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Apolipoprotein A-IV enhances cholecystokinnin secretion

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ABSTRACT

Cholecystokinin (CCK) and apolipoprotein A-IV (ApoA-IV) are gastrointestinal peptides that play an important role in controlling energy homeostasis. Lymphatic ApoA-IV and plasma CCK secretion are mediated via a chylomicron formation-dependent pathway during a dietary lipid infusion. Given their similar roles as satiating proteins, the present study examines how the two peptides interact in their function. Specifically, this study sought to understand how ApoA-IV regulates CCK secretion. For this purpose, *Cck* gene expression in the small intestines of ApoA-IV knockout (ApoA-IV-KO) and wild-type (WT) mice were compared under an array of feeding conditions. When fed with a chow or high-fat diet (HFD), basal levels of *Cck* transcripts were significantly reduced in the duodenum of ApoA-IV-KO mice compared to WT mice. Furthermore, after an oral gavage of a lipid mixture, *Cck* gene expression in the duodenum was significantly reduced in ApoA-IV-KO mice relative to the change seen in WT mice. To determine the mechanism by which ApoA-IV modulates *Cck* gene expression, STC-1 cells were transfected with predesigned mouse lysophosphatidic acid receptor 5 (LPAR5) small interfering RNA (siRNA) to knockdown *Lpar5* gene expression. In this in-vitro study, mouse recombinant ApoA-IV protein increased *Cck* gene expression in enteroendocrine STC-1 cells and stimulated CCK release from the STC-1 cells. However, the levels of CCK protein and *Cck* expression were attenuated when *Lpar5* was knocked down in the STC-1 cells. Together these observations suggest that dietary lipid-induced ApoA-IV is associated with *Cck* synthesis in the duodenum and that ApoA-IV protein directly enhances CCK release through the activation of a LPAR5-dependent pathway.

1. Introduction

Obesity is a global epidemic that increases the risk of type II diabetes mellitus, cardiovascular disease, and many cancers [2,8,15,46]. Consumption of a high-fat diet (HFD) promotes positive energy balance, which is stored as fat and leads to subsequent development of obesity and insulin resistance [19,48]. Gastrointestinal peptides such as cholecystokinin (CCK) and apolipoprotein A-IV (ApoA-IV) are important satiating proteins for controlling energy homeostasis [21,47]. Both ApoA-IV and CCK inhibit gastric emptying and food intake [11,12,18,35,45]. There are two types of CCK receptors that exist in humans and rodents: CCK A receptors (CCK-AR), which are more abundant in peripheral tissues than in the brain, and CCK B receptors (CCK-BR), which are considered the gastric receptor and are abundantly present in both peripheral tissues and the brain [40]. Peripherally administered ApoA-IV and CCK do not cross the blood-brain barrier [31,39]. CCK acts by directly binding to CCK-AR on vagal afferent neurons in the intestinal enteric plexuses to relay satiation signals to the hypothalamus via vagal nerve endings in the nucleus of the solitary tract in the hindbrain [31,32]. ApoA-IV is involved in lipid-induced activation of the vagal afferent pathway and requires vagal nerve transmission to activate neurons in the hindbrain to suppress food intake [13,28,45]. Together, ApoA-IV and CCK work synergistically to reduce food intake, specifically, ApoA-IV requires a CCK-dependent pathway to induce neuronal activation in the NTS and suppress food intake [29,50]. Thus, the interaction of ApoA-IV and CCK are important in controlling energy homeostasis through the stimulation of vagal activity.

Dietary lipids and proteins stimulate CCK release in the small intestine [24,37]. In addition, different species have different gastrointestinal physiologies in response to nutrients. In humans, dietary lipids are the major physiological stimuli of CCK secretion, whereas in rats dietary proteins are the strongest stimulant of CCK secretion [9,25]. This response occurs in a dose-dependent manner, which CCK secretion by endocrine I cells in the small intestine is elevated with higher doses of lipids [24,37]. ApoA-IV, apolipoprotein A-I (ApoA-I) and

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apolipoprotein B (ApoB) are major protein constituents of lymphatic triglycerides (TG)-rich lipoproteins and are produced by enterocytes [14,17]. The ApoA-IV protein is the only apolipoprotein that responds to varying amounts of dietary lipids, whereas lymphatic secretion of ApoA-I and ApoB proteins remains consistent [1,16,17,38,43]. During a dietary lipid infusion, lymphatic ApoA-IV and plasma CCK secretion are mediated via a chylomicron formation-dependent pathway [17,34]. These findings imply that dietary lipids are important regulators of ApoA-IV and CCK production in the small intestine and induce secretion of CCK and ApoA-IV in a co-dependent manner. However, the effect of ApoA-IV on the regulation of intestinal CCK remains unknown. Protein and protein hydrolysates (peptones) directly interact with a lysophosphatidic acid receptor 5 (LPAR5 is also known as G protein-coupled receptor 93)-dependent pathway to produce a marked elevation in CCK release [5,10,25]. It was hypothesized that ApoA-IV enhances CCK levels through the activation of a LPAR5-dependent pathway as well. The present study was designed to determine whether intestinal *Cck* gene expression in ApoA-IV-KO and WT mice would be altered after diets with different fat contents, and whether ApoA-IV-induced CCK secretion by mouse enteroendocrine STC-1 cells is mediated via a LPAR5-dependent pathway.

2. Material and methods

2.1. Animals

ApoA-IV-KO mice were kindly provided by Dr. Jan Breslow (Rockefeller University, New York, NY) and were backcrossed for > 15 generations onto a C57BL/6J genetic background. All mice were genotyped by polymerase chain reaction (PCR) analysis of tail deoxyribonucleic acid (DNA) [6,20]. ApoA-IV-KO and wild-type (WT) mice (C57BL/6 J) were housed in an AAALAC-accredited facility under conditions of controlled illumination (12:12-h light-dark cycle, lights from 0600 to 1800 h). All animal protocols were approved by the Institutional Animal Care and Use Committee in Ohio University and the University of Cincinnati.

2.2. Animal feeding and small intestine collection

Starting at 10 weeks of age, male ApoA-IV-KO and WT mice received free access to water and chow diet (6% fat, LM-485 Mouse/Rat Sterilizable Diet, Envigo, Madison, WI). To confirm that the duodenum is a major site for CCK synthesis in ApoA-IV-KO and WT mice, the small intestines of 5 h-fasted, 18 weeks old WT and ApoA-IV-KO mice maintained on a chow diet were collected. The basal *Cck* gene expression levels of these samples were then determined for experiment 1. For the study of lipid-induced CCK synthesis in ApoA-IV-KO mice in experiment 2, chow-fed WT and ApoAIV-KO mice at 15 weeks of age received a lipid mixture containing 2.4 mg triolein, 0.2 mg cholesterol (CHOL) and 0.2 mg phospholipids (200 μl) by gavage after a 5-h fast. Two h later, the small intestine was collected on dry ice for the determination of duodenal CCK levels. To examine the influence of HFD on *Cck* gene expression in the duodenum in experiment 3, WT and ApoA-IV-KO mice at 12 weeks of age were fed with either a semi-purified high-fat diet (HFD, 20% butter fat by weight) or a matched low-fat diet (LFD, 5% butter fat content, Research Diets, Inc., New Brunswick, NJ) for 8 weeks [20]. After a 5-h fast, the duodenum was collected on dry ice for the determination of *Cck* gene expression using a real-time quantitative reverse transcription (qRT-PCR) analysis.

2.3. STC-1 cells

STC-1 cells, a mouse-intestinal cell-line, were provided by Dr. Douglas Hanahan (UCSF) [3,5]. Briefly, STC-1 cells were grown in 6-well plate with Dulbecco's modified Eagles medium, 10% fetal bovine serum, penicillin (100 IU/ml) and streptomycin (100 μg/ml). To determine basal level of CCK, the STC-1 cells (80% confluency) were incubated with Hanks' balanced salt solution (HBSS) as a vehicle for 0.5, 1 and 2 h. Then the media was collected for measuring the level of CCK. To determine whether CCK release by ApoA-IV increased in a dose-dependent manner, STC-1 cells were incubated with different doses of mouse recombinant ApoA-IV proteins (50, 100, 200 and 400 μg/ml) for 0.5, 1 or 2 h. After incubation the media was collected for CCK determination. To compare CCK secretion by STC-1 cells induced by bovine serum albumin (BSA) and ApoA-IV, STC-1 cells was incubated with BSA (Sigma-Aldrich, St. Louis, MO) or mouse recombinant ApoA-IV protein at 200 μg/ml.

To determine whether the stimulation of CCK by ApoA-IV is mediated via the LPAR5 pathway, *Lpar5*-expressing STC-1 cells were transfected with predesigned mouse *Lpar5* small interfering RNA (siRNA) to knockdown *Lpar5* gene expression. In a 35-mm tissue culture plate, 2×10^5 STC-1 cells per well were seeded in 2 ml-antibiotic-free normal growth medium supplemented with fetal bovine serum 24 h before transfection. Specific siRNAs targeting mouse *Lpar5* gene (catalog number sc-75195) and control siRNAs (catalog number sc-37007) were obtained from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Inc., Dallas, TX) and utilized according to the manufacturer's instruction. Five groups for each treatment were used in this experiment, including five untransfected groups without either siRNA transfection medium or siRNA treatment as the negative controls, five groups incubated with a mixture of siRNA transfection medium plus *Lpar5* siRNA (60 pmols), and five groups incubated with mixture of siRNA transfection medium plus control siRNA (0.9 μmols). Briefly, 6 μl of control siRNAs (0.1 μmoles) were diluted in 200 μl siRNA transfection medium and 6 μl of siRNA transfection reagent (Santa Cruz) in each well of a 6-well culture plate. In parallel, 6 μl of the siRNA duplex (60 pmols siRNA) were diluted in 200 μl of the siRNA transfection Medium and 6 μl of the siRNA transfection reagent. The mixtures were incubated for 45 min at room temperature for complex formation and then added to the medium with STC-1 cells (80% confluency) without antibiotics at 37 °C. After a 7-h incubation, STC-1 cells were incubated with normal growth medium for an additional 18-h incubation. After 3 washes with ice-cold phosphate buffered saline (PBS, Gibco™, Fisher Scientific), STC-1cells were incubated with ApoA-IV (200 μg/ml) and vehicle (HBSS). After, the cells were washed with ice-cold PBS and harvested for qRT-PCR analysis, while the medium was assessed for CCK level using a CCK enzyme immunoassay kit assay.

2.4. qRT-PCR for Cck mRNA measurement

Total RNA was isolated from the duodenum, jejunum and ileum using Tri-Reagent (Ambion, Austin, TX). Total RNA was isolated, and first-strand cDNA was synthesized from 1 μg of total RNA [50]. qRT-PCR was performed using SYBR green RT-PCR master mixes at a 25-μl final reaction volume with the Bio-Rad iCycler iQ instrument (Hercules, CA) according to the manufacturer's instructions. Cyclophilin mRNA levels from each sample were used as internal controls to normalize mRNA levels. The sequences of the primers (Integrated DNA Technologies, Coralville, IA) were as follows: mouse *Cck*, 5′-CTA GCGCGATA CATCCAGCAGGTC-3′ (forward) and 5′-ACTTAATAAATAGATACTCAA ACC-3′ (reverse); mouse *Lpar5,* 5′-GCTCTGCCTGGGCGTGTGGGCTCTC ATCCTGC-3′ (forward) [5] and 5′-GCGTCGGGCCTCGCCAGTGTCCAG AAGAC-3′ (reverse); and mouse *cyclophilin*, 5′-TTCATGTGCCAGGGTG GTGACT-3′ (forward) and 5′-TCAGTCTTGGCAGTGCAGAT-3′ (reverse). Threshold cycle readings for each of the unknown samples were used, and the results were analyzed in Excel using the ΔΔCt method [27].

2.5. Measurement of the CCK release

CCK immunoreactivity was measured using an enzyme immunoassay kit (Phoenix Pharmaceuticals, Inc., Burlingame, CA) that cross-reacts at 100% with CCK-33 and CCK-8, and at 100% with

sulfated gastrin-17 [5]. 50 µl of samples or standards were added to a 96-well microtiter plate pre-coated with anti-CCK. The detection antibody was added to the captured peptide molecules according to the manufacture's specifications. After an incubation period, 100 µl of a streptavidin-horseradish peroxidase conjugate and 3,3′,5,5′-tetramethylbenzidine were added to the mixture. The absorbance was read at 450 nm using a microplate reader (Synergy HT, BioTek Instruments, Inc., Richmond, VA). According to the manufacture's specifications, the O.D. absorbance of each sample was determined at 450 nm using a microplate reader. The concentration of CCK was determined by locating the sample's optical density (O.D.) absorbance on the Y-axis, then drawing a horizontal line to intersect with the standard curve.

To verify CCK sensitivity and recovery by the enzyme immune assay kit, a series of mouse CCK-33 standard solutions (#06911, Phoenix Pharmaceuticals, Inc) were prepared according to the manufacture's specifications. Varying concentrations of CCK33 were dissolved in 500 µl Dulbecco's modified Eagles medium (GE Healthcare Life Science, #SH3002101) and 50 µl of standards and samples were added to each well of the 96-well microtiter plate. The R^2 for the CCK-33 standard curve was determined to be 0.9637 and the sample quality control was calculated to be 0.34 ng/ml (sample quality control range: 0.15–0.35 ng/ml provided by the manufacture's protocol). The CCK levels were then converted to pmol/ml CCK (i.e.CCK-33 equivalents). To determine mouse CCK-33 recovery using this assay, CCK-33 at 0.025 ng or 0.25 ng was added to 50 µl of medium. CCK recovery (%) was calculated by dividing the concentration of CCK-33 obtained from the standard curve by the known concentration of CCK-33 added (determined by using the kit). The recovery for CCK-33 at 0.5 ng was 48.0 ± 1.0% (n = 2) and the recovery for CCK-33 at 5 ng was 90.5 ± 10.5% (n = 2).

2.6. Plasma parameters

Plasma TG and cholesterol (CHOL) were determined using Randox triglyceride kits (Antrim, UK) and Infinity cholesterol kits (Thermo Electron, Noble Park, Victoria, Australia), respectively.

2.7. Data analysis and statistical analysis

All values are presented as mean ± SE. Parametric statistical analyses, one-way analysis of variance (one-way ANOVA), and two-way analysis of variance (two-way ANOVA) were performed using GraphPad™ Prism (version 6.0, San Diego, CA), followed by a post hoc Sidak correction. All differences were considered significant if the P value was < 0.05.

3. Results

3.1. Cck gene expression in ApoA-IV-KO mice

To confirm that the duodenum is a major site for CCK synthesis in ApoA-IV-KO and WT mice, *Cck* gene expression in the small intestine of 5 h-fasted WT and ApoA-IV-KO mice were determined when maintained on a chow diet in experiment 1. In the WT mice, the *Cck* mRNA level in the duodenum was 4-fold higher than that in the jejunum (P < 0.05, Fig. 1). Relative to the WT mice, ApoA-IV-KO mice had significantly lower levels of *Cck* gene expression in the duodenum, but comparable levels of *Cck* expression in the jejunum and ileum (P < 0.05, Fig. 1). These findings indicate that the duodenum is a major site for CCK synthesis at basal levels in both genotype mouse models. In addition, the absence of ApoA-IV reduces basal CCK synthesis in the duodenum when animals are maintained on a chow diet.

Dietary lipids stimulate ApoA-IV synthesis in the small intestine [43]. To investigate whether dietary lipid-induced ApoA-IV enhances CCK synthesis in the duodenum, duodenal *Cck* gene levels in WT and ApoA-IV-KO mice were determined following an acute oral gavage of

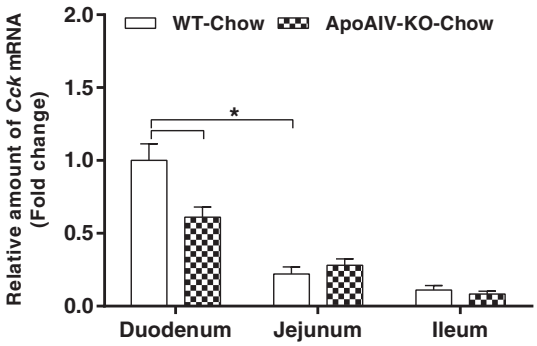

Fig. 1. Comparison of intestinal *Cck* gene expression in the duodenum, jejunum and ileum in ApoA-IV-KO and WT mice on a chow diet. Following a 5-h fast, small intestines of ApoA-IV-KO and WT were collected and *Cck* gene expression in the small intestine were determined using qPCR. Data are expressed as mean ± SEM (n = 6–7 animals per group), and *Cck* mRNA was normalized to cyclophilin housekeeping gene expression. Values with asterisks represent significant differences between different groups (P < 0.05).

lipid mixtures in experiment 2. Since TG levels are maximized at 2 h in response to a gastric gavage of dietary lipids [49], the duodenum was collected 2 h after infusion of lipid mixtures. When ApoA-IV-KO and WT mice were maintained on chow diets, no differences in body weight, fat mass, or plasma levels of TG and CHOL at 0 h were found between the two genotypes (Table 1). In the WT mice, plasma TG and CHOL were elevated 2 h after infusion of dietary lipids relative to the fasted group (P < 0.05, Table 1). Compared to the WT mice, ApoA-IV-KO mice had significantly reduced plasma TG (P < 0.05, Table 1), but comparable levels of plasma CHOL 2 h after infusion of lipid mixtures. ApoA-IV-KO mice had comparable basal levels of *Cck* gene expression in the duodenum relative to WT mice (Fig. 2). Relative to basal levels of duodenal *Cck* gene expression in the WT mice, dietary lipids induced a 17-fold increase in duodenal *Cck* gene expression in WT mice and a 8-fold increase in duodenal *Cck* gene expression in ApoA-IV-KO mice (P < 0.05, Fig. 2). These observations indicate that dietary lipids enhance CCK synthesis in the duodenum, and lipid-induced CCK synthesis is associated with ApoA-IV.

To determine the influence of a HFD on *Cck* gene expression in the duodenum, WT and ApoA-IV-KO mice were fed with either a LFD or a HFD for 8 weeks in experiment 3. HFD-fed ApoA-IV-KO mice have been reported to have comparable body weight and reduced levels of TG and CHOL in the plasma relative to WT mice [20]. In current experiment, LFD-fed ApoA-IV-KO mice had lower duodenal *Cck* gene expression than the LFD-fed WT mice, however, the difference in *Cck* levels was not statistically significant (Fig. 3). In the WT mice, a HFD increased basal levels of *Cck* gene expression relative to a LFD treatment (P < 0.05, Fig. 3). Duodenal *Cck* gene expression in the HFD-fed

Table 1
Body weight, adipose tissue weights, and plasma parameters in animals.

	WT	ApoA-IV-KO
BW (g)	31.08 ± 0.9	29.92 ± 1.7
BAT (g)	0.12 ± 0.0	0.12 ± 0.0
Epididymal Fat (g)	0.37 ± 0.1	0.63 ± 0.2
Inguinal Fat (g)	0.12 ± 0.0	0.17 ± 0.1
Triglyceride (mg/dl) 0-h	56.40 ± 4.1	33.23 ± 3.3
Triglyceride (mg/dl) 2-h	398.22 ± 61.5$^{\#}$	163.12 ± 42.6$^{*\#}$
Cholesterol (mg/dl) 0-h	68.40 ± 9.1	62.85 ± 8.7
Cholesterol (mg/dl) 2-h	155.18 ± 30.1$^{\#}$	139.6 ± 32.1

Plasma and tissues from ApoA-IV-KO and WT mice (n = 6 per group) on a chow diet were collected either 0 h or 2 h after infusion of lipid mixtures. Values represent mean ± SEM, and asterisk (*) indicates a significant difference (P < 0.05) compared to WT controls at the same treatment and the same time point. Number sign (#) indicates a significant difference (P < 0.05) compared to WT controls 0 h after infusion of dietary lipids.

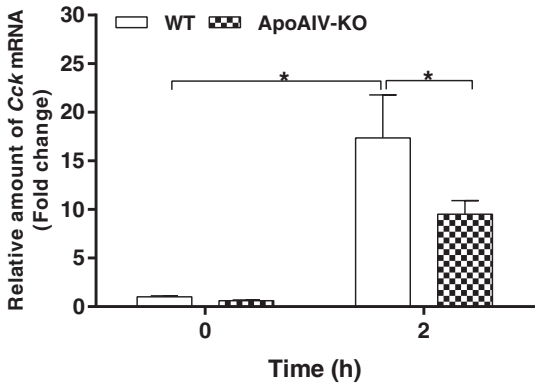

Fig. 2. The effect of lipid on duodenal *Cck* gene expression in ApoA-IV-KO and WT mice. Animals on a chow diet received a bolus of lipid mixture (200 μl) by oral gavage following a 5-h fast. The duodenum was collected prior to lipid treatment (0 h) and 2 h after infusion of lipid mixtures (2 h). Data are expressed as mean ± SEM (n = 5–6 animals per group), and *Cck* mRNA was normalized to cyclophilin housekeeping gene expression. Values with asterisks represent significant differences between different groups ($P < 0.05$).

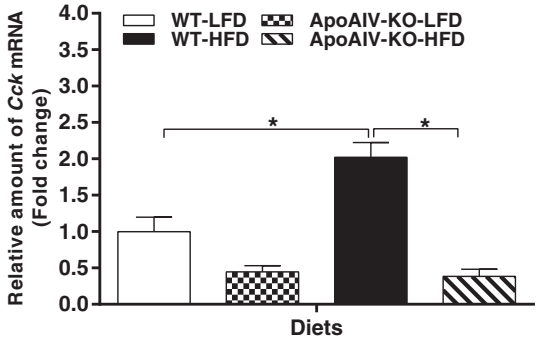

Fig. 3. The effect of chronic HFD-feeding on duodenal *Cck* gene expression in ApoA-IV-KO and WT mice. Animals at 10 weeks of age received either a LFD or a HFD for 8 weeks. Following a 5-h fast, duodenum was collected and *Cck* gene expressions were determined. *Cck* mRNA was normalized to cyclophilin housekeeping gene expression. Data are expressed as mean ± SEM, n = 6–7 animals per group and values with asterisks represent significant differences between different groups ($P < 0.05$).

ApoA-IV-KO mice was significantly lower than that in the HFD-fed WT mice ($P < 0.05$, Fig. 3). These findings suggest that ApoA-IV is partially involved in the control of duodenal *Cck* gene expression during a chronic feeding of dietary lipids.

3.2. ApoA-IV induced CCK release by STC-1 cells

To determine if ApoA-IV would dose-dependently increase the levels of CCK protein, STC-1 cells were incubated with different doses of mouse recombinant ApoA-IV (50 to 400 μg/ml) [3,5]. After a 0.5-h incubation, the CCK levels in the media of STC-1 cells were comparable between ApoA-IV- and vehicle-treated cells except for ApoA-IV treatment at 400 μg/ml (Fig. 4A). Relative to the vehicle group, ApoA-IV at or above 100 μg/ml significantly elevated CCK levels in the media of STC-1 cells after a 1-h and 2-h incubation of STC-1 cells ($P < 0.05$, Fig. 4B & C). To determine the specificity of ApoA-IV's effect on CCK secretion, STC-1 cells were incubated with BSA or ApoA-IV (both at a dose of 200 μg/ml) for 2 h. Relative to vehicle treatment (0.059 ± 0.0 pmol/ml equivalent to CCK-33), ApoA-IV significantly elevated CCK release (0.16 ± 0.03 pmol/ml) in the media of STC-1 cells ($P < 0.05$, Fig. 5). In contrast, BSA induced comparable CCK release in the medium of STC-1 cells (Fig. 5). ApoA-IV stimulated a 2-fold increase in CCK levels in the medium of STC-1 cells relative to BSA treatment at the same dose. These observations suggest that BSA is unable to stimulate CCK release by STC-1 cells.

3.3. ApoA-IV-induced CCK secretion from STC-1 cells through a LPAR5-dependent pathway

Protein hydrolysates interact with LPAR5 in the membrane of the enteroendocrine STC-1 cells to induce CCK release [5]. To determine if the LPAR5-signaling pathway also mediates the action of ApoA-IV-induced CCK release, STC-1 cells treated with *Lpar5* siRNA (knockdown) or scramble siRNA (negative control) were incubated with ApoA-IV at 200 μg/ml or vehicle. Relative to *Lpar5* gene expression in the vehicle-treated STC-1 cells without knockdown of *Lpar5*, there was an 89% reduction of *Lpar5* expression in vehicle-treated STC-1 cells with knockdown of *Lpar5* using a SiRNA treatment ($P < 0.05$, Fig. 6A). After a 2-h incubation, there was no difference in vehicle-induced *Cck* gene expression between STC-1 cells treated with or without *Lpar5* siRNA treatment (Fig. 6B), suggesting that knockdown of *Lpar5* gene did not alter basal levels of CCK synthesis in the STC-1 cells. ApoA-IV at 200 μg/ml increased *Cck* gene expression in STC-1 cells treated with scramble siRNA ($P < 0.05$, Fig. 6B). In contrast, ApoA-IV failed to elevate *Cck* expression in the STC-1 cells treated with *Lpar5* siRNA. Consistent with the induction of *Cck* gene expression by ApoA-IV in Fig. 6B, the CCK protein levels were also increased by ApoA-IV in the culture medium of STC-1 cells treated with scrambled siRNA, relative to vehicle treatment ($P < 0.05$, Fig. 6C). And assuredly, ApoA-IV-induced CCK levels in the medium of STC-1 cells transfected with *Lpar5* siRNA were attenuated. These observations suggest that ApoA-IV interacts with membrane LPAR5 to stimulate *Cck* synthesis and CCK protein secretion in STC-1 cells.

4. Discussion

CCK is a well-known satiating protein that binds with CCK-AR on intestinal vagal afferents to relay neural signals to the brain [40]. Dietary lipids and proteins are major stimulants of CCK secretion in humans and rats [24,40]. The basal concentration of CCK in human plasma is around 1 pmol/l [24,37]. After nutrients consumption, CCK levels in human plasma are increased within 20 min and then gradually reduced to reach a second peak after 1.5–2 h [24,40]. Longer fatty acids with chains greater than or equal to C12 stimulate CCK secretion much more than fatty acids with chains shorter than C12 in human and STC-1 cells [30,40]. Thus, dietary lipids play an important role in the stimulation of CCK secretion in humans and rats [24,40].

Apolipoprotein A-IV (ApoA-IV) is one of the most abundant proteins (as much as 3% of the proteins) made by the enterocytes and the hypothalamus in the presence of dietary lipids, and it functions to modulate lipid metabolism as well as glucose homeostasis in mice [21,42]. Both ApoA-IV and CCK are secreted in response to dietary lipids in human, rats and mice [9,14,21,25,26,37]. Thus, the present study investigated whether dietary lipid-induced ApoA-IV elevates CCK levels in the small intestine using ApoA-IV-KO and WT mice. ApoA-IV-KO mice fed a chow diet have normal body weight, fat mass, and *Cckar* gene expression in the duodenum [21]. In response to dietary lipids, CCK is mainly secreted by the duodenum and jejunum in humans [37]. The present experiment confirmed that duodenum was a major site for CCK synthesis in ApoA-IV-KO and WT mice. Furthermore, ApoA-IV-KO mice on a chow diet had reduced basal levels of duodenal CCK synthesis, compared with the control group [33]. Thus, mice without ApoA-IV have impaired CCK synthesis in the duodenum and normal *Cck* expression in the jejunum and ileum.

A short-term feeding of dietary lipids enhances ApoA-IV levels in the jejunum of human and rats [21,43]. In the current study, dietary lipids elevated *Cck* gene expression in the duodenum of WT mice, consistent with the observations of increased CCK protein secretion stimulated by dietary lipids in rats and human [24,34,37]. After an acute gavage of dietary lipids, the ApoA-IV-KO mice had lower levels of *Cck* mRNA level

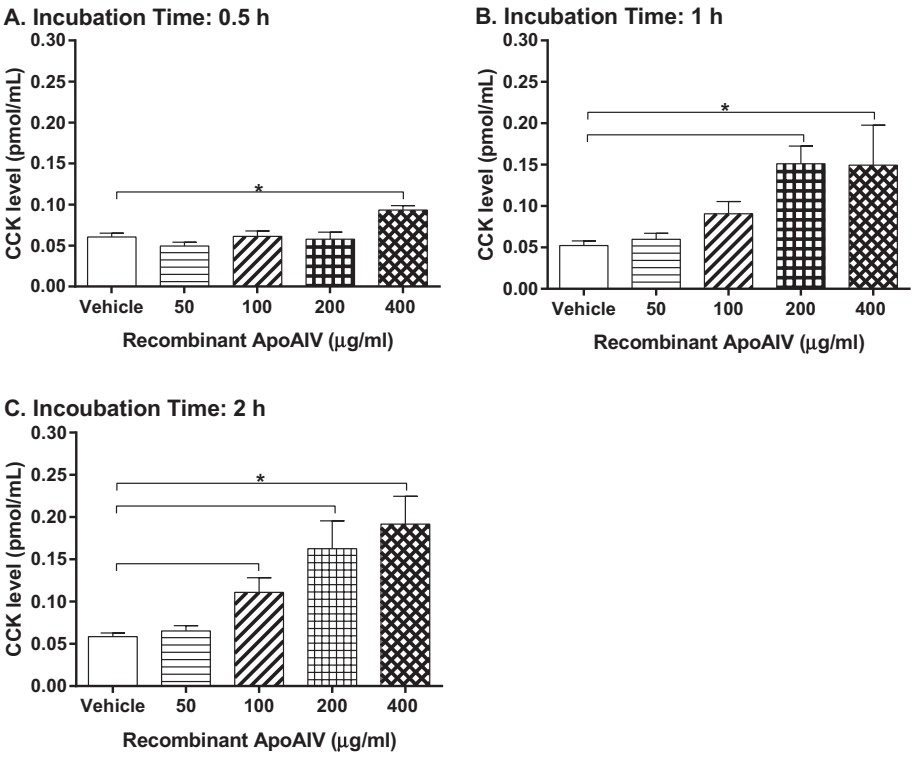

Fig. 4. ApoA-IV increased CCK levels in the media of STC-1 cells. ApoA-IV-induced CCK level after incubation of a 0.5-h (A), 1-h (B), or 2-h (C). STC-1 cells were incubated with vehicle or mouse recombinant ApoA-IV proteins, and the media were collected at different time points for the determination of CCK level (pmol/ml equivalent to CCK-33). Data are expressed as mean ± SEM for the medium of STC-1 cells from 5 to 6 wells per group. Values with asterisks represent significant differences relative to the vehicle treatment ($P < 0.05$).

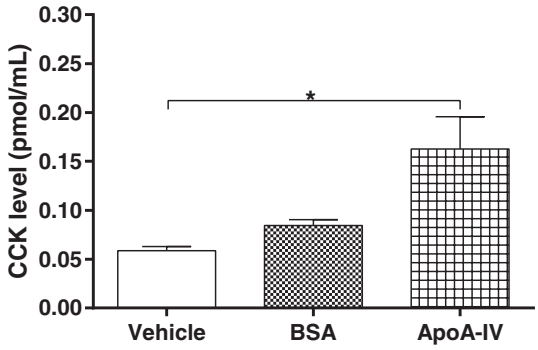

Fig. 5. Comparison of CCK levels in the media of STC-1 cells after treated with Apo-AIV and BSA. STC-1 cells were incubated with vehicle, ApoA-IV proteins or BSA at 200 μg/ml, and the media were collected after a 2-h incubation for the determination of CCK level (pmol/ml equivalent to CCK-33). Data are expressed as mean ± SEM for the medium of STC-1 cells from 5 to 6 wells per group. Values with asterisks represent significant differences relative to the vehicle treatment ($P < 0.05$).

in the duodenum compared to that of the WT mice. Thus, the elevation of intestinal CCK synthesis induced by an acute feeding of dietary lipids is associated with the presence of ApoA-IV. Chronic feeding of a HFD elevated basal levels of duodenal CCK synthesis in WT mice, consistent with the observations of increased CCK protein secretion stimulated by a HFD in rats and human [24,37]. A HFD enhances the efficiency of triglyceride digestion and luminal long-chain fatty acids trigger CCK secretion by endocrine cells in the small intestine of rats and human [24,34,37]. Thus, HFD enhances plasma CCK responses to dietary lipids. During the HFD feeding, the ApoA-IV-KO mice exhibited lower levels of *Cck* expression in the duodenum relative to WT mice *Cck* expression. Thus, these findings suggest that the elevation of duodenal *Cck* gene expression in the HFD-fed WT mice is associated with the presence of ApoA-IV. ApoA-IV and CCK are synthesized by different cells on the villi (enterocytes versus endocrine cells) and the secretion of ApoA-IV and CCK are chylomicron-dependent [17,34]. The working hypothesis for the current in-vivo study is that chylomicrons with ApoA-IV secreted by enterocytes in the villi pass through the lamina propria equipped with capillaries and nerve fibers to enter the lacteal (lymphatic vessels) located centrally in the core of the lamina propria [41]. In the lamina propria, ApoA-IV on the surface of chylomicrons may stimulate CCK release by endocrine cells via a paracrine mode before entering the lacteal [40]. However, further investigations for the site of ApoA-IV and CCK interaction through chylomicron circulation is required.

The ApoA-IV-KO mice fed a chow diet had increased body weight, comparable fat mass, and reduced levels of TG and CHOL in the fasted plasma [20]. ApoA-IV-KO mice have comparable fat absorption in the small intestine and normal transport of TG from the small intestine into the plasma [21]. ApoA-IV-KO mice produce decreased secretion of ApoA-I and ApoC-III in the lymph after an intraduodenal infusion of dietary lipids [20,44] because ApoA-IV-KO mice have a global deficiency of a gene cluster containing ApoA-IV, ApoA-I, and ApoC-III [21]. The ApoA-IV-KO mice had reduced levels of plasma lipids following an acute oral gavage of lipid mixtures, possibly due to decreased ApoC-III-induced lipolysis [20,21]. Since the role of ApoA-I and ApoC-III in the regulation of CCK levels remains unknown, further study of increased CCK release induced by ApoA-I or ApoC-III is required. Thus, these observations suggest that the downregulation of intestinal CCK synthesis in the ApoA-IV-KO mice induced by an acute feeding of dietary lipids or chronic feeding of HFD may be associated with the deficiency of ApoA-IV, ApoA-I and ApoC-III.

Since STC-1 cells do not produce detectable gastrin [30], CCK-producing STC-1 cells were used to study a direct effect of ApoA-IV on CCK released by STC-1 cells in the present experiment. Dietary proteins are the major stimulus of CCK release in rats [9,26]. The present study showed that mouse recombinant ApoA-IV proteins at or above 100 μg/ml directly induced CCK release in a dose-dependent manner after 1-h and 2-h incubations. CCK-8 release by STC-1 cells into the culture medium is induced by various secretagogues [3]. Thus, CCK-8 may be the major form of the ApoA-IV-induced CCK in the medium of STC-1 cells. In contrast, CCK-58 should be most secreted CCK by ApoA-IV in WT and ApoA-IV-KO mice because of CCK-58 is the major form in rodent plasma [36,37]. Since the ApoA-IV receptor has not yet been

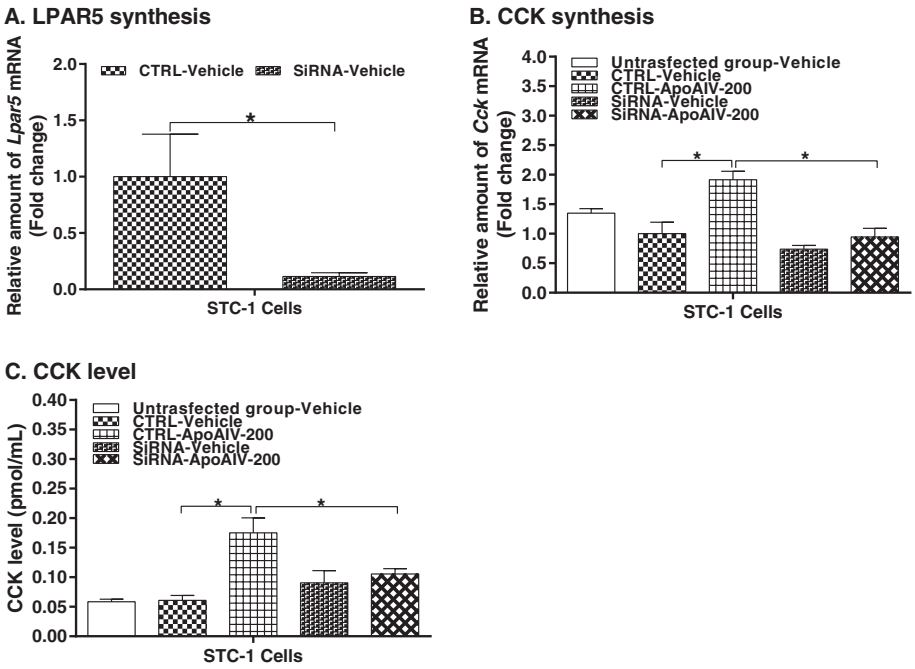

Fig. 6. ApoA-IV increased CCK level through a LPAR5-dependent pathway. *Lpar5* gene expression in STC-1 cells (A), *Cck* gene expression in STC-1 cells (B) and CCK level (pmol/ml equivalent to CCK-33) in the medium of STC-1 cells (C). STC-1 cells transfected with or without *Lpar5* siRNA were incubated with ApoA-IV or vehicle. After a 2-h incubation, the media and cells of the STC-1 cells were collected for CCK level and gene expression. Data are expressed as mean ± SEM for the cells of STC-1 cells from 7 to 8 wells per group and *Cck* mRNA was normalized to cyclophilin housekeeping gene expression. Data are expressed as mean ± SEM for the medium of STC-1 cells from 6 to 7 wells per group. Values with asterisks represent significant differences between different treatments ($P < 0.05$).

identified, the mechanism by which ApoA-IV directly stimulates CCK release by STC-1 cells remains unknown. Albumin is the major protein in the plasma and acts as a carrier protein for fatty acids [23]. BSA in the current experiment was used to mimic the action of albumin in the capillaries in the stimulation of CCK release in the lamina propria [41]. BSA at 1% has been reported to induce a moderate increase in CCK release by STC-1 cells [7]. Thus, BSA at the same dose of ApoA-IV was used to determine if ApoA-IV's effect on CCK secretion by STC-1 cells is specific. The present study demonstrated that BSA at the same dose of ApoA-IV failed to enhance CCK release by STC-1 cells, suggesting that ApoA-IV is a more potent stimulant than BSA in elevating CCK levels in the medium of STC-1 cells. LAPR5 is highly expressed in the mucosa of small intestine and is also present in STC-1 cells [4,5,22]. Protein hydrolysates (peptones) have been reported to produce a marked elevation in CCK release by STC-1 cells through the activation of LAPR5-dependent pathway [7,10]. To examine whether ApoA-IV-induced CCK release by STC-1 cells through the activation of LPAR5-dependent pathway, CCK levels in the media of STC-1 cells with knockdown of *Lpar5* and treatment of ApoA-IV were determined. In the current study, the blockade of a LPAR5-dependent pathway with siRNA significantly attenuated ApoA-IV's stimulatory effect on *Cck* gene expression and CCK secretion in STC-1 cells. Thus, the culture studies are fundamental for the connection between ApoA-IV and CCK via a LPAR5-dependnet pathway.

5. Conclusion

In light of the observations in the current study, ApoA-IV-induced CCK response to dietary lipids in mice and in STC-1 cells may provide important information about shared roles in the modulation of gastric emptying and satiation through the activation of vagal activity. Furthermore, ApoA-IV-induced CCK may be involved in lipid absorption in the lumen of the small intestine, because CCK release by enteroendocrine cells is dependent on the presence of ApoA-IV in chylomicrons during a lipid infusion. Consequently, CCK stimulates gall bladder contraction and pancreatic acini to evoke the secretions of bile acids and pancreatic lipase, which both assist in the digestion and absorption of cholesterol and fatty acid in the lumen of small intestine. Thus, ApoA-IV-induced CCK secretion plays an important role in the regulation of lipid metabolism and energy homeostasis.

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