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Lack of endogenous cholecystokinin promotes cholelithogenesis in mice

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Abstract

Background—Celiac disease is an autoimmune enteropathy caused by a permanent intolerance to dietary gluten in genetically predisposed individuals. Cholecystokinin (CCK) release from the proximal small intestine and gallbladder emptying in response to a fatty meal are greatly reduced in celiac patients before they start the gluten-free diet, showing a genetic predisposition to gallstones.

Methods—To elucidate the complex pathophysiological mechanisms determining the biliary characteristic of celiac disease, we investigated the effect of the absence of endogenous CCK on cholesterol crystallization and gallstone formation in mice fed a lithogenic diet for 28 days.

Key Results—Fasting gallbladder volumes were increased and the response of gallbladder emptying to the high-fat diet was impaired in CCK knockout mice compared to wild-type mice. Because of the absence of CCK, small intestinal transit time was prolonged and intestinal cholesterol absorption was increased. During 28 days of feeding, elevated biliary cholesterol concentrations and gallbladder stasis promoted the growth and agglomeration of solid cholesterol crystals into microlithiasis and stones. Thus, cholesterol crystallization and gallstone formation were accelerated in CCK knockout mice. In contrast, daily intraperitoneal administration of CCK-8 reduced gallstone formation in CCK knockout mice even on the lithogenic diet.

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Conclusion & Inferences—The lack of endogenous CCK enhances susceptibility to gallstones by impairing gallbladder contractile function and small intestinal motility function. These findings show that celiac disease is an important risk factor for gallstone formation and the gallbladder motility function should be routinely examined by ultrasonography and gallbladder stasis should be prevented in celiac patients.

Keywords

bile salt; celiac disease; cholesterol crystallization; cholesterol absorption; gallbladder motility; lithogenic bile

INTRODUCTION

Celiac disease is a chronic, small intestinal, immune-mediated enteropathy that is precipitated by dietary gluten in genetically predisposed individuals, and the intolerance is permanent.¹ It has been recognized that the major site of damage is in the proximal small intestine of patients with celiac disease that is characterized by villus atrophy of the small intestinal mucosa.² Although strict adherence to a gluten-free diet (GFD) leads to prompt clinical and subsequent histologic improvement, clinical and histologic relapse often occurs when gluten is reintroduced.³ Celiac disease demonstrates a wide spectrum of clinical presentations with signs and symptoms of maldigestion and malabsorption such as diarrhea, steatorrhea, weight loss, and nutritional deficiencies.^{4–7} However, recent studies have increasingly characterized celiac patients as having symptoms less typical for classic malabsorption.^{1, 8–10}

The neuroendocrine peptide hormone cholecystokinin (CCK) is synthesized and secreted by the I-cells in the mucosal epithelium of the proximal small intestine.¹¹ It is well known that CCK consists of varying numbers of amino acids depending on posttranslational modification of the CCK gene product, preprocholecystokinin.¹² As a result, CCK is actually a family of hormones identified by the number of amino acids, e.g., CCK-58, CCK-33, and CCK-8.¹² Because CCK is largely released by the I-cells in response to nutrient meals, it immediately stimulates the contraction of the gallbladder and the relaxation of the Sphincter of Oddi through its receptor, the CCK-1 receptor (CCK-1R) signaling pathway.¹³ Consequently, this promotes the delivery of gallbladder bile into the small intestine to help the digestion and absorption of dietary cholesterol, fat, and fat-soluble vitamins.¹⁴ In addition, CCK plays a critical role in regulating gastric emptying and small intestinal motility mostly through the CCK-1R signaling cascade.¹⁵ Elevated CCK may have an effect on modulating bile flow and hepatic secretion of biliary lipids.¹⁶

Many clinical studies have found that impaired postprandial gallbladder emptying in response to a fatty meal in celiac patients is attributed to a marked reduction of appropriate CCK release from the atrophic small intestinal mucosa as evidenced by low CCK concentrations in both plasma and duodenal extracts.^{12, 17–21} In addition, there may be an impairment of gallbladder responsiveness to CCK.²² As a result, the gallbladder becomes “large, lax, and lazy” in celiac patients before starting GFD,²³ and this may put them at a high risk of developing gallstones. Despite these pathophysiological alterations, it is still not

fully understood whether celiac patients have a significant predisposition to gallstone formation because there were few epidemiological investigations on gallstone prevalence rates in celiac patients and no clinical studies were reported on the pathogenesis of gallstones in celiac disease.

Although large efforts have been made in developing mouse models of celiac disease in order to exploring its pathogenesis, establishing an animal model that closely recapitulates this complex multifactorial disease has been a very challenging task. To the best of our knowledge, almost all of the animal models of celiac disease are focused on the investigation of celiac-like enteropathy,²⁴ but not its biliary feature. In the current studies, we examined the effect of endogenous CCK on cholesterol cholelithogenesis in CCK knockout (KO) mice, an excellent animal model relevant to the biliary characteristic of celiac disease. More importantly, studying the CCK KO mice on a lithogenic diet would help us to better understand the biliary and gallstone phenotypes of celiac disease. We observed that gallbladder emptying is impaired in CCK KO mice, which increases fasting and postprandial gallbladder volumes. Thus, a longer residence time of cholesterol-supersaturated bile in the gallbladder lumen is provided for rapid cholesterol crystallization and crystal growth into microlithiasis and macroscopic stones. As the deficiency of CCK also prolongs small intestinal transit times, these alterations, in turn, augment intestinal cholesterol absorption efficiency and promote the intestine-derived cholesterol for biliary hypersecretion, thereby leading to cholesterol-supersaturated bile. These findings support the novel concept that celiac disease is an important risk factor for developing gallstones and celiac patients are predisposed to gallstone formation before starting GFD.

MATERIALS AND METHODS

Animals and diets

The generation of the CCK knockout (KO) mice on the C57BL/6J genetic background has been described elsewhere.²⁵ We have established breeding colonies of CCK KO mice in-house. The inbred C57BL/6J mice of both genders from the Jackson Laboratory (Bar Harbor, ME) were bred to generate male mice as wild-type (WT) controls for the studies. All mice were housed in a temperature-controlled vivarium (22±1°C) on a 12/12 h light/dark cycle (lights on at 0600 h). Mice were fed with a normal rodent chow diet containing trace (<0.02%) amounts of cholesterol (Harlan Teklad F6 Rodent Diet 8664, Madison, WI), or a lithogenic diet containing 1% cholesterol, 15% butter fat, and 0.5% cholic acid for 28 days. All procedures were in accordance with current NIH guidelines and were approved by the Institutional Animal Care and Use Committee of Harvard University (Boston, MA).

Gallstone studies

After fasted overnight, mice (n=20 per group) were anesthetized with pentobarbital and a cholecystectomy was performed. The entire gallbladder bile was examined by phase contrast and polarizing light microscopy for the presence of mucin gel, liquid crystals, solid plate-like cholesterol monohydrate crystals, sandy stones, and gallstones according to previously published methods.²⁶ To explore whether long-term administration of CCK prevented gallstone formation, CCK KO mice (n=10 per group) were fed the lithogenic diet and

concurrently treated with 0.1 µg/kg of sulfated CCK octapeptide (CCK-8) (Tocris Bioscience, Ellisville, MO) or 100 µL of 0.9% NaCl (as a control) by intraperitoneal injection twice per day for 28 days. The images of solid cholesterol crystals and gallstones were analyzed by a Carl Zeiss Imaging System.

Measurement of biliary lipid secretion

To study biliary lipid secretion, mice (n=4 per group) were overnight fasted and anesthetized with pentobarbital. During laparotomy, a cholecystectomy was performed. After the common bile duct was successfully cannulated with a PE-10 polyethylene catheter, hepatic bile was collected by gravity during the first hour of acute fistulation for determining biliary lipid secretion rate and molecular composition of the bile salt pool according to published methods.²⁷

Dynamic measurement of gallbladder emptying function

To examine whether gallbladder contractile function was impaired in CCK KO vs. WT mice (n=4 per group), a dynamic measurement of gallbladder emptying function was performed in mice in response to a fatty meal or to exogenously administered CCK-8 after 14 days of the lithogenic diet feeding. All mice were fasted overnight but had free access to water. During laparotomy, gallbladder was clearly exposed and fasting gallbladder volume was determined according to previously published methods.²⁸ Following that, animals were intraduodenally infused with corn oil (i.e., a high-fat meal) or 0.9% NaCl (as a control) at 40 µL/minute for 5 minutes. Postprandial gallbladder volume was measured every 10 minutes for 30 minutes. The second group of mice was injected IV via the jugular vein with CCK-8 at 17 nmol/kg body weight dissolved in 100 µL of phosphate buffered saline (PBS) solution, or 100 µL of PBS solution (as a control). Gallbladder emptying function was analyzed by a difference in gallbladder volumes before and after the duodenal infusion of corn oil or the intravenous injection of CCK-8.²⁸

Biliary lipid analysis

Biliary lipid concentrations and bile salt composition, as well as cholesterol saturation index (CSI) of pooled gallbladder bile and hydrophobicity index of hepatic bile were calculated according to previously published methods.²⁹ Relative lipid composition of pooled gallbladder bile was plotted on a condensed phased diagram appropriate to mean total lipid concentration (~8 g/dL), in which the phase boundaries of the micellar zone and the crystallization pathways were extrapolated from model bile systems developed for taurocholate at 37°C.³⁰

Determination of small intestinal transit time

Because CCK also regulates small intestinal motility, we examined whether the absence of endogenous CCK influenced small intestinal transit time in CCK WT and KO mice (n=4 per group) fed the lithogenic diet for 14 days. Nonabsorbable [³H]sitostanol was used as a reference marker. Small intestinal transit time was analyzed by a geometric center method.³¹

Intestinal cholesterol absorption

The effect of lack of CCK on intestinal cholesterol absorption was investigated in chow-fed CCK WT and KO mice (n=10 per group) by a fecal dual-isotope ratio method.³² Nonfasted and nonanesthetized mice were fed by gavage an intragastric bolus of 150 μ L of medium-chain triglyceride containing 1 μ Ci of [¹⁴C]cholesterol and 2 μ Ci of [³H]sitostanol. The ratios of these two radiolabelled sterols from 4-day pooled fecal samples and in the dosing mixture were used for determining the percentage of cholesterol absorption.

Quantitative real-time PCR analysis

Total RNA was extracted from gallbladder, liver, and small intestinal tissues (n=4 per group) using RNeasy Midi (Qiagen, Valencia, CA). Total RNA (5 μ g) was reverse-transcribed to first-strand cDNA using the SuperScript II First-strand Synthesis System (Invitrogen, Carlsbad, CA). Table 1 lists the sequences of the primers and probes for ATP-binding cassette transporter A1 (*Abca1*), *Abcb4*, *Abcb11*, *Abcg5*, *Abcg8*, acyl-CoA:cholesterol acyltransferase, isoform 2 (*Acat2*), aquaporin-1 (*Aqp1*), *Aqp8*, CCK-1 receptor (*Cck-1r*), cholesterol 7 α -hydroxylase (*Cyp7a1*), 3-hydroxy-3-methylglutaryl coenzyme A reductase (*Hmgcr*), mucin gene 1 (*Muc1*), *Muc2*, *Muc3*, *Muc4*, *Muc5ac*, *Muc5b*, Niemann-Pick C1 Like 1 (*Npc1l1*), scavenger receptor class B, member 1 (*Sr-b1*), sterol 27-hydroxylase (*Cyp27a1*), and sterol-regulatory element binding protein-2 (*Srebp2*). The mRNA levels of these genes were analyzed in triplicate by quantitative real-time PCR assays.³³ Mouse β -actin mRNA levels from each sample were used as internal controls to normalize the mRNA levels.

Statistical analyses

All data are presented as means $\pm$ SD and were analyzed using Student's *t*-test, Mann-Whitney U-test, or Chi-square test, as appropriate. If the F-value was significant, comparisons among groups of mice were further analyzed by a multiple *post hoc* comparison test. Analyses were performed with a *SuperANOVA* software (Abacus Concepts, Berkeley, CA). A two-tailed probability of <0.05 was considered statistically significant.

RESULTS

Gallstone prevalence rates

Figure 1A shows representative photomicrographs of cholesterol monohydrate crystals and gallstones in CCK WT and KO mice during 28 days on the lithogenic diet. At 14 days, CCK WT mice formed a thin layer of mucin gel and solid plate-like cholesterol monohydrate crystals. In contrast, gallbladders from CCK KO mice contained a thick layer of sticky mucin gel, numerous cholesterol monohydrate crystals, and gallstones. Furthermore, at 14 days, 20% of CCK KO mice formed gallstones (Figure 1B). At 28 days, gallstone prevalence in CCK KO mice (75%) was significantly higher than that in WT mice (25%). The stone sizes (0.33 ± 0.20 mm) in CCK KO mice were significantly ($P < 0.001$) larger compared to those (0.21 ± 0.13 mm) in WT mice. The sterol extracted from randomly selected stones was pure cholesterol and constituted >99% of stone weight, as examined by HPLC analysis.

Bile flow and biliary lipid secretion rates

Figure 2 shows bile flow and biliary lipid output during the first hour of acute fistulation in CCK WT and KO mice, thus avoiding appreciable perturbation of the enterohepatic circulation of bile salts. After 28 days of the lithogenic diet feeding, bile flow rate (91 ± 56 $\mu\text{L}/\text{min}/\text{kg}$ BW) in CCK KO mice was slightly higher compared with WT mice (83 ± 23 $\mu\text{L}/\text{min}/\text{kg}$ BW). Furthermore, there was a significant increase in hepatic secretion rates of biliary cholesterol (14.5 ± 8.4 $\mu\text{mol}/\text{h}/\text{kg}$ BW) in CCK KO mice than in WT mice (9.1 ± 3.6 $\mu\text{mol}/\text{h}/\text{kg}$ BW). Biliary phospholipid secretion rates (31.8 ± 21.9 $\mu\text{mol}/\text{h}/\text{kg}$ BW) in CCK KO mice were significantly higher than those in WT mice (20.8 ± 5.8 $\mu\text{mol}/\text{h}/\text{kg}$ BW). No significant difference in biliary bile salt output was found between CCK WT (154.3 ± 31.2 $\mu\text{mol}/\text{h}/\text{kg}$ BW) and KO mice (139.6 ± 75.8 $\mu\text{mol}/\text{h}/\text{kg}$ BW).

Physical-chemical analysis of gallbladder bile

Figure 3A shows that during gallstone formation, relative lipid composition of gallbladder bile gradually moves upward and to the right of the phase diagram in both groups of mice, which is induced by an increase in biliary cholesterol and phospholipid concentrations and a reduction in bile salt concentrations. In CCK WT and KO mice, relatively biliary lipid composition at day 0 (on chow) was located within the one-phase zone, i.e., unsaturated micelles. At 14 days on the lithogenic diet, lipid composition of gallbladder bile in CCK WT mice directly entered region C from the micellar zone. After 28 days, lipid composition of gallbladder bile in CCK KO mice was located in region C of the central three-phase area denoted (Figure 3A). By phase analysis, bile was composed of liquid crystals, solid cholesterol crystals, and saturated micelles at equilibrium. Although lipid composition of gallbladder bile in CCK WT mice fed the lithogenic diet was also located in region C, they contained appreciably lower cholesterol content compared to KO mice. The CSI values of pooled gallbladder bile in CCK KO mice were markedly higher than those in WT mice (Figure 3B). The ratios of cholesterol/phospholipids and cholesterol/bile salts were markedly higher in CCK KO mice compared to those in WT mice (Table 2). These results show that the targeted disruption of the *Cck* gene induces supersaturated bile by enhancing hepatic secretion of biliary cholesterol.

Under conditions of feeding the lithogenic diet, CCK WT and KO mice displayed similar distribution of the molecular species of bile salts. Because the lithogenic diet contains 0.5% cholic acid, taurocholate became the major bile salt (53.4–55.3%) of the biliary bile salt pool, followed by taurodeoxycholate (11.8–13.1%) and tauroursodeoxycholate (9.0–17.7%). In addition, there were low concentrations of tauro- β -muricholate (3.9–10.4%), taurochenodeoxycholate (4.8–8.9%), and tauro- ω -muricholate (5.1–6.5%). Hydrophobicity indexes of individual hepatic bile were comparable between CCK WT (-0.06 ± 0.03) and KO mice (-0.07 ± 0.01).

Gallbladder volume and emptying

At 14 days on the lithogenic diet, fasting gallbladder volumes (77.7 ± 12.6 μL) in CCK KO mice were significantly ($P < 0.001$) larger compared to those in WT mice (37.3 ± 5.0 μL). As expected, 0.9% NaCl administration did not influence gallbladder contractile function in either CCK WT or KO mice. However, postprandial gallbladder responsiveness to the high-

fat meal occurred in CCK WT, but not KO mice (Figure 4), suggesting an impaired gallbladder emptying function in CCK KO mice. More interestingly, CCK-8 treatment led to a significant gallbladder emptying in both CCK WT and KO mice, and no significant difference was found in gallbladder responsiveness to CCK-8 between CCK WT and KO mice. Gallbladder emptying rates were $87.5 \pm 6.6\%$ in CCK WT mice, being significantly higher than those ($56.2 \pm 13.5\%$) in CCK KO mice. Overall, in the lithogenic state, gallbladder emptying function was dramatically impaired in CCK KO mice.

Small intestinal transit time and intestinal cholesterol absorption

Figure 5A exhibits the distributions of radioactivity along the length of the entire small intestines at 30 minutes after intraduodenal infusion of the nonabsorbable [^3H]sitostanol dissolved in medium chain triglyceride in CCK WT and KO mice fed the lithogenic diet for 14 days. The geometric center of the distribution profile of [^3H]sitostanol in the small intestine was 15.5 ± 1.0 in CCK WT mice whereas the value was 9.8 ± 0.7 in KO mice. This difference indicated that small intestinal transit time was significantly slower in CCK KO mice compared with WT mice. As shown in Figure 5B, cholesterol absorption efficiency was significantly higher in CCK KO mice than that in WT mice. These results suggest that sluggish small intestinal transit rate could be responsible for increased absorption efficiency of intestinal cholesterol in CCK KO mice because it provides a longer contacting time of cholesterol with the sterol influx transporters on the apical membrane of enterocytes for intestinal uptake and absorption.

Long-term administration of CCK-8 on the prevention of gallstones

To study whether improving gallbladder contractile function reduces the risk of developing gallstones, we examined the effect of long-term administration of CCK-8 on gallstone formation in CCK KO mice fed the lithogenic diet for 28 days. Figure 6 exhibits that the prevalence rate of gallstones was dramatically reduced from 80% in CCK KO mice administered with 0.9% NaCl to 20% in CCK KO mice treated with CCK-8.

Changes in expression of mucin genes and the genes related to lipid metabolism and transporters in the gallbladder, liver, and small intestine

The relative mRNA levels of these genes in CCK WT mice are set at 1 for all panels of Figure 7A. After 28 days on the lithogenic diet, mRNA levels of *Muc2*, *Muc5ac*, and *Muc5b* (for producing the gel-forming mucins), but not *Muc1*, *Muc3*, and *Muc4* (for producing the epithelial mucins) were significantly increased in CCK KO mice compared to those in CCK WT mice. Moreover, mRNA levels of *Acat2* (for cholesterol esterification), as well as *Abcg5* and *Abcg8*, but not *Abca1* or *Sr-b1* (the gallbladder lipid transporters) were significantly increased in CCK KO mice. By contrast, mRNA levels of *Npc1l1* and *Cck-1r* in the gallbladder were significantly reduced in CCK KO mice. Expression of *Aqp1* and *Aqp8* for regulating transcellular water absorption was comparable between two groups of mice, implying that gallbladder concentrating function may be similar in these mice.

In the liver (Figure 7B), expression of *Abcg5*, *Abcg8*, and *Abcb4*, but not *Abcb11* was significantly increased in CCK KO mice compared to those in CCK WT mice on the lithogenic diet for 28 days. It is well known that ABCG5/G8, ABCB4, and ABCB11 are

responsible for hepatic cholesterol, phospholipid, and bile salt secretion, respectively. Furthermore, expression of the *Cyp7a1* and *Cyp27a1* genes encoding two rate-limiting enzymes in the classical and the alternative pathways of bile salt synthesis was comparable between two groups of mice on the lithogenic diet. No significant differences in expression of *Acat2* (for cholesterol esterification) and *Hmgcr* and *Srebp2* (for regulating cholesterol biosynthesis) were found between two groups of mice.

In the small intestine (Figure 7C), mRNA levels of *Npc1l1* for intestinal cholesterol uptake and absorption were significantly increased in CCK KO mice. This alteration might be secondary to sluggish small intestinal transit time, leading to increased cholesterol absorption in CCK KO mice. However, mRNA levels of *Abcg5* and *Abcg8* were comparable between two groups of mice.

DISCUSSION

Whether celiac disease is a risk factor for gallstone formation was never really appreciated because there were very few studies investigating gallstone pathogenesis in patients with celiac disease in the past years. Moreover, little is known about the accurate prevalence rate of gallstones in patients with celiac disease because these patients are often recommended immediately starting GFD as soon as a positive diagnosis is made. Another possible explanation is that many celiac patients are not diagnosed because of their atypical or mild symptoms.¹ To the best of our knowledge, there was only a survey reporting that gallstone prevalence was 20% in 30 elderly celiac patients that were diagnosed by intestinal biopsy,³⁴ which is markedly higher than that in average Americans (12% of adults). Thus, more careful epidemiological investigations on gallstone prevalence rates and clinical studies on the pathogenesis of gallstones in celiac disease are desperately needed.

In the present study, we examined the gallstone phenotypes in a mouse model relevant to the biliary characteristic of celiac disease. Three important findings include: (i) the lack of CCK causes gallbladder stasis by impairing gallbladder emptying function, promoting cholesterol crystallization and gallstone formation; (ii) the absence of CCK enhances intestinal cholesterol absorption by prolonging small intestinal transit time, thus increasing hepatic availability of cholesterol for biliary hypersecretion and inducing supersaturated bile; and (iii) long-term administration of CCK-8 restores gallbladder contractile function and reduces the risk of developing gallstones in CCK KO mice.

These findings highlight the importance of early diagnosis and therapy in celiac disease to prevent the long-term impact of CCK deficiency on the biliary consequences due to the small intestinal mucosal atrophy. Clinical studies has found that in untreated celiac patients, gallbladder emptying is impaired in response to a fatty meal, which is associated with a failure of postprandial release of CCK from the small intestine into the circulation.^{12, 17–21} In the fasting state, the circulating CCK levels are also dramatically lower in untreated celiac patients than in healthy subjects.¹⁸ Immunocytochemical studies have found that the number of CCK-producing endocrine cells in jejunal biopsies is significantly higher in patients with celiac disease and in celiac patients on gluten challenge diet compared to healthy subjects.^{35–37} This suggest that a fatty meal cannot effectively stimulate release of CCK in

celiac patients, which could be attributable to severe villus atrophy, crypt hyperplasia, enterocyte disarray, epithelial cell layer, and intense inflammation of the lamina propria in celiac disease.¹ However, after eating GFD, plasma CCK concentrations are increased in celiac patients and no differences in the number of endocrine cells are observed between celiac patients on GFD and normal controls.³⁷ This implies that there is a compensated augment in the number of endocrine cells in celiac patients, which are restored to normal after GFD leads to histologic improvement.

We also found that the lack of endogenous CCK markedly increases fasting and postprandial gallbladder volumes by impairing gallbladder emptying function, leading to gallbladder stasis. This greatly facilitates the crystallization of cholesterol monohydrate crystals and their growth and agglomeration.²⁸ Because impaired gallbladder motor function is strongly linked to gallstone formation,³⁸ these abnormalities could enhance cholelithogenesis in celiac patients. Moreover, supersaturated bile increases expression of *Muc2*, *Muc5ac*, and *Muc5b* for producing the gel-forming mucins, inducing mucin gel accumulation in the gallbladder, which promotes growth and agglomeration of solid cholesterol monohydrate crystals into microlithiasis. The gallbladder epithelial cells also actively secrete and absorb biliary lipids and water to modify lipid concentrations of bile.³⁸ In the lithogenic state, gallbladder cholesterol absorption is enhanced so that the accumulation of excess cholesterol in the gallbladder wall is increased. Because gallbladder epithelial cells are not able to synthesize lipoproteins for lipid transport into the circulation, the absorbed cholesterol has to be converted to cholesteryl esters for storage in the mucosa and lamina propria. Thus, excess cholesterol in the smooth muscle stiffens the sarcolemmal membrane, disrupts CCK-1R signaling cascade, and decouples the G-protein-mediated signal transduction. These alterations further paralyze gallbladder contractile function. All of these findings indicate that impaired gallbladder emptying and increased mucin gel accumulation are two important risk factors for gallstone formation in CCK KO mice.

Miettinen and colleagues reported that hepatic secretion of biliary cholesterol, phospholipids, and bile salts, as well as bile flow are significantly increased in patients with celiac disease.³⁹ We found that hepatic secretion of biliary cholesterol and phospholipids, but not bile salts, is significantly increased in CCK KO mice. These abnormalities promote biliary lithogenicity, cholesterol crystallization, and gallstone formation in CCK KO mice fed the lithogenic diet. Consistent with these findings, hepatic expression of the sterol transporters *Abcg5/g8* is significantly higher in CCK KO mice than in WT mice. However, mRNA levels of *Hmgcr* and *Srebp2* are comparable in two groups of mice, showing that hepatic cholesterol synthesis is not a major source for biliary lithogenicity in CCK KO mice.

We also found that small intestinal transit time was significantly slower in CCK KO mice than that in WT mice. Furthermore, *Cck-1r* is expressed in the smooth muscle of small intestine and small intestinal transit time was significantly retarded in CCK-1R KO mice.³¹ It is well known that the small intestinal transit rate plays a critical role in regulating cholesterol absorption.¹⁴ More cholesterol molecules are incorporated into mixed micelles because they have a longer residence time stayed in the small intestinal lumen. This greatly increases partitioning of cholesterol monomers out of saturated micelles and renders the sterol available for intestinal uptake and absorption by the sterol influx transporter NPC1L1

on the apical membrane of enterocytes. It has been observed that sluggish small intestinal transit rate augments intestinal cholesterol absorption efficiency not only in CCK-1R KO mice,³¹ but also in humans with pharmacological intervention.⁴⁰ Thus, increased intestinal cholesterol absorption due to slower small intestinal transit time is also a critical factor for the formation of lithogenic bile and gallstones in CCK KO mice. Although small intestinal transit time is not examined, intestinal cholesterol absorption efficiency is lower in untreated celiac patients compared to healthy subjects,⁴¹ which is attributable to a mucosal damage in the proximal small intestine, a major site for cholesterol absorption.⁴² However, further clinical studies found that because biliary cholesterol secretion is almost doubled in celiac patients,³⁹ intestinal cholesterol absorption in absolute terms is high despite its low fractional absorption.⁴¹ Under these circumstances, a large amount of cholesterol that is absorbed from the small intestine enters the liver through the chylomicron pathway for hepatic secretion. This explains, in part, why hepatic cholesterol secretion is high in untreated celiac patients.

Long-term total parenteral nutrition (TPN) often causes gallbladder stasis, inducing gallstone formation in humans. A single daily intravenous injection of CCK-8 prevents TPN-induced gallstones by improving gallbladder motor function in patients.⁴³ In the current study, we observed that daily treatment of CCK-8 restores gallbladder contractility and reduces the risk of developing gallstones in CCK KO mice even on the lithogenic diet. Our findings may open up the new avenue for preventing gallstones by using oral non-peptide CCK-1R agonists, i.e., CCK-8 analogues in celiac patients.

Many clinical studies have found that after strict adherence to GFD in celiac patients, intestinal CCK release is increased and gallbladder responsiveness to CCK are restored, as well as hepatic cholesterol secretion is decreased and intestinal cholesterol absorption is restored to normal.¹⁷ Obviously, this could be attributed to a marked improvement by GFD, with well-formed villi and a return of the mucosal architecture toward normal.^{44, 45} These clinical studies show that the GFD not only leads to prompt clinical and subsequent histologic improvement, but also reduces the risk of developing gallstones in celiac patients.

Taken together, our findings have a translational value because it provides a new direction for reducing susceptibility to gallstone formation in celiac patients. Although gallstones could be prevented by strict adherence to GFD, some of the celiac patients still do not respond well to GFD.¹ Moreover, when gluten is reintroduced, clinical and histologic relapse often occurs because the intolerance is permanent.⁴⁶ Therefore, it is imperative to routinely examine whether gallbladder motor function is impaired and monitor whether biliary sludge (a precursor to gallstones) is formed in the gallbladder by ultrasonography in patients with celiac disease, regardless of whether or not they are under the GFD program. Based on our results, it is highly likely that CCK-1R agonists would prevent gallstone formation by improving gallbladder contraction function and reduce bile lithogenicity in celiac patients. In addition, CCK-1R agonists may ameliorate clinical symptoms and subsequently prompt histologic improvement of the small intestinal mucosa induced by gluten by reducing contacting time between gluten and the mucosal surface of the small intestine through accelerating small intestinal transit rate in these patients. Obviously, this

potential therapeutic effect needs to be further studied in animal models and patients with celiac disease.

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Abbreviations

ABC	ATP-binding cassette (transporter)
CCK	cholecystokinin
CCK-1R	CCK-1 receptor
CSI	cholesterol saturation index
GFD	gluten-free diet
KO	knockout
NPC1L1	Niemann-Pick C1-like protein 1
TPN	total parenteral nutrition
WT	wild-type

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Key Messages

- Patients with celiac disease display a dramatic reduction in cholecystokinin (CCK) release from the upper part of small intestine and gallbladder emptying in response to a fatty meal before they start the gluten-free diet, suggesting that celiac patients have a genetic predisposition to gallstones
- To elucidate the complex pathophysiological mechanisms determining the biliary characteristics of celiac disease, a mouse model with the lack of endogenous CCK was created
- The lack of endogenous CCK enhances cholelithogenesis by accelerating cholesterol crystallization through impairing gallbladder emptying and by providing excess amounts of intestine-derived cholesterol for biliary hypersecretion through reducing small intestinal transit time and increasing intestinal cholesterol absorption
- These findings show that celiac disease is an important risk factor for gallstone formation and it is imperative to routinely examine whether gallbladder motor function is impaired and monitor whether biliary sludge (a precursor to gallstones) is formed in the gallbladder by ultrasonography in celiac patients, regardless of whether or not they are under the gluten-free diet program

Figure 1.

(A) Representative photographs of mucin gel, liquid crystals, cholesterol monohydrate crystals, and gallstones observed in CCK WT and KO mice during lithogenic diet feeding for 28 days. All magnifications are $\times 400$, except for the left top panel, which is $\times 800$, by polarizing light microscopy. (B) It should be emphasized that the growth and agglomeration of solid cholesterol crystals, as well as the development of cholesterol gallstones are dramatically accelerated in CCK KO mice. Thus, gallstone prevalence is significantly higher in CCK KO mice compared with wild-type mice.

Figure 2.

Bile flow is comparable between CCK WT and KO mice. Hepatic output of biliary cholesterol and phospholipids, but not bile salts during the first hour of interruption of the enterohepatic circulation is significantly higher in CCK KO mice compared to that in CCK WT mice fed the lithogenic diet for 28 days.

Figure 3.

(A) The relative lipid composition of pooled gallbladder bile from CCK KO and WT mice at each time point before (day 0, on chow diet) and at 14 and 28 days on the lithogenic diet are plotted on a condensed phase diagram. The one-phase micellar zone at bottom is enclosed by a solid curved line. Above the micellar zone, two solid lines divide the two-phase zones from a central three-phase zone. Based upon the solid and liquid crystallization sequences present in bile, the left two phase and central three-phase regions are divided by dashed lines into region A to E. With passage of time, relative lipid composition of gallbladder bile shift upward and to the right in both CCK KO and WT mice. Only relative lipid composition of bile in CCK KO mice pass through region B and enter region C. In contrast, relative lipid composition of bile in CCK WT mice enter region C directly from the micellar zone. Black symbols represent CCK KO mice and white symbols for CCK WT mice. (B) The CSI values of pooled gallbladder bile reach supersaturation at 14 days on the lithogenic diet in both CCK WT and KO mice. Notably, the CSI values are markedly higher in CCK KO mice than in WT mice.

Figure 4.

Gallbladder emptying in response to duodenal infusion of corn oil or exogenously administered IV CCK-8 (as indicated by the arrows) is significant in CCK WT mice. BY contrast, gallbladder contractile function in response to duodenal infusion of corn oil is impaired in CCK KO mice fed the lithogenic diet for 14 days. Although exogenously administered CCK-8 stimulates gallbladder emptying in both CCK WT and KO mice, the percentage of gallbladder emptying in CCK WT mice is significantly greater than that in CCK KO mice.

Figure 5.

(A) Small intestinal transit times in CCK WT and KO mice fed the lithogenic diet for 14 days as determined by the distribution of radioactivity along the small intestine at 30 minutes after intraduodenal infusion of [^3H]sitostanol dissolved in medium-chain triglyceride. Each bar is the mean percentage of radioactivity in each segment. Segments 1 and 20 represent evenly divided segments from the most proximal to the distal part of the small intestine. Arrows indicate the geometric center that is significantly shorter in CCK KO mice, showing significantly slower small intestinal transit times compared with CCK WT mice. (B) CCK KO mice display significantly higher intestinal cholesterol absorption efficiency compared with CCK WT mice, as measured by the fecal dual-isotope ratio method.

Figure 6.

In the lithogenic state, gallstone prevalence is significantly reduced in CCK KO mice treated with CCK-8 twice per day for 28 days compared to that in CCK KO mice administered with 0.9% NaCl as a control.

Figure 7.

Effect of CCK on mRNA levels of gallbladder mucin genes, as well as the genes involved in lipid metabolism and transporters in (A) the gallbladder, (B) liver, and (C) small intestine in CCK WT and KO mice fed the lithogenic diet for 28 days. See text for further details and for abbreviations.

Table 1

Primer and probe sequences in mRNA quantification by real-time PCR

Gene	Accession Number	Forward	Reverse	Probe
<i>Muc1</i>	NM_013605	5'-GGTTGCTTTGGCTATCGTCTATT-3'	5'-AAAAGATGTCAGCTGCCCAT-3'	5'-AGTGTGCCAGTGCCGCCGAAAAG-3'
<i>Muc2</i>	NM_023566	5'-GTCTGCCACCTCATCATGGA-3'	5'-CAGGCAAGCTTCATAGTAGTGT-3'	5'-CCTATTCTCCAGTGCCAGGCCTTC-3'
<i>Muc3</i>	AF027131	5'-GTGGGACGGGCTCAAAATG-3'	5'-CTCTACGCTCTCCACCAGTTCCT-3'	5'-AGTGCACACAGCCTCTTCTATGGGCC-3'
<i>Muc4</i>	NM_080457	5'-TCCTTCTCTCAACTGTTGAATCAGA-3'	5'-CGTGGCCAGGATGTCAAAC-3'	5'-TGAAGACTTGGCCTCTGGGTGCAAG-3'
<i>Muc5ac</i>	L42292	5'-GTCTGGCAGAAACAGTGGAGATT-3'	5'-TCGTGGCTTCTCAGAGAACTTG-3'	5'-TGACACCTTTGTGAACCTGAGATCCAAAGG-3'
<i>Muc5b</i>	NM_028801	5'-CAGATCCATCCATCCCATTTCT-3'	5'-TATCTGACTACCACTTGTGTGATGACT-3'	5'-ATTTCCAAAAGCAACTCTATGTTGCTAGTAGGCC-3'
<i>Cck-1r</i>	NM_009827	5'-CAACCTGCTCAAGGATTTTCATCT-3'	5'-CACGGAAGTGCCCATGAAAT-3'	5'-CGGAAGTGCCGTGTGCAAGACTACC-3'
<i>Acat2</i>	AF078751	5'-TTTGCTCTATGCCTGCTTCATC-3'	5'-GGTTCCGGCTCATGTTG-3'	5'-TGGGCCGCTCTGTGTCCT-3'
<i>Abcg5</i>	AF312713	5'-CCTGCGACAGCGACGTTTTC-3'	5'-GCA TCGCTGTGTATCGCAAC-3'	5'-AGCAGCCTCACTGTGCGCGAGA-3'
<i>Abcg8</i>	AF324495	5'-TGGATAGTGCTGCATGGATC-3'	5'-AATTGAATCTGCATCAGCCCC-3'	5'-CAAGCTGTCTGTTCC TCCGTTGGTG-3'
<i>Abca1</i>	AK019548	5'-TTGAAATCTCTCGGATACAAATCGA-3'	5'-AAGCCCCATTGTTGCCCAT-3'	5'-CGTCTGACCTTTGGGATCCTCACC-3'
<i>Sr-b1</i>	U37799	5'-TTCACGGGCGTCCAGAA-3'	5'-GATCTTTGCTGAGTCCGTTTCCA-3'	5'-TTCAGCAGGATCCATCTGTGGACAA-3'
<i>Npc1l1</i>	AY437866	5'-CCACAGACCCCTGTGAACTGT-3'	5'-GCTCGTCAATGGAAGCCTTT-3'	5'-CCCCTAAAAGCCAAAGCCGGAAG-3'
<i>Aqp1</i>	NM_007472	5'-TTCCCTTTGGTCTGACTTACC-3'	5'-TCAGCACAGGGACAAATCCA-3'	5'-AGGACCTTCCCTTGAACTCACTAAGACC-3'
<i>Aqp8</i>	NM_007474	5'-CAGTCTGTGACCTAGAGATAAGTGAGTACA-3'	5'-GATCCGAGCCAGAGCTACCA-3'	5'-AGGCGAGCCGCGCAACGTC-3'
<i>Cyp7a1</i>	NM_007824	5'-TCGTGATCCTCTGGGCATCT-3'	5'-CCAAGTGCAATTAACCTGTGGGTAAA-3'	5'-AGGAGGCTCTGCGGCTCTCC-3'
<i>Cyp27a1</i>	NM_024264	5'-TGACATGGGCCCTGTACCA-3'	5'-TCCCAGGGTTATCAGCCTCTT-3'	5'-CAGAGATCCAGGAGGCTTGGAC-3'
<i>Hmgcr</i>	M62766	5'-ATTCTGGCAGTCACTGGGAAT-3'	5'-CCTCTGCTTCGATCCAAATT-3'	5'-CACCGACAAGAGCCCTGCTGCCA-3'
<i>Srebp2</i>	AF374267	5'-TGAAGCTGGCCAAATCAGAAAAA-3'	5'-CCACATCACTGTCCACCAGACT-3'	5'-CAAGCTCTGAAGGGCATCGACCTG-3'
<i>β-Actin</i>	NM_007393	5'-GCTCTGGCTCCTAGCACCAT-3'	5'-GCCACCATCCACACAGAGT-3'	5'-AAGATCATTTGCTCTCTGTAGCGCAA-3'

See text for abbreviations.

Table 2

Biliary lipid composition of gallbladder bile during gallstone formation

Day	Ch (Mole%)	PL (Mole%)	BS (Mole%)	Ch/PL Ratio	Ch/BS Ratio	[TL] (g/dL)	CSI
CCK WT							
0	2.59	11.34	86.07	0.228	0.030	7.193	0.61
14	6.13	16.81	77.06	0.365	0.080	8.465	1.06
28	6.83	16.42	76.75	0.416	0.089	9.504	1.17
CCK KO							
0	3.70	12.18	84.12	0.304	0.044	7.215	0.83
14	6.79	14.96	78.25	0.454	0.087	9.111	1.25
28	7.40	15.85	76.75	0.467	0.096	9.082	1.30

Values were determined from pooled gallbladder bile (n=10–20 per group).

Abbreviation: BS, bile salts; CCK, cholecystokinin; Ch, cholesterol; CSI, cholesterol saturation index; PL, phospholipids; [TL], total lipid concentration.