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Acylated apelin-13 amide analogues exhibit enzyme resistance and prolonged insulin releasing, glucose lowering and anorexic properties

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ABSTRACT

The adipokine, apelin has many biological functions but its activity is curtailed by rapid plasma degradation. Fatty acid derived apelin analogues represent a new and exciting avenue for the treatment of obesity-diabetes. This study explores four novel fatty acid modified apelin-13 analogues, namely, (Lys^{8}GluPAL)apelin-13 amide, pGlu(Lys^{8}GluPAL)apelin-13 amide, Lys^{8}GluPAL(Tyr^{13})apelin-13 and Lys^{8}GluPAL(Val^{13})apelin-13. Fatty acid modification extended the half-life of native apelin-13 to >24 h *in vitro*. pGlu(Lys^{8}GluPAL)apelin-13 amide was the most potent insulinotropic analogue in BRIN-BD11 cells and isolated islets with maximal stimulatory effects of up to 2.7-fold ($p < .001$). (Lys^{8}GluPAL) apelin-13 amide (1.9-fold) and Lys^{8}GluPAL(Tyr^{13})apelin-13 (1.7-fold) were less effective, whereas Lys^{8}GluPAL(Val^{13})apelin-13 had an inhibitory effect on insulin secretion. Similarly, pGlu(Lys^{8}GluPAL) apelin-13 amide was most potent in increasing beta-cell intracellular Ca^{2+} concentrations (1.8-fold, $p < .001$) and increasing glucose uptake in 3T3-L1 adipocytes (2.3-fold, $p < .01$). Persistent biological action was observed with both pGlu(Lys^{8}GluPAL)apelin-13 amide and (Lys^{8}GluPAL)apelin-13 amide significantly reducing blood glucose (39–43%, $p < .01$) and enhancing insulin secretion (43–56%, $p < .001$) during glucose tolerance tests in diet-induced obese mice. pGlu(Lys^{8}GluPAL)apelin-13 amide and (Lys^{8}GluPAL)apelin-13 amide also inhibited feeding (28–40%, $p < .001$), whereas Lys^{8}GluPAL(Val^{13}) apelin-13 increased food intake (8%, $p < .05$) in mice. These data indicate that novel enzymatically stable analogues of apelin-13 may be suitable for future development as therapeutic agents for obesity-diabetes.

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1. Introduction

Central obesity and excess visceral fat accumulation has been recognised as a significant risk factor for numerous chronic diseases, including atherosclerosis, arterial hypertension, type 2 diabetes mellitus (T2DM), as well as for certain forms of cancer [62]. Consumption of the energy dense Western diet coupled with a sedentary lifestyle, has led to a pandemic of T2DM, which has become a substantial public health problem [49]. Development of T2DM characterised by hyperglycaemia along with insulin resistance, often leads to macro- and microvascular complications, resulting in severe illness, premature death and significant economic, as well as personal costs [72]. Recent advances in pharmacological interventions of T2DM are promising new and more effective treatments, however, prevention strategies are also urgently needed to help reduce the burden of the disease [8].

Apelin is an adipokine produced and secreted by white adipose tissue (WAT) in both humans and animals [2,1]. First isolated from extracts of the bovine stomach [69], apelin is secreted as a 77-amino-acid precursor, preproapelin and subsequently cleaved into several smaller bioactive peptides, including apelin-36 and apelin-13 [69,34]. Apelin is a cognate ligand of the APJ receptor, first identified as an orphan G-protein-coupled receptor (GPCR), with the closest identity to the angiotensin II (Ang II) receptor, type AT_{1a} [48]. Apelin mRNA and its receptors are expressed in numerous tissues including heart, brain, pancreas and gastrointestinal tract [59,60,47].

Studies indicate that apelin plays an important role in regulation of a variety of metabolic processes [59]. Given its hypotensive and cardiac contractility properties, apelin appears to be involved in cardiovascular function [70,67,3]. Central administration of apelin was shown to regulate both food intake and water balance [68,66]. Apelin also improved insulin sensitivity by increasing glucose uptake in skeletal muscle of insulin-resistant obese mice [11,79],. Increased serum levels of apelin were reported in obesity in association with hyperinsulinaemia [2]. Moreover, plasma concentrations of apelin

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were increased in obese individuals [24], and both type 1 and type 2 diabetic subjects [38,23] but were reduced after bariatric surgery [33]. It is not clear if tissue apelin resistance contributes to elevated circulating apelin concentrations.

We hypothesised that activation of apelin signalling pathways may be useful in the treatment of metabolic disease including T2DM. The short half-life of native apelin analogues hampers the pharmacological impact of apelin agonism [45]. Accordingly, we modified the structure of apelin-13 and recently reported N- and C-terminally modified analogues [52,56] by addition of a fatty acid moiety. We developed four acylated synthetic apelin-13 analogues named (Lys^8GluPAL)apelin-13 amide, pGlu(Lys^8GluPAL)apelin-13 amide, Lys^8GluPAL(Tyr^{13})apelin-13 and Lys^8GluPAL(Val^{13})apelin-13. Notably, these analogues have the same fatty acid conjugated to Lys^8 as the long-acting GLP-1 receptor agonist liraglutide. These apelin analogues were assessed for plasma stability, *in vitro* insulinotropic activity, ability to stimulate glucose uptake in adipocytes, and enhancement of glucose disposal and insulin release in diet-induced obese (DIO) diabetic mice.

2. Materials & methods

2.1. Peptides

All peptides (Table 1) were purchased from EZ Biolabs (Carmel, IN, USA). Peptide purity and structural identity were confirmed as described previously [55]. Modification of native peptide was carried out to confer resistance to cleavage by angiotensin converting enzyme 2 (ACE2) and thus prolong the biological activity. Modifications included, substitution of C-terminal Phe^{13} amino acid with either Tyr^{13} or Val^{13}, amidation of the C-terminus or addition of a N-terminal pyroglutamate ($pGlu^1$) as reported previously [52,56]. Furthermore, a gamma-glutamyl spacer with palmitate adjunct (GluPAL) was added to the side-chain of Lys^8 to promote binding to plasma proteins and reduce renal clearance, thus extending *in vivo* bioactivity as reported previously for stable forms of GLP-1 [36,54,57]. We have used these type of substitution in our laboratory previously, with other natural occurring peptides and these have been tested for stability and efficacy.

2.2. In vitro stability of fatty acid apelin analogues in mouse plasma

Apelin-13 and related peptides susceptibility to proteolytic enzymes of murine plasma was measured, as described previously for other peptides [51]. Reactions were terminated by adding 10% (v/v) TFA/water (Sigma, Poole, UK) and intact or degraded products were separated by reversed-phase HPLC with molecular masses confirmed by MALDI-ToF. The half-life of apelin analogues and the percentage of intact peptides remaining after 4 h, were calculated using HPLC peak area as described previously [16,55].

2.3. Estimation of the peptide half-life using an acylated radiolabelled apelin-13 analogue

The tyrosine (Tyr^{13}) containing analogue was the only one suitable for iodination and estimation of *in vivo* clearance. Thus Lys^8-GluPAL(Tyr^{13})apelin-13 amide was labelled with ^{125}I (Perkin Elmer, Cambridge, UK) by in-house, iodogen method as previously described [58]. A dose of 4.1 nmol/kg (5 μCi) ^{125}I-apelin analogue was administered by intraperitoneal injection to normal healthy NIH Swiss mice (male, 7–8 weeks old) and blood (20 μl) collected at 0, 15, 30, 45, 60, 90, 120, 240, 480, 960 and 1440 min after injection in chilled fluoride/heparin microcentrifuge tubes (Starstedt, Numbrecht, Germany) and centrifuged immediately for 2 min at 12,000*g* at 4°C. Correction for free iodine, a product of intracellular dehalogenation, was performed by determining the radioactivity of the pellet after precipitation with 10% final concentration of trichloroacetic acid (TCA) (Sigma, Poole, UK). Radioactivity was converted into picomoles and half-life (min) of the peptide was calculated by the equation $In(2)/\lambda Z$.

2.4. In vitro insulin secretion

In vitro insulinotropic activity of fatty acid modified apelin analogues were assessed utilizing rat clonal pancreatic BRIN-BD11 cells, routinely cultured in RPMI-1640 medium supplemented with 10% (v/v) FBS and 1% (v/v) antibiotics: penicillin (100 U/mL) and streptomycin (0.1 mg/mL) (Gibco, Strathclyde, UK), as described previously [41].

In a separate set of experiments, dispersed pancreatic islet cells from NIH Swiss mice, obtained by collagenase digestion [18,42] were incubated with apelin-13 peptides and related analogues (10^{-12} to 10^{-6} M) for 1 h in Krebs Ringer bicarbonate (KRB) buffer supplemented with either 1.1 or 11.1 mM glucose. Insulin from cell and islet supernatants were measured by radioimmunoassay [15].

2.5. Intracellular Ca^{2+}

Effects of apelin-13 and selected peptide analogues on concentrations of intracellular Ca^{2+} was assessed using clonal pancreatic BRIN-BD11 cells and FLIPR calcium assay kit (Molecular Devices, Sunnyvale, CA, USA) as described previously [44]. Ca^{2+} data were collected using a FlexStation scanning fluorometer with integrated fluid transfer workstation (Molecular Devices, Sunnyvale, CA, USA) and analysed using Softmax Pro software.

Table 1
Primary structures, molecular masses and plasma degradation of apelin and related peptides.

Name	Amino acid sequence	Theoretical molecular mass (observed mass Da)	Degradation		
			% intact peptide (4 h)	Half-life ($t_{1/2}$) (h)	
				In vitro	In vivo
Apelin-13	NH_2-Q-R-P-R-L-S-H-K-G-P-M-P-F-COOH	1551.9 (1551.8)	25.4 ± 1.5	2.1	–
(Lys^8GluPAL)apelin-13-amide	NH_2- Q-R-P-R-L-S-H-K-G-P-M-P-F-**Amide**	1920.4 (1922.8)	100	>24	–
Lys^8GluPAL(Tyr^{13})apelin-13	NH_2-Q-R-P-R-L-S-H-K-G-P-M-P-**Y**-COOH	1939.4 (1939.2)	100	>24	2.5–3.0
Lys^8GluPAL(Val^{13})apelin-13	NH_2- Q-R-P-R-L-S-H-K-G-P-M-P-**V**-COOH	1875.4 (1875.0)	100	>24	–
pGlu(Lys^8GluPAL)apelin-13-amide	pGlu-R-P-R-L-S-H-K-G-P-M-P-F-**Amide**	1900.4 (1901.7)	100	>24	–

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