European Journal of Pharmacology 834 (2018) 126–135

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Endocrine pharmacology

Characterisation and antidiabetic utility of a novel hybrid peptide, exendin-4/gastrin/xenin-8-Gln

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ARTICLE INFO

Keywords:
Gastrin
Glucagon-like-peptide-1 (GLP-1)
Glucose-dependent insulinotropic polypeptide (GIP)
Glucose homeostasis
Hybrid
Xenin

ABSTRACT

Enteroendocrine derived hormones such as glucagon-like-peptide-1 (GLP-1), glucose-dependent insulinotropic polypeptide (GIP), gastrin and xenin are known to exert complementary beneficial metabolic effects in diabetes. This study has assessed the biological activity and therapeutic utility of a novel GLP-1/gastrin/xenin hybrid peptide, namely exendin-4/gastrin/xenin-8-Gln hybrid, both alone and in combination with the stable GIP mimetic, (DAla^2)GIP. Exendin-4/gastrin/xenin-8-Gln increased *in vitro* insulin secretion to a similar or superior extent, as the parent peptides. Insulinotropic effects were mainly linked to modulation of GLP-1 and neurotensin receptors. Exendin-4/gastrin/xenin-8-Gln also augmented the insulinotropic actions of (DAla^2)GIP. Acute administration of exendin-4/gastrin/xenin-8-Gln in mice induced significant appetite suppressive, glucose lowering and insulin secretory effects, with a duration of biological action beyond 8 h. Twice daily administration of exendin-4, exendin-4/gastrin/xenin-8-Gln, either alone or in combination with (DAla^2)GIP, reduced circulating glucose, increased plasma insulin as well as improving glucose tolerance, insulin sensitivity and metabolic response to GIP in high fat fed mice. Body weight, food intake, circulating glucagon and amylase activity were unaltered. All hybrid peptide treated high fat mice exhibited marked reductions in LDL-cholesterol and body fat mass. Energy expenditure and locomotor activity were increased in mice treated with exendin-4/gastrin/xenin-8-Gln in combination with (DAla^2)GIP. Interestingly, exendin-4 and exendin-4/gastrin/xenin-8-Gln treatment, but not exendin-4/gastrin/xenin-8-Gln in combination with (DAla^2)GIP, reduced pancreatic islet and beta-cell area when compared to high fat controls. These studies confirm that unimolecular multi-agonist peptide hormones exert beneficial metabolic effects in diabetes, highlighting their potential as novel treatment strategies.

1. Introduction

Type 2 diabetes mellitus (T2DM) is a human disorder linked to loss of pancreatic beta-cell mass and function over time (Meier and Bonadonna, 2013), that usually requires pharmacological intervention to maintain optimal glycaemic control (Page and Reisman, 2013). Due to the degenerative nature of T2DM, use of single-target monotherapy treatments can result in diminishing antidiabetic efficacy, leading to elevated drug doses and increased risk of adverse side effects (Campbell and Drucker, 2013). Therefore, combinatorial therapies, in particular unimolecular multi-targeting gut derived hybrid peptides, are receiving much attention as possible new pharmacological approaches for successfully controlling T2DM (Finan et al., 2015; Frias et al., 2017; Irwin et al., 2015; Jall et al., 2017; Khajavi et al., 2017; Sánchez-Garrido et al., 2017; Tschöp et al., 2016).

In this regard, early research has highlighted the beneficial additive or synergistic biological effects of various gut hormone combinations in diabetic rodents (Flatt et al., 2009; Suarez-Pinzon et al., 2008a). Although administration of separate, enzymatically stable gut peptide forms, is one potential method to modulate multiple regulatory hormone receptors (Gault et al., 2011; Irwin et al., 2013), use of hybrid peptides, created by fusion of the key bioactive regions of parent peptides, is preferred pharmaceutically as it facilitates formulation, dosing and marketing of a single entity. As such, a glucagon-like peptide-1 (GLP-1)/gastrin hybrid peptide has been shown to increase beta-cell mass, a key pathophysiological characteristic of diabetes (Dalboge et al., 2014), and combination therapy with GLP-1 and gastrin improves diabetic control in type 1 diabetic mice (Suarez-Pinzon et al., 2008a, b). Both GLP-1 and gastrin are well recognised to possess beta-cell growth actions (Liu and Habener, 2008; Rooman et al., 2002), as does the sister incretin hormone of GLP-1, namely glucose-dependent insulinotropic polypeptide (GIP) (Kim et al., 2008). Interestingly, with the upsurge in interest in hybrid peptide therapies, GIP-based antidiabetic strategies, once largely ignored due to reported inefficacy in T2DM (Nauck et al.,

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https://doi.org/10.1016/j.ejphar.2018.07.027
Received 9 May 2018; Received in revised form 29 June 2018; Accepted 13 July 2018
Available online 17 July 2018

1993), are gaining more prominence (Bhat et al., 2013; Hasib et al., 2017, 2018; Jall et al., 2017).

Further encouragement regarding the use of antidiabetic GIP peptides is revealed through recent observations with xenin, a hormone co-secreted with GIP, which significantly augments the biological actions of GIP (Martin et al., 2012; Wice et al., 2010). Notably, GIP/xenin and GLP-1/xenin hybrid peptides have been generated and shown to exhibit beneficial metabolic and GIP potentiating activity in high fat fed diabetic mice (Hasib et al., 2017, 2018), with the xenin component of these hybrid molecules based on the bioactive C-terminal octapeptide of xenin, xenin-8 (Silvestre et al., 2003). Based on this premise, in the current study a triple-acting novel hybrid peptide, exendin-4/gastrin/xenin-8-Gln, was designed, to incorporate the recognised antidiabetic and beta-cell proliferative effects of GLP-1/gastrin dual agonism (Dalboge et al., 2014), with the additive beneficial actions of an enzymatically stable xenin-8 molecule, xenin-8-Gln (Martin et al., 2016).

Initially, enzymatic resistance of exendin-4/gastrin/xenin-8-Gln was assessed, together with *in vitro* insulin secretory, and *in vivo* glucose-lowering, insulin-releasing and satiety actions. In addition, the beneficial metabolic effects of a twice-daily injection regimen with exendin-4/gastrin/xenin-8-Gln were then examined in high-fat–fed mice. In order to incorporate activation of GIP receptor signalling pathways into the therapeutic paradigm, a stable and long-acting GIP analogue, namely ($DAla^2$)GIP (Hinke et al., 2002), was added to the exendin-4/gastrin/xenin-8-Gln treatment regimen. The results reveal that concurrent modulation of GLP-1, gastrin, xenin and GIP receptor signalling pathways represents an extremely attractive therapeutic strategy for T2DM that merits further detailed consideration.

2. Material and methods

2.1. Peptides synthesis and assessment of plasma enzymatic stability

All peptides (> 95% purity) were purchased from American Peptide company (Sunnyvale, CA, USA). Prior to experimentation, peptides were characterised in-house using RP-HPLC and MALDI-TOF MS, as described previously (Martin et al., 2013). Table 1 displays the amino acid sequence of the novel hybrid peptide, and related parent peptides. The full sequence comprises the first N-terminal 28 amino acid residues of exendin-4, followed by gastrin-6, and then xenin-8-Gln, with each parent peptide coupled together by two 8-amino-3,6-dioxaoctanoic acid linker molecules (Table 1). *In vitro* enzymatic stability of test peptides was assessed using murine plasma. Briefly, test peptides (50 µg) were incubated in 50 mmol/l triethanolamine–HCl (pH 7.8) with overnight fasted mouse plasma (50 µl) for 0, 120, 240 and 480 min (at 37 °C). Degradation profiles of each peptide was followed using RP-HPLC and all collected HPLC peaks were analysed by MALDI-TOF MS to determine identity.

2.2. Insulin secretory studies

In vitro insulin secretory activity of test peptides was determined using clonal pancreatic clonal BRIN-BD11 beta-cells (McClenaghan et al., 1996) and isolated mouse islets. In brief, BRIN-BD11 cells were seeded into 24-well plates (150,000 cells per well) and allowed to attach overnight at 37 °C. Before insulin secretory tests, cells were pre-incubated (40 min; 37 °C) in Krebs–Ringer bicarbonate buffer (KRBB) (pH 7.4) supplemented with 0.5% (w/v) BSA and 1.1 mM glucose. In the first series of experiments, cells were incubated (20 min) with various concentrations (10^{-6} – 10^{-12} mol/l) of test peptides at 5.6 and 16.7 mmol/l glucose. In a second series, BRIN-BD11 cells were incubated with a range of concentrations (10^{-8} to 10^{-6} mol/l) of test peptides in the presence and absence of ($DAla^2$)GIP (10^{-7} mol/l) at 5.6 mmol/l glucose. In the final set of experiments, effects of the specific GLP-1, neurotensin, CCK-A, and CCK-B receptor antagonists, namely exendin(9-39) (Thorens et al., 1993), SR142948A (Hasib et al., 2017), SR27897, and LY288513 (each at 10^{-6} mol/l) (Irwin et al., 2015) respectively, on exendin-4/gastrin/xenin-8-Gln induced insulin secretion (10^{-7} mol/l) were examined. We employed higher doses of the antagonists than the hybrid peptide (10^{-6} *vs.* 10^{-7} mol/l), to ensure the most prominent inhibitory actions. After test incubations, assay buffer (200 µl) from each experiment was aliquoted and insulin concentrations measured by an in-house radioimmunoassay, as described previously (Flatt and Bailey, 1981). Finally, insulinotropic effects of test peptides at (60 min, 16.7 mmol/l glucose) were also examined in isolated islets of NIH Swiss mice (14 week-old), using a standard collagenase-based method of isolation (McKillop et al., 2014). Following removal of the test solutions, 200 µl of acid-ethanol solution (1.5% [v/v] HCl, 75% [v/v] ethanol, 23.5% [v/v] H_2O) was added overnight (18 h) to extract cellular insulin. Samples were stored at − 20 °C prior to assessment of insulin secretion and cellular insulin content by radioimmunoassay (Flatt and Bailey, 1981).

2.3. Animals

All studies were carried out using male NIH Swiss mice (12–14 weeks old, Envigo Ltd, UK). For acute *in vivo* studies, animals were maintained on a standard rodent maintenance diet (10% fat, 30% protein and 60% carbohydrate, Trouw Nutrition, UK) with free access to diet and water. Sub-chronic experiments were performed using NIH Swiss mice previously fed a high-fat diet (45% fat, 35% carbohydrate and 20% protein, Special Diet Services, UK) for 12 weeks. All animals were housed individually in an air-conditioned room at 22 ± 2 °C with a 12 h light:12 h dark cycle. Animal experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and approved by the University of Ulster Animal Welfare and Ethical Review Body (AWERB).

2.4. Acute in vivo studies

To assess glucose tolerance and insulin secretory effects, blood glucose and plasma insulin concentrations were measured immediately prior to and 15, 30 and 60 min after i.p. administration of glucose alone (18 mmol/kg bw) or in combination with test peptides (25 nmol/kg bw) in overnight fasted (18 h) mice. In a second series of experiments, freely fed mice were administered test peptides (25 nmol/kg bw; i.p.) or saline vehicle (0.9% w/v NaCl; i.p.), then immediately fasted, and subsequently given an i.p. glucose challenge 8 h later (18 mmol/kg bw) and blood glucose measured at 0, 15, 30 and 60 min post glucose injection. In a separate series of experiments, cumulative food intake was

Table 1
Amino acid sequence and murine plasma half-lives of exendin-4, xenin-8-Gln and the novel exendin-4/gastrin/xenin-8-Gln hybrid molecule.

Peptide	Amino acid sequence	*In vitro* half-life (murine plasma)
Xenin-8-Gln	**HPQQPWIL**-OH	> 6 h
Exendin-4	**HGEGTFTSDLSKQMEEEAVRLFIEWLKN**-G-G-P-S-S-G-A-P-P-P-S-NH_2	> 6 h
Gastrin-6	**YGWLDF**-NH_2	–
Exendin-4/ gastrin/xenin-8-Gln	**HGEGTFTSDLSKQMEEEAVRLFIEWLKN**-AEEAc–AEEAc-**YGWLDF**-AEEAc–AEEAc-**HPQQPWIL**-OH	> 6 h

Amino acid sequence of peptides using one letter amino acid nomenclature, where AEEAc is 8-amino-3,6-dioxaoctanoic acid. Common amino acid sequence of hybrid peptide derived from parent molecules are shown in bold text.