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Published in:
Journal of Lipid Research

DOI:
[10.1194/jlr.M012880](https://doi.org/10.1194/jlr.M012880)

2010

[Link to publication](#)

Citation for published version (APA):

Zhang, Y., Cheng, Y., Hansen, G. H., Niels-Christiansen, L.-L., Koentgen, F., Ohlsson, L., Nilsson, Å., & Duan, R.-D. (2010). Crucial role of alkaline sphingomyelinase in sphingomyelin digestion: A study on the enzyme knockout mice. *Journal of Lipid Research*, 52(4), 771-781. <https://doi.org/10.1194/jlr.M012880>

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Citation for the published paper:
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"Crucial role of alkaline sphingomyelinase in
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Journal of lipid research
2010 Dec 21
© the American Society for Biochemistry and
Molecular Biology

<http://dx.doi.org/10.1194/jlr.M012880>

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**Crucial role of alkaline sphingomyelinase in sphingomyelin digestion:
A study on the enzyme knockout mice**

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Running title: Sphingomyelin digestion in alk-SMase knockout mice

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Abstract

Alkaline sphingomyelinase (alk-SMase) hydrolyses sphingomyelin (SM) to ceramide in the gut. To evaluate physiological importance of the enzyme, we generated alk-SMase knockout (KO) mice by Cre-LoxP system and studied SM digestion. Both wild type (WT) and the KO mice were fed ^3H -palmitic acid labeled SM together with milk SM by gavage. The lipids in intestinal content, intestinal tissues, serum and liver were analysed by TLC. In KO mice, non-digested ^3H -SM in the intestinal content increased by 6 fold and the formation of ^3H -ceramide decreased markedly, resulting in 98% reduction of ^3H -ceramide/ ^3H -SM ratio 1 h after gavage. The absorbed ^3H -palmitic acid portion was decreased by 95%. After three hours, a small increase in ^3H -ceramide was identified in distal intestine in KO mice. In feces ^3H -SM was increased by 243% and ceramide decreased by 74% in the KO mice. The KO mice also showed significantly decreased radioactivity in liver and serum. Furthermore alkaline phosphatase activity in the mucosa was reduced by 50%, and histological comparison of two female littermates preliminarily suggested mucosal hypertrophy in KO mice. The study provides definite proofs for crucial roles of alk-SMase in SM digestion and points to possible roles in regulating mucosal growth and alkaline phosphatase function.

Key words. Alkaline sphingomyelinase, NPP7, sphingomyelin digestion, knockout mice, alkaline phosphatase, hypertrophy.

Introduction

Sphingomyelin (SM) is a major type of sphingolipid that is mainly present in membranes of mammalian cells and dietary products such as milk, egg, meat and fish. Our daily intake of SM is about 300 mg (1). SM in the diet has been shown to inhibit the colonic tumorigenesis in mice treated with chemical carcinogens (2), to promote the development of the intestinal mucosa in new born rats (3), and to inhibit cholesterol absorption in the gut (4, 5). Because most of these effects can be reproduced or linked to ceramide, a hydrolytic product of SM (6, 7), it is important to study the enzymes that hydrolyze SM and generate ceramide in the gut.

More than 40 years ago Nilsson identified a type of enzyme in the intestinal mucosa that hydrolyses SM and generates ceramide at alkaline pH (8). This enzyme was thereafter named alkaline sphingomyelinase (alk-SMase) (9). Due to the findings of important biological effects of sphingolipids in the last two decades (10-12), we performed several studies on alk-SMase. We have purified the protein (13, 14), cloned the gene (15, 16), and found potential implications of the enzyme in SM digestion (17), cell proliferation (18), colonic inflammation (19) and cholesterol absorption (7). We also found decreased alk-SMase activity in colonic diseases such as colon cancer and colitis (20-22), and identified inactive abnormal splicing forms of the enzyme in human colon and liver cancer cells (23, 24).

Besides alk-SMase, other SMases such as acid and neutral SMases were also identified in the intestinal tract (9). Cloning study showed that alk-SMase shares no structural similarities with either acid or neutral SMase but belongs to the ectonucleotide pyrophosphatase/phosphodiesterase (NPP) family (15). Being a novel member of this family, alk-SMase is also called NPP7. The gene encoding alk-SMase in human is *Enpp7* (Gene ID: 339221) that is located at chromosome 17q25. Orthologous genes have been identified in mouse (Gene ID: 238011), rat (Gene ID: 303729) (16), dog (Gene ID: 608627), chicken (Gene ID: 426249), monkey (Gene ID: 717416), frog (Gene ID: 100462924), and zebra fish (Gene ID: 557756). Alk-SMase has several unique properties

differing from other NPPs and SMases. It is expressed specifically in the intestinal mucosa in many species and additionally in human liver (25). In the gut it is abundant in the middle part of jejunum but low in the colon. Unlike acid and neutral SMases that are rapidly inactivated by pancreatic trypsin, alk-SMase is stable and resistant to trypsin digestion (26). As an ectoenzyme, alk-SMase is located on the surface of the mucosa and can be released into the lumen by both bile salt and pancreatic trypsin (27, 28). The active enzyme can be transported to the colon and high activity can be detected in the feces. The enzyme can hydrolyze SM both in the intestinal lumen and mucosa. Detailed information on the enzyme has been summarized in recent review articles (26, 29).

Based on the previous finding that SM digestion occurs mainly in the middle part of the small intestine where alk-SMase is abundant, alk-SMase is considered to be important for SM digestion (17). However, definite proof for the role of alk-SMase has not been obtained. In the present study, by Cre-LoxP system, we created alk-SMase knockout (KO) mice. Using this animal model, we examined the SM digestion and ceramide generation in the intestinal tract in wild type (WT) and the KO mice given ^3H -palmitoyl-SM. Our results show that the processes of SM digestion, ceramide formation, and fatty acid absorption are severely affected in the KO mice, providing the evidence for essential physiological roles of alk-SMase in SM digestion and ceramide generation in the gastrointestinal tract.

Materials and methods

Materials

Milk SM and (N- $^{14}\text{CH}_3$) choline-labeled milk-SM (^{14}C -SM) were provided from the Swedish Dairies' Association. ^3H -palmitoyl-labeled SM (^3H -SM) was obtained from American Radiolabeled Chemicals, Inc. (St Louis, MO, USA). Ceramide, sphingosine, palmitic acid, and triglyceride (TG) used as standard substances in TLC analysis were purchased from Sigma Chemical Co. (St. Louis, MO, USA). TLC Silica gel 60 plates (0.25 mm) were purchased from Merck (Darmstadt, Germany). Anti alk-SMase antibody was developed by AgriSera AB (Vännäs Sweden) as described before (14).

Genomic targeting and design

The Cre-LoxP system was used to generate alk-SMase KO mice. Targeting vector construction, gene targeting in embryonic stem (ES) cells, injection to generate chimeric mice and initial mouse breeding were performed by Ozgene Pty. Ltd. The gene *Enpp7* encoding alk-SMase for mouse was located on chromosome 11 and the sequence was obtained from the Ensembl Mouse genome database (Ensembl Gene ID: ENSMUSG00000046697). As shown in Fig. 1, loxP sites were inserted flanking exon 2 and a PGK-neo selection cassette was inserted downstream of exon 2, within the floxed region. Deletion of the floxed region by Cre recombinase results in a frame-shift mutation that creates an early stop codon in exon 3. The targeting vector contained an additional BglIII restriction enzyme site to allow screening for targeted clones by Southern blot hybridization.

The targeting vector was electroporated into W9.5 (129Sv/J) ES cells. Targeted ES cell clones generated by homologous recombination were identified by Southern blot hybridization using a 3' external probe (primers for 3' probe: 5'-CATCTGTAGCCTTGGGTTTCTGACG and 5'-AATGGAGGTGGGTGAGGGTTAATC). Chimeric mice were generated by injection of targeted ES cells into donor blastocysts followed by implantation of the injected blastocysts into pseudopregnant recipient mice. Chimeric mice were mated to C57BL/6 mice to produce heterozygous WT/flox mice, which were mated to OzCre mice (C57BL/6 background) to generate heterozygous WT/KO (*Enpp7*^{+/-}) mice. The genotype of the *Enpp7*^{+/-} mice was confirmed by Southern blot hybridization using the "en probe" (primers for en probe: 5'-AATGGCACTGTCCTCGCATGAATC and 5'-TGGATGTTTCAGAGTCAGACATGGTGG).

Heterozygous *Enpp7*^{+/-} mice were maintained on C57BL/6 background and backcrossed at least five times to get an inbred strain. Heterozygous *Enpp7*^{+/-} mice were then intercrossed to generate homozygous *Enpp7*^{-/-} (alk-SMase KO) mice. All mice were housed in the animal facilities in Lund University, and fed a commercial standard pellet

diet with free access to water. C57BL/6 wild type mice were obtained from Mollegaard Denmark. All experimental protocols were approved by the Animal Ethics Committee of Lund University.

Genotype analysis and identification of alk-SMase KO mice

Both Southern blot and PCR were used in genotype analysis. Genomic DNA was extracted from mouse tail using a kit purchased from Sigma GenElute™ Mammalian Genomic DNA Miniprep Kit (Helsingborg, Sweden), digested with BglII, and hybridized with the “en probe” to generate a 13.9 kb fragment for the WT allele, and a 4.5 kb fragment for the KO allele. For routine genotyping, genomic DNA was amplified by PCR using forward primer (5'-CTGCCACTTTACACTGGTCAC) and reverse primer (5'-TGGCACTGAGGCGAGAAC) to yield products of 732 bp for the WT allele and 247 bp for the KO allele, respectively (Fig.1). The locations of the PCR primers designed are shown in Fig.1 as arrows. The PCR program consisted of an initial incubation step at 95 °C for 10 min, followed by 35 cycles at 95 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 60 sec.

Animal experiment on SM digestion

Preparation of SM emulsion.

Forty μ l ^3H -SM (50 Ci/mol, 0.1 mCi/ml) dissolved in ethanol was taken to dryness under nitrogen. Fifteen mg unlabeled milk SM and 16 mg bile salt mixture (Sigma B#8756) in 10 ml 0.9% sodium chloride were added followed by sonication for 5×15 seconds. The suspension was then mixed with 5 ml skim milk followed by vortex for 1 min.

Calculating with a mean molecular mass of 775 for milk SM, the SM concentration of the emulsion was 3.2 μ mol per ml.

Animal treatment

After fasting for 16 h with free access to water, both WT and the KO mice were fed 0.3 ml emulsion containing 1 μ mol unlabeled milk-SM and 2 μ Ci ^3H -SM by gavage. After 1 and 3 h, the mice were anaesthetized with Xylazine/Ketamine. Blood samples were collected by cardiac puncture. Stomach, small intestine, colon, and liver were removed.

The small intestine was divided into three parts in equal length marked as S1, S2 and S3, which represent the proximal, middle and distal parts of small intestine, respectively. The segments were opened longitudinally, and each was rinsed in 5 ml 0.9% sodium chloride. The sodium chloride solution was then saved which represented the samples of intestinal content. Lipids in both intestinal contents and tissues were extracted as described below. In another study, the mice were fed the same amount of the SM emulsion, and the fecal samples were collected in a metabolism cage 1 and 2 days after the gavage. The total lipids in the fecal samples were extracted.

Lipid extraction and analysis

Lipids were extracted from the intestinal contents, tissues, and feces with chloroform : methanol : water according to Bligh and Dyer (30). The whole intestinal segment, 2 ml of the washout of the content, and a piece of the liver were used as the starting materials. The lipids extracted in the organic phase of the solution were collected, evaporated under nitrogen gas, and dissolved in 500 μ l of chloroform/methanol (2:1, v/v). 100 μ l of the extracted lipids were taken for TLC analysis. The plates were developed with toluene: methanol (70:30, v/v) to separate SM and ceramide. In this solvent system, R_f values of SM, sphingosine, and ceramide were 0.04, 0.14, and 0.57, respectively. To separate fatty acid and TG, the silica gel plates were developed with petroleum ether: ether: acetic acid (80:20:1, v/v). The R_f values of fatty acid and TG were 0.29 and 0.56, respectively. The bands were visualized with iodine, and the specific lipid bands were scraped according to the positions of the standards. The scraped band was mixed with 1ml of methanol/H₂O (1:1) overnight. Nine ml of Ready Value (Beckman Coulter) were added and the radioactivities in ³H-SM, ³H-ceramide, ³H-palmitic acid, and ³H-triglyceride (TG) were determined by liquid scintillation counting.

Morphological examination

Small pieces of the middle part of the small intestine from the WT and KO mice from the same littermate were excised and fixed in 4% paraformaldehyde in 0.1M sodium phosphate buffer (PB), pH 7.2 for 2 h at 4°C, briefly rinsed in PB and stored in 1%

paraformaldehyde in PB. After washing in PB the tissue samples were dehydrated and embedded in paraffin. Sections of 5 μm were cut in a Leica RM 2165 microtome. The sections were dewaxed and stained in Haematoxyline and Eosin-Phloxine. After mounting in Vecto mount H-5000 permanent the sections were examined in a Leica DM 4000 B microscope equipped with a Leica DC 300 FX camera. Pictures from the middle part of the small intestine were taken at random from different sections of WT and KO mice and the widths of the villi, the crypts and the thickness of the muscle layer under the crypts were measured.

Sphingomyelinase assay

The activities of SMases were determined as described previously (31). For alk-SMase assay, 5 μl samples were mixed with 95 μl of 50 mM Tris-HCl buffer, pH 9.0, containing 0.15 M NaCl, 2 mM EDTA, 6 mM taurocholate, 100 mM milk SM and 0.80 μM [^{14}C]-choline labeled SM to a final volume of 100 μl , followed by incubation at 37 °C for 30 min. The reaction was terminated by adding 0.4 ml of chloroform/methanol (2/1, v/v). After phase partition, the cleaved ^{14}C -phosphocholine in the upper phase was measured by liquid scintillation counting. The activities of neutral and acid SMases were measured similarly except small modifications. Neutral SMase was assayed in 50 mM Tris buffer containing 2 mM MgCl_2 , 0.15 M NaCl, and 0.12% Triton X 100, pH 7.5. Acid SMase was determined in 50 mM Tris-maleate buffer containing 0.15 NaCl and 6 mM Triton X 100, pH 5.0. Due to the low activities of acid and neutral SMase, unlabeled SM was not added in the assay system (31).

Western blotting for alk-SMase

The Western blotting for alk-SMase in the intestinal content was performed as described before (15). The intestinal content in the small intestine was rinsed in 20 ml of saline. After centrifugation, the supernatant was concentrated by about 10 fold by ultrafiltration with the cut off size of the membrane being 10 KD. Fifty μl of the concentrated intestinal content were subjected to 10% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blotted with anti-human alk-SMase antibody (1:5000 dilution) for 2 h. and then reacted with anti-rabbit IgG antibody conjugated with horseradish peroxidase

(1:50000 dilution) for 1 h at room temperature. The alk-SMase bands were identified by ECL advance reagents and the emitted light was recorded on Kodak X-ray film.

Other biochemical assays

The small intestine was divided into three parts in equal length as above and was opened longitudinally. Each segment was rinsed in 5 ml 0.9% sodium chloride solution and the collected solutions represent the samples of intestinal content. The mucosa was scraped and homogenized as described previously (9). The activities of the enzyme in both the mucosa and content were determined. The acid and alkaline phosphatase activities were analyzed using *p*-nitrophenylphosphate as a substrate (32). The neutral ceramidase was assayed using ¹⁴C-labeled C16-ceramide as a substrate (33). Bile salt stimulated lipase (BSSL) was assayed using *p*-nitrophenylacetate as substrate (34). The aminopeptidase N (APN) activity was measured by the method of Amoscato et al (35), using alanine-*p*-nitroanilide as substrate. The protein was assayed by a DC protein assay kit obtained from Bio-Rad with bovine albumin as a standard. The activities of the enzymes analyzed in mucosal samples were adjusted to the protein levels in the samples, and those in the intestinal content were adjusted with the sample volume.

Statistical analysis

Data were expressed as means \pm SEM. Statistical analysis was performed by two tails and unpaired Student *t* test. *P* < 0.05 was considered as statistically significant.

RESULTS

Generation of alk-SMase knockout mice

Screening the ES cells with the homologous recombinant gene was performed by Southern blot using the 3' probe. As shown in Fig. 2, after BglII digestion, the negative ES cells yielded a 13.9 kb band corresponding to the WT allele of *Enpp7*, whereas the targeted ES cells with the homologous recombinant yielded an additional 8.8 kb band (Fig. 2A). A targeted ES clone was used to produce chimeras and subsequently heterozygous mice. Heterozygous *Enpp7*^{+/-} mice were genotyped by Southern blot with "en probe" (Fig. 2B). The WT mice showed a band of 13.9 kb whereas the heterozygous

showed an additional band with 4.5 kb. After backcross, genotype of WT, heterozygous and homozygous KO mice was determined by Southern blot and later by PCR with primers indicated in Fig. 1. A representative PCR result is shown in Fig. 2C. WT (+/+) mice gave a 732 bp band, whereas heterozygous (+/-) mice gave an additional 247 bp band and the KO (-/-) mice, only a 247 bp band (Fig. 2C). To further confirm the successful knockout, the alk-SMase activities in the small intestinal mucosa, intestinal content and in the feces were assayed. As shown in the Fig. 3, little activity could be identified in the KO mice in mucosa, content and feces. Western blot showed no protein band in the intestinal content of the KO mice.

Interbreeding of the heterozygous *Enpp7*^{+/-} mice yielded WT, heterozygous, and homozygous offspring at expected similar Mendelian ratios, without the appearance of embryonic lethality in the mutant mice. The sexual ratio (male: female) was approximate 1:1 either for the total offspring or the homozygous mutant. Intercrossing of the homozygous KO mice produced homozygous mutant offspring, indicating that fertility of males and females was largely unaffected. The alk-SMase KO mice grew normally without obvious abnormalities. They showed no significant changes in appearance, behavior, body weight, and the length of intestine (data not shown). Microscopic examination of one KO and one WT mouse from the same littermate did not find gross disorganization of the mucosal structure, but signs of hypertrophy of the intestinal epithelium in the KO mouse was indicated (Fig.4 and Table I), as the widths of the villi and crypts, as well as the thickness of muscle layer under the crypts appear increased. However, no spontaneous tumorigenesis either in the small intestine or in the colon was identified in 6 KO mice up to the age of 10 months.

Activities of other Intestinal enzymes

Whether the deficiency of alk-SMase affects other types of SMase in the small intestine is shown in Fig. 5. Panel A and B show again a clear abolishment of alk-SMase in proximal, middle and distal intestinal mucosa and content. Panel C shows a small but statistically significant increase in acid SMase in the middle part of the small intestine. Panel D shows no significant changes in neutral SMase activity between the WT and the

KO mice. Liver is an organ with relatively high activity of acid SMase. Disruption of alk-SMase had no significant effect on acid or neutral SMase expression in the liver (panel C and D). Panel A also shows a lack of alk-SMase activity in the liver of WT mice, which is in agreement with previous finding that alk-SMase is only expressed in human liver, and not the liver of other species (16, 25).

The activities of other lipolytic enzymes including ceramidase, BSSL, alkaline phosphatase, acid phosphatase, and APN are shown in Table II. While the activities of most of the enzymes tested did not show significant alterations in the KO mice, the activities of alkaline phosphatase in KO mice were reduced in the mucosa by about half in all three segments of the small intestine compared to WT mice. However, differing from the changes of alk-SMase, no reduction of alkaline phosphatase activity in the intestinal content was identified. If anything, a slight increase of the activity was indicated towards the distal part of the small intestine.

SM digestion and ceramide generation in alk-SMase KO mice

The changes in the ability to digest SM in the KO mice were then examined. As shown in Table III, 1 h after feeding the mice 0.3 ml emulsion containing 1 μ mol unlabeled milk-SM and 2 μ Ci of fatty acid labeled 3 H-SM, the total radioactivity in the content was found mainly in stomach and small intestine but not in the colon, whereas after 3 h, significant amounts of radioactivity was present in the colon. The total radioactivity after 1 h in the small intestinal content in the KO mice was significantly higher than in the WT mice, whereas at 3 h, the difference was most obvious in the colon indicating that the non-absorbed SM had passed the small intestine and part of the radioactivity could already have been excreted.

The total 3 H activities in the serum were also measured. In 50 μ l of serum, the mean dpm for WT were 335 ± 62 and 636 ± 70 at 1 and 3 h, respectively, whereas those in the KO mice were 84 ± 12 and 162 ± 24 , respectively. The serum radioactivities in the KO mice at both time points were thus about 25% of those in the WT mice.

The lipids were then extracted in both intestinal content and mucosa and analyzed by TLC. Fig 6 shows the results for ^3H -SM, ^3H -ceramide and their ratios in the intestinal content 1 h after administration. Comparing with the WT mice, the amount of ^3H -SM in the KO mice was significantly higher in the middle and distal parts of small intestine. The amount of ^3H -ceramide was significantly lower in all three parts of small intestine. If total levels in the small intestine were calculated, ^3H -SM was 6 fold higher and ^3H -ceramide fivefold lower in the KO than in the WT mice. The ratio of ^3H -ceramide/ ^3H -SM in the intestinal content was markedly decreased. The maximal decrease occurred at the middle part of the small intestine where alk-SMase is normally enriched (9). At this part, the ratio is 3.87 for the WT mice and 0.12 for the KO mice, indicating a low rate of SM hydrolysis in the KO mice.

Since ceramide formed by alk-SMase is further hydrolyzed by mucosal ceramidase, we therefore asked whether the decreased ^3H -ceramide formation in the KO mice also decreased the amount of unesterified ^3H -fatty acid (FA) in the gut lumen. As shown in Figure 7A, the amount of ^3H found in the FA fraction in the KO mice was less than 10 % of that found in the WT mice. The absorbed ^3H -FA acid in the intestinal mucosa was decreased by 86% in the upper, 95% in the middle, and 94% in the distal third of the small intestine (Fig. 7B), accompanied by a similar decrease in ^3H labeled TG in the intestinal mucosa in the KO mice (Fig. 7C).

Three hours after gavage, the levels of ^3H -SM, ^3H -ceramide and ^3H -palmitic acid in the intestinal content and the absorbed ^3H -palmitic acid are shown in Table IV. Comparing with the results 1 h after gavage (Fig. 6), accumulation of ^3H -SM in the KO mice was found mainly occurred in the distal part of the small intestine (S3), where it was 6 fold higher than in the WT mice. Interestingly, ceramide levels in S1 and S2 segments of KO mice were lower than in the WT mice, but in S3, it was increased to a level higher than WT, although statistical significance was not reached. At this time point, the ratios of ceramide/SM were still significantly lower in the KO mice than in WT mice. The amount of ^3H -FA in the content or absorbed in the mucosa was very low in the KO mice also after 3 h.

The radioactivity in the fractions of FA and TG in the liver 1 and 3 h after administration is shown in Fig.8. The levels in the KO mice were 62% and 87% lower than in the WT mice, 1 and 3 h after administration, respectively. Radioactivities in lipid classes other than TG and FA in the liver were all negligible (data not shown).

We finally examined the excreted ^3H -SM and ^3H -ceramide in the feces collected 1 and 2 days after feeding. Comparing with WT mice, ^3H -SM was increased by 278% and ^3H -ceramide was decreased in the KO mice by about 60% in the feces collected after 1 day (Fig. 9A). On the second day, higher fecal excretion of ^3H -SM in the KO mice than in the WT mice was still observed (Fig. 9B). However, at this time, the excreted ^3H -ceramide was equal or higher in the KO mice than in the WT mice. Such an increase in ^3H -ceramide excretion was small comparing with the changes of SM, and the ratio of ceramide/SM was still significantly lower in the KO mice than in the WT ones (Fig. 9C).

Discussion

The present study reports the successful generation of alk-SMase knockout mice and proves the importance of alk-SMase in SM digestion in the intestinal tract.

The alk-SMase KO mice were generated by Cre-LoxP system. The successful knocking out of the enzyme was clearly shown by abolishment of the enzyme activity in the intestinal mucosa, intestinal content and feces; by the partial decrease of the activity in the heterozygous animals, and by a disappearance of the protein in the gut on Western blotting. Cre-LoxP system is a newly developed method that can be used for generation either conventional or tissue specific conditional KO mice. This study was performed in the conventional alk-SMase KO mice for two reasons. First the conventional KO mice exhibited no embryonic lethality and the mice had normal global phenotypes during the period they were observed i.e. up to the age of 10 months. Second, our previous studies showed that the expression of the enzyme was highly tissue specific, and the enzyme activity as well as the expressed protein were only found in the intestinal mucosa in many

species studied (25). Although the enzyme was additionally expressed in human liver and released in the bile, the liver form was not found in other species including rat, mouse, pig, rabbit, and baboon (16, 25, 36).

Although previous studies indicated that alk-SMase is an important enzyme in SM digestion, the concept was based indirectly on the finding that hydrolysis of SM occurs mainly in the middle part of the small intestine, where alk-SMase is high (17), and on the properties of the enzyme, in particular its dependence on certain bile salts (taurocholate or taurochenodeoxycholate), its protease resistance and its location at the brush border and in the gut lumen. Definite proof is, however, needed as the mucosa also contains acid and neutral SMases, and intestinal bacteria may be able to hydrolyze SM (37).

The present study is the first investigation performed in alk-SMase KO mice on SM digestion. Two time points, 1 and 3 h were examined after giving the mice SM. Our results show that SM digestion mainly occurred in 1-2 h in mice, because at 3 h, much of the remaining radioactivity had reached the colon and some may already be excreted in the feces. One hour after feeding, the KO mice showed an increased accumulation of ^3H -SM associated with a decreased ^3H -ceramide at all levels of the small intestine; the difference was most obvious in the middle part of the small intestine. Based on the ratio of ^3H -ceramide to ^3H -SM (3.87 vs. 0.12), the hydrolysis rate of SM in the WT mice is therefore much higher than that in the KO mice. As expected, the decreased formation of ^3H -ceramide caused more than 95% reduction of the absorption of ^3H -fatty acid formed from ceramide. The reduced fatty acid absorption was associated with a reduced radioactivity in TG in the mucosa, supporting the previous conclusion that dietary SM and ceramide do not penetrate into the mucosal cell but a sequential hydrolysis of SM to ceramide and then to free fatty acid and sphingosine is necessary for effective absorption (17, 38). The big change in the ^3H -ceramide/ ^3H -SM ratio in the intestinal content and big change in the amount of ^3H -palmitic acid absorbed prove that alk-SMase is a crucial enzyme in SM digestion in the intestinal tract under physiological conditions.

Comparing the data between 1 and 3 h, one finding is worth noticing. At 1 h, the levels of ^3H -ceramide in the intestinal content were lower in the KO mice than in the WT mice in all parts of the small intestine, whereas at 3 h, the levels of ceramide at the distal part of the small intestine were higher than in the WT mice. Obviously the decreased digestion of ^3H -SM in the upper two thirds of the gut increased the exposure of the lower third to ^3H -SM from which ^3H -ceramide may be formed by enzymes other than alk-SMase. The enzyme(s) could be of tissue or bacterial origin (37). To further elucidate the possible role of bacteria in the hydrolysis of dietary SM we analyzed feces collected during day 1 and day 2 after gavage. The ^3H -ceramide/ ^3H -SM ratio was much lower in the KO than in the WT mice in both cases. However, the ratio for the KO mice was slightly increased in the 2nd day comparing with the 1st day, suggesting that colon bacteria may hydrolyze some SM when transit is slow. Since previous studies showed that SMase in the bacteria is much lower than that of alk-SMase (37) and no significant SMase activity was found in the fecal samples of our KO mice, our data do not indicate a quantitatively important role of bacteria in SM digestion.

Besides bacterial enzymes, acid and neutral SMases are present in the intestinal tract. However, neither acid nor neutral SMase was upregulated significantly in the KO mice although a small increase of acid SMase was found in the middle part of the small intestine. The increase in acid SMase activity may be a response to the loss of alk-SMase activity. Indeed, opposite changes of alk-SMase and acid SMase activities in the gut were observed by us before (39). However, such an increase may not be important for SM digestion, as alk-SMase activity normally is about 200-300 times higher than acid SMase in the small intestine, and acid SMase, not like alk-SMase that is resistant to trypsin digestion, is rapidly inactivated by trypsin (9). This explains the result that in this region the formation of ^3H -ceramide and the ratio of ^3H -ceramide/ ^3H -SM were still very low.

Mucosal brush border ceramidase is an important enzyme that hydrolyses ceramide to free fatty acid and sphingoid bases. In alk-SMase KO mice, we did not identify any significant change in neutral ceramidase activity using C16-ceramide as substrate, i.e. the same ceramide species that the ^3H -SM fed to the mice contained. We also measured the

activity of BSSL, an enzyme previously shown to have ceramidase activity (40). We conclude that when alk-SMase expression is knocked out, the capacity of SM digestion by acid and neutral SMase is not upregulated to a level that can take over the function of alk-SMase, and that any ceramide formed may be normally degraded by ceramidase and BSSL.

Another interesting finding in this work is the decrease of alkaline phosphatase throughout the small intestinal tract in the KO mice. Alkaline phosphatase is a brush border enzyme in the intestinal mucosa. However, the decrease did not reflect a general reduction of brush border enzyme activities in the KO mice, as the other brush border enzymes measured i.e. ceramidase and APN, were not decreased. Since the decreased alkaline phosphatase activity in the mucosa was not associated with a similar decrease in the intestinal lumen, rather a small increase in the distal part of the small intestine, further studies should focus on the membrane binding, releasing, and translocation of the enzyme in alk-SMase KO mice. Of interest is a recent finding that lysophosphatidylcholine, also a substrate for alk-SMase (15), can stimulate alkaline phosphatase release in the intestinal tract (41). Whether the luminal levels of lysophosphatidylcholine are increased in the KO mice and thus enhancing the release of alkaline phosphatase from the mucosa is of interest for investigation. In addition, alkaline phosphatase has multiple functions including absorption of lipid, inactivation of bacterial endotoxin, and protection of the barrier function (42), and the enzyme is structurally related to the NPP family, of which alk-SMase is a member (43). Oral administration of alkaline phosphatase (44) and rectal administration of alk-SMase alleviate experimental colitis (19). The expression and function of the two enzymes may therefore be integrated and requires further investigation.

Finally, previous studies indicated that the hydrolysis of SM to generate ceramide may prevent tumorigenesis in the colon (26). Marked reduction of alk-SMase activity due to the expression of abnormal splicing forms has been reported in human colon and liver cancer cells (23, 45). In the present work with alk-SMase KO mice, we did not find spontaneous tumorigenesis either in the small intestine or colon up to 10 months of the

age. Still, the lack of spontaneous tumorigenesis is compatible with the hypothesis that alk-SMase may have protective effects, which may not be demonstrated in the absence of carcinogens. However, comparison of one KO and one WT mouse from the same littermate showed intestinal hypertrophy in the KO mouse. Obviously this is a preliminary “case report” that needs confirmation in extended morphological studies on animals with different genotypes including genders among the littermates. Furthermore the levels of SM and ceramide in the epithelial cells should be measured, and the apoptotic and the proliferative pathways in the KO mice explored. Alk-SMase was previously found to be correlated positively with caspase 3 activity in the gut of mice (39), deficiency of alk-SMase may affect the apoptosis of the epithelial cells in the gut resulting in hypertrophy.

In conclusion, this is the first study performed in alk-SMase knockout mice. The results prove that alk-SMase plays a crucial role in dietary SM digestion and ceramide formation in the gut, and may have important implications for further studying the role of alk-SMase in tumorigenesis, cholesterol absorption and other related functions in the gut.

Acknowledgement

The work was supported by the grants from Swedish Cancerfonden, Albert Pålsson foundation, the Swedish Research Council, Swedish Nutrition Foundation, and the research grants of the University Hospital of Lund.

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Figure legends

Figure 1. Genomic design. The first panel shows the Enpp7 targeting vector. The black bars indicate the exons. A PGK-Neo cassette was inserted after exon 2 and the exon together with the insert were flanked by 2 LoxP sites. An additional BglII site was constructed. The second panel shows the wild type allele and the locations of “en probe” and 3’probe used for Southern blot. The PCR primers are indicated by arrows. The 3rd panel shows the targeted allele, and the last panel, the resulting knockout (KO) allele. Cre-mediated recombination leads to deletion of exon 2 and the Neo marker. By this method, Southern blot using 3’probes can identify the homologous recombinants in ES cells by the appearance of an 8.8 kb band after digesting of the DNA by BglII. Southern blot using “en probe” can identify a single band of 4.5 kb for the KO mice, 4.5 and 8.8 kb bands for the heterozygous mice, and only 13.9 kb band for the WT mice. Using the primers as indicated, PCR can amplify 732 bp in WT mice, a 247 bp in homozygous KO mice, and both 732 and 247 bp bands for the heterozygous.

Fig 2. Genotype analysis. Panel A. A representative figure of Southern blot for identifying the homologous recombinant ES cells in the BglII-digested genomic DNA, using 3’ probe. Integration of the targeting vector generated a band of 8.8 kb in addition to the 13.9 kb for the WT allele. M: DNA marker. T: targeted ES cells, and WT: the WT ES cells. Panel B. A representative figure of Southern blot for identifying the heterozygous +/- mice upon Cre-recombinase reaction after BglII digestion using the “en probe”. A band of 13.9 kb was identified for the WT (+/+) mice, and an additional 4.5 kb was identified for the homozygous (+/-) mice. Panel C. A representative figure of genotype characterization by PCR with the primers, which amplify a 732 bp band for the WT (+/+), a 732 bp plus a 247 bp for the heterozygous (+/-) and only the 247 bp band for the KO (-/-) mice.

Figure 3. Alk-SMase activity in the intestinal mucosa, content and feces. The whole small intestine was removed from the wild-type (+/+), heterozygous (+/-), and homozygous

knockout (-/-) mice. The intestinal content was washed out in 25 ml 0.9% saline and the mucosa was scraped and homogenized in 2 ml lysis buffer. One piece of fecal sample collected from the distal part of the colon was dried, weighed, and resuspended in 0.5 ml saline. After centrifugating, the supernatants were saved for analysis. Results are from 3 mice in each group. Western blotting of alk-SMase in the intestinal content was analyzed as described in the method section.

Fig. 4. Morphology of the middle part of the small intestine from WT and KO mice. The upper panel (5 x objectives) shows an overview of the S2 region, whereas the villi are shown in higher magnification (40 x objectives) in the middle panel and the crypt region is shown in the lower panel (40 x objectives). An increased width of the villi, the crypts and the thickness of the muscle layer under the crypts were observed in the KO mice.

Fig. 5. Changes of 3 types of SMase activities in the small intestine and liver in KO mice. The small intestine was divided into three parts, which represent the proximal (S1), middle (S2) and the distal parts (S3) of the small intestine, respectively. The mucosa in each segment was scraped and homogenized in 2 ml lysis buffer and the intestinal content was washed in 5 ml 0.9% saline. The alk-SMase activity in the intestinal mucosa (A) and content (B), and the acid and neutral SMase activities in intestinal mucosa (C and D) were determined as well as those in the liver. The data are means \pm SEM, $n = 7$. * $p < 0.05$, *** $p < 0.001$ compared with the WT mice.

Fig. 6. The hydrolysis of ^3H -SM (panel A), formation of ^3H -ceramide (panel B) and the ratio of ceramide/SM (panel C) in the intestinal content 1 h after administration. Both WT and KO mice were fed ^3H -SM as described. After 1 h, the whole small intestine was removed and cut into 3 parts in equal length representing the proximal (S1), middle (S2) and distal (S3) parts of the small intestine. The intestinal contents in each part were washed with 5 ml saline. Total lipids were extracted from 2 ml of the washout and the extracted lipids were dissolved in 500 μl of the chloroform/methanol (2:1). One hundred μl of the lipids were subject to TLC. The radioactivities in the bands of ^3H -SM and ^3H -

ceramide were determined by liquid scintillation. The ratio of ceramide/SM was calculated. Results are mean \pm SEM from 6 animals in each group. * $P < 0.05$, ** $p < 0.01$, *** $p < 0.005$ as compared with the WT mice.

Fig. 7. ^3H -palmitic acid formation in the intestinal content (A), absorption in the intestinal mucosa (B) and conversion to ^3H -TG (C). The mice were fed ^3H -SM and the small intestine was removed and cut into 3 segments (S1, S2 and S3) 1 h later as described. The total lipids in the content and tissues in each segment were extracted and dissolved in 500 μl chloroform/methanol. 100 μl of the lipids were subjected to TLC. The radioactivities in the bands of ^3H palmitic acid and ^3H -TG according to the standards were determined. The results are mean \pm SEM in 6 animals in each group. ** $p < 0.01$, *** $p < 0.005$, compared with control.

Fig. 8. The radioactivity of fatty acid plus triglyceride (TG) in the liver 1 and 3 h after ^3H -SM administration. The mice were fed ^3H -SM as described. One and three h after administration, the mice were killed and the liver was removed. The total lipids were extracted and isolated by TLC as described. The radioactivities in the bands of palmitic acid and triglyceride were determined. The results were expressed as dpm per gram of the wet weight of the liver. Results are mean \pm SEM. $N = 6$ in each group for 1 h study and $n = 3$ for the 3 h. * $p < 0.05$ and ** $p < 0.01$ compared with the WT mice.

Fig. 9. Sphingomyelin and ceramide excreted in the feces. Both WT and KO mice were fed ^3H -SM as described and fecal samples were collected separately for 2 days. The total lipids in the feces were extracted in 500 μl chloroform/methanol and 100 μl of the lipids were subject to TLC analysis. The bands of SM and ceramide were scraped and the radioactivities in the bands of day 1 (panel A), day 2 (panel B) were counted. The radioactivities were calculated and expressed as the percentage of the total radioactivity given. The ratios of ceramide/SM were calculated (pane C). The results are mean \pm SEM from 6 mice in each group. * $p < 0.05$, and ** $p < 0.01$ compared with the WT mice.

Tables

Table I. The width (μm) of the villi, the crypts, and the thickness of the muscle layer in the middle of the small intestine from WT and KO mice.

Measurement	WT	KO	P value	N
Width of villi	57.18 \pm 1.25	81.92 \pm 1.64	<0.0001	96
Width of crypts	29.27 \pm 0.59	33.26 \pm 0.52	<0.0001	101
Thickness of muscle	18.90 \pm 0.96	26.90 \pm 1.34	<0.0001	39

Two female mice from the same littermate were killed. Small pieces were obtained from the middle part of the small intestine and processed for morphological analyses as described in Materials and Methods. The width of villi, crypts and the thickness of the muscle layer were measured at random. Results are means \pm SEM. P values are obtained from Student's t-test and N represents the number of the villi, crypts and the points of the muscle layer determined.

Table II. Activity changes of other intestinal enzymes in alk-SMase knockout mice.

Measurement	S1		S2		S3	
	WT	KO	WT	KO	WT	KO
CDase mucosa (nmol/h/mg)	2.83±0.20	3.23±0.33	3.80±0.26	4.23±0.3	2.79±0.31	2.74±0.26
BSSL content (nmol/min/ml)	5.19±0.83	4.71±0.85	5.58±0.87	6.44±0.76	1.38±0.32	2.15±0.33
AKP mucosa (μmol/min/mg)	4.93±0.83	2.19±0.61*	1.69±0.25	0.78±0.11**	1.09±0.21	0.56±0.09*
AKP content (μmol/min/ml)	11.98±1.58	9.71±1.42	9.89±1.38	11.55±2.50	9.27±1.15	14.29±3.59
AP mucosa (μmol/min/mg)	9.70±1.44	8.91±1.63	9.56±1.47	7.91±0.93	7.33±0.81	5.47±0.81
AP content (μmol/min/ml)	9.56±1.30	11.45±1.77	9.72±1.13	11.25±0.56	4.95±0.97	4.59±0.61
APN mucosa (nmol/min/mg)	4.69±0.70	4.14±0.54	6.41±1.32	5.75±0.88	6.93±1.46	5.22±0.78

The mice were killed and the small intestines were removed and cut into 3 segments in equal length (S1, S2, and S3) as described. The contents in each segment were rinsed in 5 ml of 0.9% saline and the mucosa were scraped. Results are means \pm SEM from 7 animals in each group. * $p < 0.05$, ** $p < 0.01$ compared with wild type animals. The activities in the mucosa were adjusted to the protein contents and those in the content, to the volume of the samples. CDase: ceramidase, BSSL: bile salt stimulated lipase, AKP: alkaline phosphatase, AP: acid phosphatase, APN: aminopeptidase N. The activity in the mucosa was adjusted to the protein levels whereas those in the content, to the sample volume.

Table III. The total ^3H radio activity (% of given dose) in the washout of the stomach, small intestine and colon 1 and 3 hours after gavage.

Time	Stomach		Small intestine		Colon	
	WT	KO	WT	KO	WT	KO
1 hour	13.6±4.0	12.5±3.4	14.3±0.5	26.4±4.9*	0.5±0.1	0.6±0.2
3 hour	5.3±0.6	2.1±1.0*	7.7±2.1	9.5±1.2	1.6±0.4	7.2±1.8*

The mouse after fasting overnight was given 0.3 ml of emulsion containing ^3H -SM by gavage and killed 1 and 3 h later. The contents of stomach, intestine and colon were rinsed and collected. The radioactivity in the washing solution was determined. The results were calculated and expressed as percentage of the total dose given. n=7 for 1 h group and 3 for 3 h group. * p< 0.05, **p<0.01 compared with the WT.

Table IV. Digestion of ^3H -SM and absorption of ^3H -fatty acid in mice 3 hours after administration of ^3H -SM.

	S1		S2		S3	
	WT	KO	WT	KO	WT	KO
^3H -SM (dpm)	310±198	201±71	344±40	616±146	301±240	1757±240***
^3H -Cer (dpm)	125±85	25±28	138±70	78±22	172±56	310±36
Cer/SM	0.25±0.07	0.05±0.02*	0.42±0.14	0.10±0.01*	0.46±0.09	0.17±0.01*
^3H -FA content (dpm)	229±175	11±5	156±64	37±22	52±18	61±10
^3H -FA absorbed (dpm)	123±43	14±2	548±152	39±4*	173±10	76±10***

Both wild type (WT) and alk-SMase knockout mice (KO) were given ^3H -SM as described. After 3 h, the lipids were extracted and dissolved in 500 μl solvent, of which 100 μl were loaded on TLC plate. The radioactivities of ^3H -SM, ^3H -ceramide, ^3H -fatty in the intestinal content and ^3H -FA absorbed in the mucosa were analyzed. Results are means \pm SEM from 3 animals in each group. * $p<0.05$, *** $p<0.005$ compared with WT. S1, S2 and S3 indicate the proximal, middle, and distal parts of the small intestine, respectively.

Fig. 1

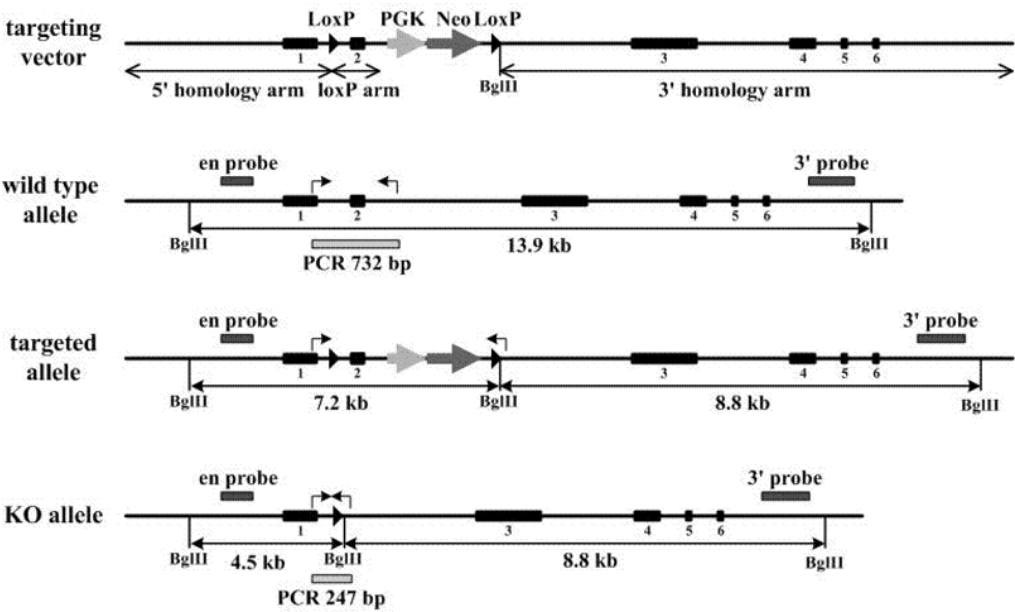


Fig. 2

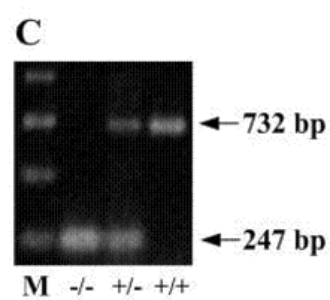
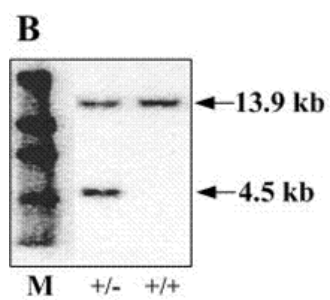
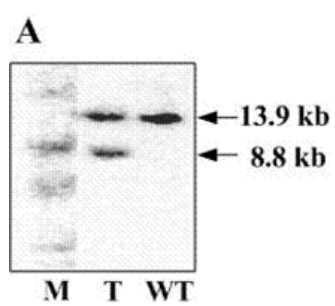


Fig. 3

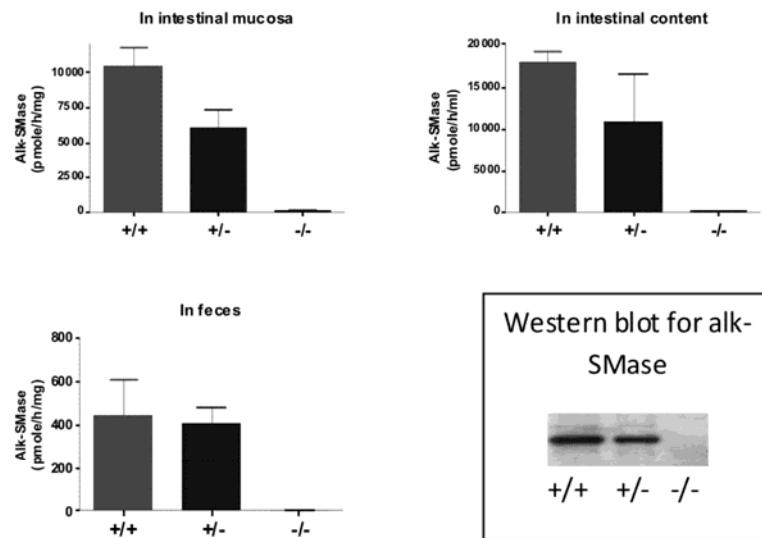


Fig. 4

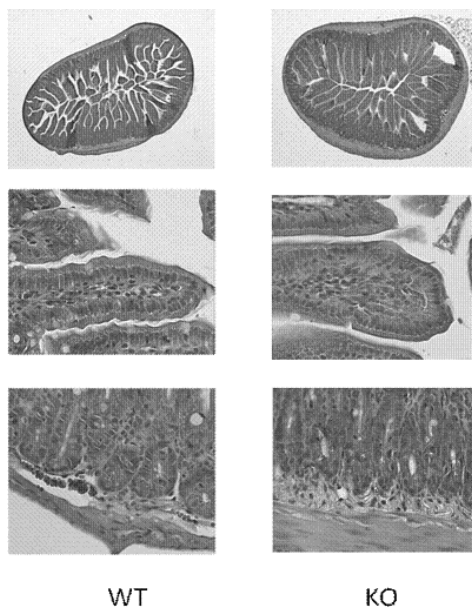


Fig. 5

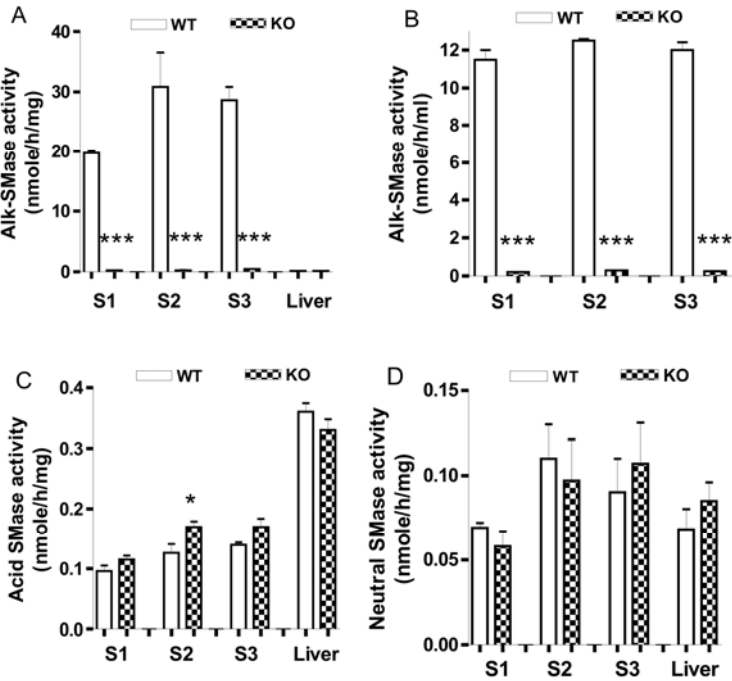


Fig. 6

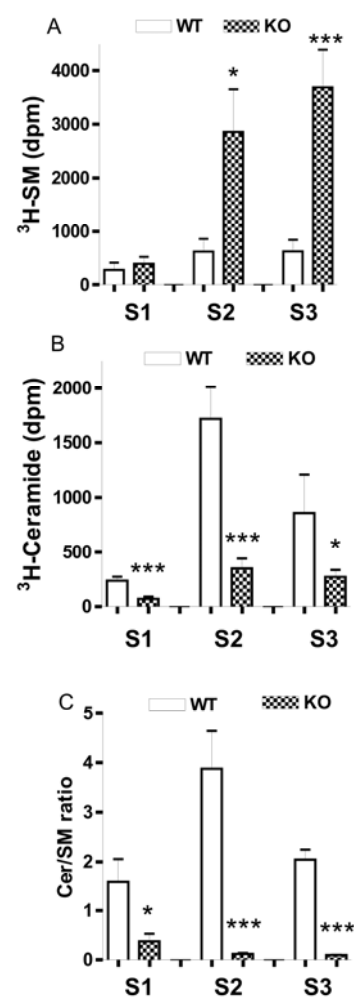


Fig. 7

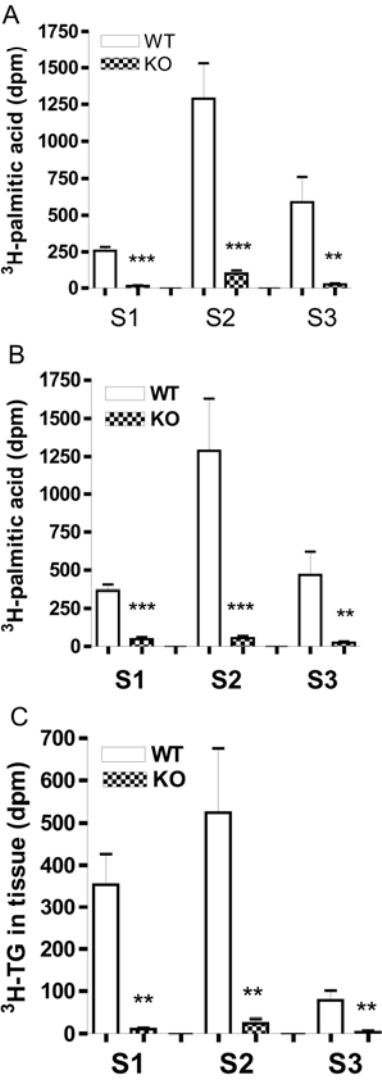


Fig. 8

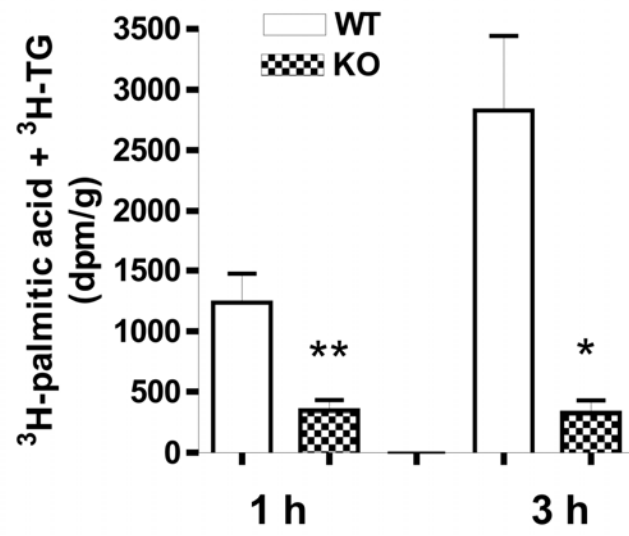


Fig. 9

