Published in final edited form as:

Neurogastroenterol Motil. 2012 July ; 24(7): e313–e324. doi:10.1111/j.1365-2982.2012.01935.x.

Disruption of Gallbladder Smooth Muscle Function is an Early Feature in the Development of Cholesterol Gallstone Disease

B. Lavoie¹, B. Nausch¹, E.A. Zane⁴, M.R. Leonard⁴, O.B. Balemba¹, A.C. Bartoo¹, R. Wilcox³, M.T. Nelson², M.C. Carey⁴, and G.M. Mawe^{1,2}

¹ Department of Anatomy and Neurobiology, University of Vermont School of Medicine, Burlington, VT

² Department of Pharmacology, University of Vermont School of Medicine, Burlington, VT

³ Department of Pathology, University of Vermont School of Medicine, Burlington, VT

⁴ Department of Medicine, Harvard Medical School, Gastroenterology Division, Brigham and Women's Hospital, Boston, MA

Abstract

Background—Decreased gallbladder smooth muscle (GBSM) contractility is a hallmark of cholesterol gallstone disease, but the interrelationship between lithogenicity, biliary stasis and inflammation are poorly understood. We studied a mouse model of gallstone disease to evaluate the development of GBSM dysfunction relative to changes in bile composition and the onset of sterile cholecystitis.

Methods—BALB/cJ mice were fed a lithogenic diet for up to 8 weeks, and tension generated by gallbladder muscle strips was measured. Smooth muscle Ca²⁺ transients were imaged in intact gallbladder.

Key Results—Lipid composition of bile was altered lithogenically as early as one week, with increased hydrophobicity and cholesterol saturation indexes; however, inflammation was not detectable until the fourth week. Agonist-induced contractility was reduced from weeks 2 through 8. GBSM normally exhibits rhythmic synchronized Ca²⁺ flashes, and their frequency is increased by carbachol (3μM). After one week, lithogenic diet-fed mice exhibited disrupted Ca²⁺ flash activity, manifesting as clustered flashes, asynchronous flashes or prolonged quiescent periods. These changes could lead to a depletion of intracellular Ca²⁺ stores, which are required for agonist-induced contraction, and diminished basal tone of the organ. Responsiveness of Ca²⁺ transients to carbachol was reduced in mice on the lithogenic diet, particularly after 4-8 weeks, concomitant with appearance of mucosal inflammatory changes.

Conclusions & Inferences—These observations demonstrate that GBSM dysfunction is an early event in the progression of cholesterol gallstone disease and that it precedes mucosal inflammation.

Correspondence G.M. Mawe Department of Anatomy and Neurobiology, University of Vermont College of Medicine 89 Beaumont Ave Burlington VT 05405 Tel: 802 656 8257 Fax 802 656 8704 gary.mawe@uvm.edu.

Author Contributions BL, BN, EAZ, MRL OBB, ACB performed the experiments, and all authors contributed to the analysis and interpretation of the data; BL, BN, OBB, ACB and GM conceived the study and designed the experiments; BL and GM generated the original draft of the manuscript; and all authors contributed to critical revisions and ultimately approved the final version. The experiments were carried out at the University of Vermont and at Harvard Medical School.

Current addresses: BN University of Calgary, Calgary, AB Canada; OBB University of Idaho, Moscow, ID; ACB Medtronics, Minneapolis, MN

Competing Interests: the authors have no competing interests.

Keywords

bile composition; cholecystitis; biliary motility; gallbladder emptying

INTRODUCTION

Cholesterol gallstone disease is one of the most common and costly digestive disorders. Although its pathogenesis is beginning to be understood, alterations in bile composition, inflammation of the gallbladder wall, and decreased contractility of gallbladder smooth muscle (GBSM) are known to be consistent features of cholesterol gallstone disease. Gallbladder biles from patients and experimental animal models of cholesterol gallstone disease contain elevated levels of cholesterol and hydrophobic bile salts (1-3). Cholesterol supersaturation of bile and increased hydrophobicity of bile salts promote mucin gel nucleation and crystallization (2, 4). Sterile cholecystitis, a consistent feature of cholesterol gallstone disease in humans, is also observed in mice, ground squirrels and prairie dogs fed a lithogenic diet (5-9). Changes also occur in GBSM in the gallbladder harboring lithogenic bile prior to inflammation and stone formation.

Gallbladder tone is maintained during filling by phasic activity of individual smooth muscle bundles. Rhythmic, spontaneous action potentials are synchronized amongst the cells within individual GBSM bundles and their associated contractions are believed to generate gallbladder tone (10). Rhythmic contractions of muscle bundles are driven by rapid transient increases in intracellular Ca^{2+} known as Ca^{2+} flashes (10). Ca^{2+} flashes also help maintain intracellular Ca^{2+} stores (10, 11), which control the responsiveness of GBSM to neurohormonal stimulation during gallbladder emptying. Another type of intracellular Ca^{2+} transient, the Ca^{2+} wave, propagates spontaneously within individual GBSM cells and reflects the intracellular Ca^{2+} release from the sarcoplasmic reticulum (12). Calcium wave activity is enhanced by IP_3 receptor activation during agonist-induced contractile responses.

Muscle contractility is diminished in gallbladders from patients with cholesterol stones and in animal models of gallstone disease. For example, agonist-induced contractility is impaired in gallbladder muscle strips from patients with cholesterol gallstones (13). In animal models of cholesterol gallstone disease, elevated biliary concentrations of cholesterol and hydrophobic bile salts are associated with biliary stasis. Ground squirrels fed a cholesterol-rich diet exhibit the key features of gallstone disease, including biliary cholesterol elevation, cholesterol stones, decreased gallbladder contractility, and cholecystitis (14). Furthermore, *ex vivo* application of cholesterol (15) or hydrophobic bile salts (16) disrupts action potentials and Ca^{2+} flashes in GBSM.

Although it is clear that GBSM contractility is diminished in cholesterol gallstone disease, the basis and time-course of this dysfunction are not clear. It is also not known whether GBSM contractility deficits precede other pathophysiological changes. The goal of the current investigation was to determine how gallstone disease affects contractile properties and Ca^{2+} transients of GBSM, and to evaluate the onset of these changes relative to alterations in gallbladder bile composition and cholecystitis during the development of cholesterol gallstone disease. To study GBSM dysfunction under pathophysiological conditions, we fed inbred mice a standard lithogenic diet (LD) and observed GBSM activity during the progression of the disease. This model of gallstone disease was used to investigate the cellular mechanisms responsible for decreased gallbladder contractility, and to evaluate the interrelationships between smooth muscle dysfunction, bile composition and gallbladder mucosal (i.e. parietal) inflammation. This work has been presented in abstract form (17).

METHODS

Animals

Male BALB/cJ mice (56± 3days, 20-30g) were fed a dairy butter diet for one to eight weeks. The lithogenic diet (LD) consisted of 15% butter, 1.23% cholesterol, 0.5% cholic acid (Dairy Butter diet; MP Biomedicals; Solon, OH). Control BALB/cJ mice were fed normal chow for the same length of time. The cholesterol- and cholic acid-rich diets were comprised of the dairy butter diet in the absence of cholic acid or cholesterol, respectively. Mice were anesthetized with isoflurane and exsanguinated according to a protocol approved by the Institutional Animal Care and Use Committee of the University of Vermont, Burlington VT.

Chemicals

All reagents used in this study, unless otherwise specified, were from Sigma-Aldrich Chemicals.

Gallbladder volume, cholesterol monohydrate crystals and histopathology

Gallbladders were gently removed and collected on ice-cold HEPES solution composed of: 134 NaCl, 6.0 KCl, 2.0 CaCl₂, 1.0 MgCl₂, 10 HEPES, 10 glucose (all in mM; pH 7.4). Excised gallbladders were measured (**length** from the cystic duct to the base of the fundus; **width** at the widest part of the body) and weighed. The gallbladders were then opened from the base to the cystic duct and bile was collected onto a microscope slide for analysis of cholesterol crystals and stone content using polarized microscopy. Once emptied fully the gallbladders were weighed again.

Gallbladder tissues to be processed for histopathology were then pinned flat in a Sylgard-coated dish and fixed in a 0.1M phosphate buffer containing 4% paraformaldehyde overnight at 4°C. Fixed tissues were then embedded in paraffin, cut into serial cross sections and processed for hematoxylin & eosin (H&E) staining.

Immunohistochemistry

Paraffin-embedded sections were processed for immunohistochemistry to detect monocytes/macrophages and neutrophils/eosinophils using rat anti-mac-2 (Cederlane, Burlington NC) and mouse anti-CD15 (Acris Antibodies, San Diego CA) antibodies, respectively. Slides were immersed in xylene, rehydrated, treated with target retrieval solution (Dako, Carpinteria, CA) according to the manufacturer's protocol and incubated for 1hr at room temperature (RT) in a blocking solution composed of 4% NGS diluted in a 0.5% Triton X-100 PBS buffer (pH 7.4). Sections were then incubated overnight at RT with either mac-2 or CD15 antibodies diluted 1:200 in the blocking solution. Following washes in PBS, sections were incubated for 1hr at RT in goat-anti rat IgG Alexa Fluor 555 (1:500; Invitrogen, Grand Island, NY) or goat anti-mouse IgM Alexa Fluor 568 (1:500; Invitrogen) diluted in the blocking solution and washes again. Slides were coverslipped using Citifluor antifadent mounting medium (CitiFluor Ltd, London UK).

Bile lipid composition

To maximize biliary volume, animals were fasted overnight with free access to drinking water. Samples for analysis of bile lipid composition were collected in Eppendorf tubes and frozen at -80°C. Bile salt species were separated and quantified on a Hichrom Ultrasphere ODS column by HPLC (18) using a Beckman HPLC system (Beckman Instruments, Fullerton, CA) consisting of a 166 UV-Vis detector, 125 pump assembly, and 32Karat software.

Biliary cholesterol was extracted into hexane (19), and the pure sterol was analyzed by HPLC (20). Cholesterol was eluted isocratically from a Beckman Ultrasphere ODS column with an acetonitrile:isopropanol (3:1, v/v) mobile phase. Bile salt concentrations were measured by 3 α -hydroxysteroid dehydrogenase enzymatic assay (21). Total phospholipids were assayed as inorganic phosphorus (22). Total Lipid Concentrations (TLCs) were calculated from the sum of bile salt, phospholipid, and cholesterol concentrations of individual bile samples. Cholesterol Saturation Indexes (CSIs) were calculated using critical tables (23) and were then urso- and muri-corrected because of the diminished solubility of cholesterol in murine bile, which contains a high percentage of hydrophilic bile salts such as muricholates and ursodeoxycholates. However, during lithogenic diet feeding most of these are replaced by cholate conjugates (21).

Myograph studies

The contractility of gallbladders was measured using a MyoMED myograph system (MED Associates, Georgia, VT) equipped with four force transducers and corresponding organ baths allowing to process 4 gallbladders side-by-side (2 controls and 2 LD-fed mice). Two silk threads were attached on the gallbladder, one at the neck and the other at the base of the fundus. Each gallbladder was then mounted by the threads onto the hooks of force transducers and lowered into the 7 ml organ bath filled with aerated physiological saline solution (PSS) containing (in mM) 119 NaCl, 7.5 KCl, 1.6 CaCl₂, 1.2 MgCl₂, 23.8 NaHCO₃, 1.2 NaH₂PO₄, 0.023 EDTA and 11 glucose (pH 7.3) at 37°C. An initial force of 5 mN was applied, and the tissue was allowed to equilibrate and accommodate to the initial load for 30 min (bath solution replaced with fresh PSS every 15 min). Agonist responses were obtained by applying the muscarinic agonist carbachol (0.1, 0.3, 1, 3, 10 and 30 μ M; applied cumulatively) directly into the organ bath every 4 minutes. Tissue was then washed for 30 min, and 80 mM KCl was added directly to the bath solution. Responses were normalized to the wet weight of the tissue.

Imaging and analysis of Ca²⁺ events

The full thickness whole mounts were stretched open and pinned serosa side up between two pieces of Sylgard connected by metal pins and processed for Ca²⁺ imaging as previously described (10). Briefly, tissues was loaded in a HEPES buffer containing 10 μ M fluo-4AM (Invitrogen) and 2.5 μ g/ml pluronic acid (Invitrogen) for 45 minutes at room temperature and washed in HEPES for at least 30 min to allow deesterification. The loaded tissues were placed in an imaging chamber and superfused with aerated PSS. Ca²⁺ transients were visualized using a Nikon TMD inverted microscope with a 60X water immersion lens attached to a Noran Oz laser confocal system. In some experiments, Ca²⁺ events were visualized using an Andor iXon^{EM+} 897 back-illuminated EMCCD camera attached to an inverted fluorescent Olympus IX70 microscope equipped with a 40X lens. After equilibrating the tissue for 20 minutes, video images of the Ca²⁺ events were captured over periods of 20-60 seconds (30 frames per second). Movies were analyzed using SparkAN, a custom software written at the University of Vermont (AD Bonev).

Statistical analyses

Statistical analyses were performed using Graph Pad Prism (GraphPad Software Inc., San Diego, CA). Data are expressed as mean $\pm$ SEM, n refers to the number of preparations obtained from different animals. *P* values $\leq$ 0.05 (using One-way ANOVA and Bonferroni's Multiple Comparison Test or Student's t-test) were considered statistically significant.

RESULTS

Maurer and colleagues (6) have described the prevalence of lithogenic bile and inflammation in the gallbladders of *Helicobacter* negative BALB/cJ mice fed a diet rich in dairy fat, cholesterol and cholic acid. Therefore we used this model to examine the progression of gallstone disease, including GBSM function, in male BALB/cJ mice fed a LD for 1-8 weeks. Weights of mice fed the LD were comparable to controls (Supplementary data). LD-fed mice developed enlarged gallbladders (Supplementary data).

Cholesterol monohydrate crystals, which were never observed in the gallbladders of chow fed mice, were observed in nearly 60% of the mice fed the LD for one week. A mixture of cholesterol crystals, sandy stones and true gallstones was observed in more than 80% of the gallbladders from mice fed the diet for two to eight weeks (Supplementary data). True gallstones were only observed in 4 of 144 mice (2.8%) and appeared between 4-8 weeks on the diet. Microscopically, viscosity of the bile was highest at 4 weeks on the lithogenic diet, at which point large amounts of extremely viscous mucin gel hampered sampling of the bile.

Bile composition

Altered bile lipid composition is a fundamental feature of cholesterol gallstone formation (3). To examine the progression of bile lipid composition changes in our model, we evaluated the biliary ratios of bile salts, cholesterol and phospholipids present in the LD-fed mice at different time points and compared them to those of mice fed standard chow. Whereas on the LD molar percentages of bile salts decreased after one week ($P<0.0001$) and remained low at all time points, molar percentage of phospholipids and cholesterol increased after one week on LD ($P<0.0001$ for both) and remained elevated (Supplementary data). Total lipid composition (TLC) was not altered by the LD, but both the cholesterol saturation index (Fig. 1A) and the bile salt hydrophobicity index (Fig. 1B) of bile samples were increased for mice fed LD for 1-2 weeks, likely contributing to the early appearance of cholesterol monohydrate crystals in the bile (Fig. 1C).

Primary and secondary bile salts present in the samples exhibit different levels of hydrophobicity and therefore can be used to assess the lithogenicity of the bile. In BALB/cJ mice fed the chow diet, normal murine bile salt profiles were observed (24), with 60-70% muricholate conjugates (tauro- α -muricholate plus tauro- β -muricholate), 30-40% taurocholate (TC), and <5% secondary bile salts. Because cholic acid was fed with the LD, as early as one week on the LD the bile became more hydrophobic, with over 85% TC, less than 10% muricholates, and <5% other bile salts, resulting in an increase of the hydrophobicity index ($P<0.0001$) (Fig. 1B,D). Tauroursodeoxycholate (TUDC) and taurochenodeoxycholate (TCDC) were minor contributors (3% and 2%) to the bile salt pool.

Histopathology and immunohistochemistry

Histopathological features of gallstone disease include gallbladder wall thickening secondary to muscle hypertrophy, epithelial hyperplasia and inflammation. In animals fed the standard chow, no signs of acute (eosinophils and neutrophils) or chronic (lymphocytes and plasma cells) inflammation were observed, and the muscularis mucosa was 2-3 muscle cells thick. After one to two weeks on the LD, the thickness of the murine gallbladder wall was not significantly different from control ($\sim 20 \mu\text{m}$; Supplementary data, Table 1) and lymphocytes were rarely observed (Fig. 2). However, the number of cell layers in the muscularis increased to 5-6 layers by the end of the second week. At four weeks on the LD, epithelial hyperplasia and muscularis hypertrophy were apparent, and overall gallbladder wall thickness increased to $79.8 \pm 10.9 \mu\text{m}$ ($P<0.0001$ compared to control). Furthermore, acute and chronic inflammation was observed at this time point, including the presence of

eosinophils, neutrophils, and lymphocytes within the lamina propria (Fig. 2). After eight weeks on the LD, increased thickness of the gallbladder wall ($69.1 \pm 12.0 \mu\text{m}$; $P < 0.001$ when compared to control) was still observed. Morphological changes associated with epithelial hyperplasia and expansion of the lamina propria, including the appearance of broad-based villi and mucosal folding were observed, along with hypertrophy of the muscle layer. Eosinophils and lymphocytes were observed throughout the gallbladder wall (Fig. 2).

We also used immunohistochemistry to evaluate the presence of inflammatory cells in the gallbladder wall. The distribution of the immunopositive cells followed a pattern similar to that observed on H&E sections. Monocytes/macrophages (anti-mac-2; Supplementary data, Figure 2) and neutrophils/eosinophils (anti-CD15, data not shown) were sparsely observed in mice fed the control chow as well as in mice on the LD for 1-2 weeks. After 4 weeks on the LD, immunopositive cells were numerous within the lamella propria and were observed extending into the muscularis propria. Collectively, these data suggest that inflammation develops after the 2 week time point.

Gallbladder Contractility

Gallbladder enlargement during cholesterol cholelithogenesis is associated with decreased contractility in human disease and animal models (1, 25). To investigate the time course of altered contractility in our model, tension assays were performed on whole gallbladders (emptied of their bile content). Spontaneous basal contractile activity and agonist-induced contraction by the muscarinic agonist carbachol were examined and responses were normalized to wet weight of the gallbladder.

In mice fed standard chow, only 12% of the gallbladders exhibited spontaneous activity, whereas in LD-fed mice, more than 35% of the gallbladders generated spontaneous contractions by four weeks on the LD. However by eight weeks on the diet, spontaneous contraction activity fell back to 12% (Fig. 3A). Although observed more frequently in mice on the LD, the spontaneous events were not significantly different from controls.

Carbachol caused concentration-dependent contractions of gallbladders from control and LD-fed animals; however, the contractile responses were attenuated in amplitude and were more transient in lithogenic animals. In gallbladders removed from mice fed the standard chow, addition of carbachol to the bath led to a rapid and sustained contraction, with a maximal response detected at concentrations as low as $3 \mu\text{M}$ ($n = 42$; Fig. 3A). In general, the gallbladders of LD-fed mice responded quickly to the addition of carbachol, reaching maximal contraction at a concentration and rate similar to controls. By 8 weeks on the LD only one out of 8 mice showed a sustained contraction between the cumulative doses of carbachol. Furthermore, the maximal contractility in response to the cholinergic agonist was significantly decreased at 2, 4, and 8 weeks on LD, as compared to control values (Fig. 3B). On the other hand, contractility in response to 80 mM KCl was not significantly affected by LD at the 2 or 8 week time points, but was suppressed at 4 weeks (Fig. 3C).

Smooth Muscle Activity

Basal activity: patterns observed at 8 weeks on the lithogenic diet—Basal activity of GBSM can be evaluated *ex vivo* by examining spontaneous Ca^{2+} transients in whole mount preparations. These events include rhythmic Ca^{2+} flashes that occur simultaneously in all of the cells of a given bundle and calcium entry during action potentials, as well as irregularly occurring, asynchronized Ca^{2+} waves that propagate within individual muscle cells (10, 12, 16). In most mice fed chow, Ca^{2+} flashes in GBSM cells fired rhythmically and synchronously with other cells in that bundle, and at a frequency of $0.61 \pm 0.05 \text{ Hz}$ ($n = 8$). In two mice, flashes were less rhythmic, but Ca^{2+} flash activity was

synchronized. Ca^{2+} waves, which are spontaneous intracellular Ca^{2+} release events that propagate within individual GBSM cells, were also observed in control mice.

Mice fed the LD for 8 weeks typically exhibited altered Ca^{2+} transient activity, and this time point was used to establish the criteria to evaluate changes in GBSM activity over time. The patterns of Ca^{2+} transients were divided into three main groups based on the Ca^{2+} flash activities that were observed (Fig. 4). The first pattern, termed “clustered flashes”, was detected in 4 of 12 preparations and consisted of brief clusters of 5-15 synchronized Ca^{2+} flashes interrupted by quiescent periods that lasted 15-20 sec. The frequency of the flashes during the cluster (0.47 ± 0.06 Hz) was not statistically different from the frequency of flashes in control tissue. The second group, called “asynchronized flashes”, was also observed in 4 of 12 preparations. In these preparations, repetitive Ca^{2+} flashes occurred in individual GBSM cells at a rate of 0.52 ± 0.02 Hz, but the activity of the cells in a given bundle was asynchronized. The third pattern, referred to as “sporadic flash activity”, was detected in 3 of 12 preparations. In this preparation, Ca^{2+} flashes were observed only sporadically in a few cells, whereas Ca^{2+} waves were still active. The infrequent flashes were generally synchronized throughout the cells of a given muscle bundle.

Basal activity and onset of abnormal Ca^{2+} activities in GBSM—After establishing the different patterns of activity in mice fed the LD for 8 weeks, spontaneous Ca^{2+} transients were evaluated at earlier time points (1-4 weeks; Fig. 5). At the end of one week on the diet, changes in Ca^{2+} flash activity were observed in 50% of mice (4 of 8). Altered Ca^{2+} activity consisted of either a pattern of clustered flashes (2 of 8 mice) or asynchronized flashes (2 of 8 mice). After 2 weeks on the LD, activity was disrupted in 45.5% of the mice (5 of 11) and in these preparations, patterns of activity included clustered, asynchronized and sporadic flashes (18.2% each; Fig. 5). After 4 weeks on the LD, Ca^{2+} transients were disrupted in 88% (15 of 17) of the mice, including all three patterns of disrupted activity (clustered, $n=5$; asynchronized, $n=4$; and sporadic, $n=6$; Fig. 5).

Agonist-induced activity—In mice fed standard chow diet, carbachol (3 μM) increased the frequency of the Ca^{2+} flashes from 0.56 ± 0.06 Hz to 0.75 ± 0.04 Hz ($P < 0.03$, paired Student's t-test, $n = 6$) (Fig. 6). In mice fed the LD for 1-2 weeks, cholinergic activation changed patterns of asynchronized Ca^{2+} flashes to more rhythmic and synchronized Ca^{2+} flashes. The agonist-induced Ca^{2+} transient closely resemble Ca^{2+} transients in control mice. At later time points (4 and 8 weeks), the frequency of synchronized Ca^{2+} flashes was not increased by carbachol; however, the agonist appeared to increase the appearance of the asynchronized flashes and waves.

Effect of diets enriched in either cholesterol or cholic acid alone

Mice fed a diet enriched in either cholic acid or cholesterol for 4-8 weeks were compared to LD-fed mice as well as mice fed the standard chow. Mice fed the cholic acid-rich diet displayed normal weight gain while mice fed the cholesterol-rich diet were significantly larger than the control mice (Supplementary data). Mice on the cholic acid-rich diet showed enlarged gallbladders, and their bile salt species, cholesterol saturation, and hydrophobicity indexes were similar to the values obtained in mice fed LD for 8 weeks, whereas gallbladders from mice on the cholesterol-rich diet were not significantly different from mice on chow, although their hydrophobicity indexes were slightly elevated (Fig. 1A, B). Neither group showed evidence of cholelithogenesis (Fig. 1C) or cholecystitis. Contractility assays (Fig. 4B) and analysis of Ca^{2+} transients (Fig. 7) for both groups were similar to controls.

DISCUSSION

The principal aim of this study was to investigate the progression of smooth muscle dysfunction in early cholesterol gallstone disease. Using an established mouse model, we determine that smooth muscle dysfunction is a very early event in the development of the disease. Decreased contractile response to an agonist and altered patterns of spontaneous and induced Ca^{2+} transients in the gallbladder were apparent by two weeks on the lithogenic diet. Along with these early changes, we observed increases in the cholesterol saturation of bile and hydrophobicity indexes of bile salts. Although hypertrophy was also detected in the muscularis propria at early time points, signs of inflammation were not obvious until the 4 week time point.

The gallbladder is a tonic organ and previous studies have suggested that its tone during filling is maintained by asynchronous spontaneous contractions of smooth muscle bundles throughout the wall of the gallbladder, whereas postprandial emptying is likely the result of synchronized contraction of these muscle bundles. The loss of tone, which contributes to gallbladder enlargement, is a feature of human gallstone disease that has also been observed in various animal models (1, 25, 26). Although gallbladder tone was not assessed directly in the current study, gallbladders from mice on the LD were enlarged. Under basal conditions, rhythmic Ca^{2+} flashes precede contractions of individual GBSM bundles, and changes in this rhythmic activity likely result in decreased tone and may contribute to gallbladder enlargement.

Analysis of the biliary lipids showed early changes in absolute as well as relative composition and increases in biliary cholesterol saturation indexes of bile and hydrophobicity indexes of bile salts within the first two weeks on the diet. *In vitro* studies have shown that both cholesterol (15) and hydrophobic bile salts (16) decrease GBSM excitability, but they act on smooth muscle through different mechanisms. Cholesterol decreases Ca^{2+} channel function and excitatory receptor activation (15, 27). These changes may also result in decreased fluidity of cholesterol-enriched membranes, particularly in caveolar regions (28, 29). For instance, an elevated concentration of cholesterol in the plasma membrane increases expression of caveolin-3 causing sequestration of CCK-1 receptors and therefore supporting the lower binding of CCK in affected smooth muscle (28, 29). Increased incorporation of cholesterol in the plasma membrane also decreases the cytoprotective effect of prostaglandin E2 (30, 31). Hydrophobic bile salts suppress excitability by activating G-protein coupled bile acid receptors (GPBAR1), resulting in the opening of K_{ATP} channels (16).

In addition to changes in basal activity of the GBSM in LD-fed mice, agonist-induced contractility of GBSM was also affected. Decreased contractility in response to CCK and cholinergic agonists has been shown in strips of gallbladder muscle from humans with gallstone disease as well as in animal models (5, 27, 32). Decreased agonist-induced contraction has been linked to disrupted receptor binding to agonist (33) and likely involves decreased Ca^{2+} channel function. Data from the present study indicate that, at an early time point (before inflammation becomes histologically evident), rhythmicity of Ca^{2+} flashes in GBSM cells can be restored by carbachol, although contractile response of the gallbladder to the agonist is reduced in mice fed the diet for two weeks. Since agonist-induced contractions involve release of Ca^{2+} from intracellular stores, and intracellular stores in GBMS require Ca^{2+} flash/action potential activity to be maintained, it is possible that a decrease in intracellular stores contributes to decreased agonist-induced contraction in the LD mice (10, 34). In addition, this decrease in contractility could involve altered Ca^{2+} sensitization in GBSM, including deficits in intracellular signaling pathways and/or in the Ca^{2+} -sensitive contractile proteins. Pozo and colleagues have previously described altered Ca^{2+}

sensitization in developing and aging guinea pig GBSM (35, 36). In the prairie dog lithogenesis model, Xu and Shaffer showed that contractility in response to a Ca^{2+} ionophore was unchanged in animals with gallstones, suggesting that the deficit is upstream of the contractile proteins (5). To better understand the cellular mechanisms responsible for the decreased contractility in gallstone disease, further studies using pharmacological approaches will be needed to tease apart the potential involvement of intracellular Ca^{2+} stores and/or Ca^{2+} sensitivity of contractile proteins.

Even though the response to membrane receptor-dependent agonist (carbachol) was decreased in the gallbladders of LD-fed mice, their response to a membrane receptor-independent stimulation (KCl) was not different from controls in the early (1-2 weeks) and late (8 weeks) time points. This supports previous reports that the intrinsic contractile apparatus is not affected in cholesterol gallstone disease. Behar and colleagues have demonstrated that stimulation of gallbladder muscle strips from gallstone patients which are non-responsive to membrane-bound receptor (CCK-1R) exhibit normal contractility to direct activation of G-protein signaling pathway by inositol triphosphate (37). This contrasts with evidence of damaged contractile machinery in acute (38, 39) and chronic acalculous cholecystitis (40). Interestingly, hypertrophy of the muscle layer early in the progression of the disease does not prevent the enlargement of the gallbladder, supporting the concept that excitability of, and communication between, GBSM cells are affected by the biliary lipid compositional changes from the lithogenic diet.

Whereas both cholesterol and hydrophobic bile salts can individually affect GBSM activity when applied directly to isolated or intact GBSM preparations (15, 16), both compounds appear to be necessary to affect GBSM activity. This is in contrast to the ground squirrel (14) and prairie dog (9) models, where cholelithogenesis develops in response to a diet enriched in cholesterol alone, suggesting that mice may handle cholesterol differently than other species. Gallbladders of mice fed a diet enriched in cholesterol alone appeared and behaved in a similarly fashion to control gallbladders, with the exception of a slight increase in bile salt hydrophobicity. This can be explained by the fact that high levels of muricholate in these mice prevent intestinal cholesterol absorption (41). Mice fed a diet rich in cholic acid possessed enlarged gallbladders with an increased bile salt hydrophobicity index but no change in cholesterol saturation index because this diet is low in cholesterol content. Neither set of mice developed cholesterol crystals or gallstones in bile, which is supported by the prediction made from the critical tables for calculating the cholesterol saturation of bile (23). Basal and agonist-induced Ca^{2+} transient patterns as well as contractile responses in these two groups were comparable to control mice. These data suggest that, although both cholesterol and hydrophobic bile salts can directly modify GBSM activity *in vitro*, specific interactions between these compounds are involved in the development of muscle dysfunction in gallstone disease.

In the current study, inflammation appeared after changes in bile composition and muscle function were detected. Histological changes of the gallbladder wall observed in this study are comparable to those described by van Erpecum and collaborators in C57L/J mice fed a lithogenic diet for two weeks (8). In both studies, major changes in gallbladder wall thickness and appearance of inflammatory infiltrates appeared following four weeks on the diet. Only one study has reported a slight increase in myeloperoxidase (MPO) and IL6 activities at time points earlier than 4 weeks (42). In the current study, increased mucin glycoprotein gel levels were particularly noticeable at 4 weeks, a condition that hampers analysis of bile lipids (43). Using $\text{Rag}2^{-/-}$ mice which are B- and T-cell deficient, Maurer and colleagues have shown that functional T-cells are crucial in the development of gallstone disease in this model, with $\text{Rag}2^{-/-}$ mice being resistant to gallstone formation when fed a lithogenic diet for 8 weeks (6). According to their reasoning, mucin secretion

and gelation promotes a liquid crystalline-to-solid cholesterol crystal phase transition that leads to cytokine activation and subsequent mucosal and muscle dysfunction. Based on our findings, we propose that early changes in smooth muscle function and associated stasis may contribute to cholesterol monohydrate crystal formation rather than being secondary to the inflammation. This possible paradigm shift needs to be explored further by comparing smooth muscle function in control mice to Rag2^{-/-} mice fed the LD or to mice fed a lithogenic diet while being treated with an inhibitor of acute sterile inflammation such as a COX-2 antagonist. Interestingly, treatment of patients with symptomatic cholesterol gallstone with the hydrophilic bile salt ursodeoxycholic acid (UDCA) prior to cholecystectomy improves both contractility and inflammatory scores, possibly by decreasing cholesterol content of the plasma membrane (44-46). Furthermore, UDCA can prevent the muscle dysfunction caused by the hydrophobic bile chenodeoxycholic acid (CDCA) in animal model and isolated cells (47, 48).

In conclusion, the results of this investigation support the concept that disruptions in GBSM function represent an early event in the development of cholesterol gallstone disease and are detectable soon after lithogenic changes occur in the lipid composition of bile. The finding that GBSM function is altered prior to the development of overt inflammation indicates that changes in the smooth muscle of the gallbladder are not a result of the inflammatory response per se, and the decrease in GBSM muscle function may actually contribute to the inevitable cholecystitis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank Todd Clason for his assistance with the imaging studies. This work is funded by NIH grants DK080480 to GMM, R37 DK036588 to MCC, R37 DK053832 to MTN, and by grants 5 P30 RR032135 from the COBRE Program of the National Center for Research Resources and 8 P30 GM 103498 from the National Institute of General Medical Sciences.

REFERENCES

1. Portincasa P, Moschetta A, Calamita G, Margari A, Palasciano G. Pathobiology of cholesterol gallstone disease: from equilibrium ternary phase diagram to agents preventing cholesterol crystallization and stone formation. *Curr Drug Targets Immune Endocr Metabol Disord*. 2003; 3(1): 67–81. [PubMed: 12570727]
2. Van Erpecum KJ. Pathogenesis of cholesterol and pigment gallstones: an update. *Clinics and research in hepatology and gastroenterology*. 2011; 35(4):281–7. [PubMed: 21353662]
3. Wang DQ, Cohen DE, Carey MC. Biliary lipids and cholesterol gallstone disease. *J Lipid Res*. 2009; 50(Suppl):S406–11. [PubMed: 19017613]
4. Wang DQ, Carey MC. Characterization of crystallization pathways during cholesterol precipitation from human gallbladder bile: identical pathways to corresponding model bile with three predominating sequences. *J Lipid Res*. 1996; 37(12):2539–49. [PubMed: 9017506]
5. Xu QW, Shaffer EA. The potential site of impaired gallbladder contractility in an animal model of cholesterol gallstone disease. *Gastroenterology*. 1996; 110(1):251–7. [PubMed: 8536864]
6. Maurer KJ, Rao VP, Ge Z, Rogers AB, Oura TJ, Carey MC, et al. T-cell function is critical for murine cholesterol gallstone formation. *Gastroenterology*. 2007; 133(4):1304–15. [PubMed: 17919501]
7. van Erpecum KJ, Wang DQ, Lammert F, Paigen B, Groen AK, Carey MC. Phenotypic characterization of Lith genes that determine susceptibility to cholesterol cholelithiasis in inbred mice: soluble pronucleating proteins in gallbladder and hepatic bile. *J Hepatol*. 2001; 35(4):444–51. [PubMed: 11682027]

8. van Erpecum KJ, Wang DQ, Moschetta A, Ferri D, Svelto M, Portincasa P, et al. Gallbladder histopathology during murine gallstone formation: relation to motility and concentrating function. *J Lipid Res.* 2006; 47(1):32–41. [PubMed: 16224116]
9. Yu P, Chen Q, Biancani P, Behar J. Membrane cholesterol alters gallbladder muscle contractility in prairie dogs. *Am J Physiol.* 1996; 271(1 Pt 1):G56–61. [PubMed: 8760107]
10. Balemba OB, Salter MJ, Heppner TJ, Bonev AD, Nelson MT, Mawe GM. Spontaneous electrical rhythmicity and the role of the sarcoplasmic reticulum in the excitability of guinea pig gallbladder smooth muscle cells. *Am J Physiol Gastrointest Liver Physiol.* 2006; 290(4):G655–64. [PubMed: 16293655]
11. Morales S, Camello PJ, Mawe GM, Pozo MJ. Characterization of intracellular Ca^{2+} stores in gallbladder smooth muscle. *Am J Physiol Gastrointest Liver Physiol.* 2005; 288(3):G507–13. [PubMed: 15499078]
12. Balemba OB, Heppner TJ, Bonev AD, Nelson MT, Mawe GM. Calcium waves in intact guinea pig gallbladder smooth muscle cells. *Am J Physiol Gastrointest Liver Physiol.* 2006; 291(4):G717–27. [PubMed: 16710055]
13. Behar J, Lee KY, Thompson WR, Biancani P. Gallbladder contraction in patients with pigment and cholesterol stones. *Gastroenterology.* 1989; 97(6):1479–84. [PubMed: 2583414]
14. Fridhandler TM, Davison JS, Shaffer EA. Defective gallbladder contractility in the ground squirrel and prairie dog during the early stages of cholesterol gallstone formation. *Gastroenterology.* 1983; 85(4):830–6. [PubMed: 6309603]
15. Jennings LJ, Xu QW, Firth TA, Nelson MT, Mawe GM. Cholesterol inhibits spontaneous action potentials and calcium currents in guinea pig gallbladder smooth muscle. *Am J Physiol.* 1999; 277(5 Pt 1):G1017–26. [PubMed: 10564108]
16. Lavoie B, Balemba OB, Godfrey C, Watson CA, Vassileva G, Corvera CU, et al. Hydrophobic bile salts inhibit gallbladder smooth muscle function via stimulation of GPCR1 receptors and activation of KATP channels. *J Physiol.* 2010; 588(Pt 17):3295–305. [PubMed: 20624794]
17. Lavoie B, Nausch B, Balemba OB, Bartoo AB, Nelson MT, Mawe GM. Gallbladder muscle disruption is an early and contributing factor in the development of gallstone disease. *Neurogastroenterol Motil.* 2011; 23(Supplement 1):7.
18. Rossi SS, Converse JL, Hofmann AF. High pressure liquid chromatographic analysis of conjugated bile acids in human bile: simultaneous resolution of sulfated and unsulfated lithocholyl amides and the common conjugated bile acids. *J Lipid Res.* 1987; 28(5):589–95. [PubMed: 3598401]
19. Jeske DJ, Dietschy JM. Regulation of rates of cholesterol synthesis in vivo in the liver and carcass of the rat measured using [^3H]water. *J Lipid Res.* 1980; 21(3):364–76. [PubMed: 7189770]
20. Duncan IW, Culbreth PH, Burtis CA. Determination of free, total, and esterified cholesterol by high-performance liquid chromatography. *Journal of chromatography.* 1979; 162(3):281–92. [PubMed: 528596]
21. Turley SD, Dietschy JM. Re-evaluation of the 3 α -hydroxysteroid dehydrogenase assay for total bile acids in bile. *J Lipid Res.* 1978; 19(7):924–8. [PubMed: 712252]
22. Bartlett GR. Phosphorus assay in column chromatography. *J Biol Chem.* 1959; 234(3):466–8. [PubMed: 13641241]
23. Carey MC. Critical tables for calculating the cholesterol saturation of native bile. *J Lipid Res.* 1978; 19(8):945–55. [PubMed: 731129]
24. Wang DQ, Paigen B, Carey MC. Phenotypic characterization of Lith genes that determine susceptibility to cholesterol cholelithiasis in inbred mice: physical-chemistry of gallbladder bile. *J Lipid Res.* 1997; 38(7):1395–411. [PubMed: 9254065]
25. Wang HH, Portincasa P, Liu M, Tso P, Samuelson LC, Wang DQ. Effect of gallbladder hypomotility on cholesterol crystallization and growth in CCK-deficient mice. *Biochim Biophys Acta.* 2009
26. Portincasa P, Di Ciaula A, vanBerge-Henegouwen GP. Smooth muscle function and dysfunction in gallbladder disease. *Curr Gastroenterol Rep.* 2004; 6(2):151–62. [PubMed: 15191695]
27. Xiao ZL, Chen Q, Amaral J, Biancani P, Jensen RT, Behar J. CCK receptor dysfunction in muscle membranes from human gallbladders with cholesterol stones. *Am J Physiol.* 1999; 276(6 Pt 1):G1401–7. [PubMed: 10362643]

28. Cong P, Pricolo V, Biancani P, Behar J. Effects of cholesterol on CCK-1 receptors and caveolin-3 proteins recycling in human gallbladder muscle. *Am J Physiol Gastrointest Liver Physiol.* 2010; 299(3):G742–50. [PubMed: 20558763]
29. Xiao Z, Schmitz F, Pricolo VE, Biancani P, Behar J. Role of caveolae in the pathogenesis of cholesterol-induced gallbladder muscle hypomotility. *Am J Physiol Gastrointest Liver Physiol.* 2007; 292(6):G1641–9. [PubMed: 17307729]
30. Xiao ZL, Biancani P, Behar J. Role of PGE2 on gallbladder muscle cytoprotection of guinea pigs. *Am J Physiol Gastrointest Liver Physiol.* 2004; 286(1):G82–8. [PubMed: 12936912]
31. Xiao ZL, Amaral J, Biancani P, Behar J. Impaired cytoprotective function of muscle in human gallbladders with cholesterol stones. *Am J Physiol Gastrointest Liver Physiol.* 2005; 288(3):G525–32. [PubMed: 15486345]
32. Xiao ZL, Chen Q, Biancani P, Behar J. Abnormalities of gallbladder muscle associated with acute inflammation in guinea pigs. *Am J Physiol Gastrointest Liver Physiol.* 2001; 281(2):G490–7. [PubMed: 11447029]
33. Xiao ZL, Chen Q, Amaral J, Biancani P, Behar J. Defect of receptor-G protein coupling in human gallbladder with cholesterol stones. *Am J Physiol Gastrointest Liver Physiol.* 2000; 278(2):G251–8. [PubMed: 10666049]
34. Morales S, Camello PJ, Alcon S, Salido GM, Mawe G, Pozo MJ. Coactivation of capacitative calcium entry and L-type calcium channels in guinea pig gallbladder. *Am J Physiol Gastrointest Liver Physiol.* 2004; 286(6):G1090–100. [PubMed: 14739141]
35. Camello-Almaraz C, Macias B, Gomez-Pinilla PJ, Alcon S, Martin-Cano FE, Baba A, et al. Developmental changes in Ca^{2+} homeostasis and contractility in gallbladder smooth muscle. *Am J Physiol Cell Physiol.* 2009; 296(4):C783–91. [PubMed: 19211915]
36. Macias B, Gomez-Pinilla PJ, Camello-Almaraz C, Pascua P, Tresguerres JA, Camello PJ, et al. Aging impairs Ca^{2+} sensitization pathways in gallbladder smooth muscle. *Age (Dordr).* 2011
37. Behar J, Rhim BY, Thompson W, Biancani P. Inositol trisphosphate restores impaired human gallbladder motility associated with cholesterol stones. *Gastroenterology.* 1993; 104(2):563–8. [PubMed: 8425698]
38. Parkman HP, James AN, Bogar LJ, Bartula LL, Thomas RM, Ryan JP, et al. Effect of acalculous cholecystitis on gallbladder neuromuscular transmission and contractility. *J Surg Res.* 2000; 88(2): 186–92. [PubMed: 10644487]
39. Parkman HP, James AN, Thomas RM, Bartula LL, Ryan JP, Myers SI. Effect of indomethacin on gallbladder inflammation and contractility during acute cholecystitis. *J Surg Res.* 2001; 96(1):135–42. [PubMed: 11181007]
40. Amaral J, Xiao ZL, Chen Q, Yu P, Biancani P, Behar J. Gallbladder muscle dysfunction in patients with chronic acalculous disease. *Gastroenterology.* 2001; 120(2):506–11. [PubMed: 11159891]
41. Wang DQ, Tazuma S, Cohen DE, Carey MC. Feeding natural hydrophilic bile acids inhibits intestinal cholesterol absorption: studies in the gallstone-susceptible mouse. *Am J Physiol Gastrointest Liver Physiol.* 2003; 285(3):G494–502. [PubMed: 12748061]
42. Rege RV, Prystowsky JB. Inflammation and a thickened mucus layer in mice with cholesterol gallstones. *J Surg Res.* 1998; 74(1):81–5. [PubMed: 9536979]
43. Lee SP, LaMont JT, Carey MC. Role of gallbladder mucus hypersecretion in the evolution of cholesterol gallstones. *J Clin Invest.* 1981; 67(6):1712–23. [PubMed: 7240416]
44. Guarino MP, Cong P, Cicala M, Alloni R, Carotti S, Behar J. Ursodeoxycholic acid improves muscle contractility and inflammation in symptomatic gallbladders with cholesterol gallstones. *Gut.* 2007; 56(6):815–20. [PubMed: 17185355]
45. Guarino MP, Carotti S, Morini S, Perrone G, Behar J, Altomare A, et al. Decreased number of activated macrophages in gallbladder muscle layer of cholesterol gallstone patients following ursodeoxycholic acid. *Gut.* 2008; 57(12):1740–1. [PubMed: 19022933]
46. Carotti S, Guarino MP, Cicala M, Perrone G, Alloni R, Segreto F, et al. Effect of ursodeoxycholic acid on inflammatory infiltrate in gallbladder muscle of cholesterol gallstone patients. *Neurogastroenterol Motil.* 2010; 22(8):866–73, e232. [PubMed: 20426797]
47. Xiao ZL, Rho AK, Biancani P, Behar J. Effects of bile acids on the muscle functions of guinea pig gallbladder. *Am J Physiol Gastrointest Liver Physiol.* 2002; 283(1):G87–94. [PubMed: 12065295]

48. Xiao ZL, Biancani P, Carey MC, Behar J. Hydrophilic but not hydrophobic bile acids prevent gallbladder muscle dysfunction in acute cholecystitis. *Hepatology*. 2003; 37(6):1442–50. [PubMed: 12774024]

Figure 1. Changes in biliary lipid compositions are observed in mice fed the lithogenic diet for only one week

Cholesterol saturation index (CSI) of bile (A) and hydrophobicity index (B) of bile salts are elevated in mice fed the lithogenic diet compared to mice fed the standard chow. CSI is corrected for urso- and muricholate because an increase in hydrophilic bile salts reduces micellar cholesterol solubility. C. Cholesterol crystals are present as early as one week, and most mice have crystals/stones by 2 weeks. D. An increase in the content of taurocholate (TC) and decrease in conjugated muricholate bile salts in LD fed-mice are responsible for the increased bile salt hydrophobicity since taurocholate (TC), taurochenodeoxycholate (TCDC) and taurodeoxycholate (TDC) (grayish shades) are more hydrophobic than tauro-alpha-muricholate (TaM), tauro-beta-muricholate (TbM), and tauroursodeoxycholate (TUDC) molecular species (bluish shades). Bile composition of mice fed a diet rich in cholic acid only resembled LD mice more closely while bile compositions of mice fed a diet enriched in cholesterol alone are similar to control mice (A, B and D). Neither the cholic acid- nor the cholesterol-enriched diets result in the presence of cholesterol crystals or stones in bile (C). Significantly different from control (chow) at $P < 0.05$ (*), $P < 0.001$ (***) and $P < 0.0001$ (****) (ANOVA with Bonferroni's multiple comparison test).

Figure 2. Mice fed a lithogenic diet do not show inflammatory changes until 4 weeks on the diet
 A. H & E stained sections of mouse gallbladder. Although subtle hyperplasia of the GBSM is observed in mice on the LD diet for 2 weeks, significant morphologic changes are seen only after 4 weeks on the LD. At 4 weeks, there is epithelial hyperplasia, more pronounced muscle hypertrophy and expansion of the lamina propria by inflammatory cells. At 8 weeks, the lamina propria continues to expand with mucosal folding into broad-based villi. There are inflammatory cells present in both the lamina propria and within the muscularis propria. In addition to hypertrophy, at 8 weeks the muscularis propria takes on a more disorganized architecture. B. Gallbladder sections immunostained with a monoclonal anti-mac-2 antiserum that detects macrophages and monocytes. Immunoreactive cells were sparse in mice fed chow or the LD for 1-2 weeks. At 4 weeks on the LD, a marked increase in the number of mac-2 positive cells is observed in the lamella propria, and extending into the muscularis propria.

Figure 3. Contractile responses of GBSM to cholinergic agonist are reduced in mice fed the LD for more than one week

A. Muscle tension recordings tension from mice fed a normal chow (upper trace) or LD (lower trace) for 8 weeks. The carbachol concentration was increased (0.1 to 10 μ M) every 4 minutes as indicated beneath the traces. LD mice have increased spontaneous basal activity, indicated as small variations in tension before the addition of the agonist (arrow). **B.** Maximal carbachol responses obtained at doses ranging from 3-10 μ M were reduced at 2 (* P <0.05), 4 (**** P <0.0001) and 8 (** P <0.01) weeks on LD (n >8). **C.** Maximal responses to KCl (80 mM) were reduced only at 4 weeks (**** P <0.0001) on LD (n >8). Contractility of GBSM in mice fed a cholic acid- or cholesterol-rich diet is comparable to control.

Figure 4. Disruption of synchronized and rhythmic activity in GBSM of mice fed a LD diet for 8 weeks

In control mice, rhythmic Ca^{2+} flashes are synchronized in a muscle bundle (traces from two different cells in one muscle bundle). After 8 weeks on the LD diet, Ca^{2+} flash activity is disorganized and three main patterns of Ca^{2+} activity are observed. Type I. Clusters of synchronized Ca^{2+} flashes interrupted by quiescent period (15-20 sec). Type II. Asynchronous Ca^{2+} flashes between GBSM cells of a single muscle bundle, with more or less rhythmic activity. Type III. Sporadic synchronized Ca^{2+} flashes with Ca^{2+} waves still active. Ca^{2+} flashes and Ca^{2+} waves throughout the recording period are indicated by orange and blue bars, respectively, beneath the traces. Disrupted flash activity would likely lead to decreased gallbladder tone and diminished intracellular Ca^{2+} stores.

Figure 5. Alterations in the occurrence of the various types of Ca^{2+} activities from 0-8 weeks on the LD

The three types of Ca^{2+} flash activity described for mice fed the LD for 8 weeks are found at earlier time points, and proportions vary with passage of time.

Figure 6. Time course of changes in basal and agonist-induced Ca^{2+} transient
A. In mice fed the standard chow, GBSM show rhythmic and synchronized Ca^{2+} flash activity as well as increased frequency of flashes in response to cholinergic activation. **B.** Basal activity was disrupted as early as the one-week time point, and at 4-8 weeks, carbachol failed to increase flash activity, but Ca^{2+} waves were observed.

Figure 7. Basal Ca^{2+} activity of GBSM in mice fed a cholic acid- or cholesterol-rich diet is comparable to mice fed a standard chow diet

In mice fed a diet enriched in cholic acid (upper trace) or cholesterol (lower trace) for 8 weeks, Ca^{2+} flashes are rhythmic and synchronized within a given bundle. In each set of traces, data from two GBSM cells from a single muscle bundle are illustrated. The orange bar under the trace represents Ca^{2+} flashes.