Metabolic effects of activation of CCK receptor signaling pathways by twice-daily administration of the enzyme-resistant CCK-8 analog, (pGlu-Gln)-CCK-8, in normal mice

Nigel Irwin, Pamela Frizelle, Finbarr P M O'Harte and Peter R Flatt

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

Correspondence
should be addressed
to N Irwin
Email
n.irwin@ulster.ac.uk

Abstract

Cholecystokinin (CCK) is a hormone that has important physiological effects on energy balance. This study has used a stable CCK₁ receptor agonist, (pGlu-Gln)-CCK-8, to evaluate the metabolic effects of prolonged administration in normal mice. Twice-daily injection of (pGlu-Gln)-CCK-8 for 28 days resulted in significantly lowered body weights ($P < 0.05$) on days 24 and 28, which was associated with decreased accumulated calorie intake ($P < 0.01$) from day 12 onward. Nonfasting plasma glucose was significantly reduced ($P < 0.05$) on day 28, while plasma insulin concentrations were increased ($P < 0.05$). After 28 days, glucose tolerance and glucose-mediated insulin secretion were not significantly different in (pGlu-Gln)-CCK-8-treated mice. However, following a 15-min refeeding period in 18-h fasted mice, glucose levels were significantly ($P < 0.05$) decreased by (pGlu-Gln)-CCK-8 despite similar food intake and nutrient-induced insulin levels. Insulin sensitivity in (pGlu-Gln)-CCK-8-treated mice was significantly ($P < 0.01$) improved compared with controls. Accumulation of triacylglycerol in liver was reduced ($P < 0.01$) but there were no differences in circulating cholesterol and triacylglycerol concentrations, as well as triacylglycerol content of pancreatic, muscle, and adipose tissue in (pGlu-Gln)-CCK-8 mice. These data highlight the beneficial metabolic effects of prolonged (pGlu-Gln)-CCK-8 administration and confirm a lack of detrimental effects.

Key Words

- ▶ cholecystokinin (CCK)
- ▶ glucose homeostasis
- ▶ insulin sensitivity
- ▶ food intake

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Introduction

Cholecystokinin (CCK) is considered to be an important hormone in terms of biological effects on food intake and overall energy balance (Rehfeld 2011). CCK was first isolated from porcine duodenal mucosa as a 33 amino acid intestinal hormone (Mutt & Jorpes 1968) that was shown to release pancreatic amylase and other enzymes

(Mutt 1980). However, CCK is now known to exist in a number of molecular isoforms, of which the C-terminal octapeptide (CCK-8) represents the most abundant molecular species and importantly retains full biological activity (Rehfeld *et al.* 2007). Moreover, CCK is now mainly recognized through its ability to stimulate

short-term satiety by activation of CCK₁ receptors in vagal afferent neurons (Strader & Woods 2005). However, in addition to this, CCK also plays a significant role in a number of other important physiological processes including insulin secretion, gastric emptying, and bowel motility (Liddle 1994). More recent evidence reveals that CCK acts as a growth factor and anti-apoptotic agent for pancreatic β -cells (Kuntz *et al.* 2004, Chen *et al.* 2007). In agreement, dual elimination of receptors for glucagon and the incretin hormone, glucagon-like peptide-1, clearly show the importance of CCK receptor signaling in the regulation of insulin secretion and glucose homeostasis (Ali *et al.* 2011).

Studies in CCK receptor-deficient rodents shed further light on the important physiological role of CCK. Thus, Otsuka Long Evans Tokushima Fatty (OLEFT) rats, which have a 6847 bp deletion within the gene for the CCK₁ receptor protein resulting in disrupted CCK₁ receptor production, present with hyperglycemia, hyperphagia, impaired glucose tolerance, and mild obesity (Moran 2008). Interestingly, in mice with genetic deletion of the CCK₁ receptor, the adverse effects are much less obvious (Bi *et al.* 2007). However, an inherent problem with models such as this is the lifetime opportunity for compensatory metabolic adaptation. Nevertheless, administration of CCK₁ receptor antagonists results in increased meal size and overall food intake (Moran *et al.* 1993), while administration of native CCK reduces food intake and results in early satiety in rodents and humans (Gibbs *et al.* 1973, Degen *et al.* 2001). In addition, in patients with bulimia, there is an impaired secretion of CCK in response to a meal (Devlin *et al.* 1997).

Thus, taken together, CCK₁ receptor activation possesses biological characteristics that would suggest potential therapeutic application for obesity and related metabolic disturbances. As such, numerous studies have shown notable therapeutic effectiveness of longer-acting CCK-based compounds (O'Harte *et al.* 1998, Verbaeys *et al.* 2007, 2008, 2009a). In particular, the recently characterized N-terminally modified, enzymatically stable CCK-8 analog, (pGlu-Gln)-CCK-8, causes sustained weight loss and improves both insulin resistance and glucose tolerance in mice with genetically and environmentally induced forms of obesity–diabetes (Irwin *et al.* 2012). Therefore, in this study, (pGlu-Gln)-CCK-8 has been used to evaluate the effects of short-term upregulation of CCK receptor signaling on energy intake, body weight regulation, glucose homeostasis, insulin secretion and sensitivity in normal mice. In view of the possible therapeutic application and increasing awareness of the

metabolic role of CCK-8, evaluation of the effects of extended (pGlu-Gln)-CCK-8 action under normal physiological conditions is of value. This study indicates that (pGlu-Gln)-CCK-8 exerts beneficial effects on body weight regulation and insulin sensitivity with no obvious signs of malaise or harmful effects on behavior.

Materials and methods

Peptides synthesis

(pGlu-Gln)-CCK-8 was obtained from American Peptide Company (Sunnyvale, CA, USA) and characterized using matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (Kerr *et al.* 2009).

Subchronic metabolic effects in normal mice

Over a 28-day period, 14- to 16-week-old male Swiss NIH mice ($n=8$) maintained on a standard rodent maintenance diet (10% fat, 30% protein, and 60% carbohydrate, Trouw Nutrition, Cheshire, UK) on reversed light cycle (lights off between 0930 and 2130 h) received twice-daily i.p. injections (0900 and 1700 h) of either saline vehicle (0.9% (w/v), NaCl) or (pGlu-Gln)-CCK-8 (25 nmol/kg body weight). Food intake, body weight, nonfasting plasma glucose, and insulin concentrations were monitored (1000 h) at intervals of 3–6 days. In addition, i.p. glucose tolerance (18 mmol/kg body weight) and insulin sensitivity (10 IU/kg body weight) tests were performed on day 28 in nonfasted mice. Mice fasted for 18 h were used to examine the metabolic response to 15-min feeding. All acute tests commenced at 1000 h. Pancreatic, liver, gastrocnemius muscle, and subcutaneous adipose tissues were excised at the end of the treatment period and processed for measurement of triacylglycerol content (Montgomery *et al.* 2010). Blood triacylglycerol and cholesterol content was also assessed at the end of the study. All animal experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and no mortalities were noted during the study.

Biochemical analysis

All blood samples were taken from the cut tip of the tail vein of conscious mice at the times indicated in the figures and immediately centrifuged using a Beckman microcentrifuge (Beckman Instruments, Galway, Ireland) for 30 s at 13 000 *g*. The resulting plasma was then aliquoted

into fresh Eppendorf tubes and stored at -20°C before glucose, insulin, and triglyceride determinations. Glucose was assayed by an automated glucose oxidase procedure using a Beckman Glucose Analyzer II (Beckman Instruments). Insulin was determined by a modified dextran-coated charcoal RIA (Flatt & Bailey 1981). Plasma and tissue lipid levels were measured as described previously (Montgomery *et al.* 2010).

Statistical analysis

Results are expressed as mean $\pm$ S.E.M. Data were compared using ANOVA, followed by a Student–Newman–Keuls *post hoc* test. Area under the curve (AUC) analyses were calculated using the trapezoidal rule with baseline subtraction. $P < 0.05$ was considered to be statistically significant.

Results

Effects of (pGlu-Gln)-CCK-8 on food intake, body weight, nonfasting plasma glucose, and insulin concentrations

Administration of (pGlu-Gln)-CCK-8 twice daily for 28 days resulted in significantly ($P < 0.05$ – < 0.01) reduced accumulated food intake from day 12 onward associated with decreased ($P < 0.05$) body weight on days 24 and 28

when compared with saline-treated controls (Fig. 1A and B). A significant decrease ($P < 0.05$) in plasma glucose and increase ($P < 0.05$) in plasma insulin concentrations were observed on day 28 of the study (Fig. 1C and D).

Effects of (pGlu-Gln)-CCK-8 on glucose tolerance, metabolic response to feeding, and insulin sensitivity

As shown in Fig. 2, (pGlu-Gln)-CCK-8 administration twice-daily for 28 days had no significant effect on plasma glucose levels or glucose-stimulated insulin concentrations following administration of an exogenous i.p. glucose load (Fig. 2). However, plasma glucose responses to 15 min feeding were significantly lowered ($P < 0.05$) at 105 min in (pGlu-Gln)-CCK-8-treated mice (Fig. 3A). Similarly, AUC glucose values were significantly ($P < 0.05$) decreased by (pGlu-Gln)-CCK-8 compared with controls (71.5 ± 23.2 vs 332.3 ± 90.3 mmol/l per min respectively; data not shown), despite similar food intakes of 0.3 ± 0.1 vs 0.4 ± 0.1 g/mouse per 15 min respectively. Oral nutrient-stimulated insulin concentrations were not significantly altered between groups (Fig. 3B). However, the hypoglycemic action of insulin was significantly augmented in terms of post-injection ($P < 0.05$ – < 0.01) and AUC ($P < 0.01$) values in mice treated twice daily with (pGlu-Gln)-CCK-8 for 28 days (Fig. 4).

Effects of (pGlu-Gln)-CCK-8 blood lipid content and triacylglycerol accumulation in muscle, liver, pancreatic, and adipose tissues

Circulating cholesterol and triacylglycerol concentrations were similar in the control and (pGlu-Gln)-CCK-8 mice after 28 days of treatment (Fig. 5A and B). Similarly, there were no significant differences in the triacylglycerol content of muscle, pancreatic, and adipose tissue in (pGlu-Gln)-CCK-8 mice (Fig. 5C, D, and E). However, (pGlu-Gln)-CCK-8 treatment twice daily for 28 days resulted in significantly ($P < 0.01$) reduced triacylglycerol accumulation in liver tissue when compared with controls (Fig. 5F).

Discussion

Genetic knockout studies have shown that annulment of CCK₁ receptor activation in rats resulted in significant hyperphagia and mild obesity associated with hyperglycemia and impaired glucose tolerance (Moran 2008). However, similar gene knockout in mice was without effect on body weight and food intake regulation (Chen *et al.* 2007). In addition, while pair-feeding is known to

Figure 1

Effects of twice-daily (pGlu-Gln)-CCK-8 administration on (A) body weight, (B) accumulated food intake, (C) nonfasting plasma glucose, and (D) insulin. (A, B, C and D) Parameters were measured for 5 days before and 28 days during (indicated by horizontal black bar) treatment with saline or (pGlu-Gln)-CCK-8 (25 nmol/kg body weight). Values are mean $\pm$ S.E.M. for eight mice. $*P < 0.05$ and $**P < 0.01$ compared with saline group.

Figure 2

Effects of twice-daily (pGlu-Gln)-CCK-8 administration on glucose tolerance and plasma insulin response to glucose. Tests were conducted after twice-daily treatment with saline or (pGlu-Gln)-CCK-8 (25 nmol/kg body weight) for 28 days. (A and C) Glucose (18 mmol/kg body weight) was administered at the time indicated by the arrow in nonfasted mice. (B and D) Plasma glucose and insulin AUC values for 0–60 min post-injection are also shown. Values are mean $\pm$ s.e.m. for eight mice.

fully normalize energy control and metabolic responses in OLETF rats (Bi *et al.* 2007), the beneficial effects of (pGlu-Gln)-CCK-8 are not reproduced by simple dietary restriction (Irwin *et al.* 2012). In the current study, we have used twice-daily administration of (pGlu-Gln)-CCK-8 to examine the overall metabolic effects under normal physiological conditions. This approach avoids the problems of lifelong compensatory actions.

Our previous *in vitro* and *in vivo* studies in high-fat and *ob/ob* mice have demonstrated that (pGlu-Gln)-CCK-8 is a potent, longer-acting agonist of the CCK₁ receptor (Irwin *et al.* 2012). In this study, twice-daily injection of normal mice with (pGlu-Gln)-CCK-8 for 28 days had no obvious adverse or toxic effects. Indeed, previous studies in our laboratory using (pGlu-Gln)-CCK-8 have established its safety in terms of lack of induction of pancreatic inflammation or development of anxiety (Irwin *et al.* 2012). Moreover, knockout rather than upregulation of the CCK₁ receptor has been shown to predispose to anxiety-like behavior in rats (Schroeder & Weller 2010). Body weights of (pGlu-Gln)-CCK-8-treated mice were substantially lower than controls by day 28 accompanied by predictable inhibitory effects on food intake, highlighting the therapeutic potential of (pGlu-Gln)-CCK-8 for obesity–diabetes (Verbaeys *et al.* 2007, Irwin *et al.* 2012). Interestingly, effects of the peptide were recently shown

not to be associated with changes in energy expenditure (Irwin *et al.* 2012), highlighting the plasticity of signaling pathways involved in energy intake and weight regulation and suggesting multiple actions of (pGlu-Gln)-CCK-8. In addition, (pGlu-Gln)-CCK-8 was shown to stimulate insulin secretion from clonal β -cells and acutely improve glucose homeostasis in mice. However, when comparing the acute insulinotropic actions of (pGlu-Gln)-CCK-8 *in vitro* and *in vivo*, it would suggest that larger doses would be required to induce any prominent insulin-releasing effect during the current treatment regimen (Irwin *et al.* 2012). In agreement, circulating glucose and insulin were almost identical to saline-treated control mice, indicating lack of detrimental metabolic effects despite significantly lowered body weights. These observations accord with other studies using longer-acting CCK agonists, where the

Figure 3

Effects of twice-daily (pGlu-Gln)-CCK-8 administration on (A) glucose and (B) insulin responses to feeding. Tests were conducted after twice-daily treatment with saline or (pGlu-Gln)-CCK-8 (25 nmol/kg body weight) for 28 days. Mice were fasted for 18 h and allowed to refeed for 15 min (black horizontal bar indicates time of feeding). Values are mean $\pm$ s.e.m. for eight mice. * P < 0.05 compared with saline group.

Figure 4

Effects of twice-daily (pGlu-Gln)-CCK-8 administration on insulin sensitivity. Tests were conducted after twice-daily treatment with saline or (pGlu-Gln)-CCK-8 (25 nmol/kg body weight) for 28 days. (A) Insulin (20 U/kg body weight) was administered at the time indicated by the arrow in nonfasted mice. (B) AUC values for 0–60 min post-injection are also shown. Values are mean $\pm$ S.E.M. for eight mice. * $P < 0.05$ and ** $P < 0.01$ compared with saline group.

occurrence of adverse effects such as intestinal discomfort, tolerance, and conditioned taste aversion was atypical and dose related (Verbaeys *et al.* 2008, 2009a,b). The finding in one study of pancreatic hyperplasia and, in some cases, pancreatitis in rats treated with a long-acting PEGylated version of CCK-9 for 14 days (Verbaeys *et al.* 2009a) was not replicated in previous investigations with (pGlu-Gln)-CCK-8, where amylase and lipase levels were actually reduced (Irwin *et al.* 2012).

Consistent with a physiological role in regulating insulin sensitivity (Lo *et al.* 2011), mice receiving twice-daily (pGlu-Gln)-CCK-8 injections displayed marked augmentation of the glucose-lowering action of insulin by day 28. This is in harmony with the observed beneficial effects of (pGlu-Gln)-CCK-8 in animal models of obesity–diabetes (Irwin *et al.* 2012). Thus, twice-daily (pGlu-Gln)-CCK-8 administration results in marked

improvement of metabolic status in high-fat-fed and obese diabetic (*ob/ob*) mice, including substantially decreased energy intake and body weight, improved glucose tolerance and insulin sensitivity, and significantly reduced triacylglycerol content of peripheral tissues (Irwin *et al.* 2012). Moreover, triacylglycerol accumulation in liver was also decreased in this study suggestive of improved peripheral insulin action. In keeping with this, when challenged with a test meal, glucose concentrations were lowered compared with those of saline-treated controls, despite similar nutrient-induced insulin levels. Interestingly, i.p. glucose tolerance was not affected by twice-daily (pGlu-Gln)-CCK-8 administration for 28 days, confirming normal glucose uptake and disposal mechanisms in these animals. In relation to these observations, it has been shown that CCK and incretin hormones may have complementary metabolic effects (Hisadome *et al.* 2011). This could account for the disparity between oral and i.p. nutrient challenge in the current setting. In agreement, there appears to be significant overlap in the hormonal expression of intestinal cells that secrete incretin hormones and CCK (Habib *et al.* 2012). In addition, assessment of the

Figure 5

Effects of twice-daily (pGlu-Gln)-CCK-8 administration on (A and B) plasma cholesterol and triacylglycerol levels and (C, D, E and F) triacylglycerol content of muscle, pancreas, adipose, and liver tissue. Parameters were measured after 28 days of treatment with saline or (pGlu-Gln)-CCK-8 (25 nmol/kg body weight). Values are mean $\pm$ S.E.M. for eight mice. ** $P < 0.01$ compared with saline group.

expression of CCK₁ and CCK₂ receptors following prolonged treatment with (pGlu-Gln)-CCK-8 may have aided with interpretation of the current data set.

A key observation from this study was the lack of discernible harmful effects following sustained administration of (pGlu-Gln)-CCK-8. Indeed, due to scarcity of specific assays, knowledge about CCK in disease is currently limited (Rehfeld *et al.* 2007), highlighting the importance of our findings. Thus, circulating triacylglycerol and cholesterol levels were identical to lean controls in (pGlu-Gln)-CCK-8-treated mice, despite the well-characterized actions of CCK on gallbladder function and bile production (Rehfeld 2011). Moreover, CCK₁ receptor activation has been shown to decrease the risk of gallstone formation in mice (Sato *et al.* 2003) and to attenuate inflammation in rats (Lubbers *et al.* 2010). In addition, this study highlights the lack of effect of sustained (pGlu-Gln)-CCK-8 treatment in normal mice on circulating glucose and insulin, glucose tolerance, and tissue and plasma triacylglycerol content. Moreover, previous studies with (pGlu-Gln)-CCK-8 have confirmed its safety in terms of lack of induction of pancreatic inflammation, development of anxiety, and preservation of islet structure (Irwin *et al.* 2012). Thus, the recent significant focus on peptide therapies, which has been based on the classical glucoregulatory incretin hormones, should be revisited (Holst *et al.* 2009), especially as meals consist of a mixture of components and nutrients that release a number of other gut-derived hormones that undoubtedly have potential therapeutic implications (Irwin *et al.* 2012). In effect, it appears highly likely that the beneficial effects of upregulation of CCK₁ receptor action extend beyond direct effects on energy balance and encompass numerous other actions including effects on glucose homeostasis (Ahrén *et al.* 1991, Lavine *et al.* 2010).

In conclusion, this study has demonstrated that twice-daily administration of (pGlu-Gln)-CCK-8 results in beneficial metabolic effects in normal mice. Observation of decreased body weight gain and an enhancement in insulin sensitivity, without change of nutrient-induced pancreatic β -cell function, demonstrates important metabolic actions that are transferred to models of obesity-diabetes (Irwin *et al.* 2012). Overall, (pGlu-Gln)-CCK-8 appears to be an effective means of improving metabolic control, which could be particularly valuable in situations of obesity and insulin resistance.

Declaration of interest

N I, F P M O, and P R F hold shares with Diabetica Ltd., which has patents for exploitation of peptide therapeutics.

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