

Effects of Dietary Sphingomyelin on Central Nervous System Myelination in Developing Rats

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ABSTRACT

Human milk contains sphingomyelin (SM) as a major component of the phospholipid fraction. Galactosylceramide (cerebroside), a metabolite of sphingolipids, increases along with CNS myelination, and is generally considered a universal marker of myelination in all vertebrates. L-Cycloserine (LCS) is an inhibitor of serine palmitoyltransferase (SPT), a rate-limiting enzyme for sphingolipid biosynthesis that is reported to show increased activity with development of the rat CNS. The present study examined the effects of dietary SM on CNS myelination during development in LCS-treated rats. From 8 d after birth, Wistar rat pups received a daily s.c. injection (100 mg/kg) of LCS. From 17 d after birth, the animals were fed an 810 mg/100g of bovine SM-supplemented diet (SM-LCS group) or a non-supplemented diet (LCS group). At 28 d after birth, the animals were killed and subjected to biochemical and morphometric analyses. The myelin dry weight, myelin total lipid content, and cerebroside content were significantly lower in the SM-LCS and LCS groups than in a group not treated with LCS (the non-LCS group). However, these levels were significantly higher in the

SM-LCS group than in the LCS group. Morphometric analysis of the optic nerve revealed that the axon diameter, nerve fiber diameter, myelin thickness, and g value (used to compare the relative thickness of myelin sheaths around fibers of different diameter) were significantly lower in the LCS group than in the other groups, but were similar in the SM-LCS and non-LCS groups. These findings suggest that dietary SM contributes to CNS myelination in developing rats with experimental inhibition of activity. (*Pediatr Res* 53: 589–593, 2003)

Abbreviations

SM, sphingomyelin
LCS, L-cycloserine
PC, phosphatidylcholine
PE, phosphatidylethanolamine
PI, phosphatidylinositol
PS, phosphatidylserine
SPT, serine palmitoyltransferase
TLC, thin-layer chromatography

SM is composed of phosphocholine as the polar head group and sphingosine as the backbone of the molecule, and it is therefore classified as one of the sphingolipids. Recent studies have demonstrated that sphingolipids are found in all eukaryotic and some prokaryotic organisms (1). These molecules are involved in the regulation of cell growth (2), cell differentiation, and diverse other functions, including cell–substratum interactions and intracellular signal transduction (3, 4). Human milk has a lower content of phospholipids compared with triglycerides. Bitman *et al.* (5) reported that human milk has a total phospholipid content of approximately 15 to 20 mg/dL, with SM accounting for approximately 37% of the phospholipid fraction. Although many foods contain a small amount of

SM (6), its nutritional and physiologic roles have not been fully examined.

CNS myelin has a higher lipid content (65–80%) than that of general cell membranes. SM and sphingolipid metabolites, such as cerebroside and sulfatide, are prominent components of the myelin sheath that surrounds the axons of some neurons. This sheath acts as an insulator for nerve impulses and controls the salutatory mode of conduction via the nodes of Ranvier. Myelination of the human CNS begins from 12 to 14 wk of gestation in the spinal cord (7, 8) and continues into the third decade of life in the intracortical fibers of the cerebral cortex (9), but the most rapid and dramatic changes occur between midgestation and the end of the second postnatal year (10, 11). Myelination accounts for a large part of the more than tripling of brain weight that occurs during this period.

Recently, Luberto and Hannun (12) reported on a metabolic pathway for sphingolipids. SPT (EC 2.3.1.50) is the first step and the rate-limiting enzyme in sphingolipid biosynthesis (13, 14), catalyzing the synthesis of 3-ketosphinganine from L-

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serine and palmitoyl-CoA (15). This enzyme is located in the endoplasmic reticulum or Golgi apparatus (16). A recent study showed that SPT activity gradually increases from the third prenatal to the third postnatal week in the hypothalamus of rats (17). As myelination begins at the same period in these animals, it is conceivable that an increment of SPT activity may be one of the major factors involved in myelinogenesis. CNS myelin has a high cerebroside content when compared with its level in other tissues (18). Cerebroside is generated from ceramide by ceramide UDP-galactosyltransferase, which is the key enzyme in the biosynthesis of cerebroside and catalyzes the transfer of galactose from UDP-galactose to ceramide (19). In rats, cerebroside is hardly detectable in the brain before 10 d after birth, but the cerebroside content increases markedly from the second to the third postnatal weeks, especially between d 14 and 23 of life (20). Because the period of maximum cerebroside biosynthesis corresponds with the time of most active myelination (21), cerebroside is generally recognized as a universal marker of CNS myelination (22–25).

Ceramides can be generated from L-serine and palmitoyl-CoA by *de novo* synthesis of SPT, and from SM by sphingomyelinase. Therefore, during the period of low SPT activity, we hypothesized that cerebroside in CNS myelin of developing rats may be mainly derived from dietary SM ingested in milk that is transformed to ceramide and then to cerebroside.

Miller and Denisova (26) reported that LCS caused a decrease of cerebroside in rat CNS myelin by inhibiting SPT activity and therefore could be useful for investigating the role of cerebroside in the formation of myelin.

The rat optic nerve has been widely used for correlative morphometric, physiologic, and biochemical studies of the CNS because of its structural and functional homogeneity (27, 28). In particular, morphometric analysis of optic nerve was performed to evaluate the myelin formation, as neonatal rat optic nerves are entirely unmyelinated and almost all of the axons undergo myelination during maturation (27).

In the present study, to examine the influence of dietary SM on the maturation of CNS myelin, we created a rat model of low SPT activity by administration of LCS and evaluated the effect of dietary SM supplementation. We found that the myelin dry weight, cerebroside content, and myelin thickness were decreased in the CNS by inhibition of SPT activity, whereas dietary supplementation of SM reversed these changes. Our results suggest that dietary SM may play an important role in CNS myelination when SPT activity is low.

METHODS

Study design. All procedures for the care and use of animals conformed to the Guidelines for Animal Experiments of Jun-tendo University. Pregnant female Wistar rats were purchased from Charles River Japan Inc. (Kanagawa, Japan) and were allowed free access to a commercial diet (CRF-1; Oriental Yeast, Co., Ltd, Tokyo, Japan) and water during pregnancy and lactation. The animals were housed in polycarbonate cages in a room with a controlled temperature (25°C) and lighting period (0800–2000 h). Because sex-based differences in sphingolipid metabolism have not been fully examined, in view of

the influence of sex hormones, only male pups were studied. On the first day after birth, 30 male pups were randomly assigned to three experimental groups: 1) the non-LCS group did not receive LCS treatment or dietary SM supplementation; 2) the LCS group received LCS treatment without dietary SM supplementation; and 3) the SM-LCS group received LCS treatment with dietary SM supplementation. The pups of each experimental group were suckled by their dams until 16 d after birth. From 8 d of life (2 d before the onset of myelination in rats), the pups in the LCS and SM-LCS groups received daily s.c. injections of LCS (100 mg/kg; Tokyo, Japan) in PBS (pH 7) (26). The pups in the non-LCS group received daily injections of PBS alone. From 17 d of life, the pups in each group were isolated from their dams and fed an experimental diet. At 28 d of age, six pups from each group were killed for biochemical analysis, and the other pups were used for morphometric analysis.

Extraction of dietary SM from buttermilk powder. Total lipids were extracted from bovine buttermilk powder by the method of Bligh and Dyer (29), and then crude phospholipids were obtained from the acetone-insoluble fraction by authentic acetone treatment. After weak alkaline hydrolysis at 37°C using 0.5 N NaOH (30), glycerophospholipids (*O*-acyl-bound lipids) were hydrolyzed, but sphingolipids (*N*-acyl-bound lipids) including SM remained intact. Next, gangliosides in the unsaponifiable matter were removed by pyridine treatment. The purity of SM obtained by this method was >90% according to TLC.

Experimental diets. The diet was based on the AIN-93G formula for rats (31), and its composition (grams per 100 g diet) was as follows: casein defatted by the method of Bligh and Dyer (29), 20; cornstarch, 39.7486; dextrinized cornstarch, 13.2; sucrose, 10; soybean oil, 7; mineral mix (AIN-93G), 3.5; vitamin mix (AIN-93G), 1; L-cystine, 0.3; choline bitartrate, 0.25; tert-butyl hydroquinone, 0.0014; and cellulose. The pups in the SM-LCS group received a diet containing 810 mg/100 g of SM derived from bovine buttermilk powder as mentioned above. The total weight of the diet was adjusted by altering the amount of cellulose.

Isolation of CNS myelin and lipid analysis. Isolation of myelin from whole rat brains was performed in accordance with the authentic method of Norton and Poduslo (32) using ultracentrifugation. At 28 d after birth, the pups were anesthetized with Nembutal (Dainippon Pharmaceutical Co., Ltd, Osaka, Japan) and perfused via the left ventricle with 50 mL of warm physiologic saline, after which the whole brain was immediately removed. The freshly isolated myelin was freeze-dried, and total lipids were extracted by the method of Norton and Poduslo (18). Glycerophospholipids were separated on TLC 60 plates with a solvent system of chloroform-methanol-acetic acid-water (25:15:4:2, vol/vol). The other sphingolipids (SM, cerebroside, and sulfatide) were separated by high-performance TLC 60 with chloroform-methanol-water (70:30:4, vol/vol). Lipids were visualized with 50% sulfuric acid spray. Eight classes of myelin lipid were measured: total sterols, PE, PI, PS, PC, SM, cerebroside, and sulfatide. The individual lipid classes were quantitatively determined using a TLC-densitometry apparatus (AE-6920M; Atto Corporation,

Tokyo, Japan) by comparison with known amounts of authentic preparations.

Morphometric analysis of optic nerve myelination. The optic nerves were removed from rats that had been perfused via the left ventricle with an ice-cold solution of 4% paraformaldehyde and 2% glutaraldehyde in 0.1 M cacodyl buffer (pH 7.4). Optic nerve specimens were fixed overnight at 4°C in the same fixative after being cut from the left optic nerve at 1.0 mm anterior to the optic chiasm. Then the specimens were fixed for 2 h in 2% glutaraldehyde buffered to pH 7.4 with Sørensen's phosphate buffer (0.1 M) and treated with 2% osmium tetroxide for 2 h in the same buffer (both procedures were carried out at 4°C). Next, the tissue was embedded in Epok 812, after which thin sections were cut on an MT 5000 ultramicrotome (Du Pont K.K., Tokyo, Japan) with a diamond knife and were stained with uranyl acetate followed by lead citrate. Electron micrographs were obtained with a JEM-1200EX electron microscope (JEOL Ltd., Tokyo, Japan) operating at 80 kV. Morphometric analysis was performed according to the method of Little and Heath (33). Electron micrographs at a magnification of 3000 were digitized using a computer image scanner (GT-8000; Seiko Epson Corp., Tokyo, Japan). Then a grid containing approximately 150 nerve fibers was identified on the electron micrograph, and 150 fibers were selected randomly from this grid using Photoshop v.6.0 software (Adobe Systems Inc., Tokyo, Japan). Myelin sheaths normally vary in shape when examined in cross-section. In this study, the actual area of each myelin sheath measured on the electron micrograph was converted to the equivalent circular shape because this approach has been demonstrated to provide the best estimate of axon and fiber diameter (33, 34). The axon area was obtained from the digitized axon perimeter using Image v.1.61 software (National Institutes of Health, Bethesda, MD, U.S.A.), and the axon diameter was calculated by the following equation:

$$\text{Axon diameter} = 2 \times \sqrt{(\text{axonal area}/\pi)}. \quad (1)$$

The fiber area was obtained using Image software to detect the digitized outermost major dense line of the myelin sheath, and the nerve fiber diameter was calculated by the following equation:

$$\text{Fiber diameter} = 2 \times \sqrt{(\text{fiber area}/\pi)}. \quad (2)$$

The thickness of each myelin sheath was calculated for each individual nerve fiber cross-section by the following equation:

$$\text{Myelin thickness} = \text{fiber diameter} - \text{axon diameter}. \quad (3)$$

Then the *g* value was calculated as the ratio of the axon diameter to the fiber diameter.

Statistical methods. Data on myelin lipids were expressed as the mean \pm SEM ($n = 6$). The morphometric data on optic nerve myelination were expressed for 150 fibers from the defined grid of each electron micrograph. One-way ANOVA followed by a *post hoc* multiple range Tukey-Kramer test was used for statistical evaluation of significant differences among the groups, and $p < 0.05$ was considered significant. Analyses were performed using StatView v.5.0 software (Japanese version; Hulinks Co., Ltd., Tokyo, Japan).

RESULTS

The brain wet weight, CNS myelin dry weight, and myelin total lipids concentrations at 28 d of life are shown in Table 1. The brain wet weight of the LCS group was significantly lower than the weights in the other two groups. CNS myelin dry weight was also significantly lower in the LCS group than in the other two groups. The myelin dry weight of the SM-LCS group was significantly higher than that of the LCS group, but was significantly lower than that of the non-LCS group. The total lipid content of whole myelin (milligrams per brain) showed a similar pattern of changes to those of myelin dry weight. However, the total lipid composition (percent) of myelin showed no significant differences among the three groups.

The lipid classes of CNS myelin are shown in Table 2. In the LCS group, the level of each lipid class, except for PI, was significantly lower than in the non-LCS group. In the SM-LCS group, the level of each lipid class (except for PI) was significantly higher than in the LCS group. The levels of total sterols, PS, PC, cerebroside, and sulfatide were significantly lower in the SM-LCS group than in the non-LCS group.

The results of morphometric analysis are shown in Table 3. The axon diameter, fiber diameter, myelin thickness, and *g* value were all significantly lower in the LCS group than in the non-LCS or SM-LCS groups, whereas no significant differences of these variables were observed between the non-LCS and SM-LCS groups.

DISCUSSION

In the present study, dietary supplementation of SM restored the brain weight and myelin dry weight that were decreased by LCS treatment. These findings suggest that orally ingested SM was transformed to ceramide or other metabolites in the intestinal tract, which were absorbed from the bowel and entered the circulation to reach the CNS across the blood-brain barrier. However, the incorporation of sphingolipids into the CNS from lipoproteins has not yet been proven. As shown in Table 1, changes of the myelin total lipid content (milligrams per brain) in each group were nearly parallel to those of myelin dry weight (milligrams per brain), but there was no significant difference of the myelin total lipid composition (percent) among the experimental groups.

Table 1. Effects of dietary sphingomyelin on brain and myelin weights and myelin lipids*

	Experimental group		
	Non-LCS	LCS	SM-LCS
Brain wet wt (g)	1.67 \pm 0.01 ^a	1.55 \pm 0.01 ^b	1.64 \pm 0.01 ^a
Myelin dry wt (mg/brain)	24.4 \pm 0.5 ^a	12.9 \pm 0.5 ^b	21.7 \pm 0.8 ^c
Myelin total lipid content (mg/brain)	17.2 \pm 0.5 ^a	8.5 \pm 0.2 ^b	14.8 \pm 0.4 ^c
Myelin total lipid composition (%)	70.5 \pm 1.8 ^a	67.0 \pm 4.3 ^a	68.4 \pm 2.2 ^a

Values are the mean \pm SEM ($n = 6$).

* On each horizontal line, values with the same superscript letters (a, b, or c) are not significantly different. Values with different letters are significantly different at $p < 0.05$.

Table 2. Effects of L-cycloserine and sphingomyelin on myelin lipid classes (mg/total myelin)*

	Experimental group		
	Non-LCS	LCS†	SM-LCS
Total sterols	4.27 ± 0.10 ^a	2.32 ± 0.07 ^b (46%)	3.87 ± 0.09 ^c
PE	3.25 ± 0.11 ^a	1.82 ± 0.05 ^b (44%)	2.97 ± 0.11 ^a
PI	0.08 ± 0.03 ^a	0.15 ± 0.02 ^b (-88%)	0.15 ± 0.02 ^b
PS	1.45 ± 0.06 ^a	0.77 ± 0.02 ^b (47%)	1.06 ± 0.04 ^c
PC	2.62 ± 0.06 ^a	1.52 ± 0.03 ^b (42%)	2.38 ± 0.06 ^c
SM	0.60 ± 0.05 ^a	0.38 ± 0.02 ^b (37%)	0.71 ± 0.04 ^a
Cerebroside	3.92 ± 0.16 ^a	1.30 ± 0.05 ^b (67%)	2.96 ± 0.10 ^c
Sulfatide	0.98 ± 0.07 ^a	0.25 ± 0.02 ^b (74%)	0.66 ± 0.04 ^c

Values are the mean ± SEM ($n = 6$).

* On each line, values with the same superscript letters (a, b, or c) are not significantly different.

Values with different letters are significantly different at $p < 0.05$.

† For the LCS group, values in parentheses indicate the percent to the non-LCS group.

Table 3. Morphometric optic nerve fiber variables*

	Experimental group		
	Non-LCS	LCS	SM-LCS
Axon diameter (μm)	0.86 ± 0.04 ^a	0.59 ± 0.03 ^b	0.80 ± 0.03 ^a
Fiber diameter (μm)	1.17 ± 0.04 ^a	0.85 ± 0.03 ^b	1.12 ± 0.04 ^a
Myelin thickness (μm)	0.31 ± 0.01 ^a	0.26 ± 0.01 ^b	0.31 ± 0.01 ^a
g value	0.71 ± 0.01 ^a	0.67 ± 0.01 ^b	0.70 ± 0.01 ^a

Values are the mean ± SEM for 150 nerve fibers.

* On each line, values with the same superscript letters (a, b, or c) are not significantly different.

Values with different letters are significantly different at $p < 0.05$.

Rohlf's *et al.* (35) reported that rat milk contains 134 μM SM. In normal developing rats, it is possible that SM from milk can supplement early *de novo* synthesis of ceramide during the period when SPT activity is low.

Analysis of the lipids in CNS myelin isolated from the whole brains of 28-d-old rats showed that the cerebroside level was reduced by LCS administration (LCS group) when compared with that in the non-LCS group, but was significantly increased by dietary SM supplementation (SM-LCS group).

In mice lacking ceramide UDP-galactosyltransferase, an enzyme required for the synthesis of cerebroside from ceramide, the cerebroside (galactosylceramide) normally found in CNS myelin is replaced by glucosylceramide, a lipid not originally identified in CNS myelin (36). Unexpectedly, the ultrastructure of myelin in this type of knockout mouse is similar to that of normal mice, but the animals show generalized tremor, mild ataxia, and defective conduction on electrophysiologic examination. In the present study, formation of myelin may have been impaired in the LCS group because *de novo* synthesis of ceramide was largely inhibited by LCS injection. If biosynthesis of cerebroside (galactosylceramide) or glucosylceramide from ceramide occurred sufficiently, then morphologically normal myelination of the CNS would be detected, whereas low ceramide levels would lead to limited myelination. Baumann (37) proposed that minimum amounts of specific myelin components, including cerebroside, might be required for the deposition of stable myelin. Accordingly, it seems that ceramide is necessary for the formation of myelin,

and cerebroside is important for the maintenance of its function.

Ceramide is synthesized from L-serine and palmitoyl-CoA by SPT, which is the rate-limiting enzyme for sphingolipid synthesis. Merrill *et al.* (38) have demonstrated SPT activity in microsomes from different tissues of mature rats, including the liver, lung, brain, kidney, intestine, spleen, muscle, heart, pancreas, testes, ovary, and stomach. However, there have been no studies on SPT activity in human diseases or during development, so further investigation of the relationship between SPT and various diseases is needed.

In the LCS group, LCS treatment decreased the levels of sphingolipids (cerebroside and sulfatide), total sterols, and glycerophospholipids (PE, PS, and PC) in CNS myelin (Table 2). Inasmuch as glycerophospholipid metabolism is unaffected by LCS, certain amounts of glycerophospholipids may need to be present in oligodendrocytes for myelination to occur. Therefore, the sphingolipids that were decreased most markedly in the LCS group (cerebroside and sulfatide were reduced by 67% and 74%, respectively, compared with the non-LCS group) might be essential for myelination. This study demonstrated that dietary SM restored the depleted levels of cerebroside and sulfatide in CNS myelin, and concomitantly restored the levels of glycerophospholipids and total sterols.

Electron microscopy showed that the axon diameter of the LCS group was significantly smaller than in the other two groups, whereas the axon diameter of the SM-LCS group was comparable to that of the non-LCS group. Ledesma *et al.* (39) have reported a role of SM in axonal maturation. The present study indicated that dietary SM supplementation restored the reduced axon diameter in LCS-treated rats.

The g value was defined as the ratio of axon diameter to fiber diameter by Bear and Schmitt (40), and it is useful for assessing the relative thickness of the myelin sheaths around fibers of different diameter. This value is influenced by a range of factors that include differences of nerve trunk diameter and species differences (33), but the theoretical optimal g value is reported to range from 0.6 to 0.7 (41). In this study, all three experimental groups had g values within the optimal range, but the LCS group had a significantly lower g value than the other two groups. The g value tended to show statistically similar changes to those of axon diameter, nerve fiber diameter, and myelin thickness.

CONCLUSION

In conclusion, the present lipid and morphometric analyses demonstrated that dietary SM can contribute to myelination of the developing rat CNS in the experimental setting of low SPT activity.

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