RESEARCH ARTICLE

Acute and chronic effects of dietary fatty acids on cholecystokinin expression, storage and secretion in enteroendocrine STC-1 cells

Katharine V. Hand[1], Christine M . Bruen[2,3], Fiona O'Halloran[2], Linda Giblin[2] and Brian D. Green[1]

[1] Institute of Agri-Food and Land Use, School of Biological Sciences, Queen's University Belfast, Belfast, UK
[2] Moorepark Food Research Centre, Teagasc, Fermoy, Co. Cork, Ireland
[3] Department of Food and Nutritional Science, University College Cork, Ireland

Received: July 17, 2009
Revised: November 1, 2009
Accepted: November 17, 2009

Cholecystokinin (CCK) is a peptide hormone secreted from the I-cells of the intestine and it has important physiological actions related to appetite regulation and satiety. In this study we used STC-1 cells to investigate the effects of common dietary-derived fatty acids (FAs) on I-cell secretory function and metabolism. We extend earlier studies by measuring the acute and chronic effects of 11 FAs on CCK secretion, cellular CCK content, CCK mRNA levels, cellular DNA synthesis, cellular viability and cytotoxicity. FAs were selected in order to assess the importance of chain length, degree of saturation, and double bond position and conformation. The results demonstrate that secretory responses elicited by dietary FAs are highly selective. For example, altering the conformation of a double bond from *cis* to *trans* (*i.e.* oleic acid *versus* elaidic acid) completely abolishes CCK secretion. Lauric acid appears to adversely affect I-cell metabolism and arachidonic acid suppresses DNA synthesis. Our studies reveal for the first time that conjugated linoleic acid isoforms are particularly potent CCK secretagogues, which also boost intracellular stores of CCK. These actions of conjugated linoleic acid may explain satiating actions observed in dietary intervention studies.

Keywords:
Cholecystokinin-8 / Endocrine / Fatty acids / Gut hormones / Secretion

1 Introduction

The actions of the gut hormone cholecystokinin (CCK) were first characterised in 1973 by Gibbs *et al.* [1] who described an important regulatory role on appetite. CCK is produced and secreted by highly specialised enteroendocrine cells (I-cells) located in the duodenal and jejunal mucosa of the gut [2] and it is also expressed in the central and enteric nervous systems [3, 4]. Basal CCK levels are typically in the low picomolar range and they increase approximately ten-fold following nutrient ingestion [5].

CCK has been associated with a number of physiological actions related to digestion, appetite and metabolism. For example, CCK infusion delays gastric emptying, [6] stimulates gallbladder contraction [7, 8] and results in the release of pancreatic enzymes [3, 9]. A commonly reported action of CCK is the ability to reduce appetite [1, 10, 11]. In rats CCK administration reduces both the size and duration of a meal while leaving water intake unchanged; however this has also been reported to increase meal frequency [1, 12–14]. Similar findings have also been reported in humans [15] with infusion of CCK-8 reportedly reducing the food intake and initiating the termination of a meal. Furthermore, pre-meal

Correspondence: Katharine V. Hand, Institute of Agri-Food and Land Use, School of Biological Sciences, Queen's University, Belfast, David Keir Building, Stranmillis Road, Belfast BT9 5AG, UK
E-mail: khand02@qub.ac.uk
Fax: +4428-9097-6513

Abbreviations: AA, arachidonic acid; **ALA,** α-linolenic acid; **CCK,** cholecystokinin; **CLA 9,11,** conjugated linoleic acid 9, 11; **CLA 10,12,** conjugated linoleic acid 10, 12; **DHA,** docosahexaenoic acid; **EA,** elaidic acid; **EPA,** eicosapentaenoic acid; **FAs,** fatty acids; **LA,** lauric acid; **LOA,** linoleic acid; **OA,** oleic acid; **SA,** stearic acid

infusion of CCK-8 resulted in decreased pre-meal hunger, reduced meal size and shortened meal duration in human volunteers [16]. There are indications that when the stomach is distended the satiety-inducing effect of infused CCK-8 is significantly greater, suggesting that there is a synergistic action [10, 11].

Other potentially beneficial metabolic actions of CCK have been reported. For example one *in vitro* study demonstrated that CCK causes insulin exocytosis [17], and in others CCK promoted the growth of pancreatic β cells [3, 9].

Relatively little is known concerning the mechanisms whereby digested nutrients trigger cellular CCK biosynthesis, storage and exocytosis. However, a few nutrients have been examined in relation to the secretion of CCK. Previous evidence does not ascribe any significant role for carbohydrates in eliciting CCK secretion [18], and glucose ingestion only has a transient effect on the release of CCK in humans [19–21]. Other studies have examined the role of protein-derived nutrients. *In vivo* [22] and *in vitro* [23, 24] studies indicate that the modest CCK secretory effects of proteins are dramatically improved when they are broken down into hydrolysates. However, complete hydrolysis of protein (*i.e.* individual amino acids) does not contribute to CCK release [7].

Lipids molecules were first noted to cause CCK release following either intraduodenal or oral administration to human male subjects [25]. It also appears that the composition of dietary fat influences the CCK secretory response. One human study demonstrated that a meal rich in unsaturated fat elicits a greater secretory response than a meal containing saturated fats [26]. Another *in vivo* study demonstrated that long-chain triglycerides cause CCK secretion, whereas medium-chain fatty acids (FAs) do not [27]. In addition, consumption of free FAs from pine nut oil elicited a 60% greater plasma CCK response compared with placebo [28]. CCK release also appeared dependent upon the load of FA infused, and not simply the concentration applied [29].

Furthermore, free FAs induce greater CCK secretion than triglycerides in humans [28, 30] and this therefore indicates that the action of dietary fat is improved if it undergoes hydrolysis and/or digestion [31]. What is not yet clear is which diet-derived FAs are most beneficial to intestinal I-cells or biosynthesis, storage and exocytosis of CCK.

Using STC-1 cells as a model we examined the individual cellular actions (acute and chronic) of a range of FAs commonly occurring in the human diet (listed in Table 1). The concentrations of FAs used were much lower than those present in food and similar to the concentrations present in the lumen of the human small intestine [32, 33]. We extend earlier work by not only comparing effects on acute CCK secretion, but also cellular CCK content, CCK mRNA, cellular DNA synthesis, cell viability and cell toxicity. Results indicate that slight changes in molecular structure dramatically affect the functionality of FAs, indicating that FA–cell interactions may be highly specific. Dietary FAs also appear to influence CCK mRNA levels, the

Table 1. Characteristics of FAs tested in the current study

Abbreviation	Name		MW	Empirical formula	Dietary sources	Omega no.	CCK secretion[a)]
LA	Dodecanoic acid	12:0	200.32	$C_{12}H_{24}O_2$	Coconut oil, palm kernel oil, milk – cow and goat	n/a	+
SA	Octadecanoic acid	18:0	284.48	$C_{18}H_{36}O_2$	Animal and vegetable fats/oils, cocoa	n/a	–
OA	*Cis*-9-octadecenoic acid	18:1	282.46	$C_{18}H_{34}O_2$	Olive oil, grape seed oil, buckthorn oil	ω9	+
EA	*Trans*-9-octadecenoic acid	18:1	282.46	$C_{18}H_{34}O_2$	Hydrogenated vegetable oils	ω9	–
LOA	*Cis, cis*-9,12-octadecandienoic acid	18:2	280.45	$C_{18}H_{32}O_2$	Vegetable oils, grass-fed cow milk, poppy seed oil	ω6	–
CLA 9,11	*Cis, trans*-9,11-octadecadienoic acid	18:2	280.45	$C_{18}H_{32}C_2$	Grass-fed ruminants, eggs	ω3	+
CLA 10,12	*Trans, cis* 10,12-octadecadienoic acid	18:2	280.45	$C_{18}H_{32}O_2$	Grass-fed ruminants, eggs	ω3	+
ALA	All *cis*-9,12,15-octadecatrienoic acid	18:3	278.43	$C_{18}H_{30}O_2$	Seed oils – rapeseed oil, soy bean, walnut, flaxseed	ω3	–
AA	All *cis*-einosa-5,8,11,14-tetraenoic acid	20:4	304.5	$C_{20}H_{32}O_2$	Dietary animal sources – meat, eggs, dairy	ω6	+
EPA	All *cis*-eicosa-5,8,11,14,17-pentaenoic acid	20:5	302.45	$C_{20}H_{30}O_2$	Fish oils, human milk	ω3	–
DHA	All *cis*-docosa-4,7,10,13,16,19-hexaenoic acid	22:6	328.49	$C_{22}H_{32}O_2$	Fish oils	ω3	+

"+" indicates stimulation of CCK secretion, "–" indicates no stimulation of CCK secretion.
a) As indicated by the present study.

amount of CCK available for secretion and the overall metabolic status of the enteroendocrine cell.

2 Materials and methods

2.1 Materials

Polyclonal primary antibody (R617–3, raised against sulphated CCK-8) was provided by the regional Regulatory Peptide Laboratory, Royal Victoria Hospital, Belfast. Monoclonal secondary antibody (AB154, anti-rabbit IgG (whole molecule) peroxidase) was obtained from Sigma-Aldrich (Poole, Dorset, UK); CCK-8s peptide was obtained from EZBiolab (Indiana, USA); plates (Nunc 96 well maxisorp plates, Nunc 12-well cell culture plates, BD Falcon 6-well cell culture plate). FAs obtained from Sigma-Aldrich included lauric acid (LA, 500 μM), stearic acid (SA, 100 μM), oleic acid (OA, 50 μM), elaidic acid (EA, 100 μM) and eicosapentaenoic acid (EPA, 100 μM). Linoleic acid (LOA, 100 μM), α-linolenic acid (ALA, 100 μM), arachidonic acid (AA, 100 μM), docosahexaenoic acid (DHA, 100 μM), conjugated linoleic acid 9,11 (CLA 9,11, 100 μM) and conjugated linoleic acid 10,12 (100 μM) were sourced from Cayman Europe (Tallinn, Estonia). FA solutions were prepared in pre-warmed buffer immediately prior to experiments and a sonicating water bath was used as necessary to aid solubility. Careful monitoring took place to ensure FAs remained in solution for the duration of experiments.

2.2 Cell culture

The STC-1 clonal cell line was received as a kind gift from Dr. B. Wice (Washington University of St. Louis) with permission from Dr. D. Hanahan (University of California, San Francisco, CA, USA). This enteroendocrine cell line originated from a double transgenic mouse tumour [34]. Cells were cultured in DMEM containing 4.5 G/L with L-glutamine, without sodium pyruvate (Gibco, Paisley, UK) and supplemented with 17.5% foetal bovine serum, 100 U/mL penicillin and 100 mg/L streptomycin and incubated in an 5% CO_2 humidified atmosphere at 37°C. Cells underwent passage upon reaching 80–90% confluence and were used between passage numbers 15–50.

2.3 Acute secretion of CCK from STC-1 cells

Approximately 2×10^6 cells were seeded into 12-well plates and incubated for 18 h at 37°C. Media was removed, the cells were washed with HEPES and then underwent 60 min pre-incubation with HEPES buffer (20 mM HEPES, 10 mM glucose, 140 nM NaCl, 4.5 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$). Buffer was aspirated off and cells were incubated for 30 min with 400 μL of vehicle or FA test solution. Following the test period 350 μL of the incubation solution was removed to a separate tube, placed on ice and centrifuged (900 *g*, 5 min) to remove any cellular debris. The supernatant was collected and stored at −80°C prior to further analysis by ELISA.

2.4 Determination of cellular CCK content and mRNA levels in STC-1 cells

Following a series of preliminary experiments a period of 72 h was selected for chronic studies. Cellular CCK peptide levels were determined in STC-1 cells incubated with FA for 72 h. For mRNA studies, cells were incubated for a period of 30 min (acute incubation) and 72 h (chronic incubation). Initially STC-1 cells (1.5×10^6) were seeded into 6-well plates and cultured overnight at 37°C in a humidified atmosphere of 5% CO_2. Media was removed and fresh media (for controls) or FA supplemented media was added. For chronic incubations, media (with or without FA) was removed and replaced with fresh media (with or without FA) every 24 h until an incubation period of 72 h was reached. Media was then removed, cells were washed and separate protocols were followed according to whether CCK content or mRNA levels were being investigated. For cellular CCK content CCK was extracted by addition of acid/ethanol (1.5% v/v HCl: 75% v/v ethanol: 23.5% v/v H_2O) and incubated overnight at 4°C. The incubation solution was removed and centrifuged (900 *g*, 5 min) to remove cellular debris. The supernatant was collected and the ethanol evaporated off using a Speedvac sample concentrator (Genevac, Ipswich, UK). Samples were reconstituted in PBST 0.1% BSA/TRIS-HCl and stored at −80°C prior to measurement. For determination of CCK-8 cellular content by ELISA thawed samples were diluted 100-fold with HEPES buffer.

For determination of CCK mRNA levels at least two wells were examined *per* treatment alongside two control wells *per* plate. Cell monolayers were initially washed with 1 mL of Hanks Balanced Salt Solution (Sigma Aldrich). Cells were then lysed using QIAzol Lysis Reagent (QIAGEN, West Sussex, UK) and RNA was isolated from cell suspensions using the QIAGEN miRNeasy Mini kit (QIAGEN) according to the manufacturer's instructions. RNA was quantified spectrophotometrically using the Nanodrop 1000 (Thermo Fisher Scientific, USA) and the integrity assessed by electrophoresis in a 1.5% glyoxyl gel with 1 × glyoxyl buffer (Ambion, Applied Biosystems, Foster City, USA). cDNA synthesis was prepared from 1 μg of RNA using the QIAGEN QuantiTect reverse transcription kit (QIAGEN).

2.5 Real-time RT-PCR

CCK mRNA was quantitated using Lightcycler and SyBr green technology (Roche Diagnostics, Mannheim, Germany) and by the principles of relative quantification. For

this, the CCK gene was quantified relative to the housekeeping gene ubiquitin-conjugating enzyme (E2D2) [35]. Primers for murine CCK were designed based on the GenBank sequence (accession NM031161) and across intron/exon boundaries (exons 2 and 3) to minimise amplification of gDNA: Forward primer 5′-ATGTCTGTGCGTGGTGAT-3′, Reverse primer 5′-AAATCCATCCAGCCCATGTA−3′. Primers for E2D2 were designed based on exon 7 of the murine mRNA sequence (GenBank accession NM019912.1): Forward primer 5′-CATAAAGAGTAGCTGACCGAACCT-3′, Reverse primer 5′-GCTGGCCTGGCTTACATTTAG-3′. PCRs were performed in a final volume of 10 μL, which included 5 μL SYBR green PCR master mix (Roche Diagnostics), 1 μL each primer (5 pmol/μL) and 1 μL of cDNA (50 ng/μL). Cycling conditions were as follows: initial denaturation at 95°C for 10 min, 40 cycles of 95°C for 10 s, 56°C for 5 s and 72°C for 15 s and finally 40°C for 30 s. Experiments were repeated in triplicate for each well.

The target (CCK) to reference (E2D2) ratio expression was calculated for each FA treatment using the $2^{-\Delta\Delta CT}$ method [36] and normalised compared with untreated (media alone) controls. Samples with a ratio of >1 indicated gene up-regulation and samples with a ratio of <1 indicated down-regulation of CCK mRNA.

2.6 Effects of FAs on DNA synthesis, cell viability and cytotoxicity

STC-1 cells (5×10^4) were seeded into 96-well plates and cultured overnight at 37°C in a humidified atmosphere of 5% CO_2. Media was removed and fresh media (for controls) or FA supplemented media was added and cells were incubated for a period of 72 h (with additional FA supplementation at 24 h intervals). Following incubation assay kits were employed to determine the effects of FAs on DNA synthesis, cell viability and cytotoxicity. For DNA synthesis cells were incubated with BrdU solution (Roche Diagnostics) for 4 h, following which they were washed and the protocol followed as stated within the kit. For cell viability 10 μL of Alamar Blue (Invitrogen, Paisley, UK) was added to each well ($n = 8$) and incubated for 3 h at 37°C. Absorbance was then measured at 570 nm. Cytotoxicity was determined by a lactate dehydrogenase kit. Briefly, media was removed and 5 μL of lysis solution added ($n = 8$) and this was incubated for 15 min (37°C). Assay buffer (100 μL) was added for a further 30 min and this was followed by stop solution (50 μL). Plates were shaken and absorbance measured at 492 nm (reference wavelength = 600 nm).

2.7 Determination of CCK concentrations using ELISA

Sulphated CCK-8 was measured in supernatant and extracts using an in-house fully optimised ELISA assay with no cross reactivity for either non-sulphated CCK or gastrin. Matrix-matched standards ranging from 15 pM to 30 nM were prepared by serial dilution using a phosphate buffered saline Tween solution containing 0.1% BSA. Primary antibody was added to the microtitre plate and incubated for 1 h at 37°C with gentle agitation. Plates were then washed four times with PBST and secondary antibody was added and incubated for 2 h at 37°C with gentle agitation. Plates were washed four times with PBST and 3,3,5,5-tetramethylbenzidine substrate was added and incubated for 10 min at 37°C, following which the reaction was stopped with the addition of 2.5 M H_2SO_4. The absorbance was then measured at 450 nm on a microplate reader (TECAN Safine, Tecan UK Ltd, Theale).

2.8 Processing and statistical analysis

CCK standard curves were analysed by non-linear regression analysis and absorbances of sample unknowns were interpolated from this curve. Data points represent means $\pm$SEM. Data for CCK secretion, cellular CCK content, DNA synthesis, cell viability and cytotoxicity were compared using the unpaired Student's *t*-test. *P*-Values less than 0.05 were deemed to be statistically significant.

3 Results

3.1 Acute effects of FAs on CCK secretion from STC-1 cells

Figure 1 shows the CCK secretory responses to FAs over a 30 min period. Similar to the work of McLaughlin *et al.* [37] LA (500 μM) increased levels of CCK in the test buffer by 2.8-fold ($p<0.001$). Other FAs (50–100 μM) significantly increased CCK secretion: 50 μM OA (6.5-fold; $p<0.001$), 100 μM CLA 9, 11 (4.1-fold; $p<0.001$), 100 μM CLA 10, 12 (4.8-fold; $p<0.001$), 100 μM AA (3.1-fold; $p<0.05$) and 100 μM DHA (5.1-fold; $p<0.001$); 100 μM LOA, 100 μM ALA, 100 μM SA, 100 μM EA and 100 μM EPA did not alter CCK secretion.

3.2 Effects of prolonged FA incubation on cellular CCK content

Figure 2 shows the total cellular CCK content of STC-1 cells following 72 h incubation with FAs. CLA 9,11 and CLA 10,12 were the only FAs tested, which increased cellular stores of CCK (2.0-fold and 1.7-fold, respectively; $p<0.05$). Incubations with LOA and ALA led to decreases in cellular content (0.6-fold and 0.6-fold, respectively; $p<0.001$). All other FAs did not significantly alter cellular CCK content.

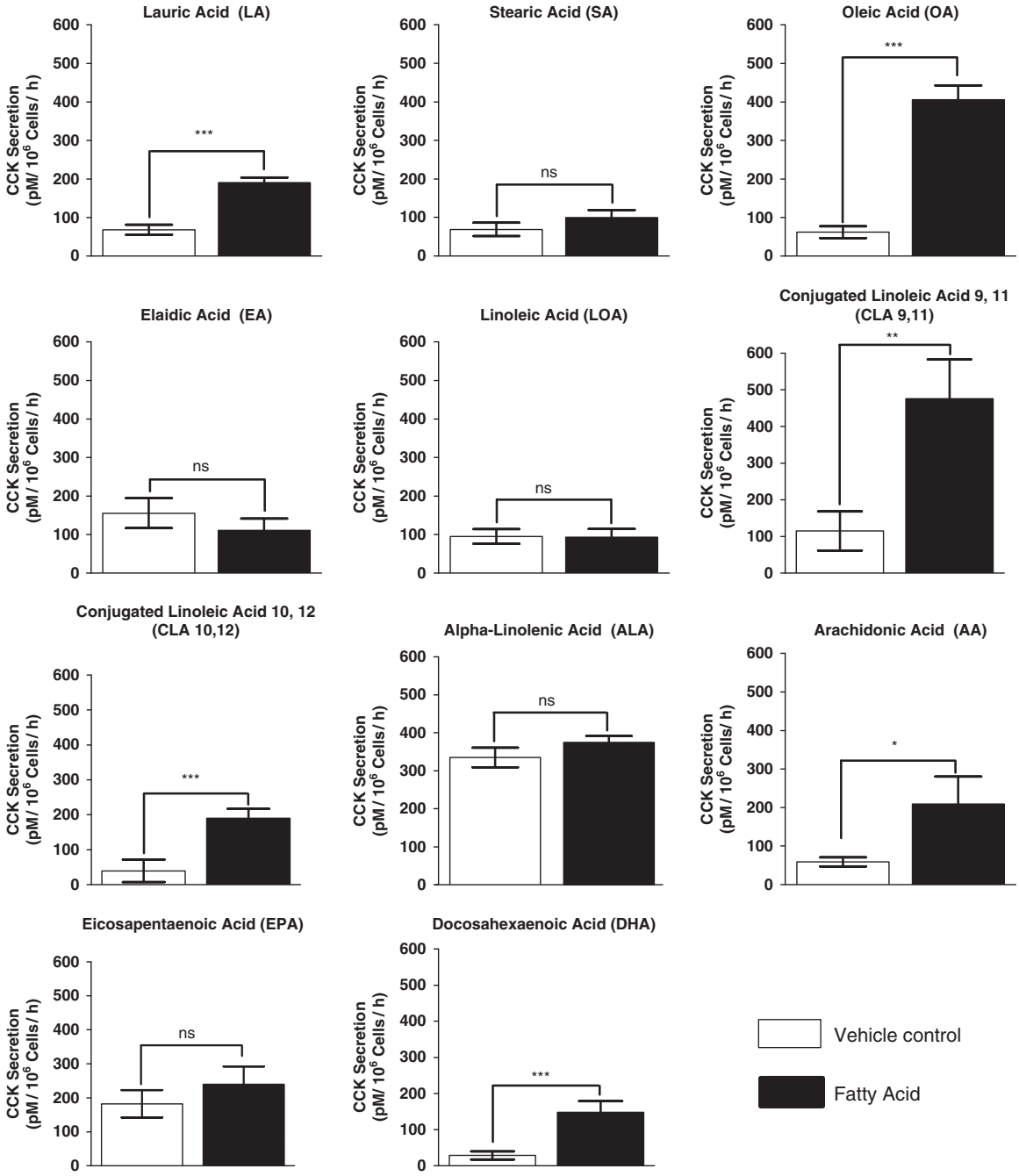

Figure 1. Effects of FAs on acute CCK secretion. Graphs show CCK release from STC-1 cells in response to 30 min incubation with FAs: LA (500 M), SA (100 μM), OA (50 μM), EA (100 μM), LOA (100 μM), ALA (100 μM), CLA 9,11 (100 μM), CLA 10, 12 (100 μM), AA (100 μM), EPA (100 μM), DHA (100 μM), or vehicle control (HEPES buffer). CCK concentrations were determined by ELISA. Each value represents mean ± SEM ($n = 16$). Groups were compared using the unpaired Student's *t*-test (*$p<0.05$; **$p<0.01$; ***$p<0.001$).

3.3 Effect of acute and prolonged FA incubations on CCK mRNA

Acute incubations with FAs (Fig. 3A) had varying effects on CCK mRNA levels when compared with the housekeeping reference gene E2D2 and to untreated controls (media alone). SA, OA, EA, AA and EPA significantly increased CCK mRNA levels with a maximum fold increase of 1.21. All other treatments had no significant effect. Prolonged incubations with FAs (Fig. 3B) also varied mRNA levels. CLA 10,12 and DHA significantly down-regulated mRNA levels, SA and AA had no net effect, while the other seven FAs significantly up-regulated CCK mRNA levels with a maximum fold increase of 1.3.

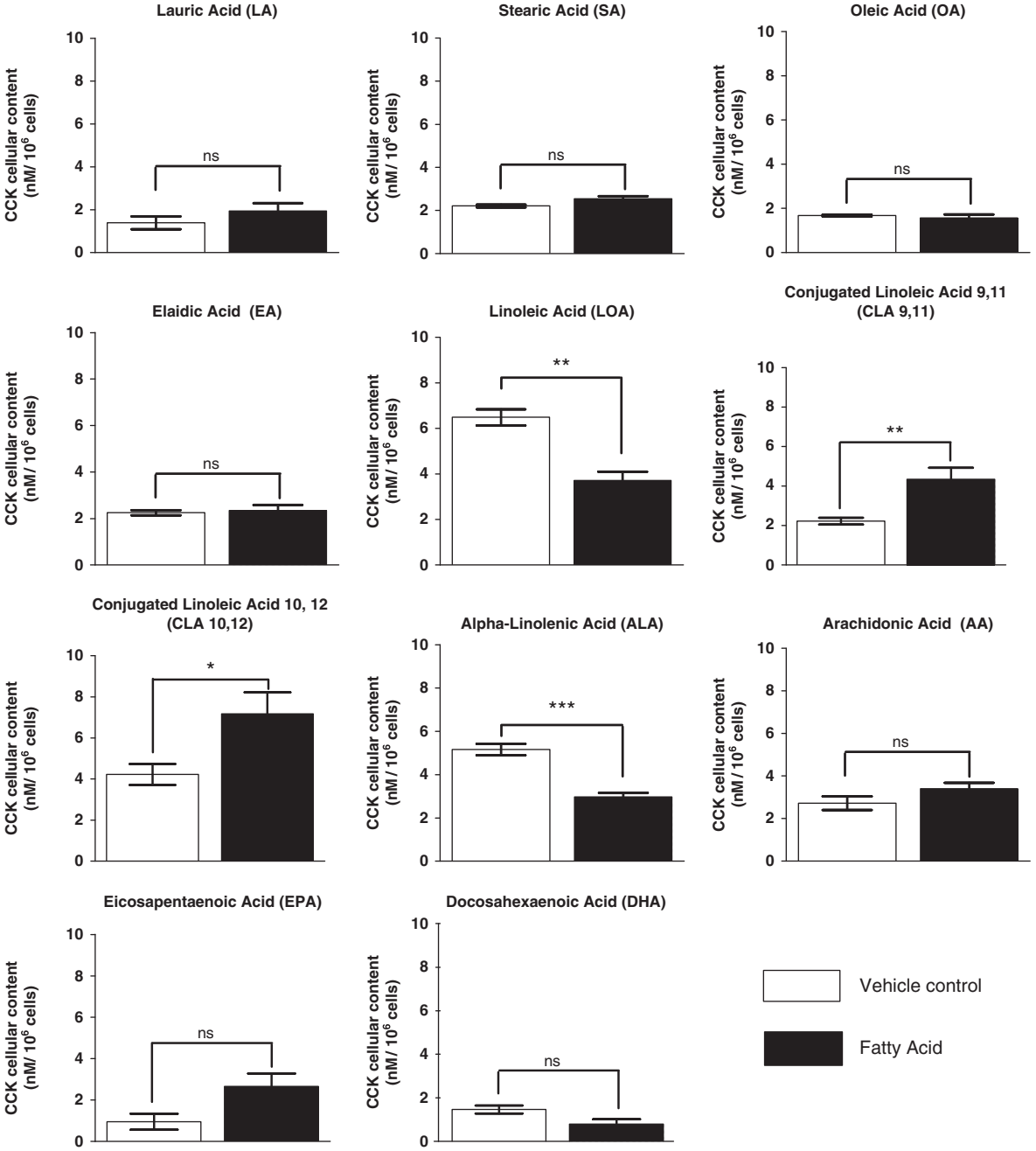

Figure 2. Effect of prolonged FA incubations on cellular CCK content. Graphs show the CCK content of STC-1 cells following 72 h incubation with FAs: LA (500 μM), SA (100 μM), OA (50 μM), EA (100 μM), LOA (100 μM), ALA (100 μM), CLA 9,11 (100 μM), CLA 10,12 (100 μM), AA (100 μM), EPA (100 μM), DHA (100 μM), or vehicle control (media). CCK was removed from cells using an acid/ethanol extraction method and CCK concentrations were determined by ELISA. Results represent mean ± SEM ($n = 16$). Groups were compared using the unpaired Student's *t*-test ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

3.4 Effect of prolonged FA incubation on DNA synthesis, cell viability and cytotoxicity

As indicated in Fig. 4A there was a tendency for dietary FAs to reduce DNA synthesis in STC-1 cells following 72 h incubations. Incubations of LOA, LA, EA, CLA 9,11, CLA 10,12 and AA significantly reduced cellular DNA synthesis. Control DNA synthesis was reduced by 70% following incubation with AA. Figures 4B and C show the status of STC-1 cells in relation to cellular viability and cytotoxicity, respectively. The viability and toxicity of STC-1 cells was generally unaffected by FA incubations. Only incubations

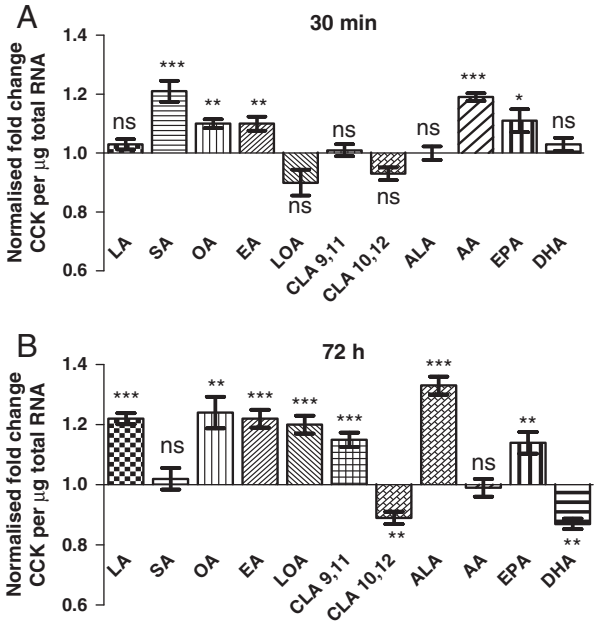

Figure 3. Effect of acute and prolonged FA incubations on CCK mRNA levels. Graphs show normalised fold change in CCK mRNA in STC-1 cells (calculated by: [(treated CCK/E2D2)/(untreated CCK/E2D2)] following incubations for (A) 30 min and (B) 72 h with FAs: LA (500 μM for (A), 100 μM FOR (B)), SA (100 μM), OA (50 μM), EA (100 μM), LOA (100 μM), ALA (100 μM), CLA 9,11 (100 μM), CLA 10,12 (100 μM), AA (100 μM), EPA (100 μM), DHA (100 μM), or vehicle control (media). Non-normalised target/ref ratios for each FA treatment were compared with non-normalised target/ref ratios for corresponding untreated controls using the unpaired Student's *t*-test (*$p<0.05$; **$p<0.01$; ***$p<0.001$; ns- not significant). Error bars represent the standard error in normalised target/ref ratios for each FA treatment.

with LA decreased cell viability (Fig. 4B; 95%; $p<0.001$) and increased cytotoxicity (Fig. 4C; 0.5%; $p<0.001$).

4 Discussion

Intestinal peptide hormones are released in response to nutrients and they trigger a number of important post-prandial physiological responses [38, 39]. One such hormone is CCK, which has important appetite-regulating actions [40]. It is important to determine which dietary components are most influential in eliciting gut hormones responses since this will provide information concerning mechanisms of action, and may uncover important health-promoting nutrients with effects beyond basic nutritional functions.

FAs are an important stimulator of postprandial CCK secretion and their chain length is a key determinant of secretory response. McLaughlin and colleagues tested saturated FAs, such as LA (12:0) and mono-unsaturated FAs such as OA (18:1) concluding that only unesterified or

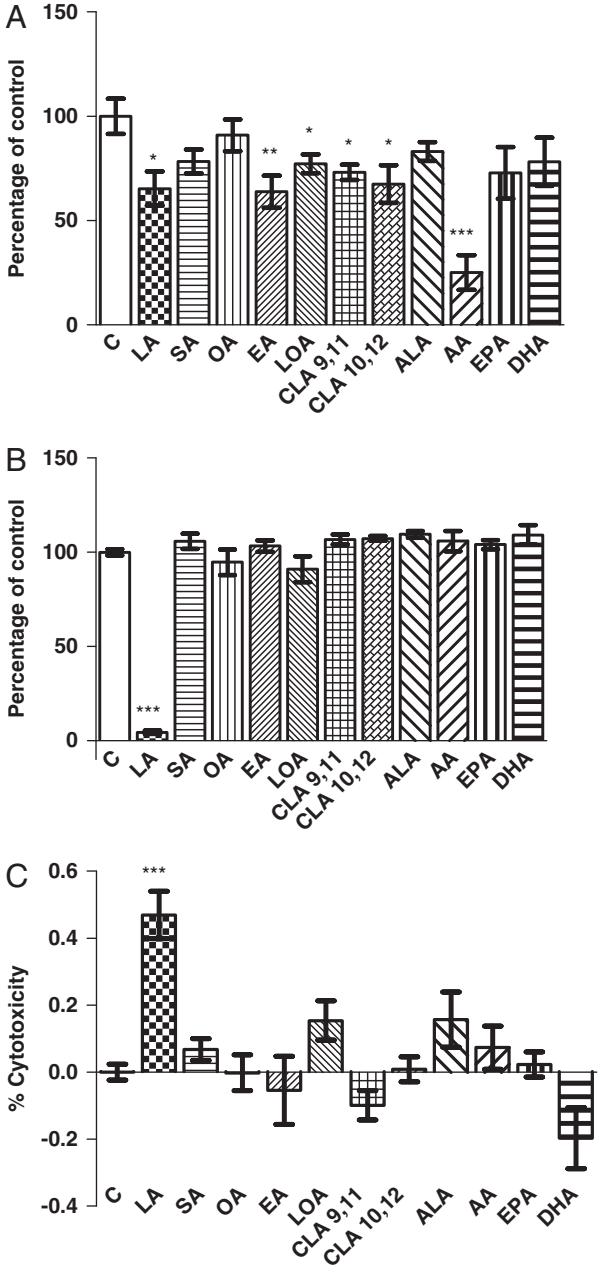

Figure 4. Effect of prolonged FA incubations on DNA synthesis, cell viability and cytotoxicity. Graphs show (A) DNA synthesis, (B) cell metabolism and (C) cytotoxicity in STC-1 cells following 72 h incubation with LA (500 μM), SA (100 μM), OA (50 μM), EA (100 μM), LOA (100 μM), ALA (100 μM), CLA 9,11 (100 μM), CLA 10,12 (100 μM), AA (100 μM), EPA (100 μM), DHA (100 μM), or vehicle control (media). Values represent mean ± SEM ($n=8$). Groups were compared using the unpaired Student's *t*-test (*$p<0.05$; **$p<0.01$; ***$p<0.001$).

unbranched FAs result in CCK release [37]. These studies also indicated that a carbon chain of 12 or more is a prerequisite for FA-induced CCK secretion to occur *in vitro*, and this appears to be supported by *in vivo* studies [41, 42]. In

this study we observed potent CCK secretory responses induced by specific FAs with chain lengths of 12, 18, 20 and 22. Our findings indicate that secretory responses are affected by other parameters particularly the position and the conformation of double bonds. Furthermore, the data significantly extend earlier observations by investigating the effects of dietary FAs on cellular CCK content, mRNA and cellular metabolism.

STC-1 cells are reasonably well established as an entero-endocrine cell model for investigating CCK secretion [43]. In this study we further characterised the cells by demonstrating that FA-induced CCK secretion ranged from 90 to 450 pM/10^6 cells, and that cellular stores of the hormone ranged from 0.8 to 7.2 nM/10^6 cells. This suggested that up to ~16% of intracellular CCK pools are exocytosed following acute exposure to free FAs.

The CCK promoter contains positive and negative regulatory elements for tissue specific, basal and regulated transcription [44] The level of CCK gene expression in intestinal I cells is most likely finely controlled to synthesize CCK peptide for immediate secretion, in response to ingested nutrients, and/or storage awaiting secretion. Observed fold changes in CCK mRNA in response to FAs ranged from 0.8- to 1.2-fold. Differences in CCK mRNA levels in response to various FAs are small (−0.87- to +1.34-fold). This degree of variation is in good agreement with previous reports where Choi *et al.* demonstrated that CCK mRNA levels in STC-1 cells increased to a maximum of 1.7-fold in the presence of 50mg/mL peptone [45]. In the human neuroepithelioma cell line, SK-N-MCIXC, CCK mRNA levels increased 1.4-fold in response to isoproterenol [46].

After 30 min SA, OA, EA, AA and EPA significantly up-regulated CCK mRNA levels compared with untreated controls albeit the differences were small with a maximum of 1.21 fold increase. After 72 h LA, OA, EA, LOA, CLA 9,11, ALA and EPA significantly up-regulated CCK mRNA levels. In contrast, CLA 10,12 and DHA significantly down-regulated CCK mRNA levels compared with untreated controls albeit the down-regulation was small at −0.87-fold.

Following transcription and translation CCK undergoes a number of posttranslational steps. On one hand the CCK peptide product becomes tyrosine sulphated, and on the other the 115 amino acid precursor is progressively cleaved to generate peptides of 58–, 33– and 8-amino acids in length [47]. In our studies mRNA levels measured the 296 bp CCK coding region and cellular stores/secretion measured sulphated-CCK-8 using a highly specific antibody. Therefore, any changes in the rate of CCK posttranslational modification are not accounted for and may explain the observed discrepancies between the measure mRNA and protein levels.

LA has been previously demonstrated to induce acute CCK release *in vitro* and *in vivo* [37, 41, 48]. This study is in agreement with these earlier reports; however, whether LA directly stimulates CCK secretion is unclear, as elevated levels may be the result of membrane damage and leakage of CCK into the extracellular buffer, as evidenced by LDH release. Over the acute period LA had negligible effects on CCK mRNA levels; however chronic incubations with LA increased them, even when LA concentrations were reduced from 500 to 100 μM. We predict this up-regulation is necessary to restock depleted cellular levels of CCK peptide in response to an LA-induced CCK secretion event. There is evidence that LA may have adverse effects on the cell since it decreased DNA synthesis, increased cytotoxicity and profoundly reduced cell viability.

We selected a number of C18 FAs in order to test important characteristics such as degree of saturation, and double bond position and conformation (Table 1). Previous work demonstrated that SA, OA and LOA (as part of an intestinally infused intralipid emulsion) significantly increased CCK release [49]. We found that the saturated FA SA had negligible effects in each of the cellular tests and minor effects on CCK mRNA over 72 h. However, a short incubation with SA does result in an up-regulation of CCK mRNA. In this study and in others the monounsaturated FA OA stimulated CCK release [50–52] and up-regulated CCK mRNA possibly to maintain cellular stores at a constant level. The effects of mono-unsaturated C18 FAs (OA and EA) were contrasting. Both FAs contain a single double bond located at position 9 but in OA the bond is in the *cis* conformation whereas in EA this is *trans*. This subtle structural difference dramatically affected CCK secretory activity with the *cis* conformation being most favourable. Surprisingly, CCK mRNA levels were up-regulated in response to EA but with no observed increase in peptide secretion or cellular pools. EA may be altering CCK peptide degradation or mRNA transcript stability. Indeed EA significantly reduced DNA synthesis whereas OA did not. While the effects of *trans* FA isomers such as EA have not been examined in these cells before, they are known to influence human health by raising LDL cholesterol and depressing HDL cholesterol [53].

Studies involving C18 polyunsaturated FAs (LOA, CLA 9,11, CLA 10,12 and ALA) clearly indicate the importance of double bond position within the chain. The functionality of FAs with "'methylene interrupted'' double bonds (*e.g.* LOA and ALA with double bonds at 9,12 and 9,12,15, respectively) was notably different from FAs with ''conjugated'' double bonds (*e.g.* CLAs with double bonds at 9,11 and 10,12). LOA and ALA did not stimulate CCK secretion but significantly reduced cellular stores of CCK. The down-regulation of CCK mRNA levels no doubt contributed to this reduction. Interestingly after chronic exposures to LOA and ALA, mRNA levels were up-regulated presumably in an effort to return CCK stores to control levels. Actions of LOA and ALA on STC-1 cells were roughly similar indicating that in this case the degree of saturation was not important. This study is the first to investigate the effects of CLAs on STC-1 cells and these initial findings are promising. The acute secretory responses of CLAs were around 400% higher than controls. Furthermore prolonged incubation periods led to a

doubling of CCK cellular stores, perhaps increasing the future capacity of STC-1 cells to release CCK. We do note divergent gene expression patterns with CLA 10,12 incubations resulting in a reduction of CCK mRNA transcript at both time frames whereas CLA 9,11 consistently up-regulated CCK mRNA levels. This is not surprising as CLA isomer-specific effects have been documented on gene expression patterns in intestinal cell lines [54].

CCK-secreting actions of CLA have not previously been described; however, they seem logical given the findings of some dietary intervention studies. In a number of animal studies CLA has been shown to decrease energy intake, increase lean body mass and reduce body fat mass [55–57]. In overweight volunteers hunger, satiety and fullness were favourably and dose-independently affected by 13-wk CLA supplementation [58]. However, dietary supplementation of CLA for regulating appetite or aiding weight maintenance remains controversial and more human studies are needed.

Polyunsaturated FAs with longer chain lengths had differing effects on STC-1 cells. After an acute incubation period beneficial effects were observed with AA and DHA causing potent CCK release and AA and EPA causing gene up-regulation. However, over a prolonged period mRNA levels and cellular CCK content remained relatively unchanged and AA reduced DNA synthesis by around 70%. This is perhaps unsurprising since AA is an important intracellular signalling molecule and causes significant reductions in DNA synthesis in other cell types [59].

In conclusion these studies significantly extend earlier observations concerning FA-induced CCK secretion. Chain length was previously deemed important to the secretory response; however, these studies now demonstrate that responses elicited by dietary FAs are highly selective in their nature. Extremely subtle structural differences between FAs (*e.g.* the conformation or the position of a double bond) are pivotal in determining the CCK secretory response. Furthermore, effects of FAs on CCK secretion appear to be separate from actions related to mRNA levels and cellular CCK content, perhaps suggesting that intracellular pathways controlling CCK synthesis and exocytosis are detached. There are indications that some free FAs, such as LA and AA, may adversely affect the metabolism of intestinal I-cells and this warrants further investigation. Of particular interest are the conjugated FAs (CLAs), which are potent secretagogues that lead to increases in the amount of CCK available for secretion. Further studies are necessary to better understand the mechanisms by which CLA FAs act on STC-1 cells.

K. V. H. and C. M. B. are currently supported by Teagasc Walsh Fellowships. We also acknowledge funding from the Irish Dept. of Agriculture, Fisheries and Food under the Food Institutional Research Measure.

The authors have declared no conflict of interest.

5 References

[1] Gibbs, J., Young, R. C., Smith, G. P., Cholecystokinin decreases food intake in rats. *J. Comp. Physiol. Psychol.* 1973, *84*, 488–495.

[2] Buchan, A. M. J., Polak, J. M., Solica, E., Capella, C. *et al.*, Pearse AGE. Electron immunohistochemical evidence for human intestinal I cell as the source of CCK. *Gut* 1978, *19*, 403–407.

[3] Reidelberger, R. D., Solomon, T. E., Comparative effects of CCK-8 on feeding, sham feeding, and exocrine pancreatic secretion in rats. *Am. J. Physiol. Reg. Integr. Comp. Physiol.* 1986, *251*, R97–R105.

[4] Bi, S., Scott, K. A., Kopin, A. S., Moran, T. H., Differential roles for cholecystokinin A receptors in energy balance in Rats and Mice. *Endocrinology.* 2004, *145*, 3873–3880.

[5] Nawrot-Porabka, K., Jaworek, J., Leja-Szpak, A., Szklarczyk, K. *et al.*, The effect of luminal ghrelin on pancreatic enzyme secretion in the rat. *Regul. Pept.* 2007, *143*, 56–63.

[6] Moran, T. H., McHugh, P. R., Cholecystokinin suppresses food intake by inhibiting gastric emptying. *Am. J. Physiol. Reg. Integr. Comp. Physiol.* 1982, *242*, R491–R497.

[7] Liddle, R. A., Goldfine, I. D., Rosen, M. S., Taplitz, R. A. *et al.*, Cholecystokinin bioactivity in human plasma. Molecular forms, responses to feeding, and relationship to gallbladder contraction. *J. Clin. Invest.* 1985, *75*, 1144–1152.

[8] Suzuki, S., Takiguchi, S., Sato, N., Kanai, S. *et al.*, Importance of CCK-A receptor for gallbladder contraction and pancreatic secretion: a study in CCK-A receptor knockout mice. *Jpn. J. Physiol.* 2001, *51*, 585–590.

[9] Reidelberger, R. D., Varga, G., Liehr, R. M., Castellanos, D. A. *et al.*, Cholecystokinin suppresses food intake by an endocrine mechanism in rats. *Am. J. Physiol. Reg. Integr. Comp. Physiol.* 1994, *267*, R901–R908.

[10] Melton, P. M., Kissileff, H. R., Pi-Sunyer, F. X., Cholecystokinin (CCK-8) affects gastric pressure and ratings of hunger and fullness in women. *Am. J. Physiol. Reg. Integr. Comp. Physiol.* 1992, *263*, R452–R456.

[11] Kissileff, H. R., Carretta, J. C., Geliebter, A., Pi-Sunyer, F. X., Cholecystokinin and stomach distension combine to reduce food intake in humans. *Am. J. Physiol. Reg. Integr. Comp. Physiol.* 2003, *285*, R992–R998.

[12] West, D. B., Fey, D., Woods, S. C., Cholecystokinin persistently suppresses meal size but not food intake in free-feeding rats. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 1984, *246*, R776–R787.

[13] Finglewicz, D. P., Stein, L. J., West, D., Porte, D. *et al.*, Intracisternal insulin alters sensitivity to CCK-induced meal suppression in baboons. *Am. J. Physiol. Reg. Integr. Comp. Physiol.* 1986, *250*, R856–R860.

[14] Moran, T. H, McHugh, P. R., Gastric and nongastric mechanisms for satiety action of cholecystokinin. *Am. J. Physiol. Reg. Integr. Comp. Physiol.* 1988, *254*, R628–R632.

[15] Kissileff, H. R., Pi-Sunyer, F. X., Thornton, J., Smith, G. P., *C*-terminal octapeptide of cholecystokinin decreases food intake in man. *Am. J. Clin. Nutr.* 1981, *34*, 154–160.

[16] Greenough, A, Cole, G., Lewis, J., Lockton, A., Untangling the effects of hunger, anxiety, and nausea on energy intake during intravenous cholecystokinin octapeptide (CCK-8) infusion. *Physiol. Behav.* 1998, *65*, 303–310.

[17] Abdel-Wahab, Y. H., O'Harte, F. P., Mooney, M. H., Conlon, J. M. *et al.*, N-terminal glycation of cholecystokinin-8 abolishes its insulinotropic action on clonal pancreatic B-cells. *Biochim. Biophys. Acta* 1999; *1452*, 60–67.

[18] Pilichiewicz, A. N., Papadopoulos, P., Brennan, I. M., Little, T. J. *et al.*, Load-dependent effects of duodenal lipids on antropyloroduodenal motility, plasma CCK and PYY, and energy intake in healthy men. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 2007, *293*, R2170–R2178.

[19] Liddle, R. A., Goldfine, I. D., Rosen, M. S., Taplitz, R. A., Williams, J. A., Cholecystokinin bioactivity in human plasma. Molecular forms, responses to feeding, and relationship to gallbladder contraction. *J. Clin. Invest.* 1985, *75*, 1144–1152.

[20] Green, G. M., Taguchi, S., Friestman, J., Chey, W. Y. *et al.*, Plasma secretin, CCK, and pancreatic secretion in response to dietary fat in the rat. *Am. J. Physiol.* 1989, *256*, G1016–G1021.

[21] Snow, N. D, Prpic, V., Mangel, A. W., Sharara, A. I. *et al.*, Regulation of cholecystokinin secretion by bombesin in STC-1 cells. *Am. J. Physiol.* 1994, *267*, G859–G865.

[22] Nishi, T., Hara, H., Hira, T., Tomita, F., Dietary protein peptic hydrolysates stimulate cholecystokinin release via direct sensing by rat intestinal mucosal cells. *Exp. Biol. Med.* 2001, *226*, 1031–1036.

[23] Cordier-Bussat, M., Bernard, C., Haouche, S., Roche, C. *et al.*, Peptones stimulate Cholecystokinin secretion and gene transcription in the intestinal cell line STC-1. *Endocrinology* 1997, *138*, 1137–1144.

[24] Beucher, S., Levenez, F., Yvon, M., Corring, T., Effects of gastric digestive products from casein on CCK release by intestinal cells in rats. *J. Nutr. Biochem.* 1994, *5*, 578–584.

[25] Wiener, I., Inoue, K., Fagan, C. J., Lilja, P. *et al.*, Release of cholecystokinin in man: correlation of blood levels with gallbladder contraction. *Ann. Surg.* 1981, *194*, 321–327.

[26] Beardshall, K., Frost, G., Morarji, Y., Domin, J. *et al.*, Saturation of fat and cholecystokinin release: Implications for pancreatic carcinogenesis. *Lancet* 1989, *2*, 1008–1010.

[27] Douglas, B. R., Jansen, J. B., de Jong, A. J., Lamers, C. B., Effect of various triglycerides on plasma cholecystokinin levels in rats. *J. Nutr.* 1990, *120*, 686–690.

[28] Pasman, W. J., Helimerikx, J., Rubingh, C. N., van den Berg, R. *et al.*, The effect of Korean pine nut oil in vitro CCK release, on appetite sensations and on gut hormones in post-menopausal overweight women. *Lipids Health Dis.* 2008, *7*, 10.

[29] Feltrin, K. L., Little, T. J., Meyer, J. H., Horowitz, M. *et al.*, Effects of Lauric acid on upper gut motility, plasma cholecystokinin and peptide YY, and energy intake are load, but not concentration, dependent in humans. *J. Physiol.* 2006, *581*, 767–777.

[30] Little, T. J., Russo, A., Meyer, J. H., Horowitz, M. *et al.*, Free fatty acids have more potent effects on gastric emptying, gut hormones, and appetite that triacylglycerides. *Gastroenterology* 2007, *133*, 1124–1131.

[31] Hayes, M. R., Miller, C. K., Ulbrecht, J. S., Mauger, J. L. *et al.*, A carbohydrate-restricted diet alters gut peptides and adiposity signals in men and women with metabolic syndrome. *J. Nutr.* 2007, *137*, 1944–1950.

[32] Murphy, E. F., Hooiveld, G. J., Müller, M., Calogero, R. A. *et al.*, The effect of trans-10, cis-12 conjugated linoleic acid on gene expression profiles related to lipid metabolism in human intestinal-like Caco-2 cells. *Gene Nutr.* 2009, *4*, 103–112.

[33] Jewell, C., Cusack, S., Cashman, K. D., The effect of conjugated linoleic acid on transepithelial calcium transport and mediators of paracellular permeability in human intestinal-like Caco-2 cells. *Prostahlandins Leukot. Essent. Fatty Acids.* 2005, *72*, 163–171.

[34] Rindi, G., Grant, S. G. N., Yiangou, Y., Ghatei, M. A., Development of neuroendocrine tumors in the gastrointestinal tract of transgenic mice. *Am. J. Pathol.* 1990, *136*, 1349–1363.

[35] Rubie, C., Kempf, K., Hans, J., Su, T. *et al.*, Housekeeping gene variability in normal and cancerous colorectal, pancreatic, oesophageal, gastric and hepatic tissues. *Mol. Cell. Probes* 2005, *19*, 101–109.

[36] Livak, K. J., Schmittgen, T. D., Analysis of Relative Gene Expression Data using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 2001, *25*, 402–408.

[37] McLaughlin, J. T., Lomax, R. B., Hall, L., Dockray, G. J. *et al.*, Fatty acids stimulate cholecystokinin secretion via an acyl chain length-specific, Ca^{2+}-dependent mechanism in the enteroendocrine cell line STC-1. *J. Physiol.* 1998, *513*, 11–18.

[38] Small, C. J., Bloom, S. R., The therapeutic potential of gut hormone peptide YY3-36 in the treatment of obesity. *Exp. Opin. Investig. Drugs.* 2005, *14*, 647–653.

[39] Green, B. D., Flatt, P. R., Incretin hormone mimetics and analogues in diabetes therapeutics. *Best Pract. Res. Clin. Endocrinol. Metab.* 2007, *21*, 497–516.

[40] D'Alessio, D., Intestinal hormones and regulation of satiety: the case for CCK, GLP-1, PYY, and Apo A-IV. *JPEN J. Parenter. Enteral. Nutr.* 2008, *32*, 567–568.

[41] McLaughlin, J. T., Luca, M. G., Jones, M. N., Thompson, D. G. *et al.*, CCK is released by C12 but not C10 saturated fatty acyl chain fatty acid in humans: evidence for a critical acyl chain length. *Gastroenterology* 1996, *110*, A1099.

[42] Feltrin, K. L., Little, T. J., Meyer, J. H., Horowitz, M., Effects of intraduodenal fatty acids on appetite, antropyloroduodenal motility, and plasma CCK and GLP-1 in humans vary with their chain length. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 2004, *287*, R524–R533.

[43] Snow, N. D., Prpic, V., Mangel, A. W., Sharara, A. I. *et al.*, Regulation of cholecystokinin secretion by bombesin in STC-1 cells. *Am. J. Physiol.* 1992, *267*, G859–G865.

[44] Hansen, T. V., Cholecystokinin gene transcription: promoter elements, transcription factors and signaling pathways. *Peptides* 2001, *22*, 1201–1211.

[45] Choi, S., Lee, M., Shiu, A. L., Yo, S. J. *et al.*, GPR93 activation by protein hydrolysate induces CCK transcription

and secretion in STC-1 cells. *Am. J. Physiol.* 2007, *292*, G1366–G1375.

[46] Mania-Farnell, B. L., Botros, I. W., Davis, T, P., Modulation of CCK mRNA in cell lines in response to isoproterenol and retinoic acid. *Neuropeptides* 1995, *29*, 221–227.

[47] Ødum, L,, Rehfeld, J. F., Expression and processing of procholecystokinin in a rat medullary thyroid carcinoma cell line. *Biochem. J.* 1990, *271*, 31–36.

[48] Benson, R. S. P., Sidhu, S. S., Jones, M. N., Case, R. M. *et al.*, Fatty acid signaling in a mouse enteroendocrine cell line involves fatty acid aggregates rather than free fatty acids. *J. Physiol.* 2002, *538*, 121–131.

[49] French, S. J., Conlon, C. A., Mutuma, S. T., Arnold, M. *et al.*, The effects of intestinal infusion of long-chain fatty acids on food intake in humans. *Gastroenterology* 2000, *119*, 943–948.

[50] Chang, C. H., Chey, W. Y., Sun, Q., Leiter, A. *et al.*, Characterization of the release of cholecystokinin from a murine neuroendocrine tumor cell line, STC-1. *Biochim. Biophys. Acta.* 1994, *1221*, 339–347.

[51] Chang, C. H., Chey, W. Y., Chang, T. M., Cellular mechanism of sodium oleate-stimulated secretion of cholecystokinin and secretin. *Am. J. Physiol. Gastrointest. Liver Physiol.* 2000, *279*, G295–G303.

[52] Miyasaka, K., Kanai, S., Masuda, M., Ibuka, T., Involvement of cholinergic processes in cholecystokinin (CCK) release by luminal oleic acid. *J. Auton. Nerv. Syst.* 1997, *63*, 179–182.

[53] Sundram, K., Ismail, A. Hayes, K. C., Jeyamalar, R. *et al.*, Trans (Elaidic) fatty acids adversely affect the Lipoprotein profile relative to specific saturated fatty acids in humans. *J, Nutr.* 1997, *127*, 514s–520s.

[54] Murphy, E. F., Hooveld, G. J., Muller, M., Calogero, R. A. *et al.*, Conjugated linoleic acid alters global gene expression in human intestinal-like Caco-2 cells in an isomer-specific manner. *J. Nutr.* 2007, *137*, 2359–2365.

[55] Park, Y., Storkson, J. M., Albright, K. J., Liu, W. *et al.*, Evidence that the trans-10, cis-12 isomer of conjugated linoleic acid induces body composition changes in mice. *Lipids* 1999, *34*, 235–241.

[56] DeLany, J. P., Blohm, F., Truett, A. A., Scimeca, J. A. *et al.*, Conjugated linoleic acid rapidly reduces body fat content in mice without affecting energy intake. *Am. J. Physiol.* 1999, *276*, R1172–R1179.

[57] West, D. B., Delany, J. P., Camet, P. M., Blohm, F., Effects of conjugated linoleic acid on body fat and energy metabolism in the mouse. *Am. J. Physiol.* 1998, *275*, R667–R672.

[58] Kamphuis, M. M., Lejeune, M. P., Saris, W. H., Westerterp-Plantenga, M. S., Effect of conjugated linoleic acid supplementation after weight loss on appetite and food intake in overweight subjects. *Eur. J. Clin. Nutr.* 2003, *57*, 1268–1274.

[59] Verlengia, R., Gorjão, R., Kanunfre, C. C., Bordin, S. *et al.*, Effect of arachidonic acid on proliferation, cytokines production and pleiotropic genes expression in Jurkat cells--a comparison with oleic acid. *Life Sci.* 2003, *73*, 2939–2951.

