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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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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-Lys^{30}-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 (desHis^{1}Pro^{4}Glu^{9})glucagon amide but not by (Pro^{3})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.

1. 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 and Drucker, 2013; Irwin and 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 and 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 and 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 and 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 and 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 and Drucker, 2013; Charron and 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

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effects on body weight, glycaemic control and lipid metabolism (Sadry and Drucker, 2013). Furthermore, a study by Pocai et al. (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 and 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 (Glu^3, Tyr^{13}, and Lys^{20}) 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).

2. Materials & methods

2.1. 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

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-P-S-amide	4186.6 (4186.3)
GLP-1(7-36) amide	H-A-E-G-T-F-T-S-D-V-S-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-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