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

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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

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[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.

2.9. in vivo glucose homeostasis and insulin secretion

Blood glucose and plasma insulin responses were measured after intraperitoneal (i.p.) administration of glucose alone (18 mmol/kg body weight) or in combination with apelin peptides (25 nmol/kg bw) in normal (4 h fasted) and DIO mice (18 h fasted) mice. To evaluate the persistent effects of peptides, glucose responsiveness was evaluated after i.p. injection of glucose alone (18 mmol/kg bw) at 1, 2, 4 and 8 h after i.p. injection of apelin peptide analogues. In a series of separate experiments plasma insulin and blood glucose response was evaluated at various doses of apelin analogues (1, 5, 25 and 100 nmol/kg body weight) in 4 h fasted normal mice.

2.10. Voluntary food intake study

Normal NIH Swiss mice were habituated to daily feeding regime of 3 h per day by progressively reducing daily feeding period over 3 weeks. In brief, on days 1–7, mice were provided with food for 10 h (10:00 h to 20:00 h), days 8–14 for 6 h (10:00 h to 16:00 h) days 15–21, food was restricted to 3 h (10:00 to 13:00 h), which was maintained throughout the experimental period. For food intake studies, habituated mice (21 h fasted) were administered i.p. injections of peptides at a dose of 1, 5, 25 and 100 nmol/kg bw. Mice were allowed free access to chow for 180 min and cumulative food intake was measured at 30 min intervals.

2.11. Immunocytochemistry for distribution of apelin (APJ) receptors

Deparaffinised, rehydrated and blocked pancreatic tissue sections or fixed BRIN-BD11 cells on slides, were probed with primary antibodies: mouse anti-insulin antibody (1:500; Abcam, ab6995) and porcine anti-glucagon antibody (1:400; Abcam, ab8055) or rabbit anti APJ receptor antibody (1:750, Abcam, ab84296) where appropriate. Following overnight incubation, sections were incubated for 1 h at 37 °C with fluorescent secondary antibody, Alexa Fluor 488 goat anti-porcine or anti mouse IgG (1:400), Alexa Fluor 594 goat anti-rabbit IgG (1:400). Slides were visualised for co-localisation under a FITC (488 nm) or TRITC filter (594 nm) using a fluorescent microscope (Olympus system microscope, model BX51) and photographed using a DP70 camera adapter system.

2.12. Statistical analysis

All results are expressed as mean ± SEM. Where appropriate, groups of data were compared using the unpaired Student's *t*-test, one-way ANOVA or, repeated measures ANOVA followed by the Student-Newman-Keuls post-hoc test. Incremental area under the curve (AUC) analyses for plasma glucose and insulin were calculated using GraphPad Prism version 5.0. software (GraphPad Software, San Diego, CA, USA). Calculation of EC_{50} values were performed from sigmoidal dose-response data using GraphPad Prism and groups of data were considered to be significantly different if $P < 0.05$.

3. Results

3.1. Peptides stability in plasma

Primary structures and molecular masses of each apelin analogue are shown (Table 1). Native apelin-13 and all analogues showed varying degrees of degradation when incubated with mouse plasma. Novel synthetic apelin-13 analogues, with C-terminal modification with Ala^{13}, Val^{13}, Tyr^{13} or amidation showed improved stability ($p < 0.001$) compared to native apelin-13 at 4 h (Table 1, Fig. 1A). Similarly, N-terminus pyroglutamate addition improved stability ($p < 0.05$) but it was further enhanced ($p < 0.001$; Table 1; Fig. 1B) when combined with the C-terminal modifications.

3.2. Effects of apelin-13 and its analogues on in vitro insulin release

The effects of apelin-13 and related analogues on insulin secretion from clonal pancreatic beta cells (BRIN-BD11) are shown in Table 2. All peptides, with the exception of Ala^{13} and Val^{13} substituted versions, showed a significant increase ($p < 0.05$ to $p < 0.001$) in insulin secretion compared to 5.6 or 16.7 mmol/L glucose alone. Peptides with C-terminal amide ($p < 0.001$; Fig. 1D) or pyroglutamate ($p < 0.001$; Fig. 1E,F), showed remarkably improved insulinotropic actions ($p < 0.001$, Table 2). As expected, the known antagonist of apelin receptors, (Ala^{13})apelin-13 as well as the structurally similar (Val^{13}) apelin-13, and their (pGlu) variants, all significantly reduced insulin secretion ($p < 0.01$ to $p < 0.001$, Table 2)

3.3. Effects of apelin analogues on intracellular Ca^{2+} and cyclic AMP production

BRIN-BD11 cells treated with native, Tyr substituted or C-terminally amidated apelin-13 along with (pGlu)apelin-amide and pGlu(Tyr^{13}) apelin-13 showed increased intracellular calcium responses (130–296%; $p < 0.05$ to $p < 0.01$, Table 1, Fig. 1G,H) and significant dose-dependent increases in cAMP production (82–165%; $p < 0.05$ to $p < 0.01$, Table 1, Fig. 1I,J) compared to glucose alone. As expected, no stimulation of intracellular calcium or cAMP was detected with the known APJ receptor antagonist (Ala^{13})apelin-13 or its structurally related analogue (Val^{13})apelin-13 (Fig. 1, Table 2). These data suggest that apelin-13 and its related analogues operate through membrane depolarisation facilitating a rise in intracellular Ca^{2+} and cAMP second messenger pathways.

3.4. Effects of apelin analogues on insulin secretion from isolated islets

Apelin-13 and related C-terminally amidated and pGlu analogues produced a concentration-dependent increase in the rate of insulin secretion from isolated mouse islets (Fig. 2D–G). The stimulatory effects of peptides, measured as the% of total insulin content released, showed apelin-13 amide as the most potent stimulator (7-fold, EC_{50} of 8.6×10^{-10}, $p < 0.001$, Fig. 2E) while, apelin-13 (2.6-fold, $p < 0.01$, Fig. 2D), (pGlu)apelin-13 (2.9-fold, $p < 0.01$, Fig. 2F) and (pGlu) apelin-13 amide (6-fold, $p < 0.001$, Fig. 2G) all showed significant dose-dependent effects. The APJ receptor antagonist, pGlu(Ala^{13}) apelin-13 and the structurally related pGlu(Val^{13})apelin-13 both inhibited insulin release from isolated mouse islets.

3.5. Effect of apelin analogues on glucose uptake from 3T3-L1 adipocytes

Glucose uptake by differentiated adipocytes was increased when cells were treated with apelin-13 amide compared to the glucose-free media (184% increase; $p < 0.05$, Fig. 2H). The effect however was not as great as the positive control insulin (100 nM; 326% increase). Similar enhancements of glucose uptake were observed with (pGlu)apelin-13 (153% increase, $p < 0.01$), pGlu(Tyr^{13})apelin-13 (161% increase, $p < 0.01$) and (pGlu)apelin-13 amide (222% increase, $p < 0.01$, Fig. 2I) compared to glucose-free media.

3.6. Expression of APJ receptor on BRIN-BD11 cells and pancreatic islets

Distribution of APJ receptors are displayed as double immuno-fluorescence showing insulin (green) and APJ (red) and co-localization (yellow) in BRIN-BD11 cells (Fig. 2A), suggesting that APJ receptors co-localize with insulin-secreting beta cells. A similar staining pattern was observed in pancreatic islets of normal (Fig. 2B) and DIO mice (Fig. 2C). Glucagon was stained on the periphery of the islet (Fig. 2B,C) though no evidence of the receptor co-localisation with glucagon-secreting alpha cells (no yellow staining) was observed.

Table 1
Primary structures, molecular masses and degradation of apelin-13 and related analogues. Half-lives were calculated by constructing a graph of percentage intact peptide against time. Linear regression "best-fit" analysis was used to calculate the time at which half of the peptide was degraded. Values are mean $\pm$ SEM for n = 2, where ***p < 0.001 is compared to native apelin-13 peptide.

Name	Amino acid sequence	Theoretical molecular mass (observed mass Da)	Degradation	
			% intact peptide (4 h)	Half-life ($t_{1/2}$) (h)
Apelin-13	NH$_2$-Q-*R*-P-*R*-L-*S*-H-K-G-P-M-P-F-COOH	1551.9 (1551.8)	25.4 $\pm$ 1.5	2.1
(Ala^{13})apelin-13	NH$_2$-Q-*R*-P-*R*-L-*S*-H-K-G-P-M-P-**A**-COOH	1474.8 (1474.8)	70.5 $\pm$ 2.2***	10.3
(Val^{13})apelin-13	NH$_2$-Q-*R*-P-*R*-L-*S*-H-K-G-P-M-P-**V**-COOH	1503.8 (1505.7)	66.5 $\pm$ 1.1***	7.7
(Tyr^{13})apelin-13	NH$_2$-Q-*R*-P-*R*-L-*S*-H-K-G-P-M-P-**Y**-COOH	1566.8 (1569.1)	76.7 $\pm$ 1.3***	8.5
Apelin-13-amide	NH$_2$-Q-*R*-P-*R*-L-*S*-H-K-G-P-M-P-F-**Amide**	1550.9 (1552.3)	80.4 $\pm$ 1.9***	11.4
(pGlu)apelin-13	**pGlu**-*R*-P-*R*-L-*S*-H-K-G-P-M-P-F–COOH	1535.8 (1535.7)	47.0 $\pm$ 3.2	3.8
pGlu(Ala^{13})apelin-13	**pGlu**-*R*-P-*R*-L-*S*-H-K-G-P-M-P-**A**-COOH	1459.7 (1461.2)	74.4 $\pm$ 5.2***	11.1
pGlu(Val^{13})apelin-13	**pGlu**-*R*-P-*R*-L-*S*-H-K-G-P-M-P-**V**-COOH	1487.8 (1488.8)	76.2 $\pm$ 1.9***	9.8
pGlu(Tyr^{13})apelin-13	**pGlu**-*R*-P-*R*-L-*S*-H-K-G-P-M-P-**Y**-COOH	1550.7 (1551.5)	79.0 $\pm$ 0.6***	12.2
(pGlu)apelin-13-amide	**pGlu**-*R*-P-*R*-L-*S*-H-K-G-P-M-P-F –**Amide**	1534.8 (1536.2)	85.0 $\pm$ 1.3***	10.4

3.7. Acute and persistent glucose-lowering and insulinotropic actions of apelin analogues in lean and DIO mice

The acute effects of apelin-13 analogues (25 nmol/kg bw) on glucose-lowering and insulinotropic actions were assessed in normal lean and DIO mice (Table 3 and Fig. 3). Intraperitoneal injection of the most effective peptides from in vitro studies, apelin-13 amide, (Tyr^{13})apelin-13, pGlu(Tyr^{13})apelin-13 and (pGlu)apelin-13, all significantly decreased plasma glucose excursions compared to glucose alone control in normal mice (Fig. 3A,C) and in DIO mice (Fig. 3E,G). Integrated responses, presented as area under the curve (AUC), showed that plasma glucose was reduced by 31% (p < 0.01), 23% (p < 0.01), 29% (p < 0.05) and 43% (p < 0.05) respectively, in lean mice and a similar trend was observed in DIO mice (26% to 49% reduction; p < 0.05 to p < 0.01; Table 3). This was accompanied by an improved integrated insulin response in lean mice (29% to 49% increase;
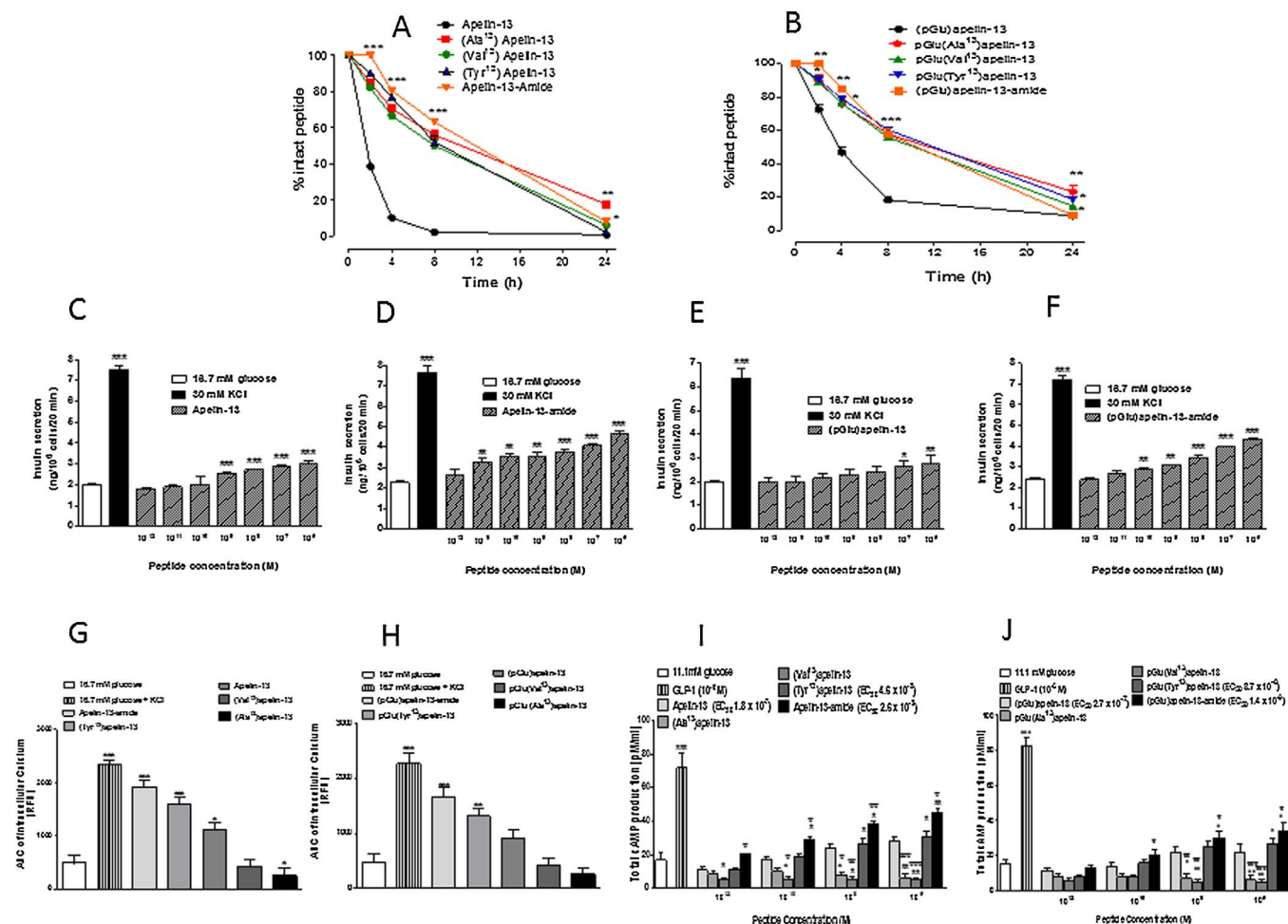

Fig. 1. (A, B) Stability of apelin-13 and related analogues after incubation with plasma from fasted normal mice for 0, 2, 4, 8 and 24 h (n = 2). Values represent mean $\pm$ SEM of intact peptide. *p < 0.05, **p < 0.01 and ***p < 0.001 compared to apelin-13 at each time point. (C–F) Effects of apelin-13 analogues on insulin secretion at 16.7 mM glucose from BRIN-BD11 cells. Values represent mean $\pm$ SEM (n = 8) where, *p < 0.05, **p < 0.01 and ***p < 0.001 is compared to respective glucose controls. (G, H) Area under the curve for intracellular calcium at 16.7 mM glucose and (I, J) total cAMP production by BRIN-BD11 cells at 11.1 mM glucose.

Table 2

Effects of apelin-13 and related analogues on in vitro insulin secretion, intracellular calcium and cAMP generation in BRIN-BD11 cell. EC_{50} values at 16.7 mM glucose are also shown with responses to 10^{-6} M peptide concentration. Values are mean ± SEM for n = 8 where *p < 0.05, **p < 0.01, ***p < 0.001 is compared to respective glucose controls for insulin secretion (5.6 mM and 16.7 mM), intracellular calcium (5.6 mM), and cAMP (11.1 mM).

Peptide (10^{-6} M)	*In vitro* insulin secretion (ng/10^6 cells/ 20 min)			Intracellular [Ca^{2+}]	Cyclic AMP
	5.6 mM glucose	16.7 mM glucose	EC_{50} (M)	Change	Change
None (glucose alone)	1.00 ± 0.08	1.99 ± 0.07	--------------	--------------------	--------------------
Apelin-13	1.37 ± 0.1*	3.03 ± 0.09 ***	6.06e-010	130% ↑ (p<0.05)	75% ↑ (p>0.05)
(Ala¹³)apelin-13	0.63 ± 0.02**	1.65 ± 0.06 *	1.74e-009	48% ↓ (p<0.05)	70% ↓ (p<0.01)
(Val¹³)apelin-13	0.72 ± 0.04**	1.19 ± 0.04 ***	1.29e-010	14% ↓ (p<0.05)	75% ↓ (p<0.01)
(Tyr¹³)apelin-13	1.56 ± 0.06**	3.92 ± 0.07 ***	9.51e-009	171% ↑ (p<0.01)	82% ↑ (p<0.05)
Apelin-13 amide	1.92 ± 0.18***	4.66 ± 0.13 ***	2.68e-008	296% ↑ (p<0.01)	165% ↑ (p<0.01)
pGlu(apelin-13)	1.28 ± 0.08*	2.76 ± 0.34 **	1.10e-008	89% ↑ (p>0.05)	47% ↑ (p>0.05)
pGlu(Ala¹³)apelin-13	0.84 ± 0.07	1.55 ± 0.06	7.68e-009	46% ↓ (p>0.05)	60% ↓ (p<0.01)
pGlu(Val¹³)apelin-13	0.62 ± 0.04***	1.19 ± 0.04 ***	1.59e-008	13% ↓ (p>0.05)	72% ↓ (p<0.01)
pGlu(Tyr¹³)apelin-13	1.57 ± 0.03***	3.67 ± 0.12 ***	1.48e-008	171% ↑ (p<0.01)	85% ↑ (p<0.05)
(pGlu)apelin-13 amide	1.65 ± 0.03***	4.33 ± 0.06 ***	9.92e-009	244% ↑ (p<0.001)	127% ↑ (p<0.05)

p < 0.05 to p < 0.01; Table 3) and in DIO mice (26% to 55% increase; p < 0.05 to p < 0.001; Table 3). In contrast, the receptor antagonist peptides (Ala¹³ and Val¹³ modified analogues) had opposite effects as it increased blood glucose (p < 0.01) and decreased plasma insulin (p < 0.01, Table 3) in both lean and DIO mice.

On the basis of the above functional and peptide stability data, apelin-13 amide and (pGlu)apelin-13 amide were selected for further in vivo evaluation of persistent and concentration dependent effects. Both peptides showed a reduction in glycemic excursion (p < 0.01 to p < 0.001; Fig. 3I) at 25 and 100 nmol/kg bw concentration which was accompanied with an overall insulinotropic response (p < 0.05 to p < 0.001; Fig. 3J). Only (pGlu)apelin-13 amide was potent at a concentration on 5 nmol/kg bw with reduction in glucose (p < 0.05, Fig. 3I) and increase in insulin secretion (p < 0.05, Fig. 3J) compared to glucose alone controls. Administration of apelin-13 amide and (pGlu)apelin-13 amide 1, 2 and 4 h earlier significantly decreased overall glucose excursions (33% reduction; p < 0.05 to p < 0.001, Fig. 3 K) after i.p. glucose injections in mice. This was associated with increased insulin release as assessed by AUC (13% to 56% increase; p < 0.05 to p < 0.01; Fig. 3L). A delay of 8 h following peptide administration, but before glucose challenge annulled the overall antihyperglycemic efficacy of the peptides, although (pGlu)apelin-13 amide showed a small increase in plasma insulin secretion (p < 0.05; Fig. 3L).

3.8. Acute in vivo food-intake studies

At a concentration of 25 nmol/kg bw, peptides (Tyr¹³)apelin-13 (up to 120 min), apelin-13 amide (up to 180 min), pGlu(Tyr¹³)apelin-13 (up to 180 min except 90 min) and (pGlu)apelin-13 amide (up to 180 min) showed a reduction in food intake when compared with saline controls (p < 0.05 to p < 0.001; Fig. 4A–C). Apelin-13 amide and (pGlu)apelin-13 amide were superior to established incretin mimetic therapies, liraglutide and exendin-4. Significant appetite suppression was observed following administration of apelin-13 amide and (pGlu)apelin-13 amide compared to GLP-1 (60–180 min; p < 0.05 to p < 0.001; Fig. 4C), liraglutide (30–180 min; p < 0.05 to p < 0.001; Fig. 4C) and exendin-4(1–39) (150–180 min; p < 0.05 to p < 0.01;

Fig. 4C). Dose dependent effect on appetite suppression showed native apelin-13 potent at a dose of 100 nmol (90 and 120 min; p < 0.05; Fig. 4 D). At doses of 25 and 100 nmol, apelin-13 amide (60–180 min; p < 0.01 to p < 0.001; Fig. 4 E) and (pGlu)apelin-13 amide (up to 180 min; p < 0.01 to p < 0.001; Fig. 4E), (pGlu)apelin-13 (only 90 min and 60–150 min; p < 0.05; Fig. 4F) significantly suppressed food intake compared to saline treated control mice. We did not observe any changes in behaviour of mice, which would might indicate food aversion responses following apelin-13 analogue administration.

4. Discussion

Native apelin is degraded via ACE2 (angiotensin-converting enzyme 2), which removes the C-terminal Phe from apelin-13 and apelin-36 resulting in truncated forms that are subject to further degradation with resulting loss of biological activity [22,37–39]. In the present study, the naturally occurring peptides, apelin-13 and (pGlu)apelin-13, were extensively degraded by in vitro incubation with mouse plasma by 50–75% within 4 h. In contrast, the novel analogues synthesized with amino acid substitutions and/or modifications at the C-terminus exhibited significantly improved metabolic stability (75–100% intact after 4 h, Table 1) suggesting that such modifications, by blocking further degradation, would confer enhanced biological activity in vivo.

Previous research has shown that amino acid substitution in native apelin significantly affects biological activity. Thus, replacement of Leu⁵, or Arg² in and Arg⁵ of the native apelin-13, resulted in significant changes in pharmacology, including cAMP generation and intracellular Ca^{2+} in human APJ receptor transfected HEK-293 cells [40]. In the present study, many of the C-terminal modifications employed also resulted in enhanced insulin releasing activity from clonal BRIN-BD11 cells and primary mouse islets. This indicates positive rather than negative effects of our C-terminal modifications on receptor binding and activation. The actions of these apelin-13 analogues on insulin secretion can be ascribed to APJ receptors present on pancreatic islet cells [41,42]. Thus, double immunostaining for insulin/APJ and glucagon/APJ revealed that APJ receptors were expressed on the β-cells of the islet without concomitant expression in glucagon secreting α-cells and
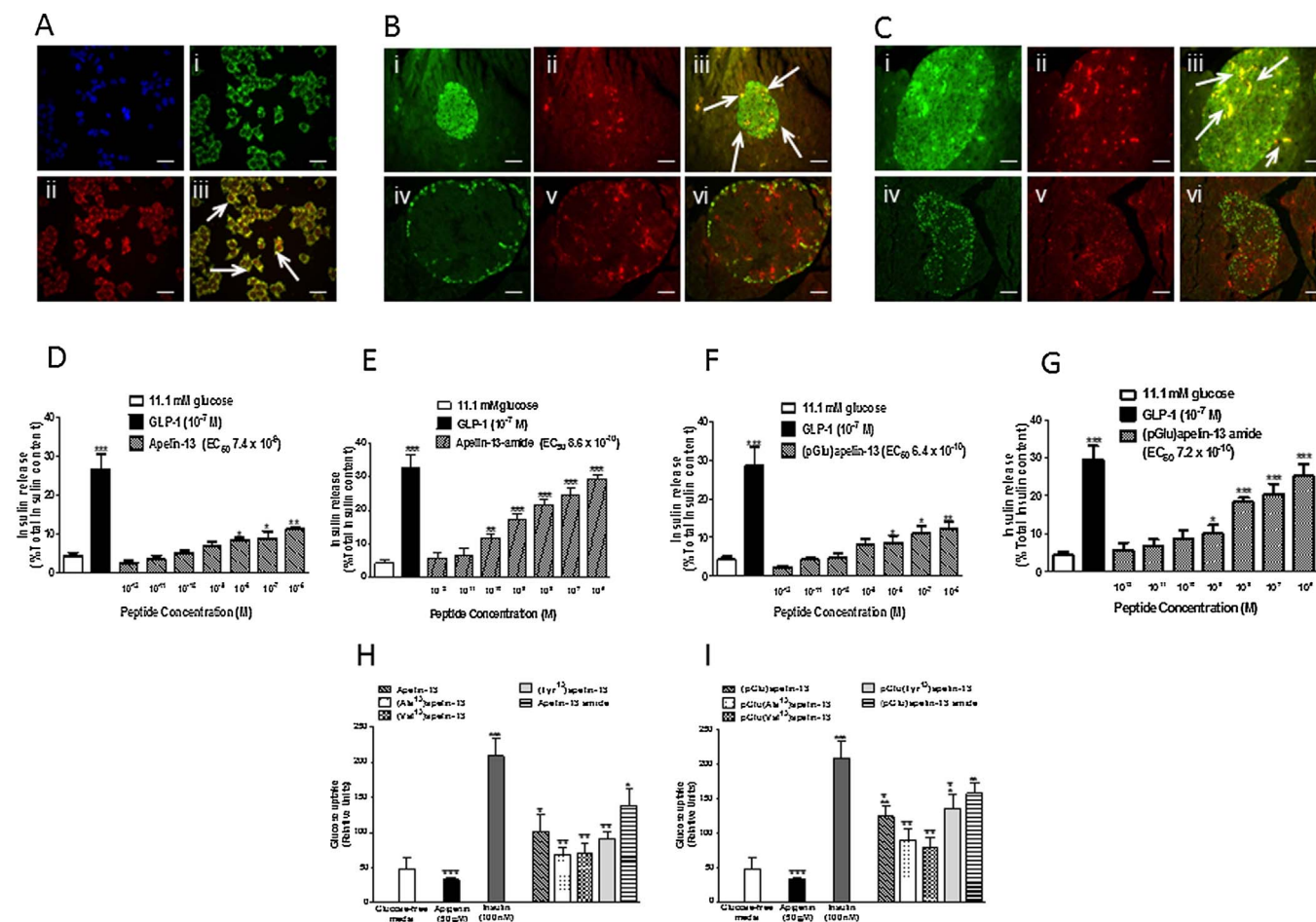
Fig. 2. Distribution of APJ in (A) BRIN-BD11 cells, (B) lean mouse pancreatic islets and (C) high fat fed mouse pancreatic islets (C). (i) insulin (ii, v) APJ receptor (iii) merge of APJ colocalised with insulin, (iv) glucagon and (vi) merge of APJ colocalised with glucagon. Examples of colocalisation are indicated by arrows. All at $\times 40$ magnification. (D–G) Insulin release by isolated mouse islets at 11.1 mM glucose in response to apelin-13 and related analogues. GLP-1 (10^{-6} M) was included as a positive control. Values represent mean $\pm$ SEM (n = 8) where, *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to glucose alone. (H, I) Glucose uptake by 3T3-L1 adipocytes in response to various apelin peptides (x 10^{-7} M). Apigenin was used as a negative control. Values represent mean $\pm$ S.E.M (n = 6). *P < 0.05 compared with glucose-free media. $^{\triangledown}$p < 0.05, $^{\triangledown\triangledown}$p < 0.01 and $^{\triangledown\triangledown\triangledown}$p < 0.001 compared with insulin (100 nM).

this contrasts with what was reported previously [23].

Earlier studies suggested that the naturally occurring peptides, apelin-13 and (pGlu)apelin-13, inhibited glucose induced insulin secretion [23,41] from INS-1 cells, activating PI3-kinase-dependent PDE3 B and subsequently suppressing cAMP levels [43]. This view sharply contrasts with the present research which clearly demonstrated that both apelin-13 and (pGlu)apelin-13, increased glucose-stimulated insulin secretion especially under hyperglycemic conditions. Indeed, we demonstrated for the first time, that novel apelin analogues, (Tyr[13]) apelin-13 and apelin-13 amide, as well as their (pGlu) versions are very good insulin secretagogues. The stimulatory effects on BRIN-BD11 cells and isolated mouse islets were both glucose-sensitive and

Table 3
Integrated glycaemic and insulin (area under the curve, AUC) responses to apelin-13 and related analogues (25 nmol/kg body weight) following i.p. administration to normal mice or high fat fed mice together with 18 mmol/kg glucose. Values are mean $\pm$ SEM for n = 8 where * p < 0.05, **p < 0.01, ***p < 0.001 is compared to the glucose control.

| Peptide (25 nmol/kg body weight)	In vivo response			
	Plasma glucose AUC$_{0-105}$ (mmol/l.min)		Plasma insulin AUC$_{0-105}$ (ng/ml.min)	
	Normal mice	High fat fed mice	Normal mice	High fat fed mice
Glucose alone	776.9 ± 56.6	1350 ± 86.5	128.9 ± 14.5	167.2 ± 9.6
Apelin-13	716 ± 42.6	1338 ± 100.3	142.6 ± 16.7	182.7 ± 21.2
(Ala[13])apelin-13	831.2 ± 66.4	1594 ± 73.9 *	87.3 ± 8.1 *	133.3 ± 8.8 *
(Val[13])apelin-13	968.6 ± 31.3 **	1672 ± 76.5 **	67.1 ± 6.8 **	114.1 ± 3.3 **
(Tyr[13])apelin-13	596.7 ± 31.5 **	1096 ± 107.1 *	165.8 ± 17.5 *	204.4 ± 12.8 *
Apelin-13 amide	507.0 ± 50.4 **	897.8 ± 87.5 **	196.6 ± 4.9 **	242.3 ± 15.0 ***
pGlu(apelin-13)	763.5 ± 45.2	1127 ± 63.9	121.8 ± 11.2	173.7 ± 16.4
pGlu(Ala[13])apelin-13	754.3 ± 45.2	1363 ± 57.62 *	101.7 ± 20.6	147.7 ± 6.9 *
pGlu(Val[13])apelin-13	867.1 ± 51.9	1440 ± 58.9 *	80.4 ± 5.1 **	128.2 ± 5.3 **
pGlu(Tyr[13])apelin-13	606.6 ± 19.8 *	899.7 ± 117.6*	177.0 ± 17.6 *	215.2 ± 9.9 **
(pGlu)apelin-13 amide	492.4 ± 59.8 *	848.4 ± 92.1 **	203.7 ± 8.2 **	233.8 ± 21.5 **
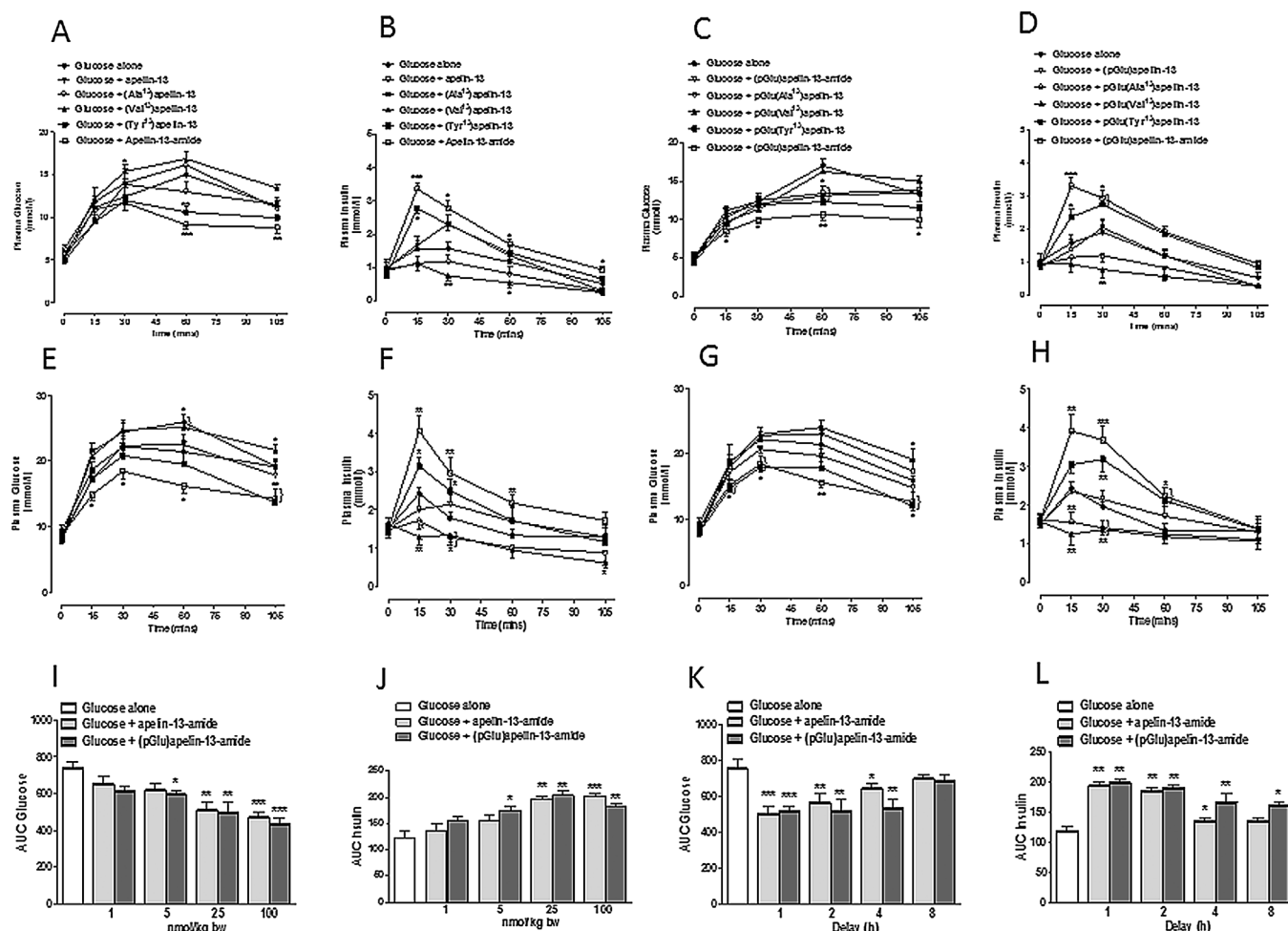
Fig. 3. Acute glucose lowering and insulin-releasing effects of apelin-13 and related analogues following i.p. administration (25 nmol/kg body weight) together with glucose (18 mmol/kg). (A, C) Plasma glucose and (B, D) plasma insulin response in normal 4 h fasted mice. (E–H) response in 18 h fasted high-fat fed mice. (I, J) Plasma glucose and insulin AUC values for 0 to 105 min post-injection for apelin-13 peptides at different doses (1–100 nmol/kg bw). (K, L) Responses to glucose (18 mmol/kg, i.p.) 1–8 h after administration of apelin peptides (25 nmol/kg bw) in 4 h fasted normal mice. Values represent mean ± SEM for eight mice (n = 8). *p < 0.05, **p < 0.01 and ***p < 0.001 compared to glucose alone.

concentration-dependent, with apelin-13 amide followed by (pGlu) apelin-13 amide appearing to be the most potent. These β-cell actions appear to be mediated through activation of more than one signal transduction pathway with elevations of both intracellular Ca^{2+} and cyclic AMP.

In contrast to these positive effects on insulin secretion, the enzyme-resistant analogues of apelin-13 with Ala^{13} or Val^{13} substitutions consistently inhibited basal and glucose-induced insulin release from BRIN-BD11 cells as well as mouse islets. Furthermore, they did not increase cyclic AMP or intracellular Ca^{2+} in BRIN-BD11 cells. (Ala^{13})apelin-13 has previously been reported to be a APJ receptor antagonist, with co-injection of the peptide completely blocking the vasodilator effects of apelin-13 in rats [44]. Interestingly (Val^{13})apelin-13 appeared to be more potent than (Ala^{13})apelin-13 at inhibiting glucose stimulated insulin release from clonal beta cells as well as primary islet tissue. These inhibitory effects were observed in the absence of any added APJ receptor agonist possibly suggesting cross talk between various signalling pathways.

In addition to their insulinotropic effects, apelin-13 amide and (pGlu)apelin-13 amide were the two most potent analogues at stimulating glucose uptake in differentiated 3T3-L1 adipocytes. This suggests that in vivo, these analogues might exert antihyperglycemic effects by addressing defects in both the secretion and action of insulin − the two most cardinal features of type 2 diabetes. This view is supported by previous demonstration that (pGlu)apelin-13 stimulated glucose uptake in both 3T3-L1 adipocytes and mouse muscle through AMP-activated

protein kinase (AMPK)-dependent and PI3 K/Akt pathways [45,46].

Consistent with their in vitro actions, (Tyr^{13})apelin-13 and apelin-13 amide peptides demonstrated marked glucose lowering and insulin-releasing activity during i.p. glucose tolerance tests in both normal mice and DIO mice. Interestingly, the natural forms apelin-13 and (pGlu) apelin-13 had minimal effects. A greater effect was observed when analogues were tested in DIO mice, possibly reflecting greater efficacy of apelin analogues to stimulate insulin release in vitro under hyperglycemic conditions. In contrast, the inhibitory analogues (Ala^{13}) apelin-13 and (Val^{13})apelin-13 both exacerbated the blood glucose excursion and blocked glucose-induced insulin release in vivo. Apelin-13 amide and (pGlu)apelin-13 amide were amongst the most potent stimulatory peptides and subsequent studies demonstrated their dose-dependent and persistent effects which were observed at doses as low as 5 nmol/kg body weight and for as long as 4–8 h. These combined data suggest that our strategies to confer enhanced stability, leading to an enhancement and extension of in vivo bioactivity, were successful as has been observed previously with other peptides including GLP-1 [47,48].

Although the data is not reported here, we undertook a PharmaScreen process with two different apelin-13 analogues using the DiscoveRx receptor model platform. This included the gpcrMAX screen which examined agonist activity against 168 known target receptors (including GLP-1, GIP and glucagon receptors), as well as against 73 orphan receptors (orphanMAX screen). The results indicated agonist activity at the recombinant human apelin APJ receptor as predicted,
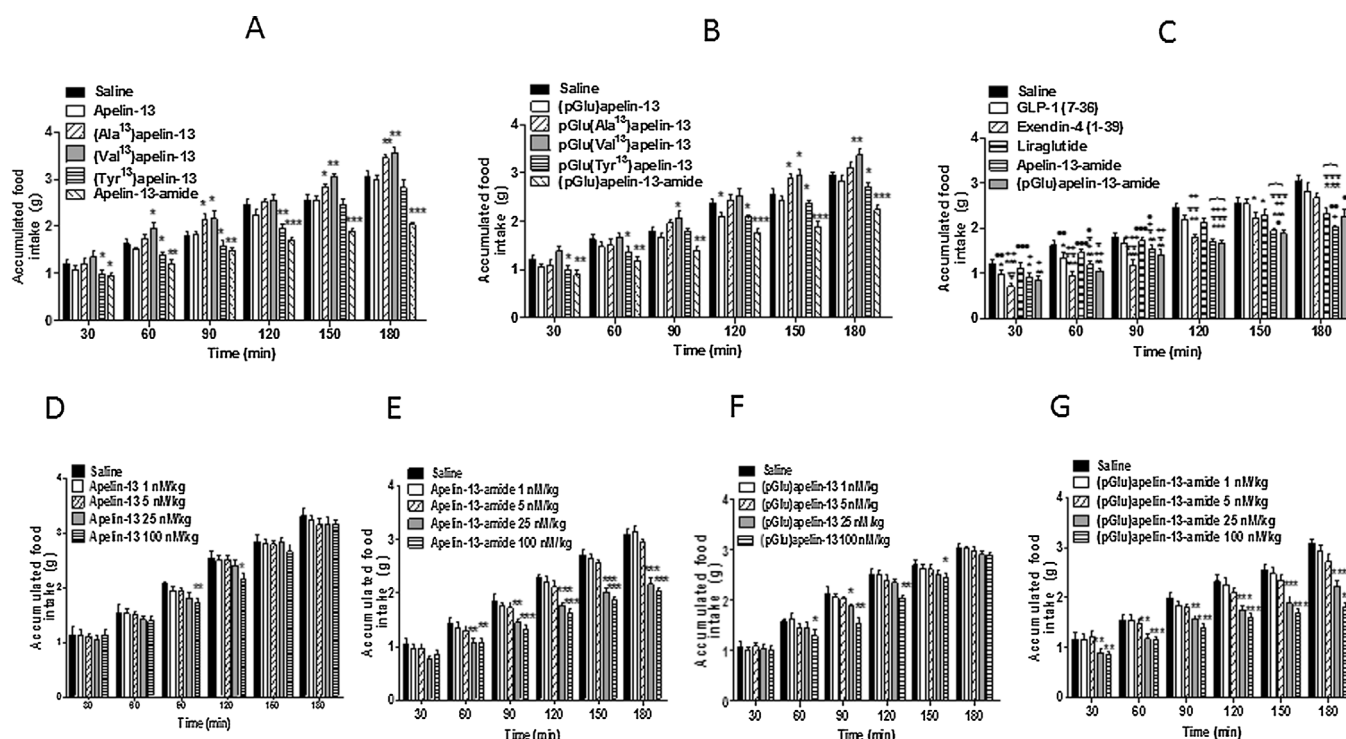
Fig. 4. Acute effects of apelin-13 and related analogues (A–C) at doses of 25 nmol/kg or (D–G) at 1–100 nmol/kg on voluntary food intake in healthy male NIH Swiss mice. Mice were acclimatised to eating for 3 h per day. Peptides were administered by intraperitoneal injection to 21 hour fasted mice and cumulative food intake was monitored at 30, 60, 90, 120, 150 and 180 min after reintroduction of food. Values are mean ± SEM (n = 8). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to saline control, $\triangledown p < 0.05$, $\triangledown\triangledown p < 0.01$ and $\triangledown\triangledown\triangledown p < 0.001$ compared to GLP-1, $^{+}p < 0.05$, $^{++}p < 0.01$ and $^{+++}p < 0.001$ compared to liraglutide, •$p < 0.05$, ••$p < 0.01$ and •••$p < 0.001$ compared to exendin-4(1-39).

but no significant agonist activity at any of the known GCPR or orphan GPCR receptors. Thus we can confidently report that no off-target receptor agonist activity was detected for our apelin-13 related analogues and that these are not acting via the GLP-1,GIP and glucagon receptors (< 1% activity).

Obesity promotes insulin resistance, making antihyperglycemic agents which also decrease appetite, such as GLP-1 mimetics [49,50] particularly useful for type 2 diabetes therapy. In the present study, apelin-13 amide and (pGlu)apelin-13 amide substantially inhibited food intake in normal mice at doses of 5–25 nmol/kg for up to 3 h. Such actions were superior to GLP-1, exendin-4 or liraglutide. The anorexigenic effect might be related to co-localisation of APJ receptors with proopiomelanocortin (POMC) in the hypothalamic arcuate nucleus (ARC) [51], which secretes α-melanocyte-stimulating hormone (α-MSH), a strong appetite suppressor [52,53]. Alternatively, apelin induced afferent vagal nerve activation via APJ receptors might impart an indirect effect via suppression of hypothalamic appetite centres [54]. Consistent with a possible physiological role of these pathways, the established APJ antagonist, (Ala^{13})apelin-13 together with its pGlu analogue and partner (Val^{13})apelin-13 analogues enhanced food intake. Irrespective of the exact mechanism by which APJ receptor activation affects feeding, suppression of appetite together with the other beneficial actions of apelin-13 analogues on both insulin release and glucose uptake by muscle and adipose tissue come together as an attractive antidiabetic repertoire of effects. Researchers have shown that APJ receptors are widely distributed in the rodent brain [40,55]. In addition, APJ receptors have been located in the hypothalamus and the PVN region which could imply that apelin is involved in important appetite control centres [51,56] with POMC neuron activation reported [51] leading to a reduction of food intake. However, the action of apelin in the hypothalamus is far from clear, since others have recently reported that icv apelin in the ARC nucleus region stimulates food intake in rats [57], and that icv apelin reduces energy expenditure in mice [58]. It remains unclear if peripheral apelin can cross the blood brain barrier to affect central brain appetite control centres. One study has examined the effect of apelin-13 on neurons within the subfornical organ, but this region of the brain does not have a normal intact blood brain barrier [59].

In conclusion, this study demonstrates that novel analogues (Tyr^{13}) apelin-13, pGlu(Tyr^{13})apelin-13, apelin-13 amide and (pGlu)apelin-13 amide are stable to enzymatic degradation and exert positive effects on glucose homeostasis through multiple physiological pathways. Emerging studies examining the effects of chronic in vivo administration of stable acylated or non-acylated forms of apelin-13 amide or (pGlu)apelin-13 amide analogues in diabetic animal models reveal promising efficacy profiles for this new family of antidiabetic peptides [60,61].

Declaration of interest

Ulster University, FOH and PRF hold patents for use of therapeutic peptides for treatment of obesity-diabetes.

Author contribution statement

FOH and PRF devised and planned the studies. CH carried out experimental work, analysed the data and commented on the manuscript. VP, FOH and PRF wrote the manuscript. All authors edited and approved the final version of the manuscript.

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