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Generating Ceramide from Sphingomyelin by Alkaline Sphingomyelinase in the Gut Enhances Sphingomyelin Induced Inhibition of Cholesterol Uptake in Caco-2 Cells

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Abstract

Background Sphingomyelin (SM) is present in dietary products and cell plasma membranes. We previously showed that dietary SM inhibited cholesterol absorption in rats. In the intestinal tract, SM is mainly hydrolyzed by alkaline sphingomyelinase (alk-SMase) to ceramide. **Aims** We investigated the influence of SM and its hydrolytic products ceramide and sphingosine on cholesterol uptake in intestinal Caco-2 cells. **Methods** Micelles containing bile salt, monoolein, and ^{14}C-cholesterol were prepared with or without SM, ceramide or sphingosine. The micelles were incubated with Caco-2 cells and uptake of radioactive cholesterol was quantified. **Results** We found that confluent monolayer Caco-2 cells expressed NPC1L1 and the uptake of cholesterol in the cells was inhibited by ezetimibe, a specific inhibitor of NPC1L1. Incorporation of SM in the cholesterol micelles inhibited cholesterol uptake dose-dependently; 38% inhibition occurred at en equal mole ratio of SM and cholesterol. The inhibition was further enhanced to 45% by pretreating the cholesterol/SM micelles with recombinant alk-SMase which hydrolyzed SM in the micelles by 85%, indicating ceramide has stronger inhibitory effects on cholesterol uptake. To confirm this, we further replaced SM in the micelles with ceramide and sphingosine, and found that at equal mole ratio to cholesterol, ceramide exhibited stronger inhibitory effect (50% vs 38%) on cholesterol uptake than SM, whereas sphingosine only had a weak effect at high concentrations. **Conclusion** Both SM and ceramide inhibit cholesterol uptake, the effect of ceramide being stronger than that of SM. Alk-SMase enhances SM-induced inhibition of cholesterol uptake by generating ceramide in the intestinal lumen.

Key words: cholesterol, sphingomyelin, ceramide, sphingosine, alkaline sphingomyelinase, Caco-2 cells.

Introduction

Cholesterol homeostasis depends on its de novo syntheses, catabolism, secretion into the bile, and intestinal absorption of biliary and dietary cholesterol. The small intestine plays an important role in regulating cholesterol homeostasis in the body. About 30% of plasma cholesterol is derived from intestinal absorption and about 37% reduction in plasma cholesterol levels could be achieved by inhibition of cholesterol absorption [1]. To control cholesterol absorption in the intestinal tract is an important strategy to lower plasma cholesterol and reduce the risk of cardiovascular diseases.

Cholesterol absorption in intestine is a multi-step process in which cholesterol is micellized by bile acids and phospholipids, taken up by the enterocytes, assembled into lipoproteins, and transported to the lymph and the circulation. The uptake of cholesterol by intestinal cells is an important step of cholesterol absorption and the process is mediated by a specific membrane protein named Niemann-Pick C1 like 1 (NPC1L1) protein, which can be specifically inhibited by ezetimibe [2]. Under physiological conditions, the intestinal absorption of cholesterol is incomplete; only approximately 50% of the ingested cholesterol is absorbed [3]. The rate-limiting factors are not well characterized.

SM is a type of sphingolipid composed of a sphingosine backbone, a fatty acid, and a polar phosphocholine headgroup. SM is particularly abundant in milk, egg and meat [4,5]. In the intestinal tract, SM and cholesterol co-exist in vesicles, liposomes and micelles.

SM is also present in the plasma membrane together with cholesterol and is enriched in the region of lipid raft. We reported previously that cholesterol absorption was inhibited by dietary SM in animals [6]. The finding was confirmed and extended by others who showed that milk SM was more effective than egg SM [6,7]. The inhibitory effect was attributed to the strong interaction of SM with cholesterol, leading to decreased thermodynamic activity of cholesterol [8].

In the intestinal tract, SM is mainly hydrolyzed by intestinal alkaline sphingomyelinase (alk-SMase), which was discovered by Nilsson and cloned by Duan et al [9,10]. The enzyme is specifically expressed in the intestinal tract and human liver and is at high levels in the middle of small intestine [11]. Alk-SMase shares no structural similarities with other SMases, and belongs to nucleotide pyrophosphatase phosphodiesterase (NPP) family, and is therefore also called NPP7 [12]. A unique property of alk-SMase is that its activity requires the presence of bile salt particularly taurocholate and taurochenodeoxycholate [13]. Hydrolysis of SM by alk-SMase generates ceramide, which is in turn further hydrolyzed by intestinal neutral ceramidase to sphingosine. Ceramide cannot be absorbed while sphingosine can be effectively absorbed by enterocytes [14] [15]. Although the inhibitory effects of SM on cholesterol absorption have been reported, the effects of hydrolytic products of SM by alk-SMase have not been closely studied. The present study is to investigate the role of ceramide, sphingosine and alk-SMase in cholesterol uptake in human intestinal Caco-2 cells.

Materials and Methods

Materials

Caco-2 cells were obtained from American Tissue Culture Collection (Manassas, VA, USA). The Modified Eagle Medium (DMEM), M199 medium, heat-inactivated fetal bovine serum (FBS), 1% non-essential amino acids and other materials were purchased from either Invitrogen or Sigma-Aldrich (Stockholm, Sweden). [^{14}C] cholesterol (50 mCi/mmol) was purchased from American Radiolabeled Chemicals Inc (St. Louis, MO, USA). C_{16}-Ceramide, sphingosine and monoolein were purchased from Larodan Fine Chemicals AB (Malmö, Sweden). SM was purified from bovine milk by The Swedish Dairies Association as described [16]. Recombinant human alk-SMase was expressed in yeast cells and purified in our laboratory as described [17]. Ezetimibe was kindly provided by Schering-Plough Research Institute (Kenilworth NJ, USA). NPC1L1 antibody was purchased from Santa Cruz (Santa Cruz, USA).

Preparations of delipidized fetal bovine serum and cholesterol micellar solution

The preparation of delipidized FBS was according to Gibson *et al* [18]. In brief, 20 g thixotropic gel powder (Cab-o-sil, Kodak) was added to 1 liter FBS and stirred overnight at 4 ℃. The mixture was then centrifuged at 15,000 rpm for 1 h at 4 ℃ and the supernatant was collected and sequentially filtered through 0.20 μm filter. For preparing micellar cholesterol solutions, M199 culture media containing 3 mM sodium taurocholate, 30 μM monoolein and 1 nM (1.25×10^5dpm) [^{14}C]cholesterol were mixed and sonicated, according to Field et al [19,20]. The micellar solution was then passed through a 0.20 μm filter before use.

Studies on uptake of micellar cholesterol by Caco-2 cells

CaCo-2 cells were cultured in DMEM, containing 10% FBS, 1% penicillin-streptomycin, 2 mM L-glutamate, 1% non-essential-amino acids and 3.7 g/l $NaHCO_3$ to 100% confluence. The expression of NPC1L1 was checked by Western blot. The cholesterol uptake was examined by the methods of Ikeda et al [21]. Before the experiment, the culture medium containing 10% FBS was replaced with the medium containing the delipidized FBS followed by culturing the cells for 24 h. The medium was then removed and the cells were washed three times with M199 buffer and then cultured in the fresh medium containing the cholesterol micelles for different times. After incubation, the medium was removed and the cells were washed three times with ice-cold PBS. The cell pellets were dissolved in 0.1 M NaOH and an aliquot of 0.1 ml of the lysate was taken for liquid scintillation counting. To confirm that the uptake of cholesterol under the conditions was mediated by NPC1L1 not a passive diffusion, some cells were pretreated with ezetimibe, the inhibitor of NPC1L1, and the changes of cholesterol uptake was examined. The results were expressed as dpm/mg protein in the lysate. The protein was quantified by a kit from Bio-Rad using serum albumin as a standard.

Effects of SM, ceramide and sphingosine on cholesterol uptake

To investigate the effects of SM and its hydrolytic products on cholesterol uptake, SM, or ceramide or sphingosine was incorporated into the cholesterol micelles at different ratios to that of cholesterol by the methods described above. The cells were then incubated with the micelles containing the individual sphingolipids added for 2 h and the cholesterol

uptake was analyzed as above. The changes of cholesterol uptake from the micelles with and without sphingolipids were examined.

To investigate whether the ceramide generated by alk-SMase could have any influence on cholesterol absorption, we treated the cholesterol/SM micelles with human recombinant alk-SMase expressed from yeast cells [17] at 37 ^{o}C for 24 h. The alk-SMase treated micelles were then incubated with the cells for 2 h as above. The uptake of cholesterol by the cells was determined, comparing with the micelles that had not been treated with alk-SMase.

Isolation of lipids and quantification of SM

To examine whether treating the cholesterol/SM micelles with alk-SMase induced hydrolysis of SM in the micelles, a small amount of [^{14}C]-choline labeled SM (25 pmole) was mixed with 1 nM unlabeled SM and incorporated into the cholesterol micelles. After treating the micelles with alk-SMase, the total lipids in the micelle solution were extracted according to the method of Bligh and Dyer [22] and subjected to silica gel plates (60 F, 0.25 mm) for TLC, with an internal standard. The plates were developed in chloroform/methanol/25% ammonium hydroxide (65:25:4, v/v/v), and the SM bands were visualized by iodine vapour. The bands were scraped according to the standard position of SM. The radioactivity of SM was measured by liquid scintillation counting [9].

Western blot Analysis

Caco-2 cells were lysed and the cell free extract was prepared as described [9]. 40 µg of proteins in cell lysate were subjected to 7.5% SDS PAGE. The resolved proteins in the gel were transferred to a nitrocellulose membrane electrophoretically overnight. The membranes were incubated with anti NPC1L1 antibody (1:5000) and then with second antibody (1:50000) conjugated with horseradish peroxidase. The specific NPC1L1 bands (145 kD) were identified by enhanced chemiluminescence advance reagent. The membranes were then stripped and re-probed with anti-actin antibody.

Statistical analysis

The results are presented as mean ± S.E.M. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by the Bonferroni posttest for multiple comparisons. Differences were considered significant at $P<0.05$. All analyses were performed using GraphPad Prism 4.0 software Inc.

Results

NPC1L1 was expressed in monolayrer Caco-2 cells and mediating uptake of micellar cholesterol into the cells

Since the cholesterol uptake is known to be mediated by NPC1L1, we first examined whether the monolayer Caco-2 cells express NPC1L1 and whether the uptake of cholesterol can be inhibited by ezetimibe, a specific inhibitor for NPC1L1. As shown in penal C of Fig. 1, Western blot clearly demonstrated the expression of NPC1L1 protein in these cells. We then incubated the cells with the micelles containing 0.02 µCi/ml

[^{14}C] cholesterol, and found that the uptake of the cholesterol increased with the time linearly (panel A). Such an uptake was significantly inhibited by ezetimibe in a dose dependent manner (panel B).

Cholesterol uptake was inhibited by SM, and the inhibition was enhanced by treating the micelles with alk-SMase.

To elucidate the effects of SM on cholesterol uptake in Caco-2 cells, milk SM was incorporated into the micelles. The influences of cholesterol uptake were demonstrated in Fig. 2. There was a significant decrease in cholesterol uptake in the presence of SM, a 38% inhibition occurred at the cholesterol: SM mole ratio of 1.0. Since alk-SMase co-exists with micelles in the intestinal lumen, we treated the cholesterol/SM micelles with alk-SMase followed by incubating the treated micelles with Caco- 2 cells. We found that pretreating the micelles with alk-SMase did not reverse SM-induced inhibition rather enhanced SM-induced inhibition of cholesterol uptake (Fig. 3). To confirm that alk-SMase did hydrolyze the SM in the micelles, the SM levels in the micelles with and without alk-SMase treatment were assayed by TLC. As shown in the lower panel of Fig. 3, SM levels in the alk-SMase treated micelles were decreased by about 85%. The results indicate that ceramide generated by alk-SMase may have stronger inhibitory effects on cholesterol uptake than SM.

Ceramide strongly and sphingosine weakly inhibits cholesterol absorption

To further confirm that ceramide is also an inhibitor of cholesterol uptake, we replaced SM in the micelles with C16-ceramide or sphingosine at different concentrations and

found that ceramide in the micelles did inhibit cholesterol uptake in a dose-dependent manner (Fig.4 upper panel). At the equal molar ratio to cholesterol, the inhibitory effect induced by ceramide was stronger than that induced by SM (50% vs. 38%). However, sphingosine, when incorporated into the micelles showed no inhibitory effects on cholesterol uptake at equal mole ratio to cholesterol (Fig. 4, lower panel). But at high concentrations sphingosine could also inhibit cholesterol uptake.

4. Discussion

This study aimed to examine the effects of SM hydrolytic products on cholesterol uptake in monolayer Caco-2 cells. Since the specific transporter for cholesterol uptake in the enterocytes is NPC1L1 [23], we first try to confirme whether there is a functional NPC1L1 in Caco-2 cells. A recent study performed by Garmy et al [24] showed the presence of mRNA of NPC1L1 in Caco-2 cells. In the present study, we extended their findings by Western blot analysis showing that the mRNA is translated to NPC1L1 protein and that the NPC1L1 protein functions effectively in nonolayer Caco-2 cells, as the uptake of cholesterol could be inhibited by ezetimibe in a dose dependent manner. Such inhibitions also indicate that the uptake of cholesterol in our system was mainly mediated by NPC1L1, not by passive diffusion or exchange of the cholesterols in the micelles and enterocyte plasma membrane.

Previous studies showed that feeding dietary SM in rat inhibited cholesterol absorption [6,7] by a mechanism related to the decreased thermodynamic activity of cholesterol in micelles by SM [8], due to close interaction between SM and cholesterol [25,26]. The

present study showed that not only SM but also ceramide, the hydrolytic product of SM in the micelles, inhibited cholesterol uptake. Both treating cholesterol/SM micelles with alk-SMase that hydrolysed 85% SM and replacing SM in the micelles with ceramide inhibited cholesterol uptake. Furthermore, the inhibition induced by ceramide appears more potent than that of SM, as at eaual molar ratio to cholesterol, the inhibition induced by SM and ceramide was 38% and 45%, respectively.

The finding that ceramide inhibits cholesterol uptake is of physiological importance. Ceramide is the backbone of all types of sphingolipids and in the gut it can be generated from hydrolysis of SM and other dietary sphingolipids. Ceramide cannot be absorbed readily but in turn be further degraded by ceramidase to sphingosine [14,27]. However, the process of ceramide degradation in the intestine is slow and incomplete. Considerable amount of non hydrolyzed ceramide is present along the GI tract including the colon [28,29]. Such a long-term existing in the gut can play important roles in inhibition of cholesterol uptake and be one of the factors that contribute the partial absorption of cholesterol in the intestine.

In the intestinal tract, alk–SMase is the key enzyme that generates ceramide [30]. It is an ecto enzyme on the apical surface of the enterocytes [9,31] and can be dissociated into the intestinal lumen in an active form by either bile salt [32] or pancreatic trypsin [33]. The influences of alk–SMase on colon cancer and intestinal inflammation have been examined in the recent years [17,30,34,35], its role in cholesterol absorption has not been examined before. In this study, we found that alk-SMase can enhance SM induced

inhibition of cholesterol uptake by generating ceramide in the lumen. This finding is also of physiological relevance, as alk-SMase activity is at the highest levels in the middle of small intestine where absorption of cholesterol occurs.

SM is also present in the plasma membrane. Previous study by Chen et al showed a significant decrease of cholesterol uptake when Caco-2 cells were treated with bacterial SMase [20] and that the contents of SM and cholesterol in the membrane also affect the synthesis of cholesterol in the cells [36]. Whether alk-SMase can also inhibited cholesterol uptake by hydrolyzing membrane bound SM in the intestinal cells is unknown. Such a question cannot be easily addressed in cell culture system, as the activity of alk-SMase is specifically depending on the presence of bile salts, particularly taurocholate at critical micellar concentration [13]. Such a concentration of bile salt can be toleranted by the mucosal cells in the gut under physiological conditions but not in the cell culture system. Future studies in alk-SMase knockout mice may give us important information.

The exact mechanism by which ceramide inhibited cholesterol uptake remains elusive. It might be related with the changed solubility of cholesterol in the micelle system. SM has been shown to facilitate the solubility of cholesterol [37] whereas formation of ceramide in bilayers may drive cholesterol from bilayer order phase to a crystal phase, thus decrease the solubility of cholesterol [38]. Further biophysical studies are required to disclose the mechanism. In this experiment we also found that sphingosine when incorporated into the micelles had inhibitory effect on cholesterol uptake. The finding is in agreement with the report of Garmy et al [24]. However, comparing the effects of SM

and ceramide, the efficacy of sphingosine was less potent and the inhibition required a relatively high concentration of sphingosine. This may be related to the different physical properties between ceramide and sphingosine, as sphingosine is water soluble and can be rapidly absorbed by intestinal cells [28]. Based on our findings, hydrolysis of SM to ceramide by alk-SMase may enhance whereas hydrolysis of ceramide by neutral ceramidase may decrease the inhibitory effect of sphingolipids on cholesterol uptake. The interplay of alk-SMase and neutral ceramidase may be a physiological factor that influences the cholesterol uptake.

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Legend for Figures

Fig. 1. Cholesterol uptake by monolayer Caco-2 cells is mediated by NPC1L1. The cells were incubated with radioactive micellar cholesterol for different times. After washing and lysis, the uptake of radioactive cholesterol in the cells was counted (panel A). In panel B. the cells were pretreated with ezetimibe at different concentrations for 2 h, and then incubated with radioactive micellar cholesterol for 2 h. The uptake of cholesterol in the absence of ezetimibe was normalized to 100%. Results are mean ± SEM of three separate determinations. $^{\#}P < 0.05$, $^{*}P < 0.01$. Panel C shows the expression of NPC1L1 in Caco-2 cells. The cells were cultured to 100% confluence. The cellular protein was extracted and subjected to Western blot for the expression of NPC1L1 and β-actin.

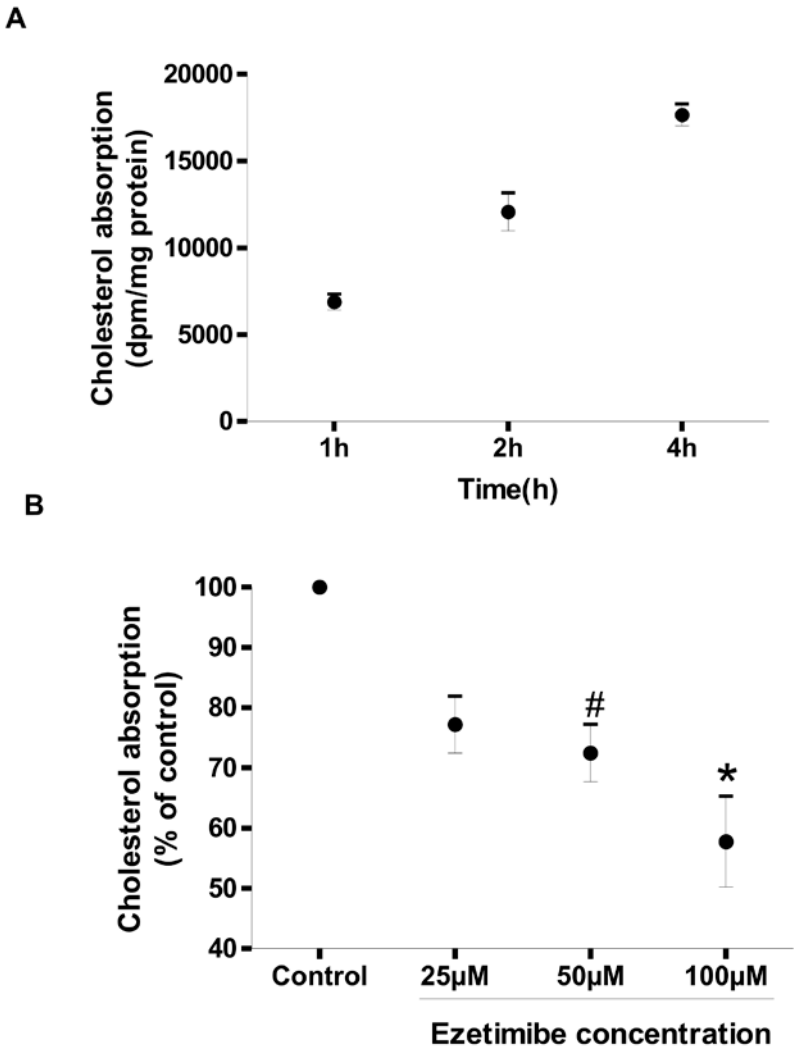
Fig. 2. Inhibitory effect of sphingomyelin (SM) on cholesterol uptake in Caco-2 cells. The cells were incubated with radioactive micellar cholesterol in the absence or presence of SM at different concentrations for 2 h. The uptake of cholesterol in the absence of SM was used as 100 %. Results are mean ± SEM from triplicate determinations in three separate experiments.

Fig. 3. Treating SM/cholesterol micelles with alk-SMase further inhibited cholesterol absorption. The [^{14}C]cholesterol/SM micelles were incubated with or without human recombinant alk-SMase at 37℃ for 24 h and then transferred to the cell culture system and incubated with the cells for 2 h. The uptakes of cholesterol in the cells from cholesterol micelles, cholesterol/SM micelles and cholesterol/SM micelles treated with

alk-SMase are shown in panel A. The cholesterol absorption with cholesterol micelles was taken as 100%. Results are mean ± SEM from triplicate determinations in three separate experiments. B. The change of SM amount in the micelles after alk-SMase treatment. A small amount (25 pmole) of [^{14}C]-SM was incorporated into the [^{14}C]cholesterol/SM micelles, followed by treating the micelles with human recombinant alk-SMase as above. The lipids were extracted and subject to TLC. The radioactivity of SM was determined as described in Methods. Results are expressed as mean ± SEM of three determinations.

Fig. 4. Effect of replacing SM in the cholesterol micelle with ceramide and sphingosine on cholesterol uptake in Caco-2 cells. The cells were incubated for 2 h with radioactive micellar cholesterol in the absence or presence of ceramide (A) or sphingosine (B) at different concentrations. The uptake of cholesterol in the absence of ceramide or sphingosine was used as 100%. Results are mean ± SEM from triplicate determinations in three separate experiments.

Fig.1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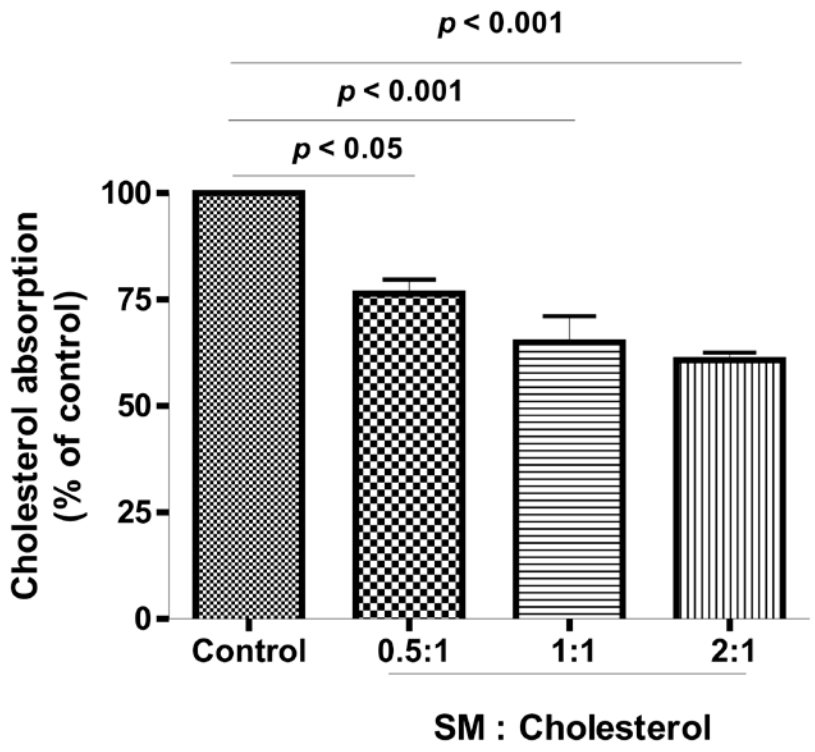
15000
10000
5000
0
1h
2h
4h
Time(h)
B
Cholesterol absorption (% of control)
100
90
80
70
60
50
40
#
*
Control
25μM
50μM
100μM
Ezetimibe concentration
C
NPC1L1
β-actin

Fig. 2.


p < 0.001
p < 0.001
p < 0.05
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50
25
0
Control
0.5:1
1:1
2:1
SM : Cholesterol

Fig. 3.

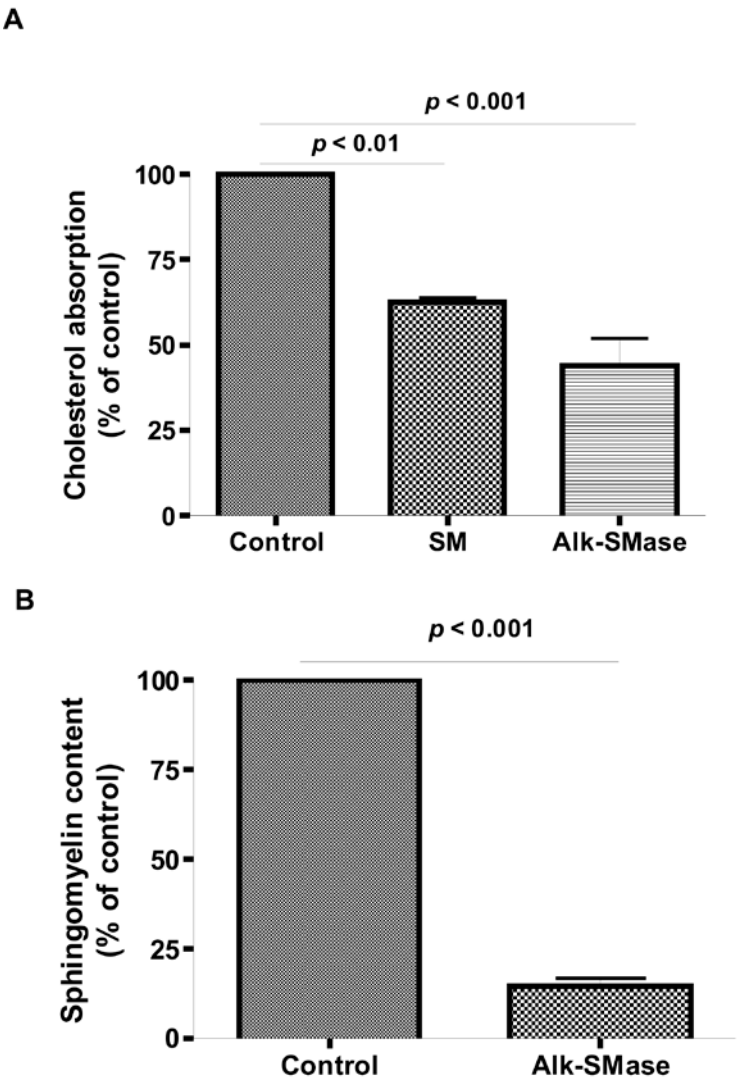
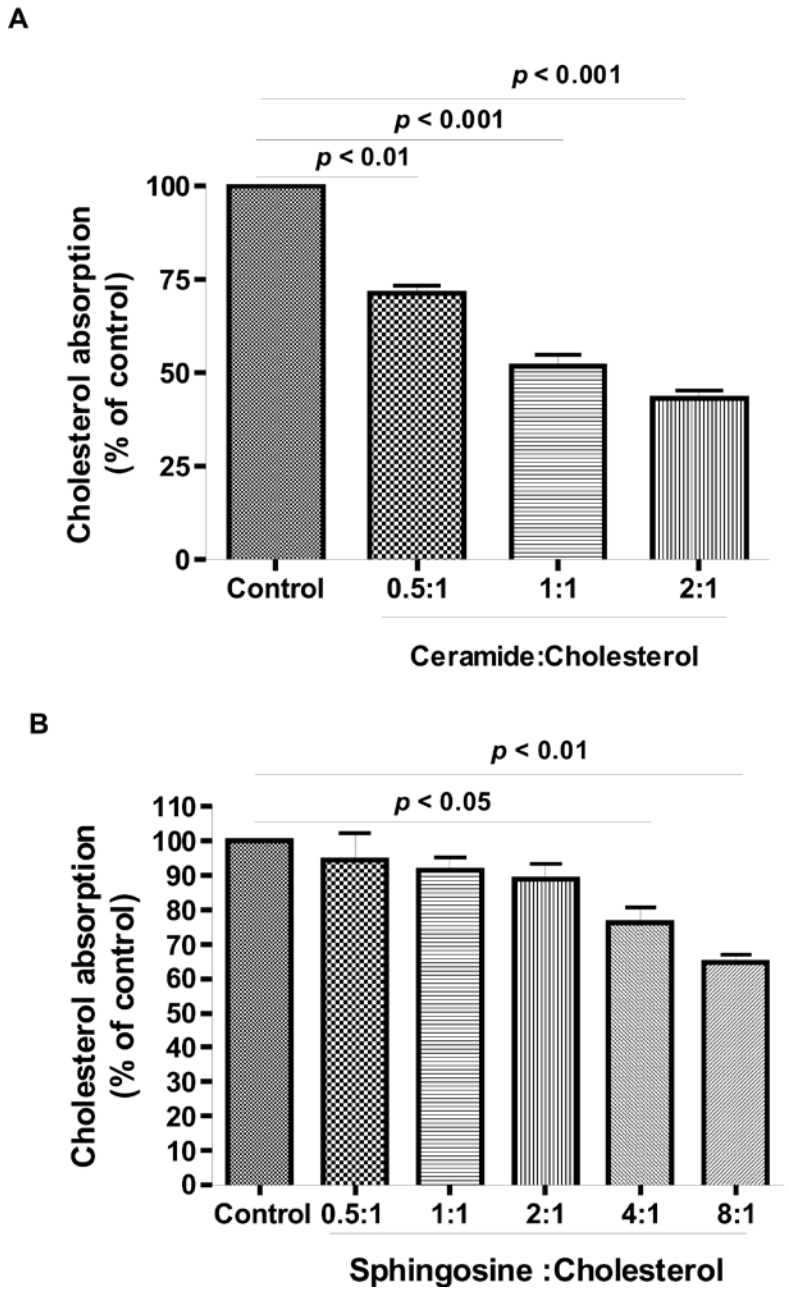

A
p < 0.001
p < 0.01
Cholesterol absorption (% of control)
100
75
50
25
0
Control
SM
Alk-SMase
B
p < 0.001
Sphingomyelin content (% of control)
100
75
50
25
0
Control
Alk-SMase

Fig. 4.


A
p < 0.001
p < 0.001
p < 0.01
Cholesterol absorption (% of control)
100
75
50
25
0
Control
0.5:1
1:1
2:1
Ceramide:Cholesterol
B
p < 0.01
p < 0.05
Cholesterol absorption (% of control)
110
100
90
80
70
60
50
40
30
20
10
0
Control
0.5:1
1:1
2:1
4:1
8:1
Sphingosine :Cholesterol