Effects of a Cholesterol-Enriched Diet on Intestinal Smooth Muscle Contraction: Inhibition of Muscarinic Receptors and their Disinhibition by the 5-HT₄ Agonist - Tegaserod

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Abstract

Background
Excess cholesterol in bile and in blood is a major risk factor for the respective development of gallbladder disease and atherosclerosis. This lipid in excess negatively impacts the functioning of other smooth muscles, including the intestine. Serotonin is an important mediator of the contractile responses of the small intestine. Drugs targeting the serotonin receptor are used as prokinetic agents to manage intestinal motor disorders, in particular irritable bowel syndrome. Thus, tegaserod, acting on 5-HT$_4$ serotonin receptor, ideally should obviate detrimental effects of excessive cholesterol on gastrointestinal smooth muscle. In this study we examined the effect of tegaserod on cholesterol-induced changes in the contractile responses of intestinal smooth muscle.

Methods
The effects of a cholesterol-enriched (1%) diet on the in vitro contractile responses of jejunal longitudinal smooth muscle from Richardson ground squirrels to the cholinergic agonist carbachol were examined in the presence or absence of tetrododotoxin (TTX). Two groups of animals, fed either low or 1% cholesterol supplemented rat chow diet, were further divided into two subgroups and treated for 28 days with either vehicle or tegaserod.

Results
The high cholesterol diet increased, by nearly 2-fold, contractions of the jejunal longitudinal smooth muscle elicited by carbachol. These cholinergic contractions were mediated by muscarinic receptors since they were blocked by scopolamine, a muscarinic receptor antagonist, but not by the nicotinic receptor antagonist, hexamethonium. Tegaserod treatment, which did not affect cholinergic contractions
of tissues from low cholesterol fed animals, abrogated the increase caused by the high cholesterol diet. With low cholesterol diet TTX enhanced carbachol-evoked contractions, whereas this action potential blocker did not affect the augmented cholinergic contractions seen with tissues from animals on the high cholesterol diet. Tegaserod-treatment removed the inhibitory effects of a cholesterol-enriched diet on neuronal muscarinic receptors, as the potentiating effect of TTX on carbachol-elicited contractions was maintained in these animals.

Conclusions
An enriched cholesterol diet causes significant changes to cholinergic neurotransmission in the enteric nerves of the jejunum. The mechanisms by which these effects of cholesterol are reversed by tegaserod are unknown, but may be related to less production of deoxycholic acid conjugates that inhibit muscarinic receptors.
Background

In cholesterol gallstone formation, gallbladder smooth muscle develops reduced contractions, and so impairs emptying and leads to stasis in the late stages. This dysfunction retains the cholesterol crystals that have nucleated from the supersaturated bile; the microcrystals agglomerate and stone growth follows [1,2]. Patients with gallstones also exhibit delayed small intestinal [3] and colonic [4] transit, which results in decreased enterohepatic cycling of bile salts [5] and so increases deoxycholic acid formation from cholic acid [6]. The deoxycholic acid and the reduced enterohepatic cycling adversely influence hepatic bile salt and cholesterol secretion and so contribute to the formation of bile supersaturated with cholesterol.

A participatory role of the intestine in gallstone formation has also been established in animal models of gallbladder disease. The Richardson ground squirrel and the prairie dog on a high cholesterol diet develop lithogenic bile containing excess cholesterol relative to its solubilizing capacity [7,8]. Smooth muscle function of these animals is altered negatively: their gallbladders exhibit reduced contractile responses to CCK8 and acetylcholine [8], while small intestine transit and the cycle period of migrating myoelectric complexes are prolonged [9,10]. The basis for these intestinal motility changes is unknown, but they might relate to the excess biliary cholesterol excreted into the duodenum and/or the hypercholesterolemic state of the animal model fed a high cholesterol diet.

From a therapeutic perspective, “prokinetic” agents, such as indoramin, an alpha-adrenergic antagonist, cisapride, a 5-HT_{4}-agonist/5-HT_{3}-antagonist, and erythromycin, a motilin agonist, promote small and large bowel transit, and enhance
gallbladder emptying and so hinder gallstone formation [10,11]. In Richardson ground squirrels, cisapride reverses the defect in gallbladder contractility, enhances bile salt secretion and lowers cholesterol saturation [11]. Cisapride is now known to cause cardiac rhythm disturbances [12]. A new prokinetic agent, tegaserod, a partial 5-HT₄-agonist [13], which enhances peristalsis, was approved for the treatment of patients exhibiting symptomatic IBS with constipation. IBS generally is associated with an increased risk of abdominal surgery, including cholecystectomy [14]. Hence, drugs used to treat this condition should have minimal impact on gallbladder function. In a recent human study, tegaserod did not affect gallbladder contractility or the diameter of the common bile and hepatic ducts [15]. Nonetheless, the effects of tegaserod on small bowel function when there is a predisposition to gallstone formation are not known.

Since serotonin agonists contribute to the contractile responses of the small intestine [16, 17] and are used to manage intestinal motor disorders [18, 19], we postulated that tegaserod would modify cholesterol-induced changes in the contractile responses of intestinal smooth muscle. To test this hypothesis we examined the effects of tegaserod on cholinergic-induced contractions of the longitudinal smooth muscle of jejunal and ileal tissues obtained from Richardson ground squirrels that were maintained for 28 days on a 1% cholesterol diet.

**Methods**

**Experimental Animals**

The University of Calgary Animal Care Committee approved the research protocol, which conforms to the guidelines of the Canadian Council on Animal Care. 96 Richardson ground squirrels (*Spermophilus richardsoni*) were trapped wild near
Calgary, Alberta and acclimatized for a minimum period of four weeks by caging them individually in thermoregulated rooms on a 12 h/12 h day/night light cycle with free access to a standard rat chow diet. After acclimatization, the animals were fed, under the same holding conditions for an additional four weeks, either a normal diet containing only trace amounts of cholesterol (0.027%, Dyets Inc., Bethlehem, PA) or a high cholesterol diet (Dyets Inc.), comprising an identical chow enriched with 1% cholesterol by weight. During this diet period the animals were injected subcutaneously, twice daily, with either vehicle or 0.1 mg/kg of tegaserod (kindly supplied by Novartis Pharma, Basel, Switzerland). Tegaserod was dissolved in vehicle (100% 1-methyl-2-pyrrolidinone) immediately before use, and then diluted into 154 mM NaCl such that the final concentration of 1-methyl-2-pyrrolidinone was 2.7%. The vehicle-treated animals received 2.7% 1-methyl-2-pyrrolidinone (Sigma-Aldrich, St. Louis, MO) in 154 mM NaCl. The animals were randomly divided into four groups (19 in each) and received the following combination of diet and drug: (1) vehicle + normal diet, (2) vehicle + cholesterol diet, (3) tegaserod + normal diet, and (4) tegaserod + cholesterol diet.

**Cholesterol Concentrations in Hepatic and Gallbladder Bile**

Acute terminal experiments in vivo directly measured bile composition. After a 14-hour overnight fast, animals were anesthetized and maintained on a low concentration (1.5 to 2%) isoflurane (Ayerst Laboratories, Montreal, Canada). At laparotomy the cystic duct was exposed and ligated, the gallbladder removed and the bile collected for subsequent analysis. The common bile duct was cannulated with a polyethylene catheter (PE50), and hepatic bile was collected into tubes. During this period 154 mmol/L NaCl was infused continuously IV at 2.5 mL/h via a femoral venous cannula for fluid replacement.
Cholesterol (was measured using the Liebermann-Burchard reaction (Cholesterol/Cholesteryl Ester Quantitation Kit™ of Biovision; Mountain View, CA).

**Intestinal Contractility in vitro**

After 4 weeks on their respective diets, 2 animals in each diet group were fasted for 16 hours and sacrificed. Intestinal contractile responses were performed and evaluated as described previously [9]. At laparotomy four 2 cm lengths of intestine were obtained in each animal, two from the proximal jejunum at a point 5 cm distal to the ligament of Treitz, and two from the distal ileum at a location 15 cm proximal to the ileocecal valve. Without occluding the lumen, 5-0 silk threads were tied to opposite ends of each segment with one end fixed to the bottom of a 20 mL organ bath, and the other to an isometric force-displacement transducer (FT03; Grass Telefactor, West Warwick, RI, USA)). This montage measured the contractile response of the longitudinal smooth muscle layer. The baths were filled with a modified Krebs solution of the following composition (in mmol/L): NaCl 103, KCl 4.7, MgCl₂ 1.13, NaHCO₃ 25, NaH₂PO₄ 1.15, CaCl₂ 2.56, D-glucose 2.8, sodium pyruvate 4.9, sodium fumarate 2.7, and sodium glutamate 4.9; at pH 7.4, continuously gassed with a mixture of 95% O₂ and 5% CO₂.

The tissues were stretched to 1 gm of tension, and equilibrated for 40 min with a change of physiological buffer every 10 min, and appropriate adjustment of tension. After equilibration, the longitudinal intestinal segments underwent dose-responses (10⁻⁸ to 10⁻⁴ M) to carbamylcholine chloride (carbachol; Sigma-Aldrich), performed either in the presence or absence of 10⁻⁷ mol/L TTX (Sigma-Aldrich). The TTX permitted evaluation of smooth muscle contractility in the absence of neural influences. Also, hexamethonium (Sigma-Aldrich), a selective nicotinic antagonist,
was used to determine the contribution of nicotinic receptors to the increased tone. At the end of each experiment, the tissue preparation were lightly blotted and weighed. The contractile response for each preparation was calculated as stress (Newtons/m²), normalized to the cross-sectional area of the longitudinal smooth muscle layer as previously described [9].

Data Analysis

The results are presented as the mean ± SEM. The statistical functions used that associated with Excel (Microsoft Office XP, Redmond, WA). Comparisons between two groups were made using the unpaired Student's t-test, and where appropriate, analysis of variance. Statistical values reaching probabilities of p<0.05 were considered significant.

Results

Bile Cholesterol

For animals on a low cholesterol diet the concentration of cholesterol in gallbladder and hepatic duct bile were 3.7±0.3 and 1.3±0.2 mmoles/L, respectively. Tegaserod treatment of low cholesterol diet animals did not modify cholesterol concentrations in the gallbladder (4.0±0.5 mmoles/L) or the hepatic duct (0.9±0.2 mmoles/L) relative to vehicle-treated animals. Feeding the animals a high cholesterol diet for 28 days significantly increased the bile cholesterol concentrations to 5.7±0.5 and 2.6±0.3 mmoles/L in the gallbladder and hepatic duct, respectively (p<0.05). Treating the animals on the cholesterol-enriched diet with tegaserod did not alter the concentration of cholesterol in the gallbladder (4.9±0.6 mmoles/L), although hepatic duct bile concentrations were reduced to control values (1.5±0.3 mmoles/L; p<0.05).
Cholinergic Contractions of Jejunal Longitudinal Smooth Muscle

Isolated jejunal segments from the ground squirrels on a low cholesterol diet responded in a dose-dependent manner to carbachol with increasing in tonic contractions to a maximal force of 2.1±0. X 10^2 Newtons/m^2 at an optimal concentration carbachol of 10^-4M (Figure 1A). In contrast, animals on the high cholesterol diet receiving the vehicle exhibited significantly greater responses to the cholinergic agent carbachol even above 10^-7M, with a maximal response of 3.6±0.3 (x 10^2) Newtons /m^2 (p<0.05).

After 4 weeks of tegaserod treatment of animals on a low cholesterol diet, 10^-4M carbachol generated a maximal force of 1.9±0.2 X 10^2 Newtons)/m^2 (Figure 1B), which was similar to the response in vehicle-treated animals. In contrast, jejunal tissues from tegaserod-treated animals on the high cholesterol diet did not exhibit any heightened contractions to carbachol; the maximal response of 2.3±0.3 x 10^2 Newtons /m^2 was 60% smaller than that seen with vehicle-treated animals on the high cholesterol diet (p<0.05).

Effect of Action Potential Blockade on Carbachol-Evoked Contractions

To determine if the cholinergic contractions of the jejunal longitudinal smooth muscle had a neural component, contractions of jejunal smooth muscle were measured in the presence of TTX, a blocker of action potential conduction. Following TTX exposure, the dose-response relationships to carbachol on tissues from the vehicle-treated animals on a low cholesterol diet increased approximately 2-fold to 4.4±0.4 (x 10^2) Newtons/m^2 (Figure 2A). Thus, the carbachol-produced contractions of jejunal smooth muscle reflects the net action of two processes; a direct contractile action on smooth muscle, and a relaxation/inhibition caused by activation of intramural nerves,
an event abrogated by the TTX. Since the nicotinic receptor antagonist, hexamethonium, did not increase carbachol-elicited contractions (Figure 3A), the potentiation of the cholinergic contractions after TTX exposure was independent of neuronal nicotinic receptors. Scopolamine, a muscarinic antagonist, used at a concentration of $10^{-5}$M, totally blocked the contractile actions of carbachol (data not shown).

Animals treated with tegaserod, like the vehicle-treated animals on the low cholesterol diet, exhibited increased contractile responses to carbachol (Figure 2C) in the presence of TTX. Hexamethonium did not modify carbachol-elicited contractions (Figure 3B). As before, scopolamine ($10^{-5}$M) totally blocked any contractile response to carbachol (not shown). Thus, with tegaserod treatment carbachol contractions of jejunal smooth muscle from normal diet animals also reflects the net action of two processes; a direct contractile action on smooth muscle and a relaxant action caused by activation of intramural nerves.

Ileal contractions elicited by carbachol were not modified by the cholesterol diet, TTX or hexamethonium (not shown), indicating that nerve-dependent potentiation of smooth muscle contractions to carbachol occurred exclusively in the jejunum, the intestinal segment receiving the cholesterol from the bile ducts and the diet. Consequently, only the contractile responses to the jejunum were considered.

Interactions between Cholinergic Contractions, Action Potential Blockade and a High Cholesterol Diet

In contrast to the animals on the low cholesterol diet, cholinergic contractions of tissues from animals on the high cholesterol diet were not potentiated after exposure
to TTX (Figure 2B). Since carbachol contractions, in the presence of TTX, were of a similar magnitude in tissues obtained from both low and high cholesterol diet animals, the high cholesterol diet probably increases carbachol-induced contractions by removing a cholinergic neurally-mediated inhibition of smooth muscle contractions. This inhibition is muscarinic receptor-mediated since hexamethonium did not alter carbachol-evoked contractions, even in cholesterol-fed animals (not shown).

For animals on a high cholesterol diet and treated with tegaserod a significant potentiation of the carbachol-elicited contractions following TTX (Figure 2D), which is comparable to that seen with tissues from low cholesterol diet animals receiving either vehicle or tegaserod. Thus, tegaserod treatment restores an inhibitory component of carbachol that was abrogated by the high cholesterol diet.

**Discussion**

This study reveals that a cholesterol-enriched diet modifies the properties of cholinergic contractions of jejunal smooth muscle through down regulation of inhibitory cholinergic receptors located on enteric nerves. Treating the animals with tegaserod prevented this down regulation caused by the cholesterol-enriched diet. The cholinergic contractions of the ileum were not affected by the high cholesterol diet, which may reflect the fact that very little cholesterol from either the diet or bile reaches the ileum, whereas the jejunum is exposed to higher concentrations of cholesterol.

Cholesterol is an essential membrane component serving as a cofactor for signalling molecules and as a precursor for steroid hormones. Cholesterol influences many of the biophysical properties of membranes, and depending on the receptor, cholesterol...
influences the affinity, binding capacity and signal transduction. When inappropriately regulated or excessive, by altering plasma membrane structure-function, cholesterol can play a crucial role in many diseases. Cholesterol's participation in cardiovascular disease is well established, and some neurological [20], immunological [21,22] and gastrointestinal disorders have cholesterol-related components. Cholesterol also has established roles in gallbladder disease [23,24,25,26] and modifies other gastrointestinal functions. Although acute exposure to cholesterol decreases pressure in rabbit intestinal loops [27] and reduces nitrergic relaxation of the rabbit sphincter of Oddi muscle [28], most cholesterol-related abnormal events probably result from prolonged exposure to this lipid, and its associated metabolites. Supplementing dietary cholesterol to 1% for 28 days has a significant impact on intestinal function with reported increases in the contractile responses of intestinal smooth muscle to cholinergic agonists [9], as confirmed in the present study (Figure 1A). The current report elucidates, in part, the intestinal defect caused by extended exposure to dietary cholesterol supplements.

A nervous system-dependent component to cholinergic agonist contractions of the intestinal tissues from Richardson ground squirrels was revealed. Since TTX removed an inhibitory component of cholinergic contractions of the jejunum from animals on a trace cholesterol diet (Figures 2A and 2C), a part of carbachol-elicited contractions, in normal animals, are neuronally mediated. Given that carbachol is a mixed muscarinic-nicotinic receptor agonist [29], the neuronal component to cholinergic contractions could be mediated by either nicotinic or muscarininc receptors on neurons and nerve terminals [30,31,32,33,34,35]. Since hexamethonium, unlike TTX, did not modify carbachol contractions (Figure 1B & 2B) the potentiation of the carbachol-elicited
contractions did not involve nicotinic receptors, but rather inhibitory neuronal muscarinic receptors. This conclusion is supported by the observation that scopolamine blocked completely all carbachol-induced contractions. This modulation by TTX of carbachol-elicited contractions was not evident in the ileum, and thus neuronal muscarinic receptors are either absent or obscured in this intestinal segment by the simultaneous activation of inhibitory and stimulatory receptors.

Cholesterol has a broad spectrum of effects on neurotransmission, ranging from having no effect on sympathetic transmission in mesenteric arteries [36], to inhibition of purinergic transmission in mesenteric arteries [36], noradrenergic transmission in the tail vein [37] and cholinergic responses in the corpus cavernosum [38], or potentiation of nitrergic transmission in the corpus cavernosum [38], and activation of the muscarinic [39] and nicotinic [40] receptors. The present study suggests that dietary cholesterol inhibits neuronal muscarinic receptors. The diverse actions of cholesterol may reflect tissues and receptor types, duration of exposure to this lipid, or could be related to increases in bile salt concentrations, which are known to interact with muscarinic receptors. The increase in plasma membrane content of cholesterol [9,10,41] may account for the detrimental effects of this lipid on neurotransmission. Lithocholyltaurine, the taurine conjugate of lithocholic acid, is a partial muscarinic receptor agonist [42], whereas the conjugates of deoxycholic acid act as cholinergic muscarinic receptor antagonists [43]. The available data does not allow definition of the precise mechanism by which a high cholesterol diet inactivates a neuronal muscarinic receptor in the intestine of the ground squirrel, but the activity of bile salt conjugates on muscarinic receptors either as weak, desensitizing, partial agonist
activity of lithocholytaurine or the antagonist activity of conjugates of deoxycholic acid are worthy of further investigation.

In the small intestine 5-HT\textsubscript{4} receptors, targets for tegaserod action, are found exclusively on enteric nerves [44]. The 5-HT\textsubscript{4} receptors, localized to cholinergic nerves, are involved in the contraction of smooth muscle from canine and human large intestines [45]. Mucosal release of 5-HT with activation of 5-HT\textsubscript{4} receptors on sensory neurons that relay via enteric interneurons to cholinergic motor neurons regulates the peristaltic reflex in mice [46], as with other species [47,48]. The rationale for the therapeutic use of agents acting at 5-HT\textsubscript{4} receptors to modulate visceral hypersensitivity supposes that continuous mucosal stimuli cause auto-inhibition and desensitization of 5-HT\textsubscript{4} receptors [49], and intramural sensory pathways [50]. In the present study we did not investigate this sensory-effector pathway for smooth muscle activation, but rather we examined the actions of the 5-HT\textsubscript{4} receptor agonist, tegaserod, on cholinergic receptor activation with carbachol. In keeping with a previous study with the guinea pig ileum [51], which reported no effect of serotonin on acetylcholine-elicited contractions, we found that long-term (4 weeks) tegaserod treatment did not affect cholinergic contractions of the jejunum or ileum of the ground squirrel on a normal (trace cholesterol) diet.

The current study revealed that tegaserod treatment removed the inhibitory effects of cholesterol diet on muscarinic receptors and enteric nerve dependent-mediated inhibition of jejunal contractions (Figures 1B & 2D). These effects of tegaserod may also have contributions from unexplored variables such as changes in cholesterol
metabolism and contraction-dependent reflex-induced release of 5-HT from enteric nerves.

Conclusions

Maintaining ground squirrels on a cholesterol-enriched diet for 4 weeks resulted in the removal of an enteric nervous system-dependent, inhibitory muscarinic component to cholinergic contractions of jejunal smooth muscle. This cholesterol-mediated inhibition of muscarinic neuronal transmission was blocked by tegaserod, a 5-HT₄-partial agonist, an agent that modulates intestinal contractions through actions on enteric nerves.

Abbreviations

5-HT – serotonin; CCK8 - cholecystokinin-octapeptide; CSI- cholesterol saturation index; IBS – irritable bowel syndrome; TTX - tetrodotoxin

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All authors participated in study design and read and approved the final manuscript. ES initiated the study and developed the protocol. RM coordinated the study, performed experiments, analyzed the data with statistical analysis and prepared the manuscript in conjunction with ES.
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Legends for Figures

Figure 1. Cholesterol diet and cholinergic contractions of the longitudinal muscle of the jejunum.

Effects of cholesterol diet on cholinergic (carbachol) - elicited contractions of the jejunum for ground squirrel on a trace (0.027%; Low) vs. enriched (1%; High) cholesterol diet for animals treated with either vehicle (A) or tegaserod (B). Cumulative dose-response curve to carbachol were constructed.

* High greater than Low (P<0.05); N=8-9.

Figure 2. Action potential blockade and contractions of the longitudinal muscle of the jejunum.

Effects of tetrodotoxin (TTX) on cholinergic contractions of the jejunum of ground squirrel fed either a low (0.027%; A & C) or a high (1%; B & D) cholesterol diet. The animals were treated with either vehicle (A & B) or tegaserod (C & D). The action potential blocker tetrodotoxin (TTX; 10^{-7} M) was added to the jejunal tissue segments 10 min before constructing a cumulative dose-response curve to the cholinergic agonist, carbachol. * TTX greater than No TTX (P<0.05). N= 8-9.

Figure 3. Antagonism of nicotinic receptors and contractions of the longitudinal muscle of the jejunum.

Absence of an effect of the nicotinic receptor antagonist, hexamethonium (10^{-5} M) on cholinergic contractions of the jejunum of vehicle-treated ground squirrel fed either a low (0.027%; A) or a high (1%; B) cholesterol diet. Hexamethonium was added to the jejunal tissue segments 10 min before constructing a cumulative dose-response curve to the cholinergic agonist, carbachol. N= 4-5.
Effect of Cholesterol Diet

A. Vehicle

B. Tegaserod

Figure 1
Effect of Tetrodotoxin

Vehicle Treatment

A. Low Cholesterol

B. High Cholesterol

Tegamerod Treatment

C. Low Cholesterol

D. High Cholesterol

Figure 2
Nicotinic Receptor Blockade

Vehicle Treatment

Figure 3