Peptides 30 (2009) 699–704

Contents lists available at ScienceDirect

Peptides

journal homepage: www.elsevier.com/locate/peptides

Lack of tolerance development with long-term administration of PEGylated cholecystokinin

Isabelle Verbaeys [a,*], Fabián León-Tamariz [b], Johan Buyse [c], Eddy Decuypere [c], Hans Pottel [a], Marnix Cokelaere [a]

[a] Interdisciplinary Research Center, Katholieke Universiteit Leuven, Campus Kortrijk, Etienne Sabbelaan 53, B-8500 Kortrijk, Belgium
[b] Laboratory of Toxicology and Bromatology, Cuenca University, av. 12 de Abril s/n Ciudadela Universitaria, Ecuador
[c] Laboratory for Livestock Physiology, Immunology and Genetics, Katholieke Universiteit Leuven Kasteelpark Arenberg 30, B-3001 Leuven, Belgium

ARTICLE INFO

Article history:
Received 2 October 2008
Received in revised form 14 November 2008
Accepted 14 November 2008
Available online 25 November 2008

Keywords:
Cholecystokinin
PEGylated Cholecystokinin
Satiety
Long-term administration
Tolerance
Pancreas proliferation

ABSTRACT

Cholecystokinin (CCK) is a short acting satiating peptide hormone produced in the proximal small intestine. Daily CCK injection in rats initially inhibits food intake, but after several days, food intake is no longer affected, suggesting development of tolerance. Previously, we covalently coupled CCK to a 10 kDa polyethylene glycol (mPEG-OH) and showed that this conjugate, PEG-CCK_9, produced a significantly longer anorectic effect than unmodified CCK_9. The present study examined whether tolerance to the anorectic effect develops during long-term administration of PEG-CCK_9. For 14 consecutive days, male Wistar rats ($n = 12$) received a daily i.p injection of 8 μg kg^{-1} of PEG-CCK_9 and a control group received a daily control injection of mPEG-OH. Body weight and food intake were monitored daily during the experiment. Effects on the pancreas were investigated. On each day, injection of PEG-CCK_9 induced an anorectic effect lasting 3–6 h, but failed to significantly reduce daily total food intake compared to controls. The body weight gain of the PEG-CCK_9-treated animals was not different from controls. The PEG-CCK_9-treated group had a significantly higher pancreas weight, mainly due to hyperplasia. In conclusion, PEG-CCK_9 continued to have a daily suppressive effect on food intake when administered for 14 consecutive days, showing there was no development of tolerance.

1. Introduction

Cholecystokinin (CCK), a gut peptide released by the endocrine cells of the duodenum and jejunum in response to the intraluminal presence of nutrient digestive products, is known to induce a state of post-prandial satiety. In a variety of species, including humans, administration of CCK results in a significant short-lasting reduction in food intake [2,13,18,32]. Although the physiological role of endogenous CCK in the control of meal size has been demonstrated [4], the functions of CCK in the regulation of energy balance, particularly the overall meal-to-meal regulation, have not yet been elucidated. Chronic infusion of CCK does not change food intake after the first day [7] nor does it affect body weight [22]. One explanation for the inability of chronic CCK administration to reduce body weight is the rapid development of tolerance to the actions of CCK on eating behavior [7]. On the contrary, West et al. [39] demonstrated that meal-dependent CCK administration persistently reduced meal size, but then resulted in a compensatory increase in meal frequency such that overall food intake was no longer affected. The short action time of CCK and the rapid loss of its effectiveness when chronically administered remain obstacles to identify its mechanism of action and possible applications for body weight management. In order to overcome these difficulties, we successfully prolonged the anorectic effect of CCK by PEGylating the peptide [20]. PEGylation is a method in which a flexible strand or strands of polyethylene glycol (PEG) is covalently attached to a protein or small drug molecule, improving the pharmacokinetics of the PEGylated molecule [15,16,38]. Our recent study demonstrated that the optimal PEGylated conjugate, 10 kDa PEG-CCK_9, dose-dependently increases the duration of the anorectic effect compared to the unmodified molecule by stimulation of CCK_1-receptors [36].

Besides inducing satiation, CCK plays a role in a number of other gastrointestinal functions, including inhibition of gastric emptying, maintenance of mucosal immunity [7] and stimulation of gallbladder contraction, intestinal motility, pancreatic growth and its secretory activities. Several studies have shown that continuous stimulation of CCK_1-receptors by infusion of CCK_8 resulted in increased proliferation of pancreatic acinar cells with a persistent increase in pancreatic weight [27,33,41]. However, the effect of

* *Corresponding author*. Tel.: +32 56 246240; fax: +32 56 246997.
E-mail addresses: isabelle.verbaeys@kuleuven-kortrijk.be (I. Verbaeys), fleontamariz@gmail.com (F. León-Tamariz), johan.buyse@biw.kuleuven.be (J. Buyse), eddy.decuypere@biw.kuleuven.be (E. Decuypere), marnix.cokelaere@kuleuven-kortrijk.be (M. Cokelaere).

0196-9781/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.peptides.2008.11.010

long-term administration of the newly synthesized peptide PEG-CCK_9 on food intake, body weights and pancreatic proliferation has not yet been studied. The aims of this study were therefore (1) to investigate whether the anorectic effectiveness of PEG-CCK_9 was lost during long-term administration due to tolerance development, (2) to monitor its effect on body weight and (3) to study its effect on the pancreas. Rats were given a daily intraperitoneal (i.p.) injection of PEGylated CCK_9 for 14 consecutive days and the effects on food intake, body weight and pancreas proliferation were monitored.

2. Materials and methods

2.1. Drugs, chemical reagents and other materials

The 10 kDa PEG-CCK_9 conjugate was prepared as described by Léon-Tamariz [20] and was quantified by analytical HPLC, calibrated using pure CCK_9 (Bachem, Bubendorf, Switzerland). The amounts of PEG-CCK_9 ($\mu g\ kg^{-1}$) mentioned in the experiments indicate the amount of peptide present in the conjugate. The 10 kDa non-active linear methoxy polyethylene glycol [mPEG-OH or CH_3-$(OCH_2CH_2)n$-OH], used for control injections, was purchased from Nektar Therapeutics (Huntsville, AL, USA). The PEG-CCK_9 conjugate consisted of 84.2% PEG and 15.8% CCK_9 by weight. The amount of mPEG-OH given in the control injection was the same as the amount of PEG injected into the PEG-CCK_9-treated animals.

2.2. Animals

The experiments were performed on 7-week-old male Wistar rats with an initial body weight of 254 ± 22 g (Janvier, Le Genest Saint Isle, France). The animals (n = 24) were housed individually in iron wire cages under standardized conditions (room temperature of 21 ± 0.2 °C, 40–60% relative humidity, reversed 12:12 h light-dark cycle with lights on at 10.00 p.m. and lights off at 10.00 a.m.), according to European guidelines on animal care. The rats had free access to water. Complete powdered rodent food (Sniff, Bioservices, Schaijk, The Netherlands), presented in special feeders to avoid spillage, was provided as described in the experimental design. The animals were adapted to the experimental conditions and to i.p. injections 2 weeks before the start of the experiment.

2.3. Experimental design

2.3.1. In vivo experiment

After the 2-week adaptation period, the young rats were divided into two groups of 12. One group received a daily i.p. bolus injection of 8 $\mu g\ kg^{-1}$ of PEG-CCK_9 in 0.5 ml of saline at 15 min before dark onset for 14 consecutive days. Each time the PEG-CCK_9-treated animals received their daily injection, a control group received an i.p. injection of 42.6 $\mu g\ kg^{-1}$ of mPEG-OH dissolved in 0.5 ml of saline. Both treatment groups had free access to food, except for the hour before dark onset during which the mangers were withdrawn to synchronize the beginning of food intake. The food intake of the two groups was measured at 3, 6 and 23 h after dark onset. Body weights were measured daily, half-hours before drug injection, in order to adjust the amount of injected drug to the changed body weight.

2.3.2. Pancreatic tissue sampling and analysis

The pancreas was excised, dissected free from fat, connective tissue and lymph nodes and weighed. Part of the pancreas was fixed in buffered 4% formaldehyde (Sigma) and sections cut and stained with hematoxylin-eosin. The other part of the pancreas was snap frozen in liquid nitrogen and stored at −80 °C for analysis of water, protein and DNA content. The tissue was homogenized in RNase/DNAse-free distilled water using a Tissuelyser mixer-mill disruptor (Qiagen, Venlo, Netherlands). The protein concentration in the homogenate was measured by the method of Lowry et al. [21] with bovine plasma albumin as the standard (BCA protein assay and standard, Pierce, Rockford, IL, USA). Pancreatic DNA was measured fluorimetrically by the Hoechst DNA assay (Sigma–Aldrich, Bornem, Belgium) [19], using calf thymus DNA (Pierce Rockford, IL, USA) as the standard. To measure the pancreatic water content, tissue from each pancreas was weighed before and after freeze-drying in a Heto Drywinner system and the water content calculated as a percentage of the wet weight.

2.3.3. Blood analysis

After drug injection on day 14, the rats were anaesthetized with ether and blood and serum samples were collected in appropriate collection tubes (Vacutainer, BD, Belgium). Serum samples were prepared by centrifugation, then aliquoted and frozen at −80 °C until analysis. Blood substrates and enzymes (kidney, pancreas and liver tests) were determined in serum by a clinical assay procedure using an Integra 400 calibrated with a calibrator for automatic systems (Roche, Basel, Switzerland).

2.4. Data analysis and statistical procedures

The results are shown as the mean ± S.E.M., unless otherwise specified. The 3, 6 and 23 h daily food intake data was analyzed by two-way analysis of variance, with one repeated measures factor (time). To evaluate possible changes of effectiveness of the anorectic effects of PEG-CCK_9 during the experimental period, the difference of food intake between the control animals and PEG-CCK_9-treated animals at 3, 6 or 23 h was plotted over time (days) and approximated by linear regression. To analyze the body weight change of the growing rats, cumulative growth (Y) was plotted vs. time (X) and was fitted by linear regression. A t-test was used for comparisons between the two unpaired treatment groups. Because of the presence of outliers, lipase and amylase serum levels were analyzed by a non-parametric Mann Whitney test. All statistical analysis were performed using GraphPad Prism (Version 4, San Diego, CA, USA). A P-value <0.05 was considered statistically significant.

3. Results

3.1. In vivo feeding experiment: food intake analysis

The cumulative food intake measured at 3, 6 or 23 h after a daily i.p. bolus injection of 8 $\mu g\ kg^{-1}$ of PEG-CCK_9 or control vehicle is depicted in Fig. 1. Two-way ANOVA revealed a significant treatment effect on the 3 h (P = 0.0003) and 6 h food intake (P = 0.0057), but not on the 23 h food intake (P = 0.164). There was a significant day effect ($P < 0.05$), but not a significant interaction for the 3, 6 and 23 h food intake ($P > 0.05$).

A non-significant 5% difference (P = 0.164) was observed in overall food intake during the entire experiment, the control group consuming on average 359.2 ± 10.0 g during the experimental period compared to 341.3 ± 7.4 g for the PEG-CCK_9 group.

In order to assess whether the effect of PEG-CCK_9-treatment was habituating over the course of the experiment, the amount eaten on the first and the thirteenth day was compared. The PEG-CCK_9-group showed a significant decrease from the first to the thirteenth day in both the 3 and 6 h food intake as well as in the 23 h food intake (t-test; P(3 h) = 0.008; P(6 h) = 0.008; P(23 h) = 0.004), but the saline groups did not ($P > 0.05$). In addition, a linear regression analysis of the food intake inhibition represented as the differences in food intake between both treatment groups (Fig. 2), revealed a significant upward trend for the 6 and 23 h food intake measurements (slope (6 h) = 0.09125 ± 0.02649;

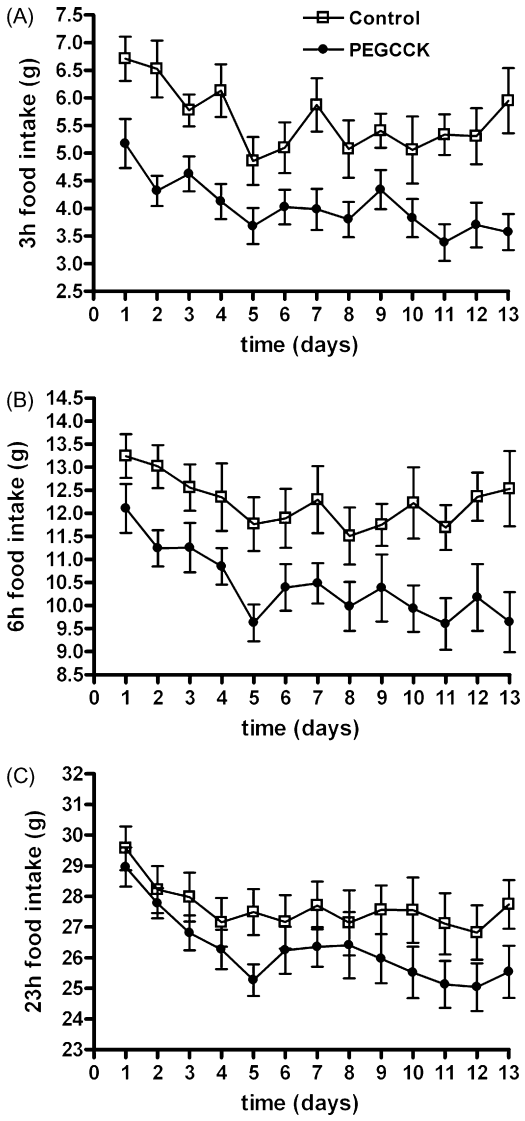

Fig. 1. Food intake over the period 0–3 h (A), 0–6 h (B) and 0–23 h (C) of the control and PEG-CCK_9-treated animals plotted vs. time. A significant effect of the PEG-CCK_9-treatment was observed by two-way repeated measures ANOVA for the 0–3 h ($P = 0.0003$) and 0–6 h period ($P = 0.0057$), but not for the 0–23 h period ($P = 0.164$). No interaction was observed for all three periods. Means ± S.E.M. are depicted.

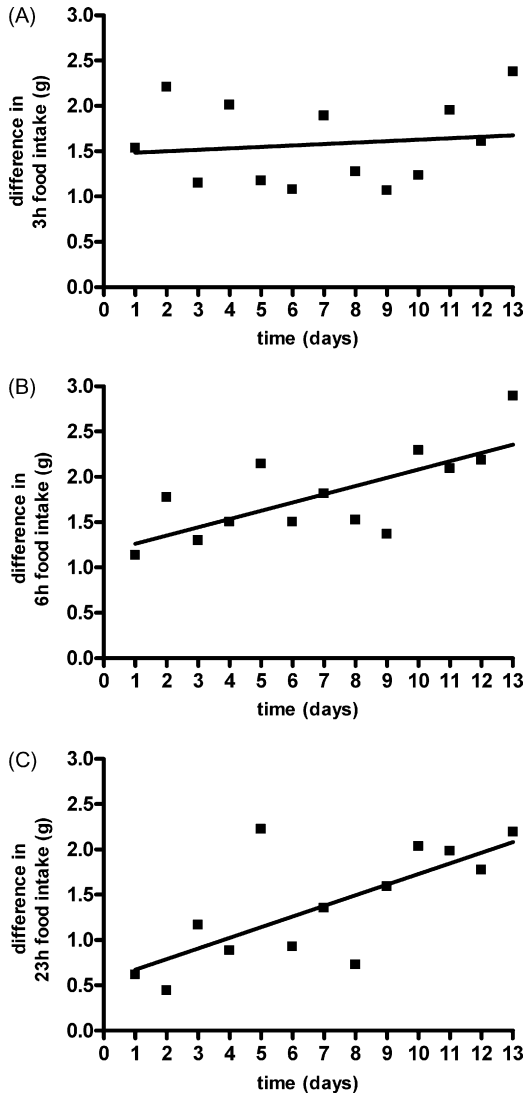

Fig. 2. Linear regression of the daily food intake reductions over the period 0–3 h (A), 0–6 h (B) and 0–23 h (C), expressed as the differences between the controls and the PEG-CCK_9-group. A significant upward trend for the 6 and 23 h food intake measurements was observed (slope (6 h) = 0.09125 ± 0.02649; P(6 h) = 0.006; slope (23 h) = 0.1174 ± 0.03351; P(23 h) = 0.005), but not for the 3 h food intake (slope (3 h) = 0.01598 ± 0.03521; P(3 h) = 0.659).

P(6 h) = 0.006; slope (23 h) = 0.1174 ± 0.03351; P(23 h) = 0.005). No upward or downward trend was observed for the 3 h food intake as the slope was not significant different from zero (slope (3 h) = 0.01598 ± 0.03521 P(3 h) = 0.659).

3.2. In vivo feeding experiment: body weight change

Linear regression analysis of the cumulative body weight gain revealed a linear relationship between growth and time for both treatment groups (control: slope = 5.63 ± 0.12; $P < 0.0001$; PEG-CCK_9: slope = 5.40 ± 0.12; $P < 0.0001$ (Fig. 3). No significant difference in slope between the control and PEG-CCK_9-injected animals was observed. This indicates that both treatment groups have an identical growth rate. The body weights at day 14 and the body weight gain over 14 days were not different (Table 1).

3.3. Pancreatic tissue analysis

As shown in Table 1, daily i.p injection of 8 μg kg^{-1} of PEG-CCK_9 for 14 consecutive days resulted in a significant increase in

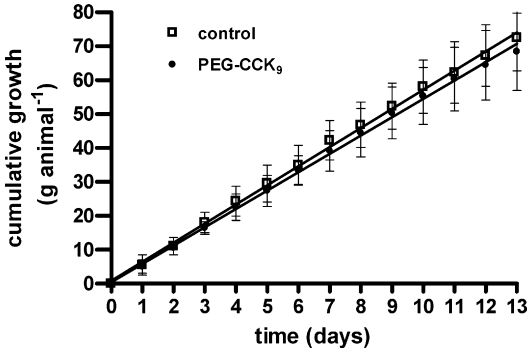

Fig. 3. Effect of daily i.p injection of 8 μg kg^{-1} of PEG-CCK_9 or mPEG-OH on body weight change. The results are presented as cumulative body weight gain (g $animal^{-1}$) over time approximated by linear regression (mean ± S.D. are depicted). A linear relationship between growth and time is present (control: slope = 5.63 ± 0.12; $P < 0.0001$; PEG-CCK_9: slope = 5.40 ± 0.12; $P < 0.0001$). There is no significant difference in slope between the control and PEG-CCK_9-injected animals.

Table 1
Effect of a daily i.p injection of 8 μg kg^{-1} of PEG-CCK$_9$ or mPEG-OH (control) for 14 consecutive days on body weight, pancreatic wet weight, pancreatic DNA, protein and water contents measured on day 14[a].

	Controls	PEG-CCK$_9$	Statistical significance *P*-value
Body weight (g)	335.0 ± 9.9	330.8 ± 5.6	0.717
Body weight gain over 14 days (g)	72.5 ± 2.8	68.3 ± 2.3	0.346
Pancreatic wet weight (g)	1.43 ± 0.03	2.32 ± 0.09	<0.0001
DNA (mg g^{-1} pancreas)	2.56 ± 0.2	6.20 ± 0.5	<0.0001
Protein (mg g^{-1} pancreas)	125.70 ± 4.3	149.30 ± 7.1	0.007
Protein/DNA ratio	57.43 ± 4.6	28.20 ± 2.7	<0.0001
Water content (%)	74.98 ± 1.1	76.98 ± 0.7	0.1463

[a] Values are expressed as the mean ± S.E.M.

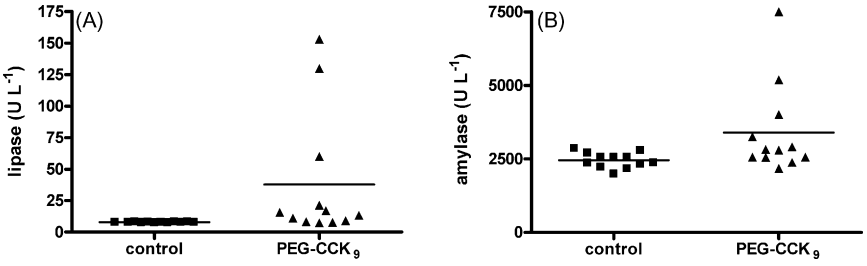

Fig. 4. Effect daily i.p injection of 8 μg kg^{-1} of PEG-CCK$_9$ or mPEG-OH on lipase serum levels (A) and amylase serum levels (B) represented as a vertical scatter plot. The means are displayed as horizontal lines. A highly significant increase in serum concentrations of both lipase and amylase is observed in the PEG-CCK$_9$ group.

pancreatic-wet weight. In addition, pancreatic protein and DNA contents of the PEG-CCK$_9$ group were significantly increased, while the protein/DNA ratio was significantly decreased. No significant differences were observed in pancreatic water content. A markedly significant increase in serum concentrations of both lipase ($P = 0.0036$) and amylase ($P = 0.0209$) activities was observed in the PEG-CCK$_9$ group (Fig. 4A and B). This was mainly due to the results for 3 of the 12 PEG-CCK$_9$-treated rats, which had extremely high values and pathological study of the pancreas revealed marked inflammation in these three rats. However, a student *t*-test after exclusion of the 3 aberrant values revealed that serum concentrations of lipase were still significantly higher in the other 9 rats than in the control ($P = 0.0050$). This was not the case for amylase ($P = 0.106$).

3.4. Kidney and liver tests

Table 2 shows the effects of a daily i.p. injection of 8 μg kg^{-1} PEG-CCK$_9$ or mPEG-OH (control) for 14 consecutive days on several serum parameters, in particular kidney and liver function tests. Statistical analysis showed there were no differences in terms of urea, creatinine, uric acid and total protein. There were also no differences in bilirubin, aspartate aminotransferase (AST) and alkaline phosphatase (ALP), but alanine aminotransferase (ALT) activity was significantly decreased in the PEG-CCK$_9$-treated group.

4. Discussion

The present study demonstrated that once-daily i.p. injection of 8 μg kg^{-1} of PEG-CCK$_9$ induced a significant inhibition of the 3 and 6 h food intake every day for 14 consecutive days. The total food intake (23 h) was, relative to controls, daily reduced with 5 ± 0.6%, but this reduction was not statistically discernable at the 5% level. An important goal of this study was to observe whether rats develop a tolerance to PEG-CCK$_9$-administration when injected on a daily basis. Tolerance is defined by Goodman and Gilman [14] as "when, after repeated administration, a given dose of a drug produces a decreasing effect, or conversely, when increasingly larger doses must be administered to obtain the effects observed with the original dose." No evidence for the development of tolerance to the PEG-CCK$_9$-administration was found in our experimental set-up. On the contrary, linear regression of the daily food intake reductions revealed that the anorectic effects of PEG-CCK$_9$ may even increase during the course of the experiment. The effectiveness of the PEG-CCK$_9$ injection on the 3 h food intake did not change during the 14 consecutive injection days (Fig. 2A) whereas the inhibitory effects on the 6 h and 23 h increased as the experimental period proceeded

Table 2
Effects of a daily i.p. injection of 8 μg kg^{-1} of PEG-CCK$_9$ or mPEG-OH (control) for 14 consecutive days on several serum parameters[a].

	Controls	PEG-CCK$_9$	Statistical significance *P*-value
Kidney tests			
Urea (mg dl^{-1})	38.85 ± 0.89	42.61 ± 1.80	0.075
Creatinine (mg dl^{-1})	0.24 ± 0.01	0.23 ± 0.01	0.871
Uric acid (mg dl^{-1})	1.59 ± 0.18	1.30 ± 0.14	0.204
Total protein (g l^{-1})	55.39 ± 0.60	55.28 ± 0.38	0.871
Liver tests			
Bilirubin, indirect (mg dl^{-1})	0.088 ± 0.01	0.081 ± 0.01	0.489
Aspartate aminotransferase (AST) (mg dl^{-1})	85.58 ± 3.13	84.42 ± 3.76	0.814
Alanine aminotransferase (ALT) (mg dl^{-1})	57.58 ± 2.38	49.92 ± 2.19	0.027
Alkaline phosphatase (ALP) (mg dl^{-1})	203.80 ± 10.14	194.80 ± 10.62	0.543

[a] Values are expressed as the mean ± S.E.M.

(Fig. 2B and C). The observation of the lack of tolerance development to PEG-CCK_9 when daily injected is opposed to reports using the unmodified peptide. Mineka and Snowdon [23] showed that the effect of CCK habituated with repeated daily injection, or, in other words, a tolerance to the drug developed. Crawley and Beinfeld [7] showed that chronic infusion of CCK via an osmotic minipump did not affect food intake beyond the first administration day and suggested that a rapid development of behavioral tolerance to the infused peptide was responsible for the observed loss of effectiveness. Others [22] suggested that the low dose used in this experiment ($1\ \mu g\ kg^{-1}\ h^{-1}$) was probably insufficient to induce a satiety response and could therefore not explain the loss of food intake inhibition after 1 day. These authors demonstrated that continuous infusion of high enough doses of CCK ($11.6\ \mu g\ kg^{-1}\ h^{-1}$) over a 7-day period affected food consumption for 4 days, but an increase in the number of meals resulted in a normalization of daily food intake to control levels by day 4. West et al. [39] found that meal-dependent CCK administration persistently reduces meal size, but, after 4 days of administration, ceased to affect the 24 h food intake due to a compensatory increase in meal number. Although the meal-contingent injections of CCK in the experiment of West et al. [39] did persistently reduce meal size, the average meal size did gradually increase, indicating that the effectiveness of the peptide in inhibiting food intake did diminish during the experimental course. This is in contrast with the observed sustained effectiveness with repeated PEG-CCK_9-administration. The reason for the persistent food intake inhibition with PEG-CCK_9-administration, lasting 3–6 h every day, could be diverse. First of all, the prolonged and sustained anorectic effectiveness of the conjugate might be a result of the PEGylation process. PEGylation is known to increase the circulation time in the body by protecting the active molecule from proteolytic enzymes and shielding immunoreactive sites [15,16]. The increased half-life of the PEGylated CCK_9, due to the higher resistance of the peptide to proteolysis, results in a prolonged anorectic effect of the conjugate in comparison to the unmodified peptide [20,36]. A dose of $8\ \mu g\ kg^{-1}$ of unmodified CCK_9 induces an anorectic effect lasting less than an hour whereas an identical dose of PEG-CCK_9 results in a 6 h lasting food intake reduction [36]. Moreover, the presence of the PEG-tail, making the protein more non-immunogenic and non-antigenic [1,15,16,31,38], might also be responsible for the lack of tolerance development. The PEG-tail might prevent the development of CCK receptor sub sensitivity, a mechanism which might be responsible for the observed tolerance development using the unmodified CCK [7,28,42]. Secondly, we also need to consider the possibility that part of the persistent anorectic effects of daily dosing PEG-CCK_9 could be the result of malaise due to the drug instead of satiety. Some authors provided evidence that the unmodified CCK may induce condition taste aversion and as such suppresses feeding due to temporary malaise [3,8,9,24]. However the proof of a CCK-induced condition taste aversion is not that clear-cut and remains a controversial issue [11,12,30,34,40]. We therefore previously investigated the ability of PEG-CCK_9 to induce a conditioned taste aversion (CTA). We demonstrated that PEG-CCK_9 induces both satiety and CTA and that both effects increase with dose [37]. A dose of $8\ \mu g\ kg^{-1}$ was the minimal effective dose to produce CTA. However, even at this dose, the PEGylated conjugate is more potent in inducing satiety, suggesting that the anorexia cannot be completely attributed to the aversiveness of the drug [37]. Experiments using smaller doses of PEG-CCK_9 and thus inducing a lesser degree of aversiveness, might be useful to unravel the contribution of the PEG-CCK_9-induced CTA in the observed lack of tolerance.

PEG-CCK_9-treatment failed to significantly suppress the 23 h food intake compared to controls. The absence of a suppressive effect on total food intake implies a compensatory eating behavior in the period 6–23 h, probably due to a change in meal pattern. An increased meal frequency, the compensatory behavior observed after unmodified CCK administration [39], might, in the period 6–23 h, be responsible for the catch-up in overall food intake. Future studies, monitoring the detailed microstructural feeding pattern of the PEG-CCK_9 rats might reveal the compensatory feeding behavior in the 6–23 h interval.

The body weight change in the PEG-CCK_9-treated rats was not different from that in the control group. This observation is not surprising, as the daily total food intake of the PEG-CCK_9-treated rats was not significantly suppressed. Earlier studies showed that chronic administration of unmodified CCK did not have a significant effect on body weight beyond the first day of infusion [7,22,39]. This lack of effect on body weight was ascribed to the rapid normalization of food consumption to control levels.

Daily i.p injection of $8\ \mu g\ kg^{-1}$ of PEGylated CCK_9 for 14 consecutive days resulted in increased serum levels of both lipase and amylase. Furthermore, a significant increase in pancreatic wet weight was observed, which was mainly due to hyperplasia, as both the DNA and protein contents were significantly increased and the protein/DNA ratio was significantly decreased. These observations are in concordance with studies using the unmodified peptide, reporting that CCK stimulates pancreatic secretion in animals and man [10,35] and to be a trophic factor inducing exocrine pancreatic hypertrophy and hyperplasia [5]. Several studies have demonstrated that the effect produced in the pancreas depends on the CCK administration method. Intermittent injection of CCK did not result in an increased pancreatic wet weight, as cell proliferation was outweighed by apoptosis and disruption of CCK receptors, while continuous CCK infusion induces a transient increase in acinar cell proliferation with an ensuing persistent increase in pancreatic wet weight [29,33]. Increased down regulation of CCK_1-receptors might explain the transient increase in proliferation [28]. In our study, it seems that intermittent injection of the PEGylated conjugate of CCK induced a similar trophic effect on the pancreas to that seen with continuous infusion of the unmodified peptide. The occurrence of apoptosis could not be excluded in this study, as no apoptotic parameters were measured, but, if present, proliferation certainly dominated both apoptosis and CCK1 receptor down regulation.

Administration of large doses of CCK analogues causes pancreatitis in rodents [6,25,26]. In the present study, pathological signs of pancreatitis were observed in 3 of the 12 PEG-CCK_9-treated rats, which displayed marked leukocytic infiltration of the exocrine part of the gland and abnormally increased serum lipase and amylase activity. The other 9 rats also displayed a significant increase in serum lipase but not in amylase activity, although no pathological signs were observed.

There were no significant differences in urea, creatinine, uric acid and total protein, suggesting normal kidney functioning after long-term administration of the PEGylated conjugate.

Furthermore, liver function tests, including bilirubin, ALT, AST and ALP, indicated that long-term administration of PEG-CCK_9 did not cause any liver damage or malfunction. Significantly lower ALT levels were observed in the PEG-CCK_9-group compared to the controls. The reason for this lower ALT value remains to be clarified.

The findings that this long-acting CCK-derivative does not significantly reduce body weight nor total daily food intake and may cause pancreatitis cast doubt about the usefulness of the this conjugate as a therapeutic agent in obesity. Nevertheless, this PEGylated peptide may be a useful investigative tool for studies such as researching pancreatic function and disease or neuropeptide interactions in the regulation of the energy balance. Moreover, PEG-CCK_9 might contribute to the further investigation of CCK's role in regulation of the gut-associated lymphoid tissue (GALT) [17].

In conclusion, $8\ \mu g\ kg^{-1}$ of PEG-CCK_9 continued to have a daily 3–6 h suppressive effect on food intake when administered for 14 consecutive days, suggesting there was no development of

tolerance. PEG-CCK_9-treatment at this dose had no significant effect on body weight. Long-term administration of PEG-CCK_9 at the dose used had no pathological effects on the liver and kidney, but induced pancreatic hyperplasia and, in some cases, pancreatitis.

Acknowledgements

This work is supported by Onderzoeksraad van de K.U. Leuven, OT 04/32 and Fonds Wetenschappelijk Onderzoek, FWO G. 0519.05. We thank K. Cokelaere for the pathological analysis and F. Martens for the blood analysis.

References

[1] Abuchowski A, van Es T, Palczuk NC, Davis FF. Alteration of immunological properties of bovine serum albumin by covalent attachment of polyethylene glycol. J Biol Chem 1977;252:3578–81.
[2] Bado A, Rodriguez M, Lewin MJ, Martinez J, Dubrasquet M. Cholecystokinin suppresses food intake in cats: structure-activity characterization. Pharmacol Biochem Behav 1988;31:297–303.
[3] Baldwin BA, Cooper TR, Parrott RF. Intravenous cholecystokinin octapeptide in pigs reduces operant responding for food, water, sucrose solution or radiant heat. Physiol Behav 1983;30:399–403.
[4] Baldwin BA, Parrott RF, Ebenezer IS. Food for thought: a critique on the hypothesis that endogenous cholecystokinin acts as a physiological satiety factor. Prog Neurobiol 1998;55:477–507.
[5] Baldwin GS. The role of gastrin and cholecystokinin in normal and neoplastic gastrointestinal growth. J Gastroenterol Hepatol 1995;10:215–32.
[6] Beglinger C. Potential role of cholecystokinin in the development of acute pancreatitis. Digestion 1999;60(Suppl. 1):61–3.
[7] Crawley JN, Beinfeld MC. Rapid development of tolerance to the behavioural actions of cholecystokinin. Nature 1983;302:703–6.
[8] Deupree D, Hsiao S. Cholecystokinin octapeptide, proglumide, and conditioned taste avoidance in rats. Physiol Behav 1987;41:125–8.
[9] Deutsch JA, Hardy WT. Cholecystokinin produces bait shyness in rats. Nature 1977;266:196.
[10] Dockray GJ. The action of secretin, cholecystokinin-pancreozymin and caerulein on pancreatic secretion in the rat. J Physiol 1972;225:679–92.
[11] Ervin GN, Birkemo LS, Johnson MF, et al. The effects of anorectic and aversive agents on deprivation-induced feeding and taste aversion conditioning in rats. J Pharmacol Exp Ther 1995;273:1203–10.
[12] Ervin GN, Teeter MN. Cholecystokinin octapeptide and lithium produce different effects on feeding and taste aversion learning. Physiol Behav 1986;36:507–12.
[13] Gibbs J, Young RC, Smith GP. Cholecystokinin decreases food intake in rats. J Comp Physiol Psychol 1973;84:488–95.
[14] Goodman LS, Gilman A. The pharmacological basis of therapeutics, 4th ed., New York: The Macmillan Company; 1970.
[15] Harris JM, Chess RB. Effect of pegylation on pharmaceuticals. Nat Rev Drug Discov 2003;2:214–21.
[16] Harris JM, Martin NE, Modi M. Pegylation: a novel process for modifying pharmacokinetics. Clin Pharmacokinet 2001;40:539–51.
[17] Keith HM, Zarzaur Jr BL, Fukatsu K, Chance DR, Renegar KB, Sherrell C, et al. Individual neuropeptides regulate gut-associated lymphoid tissue integrity, intestinal immunoglobulin A levels, and respiratory antibacterial immunity. J Parenter Enteral Nutr 2000;24:261–8.
[18] Kissileff HR, Pi-Sunyer FX, Thornton J, Smith GP. C-terminal octapeptide of cholecystokinin decreases food intake in man. Am J Clin Nutr 1981;34:154–60.
[19] Labarca C, Paigen K. A simple, rapid, and sensitive DNA assay procedure. Anal Biochem 1980;102:344–52.
[20] Léon-Tamariz F, Verbaeys I, Van Boven M, et al. PEGylation of cholecystokinin prolongs its anorectic effect in rats. Peptides 2007;28:1003–11.
[21] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265–75.
[22] Lukaszewski L, Praissman M. Effect of continuous infusions of CCK-8 on food-intake and body and pancreatic weights in rats. Am J Physiol 1988;254: R17–22.
[23] Mineka S, Snowdon CT. Inconsistency and possible habituation of CCK-induced satiety. Physiol Behav 1978;21:65–72.
[24] Moore BO, Deutsch JA. An antiemetic is antidotal to the satiety effects of cholecystokinin. Nature 1985;315:321–2.
[25] Niederau C, Grendell JH. Role of cholecystokinin in the development and progression of acute pancreatitis and the potential of therapeutic application of cholecystokinin receptor antagonists. Digestion 1999;60(Suppl. 1):69–74.
[26] Niederau C, Liddle RA, Ferrell LD, Grendell JH. Beneficial effects of cholecystokinin-receptor blockade and inhibition of proteolytic enzyme activity in experimental acute hemorrhagic pancreatitis in mice. Evidence for cholecystokinin as a major factor in the development of acute pancreatitis. J Clin Invest 1986;78:1056–63.
[27] Ohlsson B, Axelson J, Sternby B, Rehfeld JF, Ihse I. Time-course of the pancreatic changes following long-term stimulation or inhibition of the CCK-A receptor. Int J Pancreatol 1995;18:59–66.
[28] Ohlsson B, Borg K, Mulder H, et al. Continuous infusion of cholecystokinin leads to down-regulation of the cholecystokinin-A receptor in the rat pancreas. Scand J Gastroenterol 2000;35:612–8.
[29] Ohlsson B, Borg K, Rehfeld JF, Axelson J, Sundler F. The method of administration of cholecystokinin determines the effects evoked in the pancreas. Pancreas 2001;23:94–101.
[30] Perez C, Sclafani A. Cholecystokinin conditions flavor preferences in rats. Am J Physiol 1991;260:R179–85.
[31] Roberts MJ, Bentley MD, Harris JM. Chemistry for peptide and protein PEGylation. Adv Drug Deliv Rev 2002;54:459–76.
[32] Savory CJ, Gentle MJ. Intravenous injections of cholecystokinin and caerulein suppress food intake in domestic fowls. Experientia 1980;36:1191–2.
[33] Trulsson LM, Svanvik J, Permert J, Gasslander T. Cholecystokinin octapeptide induces both proliferation and apoptosis in the rat pancreas. Regul Pept 2001;98:41–8.
[34] Vanderweele DA, Oetting RL, Jones RE, Deems DA. Sham feeding, flavor associations and diet self-selection as indicators of feeding satiety or aversive effects of peptide hormones. Brain Res Bull 1985;14:529–35.
[35] Vaysse N, Laval J, Duffaut M, Ribet A. Effect of secretin and graded doses of CCK-PZ on pancreatic secretion in man. Am J Dig Dis 1974;19:887–94.
[36] Verbaeys I, Léon-Tamariz F, Buyse J, et al. PEGylated cholecystokinin prolongs satiation in rats: dose dependency and receptor involvement. Br J Pharmacol 2007;152:396–403.
[37] Verbaeys I, Léon-Tamariz F, Pottel H, et al. PEGylated cholecystokinin is more potent in inducing anorexia than conditioned taste aversion in rats. Br J Pharmacol 2008;155:417–23.
[38] Veronese FM, Pasut G. PEGylation, successful approach to drug delivery. Drug Discov Today 2005;10:1451–8.
[39] West DB, Fey D, Woods SC. Cholecystokinin persistently suppresses meal size but not food intake in free-feeding rats. Am J Physiol 1984;246:776–87.
[40] West DB, Greenwood MR, Marshall KA, Woods SC. Lithium chloride, cholecystokinin and meal patterns: evidence that cholecystokinin suppresses meal size in rats without causing malaise. Appetite 1987;8:221–7.
[41] Yamamoto M, Otani M, Jia DM, et al. Differential mechanism and site of action of CCK on the pancreatic secretion and growth in rats. Am J Physiol-Gastroint Liver Physiol 2003;285:681–7.
[42] Zarbin MA, Wamsley JK, Innis RB, Kuhar MJ. Cholecystokinin receptors: presence and axonal flow in the rat vagus nerve. Life Sci 1981;29:697–705.