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Proceeding Paper

# Peptide Co-Agonists for Combined Activation of the APJ and GLP-1 Receptors with Insulinotropic and Satiety Actions Show Potential for Alleviation of Metabolic Dysfunction in Type 2 Diabetes <sup>†</sup>

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**Abstract:** Stable analogues of the adipokine apelin-13 have shown promising therapeutic potential via APJ receptor activation in isolated  $\beta$ -cells and in animal models of obesity-related diabetes. Incretin mimetics such as exenatide that bind to GLP-1 receptors are well-established Type 2 diabetes treatment options. We developed novel hybrid co-agonist peptide analogues incorporating both exendin-4(1-30) covalently linked to apelin (ELA). The dose-dependent ( $10^{-12}$  to  $10^{-6}$  M) actions of ELA and component peptides were tested on acute (20 min) insulin secretion from cultured pancreatic BRIN-BD11  $\beta$ -cells at 5.6 mmol/L glucose. In addition, separate tests were performed in the presence or absence of specific APJ and GLP-1 receptor antagonists. The co-agonist ELA peptide showed markedly greater insulinotropic actions (1.6 to 3.3-fold) than equimolar concentrations of either component peptide alone or in combination ( $p < 0.001$ ). ELA and related acylated analogues (25 nmol/kg i.p. injection) were also tested on cumulative food intake in trained 21 h-fasted adult mice ( $n = 8$ ), with food intake measured at 30 min intervals up to 180 min. The ELA co-agonist peptides significantly reduced food intake (3.1-fold by 180 min) in mice ( $p < 0.001$ ) versus saline-treated controls. ELA peptides showed marked improvements in both insulin secretion and appetite control, raising interest in their therapeutic potential.

**Keywords:** apelin; incretin action; diabetes therapy; insulin secretion; hybrid peptides



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## 1. Introduction

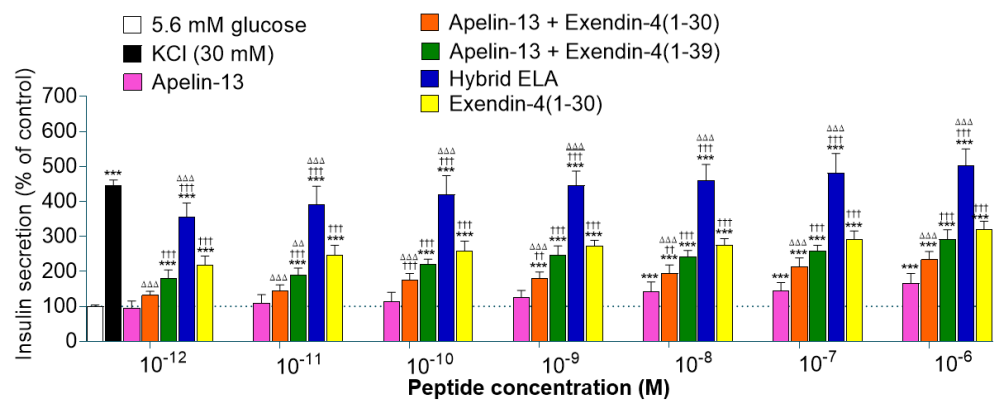
Apelin is an adipokine that is mainly produced and secreted from white adipose tissue in man [1]. Apelin has been reported to be involved in the regulation of glucose homeostasis, improving insulin sensitivity as well as energy metabolism and obesity [2]. A role for apelin in glucose homeostasis was confirmed following the adverse metabolic effects of APJ receptor knockout in mice [3]. Circulating apelin isoforms range from 36 to 13 amino acids in length [4], and the latter has promising beneficial metabolic effects [5] but requires an improved pharmacological profile due to its rapid enzymatic degradation [6]. Stable incretin mimetic peptides which activate glucagon-like peptide-1 (GLP-1) receptors such as exendin-4 (exenatide), provide well-established and efficacious therapy for Type 2 diabetes management [7,8]. More recently, longer-acting acylated peptides like liraglutide, semaglutide, and tirzepatide have gained clinical approval [9]. Here, we examined the insulinotropic actions of a novel hybrid co-agonist peptide formed by covalently linking exendin-4(1-30) to apelin-13 in a linear unimolecular sequence to produce exendin-linker-apelin (ELA). ELA plus some longer-acting acylated ELA analogues were also assessed for their actions on food intake over a 180 min interval in 21 h-pre-fasted trained mice.

## 2. Materials and Methods

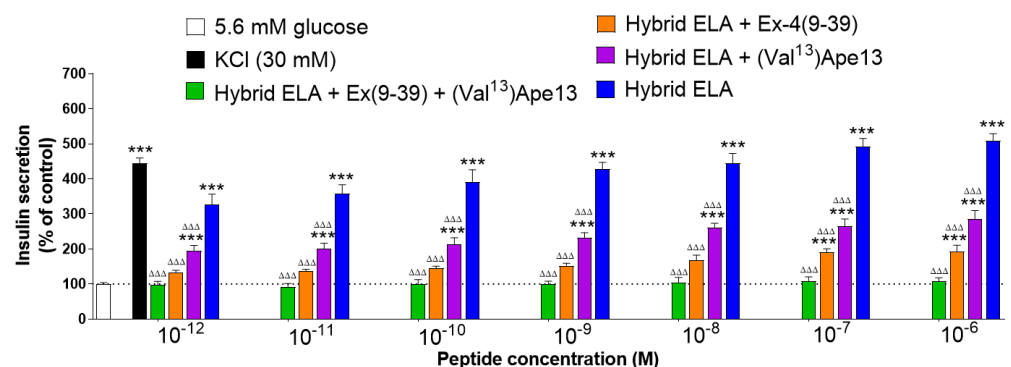
Synthetic peptides (>95% purity) were purchased from Synpeptide Co., Ltd., Shanghai, China, and structures were confirmed via HPLC and mass spectrometry as described previously [10]. Briefly, clonal pancreatic BRIN-BD11 cells (200,000 per 24-well plate; n = 8) were cultured in RPMI-1640 media (1.0 mL) containing 5.6 mmol/L glucose [10] and exposed to ELA ( $10^{-12}$ – $10^{-6}$  M) or peptides for 20 min in a static incubation. Duplicate supernatant (200  $\mu$ L) was taken for analysis with  $I^{125}$ -labelled insulin using an in-house RIA standard curve from 0.019–20 ng/mL [11] with potassium chloride (KCl, 30 mmol/L) as a positive control.

Male NIH Swiss mice (Envigo Ltd., Bicester, UK), aged 8–12 weeks, were trained on a daily feeding regime of 3 h/day (10.00–13.00 h) over a 3 week period, as described previously [12], with free access to water. Food was removed at 13.00 h daily and mice fasted overnight for 21 h. Mice (n = 8) were given an i.p. injection of saline (0.9% w/v NaCl) or peptide analogue (25 nmol/kg bw) at 10:00 h and food returned. Food was weighed prior to peptide injection (0 min) and at 30 min intervals up to 180 min, recording cumulative food intake.

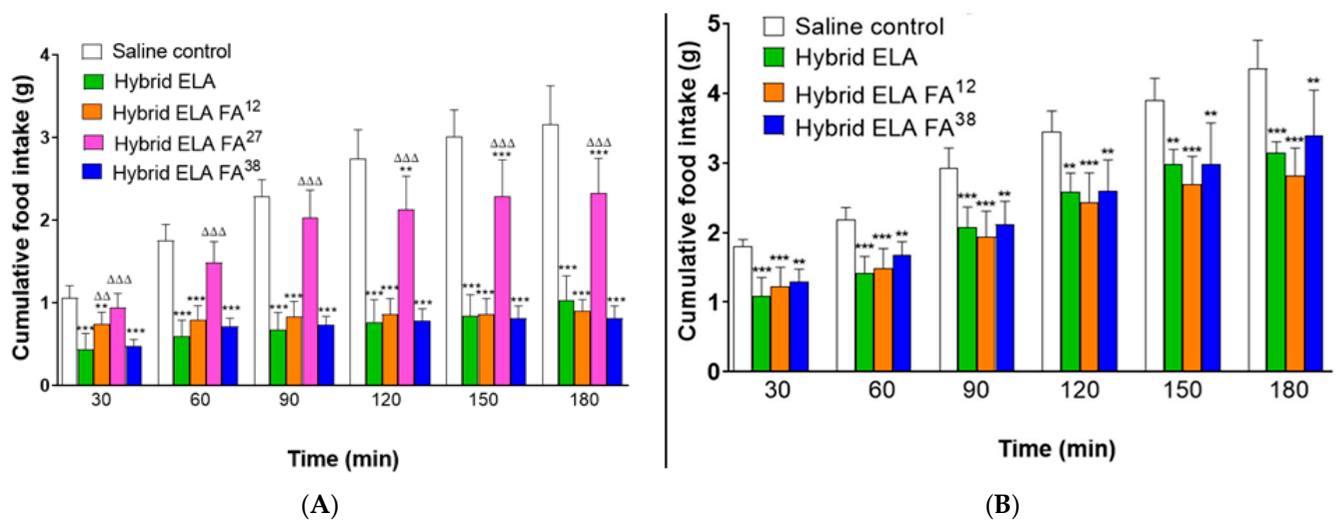
Results are expressed as mean  $\pm$  SD with either two-way ANOVA (Figures 1 and 2) or one-way ANOVA (Figure 3) followed by the Tukey post- hoc test using GraphPad Prism version 8.0. software (San Diego, CA, USA).



**Figure 1.** Acute effect of apelin/exendin peptides and their analogues on insulin secretion from BRIN-BD11 cells. Cells were co-incubated with peptides ( $10^{-12}$  to  $10^{-6}$  M), as shown for 20 min, followed by insulin measurement by RIA. Values are expressed as mean  $\pm$  SD (n = 8). \*\*\*  $p < 0.001$  versus 5.6 mmol glucose control (dashed line),  $\Delta\Delta\Delta p < 0.001$  versus exendin(1-30), and  $+++ p < 0.001$  versus apelin-13,  $++ p < 0.01$  versus 5.6 mmol glucose control (dashed line),  $\Delta\Delta p < 0.01$  versus exendin(1-30).



**Figure 2.** The effect of ELA peptide analogue on insulin secretion in combination with specific GLP-1r and APJr antagonists ( $10^{-8}$  M). Cells were co-incubated with peptides for 20 min followed by insulin content analysis using radioimmunoassay. Values are expressed as mean  $\pm$  S.E.M. (n = 8). \*\*\*  $p < 0.001$  vs. 5.6 mmol glucose basal control, and  $\Delta\Delta\Delta p < 0.001$  vs. hybrid ELA.



**Figure 3.** Cumulative food intake was measured in 21 h-fasted trained mice at 30 min intervals up to 180 min following i.p. injection of saline vehicle (0.9% *w/v* NaCl) or test peptides (25 nmol/kg body weight) either (A) immediately before ( $t = 0$ ) or (B)  $t = -21$  h prior to returning food. Values represent means  $\pm$  SEM ( $n = 7-8$  mice). \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$  compared to respective saline control.  $\Delta\Delta$   $p < 0.01$  and  $\Delta\Delta\Delta$   $p < 0.001$  compared to remaining hybrid ELA peptides.

### 3. Results

BRIN-BD11 cells exposed to KCl (30 mM) showed a 4.4-fold rise in insulin secretion versus glucose control (Figure 1). Apelin-13 showed a modest insulinotropic response from  $10^{-8}$  to  $10^{-6}$  M conc. (1.3- to 1.5-fold rise). Apelin in combination with exendin-4(1-30) or exendin-4(1-39) showed a dose-dependent increase in insulin secretion, reaching a high of 2.3-fold and 2.7-fold at the top conc. ( $10^{-6}$  M) tested, respectively (Figure 1). Exendin-4(1-30) was slightly more effective, reaching a maximum 2.9-fold increase at  $10^{-6}$  M. However, exendin-linker-apelin (ELA) achieved a marked 3.5- to 4.8-fold rise in insulin secretion ( $p < 0.001$ ) across the entire concentration range. ELA was more effective ( $p < 0.001$ ) than a combination of both exendin-4 and apelin-13 peptides at the same molar concentrations (Figure 1).

ELA and component peptides were then tested for their effect up insulin secretion from BRIN-BD11 cells in the presence of specific receptor antagonists for the APJ ((Val<sup>13</sup>)apelin-13) and GLP-1 (exendin-4(9-39)) receptors, used either alone or in combination (Figure 2). ELA produced a 3.3- to 4.8-fold increase in insulin secretion from  $10^{-12}$  to  $10^{-6}$  M. However, a marked 45% reduction in insulin secretion was observed with ELA over the test range in the presence of the APJ receptor antagonist (Val<sup>13</sup>)apelin-13, with a 43–50% reduction in the presence of the GLP-1 receptor antagonist exendin-4(9-39) (Figure 2). Notably, when both receptor antagonists were used together, the insulinotropic action of ELA was obliterated at all concentrations tested, bringing the response back to the 5.6 mM glucose baseline (Figure 2).

Next, we examined the effects of ELA and 3 acylated analogues ( $\gamma$ -glutamyl palmitate) modified by the addition of a fatty acid (FA) at Lys residues 12, 27, and 38 of these hybrid ELA peptides. The acylated ELA analogues were tested after i.p. injection in 21 h-fasted trained mice, and food intake was monitored at 30 min intervals up to 180 min (Figure 3A). When the ELA, ELA FA<sup>12</sup>, and ELA FA<sup>38</sup> were administered immediately before food was returned to mice at 10.00 h, there was a marked reduction in food intake at all test intervals, leading to a 70–78% reduction of food intake by 180 min versus saline injected controls (Figure 3A). ELA FA<sup>27</sup> was less potent than ELA and related analogues ( $p < 0.001$ ) but nevertheless reduced cumulative food intake by 27% after 180 min (Figure 3A,  $p < 0.001$ ). The longevity of the efficacy of these peptides (25 nmol/kg) with respect to food intake was examined. ELA and two acylated analogues (ELA FA<sup>12</sup> and ELA FA<sup>38</sup>) were administered

via i.p. injection 21 h in advance of the food being returned to mice. Figure 3B shows that a marked reduction in cumulative food intake was retained by ELA and related analogues (31–38% lower) over the 30–180 min period despite the 21 h-earlier administration of test peptides versus saline controls ( $p < 0.001$ ).

Figure 1 shows the effects of apelin-13, exendin-4 peptides, ELA, and positive control KCl on acute (20 min) insulin secretion from cultured pancreatic BRIN-BD11 cells.

Figure 2 shows the effects of ELA and selective APJ or GLP-1 receptor antagonists alone or in combination on acute insulin secretion from cultured pancreatic BRIN-BD11 cells.

Figure 3 shows the action of ELA and related acylated analogues on food intake when injected (Figure 3A) immediately before or 21 h before (Figure 3B) the return of food to mice.

#### 4. Discussion

Here, we examined the potential of hybrid co-agonist peptides formed by combining the actions of separate APJ and GLP-1 receptors agonists into a single, unimolecular peptide. The exendin-linker-apelin (ELA) co-agonist was a very potent insulinotropic agent in cultured pancreatic BRIN-BD11  $\beta$ -cells. ELA is more effective than equimolar concentrations of component exendin-4(1-30) and apelin-13 peptides (Figure 1). Furthermore, selective blockade of the APJ and GLP-1 receptor using specific antagonists nullified the action of ELA, demonstrating that this co-agonist molecule operates only through receptor-mediated pathways (Figure 2). Next, we examined ELA and three additional acylated forms of ELA for their actions on appetite control and food intake in 21 h-fasted mice, pre-trained to eat during a 3 h window daily. These data (Figure 3A,B) showed a marked reduction in food intake, with activity of ELA, ELA FA<sup>12</sup>, and ELA FA<sup>38</sup> sustained for up to 21 h-post injection ( $p < 0.001$ ). ELA FA<sup>27</sup> was less potent than its related acylated analogues.

In conclusion, these data concur with previous findings related to the insulinotropic and satiety effects of apelin analogues [12–14]. This work goes on to demonstrate the potential of novel unimolecular ELA co-agonist analogues for improving diabetes management through stimulation of insulin secretion and reduction of food intake, actions which should help improve metabolic control in Type 2 diabetes. Pre-clinical work is needed to further develop these peptide candidates for use in the treatment of obesity-related diabetes.

**Author Contributions:** Conceptualization, F.O. and N.I.; methodology, V.P., S.C. and E.P.; formal analysis, F.O. and N.I.; writing—original draft preparation, F.O. writing—review and editing, supervision, F.O. and N.I.; funding acquisition, F.O. and N.I. All authors have read and agreed to the published version of the manuscript.

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**Institutional Review Board Statement:** All experiments were conducted according to U.K. Animals (Scientific Procedures) Act 1986. The animal study protocol was approved by the Institutional Review Board (AWERB) of Ulster University (protocol code PPL2902 with approval date October 2019).

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Data will be available through the Ulster University Institutional Repository following formal publication.

**Conflicts of Interest:** The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

**Patents:** A patent was filed in the UK in June 2023 using some of that data presented here. Application No. 2308304.1 covers co-agonist hybrid peptides targeting APJ and GLP-1 receptors.

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