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desHis[1]Glu[9]-glucagon-[mPEG] and desHis[1]Glu[9](Lys[30]PAL)-glucagon: Long-acting peptide-based PEGylated and acylated glucagon receptor antagonists with potential antidiabetic activity

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ABSTRACT

Glucagon is hormone secreted from the pancreatic alpha-cells that is involved in blood glucose regulation. As such, antagonism of glucagon receptor signalling represents an exciting approach for treating diabetes. To harness these beneficial metabolic effects, two novel glucagon analogues, desHis[1]Glu[9]-glucagon-[mPEG] and desHis[1]Glu[9](Lys[30]PAL)-glucagon, has been evaluated for potential glucagon receptor antagonistic properties. Both novel peptides were completely resistant to enzymatic breakdown and significantly ($P<0.05$ to $P<0.001$) inhibited glucagon-mediated elevations of cAMP production in glucagon receptor transfected cells. Similarly, desHis[1]Glu[9]-glucagon-[mPEG] and desHis[1]-Glu[9](Lys[30]PAL)-glucagon effectively antagonised glucagon-induced increases of insulin secretion from BRIN BD11 cells. When administered acutely to normal, high fat fed or *ob*/*ob* mice, both analogues had no significant effects on overall blood glucose or plasma insulin levels when compared to saline treated controls. However, desHis[1]Glu[9]-glucagon-[mPEG] significantly ($P<0.05$) annulled glucagon-induced increases in blood glucose and plasma insulin levels in normal mice and had similar non-significant tendencies in high fat and *ob*/*ob* mice. In addition, desHis[1]Glu[9](Lys[30]PAL)-glucagon effectively ($P<0.05$ to $P<0.001$) antagonised glucagon-mediated elevations of blood glucose levels in high fat fed and *ob*/*ob* mice, but was less efficacious in normal mice. Further studies confirmed the significant persistent glucagon receptor antagonistic properties of both novel enzyme-resistant analogues 4 h post administration in normal mice. These studies emphasise the potential of longer-acting peptide-based glucagon receptor antagonists, and particularly acylated versions, for the treatment of diabetes.

1. Introduction

Glucagon, a 29 amino acid peptide secreted from pancreatic alpha-cells, is the major counter regulatory hormone to insulin and plays a central role maintaining adequate glucose control (Jiang and Zhang, 2003; Dunning and Gerich, 2007). Thus, despite the widely accepted view that the pathophysiology of type 2 diabetes relates predominantly to insulin resistance and beta-cell dysfunction, numerous studies indicate that hyperglucagonaemia and lack of glucagon suppression following feeding are equally as critical (Dunning and Gerich, 2007; Unger and Orci, 1981). In addition, inappropriately elevated glucagon levels play a key role in the development of hyperglycaemia in type 2 diabetes (Bagger et al., 2011).

Therefore, inhibition of glucagon signalling represents a potential new therapeutic approach for type 2 diabetes. Consistent with this, glucagon receptor knockout mice and mice treated with glucagon receptor antisense oligonucleotides exhibit improved fasting glucose, glucose tolerance and pancreatic beta-cell function (Gelling et al., 2003; Liang et al., 2004; Sloop et al., 2004). In addition, studies utilising prolonged administration of glucagon receptor neutralising monoclonal antibodies or small molecular weight glucagon receptor antagonists reveal similar metabolic improvements (Winzell et al., 2007; Gu et al., 2010; Mu et al., 2011; Xiong et al., 2012). However, given concerns regarding safety, tolerability and potential for induction of immune responses with the above approaches, peptide-based glucagon receptor antagonists may offer a more favourable therapeutic option (Unson et al., 1989; Franklin et al., 2012).

In this regard, there are a number of reports describing and characterising peptide-based glucagon receptor antagonists. These studies focused on specific amino acid modifications to the native glucagon. As such, His[1] and Asp[9] are recognised as important amino acids involved in signal transduction, whereas Arg[17], Arg[18] and Asp[21] are crucial for receptor binding (Unson et al., 1994, 1998; Sturm et al., 1998). Deletion of His[1] in combination with

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replacement of Asp^9 for Glu^9 yields an effective peptide-based glucagon receptor antagonist (Unson et al., 1989). Despite the promise of this peptide, such compounds are still susceptible to rapid enzymatic degradation and efficient renal filtration (Holst, 1991). Many studies have demonstrated that PEGylation or fatty acid derivatisation of regulatory peptides results in longer-acting analogues, largely by increased enzymatic stability and reduced renal clearance through binding to plasma proteins (Knudsen et al., 2000; Lee et al., 2006; Gault et al., 2008; Kerr et al., 2009, 2010). Building on these approaches to stabalise related regulatory hormones, we have developed two novel glucagon analogues, namely desHis^{1}Glu^9-glucagon-[mPEG] and desHis^{1}Glu^9(Lys^{30}PAL)-glucagon. Both peptides are based on the well characterised glucagon receptor antagonist, desHis^{1}Glu^9-glucagon (Unson et al., 1989), with additional C-terminal modifications to prolong circulating half-life.

The present study has assessed the *in vitro* and *in vivo* actions of desHis^{1}Glu^9-glucagon-[mPEG] and desHis^{1}Glu^9(Lys^{30}PAL)-glucagon. The results provide the experimental evidence that desHis^{1}Glu^9-glucagon-[mPEG] and desHis^{1}Glu^9(Lys^{30}PAL)-glucagon may provide an effective means of treating type 2 diabetes.

2. Materials and methods

2.1. Peptide synthesis

Native glucagon, desHis^{1}Glu^9-glucagon-[mPEG] and desHis^1-Glu^9(Lys^{30}PAL)-glucagon were obtained from GL Biochem Ltd. (Shanghai, China). All peptides were characterized using matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry as described previously (Kerr et al., 2009). The theoretical molecular masses of native glucagon, desHis^{1}Glu^9-glucagon-[mPEG] and desHis^{1}Glu^9(Lys^{30}PAL)-glucagon are 3482.8, 3503.8 and 3725.3; respectively, and the measured molecular masses were 3480.4, 3503.6 and 3726.8; respectively.

2.2. Degradation studies

Native glucagon, desHis^{1}Glu^9-glucagon, desHis^{1}Glu^9-glucagon-[mPEG] and desHis^{1}Glu^9(Lys^{30}PAL)-glucagon were incubated at 37 °C in triethanolamine-HCL (pH 7.8) with purified porcine DPP-4 (5mU; Sigma-Aldrich, UK) for 0, 2, 8 and 24 h. Reactions were terminated by the addition of trifluoroacetic acid (TFA) in water (10 μl, 10%v/v). Reaction products were applied to a Synergi C-12 column (4.6 × 250 mm, Phenomonex, Cheshire, UK) and intact peptide separated from the major degradation products. The column was equilibrated with 0.12% (v/v) TFA/water at 1 ml/min using 0.1% (v/v) TFA in 70% acetonitrile/water. The concentration of acetonitrile in the eluting solvent was raised from 0 to 40% over 10 min, and from 40 to 100% over 30 min. Absorption was measured at 214 nm using a SpectraSystem UV2000 detector (Thermoquest Ltd., Manchester, UK). HPLC peak area data was used to calculate percentage intact peptide remaining at the time points recorded.

2.3. Effects of glucagon, desHis^{1}Glu^9-glucagon-[mPEG] and desHis^{1}Glu^9(Lys^{30}PAL)-glucagon on in vitro cAMP production and insulin secretion

For cAMP measurements, glucagon receptor transfected HEK293GnT1-cells were harvested, seeded into 96 well plates (5×10^4 cells per well) and grown for 16 h. The origin and characteristics of this transfected cell line are described elsewhere (Unson et al., 1989). Cells were washed twice in Hanks Buffered Saline (HBS) buffer and incubated (20 min; 37 °C) with varying concentrations (10^{-11} to 10^{-6} M) of desHis^{1}Glu^9-glucagon-[mPEG] or desHis^{1}Glu^9(Lys^{30}PAL)-glucagon in the presence and absence of stimulatory glucagon (10^{-7} M) in HBS buffer containing 1 mM IBMX. Medium was subsequently removed, cells lysed and cAMP levels in the lysate measured using a HTS chemiluminescent immunoassay kit (Millipore, Watford, England). For assessment of insulin-release, BRIN-BD11 cells were seeded into 24 well plates (10^5 cells per well) and allowed to attach overnight at 37 °C. The origin and characteristics of this insulin-secreting cell line are described elsewhere (McClenaghan et al., 1996). Prior to acute tests, cells were pre-incubated (40 min; 37 °C) in Krebs Ringer Bicarbonate (KRB) buffer (pH 7.4) supplemented with 0.5% (w/v) BSA and 1.1 mM glucose. Test incubations were performed in the presence of 5.6 mM glucose with a range of concentrations (10^{-12} to 10^{-6} M) of desHis^{1}Glu^9-glucagon-[mPEG] or desHis^{1}Glu^9(Lys^{30}PAL)-glucagon in the presence and absence of stimulatory glucagon (10^{-7} M). After incubation (20 min; 37 °C) buffer was removed from each well and aliquots (200 μl) stored at −20 °C prior to measurement of insulin by radioimmunoassay (Kerr et al., 2009).

2.4. Acute in vivo effects of glucagon, desHis^{1}Glu^9-glucagon-[mPEG] and desHis^{1}Glu^9(Lys^{30}PAL)-glucagon on glycaemic and insulinotropic responses

The effects of native glucagon, desHis^{1}Glu^9-glucagon-[mPEG], desHis^{1}Glu^9(Lys^{30}PAL)-glucagon, or a combination of glucagon with either desHis^{1}Glu^9-glucagon-[mPEG] or desHis^{1}Glu^9(Lys^{30}PAL)-glucagon, was examined in 12–14 week-old normal NIH Swiss male mice, high fat fed NIH Swiss male mice and 12–14 week-old obese diabetic (*ob*/*ob*) mice. Experimental animals had free access to drinking water and standard rodent maintenance (10% fat, 30% protein and 60% carbohydrate, Trouw Nutrition, Cheshire, UK) or a high fat (45% fat, 35% carbohydrate and 20% protein, Special Diet Services, UK) diets as appropriate. Prior to commencement of studies, high-fat fed experimental animals were maintained on high fat diet from 6 weeks of age for 140 days. Obesity and insulin resistance were clearly manifested compared to mice maintained on normal laboratory diet as judged by body weight and plasma insulin analyses. Obese–diabetic (*ob*/*ob*) mice were derived from the colony originally maintained at Aston University (Bailey et al., 1982). All animal experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986. The animals were housed individually in an air-conditioned room at 22 ± 2 °C with a 12 h light: 12 h darkness cycle.

A common experimental protocol was employed for all three species of mice. Thus, groups of mice ($n=8$) received an intraperitoneal (i.p.) injection of saline vehicle alone (0.9% (w/v) NaCl) or in combination with native glucagon, desHis^{1}Glu^9-glucagon-[mPEG] or desHis^{1}Glu^9(Lys^{30}PAL)-glucagon (each 25 nmol/kg body weight). In a second series of experiments, mice ($n=8$) received an i.p. injection of glucagon alone (25 nmol/kg b w) or in combination with either desHis^{1}Glu^9-glucagon-[mPEG] or desHis^{1}Glu^9(Lys^{30}PAL)-glucagon (each at 25 nmol/kg b w). A dose of 25 nmol/kg was chosen based pilot studies revealing ineffectiveness of glucagon analogues to annul glucagon mediated metabolic effects at doses of 0.25 and 2.5 nmol/kg [data no shown]. The intraperitoneal route was chosen as the method of drug delivery as this bypasses the stomach and intestines and so does not stimulate release of the plethora of peptides and hormones involved in glucose regulation. Thus, the sole effects of desHis^{1}Glu^9-glucagon-[mPEG] and desHis^{1}Glu^9(Lys^{30}PAL)-glucagon treatment can be more closely examined. Normal and high fat mice were fasted 4 h, and *ob*/*ob* mice 18 h, prior to peptide administration and blood glucose and plasma insulin