Published in final edited form as:

Clin Nutr. 2016 December ; 35(6): 1374–1379. doi:10.1016/j.clnu.2016.03.003.

Beneficial effects of β -sitosterol on type 1 cholecystokinin receptor dysfunction induced by elevated membrane cholesterol

Aditya J. Desai, Maoqing Dong, and Laurence J. Miller

Department of Molecular Pharmacology and Experimental Therapeutics, Mayo Clinic, Scottsdale, AZ 85259

Abstract

Background & Aims—The type 1 cholecystokinin receptor (CCK1R) mediates the actions of CCK to support nutritional homeostasis, including post-cibal satiety. However, elevated levels of membrane cholesterol, such as have been observed in metabolic syndrome, interfere with CCK stimulus-activity coupling at the CCK1R, thereby disrupting this important servomechanism. We hypothesize that reversal of the negative impact of cholesterol on this receptor could be useful in the management of obesity.

Methods—We have studied the effects of β -sitosterol, a phytosterol structurally related to cholesterol, on CCK receptor function. This included CCK binding and biological activity at wild type CCK1R and CCK2R, as well as at CCK1R in a high cholesterol environment, and at a CCK1R mutant, Y140A, which mimics the behavior of wild type receptor in high cholesterol.

Results— β -sitosterol (100 μ M and 10 μ M) significantly improved the defective signaling of the CCK1R present in high cholesterol ($p < 0.05$), without affecting CCK binding affinity. This effect was absent at the CCK1R present in a normal cholesterol environment, as well as at the structurally-related CCK2R. Furthermore, the cholesterol-insensitive Y140A mutant of CCK1R was resistant to the effects of β -sitosterol.

Conclusion—These data suggest that β -sitosterol affects CCK1R function in high cholesterol by competing with cholesterol at a receptor cholesterol-binding site and may shift its conformation toward normal. This phytosterol extends our understanding of the structure-activity relationships for developing a drug that can target the external surface of CCK1R. Since the concentrations of β -sitosterol shown to be effective in this study are similar to serum levels of this compound achievable during oral administration, it may be worthwhile to study possible beneficial effects of β -sitosterol in metabolic syndrome.

Address for correspondence: Laurence J. Miller, M.D., Mayo Clinic, 13400 East Shea Blvd, Scottsdale, AZ 85259, Tel.: (480) 301-4217, Fax: (480) 301-8387, miller@mayo.edu.

STATEMENT OF AUTHORSHIP

AJD, MD, LJM conceptualized and designed the study. AJD and MD performed the experiments and acquired the data. AJD, MD and LJM analyzed and interpreted the data. AJD and LJM wrote the manuscript. All work was supervised by LJM.

COMPETING INTERESTS

The authors declare no competing interests.

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Keywords

cholecystokinin receptor; cholesterol; metabolic syndrome; phytosterols

INTRODUCTION

The type 1 cholecystokinin receptor (CCK1R) is a G protein-coupled receptor, which binds the peptide hormone cholecystokinin (CCK) that plays important roles in stimulating gallbladder contraction and pancreatic exocrine secretion to facilitate digestion, regulating gastric emptying and intestinal transit to titrate nutrient delivery, and inducing post-cibal satiety (1, 2). This receptor is reported to exhibit dysfunction in the setting of high membrane cholesterol, such as has been reported in metabolic syndrome and cholesterol gallstone disease. Several *in vitro* and *in vivo* studies have demonstrated that the CCK1R is uniquely sensitive to an increase in cellular membrane cholesterol, which negatively affects effective coupling between Gq and this receptor, thereby resulting in reduced biological responses to CCK (see review (3)). Included among the defective biological responses is post-cibal satiety, contributing to increased weight gain and increasingly severe obesity. However, the closely structurally-related CCK2R functions entirely normally in the same membrane environment (4, 5). Consequently, CCK1R is considered as a potential target for the treatment of obesity, with several pharmaceutical efforts directed toward developing a potent agonist (6–9). No such agents have yet been approved for clinical use, based on their lack of efficacy, as well as concerns about their side effects and potential toxicity (10–12).

We have proposed an alternate approach, seeking the advantages of a positive allosteric modulator that does not possess intrinsic agonist activity (13). Such an agent might also have the capability to correct the abnormal conformation of the CCK1R that is postulated to exist in a high cholesterol environment (5). Reduced or absent intrinsic biological activity would theoretically reduce the chances of side effects and toxicity, by acting only during the short and finite period after a meal when endogenous CCK would be released and elevated in the circulation. One approach toward this goal might be to target the well-defined intramembranous allosteric site high in the helical bundle (14–16). Another site to target might be outside of this helical bundle, also within the lipid bilayer, where cholesterol has been postulated to interact with CCK1R to exert its negative impact on receptor function (4). We recently reported a proof-of-principle for the latter approach by studying CCK1R function in the presence of bile acids that are structurally similar to cholesterol (17). In that work, ursodeoxycholic acid was observed to correct the defective biological response of this receptor to CCK in the setting of high cholesterol (17).

In the current study, we have focused our attention on a prominent phytosterol, β -sitosterol, which is a well-recognized and safe cholesterol-lowering agent that has been extensively studied in cardiovascular diseases (18, 19). Like bile acids, β -sitosterol is structurally related to cholesterol (Fig. 1). The findings in this work support the potential usefulness of a phytosterol to correct the abnormal CCK1R function present in the setting of elevated membrane cholesterol.

MATERIALS AND METHODS

Materials

β -sitosterol and methyl- β -cyclodextrin (M β CD) were from Sigma-Aldrich (St. Louis, MO); Quest Fluo-8-AMTM was from AAT Bioquest (Sunnyvale, CA); soybean trypsin inhibitor was from Gibco Life Technologies (Grand Island, NY); lipoprotein-deficient serum was from Intracel Resources (Frederick, MD); and bovine serum albumin was from Equitech-Bio (Kerrville, TX). Synthetic CCK-26-33 (CCK-8) was from Bachem (Torrance, CA). All other reagents were analytical grade.

Cell lines

All of the CCK receptor-bearing cell lines used in this study have been previously characterized. Chinese hamster ovary (CHO) cell lines stably expressing the type 1 human CCK receptor (CHO-CCK1R) and type 2 CCK receptor (CHO-CCK2R) (4, 5), Y140A (CHO-CCK1R-Y140A) (5), W166A (CHO-CCK1R-W166A) (4) and Y237A (CHO-CCK1R-Y237A) (4) mutant constructs were used. Cells were maintained in Ham's F-12 medium (Gibco Life Technologies) containing 5% Fetal Clone II (Hyclone, Logan, UT) at 37°C in a humidified environment containing 5% CO₂. The CCK1R-expressing SRD15-CHO cell line that has elevated levels of membrane cholesterol (20) was also used to stably express CCK1R (SRD15-CCK1R), as previously characterized (21). This was cultured in similar conditions except 5% lipoprotein-deficient serum and 2.5 μ M 25-hydroxy cholesterol were used as supplements in DMEM/F-12 medium (Gibco Life Technologies).

Preparation of M β CD- β -sitosterol complex

M β CD- β -sitosterol complex stock solution was prepared using a previously-described method (5, 22). In brief, 12 mg of β -sitosterol was dissolved in 80 μ l of isopropanol:chloroform (2:1 v/v) solution, which was then added drop-wise to a solution containing 200 mg M β CD in 2.2 ml of Krebs-Ringer/HEPES (KRH) medium (25mM HEPES, pH 7.4, 104 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM KH₂PO₄, 1.2 mM MgSO₄) heated to 60–80°C with constant stirring.

Receptor binding assays

In order to test the effect of β -sitosterol on CCK binding characteristics, whole cell radioligand binding assays were performed, with a similar treatment protocol as described previously for bile acids (17). Briefly, cells were seeded and grown to achieve 80–85 percent confluence in 24-well tissue culture plates. On the day of the assay, cells were washed once with 400 μ l KRH medium containing 0.01% soybean trypsin inhibitor and 0.2% bovine serum albumin. The cells were then treated with a specific concentration of M β CD- β -sitosterol complex for 15 min at 37°C, following which a constant amount (~10 pM) of the CCK-like radioligand ¹²⁵I-*D*-Tyr-Gly-[(Nle^{28,31})CCK-26-33] (23), and increasing concentrations of unlabeled CCK (ranging from 0 to 1 μ M) were added to each well. The reaction was terminated after 1 h by washing the cells twice with ice-cold KRH medium containing 0.01% soybean trypsin inhibitor and 0.2% bovine serum albumin. Free radioligand was separated from cell-bound radioligand by aspiration of the medium,

followed by lysis of the cells with 0.5 M NaOH. Bound radioactivity was quantified using a γ -counter. Nonspecific binding was quantified in the presence of 1 μ M CCK, and represented less than 10% of total binding. Values represent means $\pm$ S.E.M. from three to eight independent experiments performed in duplicate.

Intracellular calcium assays

Assays were performed as described previously (5), using 96-well black-walled clear bottom plates in which the cells were approximately 80 percent confluent. Cells were treated with a fixed concentration of M β CD- β -sitosterol in KRH medium containing 1.2 mM MgCl₂, 0.2% bovine serum albumin, 2.5 mM probenecid and 0.75 μ M Fluo-8AM for 1 h at 37°C. They were then washed with KRH medium and the responses to CCK were quantified at 37°C following robotic addition of CCK in a Flexstation 3.0 plate reader (Molecular Devices, Sunnyvale, CA) equipped with Softmax Pro 5.4 software. Intracellular calcium responses were measured at fluorescence emission intensity of 525 nm after exciting the samples at 485 nm, with data collected every 4 s over a 120 s period. Values represent means $\pm$ S.E.M. from three to eight independent experiments performed in duplicate.

Data analysis

Non-linear regression analysis was used to analyze and plot the competition-binding and intracellular calcium response data (Prism, version 6, GraphPad Software, San Diego, CA). Two-tailed *t*-tests were used to evaluate differences between conditions, with *p* values less than 0.05 considered to be significant.

RESULTS

Effects of β -sitosterol on CCK1R expressed in a high cholesterol membrane environment

The structure of β -sitosterol is closely related to that of cholesterol, with the presence of an additional ethyl group at position 24 (Fig. 1). We used CCK1R-expressing SRD15 cells (SRD15-CCK1R cells) containing a defect in the cholesterol biosynthetic pathway that results in elevated cholesterol levels (20) as a model system to study the function of this receptor in such an environment (21). We previously showed that the CCK1R expressed in this environment exhibits a higher binding affinity for CCK than in a normal membrane, yet this binding is less productive, associated with a significant reduction in potency to stimulate a biological response than is normally observed (4, 5, 17, 21, 24). We examined the concentration dependence of β -sitosterol on the function of CCK1R stably expressed in these cells, and observed a significant improvement in the potency of CCK-induced intracellular calcium responses at 100 μ M and 10 μ M concentrations by 3.1- and 1.7-fold, respectively (*p*<0.05) (Fig. 2A, Table 1). Of note, CCK continued to be a fully efficacious agonist in this setting. In contrast to its effect on biological activity, β -sitosterol at the highest concentration of 100 μ M showed no significant effects on CCK binding, with total saturable binding and binding affinity unchanged (Fig. 2B, Table 1).

Effects of β -sitosterol on CCK1R and CCK2R expressed on CHO cells with normal membrane cholesterol

We tested the effects of this same high concentration of β -sitosterol that gave a left-shift in potency of CCK responses in SRD15-CCK1R cells (Fig. 2A, Table 1) on the function of wild type CCK1R stably expressed in a normal cholesterol environment present in CHO cells. β -sitosterol (100 μ M) did not exhibit any significant impact on CCK binding affinity or potency under these conditions (Fig. 3A, C, Table 1). These observations are different from the effects of elevated cholesterol, which increases CCK binding affinity and reduces the potency of CCK-stimulated intracellular calcium responses at the CCK1R (4, 5, 17, 21, 24). We further showed that 100 μ M β -sitosterol had no effect on the function of structurally-related wild type CCK2R stably expressed on CHO cells (Fig. 3B, D, Table 1), indicating that the corrective effect observed on CCK1R function in the SRD15 cells is specific to CCK receptor subtype, similar to the effect of cholesterol on these receptors (4).

Effects of β -sitosterol on mutant CCK1R constructs that disrupt recognized cholesterol-binding motifs

The cholesterol recognition/interaction amino acid consensus pattern (CRAC) and the cholesterol consensus motif (CCM) have been previously reported to be present in CCK receptors (4). We have demonstrated that ursodeoxycholic acid competes with cholesterol to act on the CCK1R through a cholesterol-binding motif (17). In order to test whether β -sitosterol also interacts with this receptor at a site of cholesterol interaction, we used the same CCK1R mutant constructs, Y140A and Y237A disrupting the CRAC motifs in TM3 and TM5, respectively, and W166A disrupting the CCM motif in TM4 (4).

Of these mutants, Y140A has been shown to mimic the conformation and behavior of CCK1R present in a high membrane cholesterol environment (5), and has been shown to be insensitive to a reduction (4) or an increase (5) in membrane cholesterol levels. The effect of 100 μ M β -sitosterol on CCK signaling was also abolished at this mutant (Fig. 4A, Table 1), without having any effect on CCK binding (Fig. 4B, Table 1). This could be interpreted that, although the Y140A mutant mimics the conformation of the CCK1R in a high cholesterol environment, this mutation probably disrupts the ability of the β -sitosterol to bind to that site. Other site mutants for cholesterol-binding motifs that are not critical for the effect of cholesterol on CCK1R function (4) were also utilized as controls. These constructs, CCK1R-Y237A and CCK1R-W166A, also did not exhibit any change in function upon treatment with 100 μ M β -sitosterol (Fig. 4C, D, Table 1). Of note, these mutants did not mimic the conformation of CCK1R in high membrane cholesterol and have been shown to be sensitive to changes in membrane cholesterol (4).

DISCUSSION

CCK is a peptide hormone that is released from enteroendocrine I cells in the small intestine in response to dietary fat and protein. This hormone is known to play important roles in nutritional homeostasis by acting on the CCK1R. CCK is known to stimulate post-cibal satiety by acting on this receptor expressed on vagal afferent neurons (1, 2). Indeed, this has been the rationale for multiple efforts to develop CCK1R agonists for the management of

obesity (6–9). Several drug candidates have failed to reach regulatory approval, mainly due to their lack of efficacy in clinical trials, and due to concerns about side-effects and potential toxicity (10–12). However, this experience does not eliminate CCK1R as a potential drug target for weight management, since the past efforts have focused exclusively on developing highly potent full agonists. We now introduce the possibility of developing a different type of drug, a positive allosteric modulator devoid in intrinsic agonist activity. Such an agent would not have been identified in typical screens for agonist drug candidates acting at this receptor.

Studies of both humans and animals have demonstrated that elevated membrane cholesterol levels present in cholesterol gallstone disease negatively affect CCK1R function, with abnormal CCK stimulus-activity coupling resulting in reduced contractile responses (25, 26). Of note, the closely-related CCK2R is not affected (4). This defect in CCK1R function is not observed in patients with pigment gallstones (3, 25), who are known to have similar levels of gallbladder inflammation, yet normal levels of cholesterol in their bile (27). Our previous *in vitro* studies using site-directed mutagenesis of CCK1R and model cell systems have suggested that the negative impact of elevated cholesterol on CCK1R function was mediated by a direct interaction between the two molecules resulting in an abnormal receptor conformation (5, 21).

We have hypothesized that it is possible to correct this defective conformation by using a positive allosteric modulator which can act either inside (13) or outside (17) the helical bundle. Small molecule CCK1R ligands have been shown to act within the bundle (14–16), while cholesterol has been shown to act outside the bundle (4). To extend the latter approach, we utilized bile acids as a tool, due to their high structural similarity to cholesterol. We demonstrated that the hydrophilic bile acid, ursodeoxycholic acid, corrected the defective signaling of the CCK1R in elevated cholesterol, most likely by competing with cholesterol at its site of interaction with the receptor (17). That study was the first of its kind to show that it is possible to allosterically modulate the function of this receptor by targeting the extrahelical surface of the receptor (17).

In the current study, we utilize the phytosterol β -sitosterol, also based on its structural similarity to cholesterol, thereby adding to our understanding of structure-activity relationships (17). Phytosterols are sterol compounds found in plant membranes that differ from cholesterol by the addition of alkyl substituents at position 24 and/or a double bond at position 22. Findings from the current study show that, similar to the effects of cholesterol on CCK receptors, β -sitosterol had effects on CCK1R, while not having recognizable effects on the structurally-related CCK2R. This is strongly suggestive that the β -sitosterol works directly on the CCK1R. Further, β -sitosterol did not affect CCK1R function in a normal membrane cholesterol environment, while it showed a significant protective effect against excess cholesterol, reversing the right shift in the CCK dose-response curve observed in the presence of elevated cholesterol. β -sitosterol achieves this without changing the elevated CCK binding affinity typical of the high cholesterol environment.

In order to determine the molecular basis of action of β -sitosterol, we utilized the same approach used previously, in which we tested the effect of cholesterol (4), and bile acids (17)

at single amino acid mutants that disrupted the CRAC and CCM cholesterol-association motifs in an effort to localize their sites of action. These studies showed that the Y140 residue in the CRAC motif of CCK1R, at the bottom of TM3 near the DRY ionic lock mechanism, was shown to be important in binding of cholesterol (4) and bile acids (17) to the CCK1R. We have also reported that this mutant mimics the function of the wild type CCK1R in a high cholesterol environment (5). In the current study, we show that the effects of β -sitosterol observed in wild type CCK1R in high membrane cholesterol were absent in the Y140A construct. These data may imply that although the Y140A mutant mimics the conformation of the CCK1R in a high cholesterol environment, this mutation probably disrupts the ability of the β -sitosterol to bind to that site, much like its impact on the effects of cholesterol (4) and bile acids (17). This observation may suggest that β -sitosterol interacts with the CCK1R at the same site as cholesterol, and competitively inhibits the cholesterol interaction with this receptor. It can be inferred that β -sitosterol can correct the defective receptor conformation caused by the high membrane cholesterol, which is reflected in significant improvement in CCK signaling in this setting.

The observations from this study using a phytosterol are similar to those demonstrating the protective effect of ursodeoxycholic acid in the same setting (17), further strengthening the use of a therapeutic approach that targets this site of action of cholesterol on CCK1R. Phytosterols represent a small fraction of dietary sterols, but can be safely administered in substantial quantity, often in an esterified form, as dietary supplements (19, 28–33). Administration of up to 9 g/day has been shown to elicit no side effects or toxicity (33). Clinical evidence including meta-analysis also shows that β -sitosterol supplementation does not result in any increased risk of developing cardiovascular disease (19, 28).

Phytosterol administration has been extensively studied as potentially useful for cardiovascular disease. Administration of 2–9 g β -sitosterol per day has been shown to lower serum LDL-cholesterol levels by 10–15% in subjects with both normal and elevated levels of serum cholesterol without any significant effects on HDL-cholesterol or triacylglycerol levels (18, 31–33). The predominant mechanism of action of phytosterols is believed to relate to reduced intestinal absorption of cholesterol, affecting this lipid in the diet as well as that in the enterohepatic circulation (34). Phytosterols are believed to displace cholesterol in micelles, and to affect membrane ABC transporters (35), however, they are poorly incorporated into chylomicrons and rapidly excreted into bile.

While net absorption of phytosterols seems to be quite low and inefficient, because they can be administered safely in high amounts, substantial increases in their serum levels can be achieved. Data from clinical studies demonstrating an LDL-lowering effect of β -sitosterol in hypercholesteromic patients have reported serum levels of β -sitosterol of 6–30 μ M before treatment, and 8–75 μ M after administration (19, 28–32). This is particularly noteworthy, since the serum levels of β -sitosterol achieved are close to the concentrations that showed an improvement in CCK1R signaling in the setting of high membrane cholesterol *in vitro* in the present study. Accordingly, it can be hypothesized that β -sitosterol administration has the potential to show a beneficial effect on CCK1R function in metabolic syndrome.

Acknowledgments

The authors acknowledge the excellent technical assistance of M.L. Augustine and A.M. Ball.

GRANT SUPPORT

This work was supported by a grant from the National Institutes of Health, DK032878, and by the Mayo Clinic.

Abbreviations

CCK	Cholecystokinin
CCK1R	type 1 cholecystokinin receptor
MβCD	methyl- β -cyclodextrin
CRAC	Cholesterol Recognition/Interaction Amino Acid Consensus Pattern
CCM	Cholesterol Consensus Motif

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Fig. 1. Structural comparison of β -sitosterol and cholesterol

Shown is structural similarity of β -sitosterol with cholesterol. The structure of β -sitosterol has an additional ethyl group at position 24 (highlighted by grey circle).

Fig. 2. Effects of β -sitosterol on CCK1R expressed in a high cholesterol membrane environment Shown are intracellular calcium responses to increasing concentrations of CCK peptide at the CCK1R expressed in SRD15-CCK1R cells containing elevated membrane cholesterol, in the absence (control) or presence of increasing concentrations of β -sitosterol up to 100 μ M (A). Also shown are the competitive-binding curves at the CCK1R in the same cells, in the absence (control) or presence of 100 μ M β -sitosterol using 125 I-CCK as radioligand, with values presented as percentages of saturable binding of each curve (B). Values are expressed as means $\pm$ S.E.M. of at least three independent experiments performed in duplicate.

Fig. 3. Effects of β -sitosterol on natural ligand binding to and biological activity at the CCK1R and the CCK2R present in a normal membrane environment

Shown are effects of 100 μ M β -sitosterol on intracellular calcium responses to increasing concentrations of CCK at the CCK1R (A) or the CCK2R (B) stably expressed in CHO cells. Values are expressed as means $\pm$ S.E.M. from four to eight independent experiments performed in duplicate. Also shown are the competitive-binding curves at the CCK1R (C) and CCK2R (D) in the same cells, in the absence (control) or presence of 100 μ M β -sitosterol using 125 I-CCK as radioligand, with values presented as percentages of saturable binding of each curve. Values represent means $\pm$ S.E.M. of at least three independent experiments performed in duplicate.

Fig. 4. Effects of β -sitosterol at CCK1R with mutations in cholesterol-recognition motifs
 Shown are the effects of 100 μ M β -sitosterol on CCK-dose-dependent intracellular calcium responses (A) and CCK competitive-binding curves (B) on CCK1R Y140A expressed stably in CHO cells. Values represent means $\pm$ S.E.M. of three to five independent experiments performed in duplicate. Also shown are the effects of 100 μ M β -sitosterol on intracellular calcium responses to CCK at CCK1R Y237A (C) and CCK1R W166A (D) constructs stably expressed in CHO cells. Values represent means $\pm$ S.E.M. of results from at least five independent experiments performed in duplicate.

Table 1Impact of β -sitosterol on ligand binding and biological activity.

Receptor		pIC ₅₀	pEC ₅₀
CCK1R	Control	8.94 ± 0.07	10.83 ± 0.10
	β -sitosterol, 100 μ M	8.92 ± 0.09	10.57 ± 0.15
CCK2R	Control	9.24 ± 0.12	10.17 ± 0.10
	β -sitosterol, 100 μ M	9.22 ± 0.09	10.21 ± 0.16
SRD15- CCK1R	Control	8.81 ± 0.05	9.38 ± 0.06
	β -sitosterol, 100 μ M	8.80 ± 0.08	9.85 ± 0.17 [*]
	β -sitosterol, 10 μ M		9.83 ± 0.08 [*]
	β -sitosterol, 1 μ M		9.66 ± 0.21
	β -sitosterol, 0.1 μ M		9.50 ± 0.26
CCK1R- Y140A	Control	9.39 ± 0.04	9.60 ± 0.13
	β -sitosterol, 100 μ M	9.52 ± 0.02	9.53 ± 0.28
CCK1R- Y237A	Control		11.26 ± 0.11
	β -sitosterol, 100 μ M		11.35 ± 0.16
CCK1R- W166A	Control		9.13 ± 0.18
	β -sitosterol, 100 μ M		8.79 ± 0.35

Binding was done using ¹²⁵I-CCK radioligand. All values are means ± S.E.M.^{*} denotes values significantly different from control: p < 0.05.