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Nilsson, Åke

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Role of sphingolipids in infant gut health and immunity

Ake Nilsson

Department of Clinical Sciences, Division of Medicine, Gastroenterology, Southern University Hospital, S-24185 Lund, Sweden

ake.nilsson@med.lu.se

+46-46-2220708

Abstract

Sphingomyelin (SM), glycosphingolipids, and gangliosides are important polar lipids in the milk fat globule membrane, but are not found in standard milk replacement formulas. Because digestion and absorption of SM and glycosphingolipids generate the bioactive metabolites ceramide, sphingosine, and sphingosine-1-phosphate (S1P), and because intact gangliosides may have beneficial effects in the gut, this may be important for gut integrity and immune maturation in the neonate. The brush border enzymes that hydrolyze milk SM,alkaline sphingomyelinase (Nucleotide phosphodiesterase pyrophosphatase 7, NPP7), and neutral ceramidase (NC), are expressed at birth in both term and preterm infants. Released sphingosine is absorbed, phosphorylated to S1P, and converted to palmitic acid via S1P-lyase in the gut mucosa. Hypothetically, S1P may also be released from absorptive cells and exert important paracrine actions favoring epithelial integrity and renewal, as well as immune function, including secretory IgA production and migration of T lymphocyte subpopulations.

Gluco-, galacto-, and lactosylceramide are hydrolyzed to ceramide by lactase-phlorizin hydrolase, which also hydrolyzes lactose. Gangliosides may adhere to the brush border and be internalized, modified, and possibly transported into blood, and may exert protective functions by their interactions with bacteria, bacterial toxins, and the brush border.

Key Words sphingomyelin, milk fat globule membrane, sphingolipids, neonatal health, NPP7, ceramide, sphingosine-1-phosphate

Introduction

The milk fat globule membrane (MFGM) consists of amphiphilic lipids, cholesterol, and proteins. In addition to the major glycerophospholipids (PL), phosphatidylcholine (PC) and phosphatidylethanolamine, MFGM contains sphingomyelin (SM), glucosyl- and lactosylceramides, and gangliosides.[1] (Figure 1) Thus, about half of the polar lipids in MFGM are sphingolipids (SL), but that is not the case in standard milk replacement formulas, which usually contain soy lecithin i.e., mainly PC as amphiphilic lipid emulgator. The polar lipids supply choline, ethanolamine, and fatty acids, which are needed for synthesis of cell membrane PL and acetylcholine during growth and expansion of tissue PL pools in the neonate. In addition, MFGM SL have biological effects that could contribute to the beneficial effects of mothers' milk.

Digestion of SM, the major SL in milk, by nucleotide phosphodiesterase pyrophosphatase 7 (NPP7), a protease-resistant, bile salt-dependent brush border enzyme, generates ceramide, sphingosine, and S1P.[2] These compounds are both metabolic intermediates during synthesis and degradation of SL, and bioactive compounds with numerous signaling functions mediated by intracellular pathways in the case of ceramide, and by well characterized plasma membrane G-protein coupled receptors in the case of S1P.[3] Because many of these effects are

related to regulation of cell growth, differentiation, apoptosis, and immune cell migration, the question arises as to whether SL in milk may influence mucosal function and immune maturation in the gut.

NPP7 also has anti-inflammatory properties that may be related to its ability to inactivate the proinflammatory messenger platelet activating factor (PAF).[4] The sialic-acid-containing SL in milk i.e., the gangliosides, may have multiple effects including an influence on gut bacterial flora, interactions with pathogens, and effects on mucosal epithelial and immune functions.[5, 6] This article summarizes current knowledge on the digestion and absorption of SL and how it may be related to biological effects in the neonatal gut. There are, however, few neonatal studies in this area. Some general aspects on SL metabolism in relation to gut inflammation and tumorigenesis are therefore also discussed.

Sphingolipids in milk fat globule membrane

The human infant fed breast milk, ingests about 150 mg of SM per day,[7] which accounts for about 40% of the polar MFGM lipids. The MFGM also contains glucosylceramide, lactosylceramide, and gangliosides. In human milk, the content of glycosphingolipids is much lower than that of SM. Bovine milk MFGM contains more lactosylceramide than human milk.[8] The mucosal brush border contains significant amounts of SM, ceramides, glyco-SLs, and gangliosides, which are synthesized in the epithelium during differentiation along the crypt-villous axis.[9]

Digestion of sphingomyelin and glycosylceramides

As previously reviewed, [2] dietary SM is sequentially hydrolyzed by NPP7 and a neutral ceramidase (NC) acting at the brush border of the intestinal epithelium and in the gut lumen. In contrast to SM and ceramide, sphingosine is rapidly absorbed and most is converted to palmitic acid in the mucosa and transported in chyle triacylglycerol.[10] (Figure 2) Digestion

and absorption of glucosylceramide exhibit similar features. The digestion of SM in the rat is extended throughout the gut; in humans with an ileostomy most is digested and absorbed.[11]

In rodents, NPP7 occurs only in the gut, but in humans it is also expressed in the liver and secreted in bile.[12] It has been purified, cloned, and identified as a novel member of the nucleotide phosphodiesterase (NPP) family.[13] In the gut, levels of NPP7 are highest in the jejunum and ileum, and are lower in the colon. Studies in NPP7-/- mice confirmed its central role in SM digestion.[14] NPP7 was also shown to have some phospholipase C activity against PC and lyso-PC and against the proinflammatory lipid messenger PAF (platelet activating factor, 1-alken-2-acetyl-glycerophosphocholine).[4] PAF can be produced by epithelial and immunocompetent cells in the gut and has been ascribed a pathogenic role in both inflammatory bowel disease (IBD) and in neonatal NEC. [15]

Gut NC from rats and humans has been purified,[16] and studies in NC KO mice confirmed its role in ceramide digestion.[17] Interestingly NC KO mice exhibit normal growth and phenotype. Bile-salt stimulated lipase (BSSL) hydrolyzes ceramide,[18] but the physiological importance of this is uncertain. Both NPP7 and NC are protease resistant, and remain active in the gut lumen. They are released by bile salts and, and in the case of NPP7 , by tryptic cleavage as well. These features make it possible to use NPP7 and NC meconium levels to measure neonatal expression.[19]

Like SM, glucosyl- and galactosylceramide are not hydrolyzed by pancreatic enzymes, but degraded in the gut to ceramide and sphingosine.[20] The brush border enzyme lactase-phlorizine hydrolase, which hydrolyzes the lactose in milk, also hydrolyzes glycosylceramides to ceramide.[21] The absorption of gangliosides is not well characterized. Studies in Caco2 cells indicate that the intact molecule can be associated with the brush border side and converted to other gangliosides by glycosyltransferases. A transcellular transport and intracellular

degradation may also occur.[22] In rats fed ganglioside GD3, levels of this ganglioside increased in lipid rafts from the brush border and in plasma.[23]

Metabolism of sphingoid bases in epithelial cells

Released sphingosine is absorbed and most is converted to S1P by sphingosine kinases. S1P is converted to hexadecanal and ethanolamine phosphate by S-1-P lyase. Hexadecanal is oxidized to palmitic acid, which is incorporated into chylomicron triacylglycerols.[10] (Figure 2) Some sphingosine is reacylated to ceramide and used for SL synthesis, rehydrolyzed, or converted to ceramide phosphate, which has also been implicated in lipid signaling.[3]

Sphingosine kinases 1 and 2 and S1P lyase are highly expressed in the gut mucosa, which also contains higher levels of S1P lyase than any other tissue.[24] Thus, in the gut, S1P is a key intermediate in the irreversible conversion of sphingosine to palmitic acid and ethanolaminephosphate. A key question is to what extent intestinal S1P derived from dietary SLs reaches paracrine signaling targets in the epithelial and immunocompetent cells of the gut. (Figure 3) Both sphingosine kinases and S1P-lyase have been cloned and gene knockout mice developed for sphingosine kinase 1 and 2 have been shown to exhibit normal phenotypes, whereas double knockout of both is lethal at the embryonic stage.[25] S1P lyase KO mice have severely retarded development and exhibit drastically changed lymphocyte traffic and lymphopenia.[26] Interestingly, gut-specific S1P lyase KO increases S1P level in the mucosa, but normal mucosal morphology is retained and the animals develop normally, but are more vulnerable to inflammation-related colon cancer (CRC).[27]

SM digestion in the neonate

Because SM accounts for almost 40% of the polar lipids in human and cow's milk, [8] it is important to know how well the neonate can utilize components of the SM molecule. In the fetal rat, the gut epithelium undergoes rapid transformation with formation of mature villus

cells and distinct villus and crypt structures soon before birth at day 23 of gestation. Expression of NPP7 coincides with this differentiation.[28] In a human study, significant levels of both NPP7 and NC were found in meconium from both preterm and term infants.[19] Both palmitoylsphingosine and sphingosine, which may be products of NPP7 and NC, were also present in meconium.

Some acid SMase is secreted in milk. Gastric and duodenal intubation studies of 11 suckling newborns, however, indicated that digestion of milk SM in the stomach and upper duodenum is negligible, whereas analyses of jejunal and ileal samples obtained from two babies undergoing surgery indicated ceramide formation in these regions of the gut. Three-week-old suckling pigs had high levels of alkaline SMase in the jejunum and ileum. The conclusion was that the enzymes digesting SM are present at birth in both preterm and term infants, pigs and rats.[2] The transfer of nervonic acid, which occurs specifically in SLs, to tissues of newborn rats from mother's milk, supports the concept that milk SM is indeed digested and the fatty acids absorbed.[29] Lactase-phlorizin hydrolase is detected from the 12th gestational week, although at week 26-34 the level was lower than at full-term birth.[30] The conclusion was that both premature and term infants can digest SM and glycosylceramides of breast milk. The findings also bring the attention to the ability of NPP7 to hydrolyze and inactivate PAF, which may have a pathogenic role in NEC.[4] Although the NPP7 is crucial for SM digestion, the NPP KO mice grow normally during suckling and exhibit a normal gross phenotype.[14]

Influence of SM on lipolysis and cholesterol absorption

The unique polar surface coat milk fat particles may influence the course of digestion of milk triacylglycerols (TAG) during suckling. SM tends to favor the rate of lipolysis with gastric lipase, but inhibits colipase-dependent pancreatic lipase. (X) In rats, milk SM inhibits absorption of both exogenous[31] and endogenous cholesterol.[32] In humans, however, the effects

of milk polar lipids and SM on cholesterol absorption and plasma lipids are small,[33, 34] Extrapolating these data to infants, it seems unlikely that the SM in the MFGM would markedly inhibit cholesterol absorption in suckling neonates.

Biological effects of SL and its metabolites in the gut.

Generally, the gut grows rapidly after birth and the immune system expands. In the pig this development is enhanced by breastfeeding. Gut function is important for resistance to infections, particularly in premature infants, where mucosal integrity is crucial. Milk SL may be one of several components that contribute to the advantages of mothers milk, but there are few neonatal studies on the subject. Motouri et al[35] artificially reared 7-day-old rats with gastric infusion of a formula containing either 0.5 % SM or 0.5 % PC for seven days. Morphological examination of the gut revealed remaining vacuolation of epithelial cells only at the villous tip of the SM tip, better development of the Auerbachs nerve plexa, and lower lactase levels in the SM group. The findings may be interpreted as an enhancement of gut maturation, but it is unknown whether the effects were caused by SM itself or to its metabolites.

At present, hypotheses as to how milk SL might act in the neonatal gut must thus be based on current knowledge about SL metabolism and its effects in the mature gut. Ceramide has numerous signaling functions related to regulation of cell growth, induction of apoptosis, and inflammation.[3] It is formed both by synthesis and by degradation of SL. (Figure 3) Signaling actions are mediated by multiple intracellular pathways. Some actions have been linked to sequential activation of phosphatases e.g., ceramide-activated Ser–Thr phosphatases (CAPPs), such as PP1 and PP2A. Other effects might be mediated by ceramide- induced alterations in specific domains of the plasma or brush border membrane.[36] Ceramide synthesis in gut epithelial cells by acylation of absorbed sphingosine may be increased by ingestion of milk

SL. The high content of palmitic acid in milk provides an excess of substrates for de novo synthesis of dihydrosphingosine, which is reacylated to dihydroceramide and desaturated to ceramide. In both the mature and neonatal gut, mature villus cells undergo apoptosis during mucosal renewal and ceramide may affect this process. Ceramide formed during SL digestion permeates poorly into the absorptive cells and it is unknown whether it exerts signaling effects. [2] NC KO mice develop normally and have normal gut morphology, but exhibit increased apoptosis of intestinal epithelium when challenged orally with short-chain ceramide, which is absorbed intact by mucosal cells.[17]

Sphingosine is formed in the lumen and at the brush border by the action of NC. Sphingosine has been linked to cellular processes, such as inducing cell cycle arrest and apoptosis by modulation of protein kinases and other signaling pathways. It has roles in regulating the actin cytoskeleton and endocytosis and has been shown to inhibit protein kinase C, inducing apoptosis in colon carcinoma cell lines.[37] Due to the rapid metabolism to S1P and ceramide, any specific signaling function of sphingosine itself is difficult to elucidate in vivo.

Recent reviews highlight the important signaling functions of S1P.[3, 38] It favors endothelial cell survival and angiogenesis. It affects differentiation and migration of lymphocytes, dendritic cells, macrophages, and white blood cells i.e., both innate and specific immunity. It influences cell growth and the balance between apoptosis and cell proliferation/differentiation in numerous cell types. In the gut, it has been linked to effects on gut integrity and on proliferation of epithelial cells.[39, 40] Generally, S1P is present in low nanomolar concentrations in tissues. Upon exposure to stimuli sphingosine kinases are activated and S1P released and acts extracellularly on 5 different G-protein coupled plasma membrane receptors, which have been cloned. These receptors display selective tissue expression that is crucial for their biological functions and use well-known intracellular signaling pathways to mediate their

specific effects. S1P also seems to exert S1PR-independent actions intracellularly e.g., calcium release.[3]

Blood S1P, which is present in the 200 nM concentration range, is bound to albumin and apolipoprotein M, and originates mainly from erythrocytes, platelets, and endothelial cells. The high blood concentration creates a gradient that has a central role in egress of lymphocytes from secondary lymphoid tissues and lymphatic glands. The amount of S1P formed in the gut increases with the amount of SL ingested. It is unknown whether this increases only the formation of palmitic acid in the epithelium or also the amount of S1P released into compartments where it may have paracrine actions on epithelial, immunocompetent and endothelial cells in the mucosa. A recent review emphasizes the potential importance of S1P in the regulation of the gut immune system.[41] (Figure 1) The sequence of events leading to IgA production by committed B cells after processing the antigen in the Peyers patches dendritic cells, involve S1P signaling and the regulated expression of S1P1 receptor. Furthermore, peritoneal B1 cells involved in production of non-specific antibodies of the innate immune system require S1P and the expression of the S1P1 receptor for their migration to the gut epithelium. Appearance of certain subtypes of intraepithelial T lymphocytes is S1P dependent. S1P is also involved in migration of dendritic cells, macrophages, and mast cells. How all these aspects are related to maturation of the gut immunity in the newborn term and preterm baby is so far unknown. Generally the premature infant has lymphopenia and attenuated macrophage and dendritic cell function and it is not known whether increased mucosal S1P production from absorbed milk SM metabolites enhances the normalization of this immature situation.

Studies of SL's effects in gut inflammation and CRC strongly support the concept that SL signaling is indeed biologically important in the gut.[37 42] SL retard tumor growth in animal models of CRC and human CRC ,and longstanding cases of colitis have been linked to low

levels of NPP7. The role of S1P as a growth-stimulating signal in CRC cells and the high sphingosine kinase/S1P lyase ratio that colon tumors exhibit have been emphasized. Interestingly, selective targeting of the S1P lyase gene in the intestine, enhanced colitis-associated tumorigenesis, confirming that the S1P/S1P lyase ratio is crucial.[27] S1P is increased in experimental colitis and human IBD, and FTY720, a sphingosine analogue that is phosphorylated and binds to S1P receptors and enhances their degradation, was found to alleviate experimental colitis in the IL10 knockout model. Vitamin B6 is essential for S1P lyase action and vitamin B6 deficiency was found to make experimental colitis worse.[43] Studies on gene knockout mice sphingosine-kinase 1 and 2 and inflammation have yielded contradictory results.[3]

Dietary SM has been shown both to alleviate [44] and aggravate experimental colitis induced by DSS or IL10 knockout due to induction of cathepsin D by ceramide.[45] A third study[46] reported suppression by SM of inflammation-driven CRC. Increased apoptosis in colon and intestinal epithelium has been reported in IBD. It can be speculated that with barrier damage and invasion of inflammatory cells, metabolites of dietary SL may have access to signaling compartments that do not reach in the normal gut. With many mechanisms involved, the effect may be unpredictable and depend on the stage of the inflammation.

Recombinant NPP7 given rectally alleviates DSS colitis.[47] NPP7 KO mice[14] develop normally but develop mucosal hyperplasia. When exposed to a combination of DSS and the colon carcinogen azoxymethane, the NPP7 KO mice developed more, larger and more malignant tumors.[48] These findings could be linked to increased S1P levels in the gut of untreated NPP7 KO mice and to increased PAF level in NPP7 KO mice exposed to DSS. Thus, the positive effect of NPP7 in colitis may be due to its ability to inactivate PAF. [4] Increased PAF levels are seen in NEC, IBD and ischemic colitis. NPP7 is a protease resistant enzyme that survives the acid environment of the stomach and may degrade PAF both in the gut of premature

children at risk for NEC and in IBD patients. Also NC KO mice were found to develop more severe DSS-induced colitis.[49]

Potential effects of other sphingolipids in milk

Due to their sialic acid content, the gangliosides are hydrophilic amphiphiles that are water soluble and form mixed micelles, whereas SM and glycosylceramides depend on bile salts for their solubilization. It is expected that they may penetrate the mucus layer and interact with bacterial toxins, bacteria, and brush border structures.

Gangliosides interact with bacterial toxins to exert a protective role e.g., in cholera and enterotoxic E coli infections. This function has, however, been ascribed to GM1, which is not a major ganglioside in human milk, which contains predominantly GD3 and GM3. Nevertheless, milk gangliosides may act as "unintended receptors" for pathogens, alleviating their action and possibly influencing gut bacterial flora in preterm neonates, increasing bifidobacteriae and decreasing E coli. Experimental data also suggest an influence on immunological maturation, as indicated by effects on T cell differentiation and IgA production.[5] Gangliosides have been shown to protect against LPS-induced bowel inflammation and necrosis similar to necrotizing enterocolitis.[5, 50, 51] Thus, dietary gangliosides may reduce proinflammatory signaling in the gut by several mechanisms, as recently reviewed.[6] Yet, in premature piglets, a formula enriched with gangliosides could not replace colostrum, regarding anti-NEC effects.[52] The effects of milk gangliosides on brain development are so far uncertain.[53]

Sphingomyelin, NPP7 and choline (Figure 4)

Large amounts of choline are required in neonates for PL synthesis during organ growth, and for acetylcholine formation. Acetylcholine receptors are also expressed on lymphocytes and intestinal epithelial cells and acetylcholine has anti-inflammatory and trophic effects in the

gut. Adults secrete large amounts of PC in bile (6-10 g/d) and although bile secretion is not fully developed in the neonate, bile from neonates contains bile salts and PC in ratios similar to that in adults. Thus, choline from bile PC must recycled in the neonate as well. Choline occurs in human milk as free choline, choline phosphate, glycerophosphocholine, PC, and SM.[54] SM digestion thus must also be viewed in relation to choline. In adult rats fed 3H-choline labeled SM, 30 % of the radioactivity was in liver PC after 4 h, indicating that choline released during SM digestion is extensively reutilized for hepatic PC synthesis.[2] Furthermore metabolism of sphingoid bases in the gut generates ethanolamine that can be used for synthesis of PE, some of which is methylated to PC in the liver.

In adults, pancreatic phospholipase A2 hydrolyzes the 2-ester bond of PC to generate lyso-PC, which is absorbed and reacylated or degraded further in the mucosa. Interestingly, PLA2 -/- mice absorb PC fatty acids normally and are less prone to develop obesity, hyperlipidemia, and glucose intolerance on a high-fat diet.[55] In the neonate PLA2 like the colipase-dependent lipase, is poorly expressed at birth. Human gastric lipase, milk and pancreatic BSSL, and PLRP2, that are expressed early [56, 57] lack phospholipase activity. The brush border contains a phospholipase B/lipase with activity against both PC and lyso-PC, glycerides and retinylester.[58] This enzyme has optimum pH , bile-salt dependence, and longitudinal extension in the gut similar to NPP7. One may ask whether PC digestion in the neonate is also dependent on brush border enzymes. Interestingly, the mucosal phospholipase B/lipase also has broad bactericidal effects. Choline homeostasis in adults and neonates was recently reviewed.[59]

Systemic effects of sphingolipids

Details in this area are outside the scope of this article. An interesting example is that polar lipids in milk were shown to increase neuronal plasticity in the brain of rats [60] and improve

288 spatial memory learning, which could be linked to astrocyte and synaptic functions in a region
289 of the Hippocampus. Cognitive behavior improved in a small pilot study of SM-fortified milk
290 in low birth weight infants.[61]

291 **Conclusions**

292 Milk SM is degraded in the intestine to metabolites that are both signaling substances and
293 intermediary metabolites, and are an important source of choline and ethanolamine. These
294 metabolites may affect mucosal growth and immune maturation. Particularly intriguing are
295 findings that gene-targeted animals lacking either NPP7, NC, or intestinal S1P lyase are more
296 susceptible to induction of gut inflammation and inflammation-related tumors. This strongly
297 suggests an important role of SL digestion in the intestinal adaption to external challenges.
298 Current knowledge about the effects of dietary SL provide well founded hypotheses for
299 potentially beneficial effects of MFGM, but further studies in neonatal piglets and in neonates
300 are necessary for definite proof.

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458 sphingomyelin-fortified milk has a positive association with the neurobehavioural

459 development of very low birth weight infants during infancy, randomized control trial. Brain
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461 Figure Legends

462 Figure 1 Structure of sphingolipids.

463 Sphingosine is a hydrophobic amino alcohol with 18 carbons and a terminal OH-group at
464 position 1. At position 2, an amino group forms an amide bond with a long chain fatty acid in
465 ceramide. The OH group at position1forms an ester with phosphocholine in SM and a
466 glycosidic bond with glucose or galactose in glycosylceramide. In lactosylceramide, the bond
467 is formed with lactose and in gangliosides with a sialic acid-containing carbohydrate For
468 nomenclature of individual gangliosides, see ref 5.

469 Figure 2 Scheme of sphingomyelin absorption.

470 SM is not digested by any pancreatic enzyme but by the brush border enzyme NPP7, which
471 generates ceramide and then is hydrolyzed to sphingosine (sph) and free fatty acids (FA). Sph
472 is phosphorylated to S1P by sphingosine kinase and converted to palmitic acid and
473 ethanolamine phosphate by S1P lyase, which is highly expressed in the gut. The fatty acids
474 formed are incorporated mainly into chylomicron triglycerides.

475 Figure 3 Actions of S1P in the gut.

476 In addition to its role as a key intermediate in the conversion of sphingosine to palmitic acid
477 in the gut epithelial cells, S1P may also be released extracellularly via a plasma membrane
478 transporter to have paracrine actions.

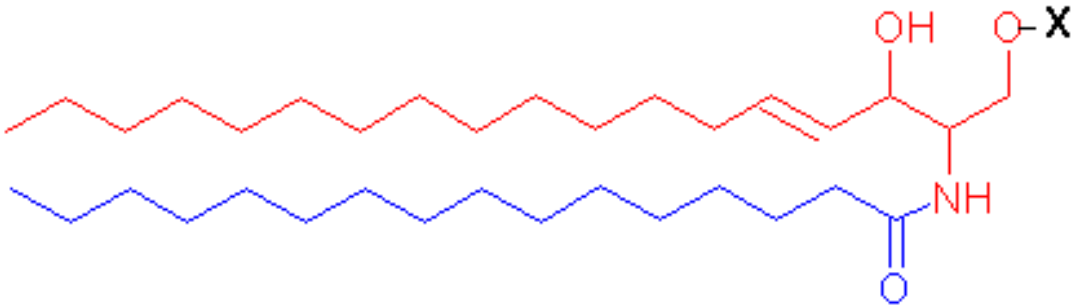
479 Figure 4 De novo synthesis of ceramide.

480 Ceramide may be formed by hydrolysis of SM and glycosphingolipids, but is also synthesized
481 de novo. Most of the sphingoid bases in the mucosal SL in the newborn are likely formed by
482 de novo synthesis from palmitic acid, which may be supplied, in part, by mothers milk.

483

Fig 1

Structure of sphingolipids



Headgroup X	Sphingolipid
H	Ceramide
Phosphocholine	Sphingomyelin
Glucose or galactose	Glycosylceramide
Lactose	Lactosylceramide
Glucose+monosaccharides+sialic acid	Ganglioside

Fig 2

Scheme of sphingomyelin absorption.

Nilsson and Duan, J Lipid Res 2006; 47:154-71

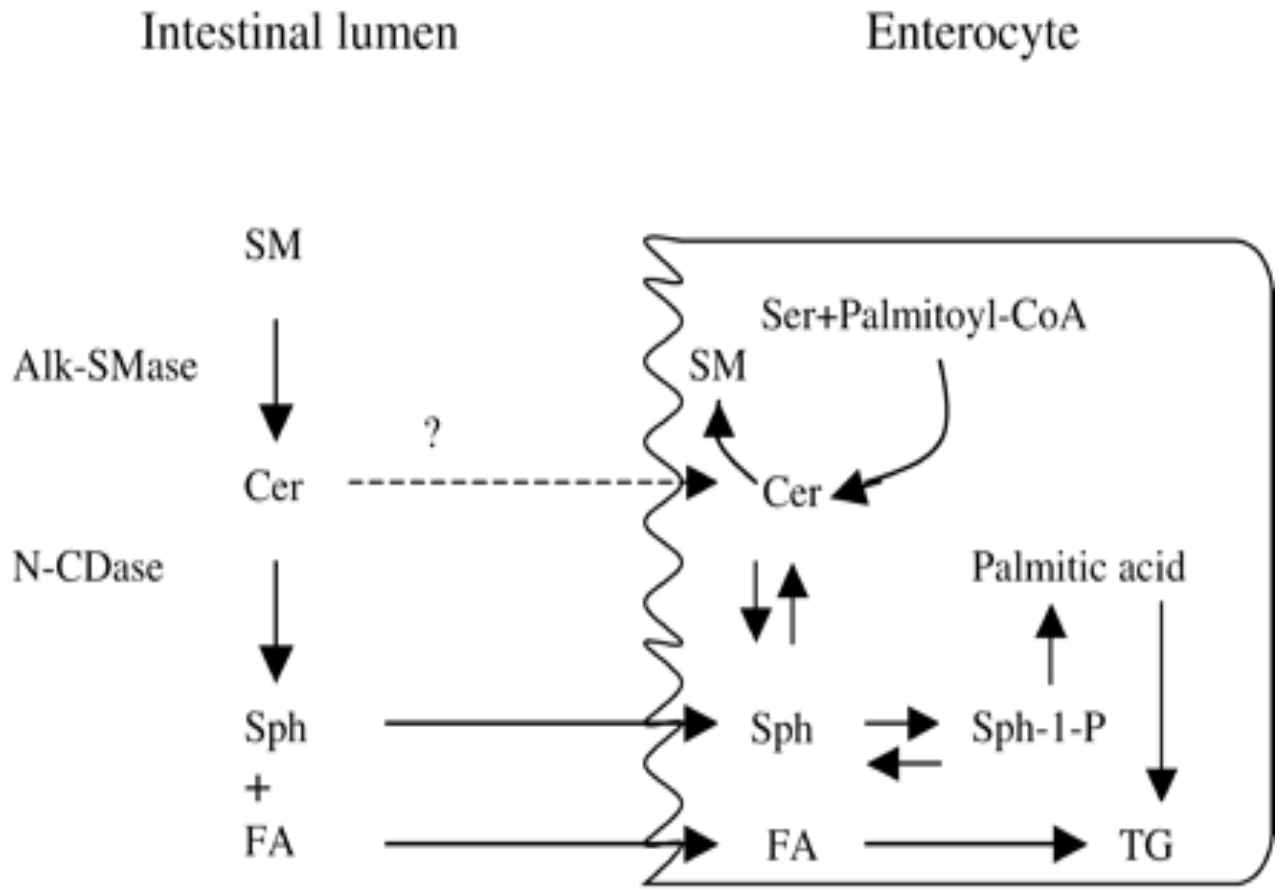


Fig 3

Actions of S1P in the gut

S1P acts extracellularly via five different receptors. Low tissue cncentrations . Paracrine actions.3

Important intermediary metabolite in sphingposine conversion to palmitic acid. Partitioning to signalling pool crucial.2

Key functions in lymphocyte traffic and homing of gut lymphocyte.41

Trophic effects on mucosal cell proliferation and barrier integrity.37

Recruitment and proliferation of IgA producing cells and intraepithelial lymphocytes41

Fig 4

De novo synthesis of ceramide