in an attempt to uncover and test novel approaches to diabetes and obesity therapy (Irwin & Flatt 2015). The elasmobranchs, represented in the present day by sharks, dogfishes, rays and skates represent the first vertebrates in evolution to develop a pancreas containing the four kinds of islet hormone cells (β , α , δ , and PP) found in mammals (Falkmer and Van Noorden 1983). Glucagon has been isolated from the intestine of the European common dogfish *Scyliorhinus canicula* (Elasmobranchi) (Conlon *et al.* 1987 and the peptide is of particular interest because it exhibits structural similarities with human GLP-1, GIP and glucagon (Table 1). Comparison of the primary structures indicates that dogfish glucagon shares three amino acid residues (Glu³, Tyr¹³, and Lys²⁰) with human GLP-1 that are not found in human glucagon. This led us to the hypothesis that dogfish glucagon may represent a template for the design of new antidiabetic peptides that may possess multiple agonist activity. In the present paper we report the insulin-releasing and anti-hyperglycaemic activities of dogfish glucagon and

analogues designed to exhibit stability to and extended bioactivity *in vivo* (Table 1). We have looked at their effects using clonal beta cells, incretin and glucagon receptor transfected cells, specific receptor antagonists and GLP-1 and GIP receptor knockout mice. Furthermore, acute actions on glucose homeostasis and insulin release were examined in lean and high-fat fed mice with glucose intolerance and insulin resistance (Winzell and Ahren, 2004).

Materials & Methods

Peptides. Table 1 displays the amino acid sequences of dogfish glucagon, dogfish glucagon analogues, exendin-4(1-39) and the human peptides used. Preliminary studies indicated that dogfish glucagon was cleaved in plasma at positions 2, 7, 12, 13 and 21 producing smaller fragment peptides. The basic structure of dogfish glucagon was modified at each of these positions including substitution of a D-Ala for Ser at position 2 (i.e. [S2a]), in an attempt to confer improved enzyme resistance. In addition, we added extended C-terminus of dogfish glucagon with the last 9 amino acid residues of exendin-4(1-39) (P-S-S-G-A-P-P-P-S-amide) to produce [S2a]dogfish glucagon-exendin-4(31-39) in order to help stabilize the peptide and improve its ability to interact with the GLP-1 receptor. Finally, a gamma-glutamyl spacer with palmitate adjunct was added to the side-chains of the lysine residues at positions 12, 20, or 30 to promote albumin binding and extend *in vivo* bioactivity as previously reported for stable forms of GLP-1 and GIP (Green *et al.* 2004; Irwin *et al.* 2005; O'Harte *et al.* 2007; Kerr *et al.* 2009; Lennox *et al.* 2013; Pathak *et al.* 2015). Mammalian (human) forms of GLP-1, GIP, glucagon, [S2a]GIP, [S2s]glucagon, [S2a]GLP-1, (Pro³)GIP and desHis¹Pro⁴Glu⁹-glucagon amide and exendin-4(9-39) were also used in some of the tests as indicated in the text. All peptides were purchased from EZBiolabs (Carmel, IL, USA; greater than 95% purity). In addition to QC data supplied, all peptides were checked for purity and their molecular mass was confirmed using in-house matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-ToF MS) as described previously (Gault *et al.* 2011).

Plasma degradation assay. Peptides (20 µg) were incubated at 37°C in 50 mmol/L TEA-HCl (pH 7.8) with pooled mouse plasma in order to test their enzymatic stability. Degradation profiles were obtained using RP-HPLC analysis as described previously (Gault *et al.* 2011) and HPLC peak area data were used to calculate percentage intact peptide remaining after 8 h incubation.

In vitro insulin secretion. Effects of peptides on *in vitro* insulin secretion were examined using clonal rat BRIN-BD11 beta cells. The generation and characteristics of this cell line have been reported previously (McClenaghan *et al.* 1996). Briefly, BRIN-BD11 cells were seeded (150,000 cells per well) into 24-well plates (Nunc, Roskilde, Denmark) and allowed to attach overnight at 37°C. Following 40 min pre-incubation (1.1 mmol/L glucose; 37°C), cells were incubated (20 min; 37°C) in the presence of 5.6 and 16.7 mmol/L glucose with a range of peptide concentrations (10^{-12} to 10^{-6} mol/L). Insulin secretion was also measured in the presence of specific receptor antagonists including exendin-4(9-39), (Pro³)GIP and desHis¹Pro⁴Glu⁹-glucagon amide which block GLP-1, GIP and glucagon receptors, respectively (Green *et al.* 2005; Parker *et al.* 2007; O'Harte *et al.* 2013). After 20 min incubation, buffer was removed from each well and aliquots (200 µl) stored at -20°C prior to determination of insulin by radioimmunoassay (Flatt & Bailey 1981).

In vitro cAMP production. Effects of dogfish glucagon analogues as well as exendin-4, GLP-1, GIP and glucagon on cellular cAMP production were assessed in Chinese Hamster Lung (CHL) cells transfected with either the human GIP- or GLP-1-R, as well as human embryonic kidney (HEK293) cells transfected with the human glucagon-R (Bhat *et al.* 2013b). The generation and properties of these cells have been reported previously (Thorens *et al.* 1993; Gremlich *et al.* 1995). Cells were seeded (200,000 cells per well) into 96-well plates (Nunc) and washed with

Hank's Balanced Salt Solution (HBSS) buffer before incubation with test peptides (10^{-6} to 10^{-12} mol/L) in the presence of 200 μ mol/l 3-isobutyl-1-methylxanthine (IBMX) for 20 min at 37°C. After incubation, medium was removed and cells lysed before measurement of cAMP using Parameter cAMP assay (R&D Systems, Abingdon, UK) according to the manufacturer's instructions (Hogan *et al.* 2011).

Animals. Acute animal studies were carried out in male NIH Swiss mice (Harlan Ltd., Blackthorne, UK; 8 to 20 weeks old), and in C57BL/6 mice with genetic deletion of either of the incretin receptors GIP-R or GLP-1R. The background and generation of GIP-R and GLP-1R have been previously described (Scrocchi *et al.* 1996; Miyawaki *et al.* 1999). Acute experiments were also performed in NIH Swiss TO mice previously fed a high-fat diet for 100 days composed of 45% fat, 20% protein and 35% carbohydrate (total energy 26.15 KJ/g; Special Diet Services, Essex, UK). Animals were housed in a 12:12 h light/dark cycle and had free access to drinking water and food. All animal experiments were conducted according to UK Home Office Regulations (UK Animals Scientific Procedures Act 1986) and the "Principles of Laboratory Animal Care" (NIH Publication Number 86-23, revised 1985). No adverse effects were observed following administration of any of the peptides.

Acute actions of peptides on plasma glucose and insulin concentrations in vivo. Plasma glucose and insulin responses were evaluated after intraperitoneal (i.p.) injection of glucose alone (18 mmol/kg body weight) or in combination with test peptides (25 nmol/kg body weight) in normal and DIO mice. In a separate series of experiments, plasma glucose and insulin responses were evaluated after i.p. injection of glucose alone (18 mmol/kg body weight) at 0 h or 4 h after an i.p. injection of dogfish glucagon or related analogues, glucagon, GLP-1 or exendin-4 (all at 25 nmol/kg body weight) in normal lean Swiss TO mice or DIO mice. All test solutions were administered in a final volume of 8 ml/kg body weight.

Acute effect of dogfish glucagon and its analogues on plasma glucose and insulin in vivo with C57BL/6J control, GIPr-KO and GLP-1r-KO mice

For better understanding of receptor specificity of dogfish glucagon and its analogues C57BL/6J wild-type, GIPr-KO and GLP-1r-KO mice were used. Mice received an i.p. injection of glucose alone (18 mmol/kg body weight), or combined with either dogfish glucagon, dogfish glucagon analogues or exendin-4(1-39) (all at 25 nmol/kg body weight). Blood glucose and plasma insulin was measured at times 0, 15, 30, 60, 90 and 120 min.

Biochemical analyses. Blood samples were collected from the cut tip on the tail vein of conscious mice into chilled fluoride/heparin glucose micro-centrifuge tubes (Sarstedt, Numbrecht, Germany) at the time points indicated in the Figures. Samples were immediately centrifuged using a Beckman microcentrifuge (Beckman Instruments, Galway, Ireland) for 1 min at 13,000 \times g. Plasma glucose was assayed by an automated glucose oxidase procedure using a Beckman Glucose Analyzer II (Beckman Instruments, Galway, Ireland). Plasma insulin was assayed by a modified dextran-coated charcoal RIA (Flatt & Bailey 1981).

Statistical analysis. Results are expressed as means \pm SEM and data compared using the unpaired Student's *t*-test. Where appropriate, data were compared using repeated measures ANOVA or one-way ANOVA, followed by the Student-Newman-Keuls *post-hoc* test. Incremental area under the curve (AUC) analyses for plasma glucose and insulin were calculated using GraphPad Prism version 5.0. EC₅₀ values were calculated from sigmoidal dose-response data using GraphPad Prism version 5.0. Groups of data were considered to be significantly different if $P < 0.05$.

Results

Stability of peptides in plasma.

Following exposure of dogfish glucagon to mouse plasma, various fragment peptides including 14-29, 1-17, 1-22 were identified by a combination of HPLC and MS analysis (data not shown). Since we were aware that His-Ala is rapidly removed by DPP-4 in the case of GLP-1 and we were interested in testing Ala in the penultimate position we made additional modifications at this position within the analogues tested (Table 1). Dogfish glucagon and all analogues were degraded to varying degrees when incubated with mouse plasma for 8 h (data not shown). Preliminary data indicated that substitution of the Ser² residue in native dogfish glucagon (39% degraded after 8 h) by D-Ala, or by 2-aminoisobutyric acid (Aib) or 2-aminoisobutyric acid (Abu) (Table 1) showed no significant improvement in the rate of peptide degradation. However, peptides that contained a D-Ala² substitution together with either a C-terminal extension by exendin-4(31-39) or a palmitate coupled via a γ -glutamyl linker to the ϵ -amino group of the Lys³⁰ residue showed enhanced stability in plasma at 8 h (18% and 12% degraded, respectively). In contrast, the derivatives containing an acylated modification at Lys²⁰ or at position Lys¹², did not show increased resistance to cleavage by plasma peptidases with 34% and 38% degraded after 8 h, respectively.

In vitro insulin secretion. Table 2 displays the effects of dogfish glucagon and related analogues on insulin secretion in BRIN-BD11 cells. All peptides (10^{-10} to 10^{-6} mol/l), significantly ($P < 0.001$) increased insulin secretion compared to 5.6 mmol/l glucose control. A range of insulin secretory potencies were observed (Table 2) with [S2a][S2a]dogfish glucagon, [S2a][S2a]dogfish glucagon-exendin-4(31-39), and [S2a]dogfish glucagon-Lys³⁰- γ -glutamyl-PAL exhibiting impressive insulintropic actions ($P < 0.05$ to $P < 0.01$) when compared to the native dogfish glucagon (Table 2; Fig. 1). The effects observed were at least comparable with the insulintropic actions of equimolar GLP-1 and more efficacious than either human glucagon or GIP (Fig. 1).

Acute glucose-lowering and insulinotropic actions of dogfish glucagon peptide analogues in lean and high-fat fed mice.

The acute *in vivo* effects of dogfish glucagon, dogfish glucagon analogues and human glucagon on glucose homeostasis were studied in normal healthy NIH Swiss mice (Fig. 2 and Table 2). Intraperitoneal injection of glucagon (25 nmol/kg body weight) together with glucose (18 mmol/kg/body weight) resulted in a significant increase ($p < 0.05$) in plasma glucose concentration at 60 min post injection compared to mice receiving glucose alone (Fig. 2A). Consistent with this, plasma glucose area under the curve (AUC) was also increased ($p < 0.05$) (Fig. 2A). In contrast, dogfish glucagon, [S2a]dogfish glucagon, and [S2a]glucagon-exendin-4(31-39) effectively lowered blood glucose ($AUC_{0-120 \text{ min}}$, $p < 0.001$) when compared with glucose alone (Fig. 2A). This anti-hyperglycaemic action was reflected in the enhanced insulinotropic response produced by the same analogues (Fig. 2B; ($p < 0.01$ to $p < 0.001$)). Plasma glucose and insulin AUC data for all dogfish peptides are shown in Table 2. Analogues modified at position 2 and C-terminally modified analogues of dogfish glucagon are identified as being amongst the most effective. The effects observed, including EC_{50} and E_{max} values, were at least comparable with the insulinotropic actions of equimolar GLP-1 and more efficacious than either human glucagon or GIP (Fig. 1). On the basis of these data together with measures of stability in plasma and insulin-releasing activity (Tables 1 and 2), dogfish glucagon (control) together with [S2a]dogfish glucagon, [S2a]dogfish glucagon-exendin-4(9-39), and [S2a]dogfish glucagon-Lys30- γ -glutamyl-PAL were carried forward for further evaluation.

Although [S2a]dogfish glucagon-Lys³⁰- γ -glutamyl-PAL showed no appreciable effect on both plasma glucose and plasma insulin in the acute *in vivo* tests (Fig. 2A and Fig. 2B), the acylated peptide showed enhanced anti-hyperglycaemic and insulinotropic actions when a 4 h delay was incorporated into the ipGTT (Fig. 3A and Fig. 3B). Dogfish glucagon, [S2a]dogfish glucagon, and [S2a]dogfish glucagon-exendin-4(31-39) also displayed significant glucose lowering and insulinotropic capabilities (Fig. 3A and Fig. 3B).

Feeding a high fat diet for 16 weeks resulted in progressive body weight gain (54.6 ± 2.6 vs. 47.0 ± 2.7 g; $P < 0.01$) and non-fasting hyperglycemia (8.6 ± 1.2 vs. 4.6 ± 0.3 mmol/l; $P < 0.05$) compared with age-matched controls on normal laboratory chow (data not shown). When glucose tolerance tests were performed in the high-fat fed mice, neither dogfish glucagon nor [S2a]dogfish glucagon-Lys³⁰- γ -glutamyl-PAL reduced the glycaemic excursion compared to mice given glucose alone (Fig. 4A). In contrast, the dogfish glucagon analogues [S2a]dogfish glucagon and [S2a]dogfish glucagon-exendin-4(31-39) reduced the plasma glucose excursion (Fig. 4A, AUC_{0-120 min}, $p < 0.05$ to $p < 0.001$). However, dogfish glucagon and all analogues tested did enhance the overall insulinotropic response (Fig. 4B, $p < 0.05$ to $p < 0.001$). These effects were equivalent to those observed with human GLP-1 or exendin-4(1-39) (Fig. 4B).

Measurement of intracellular cAMP in GIP, GLP-1 transfected cell lines and HEK293SGnT1-cells

To gain insight into the receptors mediating the actions of dogfish glucagon and its analogues, their ability to stimulate cAMP production was investigated using GLP-1, GIP and glucagon receptor transfected cells (Fig. 5). A significant and dose-dependent increase ($p < 0.05$ to $p < 0.001$) in intracellular cAMP was observed in GLP-1 and glucagon receptor transfected cells (Fig. 5A and Fig. 5C). The actions were broadly equivalent to those of human GLP-1 or glucagon with approximately similar EC₅₀ and E_{max} values. This pattern contrasted sharply with the cAMP response observed with GIP receptor transfected cells where no stimulation of intracellular messenger was detected (Fig. 5B). These data suggest that dogfish glucagon and related analogues operate through activation of the GLP-1 and glucagon receptors but not via the GIP receptor.

Effect of dogfish glucagon and dogfish glucagon analogues on insulin secretion from BRIN-BD11 cells in the presence of specific receptor antagonists.

To assess further the mechanism of action, insulinotropic effects of dogfish glucagon and the chosen 3 analogues at a concentration of 10^{-7} M were investigated using BRIN-BD11 cells in the absence and presence of various receptor antagonists. These included exendin-4(9-39), (Pro³)GIP and (desHis¹Pro⁴Glu⁹)glucagon amide (peptide O) which block GLP-1, GIP and glucagon mediated actions, respectively. None of the three antagonist peptides tested had any effect on insulin secretion when tested alone (Fig. 6). The established secretagogue alanine (10 mM) was used as positive control. All dogfish glucagon-related peptides significantly stimulated insulin release ($p < 0.001$). Exendin-4(9-39) (10^{-6} M and 10^{-7} M) consistently abolished dogfish glucagon-stimulated insulin release ($p < 0.05$ – $p < 0.001$) (Fig. 6A-D). The glucagon receptor antagonist Peptide O ((desHis¹Pro⁴Glu⁹)glucagon amide) also inhibited insulin release by [S2a]dogfish glucagon ($p < 0.05$ – $p < 0.01$) (Fig. 6B), [S2a]dogfish glucagon-exendin-4(31-39) ($p < 0.05$) (Fig. 6C), and [S2a]dogfish glucagon-Lys³⁰- γ -glutamyl-PAL ($p < 0.01$) compared to results obtained for dogfish glucagon analogues in the absence of this antagonist (Fig. 6D). In contrast, (Pro³)GIP failed to inhibit the insulin release produced by the various dogfish glucagon analogues (Fig. 6A-D), further confirming that these peptides did not operate via the GIP receptor.

Acute effect of dogfish glucagon and dogfish glucagon analogues on plasma glucose and insulin in vivo using incretin receptor knockout mice

Possible involvement of GLP-1 and GIP receptors in mediating *in vivo* actions of dogfish glucagon and analogues was examined using wild-type C57BL/6 mice and corresponding incretin receptor KO mice (Fig. 7). When injected acutely together with glucose (18 mmol/kg) in wild-type controls, dogfish glucagon, [S2a]dogfish glucagon, [S2a]dogfish glucagon-exendin-4(31-39) and [S2a]dogfish glucagon-Lys³⁰- γ -glutamyl-PAL significantly decreased plasma glucose concentrations at 30 min and 60 min ($p < 0.05$ - $p < 0.01$). The integrated plasma glucose concentration (AUC_{0-120} min) was also significantly lowered ($p < 0.001$) compared with glucose alone (Fig. 7A) and this was reflected in an enhanced insulinotropic response (Fig. 7B). A

similar pattern was observed in GIPr-KO mice (Fig. 7A and Fig. 7B) but not in GLP-1 KO mice (Fig. 7A and Fig. 7B). From these results, it is concluded that dogfish glucagon and the three analogues selected operate through the GLP-1 receptor but not the GIP receptor thereby confirming the findings from the *in vitro* cell studies using specific incretin receptor antagonists.

Discussion

Multiple-acting co-agonist peptides represent novel therapeutic agents which could be beneficial in the ongoing fight against the growing global health challenge of T2DM, obesity and related metabolic diseases (Sadry and Drucker, 2013; Pocai *et al.*, 2009). In this study, we examined insulin secretory and metabolic responses to dogfish glucagon which we hypothesized might replicate actions of glucagon together with other important members of the glucagon-secretin family of peptides (Kopin *et al.* 1991; Inagaki *et al.* 1996). Indeed, dogfish glucagon shares 52%, 55% and 83% structural identity with human GIP, GLP-1 and glucagon, respectively. From an evolutionary perspective, it has been suggested that the glucagon sequence first arose approximately 1000 million years ago (MYA) while GLP-1 and GLP-2 diverged from each other approximately 700 MYA (Irwin *et al.* 1999). The physiological roles of the proglucagon-derived peptides in the ancient fishes such as the dogfish have not been investigated but, in teleosts, the responsiveness of hepatocytes to glucagon is limited to high concentrations, while physiological concentrations of GLP-1 effectively regulate hepatic metabolism. In contrast to mammals, GLP-1 shows very weak insulinotropic activity in teleosts (Plisetskaya and Mommsen, 1996).

Mammalian glucagon is subjected to rapid degradation *in vivo* by dipeptidyl peptidase-4 (DPP-4), with cleavage of Ser²-Gln³ (producing the major fragment glucagon 3-29), followed by further removal of the following dipeptide (producing glucagon 5-29) (Hinke *et al.* 2000; Pospisilik *et al.* 2001) as well as by neutral endopeptidase 24.11 (Trebbien *et al.*, 2004). Short half-life is a problem for potential therapeutic peptides (Ahren 2004; Werle and Bernkop-Schnürch 2006) and to circumvent this difficulty, we designed a series of dogfish glucagon

analogues modified at positions 2, 13, 21 (Table 1) in order to confer peptidase resistance (Werle and Bernkop-Schnürch 2006). As expected native dogfish glucagon was degraded in plasma and several of the structural modifications made afforded significant resistance to degradation *in vitro*. In particular, we synthesized two daughter analogues of [S2a]dogfish glucagon with C-terminal extension using the last 9 C-terminal amino acids of exendin-4 and other analogues with acylation at Lys¹², Lys²⁰ or Lys³⁰. The latter is a well-established approach to extend *in vivo* bioactivity through enhanced binding to albumin in blood (Kurtzhals *et al.* 1995; Holz & Chepurny 2003), whereas the former is thought to enhance the peptide's affinity for the GLP-1 receptor (Al-Sabah and Donnelly, 2003). These peptides exhibited the lowest rates of degradation observed presumably due to protein binding resulting in steric hindrance at target sites of plasma enzymes as well as the enhanced ability of the peptide to self-associate in solution in a manner similar to liraglutide (Knudsen 2004).

Dogfish glucagon and all analogues tested stimulated insulin release from clonal BRIN-BD11 cells. Whereas the native peptide was slightly less potent than equimolar concentrations of human glucagon, GLP-1 and GIP, the analogues [S2a]dogfish glucagon, [S2a]dogfish glucagon-exendin-4(31-39) and [S2a]dogfish glucagon(Lys30)PAL exhibited greater potency and efficacy than one or more of these human peptides as indicated by EC₅₀ and E_{max} values. Similarly, the native form and each of the modified dogfish glucagon peptides demonstrated significant glucose lowering and insulin-releasing activity during glucose tolerance tests in normal mice. This was in marked contrast to human glucagon which was hyperglycaemic and only prompted a small rise in circulating insulin concentrations. This response is consistent with its counter regulatory role in mammals whereas in teleost fish plasma concentrations of GLP-1 are higher than those of glucagon and both GLP-1 and glucagon exert similar effects on glucose metabolism (Plisetskaya and Mommsen, 1996). [S2a]dogfish glucagon, [S2a]dogfish glucagon-exendin-(31-39) and [S2a]glucagon(Lys³⁰)PAL were again amongst the most active peptides in lean mice and subsequent studies demonstrated that their effect persisted for at least 4 h post injection. This

suggests the success of strategies employed to confer enzymatic stability and extend *in vivo* bioactivity as also observed for stable incretin peptide hormones (Holz & Chepurny 2003; Knusen 2004). In the high-fat fed mouse model of obesity-diabetes (Surwit *et al.* 1988), dogfish glucagon lacked glucose lowering ability despite a small increase of insulin. This presumably reflect the insulin resistance in this model (Turner *et al.* 2013). However, the three analogues of dogfish glucagon tested here exhibited anti-hyperglycaemic effects similar to GLP-1 or exendin-4 in high-fat fed mice.

From these observations, it appears that dogfish glucagon does not operate in a manner analogous to mammalian glucagon when administered to mice. The action profile more resembles that of oxyntomodulin which is a naturally occurring C-terminally extended glucagon molecule known to activate both glucagon and GLP-1 receptors (Pocai 2012; Irwin & Flatt 2015). Dogfish glucagon, like human glucagon, has 29 amino acid residues so there is not a great similarity in C-terminal structure compared with oxyntomodulin itself. However, when native dogfish glucagon and the three lead analogues were tested on cells transfected with human receptors for glucagon or GLP-1, cyclic AMP production was enhanced as was observed with the native human peptide with broadly similar EC_{50} and E_{max} values. In contrast, no activity was observed in GIPR transfected cells. This receptor specificity was confirmed by blockade of *in vitro* insulinotropic activity by established antagonists of GLP-1 and glucagon but not by an antagonist of GIP. Furthermore, the peptides exhibited anti-hyperglycaemic and insulinotropic effects in GIPR KO mice, whereas these actions were abolished in mice without functional GLP-1 receptors. The results of the present study showing that dogfish glucagon operates through both glucagon and GLP-1 mediated pathways in mammals thereby offers a possible new candidate molecule for T2DM treatment by co-agonistic action. Evidence has been obtained from preclinical studies to support a positive role for glucagon acting as a satiety factor, increasing resting energy expenditure and promoting lipolysis in adipose tissue (Habegger *et al.*, 2010). As highlighted in recent years, GLP-1 has many physiological actions which can help ameliorate diabetes,

including reduction of food intake, slowing of gastric emptying, promotion of glucose uptake in peripheral tissues, inhibition of glucagon release and stimulation of glucose dependent insulin secretion and enhancement of pancreatic β -cell function (Campbell & Drucker, 2013). Further long-term studies in animal models of diabetes are required to test effectiveness of long-acting dogfish glucagon analogues in animal models and whether the above and any other actions can afford new and improved therapeutic options.

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Conflict of Interest

Ulster University and FOH, PRF and JMC hold patents for use of therapeutic peptides for treatment of obesity-diabetes.

Contributions

FOH and PRF devised and planned the studies and wrote the manuscript. NG and AL carried out experimental work, analysed the data and commented on the manuscript. JMC contributed to analysis of data and revision of manuscript. All authors approved the final version of the manuscript.

ACCEPTED MANUSCRIPT

Legends to Figures

Figure 1. Effects of dogfish glucagon (A), [S2a]dogfish glucagon (B), [S2a]dogfish glucagon-exendin-4(31-39) (C) and [S2a]dogfish glucagon-Lys³⁰- γ -glutamyl-PAL (D) on insulin release from BRIN-BD11 cells. Values are mean \pm SEM for n=8 for insulin release. *p<0.05, **p<0.01 and ***p<0.001 compared to 5.6 mM glucose alone. +p<0.05, ++p<0.01 and +++p<0.001 compared to 10⁻⁷ M dogfish glucagon, [S2a]dogfish glucagon, [S2a]dogfish glucagon-exendin-4(31-39) or [S2a]dogfish glucagon-Lys³⁰- γ -glutamyl-PAL in parallel A-D, respectively.

Figure 2. Effects of dogfish glucagon, [S2a]dogfish glucagon, [S2a]dogfish glucagon –exendin-4(31-39) and [S2a]dogfish glucagon-Lys³⁰- γ -glutamyl-PAL on plasma glucose (A) and insulin response (B) in lean mice. Blood glucose and plasma insulin were measured before and after intraperitoneal administration of glucose (18 mmol/kg body weight) alone or in combination with glucagon, dogfish glucagon, [S2a]dogfish glucagon, [S2a]dogfish glucagon-exendin-4(31-39) or [S2a]dogfish glucagon-Lys³⁰- γ -glutamyl-PAL (each at 25 nmol/kg body weight). Integrated blood glucose (B) and plasma insulin (D) area under the curve (AUC_{0-120 min}) values are also included. Values represent mean \pm SEM for 8 mice. *p<0.05, **p<0.01 and ***p<0.001 compared to glucose alone. ++p<0.01 and +++p<0.001 compared to glucagon treated mice.

Figure 3. Acute effects of dogfish glucagon, [S2a]dogfish glucagon, [S2a]dogfish glucagon-exendin-4(31-39) and [S2a]dogfish glucagon-Lys³⁰- γ -glutamyl-PAL on blood glucose (A) and insulin response (B) in lean mice, 4 h post injection. Blood glucose and plasma insulin were measured after a glucose load (18 mmol/kg body weight) 4 hours after intraperitoneal administration of saline, dogfish glucagon, [S2a]dogfish glucagon, [S2a]dogfish glucagon-exendin-4(31-39) or [S2a]dogfish glucagon-Lys³⁰- γ -glutamyl-PAL (each at 25 nmol/kg body weight). Integrated blood glucose (B) and plasma insulin (D) (area under the curve, AUC) responses are also included. Values represent mean \pm SEM for 8 mice. * p <0.05 and ** p <0.01 compared to saline.

Figure 4. Effects of dogfish glucagon, [S2a]dogfish glucagon, [S2a]dogfish glucagon-exendin-4(31-39) and [S2a]dogfish glucagon-Lys³⁰- γ -glutamyl-PAL on blood glucose (A) and insulin response (C) in high fat fed mice. Plasma glucose and insulin were measured before and after intraperitoneal administration of glucose (18 mmol/kg body weight) alone or in combination with dogfish glucagon, [S2a]dogfish glucagon, [S2a]dogfish glucagon-exendin-4(31-39), [S2a]dogfish glucagon-Lys³⁰- γ -glutamyl-PAL, GLP-1 or exendin-4 (each at 25 nmol/kg body weight). Integrated blood glucose (B) and plasma insulin (D) (area under the curve, AUC) responses are also included. Values represent mean \pm SEM for 8 mice. * p <0.05, ** p <0.01 and *** p <0.001 compared to glucose alone.

Figure 5. Effects of dogfish glucagon and related peptides on intracellular cAMP production in Chinese hamster lung cells transfected with human GLP-1 (A), GIP (B) and glucagon (C) receptors. Intracellular cAMP was measured from GLP-1, GIP or glucagon receptor transfected cells exposed to dogfish glucagon and related peptides (10^{-9} to 10^{-6} M) for 60 min. EC₅₀ values are shown for peptides with significant effects on cAMP production. Values are mean \pm SEM for $n=8$. * p <0.05, ** p <0.01 and *** p <0.001 compared to none (control).

Figure 6. Effects of 10^{-7} M dogfish glucagon (A), [S2a]dogfish glucagon (B), [S2a]dogfish glucagon-exendin-4(31-39) (C) and [S2a]dogfish glucagon-Lys³⁰- γ -glutamyl-PAL (D) in the presence of different antagonists, exendin-4(9-39), (Pro³)GIP and peptide O ((desHis¹Pro⁴Glu⁹)glucagon amide) on insulin secretion from BRIN-BD11 cells. Values are mean \pm SEM for n=8 for insulin release. *p<0.05, **p<0.01 and ***p<0.001 compared to 5.6 mM glucose alone. +p<0.05, ++p<0.01 and +++p<0.001 compared to 10^{-7} M (A) dogfish glucagon, (B) [S2a]dogfish glucagon, (C) [S2a]dogfish glucagon-exendin-4(31-39) or (D) [S2a]dogfish glucagon-Lys³⁰- γ -glutamyl-PAL.

Figure 7. Acute effects of dogfish glucagon, [S2a]dogfish glucagon, [S2a]dogfish glucagon-exendin-4(31-39) and [S2a]dogfish glucagon-Lys³⁰- γ -glutamyl-PAL on plasma glucose (A) and insulin responses (B) in C57BL/6J mice, GIPr-KO mice or GLP-1r-KO mice. Blood glucose and plasma insulin were measured prior to and after intraperitoneal administration of glucose (18 mmol/kg body weight) alone or in combination with dogfish glucagon, [S2a]dogfish glucagon, [S2a]dogfish glucagon-exendin-4(31-39), [S2a]dogfish glucagon-Lys³⁰- γ -glutamyl-PAL or exendin-4 (each at 25 nmol/kg body weight). Integrated blood glucose and plasma insulin (area under the curve, AUC) responses are also included. Values represent mean \pm SEM for 8 mice. *p<0.05, **p<0.01 and ***p<0.001 compared to glucose alone. +p<0.05 and ++p<0.01 compared to exendin-4. ^Δp<0.05, ^{ΔΔ}p<0.01 and ^{ΔΔΔ}p<0.001 compared with dogfish glucagon.

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TABLE 1 Primary structures and molecular masses of dogfish glucagon and related peptides

Name	Amino acid sequence	Theoretical molecular mass (Observed mass Da)
Dogfish glucagon (1-29)	H-S-E-G-T-F-T-S-D-Y-S-K-Y-M-D-N-R-R-A-K-D-F-V-Q-W-L-M-N-T	3528.9 (3528.1)
Exendin-4	H-G-E-G-T-F-T-S-D-L-S-K-Q-M-E-E-E-A-V-R-L-F-I-E-W-L-K-N-G-G-P-S-S-G-A-P-P-S-amide	4186.6 (4186.3)
GLP-1(7-36) amide	H-A-E-G-T-F-T-S-D-V-S-Y-L-E-G-Q-A-A-K-E-F-I-A-W-L-V-K-G-R-amide	3297.7 (3296.0)
GIP(1-30)	Y-A-E-G-T-F-I-S-D-Y-S-I-A-M-D-K-I-H-Q-Q-D-F-V-N-W-L-L-A-Q-K	3551.1 (3550.9)
Human glucagon	H-S-Q-G-T-F-T-S-D-Y-S-K-Y-L-D-S-R-R-A-Q-D-F-V-Q-W-L-M-N-T	3482.8 (3481.5)
[S2a] dogfish glucagon	H-a-E-G-T-F-T-S-D-Y-S-K-Y-M-D-N-R-R-A-K-D-F-V-Q-W-L-M-N-T	3512.9 (3514.0)
[S2Aib] dogfish glucagon	H-Aib-E-G-T-F-T-S-D-Y-S-K-Y-M-D-N-R-R-A-K-D-F-V-Q-W-L-M-N-T	3527.0 (3527.4)
[S2Abu] dogfish glucagon	H-Abu-E-G-T-F-T-S-D-Y-S-K-Y-M-D-N-R-R-A-K-D-F-V-Q-W-L-M-N-T	3527.0 (3527.0)
[S2a] dogfish glucagon-exendin-4(31-39)	H-a-E-G-T-F-T-S-D-Y-S-K-Y-M-D-N-R-R-A-K-D-F-V-Q-W-L-M-N-T-P-S-S-G-A-P-P-S-amide	4289.8 (4290.3)
[S2a.T7I] dogfish glucagon	H-a-E-G-T-F-I-S-D-Y-S-K-Y-M-D-N-R-R-A-K-D-F-V-Q-W-L-M-N-T	3525.0 (3525.0)
[S2a,K12I] dogfish glucagon	H-a-E-G-T-F-T-S-D-Y-S-I-Y-M-D-N-R-R-A-K-D-F-V-Q-W-L-M-N-T	3497.9 (3496.0)
[S2a,Y13A] dogfish glucagon	H-a-E-G-T-F-T-S-D-Y-S-K-A-M-D-N-R-R-A-K-D-F-V-Q-W-L-M-N-T	3420.8 (3418.6)
[S2a,Y13y] dogfish glucagon	H-a-E-G-T-F-T-S-D-Y-S-K-y-M-D-N-R-R-A-K-D-F-V-Q-W-L-M-N-T	3512.9 (3513.4)
[S2aD21d] dogfish glucagon	H-a-E-G-T-F-T-S-D-Y-S-K-Y-M-D-N-R-R-A-K-d-F-V-Q-W-L-M-N-T	3512.9 (3513.6)
[S2a] dogfish glucagon-Lys ¹² -γ-glutamyl-PAL	H-a-E-G-T-F-T-S-D-Y-S-K-Y-M-D-N-R-R-A-K-D-F-V-Q-W-L-M-N-T -γ-Glutamyl-PAL	3779.3 (3780.7)
[S2a] dogfish glucagon-Lys ²⁰ -γ-glutamyl-PAL	H-a-E-G-T-F-T-S-D-Y-S-K-Y-M-D-N-R-R-A-K-D-F-V-Q-W-L-M-N-T -γ-Glutamyl-PAL	3880.5 (3881.5)
[S2a] dogfish glucagon-Lys ³⁰ -γ-glutamyl-PAL	H-a-E-G-T-F-T-S-D-Y-S-K-Y-M-D-N-R-R-A-K-D-F-V-Q-W-L-M-N-T-K-γ-Glutamyl-PAL	4008.7 (4009.1)

PAL = palmitate

TABLE 2 *In vitro* insulin secretory activity and *in vivo* glucose lowering and insulin releasing actions of dogfish glucagon and related analogues

Peptide (10 ⁻⁷ M)	<i>In vitro</i> insulin secretion		<i>In vivo</i> responses	
	EC ₅₀ (M)	5.6 mM glucose (ng/10 ⁶ cells/20 min)	Plasma glucose AUC _{0-60min} (mmol/l.min)	Plasma insulin AUC _{0-60min} (ng/ml.min)
None (glucose alone)	-	1.010±0.0+	641.9±27.6	13.3±1.1
Human glucagon	-	3.140±0.2***	757.4±38.1*	12.2±1.2
Dogfish glucagon (1-29)	8.6620e-008	3.329±0.6***	365.8±61.7***+++	20.32±2.59***++
[S2a] dogfish glucagon	1.5740e-008	5.875±1.4***++	290.6±51.5***+++	33.72±12.5***+++
[S2Aib] dogfish glucagon	3.9010e-008	4.278±0.8***	263.7±32.2***+++	27.77±6.4***+++
[S2Abu] dogfish glucagon	7.4080e-008	5.336±1.0***+	629.4±119.2	17.58±3.0*+
[S2a] dogfish glucagon-exendin-4(31-39)	1.2350e-008	4.191±0.5***++	215.0±21.8***+++	46.97±12.5***+++
[S2a,T7I] dogfish glucagon	2.5460e-010	1.807±0.2***	598.8±85.5	11.28±2.9
(S2a,K12I) dogfish glucagon	1.3760e-007	2.601±0.7***	451.4±126.3***+++	18.58±3.9*+
[S2a,Y13a] dogfish glucagon	3.0780e-008	4.163±0.5***++	573.8±69.8++	14.30±3.0
[S2a,Y13y] dogfish glucagon	7.6970e-007	1.990±0.5***	541.8±122.4++	17.72±4.1+
[S2a.D21d] dogfish glucagon	2.5260e-008	3.088±0.3***	526.2±165.4+	16.63±2.0+
[S2a] dogfish glucagon-Lys ¹² -γ-glutamyl-PAL	9.6230e-008	2.823±0.6***	579.8±49.9*++	16.25±2.2
[S2a] dogfish glucagon-Lys ²⁰ -γ-glutamyl-PAL	2.7530e-008	2.422±0.7***	614.3±35.0+	15.62±4.0
[S2a] dogfish glucagon-Lys ³⁰ -γ-glutamyl-PAL	6.8510e-008	3.825±0.5***+	654.8±44.0	15.00±3.7

Values for *in vitro* insulin secretion and *in vivo* responses are mean ± SEM for n=8. *p<0.05, **p<0.01 and ***p<0.001 compared to 5.6 mM glucose control. +p<0.05, ++p<0.01 and +++p<0.001 compared to human glucagon. EC₅₀ values are shown together with responses at 10⁻⁷ M peptide. Integrated glycaemic

and insulin (area under the curve, AUC) responses to peptides (25 nmol/kg body weight) are shown following i.p. administration to normal mice together with 18 mmol/kg glucose.

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FIGURE 1 Effects of dogfish glucagon (A), [S2a]dogfish glucagon (B), [S2a]dogfish glucagon-exendin-4(31-39) (C) and [S2a]dogfish glucagon-Lys³⁰- γ -glutamyl-PAL (D) on insulin release from BRIN-BD11 cells

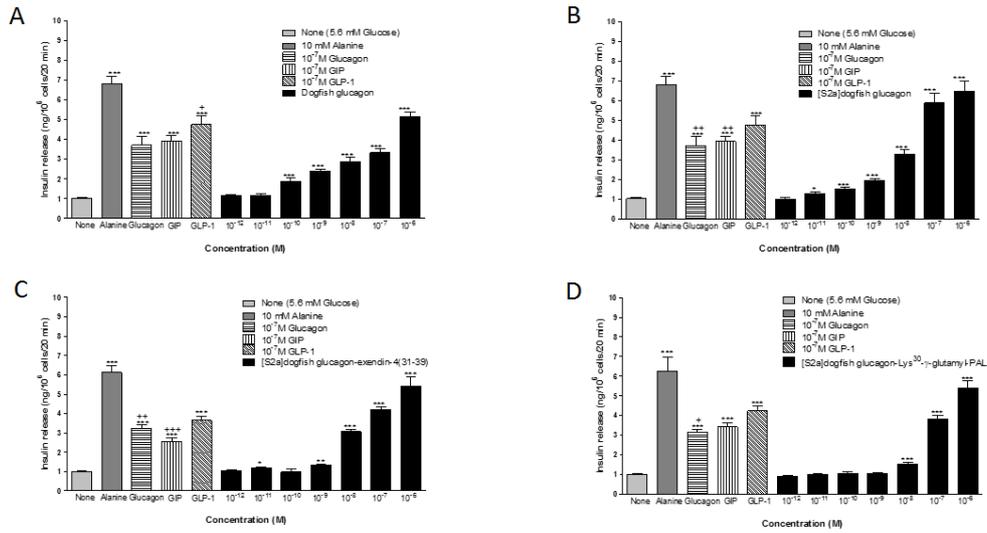


FIGURE 2 Effects of dogfish glucagon, [S2a]dogfish glucagon, [S2a]dogfish glucagon–exendin-4(31-39) and [S2a]dogfish glucagon-Lys³⁰- γ -glutamyl-PAL on plasma glucose (A+B) and insulin responses (C+D) in lean mice

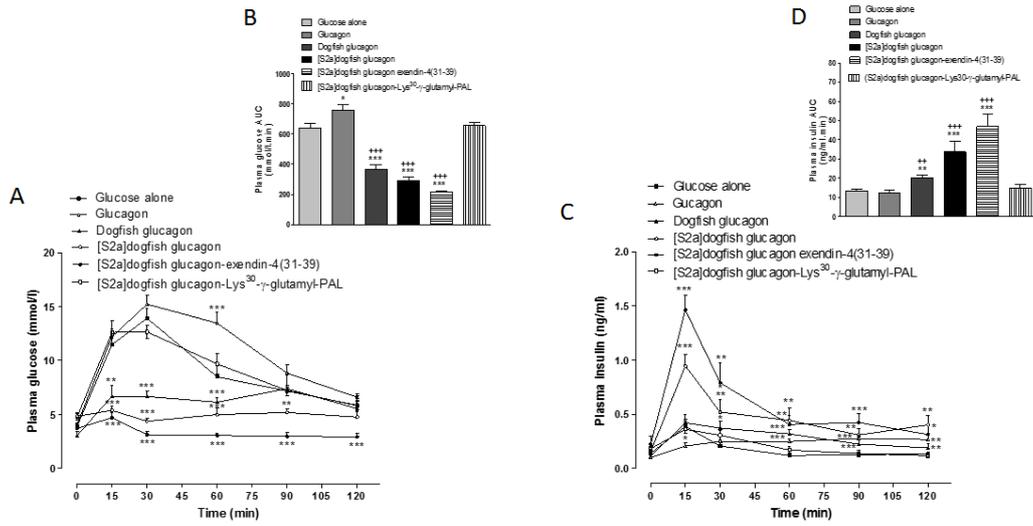
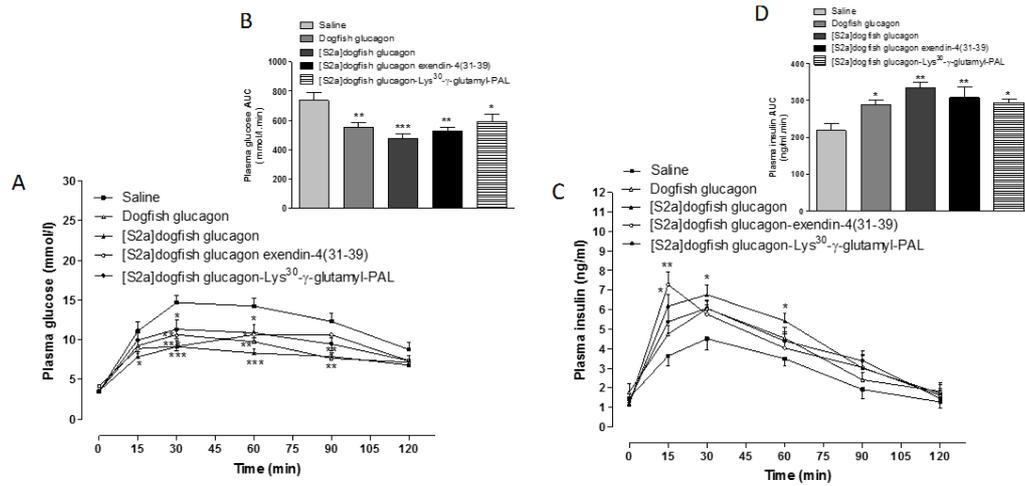


FIGURE 3 Acute effects of dogfish glucagon, [S2a]dogfish glucagon, [S2a]dogfish glucagon-exendin-4(31-39) and [S2a]dogfish glucagon-Lys³⁰- γ -glutamyl-PAL on blood glucose (A+B) and insulin response (C+D) in lean mice, 4 h post injection



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FIGURE 4 Effects of dogfish glucagon, [S2a]dogfish glucagon, [S2a]dogfish glucagon-exendin-4(31-39) and [S2a]dogfish glucagon-Lys³⁰- γ -glutamyl-PAL on blood glucose (A+B) and insulin responses (C+D) in high fat fed mice

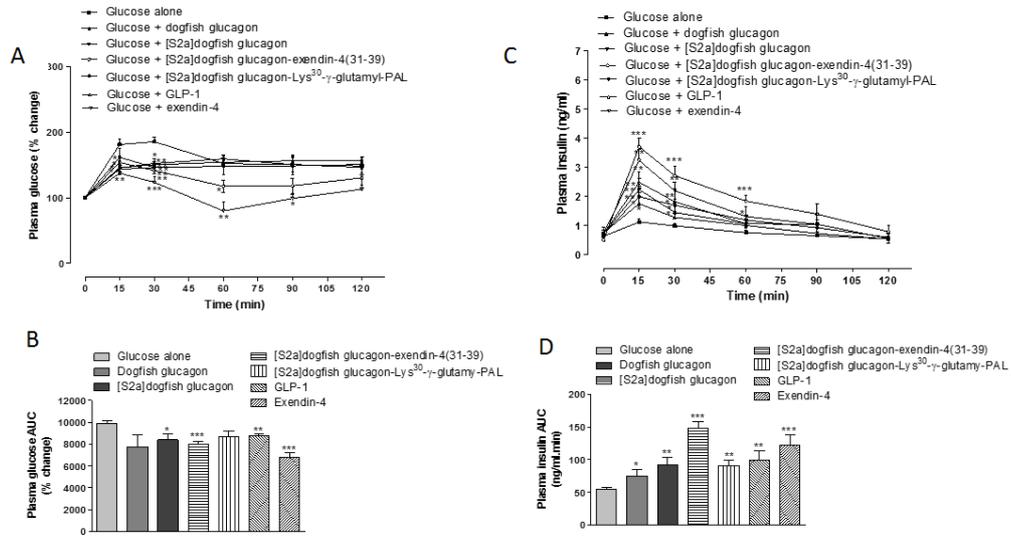
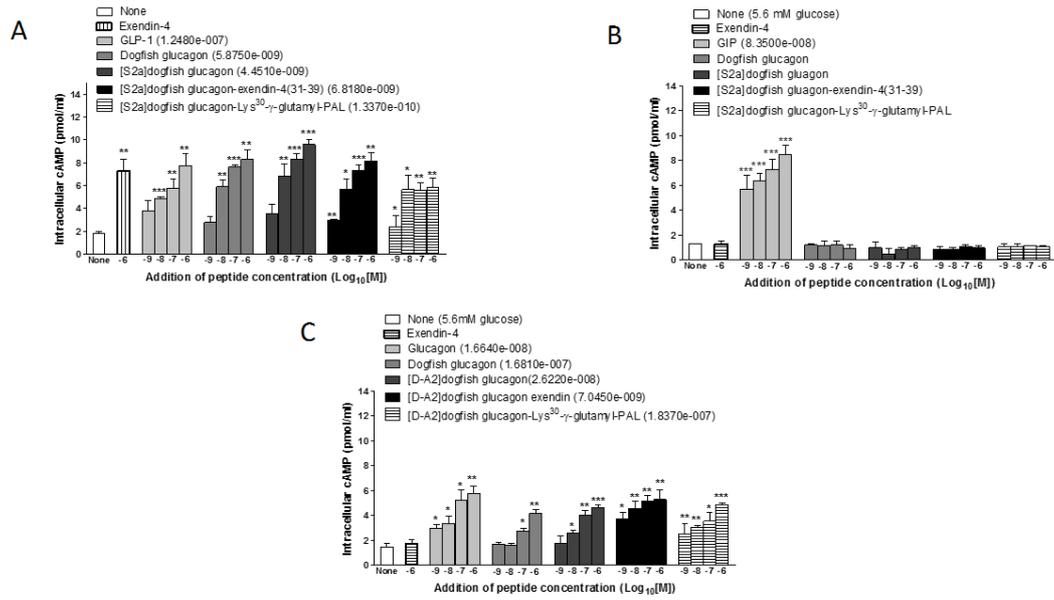


FIGURE 5 Effects of dogfish glucagon and related peptides on intracellular cAMP production in GLP-1 (A), GIP (B) and glucagon (C) receptor transfected cells

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FIGURE 6 Effects of 10^{-7} M dogfish glucagon (A), [S2a]dogfish glucagon (B), [S2a] dogfish glucagon-exendin-4(31-39) (C) and [S2a]dogfish glucagon-Lys30- γ -glutamyl-PAL (D) in the presence of different antagonists, exendin-4(9-39), (Pro³)GIP and peptide O on insulin secretion from BRIN-BD11 cells

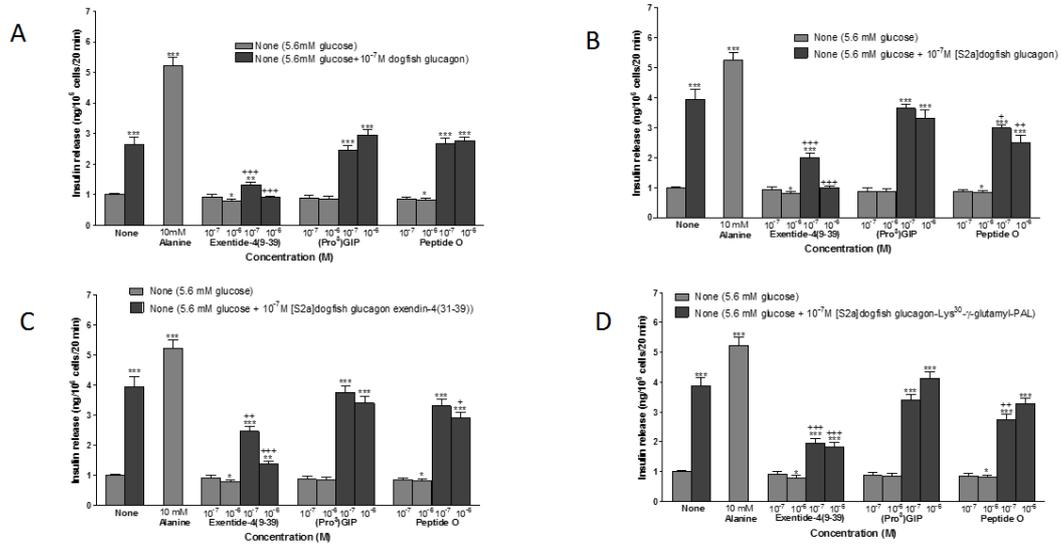
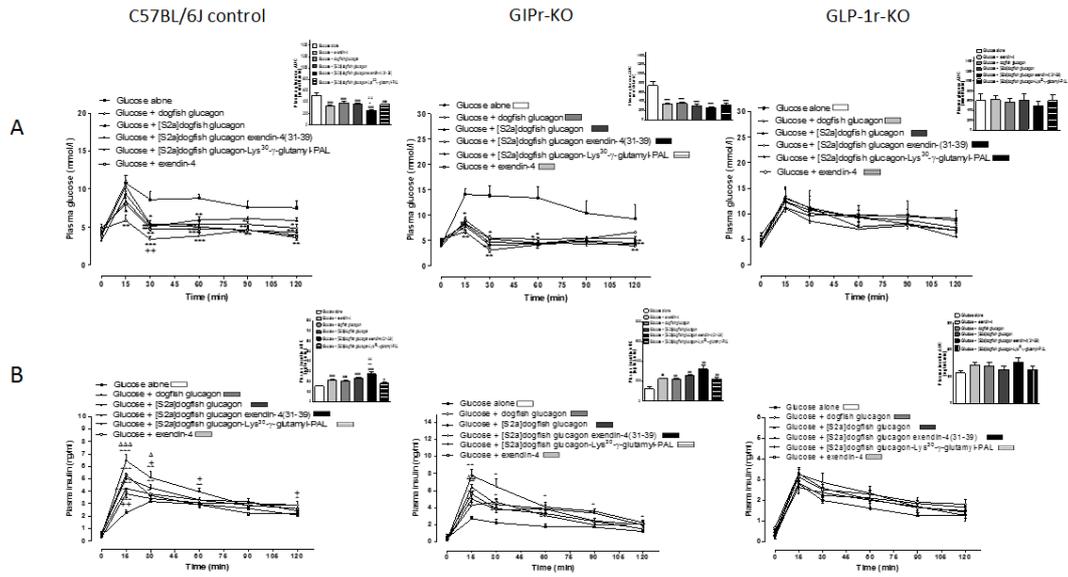


FIGURE 7 Acute effects of dogfish glucagon, [S2a]dogfish glucagon, [S2a]dogfish glucagon-exendin-4(31-39) and [S2a]dogfish glucagon-Lys³⁰⁻³¹-glutaryl-PAL on plasma glucose (A) and insulin responses (B) in C57BL/6J mice, GIPr-KO mice or GLP-1r-KO mice



Highlights:

- Dual agonist activity recognised in dogfish glucagon analogues
- Dogfish glucagon analogues operate through GLP-1 and glucagon receptors
- Peptide analogues improve glycaemic control in diet induced obese diabetic mice

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