A novel CCK-8/GLP-1 hybrid peptide exhibiting prominent insulinotropic, glucose-lowering and satiety actions with significant therapeutic potential in high-fat fed mice

Nigel Irwin*, Varun Pathak and Peter R. Flatt

From the SAAD Centre for Pharmacy and Diabetes, School of Biomedical Sciences, University of Ulster, Coleraine, Northern Ireland, UK.

*Address correspondence to: Nigel Irwin, SAAD Centre for Pharmacy and Diabetes, University of Ulster, Coleraine, Northern Ireland, UK. E-mail: n.irwin@ulster.ac.uk. Tel: ++44 (0) 28 70 124754; Fax: ++44 (0) 28 70 123939

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Abstract

Glucagon-like-peptide-1 (GLP-1) and cholecystokinin (CCK) exert important complementary beneficial metabolic effects. The present study has assessed the biological actions and therapeutic utility of a novel (pGlu-Gln)-CCK-8/exendin-4 hybrid peptide in comparison with the stable GLP-1 and CCK mimetics, exendin-4 and (pGlu-Gln)-CCK-8 respectively. All peptides significantly enhanced in vitro insulin secretion. Administration of the peptides, barring (pGlu-Gln)-CCK-8 alone, in combination with glucose significantly lowered plasma glucose and increased plasma insulin in mice. All treatments elicited appetite suppressive effects. Twice-daily administration of the novel (pGlu-Gln)-CCK-8/exendin-4 hybrid, (pGlu-Gln)-CCK-8 alone or in combination with exendin-4, for 21 days to high fat fed mice significantly decreased energy intake, body weight and circulating plasma glucose. HbA1C was reduced in the (pGlu-Gln)-CCK-8/exendin-4 hybrid and combined parent peptide treatment groups. Glucose tolerance and insulin sensitivity were also improved by all treatment modalities. Interestingly, locomotor activity was decreased in the hybrid peptide group, and these mice also exhibited reductions in circulating triglyceride and cholesterol levels. Pancreatic islet number and area, as well beta-cell area and insulinotropic responsiveness were dramatically improved by all treatments. These studies highlight clear potential of dual activation of GLP-1 and CCK1 receptors for the treatment of type 2 diabetes.
INTRODUCTION

Glucagon-like peptide-1 (GLP-1) and cholecystokinin (CCK) are hormones released from the gut following feeding that act as important regulators of postprandial glucose homeostasis and overall energy balance (1). CCK exists in multiple molecular forms, but the carboxy-terminal octapeptide, CCK-8, is well conserved among species and is the smallest form that retains the full range of biological actions (2). The most accepted biological action of GLP-1 is as an incretin hormone stimulating glucose-dependent insulin secretion to control postprandial glucose levels (3). Whereas for CCK, principal effects are gallbladder contraction together with the short-term regulation of energy balance, mediated through induction of satiety (4). Thus, it is clear that CCK-8 and GLP-1 possesses numerous complementary biological actions that suggest potential synergistic therapeutic application for obesity-diabetes (5,6).

Over the past decade, the major focus on gut hormone therapies has been directed predominantly towards single molecules that modulate individual peptide receptor targets, highlighted by the successful clinical development of long-acting GLP-1 mimetics (7). Despite this success, glycaemic control and weight reduction achieved with certain types of gastric bypass surgery are noticeably superior to GLP-1 therapy (1). Thus, multiple regulatory peptide hormones are inherently involved in glucose regulation and energy balance (8). It follows that combining the activity of gut hormones with complementary biological actions, such as GLP-1 and CCK-8, offers a more favourable approach for the treatment of obesity-related forms of diabetes. To enhance therapeutic utility, design of a single hybrid peptide molecule, capable of simultaneous activation of GLP-1 and CCK receptors, would hold promise for obesity-diabetes, facilitating formulation and dosing with a single molecule.

Following on from detailed interrogation of stable forms of CCK-8 for obesity diabetes (1,5,9-13), we have now constructed a novel CCK-8/GLP-1 hybrid molecule through fusion of the key amino acid sequences of the well characterised, stable and specific CCK-8 and GLP-1
analogues, (pGlu-Gln)-CCK-8 (12) and exendin-4 (14). The bioactive domains of the parent peptides have been fused through use of an {2-[2-aminoethoxy]ethoxy}acetic acid (AEEAc) linker molecule (15). We initially assessed enzymatic resistance, \textit{in vitro} insulin secretion and \textit{in vivo} glucose-lowering, insulino tropic and satiety actions of the parent peptides and novel (pGlu-Gln)-CCK-8/exendin-4 hybrid. We then examined the beneficial metabolic effects of a twice daily injection regimen in high-fat fed mice. The results reveal that sustained activation of CCK\textsubscript{1} and GLP-1 receptors, with a single novel (pGlu-Gln)-CCK-8/exendin-4 hybrid molecule or combined administration of the parent peptides, exerts a spectrum of beneficial metabolic effects in high fat fed mice that requires further consideration as a treatment option for obesity-diabetes.

\textbf{RESEARCH DESIGN AND METHODS}

\textbf{Peptides.} Supplementary Table 1 displays the amino acid sequence of (pGlu-Gln)-CCK-8, exendin-4 and the novel (pGlu-Gln)-CCK-8/exendin-4 hybrid molecule. All peptides were purchased from American Peptide Company (Sunnyvale, CA, USA; greater than 95% purity). Peptides were characterised in-house using matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-ToF MS), as described previously (16).

\textit{In vitro} insulin secretion. Effects of peptides on \textit{in vitro} insulin secretion were examined using BRIN-BD11 cells whose characteristics have been reported previously (17). BRIN-BD11 cells were seeded (150,000/well) into 24-well plates (Nunc, Roskilde, Denmark) and allowed to attach overnight at 37°C. Following 40 min pre-incubation (1.1 mmol/L glucose; 37°C), cells were incubated (20 min; 37°C) in the presence of 5.6 mmol/L glucose with a range of test peptide concentrations (10\textsuperscript{-12} to 10\textsuperscript{-6} mol/L). The effects of the specific GLP-1, CCK\textsubscript{1} and CCK\textsubscript{2} receptor antagonists, exendin(9-39), SR27897 and LY288513; respectively, on (pGlu-
Gln)-CCK-8/exendin-4 hybrid induced insulin secretion were also examined. After 20 min incubation, buffer was removed from each well and aliquots stored at -20°C prior to determination of insulin by radioimmunoassay (18).

**Animals.** Acute animal studies were carried out in male NIH Swiss mice (Harlan Ltd., Blackthorne, UK; 12 to 14 weeks old) maintained on standard rodent maintenance diet that contained 10% fat, 30% protein and 60% carbohydrate, with percent of total energy of 12.99 kJ/g (Trouw Nutrition, Cheshire, UK). Longer-term experiments were performed in NIH Swiss mice previously fed a high-fat diet for 130 days composed of 45% fat, 20% protein and 35% carbohydrate (total energy 26.15 KJ/g; Special Diet Services, Essex, UK). Animals were housed in a 12:12 h light/dark cycle (lights on at 08:00 h and off at 20:00 h) and had free access to drinking water and food. All animal experiments were conducted according to UK Home Office Regulations (UK Animals Scientific Procedures Act 1986).

**Acute in vivo effects in normal mice.** Plasma glucose and insulin responses were evaluated after intraperitoneal (i.p.) injection of glucose alone (18 mmol/kg body weight) or in combination with test peptides (25 nmol/kg body weight) in overnight (18 h) fasted normal NIH mice. Peptide doses were chosen based on our previous studies with (pGlu-Gln)-CCK-8 and exendin-4 based peptides (5). In a second series of experiments, 18 h fasted normal mice were used to assess the effects of respective test peptides on food intake. Mice received an i.p. injection of saline alone (0.9 % (w/v) NaCl) or in combination with test peptide (25 nmol/kg) and food intake measured at 30 min intervals.
Sub-chronic effects of in high-fat fed mice. Twice-daily (09:30 and 17:30 h) injections of saline vehicle (0.9% (w/v) NaCl; i.p.), novel (pGlu-Gln)-CCK-8/exendin-4 hybrid (25 nmol/kg bw), (pGlu-Gln)-CCK-8 (25 nmol/kg bw), exendin-4 (25 nmol/kg bw) or a combination of both peptides (25 + 25 nmol/kg bw) were administered over 21 days to high-fat fed mice. Energy intake, body weight, non-fasting plasma glucose and insulin concentrations were monitored at 3-6 day intervals. Intraperitoneal glucose tolerance (18 mmol/kg bw) and insulin sensitivity (15 U/kg bw) tests were performed after 21 days of treatment. At the end of the treatment period, glycated haemoglobin (Chirus Ltd), circulating plasma glucagon and amylase, and a blood lipid profile were also assessed. All blood samples were collected before routine morning injection of peptide. For assessment of metabolic rate and locomotor activity on day 21, mice were placed in Complete Laboratory Animal Monitoring System (CLAMS) metabolic chambers (Columbus Instruments, OH, USA) following the normal 09:30 daily injection and consumption of O₂, production of CO₂, respiratory exchange ratio, energy expenditure and ambulatory locomotor activity were calculated as described previously (12). HOMA-IR was determined using the equation HOMA-IR = fasting glucose (mmol/l) x fasting insulin (ng/ml)/22.5, and HOMA-β was calculated using the equation HOMA-β = 20 x fasting insulin (ng/ml)/fasting glucose (mmol/l) - 3.5.

Terminal analyses.

Terminal blood samples were taken and pancreatic tissue was extracted and processed appropriately for isolated islet insulin release studies, histological analysis or assessment of hormone content following acid ethanol (750 ml ethanol, 235 ml water, 15 ml concentrated HCl) extraction (12). For histological analysis, pancreata were fixed and processed as described previously (19). Tissue sections were deparaffinised, rehydrated and probed with primary antibodies: rabbit anti-insulin antibody (1:200; Santa Cruz, Heidelberg, Germany) or guinea-
pig anti-glucagon antibody (PCA2/4, 1:200; raised in-house) (19). In a separate series, pancreatic islets were isolated from respective treatment groups by collagenase digestion, as fully described previously (20). Insulin secretion was determined as described above for BRIN BD11 cells. Following removal of the test solution, 200 µl of acid–ethanol solution (1.5% (v/v) HCl, 75% (v/v) ethanol, 23.5% (v/v) H₂O) was added with overnight extraction of cellular insulin. Samples were stored at -20 °C for measurement of insulin content by radioimmunoassay (18).

**Biochemical analyses.** Blood samples were collected from the cut tip on the tail vein of conscious mice into chilled fluoride/heparin glucose micro-centrifuge tubes (Sarstedt, Numbrecht, Germany) at the timepoints indicated in the Figures. Blood glucose was measured directly using a hand-held Ascencia Contour blood glucose meter (Bayer Healthcare, Newbury, Berkshire, UK). For plasma insulin analysis, blood samples were immediately centrifuged using a Beckman microcentrifuge (Beckman Instruments, Galway, Ireland) for 1 min at 13,000 x g and stored at -20 °C. Plasma and pancreatic insulin was assayed by a modified dextran-coated charcoal RIA (18). Plasma and pancreatic glucagon were measured by a commercially available ELISA kit (Merck Millipore, Germany), according to the manufacturer’s instructions. Total-, HDL-, and LDL-cholesterol, triglycerides and plasma amylase levels were measured using an Hitachi Automated Analyzer 912 (Boehringer, Mannheim, Germany).

**Statistical analysis.** Results are expressed as means ± SEM and data compared using the unpaired Student’s t-test. Where appropriate, data were compared using repeated measures ANOVA or one-way ANOVA, followed by the Student-Newman-Keuls post-hoc test. Groups of data were considered to be significantly different if \( P<0.05 \).
RESULTS

*In vitro insulin secretion.* All test samples induced significant ($P<0.05$ to $P<0.001$) concentration-dependent elevations of insulin secretion from BRIN BD11 cells (Figure 1A). The combination of (pGlu-Gln)-CCK-8 plus exendin-4, and the hybrid, were the most effective in terms of insulin secretion (Figure 1A). Further studies with specific GLP-1, CCK$_1$ and CCK$_2$ receptor antagonists confirmed that the insulinotropic activity of the (pGlu-Gln)-CCK-8/exendin-4 hybrid was dependent on both GLP-1 and CCK$_1$ receptor activity (Figure 1B). However, it is clear, particularly at elevated concentrations, that the insulin secretory action of the (pGlu-Gln)-CCK-8/exendin-4 hybrid peptide is very much dependent upon intact GLP-1 receptor signalling pathways (Figure 1B).

*Acute in vivo effects in normal mice.* (pGlu-Gln)-CCK-8 failed to elicit significant insulin-releasing or glucose modulating actions at the dose employed (Figure 2A,B). In contrast, exendin-4 significantly reduced ($P<0.05$) overall 0-60 min AUC plasma glucose values and increased ($P<0.05$) the overall insulin secretory response (Figure 2A,B). In addition, combination of (pGlu-Gln)-CCK-8 with exendin-4, and the (pGlu-Gln)-CCK-8/exendin-4 hybrid, also significantly reduced ($P<0.01$) the overall plasma glycaemic excursion and increased ($P<0.05$) the insulin secretory response (Figure 2A,B). All peptides induced significant ($P<0.05$ to $P<0.001$) appetite suppressive effects when administered to overnight fasted mice (Figure 2C). The most effective treatments to inhibit feeding were (pGlu-Gln)-CCK-8 in combination with exendin-4, and the (pGlu-Gln)-CCK-8/exendin-4 hybrid (Figure 2C).
Effects of treatment regimens on energy intake, body weight, HbA1c and circulating glucose, insulin, glucagon and amylase in high-fat fed mice. All groups of high fat fed mice had significantly \((P<0.001)\) increased energy intake compared to lean controls during the 21 day study (Figure 3A). Exendin-4 therapy had no effect on energy intake at the same dose employed (Figure 3A). However, twice daily treatment with \((\text{pGlu-Gln})\)-CCK-8 alone, and in combination with exendin-4, or the novel hybrid, significantly \((P<0.05 \text{ to } P<0.001)\) reduced energy intake from day 11 onwards (Figure 3A). All treatment regimens, barring exendin-4 alone, significantly \((P<0.05 \text{ to } P<0.001)\) reduced body weight compared to high fat controls (Figure 3B). Indeed, treatment with the \((\text{pGlu-Gln})\)-CCK-8/exendin-4 hybrid returned body weights to levels of lean controls from day 15 onwards (Figure 3B).

A similar scenario was evident in terms of circulating blood glucose levels, with all treatments, except exendin-4, significantly \((P<0.05 \text{ to } P<0.001)\) lowering non-fasting glucose levels compared to high fat controls (Figure 3C). Circulating plasma insulin concentrations were significantly \((P<0.05 \text{ to } P<0.001)\) increased at numerous observation points in all treatment groups when compared to lean controls, but where similar to high fat controls on day 21 (Figure 3D). All high fat fed mice, barring those treated with exendin-4 alone, had significantly \((P<0.05 \text{ to } P<0.001)\) elevated glucagon concentrations compared to lean controls on day 21 (Figure 3E). Indeed, exendin-4 treated mice had significantly \((P<0.05)\) reduced plasma glucagon concentrations compared to mice treated with \((\text{pGlu-Gln})\)-CCK-8 (Figure 3E). Plasma amylase levels were not different in any of the treatment groups when compared to high fat controls on day 21 (Figure 3F). However, exendin-4 treatment increased \((P<0.05)\) amylase concentrations when compared to lean controls (Figure 3F). Glycated haemoglobin values in all treatment groups, barring exendin-4, returned to levels similar to lean controls by day 21 (Figure 3G).
Effects of treatment regimens on glucose tolerance, plasma insulin response to glucose, insulin sensitivity and pancreatic hormone content in high-fat fed mice. Following an intraperitoneal glucose challenge on day 21, overall plasma glucose levels were significantly ($P<0.05$ to $P<0.001$) reduced in all treatment groups compared to high fat controls (Figure 4A). Furthermore, there was a significant ($P<0.05$ to $P<0.01$) decrease in overall AUC glucose concentrations in high fat fed mice treated with (pGlu-Gln)-CCK-8 in combination with exendin-4, or the hybrid, when compared to (pGlu-Gln)-CCK-8 or exendin-4 treatment alone (Figure 4B). Corresponding individual plasma insulin levels were elevated ($P<0.05$ to $P<0.01$) in all high fat treatment groups compared to lean controls (Figure 4C). This was corroborated by AUC data, that revealed all treatments induced a significantly ($P<0.05$ to $P<0.001$) elevated overall insulin secretory response compared to control mice (Figure 4D).

The hypoglycaemic action of insulin, in terms of percentage fall from basal glucose values, was superior in all treatment groups compared to high fat controls (Figure 4E). However, the action of insulin was particularly enhanced ($P<0.001$) in groups with dual activation of GLP-1 and CCK$_1$ receptors in terms of 0-60 min overall values (Figure 4F). Calculation of HOMA-IR also indicated superior ($P<0.05$ to $P<0.01$) insulin sensitivity compared to other groups (0.50±0.06 and 0.52±0.04, respectively for mice treated with (pGlu-Gln)-CCK-8 in combination with exendin-4 or the novel hybrid peptide, compared with 0.71±0.04 for high fat controls). Pancreatic insulin concentrations were significantly ($P<0.05$ to $P<0.01$) reduced in all treatment groups, barring the combination of (pGlu-Gln)-CCK-8 plus exendin-4, when compared to lean control mice (Figure 4G). Pancreatic glucagon content was elevated ($P<0.01$) in (pGlu-Gln)-CCK-8 treated mice compared to lean controls, whereas both other treatment groups undergoing sustained CCK$_1$ receptor activation had similar pancreatic glucagon concentrations compared to control mice (Figure 4H). Interestingly, mice treated with exendin-
4 alone had reduced ($P<0.01$) pancreatic glucagon content compared to high fat controls (Figure 4H).

**Effects of treatment regimens on metabolic rate, locomotor activity and blood lipid profile in high-fat fed mice.** There were no differences in $O_2$ consumption, $CO_2$ production, RER and energy expenditure in any of the high fat fed groups of mice on day 21 (Supplementary Figure 1A-D). However, there was a significant ($P<0.05$) decrease in overall energy expenditure in high fat fed mice when compared to lean controls, that was normalised by all treatment regimens (Supplementary Figure 1D). Ambulatory activity, as assessed by X beam breaks, was similar in all groups during the light phase (Figure 5A), but significantly ($P<0.05$) decreased during the dark phase in mice receiving (pGlu-Gln)-CCK-8 or hybrid therapy when compared to lean controls (Figure 5B). Rearing and jumping episodes during the light phase, as assessed by Z beam breaks, were elevated ($P<0.05$) in mice receiving exendin-4 alone, or in combination with (pGlu-Gln)-CCK-8 (Figure 5C). During the dark phase, Z beam breaks were significantly ($P<0.05$ to $P<0.001$) increased in all treatment groups compared to high fat controls (Figure 5D).

Assessment of blood lipid profile on day 21 revealed significant ($P<0.05$ to $P<0.001$) reductions of total cholesterol concentrations in all treatment groups (Figure 6A). HDL-cholesterol was unaltered in all groups, barring the hybrid treated mice, which had reduced ($P<0.05$ to $P<0.01$) levels compared to all groups expect those mice receiving (pGlu-Gln)-CCK-8 alone (Figure 6B). Interestingly, LDL-cholesterol levels were significantly ($P<0.05$ to $P<0.01$) decreased in all groups compared to high fat controls (Figure 6C). Plasma triglyceride concentrations were reduced ($P<0.001$) by (pGlu-Gln)-CCK-8 and hybrid treatment when compared to high fat controls, and where similar to lean controls (Figure 6D). Treatment with exendin-4 alone, or in combination with (pGlu-Gln)-CCK-8 decreased ($P<0.05$ to $P<0.01$)
triglyceride levels compared to high fat controls, but these were still ($P<0.05$ to $P<0.01$) increased compared to lean controls (Figure 6D).

**Effects of treatment regimens on pancreatic beta-cell insulinotropic responses in high-fat fed mice.** Islets isolated from high fat mice had a significantly ($P<0.05$ to $P<0.001$) reduced insulin secretory responses when compared to lean controls (Figure 7A&B). In contrast, islets isolated from all high fat fed treatment groups had similar insulin secretory capacity as lean controls in response to glucose, tolbutamide and elevated Ca$^{2+}$ (Figure 7A&B). Moreover, there was no sign of pancreatic beta-cell tachyphylaxis following 21 days twice daily administration with any of the peptide treatment regimens (Figure 7A). The insulin secretory response of isolated islets to PMA was reduced ($P<0.05$ to $P<0.001$) in the hybrid group and in mice treated with (pGlu-Gln)-CCK-8 alone, or in combination with exendin-4, when compared to lean controls (Figure 7B). The secretory response to forskolin was also reduced ($P<0.01$ to $P<0.001$) in islets isolated from (pGlu-Gln)-CCK-8 and exendin-4 treated mice when compared to lean controls, but not in mice treated with the novel hybrid or a combination of (pGlu-Gln)-CCK-8 and exendin-4 (Figure 7B). The benefits of the latter treatments were also evidenced *in vivo* as indicated by the superior HOMA-β values (Figure 7C).

**Effects of treatment regimens on pancreatic islet histology in high-fat fed mice.** Representative images from lean control, high fat fed and all treatment groups are depicted in Figures 8A-F. Importantly, the structure and cellular organisation of islets in all treatment groups was essentially similar to lean controls, and there was no obvious evidence of pancreas fatty infiltration. All treatment regimens returned pancreatic islets numbers to similar levels as lean controls (Figure 8G). Similarly, islet area was reduced ($P<0.05$) by high fat feeding, with decreases in small medium and large islets (Figure 8H,I). All peptide treatments corrected this
detrimental effect (Figure 8H,I). Moreover, treatment with (pGlu-Gln)-CCK-8 alone, or in combination with exendin-4, significantly ($P<0.01$) increased islet area compared to lean control mice (Figure 8H). Pancreatic beta-cell area was largely unaffected by high fat feeding, but all treatments increased ($P<0.001$) beta-cell area compared to both high fat and lean control mice (Figure 8J).

**DISCUSSION**

Despite encouraging preclinical data (5,9-12), the development of monotherapy CCK-based drugs for human obesity-diabetes remains elusive (21). In contrast, GLP-1 mimetics have been adopted into the diabetic clinic with great vigour, although weight loss and metabolic control are not as impressive as first hoped (7). In this regard, several recent studies now reveal that dual agonism of CCK$_1$ and GLP-1 receptors has marked synergistic metabolic and weight reduction benefits (5,6). Although, it should be noted that earlier clinical studies, employing infusions of native GLP-1 and CCK peptides in normal overnight fasted human subjects, failed to reveal clear synergistic effects (22). This is likely related to important differences between these studies that include; use of infusion rather than bolus injection, normal fasted subject as opposed to a freely fed diabetic animal model and employing native metabolically liable peptides rather than enzymatically stable peptide forms (5,6,22). Therefore, in the present research we have evaluated the biological actions and therapeutic applicability of a novel stable (pGlu-Gln)-CCK-8/exendin-4 hybrid peptide in comparison to combined administration of the parent peptides.

The hybrid peptide was engineered to combine the satiety and energy regulating action of CCK-8 (4), with the robust insulin-releasing and antidiabetic actions of GLP-1 (3), in a single compound. Importantly, pharmacological studies reveal that CCK$_1$, as opposed to CCK$_2$, receptor activity is critical for synergy with GLP-1 receptor action (6). We confirmed through
the use of specific GLP-1, CCK₁ and CCK₂ receptor antagonists that the insulinotropic effects of the hybrid were mediated via both GLP-1 and CCK₁ receptors. Indeed, our data revealed that the hybrid peptide retained full ability to activate CCK₁ and GLP-1 receptor signalling pathways involved in glucose homeostasis, insulin secretion and appetite suppression (1). Even combination of the parent peptides, both individually at the same concentration as the single hybrid, was not superior in efficacy to the novel hybrid. Notably, biological effects of (pGlu-Gln)-CCK-8/exendin-4 were significantly enhanced when compared to either parent peptide alone, with the prominent glucose regulatory effects of the hybrid appearing largely dependent on GLP-1 receptor activation and appetite suppression predominantly driven by CCK₁ receptor signalling (5). In addition, the fact that CCK₂ receptor inhibition did not perturb the insulinotropic effect of (pGlu-Gln)-CCK-8/exendin-4 is encouraging given that activation of this receptor has been associated with panic and anxiety attacks (23,24), which has detracted from therapeutic potential of previous non-specific CCK-based drugs (25).

In harmony with previous findings (5), chronic twice daily treatment with (pGlu-Gln)-CCK-8 alone, and particularly in combination with exendin-4, resulted in sustained and significant reductions of energy intake and body weight in high fat fed mice. In relation to this, vagal afferents co-express CCK₁ and GLP-1 receptors (26), and might represent a target synergistic interaction between the two hormones. Moreover, it has been suggested that the effects of CCK and GLP-1 on the central regulation of food intake operate via different, but complementary autonomic circuits (27-29). Tolerability and induction of nausea has previously been suggested as an important limiting factor in the development of CCK₁ receptor activating drugs (30). However, in agreement with previous studies (12), we clearly show a lack of desensitisation to the anorectic or insulin-releasing effects of (pGlu-Gln)-CCK-8, either alone or in combination with GLP-1 receptor activation. Crucially, the novel hybrid peptide, (pGlu-Gln)-CCK-8/exendin-4, had equally effective beneficial metabolic actions when compared to
combined administration of the parent peptides. Indeed, reductions in HbA1c were only observed in those mice subjected to sustained activation of both CCK1 and GLP-1 receptors (5,6). Development of specific assays to directly measure each peptide in plasma would be useful to provide more precise details of circulating half-life. Surprisingly, exendin-4 did not obviously decrease energy intake, body weight or circulating glucose levels at the dose employed, although similar observations have been reported previously by us and others (5,31). The limited effect of exendin-4 could possibly reflect up-regulation of adaptive mechanisms when, although it may simply be dose, species or animal model specific (5).

Glucose tolerance was improved to a similar extent by 21-days twice daily treatment with (pGlu-Gln)-CCK-8 or exendin-4 alone. Notably, there was a much greater enhancement of glucose disposal in mice treated with (pGlu-Gln)-CCK-8 in combination with exendin-4 or the novel hybrid, again highlighting the distinct benefit of sustained dual activation of CCK1 and GLP-1 receptors (5,6). This effect was associated with significantly increased insulin levels, indicating that benefits of combined (pGlu-Gln)-CCK-8 and exendin-4 therapy, or the novel (pGlu-Gln)-CCK-8/exendin-4 hybrid, are partly mediated by direct actions on pancreatic beta-cell function (1). This was confirmed on day 21, where islets isolated from high fat fed mice treated with (pGlu-Gln)-CCK-8 in combination with exendin-4, or the novel hybrid peptide, exhibited a marked improvement in the insulin secretory response to glucose, each of the individual peptides and a variety of other agents with diverse actions on beta cell signalling pathways. This corresponded with improvements in HOMA-β values. Interestingly the action of PMA in vitro was not enhanced, suggesting that pathways downstream of protein kinase C that are usually activated by CCK (32) make a relatively small contribution to the effects of the hybrid peptide.

Prominent in vivo effects on beta-cell function are consistent with the recognised insulin-releasing action of GLP-1 and CCK-8 at pharmacological levels (3,33). In harmony
with this, pancreatic insulin levels where augmented by combined (pGlu-Gln)-CCK-8 and exendin-4 treatment. Pancreatic glucagon levels in these mice were unchanged by dual activation of CCK\textsubscript{1} and GLP-1 receptors, and actually reduced together with circulating levels by exendin-4 therapy. In contrast, (pGlu-Gln)-CCK-8 increased pancreatic glucagon as noted previously (9). In addition, we observed consistent improvement of insulin sensitivity with both the hybrid peptide and (pGlu-Gln)-CCK-8 in combination with exendin-4 by the end of the treatment period. This is in agreement with the well-known insulin sparing actions of GLP-1 (34), and evidence also suggests an integral role for CCK as a regulator of insulin action, especially under conditions of high fat feeding (35). In the present study, islet number together with both islet area and beta-cell area were increased by all treatment modalities. However, assessment of pancreatic cell volumes would be required to fully confirm islet proliferative effects of the treatment regimens. Further to this, notable reductions in total and LDL-cholesterol concentrations were observed with all treatment regimens, in agreement with effects of individual peptides in previous studies (5,9-11). HDL-cholesterol levels were also reduced by the hybrid, which was unique to this treatment modality and necessitates further investigation. While it should be noted that decreased circulating HDL-cholesterol is generally considered to be a cardiovascular disease risk factor (36), this rather one-dimensional hypothesis has been questioned of late (37). Encouragingly circulating triglyceride levels were markedly reduced by all treatments, barring exendin-4 alone, signifying improved metabolic control and insulin resistance (38).

In order to further clarify possible mechanisms behind the weight reduction observed with dual CCK\textsubscript{1} and GLP-1 receptor activation, we assessed aspects of metabolic rate and locomotor activity. Given the prominent effects of GLP-1 and CCK on energy balance (3,26), alterations of energy expenditure may have been predicted in the current study. However, consistent with other studies using (pGlu-Gln)-CCK-8 (12), metabolic benefits in the current study were not
associated with changes in energy expenditure or metabolic rate. There was a mild reduction in physical activity levels during the dark phase in mice treated with (pGlu-Gln)-CCK-8 or the hybrid, which might accompany the period of reduced energy intake. In addition, differences in locomotor activity between the hybrid and combined treatment group are intriguing. Thus, the overall significance of these centrally mediated effects requires further detailed elucidation. Moreover, assessment of gene or protein expression could also aid in uncovering the mechanisms behind the beneficial effects observed with independent and combined CCK₁ and GLP-1-receptor activation. Nonetheless, taken together, these observations suggest that decreased energy intake is the driving force behind weight loss in the present study. Importantly, there were no obvious signs of malaise in these mice and weight reduction was robust and durable.

Given the recent controversy relating to detrimental GLP-1 effects on the exocrine pancreas (39), and the fact that extremely large doses of CCK have been shown to cause pancreatitis (40), further consideration of this safety aspect is required for any combinational therapeutic approach involving GLP-1 and CCK compounds. Indeed, plasma amylase levels were increased with exendin-4 monotherapy in the current study. Nevertheless and most importantly, we did not observe any evidence of pancreatic inflammation with the (pGlu-Gln)-CCK-8/exendin-4 hybrid peptide, (pGlu-Gln)-CCK-8 alone or in combination with exendin-4, as reflected by unaltered plasma amylase levels and pancreatic histology. This is in agreement with similar studies using (pGlu-Gln)-CCK-8 (13), or CCK-8 and GLP-1 combination therapies (6). Moreover, it is likely that the CCK₁ receptor is expressed at much lower levels in pancreatic anicar cells in humans as opposed to rodents (41). However, more detailed and extended studies looking at other side-effects and related toxicology are needed before testing in man.
In conclusion, the present study has demonstrated the novel hybrid peptide analogue, (pGlu-Gln)-CCK-8/exendin-4, is a dual acting CCK₁ and GLP-1 receptor agonist, with equivalent or superior therapeutic efficacy when compared to combined administration of the parent peptides. (pGlu-Gln)-CCK-8/exendin-4 has robust satiety, glucose homeostatic and insulin secretory actions and improves glucose tolerance, insulin resistance, pancreatic beta-cell function and islet morphology in high-fat fed mice. The synergistic interplay that exists between CCK₁ and GLP-1 receptor signalling merits further consideration as a new treatment paradigm for type 2 diabetes.

AUTHOR CONTRIBUTIONS

NI and PRF. conceived the study and drafted the manuscript. VP participated in the analysis and interpretation of data. All authors revised the manuscript critically for intellectual content and approved the final version of the manuscript. VP is the guarantor of this work, had full access to all the data, and takes full responsibility for the integrity of data and the accuracy of data analysis.

DISCLOSURES

None.

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References


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Figure legends

FIG. 1. Effects of (pGlu-Gln)-CCK-8/exendin-4 hybrid, (pGlu-Gln)-CCK-8, exendin-4 or a combination of both parent peptides on insulin secretion from BRIN BD11 cells. (A) BRIN-BD11 cells were exposed to a range of concentrations (10^{-12} – 10^{-6} M) of test peptides at 5.6 mmol/l glucose during 20 min incubations (n=8). (B) Effects of specific GLP-1 (exendin(9-39); 10^{-7} M), CCK_1 (SR27897; 10^{-7} M) and CCK_2 (LY288513; 10^{-7} M) receptor antagonists on (pGlu-Gln)-CCK-8/exendin-4 induced insulin secretion at 5.6 mmol/l glucose during 20 min incubations (n=8). Values represent means ± S.E.M. (n=8). (A) *P<0.05, **P<0.01, ***P<0.001 compared with 5.6 mmol/l glucose control. ∆∆∆P<0.001 compared with (pGlu+Gln)+CCK+8. (A) ΨΨP<0.01, ΨΨΨP<0.001 compared to exendin+4, additionally (B) δP<0.05 compared with (pGlu-Gln)-CCK-8/exendin-4 in combination with SR+27897. θP<0.05, θθP<0.01 and θθθP<0.01 compared with (pGlu-Gln)-CCK-8/exendin-4 in combination with LY-288513.

FIG. 2. Acute effects (pGlu-Gln)-CCK-8/exendin-4 hybrid, (pGlu-Gln)-CCK-8, exendin-4 or a combination of both parent peptides on (A) glucose-lowering, (B) insulin responses and (C) food intake in normal mice. Plasma glucose (A) and insulin (B) concentrations were measured after administration of glucose alone (18 mmol/kg bw) or together with (pGlu-Gln)-CCK-8, exendin-4 (both at 25 nmol/kg bw), a combination of both peptides (each at 25 nmol/kg bw) and (pGlu-Gln)-CCK-8/exendin-4 hybrid (25 nmol/kg bw) at t=0. Plasma glucose and insulin AUC values for 0-60 min post injection are shown in insets. (C) Food intake was monitored in overnight (18 h) fasted mice after administration of saline vehicle (0.9% (w/v) NaCl) or peptides at the doses employed above. Data are expressed as means ± SEM for 8 mice. *P<0.05, **P<0.01 and ***P<0.001 compared to respective glucose alone (A,B) or
FIG. 3. Effects of twice daily administration of (pGlu-Gln)-CCK-8/exendin-4 hybrid, (pGlu-Gln)-CCK-8, exendin-4 or a combination of both parent peptides on accumulated (A) energy intake, (B) body weight, (C) non-fasting plasma glucose, (D) insulin, (E) glucagon, (F) amylase and (G) glycated haemoglobin concentrations in high-fat fed mice. (A-D) Parameters were measured for 6 days prior to and 21 days during (indicated by horizontal black bar) treatment with saline vehicle, (pGlu-Gln)-CCK-8, exendin-4 (both at 25 nmol/kg bw), a combination of both peptides (each at 25 nmol/kg bw) and (pGlu-Gln)-CCK-8/exendin-4 hybrid (25 nmol/kg bw). (E-G) Parameters were measured on day 21. Data are expressed as means ± SEM for 8 mice. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ compared to lean controls. $^\Delta P<0.05$, $^{\Delta\Delta} P<0.01$, $^{\Delta\Delta\Delta} P<0.001$ compared to high fat controls. $^{\Psi} P<0.05$ compared to (pGlu+Gln)+CCK+8. $^\theta P<0.05$, $^{\theta\theta} P<0.01$, $^{\theta\theta\theta} P<0.001$ compared to exendin-4.

FIG. 4. Effects of twice daily administration of (pGlu-Gln)-CCK-8/exendin-4 hybrid, (pGlu-Gln)-CCK-8, exendin-4 or a combination of both parent peptides on (A,B) glucose tolerance, (C,D) insulin response to glucose, (E,F) insulin sensitivity and (G) pancreatic insulin and (H) glucagon content in high-fat fed mice. Tests were conducted after twice daily treatment for 21 days with saline vehicle, (pGlu-Gln)-CCK-8, exendin-4 (both at 25 nmol/kg bw), a combination of both peptides (each at 25 nmol/kg bw) and (pGlu-Gln)-CCK-8/exendin-4 hybrid (25 nmol/kg bw). (A,C) Glucose (18 mmol/kg bw) was administered intraperitoneally at t=0 min in non-fasted mice. (B,D) Plasma glucose and insulin AUC values for 0-105 min post injection are also shown. (E) Insulin (10 U/kg bw) was administered intraperitoneally at t=0 min in non-fasted mice. (F) Plasma glucose AUC values for 0-60 min
post injection are also shown. (G,H) Pancreatic hormone content was measured on day 21. Data are expressed as means ± SEM for 8 mice. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ compared to lean controls. $\Delta P<0.05$, $\Delta\Delta P<0.01$, $\Delta\Delta\Delta P<0.001$ compared to high fat controls. $\Psi P<0.05$, $\Psi\Psi\Psi P<0.001$ compared to (pGlu-Gln)-CCK-8. $\theta P<0.05$, $\theta\theta P<0.01$ compared to exendin-4.

**FIG. 5.** Effects of twice daily administration of (pGlu-Gln)-CCK-8/exendin-4 hybrid, (pGlu-Gln)-CCK-8, exendin-4 or a combination of both parent peptides on locomotor activity in high-fat fed mice. Parameters were measured on day 21 following twice daily treatment with saline vehicle, (pGlu-Gln)-CCK-8, exendin-4 (both at 25 nmol/kg bw), a combination of both peptides (each at 25 nmol/kg bw) and (pGlu-Gln)-CCK-8/exendin-4 hybrid (25 nmol/kg bw). Mice were placed in CLAMS metabolic chambers and locomotor activity measured using optical beams. Activity counts in X and Z axes were recorded every minute for 22 hours. Data are expressed as means ± SEM for 8 mice. *$P<0.05$ compared to lean controls. $\Delta P<0.05$, $\Delta\Delta P<0.01$, $\Delta\Delta\Delta P<0.001$ compared to high fat controls. $\Psi P<0.05$ compared to (pGlu-Gln)-CCK-8. $\theta P<0.05$ compared to exendin-4. $\delta P<0.05$ compared to (pGlu-Gln)-CCK-8 plus exendin-4.

**FIG. 6.** Effects of twice daily administration of (pGlu-Gln)-CCK-8/exendin-4 hybrid, (pGlu-Gln)-CCK-8, exendin-4 or a combination of both parent peptides on plasma lipid profile in high-fat fed mice. Parameters were measured on day 21 following twice daily treatment with saline vehicle, (pGlu-Gln)-CCK-8, exendin-4 (both at 25 nmol/kg bw), a combination of both peptides (each at 25 nmol/kg bw) and (pGlu-Gln)-CCK-8/exendin-4 hybrid (25 nmol/kg bw). Data are expressed as means ± SEM for 8 mice. *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared to lean controls. $\Delta P<0.05$, $\Delta\Delta P<0.01$, $\Delta\Delta\Delta P<0.001$ compared to high fat controls.
controls. $^\theta P<0.05$, $^{\theta\theta} P<0.001$ compared to exendin-4. $^\delta P<0.05$, $^{\delta\delta} P<0.01$ compared to (pGlu-Gln)-CCK-8 plus exendin-4.

**FIG. 7. Effects of twice daily administration of (pGlu-Gln)-CCK-8/exendin-4 hybrid, (pGlu-Gln)-CCK-8, exendin-4 or a combination of both parent peptides on pancreatic beta-cell responsiveness in high-fat fed mice.** Pancreatic islets were isolated on day 21 by collagenase digestion following twice daily treatment with saline vehicle, (pGlu-Gln)-CCK-8, exendin-4 (both at 25 nmol/kg bw), a combination of both peptides (each at 25 nmol/kg bw) and (pGlu-Gln)-CCK-8/exendin-4 hybrid (25 nmol/kg bw). Islets were exposed to 1.4 or 16.7 mmol/l glucose and (A) each individual peptide treatment regimen and (B) various established insulin secretagogues, and insulin release (% of insulin content) measured. (C) HOMA-$\beta$ values calculated from in vivo data are also shown. Data are expressed as means ± SEM for 8 mice. $^\ast P<0.05$, $^{\ast\ast} P<0.01$, $^{\ast\ast\ast} P<0.001$ compared to lean controls. $^\Delta P<0.05$, $^{\Delta\Delta} P<0.01$, $^{\Delta\Delta\Delta} P<0.001$ compared to high fat controls. $^\Psi P<0.05$, $^{\Psi\Psi} P<0.01$ compared to (pGlu-Gln)-CCK-8. $^\theta P<0.05$, $^{\theta\theta} P<0.01$, $^{\theta\theta\theta} P<0.001$ compared to exendin-4.

**FIG. 8. Effects of twice daily administration of (pGlu-Gln)-CCK-8/exendin-4 hybrid, (pGlu-Gln)-CCK-8, exendin-4 or a combination of both parent peptides on pancreatic islet morphology in high-fat fed mice.** Parameters were measured on day 21 following twice daily treatment with saline vehicle, (pGlu-Gln)-CCK-8, exendin-4 (both at 25 nmol/kg bw), a combination of both peptides (each at 25 nmol/kg bw) and (pGlu-Gln)-CCK-8/exendin-4 hybrid (25 nmol/kg bw). (A-F) Representative images of islets from (A) lean control, and high fat fed mice treated with (B) saline, (C) (pGlu-Gln)-CCK-8, (D) exendin-4 (E) a combination of both peptides and (F) (pGlu-Gln)-CCK-8/exendin-4 hybrid. (G-H) Images were analysed using Cell^F analysis software and islet area together with related parameters were measured.
using the closed polygon tool. Approximately 20-30 islets per mouse were analysed. Data are expressed as means ± SEM for 8 mice. *P<0.05, **P<0.01, ***P<0.001 compared to lean controls. ∆P<0.05, ∆∆∆P<0.001 compared to high fat controls. ΨP<0.05, ΨΨP<0.01 compared to (pGlu-Gln)-CCK-8. θP<0.05 compared to exendin-4.
**Figure 2**

**A**
- Plots showing plasma glucose AUC (mmol/L min) over time (min) for different treatments:
  - Glucose alone
  - (pGlu-Gln)-CCK-8
  - Exendin-4
  - (pGlu-Gln)-CCK-8 plus exendin-4
  - (pGlu-Gln)-CCK-8/exendin-4 hybrid

**B**
- Plots showing plasma insulin AUC (ng/ml min) over time (min) for different treatments:
  - Glucose alone
  - (pGlu-Gln)-CCK-8
  - Exendin-4
  - (pGlu-Gln)-CCK-8 plus exendin-4
  - (pGlu-Gln)-CCK-8/exendin-4 hybrid

**C**
- Bar charts showing accumulated food intake (g) over time (min) for different treatments:
  - Saline control
  - (pGlu-Gln)-CCK-8
  - Exendin-4
  - (pGlu-Gln)-CCK-8 plus exendin-4
  - (pGlu-Gln)-CCK-8/exendin-4 hybrid
Figure 3

A. Accumulated energy intake (kJ)

B. Body weight (g)

C. Blood glucose (mmol/L)

D. Plasma insulin (ng/ml)

E. Plasma glucagon (ng/ml)

F. Plasma amylase activity (U/L)

G. HbA1c (%)

Diabetes
Figure 5

**Light phase**

A

![Graph showing X-beam ambulatory activity (counts/h) for different groups under Light phase conditions.](image)

**Dark phase**

B

![Graph showing X-beam ambulatory activity (counts) for different groups under Dark phase conditions.](image)

**C**

![Graph showing Z-beam ambulatory activity (counts/h) for different groups under Light phase conditions.](image)

**D**

![Graph showing Z-beam ambulatory activity (counts) for different groups under Dark phase conditions.](image)
Figure 6

A. Plasma total cholesterol (mmol/L)

B. Plasma HDL-cholesterol (mmol/L)

C. Plasma LDL-cholesterol (mmol/L)

D. Plasma triglycerides (mmol/L)
Figure 7

A

Lean control
High fat control
(pGlu-Gln)-CCK-8
Exendin-4
(pGlu-Gln)-CCK-8 plus exendin-4
(pGlu-Gln)-CCK-8/exendin-4 hybrid

Insulin release (% islet insulin content/60 min)

1.4 mM glucose
16.7 mM glucose
(pGlu-Gln)-CCK-8 (10^{-6} M)
Exendin-4 (10^{-6} M)
(pGlu-Gln)-CCK-8 plus exendin-4 (each at 10^{-6} M)
(pGlu-Gln)-CCK-8/exendin-4 hybrid (10^{-6} M)

B

Lean control
High fat control
(pGlu-Gln)-CCK-8
Exendin-4
(pGlu-Gln)-CCK-8 plus exendin-4
(pGlu-Gln)-CCK-8/exendin-4 hybrid

Insulin release (% islet insulin content/60 min)

1.4 mM glucose
16.7 mM glucose
Tolbutamide (200 µM)
CaCl_2 (7.68 mM)
PMA (10 nM)
Forskolin (25 µM)

C

Lean control
High fat control
(pGlu-Gln)-CCK-8
Exendin-4
(pGlu-Gln)-CCK-8 plus exendin-4
(pGlu-Gln)-CCK-8/exendin-4 hybrid

HOMA-β
Figure 8

(A) (pGlu-Gln)-CCK-8

(B) Exendin-4

(C) (pGlu-Gln)-CCK-8 plus exendin-4

(D) (pGlu-Gln)-CCK-8/exendin-4 hybrid

(E) Lean control

(F) High fat control

(G) Number of islets per mm² of pancreas

(H) Islet area (μm²)

(I) Islet size distribution (% total islets)

(J) Beta cell area (μm²)
**Supplementary Table 1** Amino acid sequence of (pGlu-Gln)-CCK-8, exendin-4 and the novel (pGlu-Gln)-CCK-8/exendin-4 hybrid molecule

<table>
<thead>
<tr>
<th>Peptide Name</th>
<th>Amino acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>(pGlu-Gln)-CCK-8</td>
<td>H-pE-Q-D-Y(SO$_3$H)-M-G-W-M-D-F-NH$_2$</td>
</tr>
</tbody>
</table>

Where AEEAc is \{2-[2-aminoethoxy]ethoxy\}acetic acid. Amino acid sequence of hybrid peptide derived from parent molecules are shown in bold text.
Supplementary Fig. 1. Effects of twice daily administration of (pGlu-Gln)-CCK-8/exendin-4 hybrid, (pGlu-Gln)-CCK-8, exendin-4 or a combination of both parent peptides on metabolic rate in high-fat fed mice. Mice were placed in CLAMS metabolic chambers and (A) O₂ consumption (B) CO₂ production (C) respiratory exchange ratio and (D) energy expenditure were measured on day 21 following twice daily treatment with saline vehicle, (pGlu-Gln)-CCK-8, exendin-4 (both at 25 nmol/kg bw), a combination of both peptides (each at 25 nmol/kg bw) and (pGlu-Gln)-CCK-8/exendin-4 hybrid (25 nmol/kg bw). Insets depict overall effect during the 22 hour recording period. Data are expressed as means ± SEM for 8 mice. *P<0.05 compared to lean controls.