Peptides 100 (2018) 219–228

Contents lists available at ScienceDirect

Peptides

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

Apelin-13 analogues show potent in vitro and in vivo insulinotropic and glucose lowering actions

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ARTICLE INFO

Keywords:
Apelin-13 analogues
Diabetes
Insulin secretion
Glucose homeostasis

ABSTRACT

Nine structurally modified apelin-13 analogues were assessed for their in vitro and acute in vivo antidiabetic potential. Stability was assessed in mouse plasma and insulinotropic efficacy tested in cultured pancreatic BRIN-BD11 cells and isolated mouse pancreatic islets. Intracellular Ca^{2+} and cAMP production in BRIN-BD11 cells was determined, as was glucose uptake in 3T3-L1 adipocytes. Acute antihyperglycemic effects of apelin analogues were assessed following i.p. glucose tolerance tests (ipGGT, 18 mmol/kg) in normal and diet-induced-obese (DIO) mice and on food intake in normal mice. Apelin analogues all showed enhanced in vitro stability (up to 5.8-fold, t½ = 12.8 h) in mouse plasma compared to native apelin-13 (t½ = 2.1 h). Compared to glucose controls, stable analogues exhibited enhanced insulinotropic responses from BRIN-BD11 cells (up to 4.7-fold, $p < 0.001$) and isolated mouse islets (up to 5.3-fold) for 10^{-7} M apelin-13 amide (versus 7.6-fold for 10^{-7} M GLP-1). Activation of APJ receptors on BRIN-BD11 cells increased intracellular Ca^{2+} (up to 3.0-fold, $p < 0.001$) and cAMP (up to 1.7-fold, $p < 0.01$). Acute ipGTT showed improved insulinotropic and glucose disposal responses in normal and DIO mice ($p < 0.05$ and $p < 0.01$, respectively). Apelin-13 amide and (pGlu)apelin-13 amide were the most effective analogues exhibiting acute, dose-dependent and persistent biological actions. Both analogues stimulated insulin-independent glucose uptake by differentiated adipocytes (2.9–3.3-fold, $p < 0.05$) and inhibited food intake (26–33%, $p < 0.001$), up to 180 min in mice, versus saline. In contrast, (Ala^{13})apelin-13 and (Val^{13})apelin-13 inhibited insulin secretion, suppressed beta-cell signal transduction and stimulated food intake in mice. Thus, stable analogues of apelin-13 have potential for diabetes/obesity therapy.

1. Introduction

Most recent figures suggest that there are at least 415 million people currently living with diabetes mellitus, a number predicted to grow to 642 million by 2040 [1,2]. The predominant form, type 2 diabetes, is a chronic disorder characterised by metabolic disarray and hyperglycemia primarily due to dysregulated insulin secretion and impaired insulin action [3,4]. Long-term complications of poor control can be devastating and include both microvascular and macrovascular pathologies [5,6]. Additionally, hypertension and abnormalities of lipoprotein metabolism are common in people with diabetes [7].

The immense challenge of diabetes has been accompanied by the introduction of a number of important novel antihyperglycemic drug classes, such as GLP-1 mimetics, DPP-4 inhibitors and SGLT2 inhibitors, which have been added recently to the established armamentum of sulphonylureas, metformin and thiazolidenediones. Despite clear-cut benefit, none of these drugs provides the complete answer to diabetes, but the diversity of their actions illustrates the value of exploiting single drug entities that act through multiple drug targets to mediate a variety of actions which converge to counter hyperglycemia. This is well illustrated by GLP-1 mimetics which act at pancreatic and extra-pancreatic sites, to not only stimulate insulin secretion but also to inhibit glucagon secretion, gastric emptying and feeding activity [8,9]. Further research is also developing dual or triple acting peptide agonists to combine the actions of GLP-1 with and other gut hormones, such as GIP, CCK and oxyntomodulin [10–15].

The role of the gut in metabolic control is well recognised [16,17], but despite the discovery that adipose tissue is not merely a depository for fat and produces a range of metabolically active peptides (adipokines) [18], the possibility of exploiting them in the context of diabetes has been largely overlooked. Apelin, a circulating adipokine, mainly produced and secreted by adipocytes [19] was discovered in bovine stomach extracts [20]. It is a 36-amino acid peptide which targets the APJ receptor, which was originally classified as an orphan receptor

Abbreviations: APJ, apelin receptor; CCK, cholecystokinin; DIO, diet induced obese; DPP, 4 dipeptidylpeptidase-4; GIP, glucose-dependent insulinotropic polypeptide; GLP-1, glucagon-like peptide-1; KRB, krebs ringer bicarbonate; pGlu, pyroglutamyl; SGLT-2, sodium glucose co-transporter-2; T2DM, type 2 diabetes mellitus; WHO, world health organisation

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https://doi.org/10.1016/j.peptides.2017.12.004
Received 20 October 2017; Received in revised form 1 December 2017; Accepted 4 December 2017

[21]. Interestingly, apelin is reported to exert multiple biological actions on feeding behaviour, glucose utilisation and insulin secretion with APJ receptors being reported on pancreatic β-cells with some expression in α-cells [22,23]. Apelin and its receptors are widely expressed in various organs such as the heart, lung, adipose tissue, gastrointestinal tract, brain, kidney, liver, adrenal glands, and endothelium [24,25]. Apelin/apelinergic receptors serve important functions in vasopressin (anti-diuretic hormone: ADH) and histamine secretion, angiogenesis, glucose fluid balance and regulation of cardiovascular system [26,27]. A further link to diabetes is that apelin production in adipose tissue is strongly upregulated by insulin, and plasma concentrations are increased in obese and hyperinsulinemic mice and humans [22].

The diabetes pandemic requires the development of new and effective pharmacological treatments [28]. We considered the biological activity profile of apelin to be encouraging as a potential new multi-acting therapeutic approach to type 2 diabetes. The present paper documents the design, development and actions of stable enzyme resistant apelin-13 analogues, which are suitable for further preclinical testing in animal models of diabetes and possible development into new drugs.

2. Materials & methods

2.1. Peptides

Apelin and its analogues were purchased from EZ Biolabs (Carmel, IN, USA) at > 95% purity. Peptide purity and structural identity were checked using RP-HPLC and MALDI-ToF MS as described previously [29]. Previous studies have shown that cleavage of the C-terminal Phe^{13} amino acid was the target for ACE2 enzymatic degradation, which was substituted with Tyr, Ala or Val to confer improved enzyme resistance. In addition, we also amidated the C-terminus of native apelin-13 and protected the N-terminus by addition of pyroglutamate which contains no free amino group to further aid the stability of apelin-13 analogues against aminopeptidase attack.

2.2. Assessment of metabolic stability

For assessment of plasma stability, peptides (20 μg) were incubated with fasted pooled mouse plasma in the presence of 50 mmol/L TEA-HCl buffer for 0, 2, 4, 8, 24 h. Degradation profiles using RP-HPLC were obtained and chromatograph peak areas used for calculation of percentage intact peptide and half-life as described previously [30,29].

2.3. in vitro insulin secretion

The effects of apelin peptide analogues on insulin secretion in vitro were examined using clonal pancreatic BRIN-BD11 β-cells [31]. Briefly, cells were seeded into 24 well plates (150,000 cells/well) and allowed to attach overnightat 37 °C. Following pre-incubation (1.1 mmol/L glucose, 40 min; 37 °C) cells were treated with various concentration of peptides (10^{-12} to 10^{-6} M) in the presence of 5.6 and 16.7 mmol/L glucose. After 20 min incubation, the supernatant was removed from each well and aliquots (200 μl) stored at −20 °C prior to determination of insulin release by radioimmunoassay [32].

2.4. Intracellular calcium ($[Ca^{2+}]_i$)

Effects of apelin peptides on intracellular Ca^{2+} in vitro were examined with monolayers of BRIN-BD11 cells using a fluorimetric intracellular Ca^{2+} assay kit (Molecular Devices, Sunnyvale, CA, USA) as per manufacturer's protocol previously described [33]. Briefly, following pre-incubation at 1.1 mmol/L glucose (40 min), cells were incubated at 37 °C for 10 min with peptides and control incubations in the presence of 16.7 mmol/L glucose. A positive control was used comprising 16.7 mmol/L glucose plus KCl (30 mmol/L). Data were acquired using Flexstation scanning fluorimeter with integrated fluid transfer workstation (Molecular Devices, Sunnyvale, USA).

2.5. in vitro cyclic AMP production

Effects of apelin peptides on cAMP production in vitro was examined in clonal pancreatic BRIN-BD11 cells. Briefly, cells were seeded into 96-well plates (10,000 cells/well) and allowed to attach overnight. Following pre-incubation with Krebs Ringer Bicarbonate (KRB) buffer (1.1 mmol/L glucose, 40 min; 37 °C), cells were treated with test peptides for 20 min, the supernatant discarded and cells were lysed by repeated freezing and thawing cycles. Cyclic AMP production by apelin analogues were measured using a cAMP immunoassay kit (R&D Systems Ltd, Abingdon, UK) following the manufacturer's recommended protocol.

2.6. Ex vivo insulin secretion from isolated islets

Pancreatic islets were isolated from adult male C57BL/6 mice (8–10 weeks old, Harlan Ltd., Blackthorne, UK) by digestion with collagenase P obtained from *Clostridium histolyticum* (Sigma-Aldrich, Poole, Dorset, UK) as described previously [34,35]. Following 48 h culture, groups of 10 islets were pre-incubated with 500 μl KRB buffer containing 1.1 mmol/L glucose for 1 h at 37 °C. Test incubations with peptides and GLP-1 (10^{-7} M) were carried out in KRB buffer supplemented with 11.1 mmol/L glucose for 1 h at 37° C. Insulin release and insulin content of islets treated overnight with acidified ethanol [36] were determined by radioimmunoassay.

2.7. Glucose uptake from 3T3-L1 adipocytes

3T3-L1 adipocytes were obtained from American Type Culture Collection (Manassas, Virginia, USA). Cells were seeded into 96 well plates at a density of 35,000 cells/well and fed every 2 days with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) heat inactivated FBS until confluence, and then maintained in the same media for an additional 2 days. Two-day post-confluent cell were differentiated in DMEM containing 10% FBS, 15 μg/ml insulin, 1 μM dexamethasone and 0.5 mM 3-isobutyl-L-methylxanthine (IBMX). Subsequently, cells were cultured in DMEM containing 10% FBS and 15 μg/ml of insulin. Glucose uptake studies were carried out according to manufacturer's protocol (Cayman Chemical Company, Ann Arbor, MI, USA). In brief, cells were incubated with fluorescently-tagged glucose derivative (2-NBDG) in serum-free medium in combination with peptides (10^{-7} M), centrifuged and washed thrice and fluorescence read (excitation/emission = 485/535 nm) using Flexstation scanning fluorimeter (Molecular Devices, Sunnyvale, USA).

2.8. Animals

Male NIH Swiss mice (8–10 weeks old, Harlan Ltd., Blackthorne, UK) were maintained on standard rodent diet (10% fat, 30% protein and 60% carbohydrate; percent of total energy 12.99 kJ/g; Trouw Nutrition, Cheshire, UK) or high fat diet (45% fat, 20% protein and 35% carbohydrate; percent of total energy 26.15 kJ/g; Special Diet Service, Essex, UK) for 21 weeks for induction of dietary-induced obesity-diabetes. Progressive body weight gain and overt hyperglycemia were observed in DIO mice after 21 weeks. Animals were housed in individual cages in an air-conditioned room (22 ± 2 °C with a 12 h light:12 h dark cycle) with food and water provided ad libitum. All experiments were performed in accordance with UK animals (Scientific Procedures) Act 1986 and "Principles of Laboratory Animal Care" (NIH Publication Number 86-23, revised 1985). No adverse effects were observed in animals following administration of any of the peptides.