Preparation of ^{13}C-labeled ceramide by acetic acid bacteria and its incorporation in mice

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Abstract We prepared 2-hydroxypalmitoyl-sphinganine (dihydroceramide) labeled with a stable isotope by culturing acetic acid bacteria with ^{13}C-labeled acetic acid. The GC/MS spectrum of the trimethylsilyl derivative of ^{13}C-labeled dihydroceramide gave molecular ions with an increased mass of 12–17 Da over that of nonlabeled dihydroceramide. The fragment ions derived from both sphinganine base and 2-hydroxypalmitate were confirmed to be labeled with the stable isotope in the spectrum. Therefore, ^{13}C-labeled dihydroceramide can be an extremely useful tool for analyzing sphingolipid metabolism. The purified [^{13}C]dihydroceramide was administered orally to mice for 12 days, and the total sphingoid base fractions in various tissues were analyzed by GC/MS. The spectrum patterns specific to ^{13}C-labeled sphingoids were detected in the tissues tested. Sphinganine pools in skin epidermis, liver, skeletal muscle, and synapse membrane in brain were replaced by [^{13}C]sphinganine at about 4.5, 4.0, 1.0, and 0.3%, respectively. Moreover, about 1.0% of the sphingosine pool in the liver was replaced by [^{13}C]sphingosine, implying that exogenous dihydroceramide can be converted to sphingosine. These results clearly indicate that ingested dihydroceramide can be incorporated into various tissues, including brain, and metabolized to other sphingolipids.—Fukami, H., H. Tachimoto, M. Kishi, T. Kaga, H. Waki, M. Iwamoto, and Y. Tanaka. **Preparation of ^{13}C-labeled ceramide by acetic acid bacteria and its incorporation in mice.** *J. Lipid Res.* 2010. **51:** 3389–3395.

Supplementary key words *Acetobacter* • sphingolipid • dihydroceramide • stable isotope • skin epidermis • brain

Sphingolipids, a diverse class of lipids that contain a long-chain base (sphingoid) as a basic building unit, are widely distributed in eukaryotes and in some prokaryotes. These types of lipids are involved in various cellular events, such as growth, differentiation, proliferation, and apoptosis, through the signal transduction pathways (1). In animals, sphingolipids are particularly abundant in skin and brain tissue and are implicated many important physiolog-

ical functions. Indeed, disruption of sphingolipid metabolism is associated with several diseases (1, 2). Ceramide, a major component of the stratum corneum, helps cells adhere to one another and prevents the loss of moisture, thereby maintaining flexibility (3–5). Many reports indicate the importance of sphingolipids in the central nervous system (6). Gangliosides, acidic glycosphigolipids that contain sialic acids, are known to accelerate synaptic transduction through the release of acetylcholine from synapses (7). Ceramide and sphingomyelin are reported to function as nerve growth factors that affect the development and survival of nerve cells (8). In other tissues, ceramide is involved in exercise-induced stress in the muscular system (9). Sphingolipids also affect cell membrane structure and function. Gangliosides can enhance glucose uptake in intestinal tissue (10). Sphingomyelin alters cholesterol transport and lipoprotein structures in serum (11–13). In addition, ceramide exhibits an anti-cancer effect in several cell types, such as colon cancer cells, through the activation of apoptosis (2, 14). However, few reports show a correlation between the incorporation of orally ingested sphingolipids into tissues and the associated physiological functions.

Nilsson reported the absorption of orally administered radiolabeled complex sphingolipids or sphingoid bases using rats, and the absorption of sphingolipids in the intestines of rats was confirmed (15–17). However, few studies evaluating the kinetics of sphingolipid metabolites in tissues after their absorption have appeared in the literature. Although compounds labeled with a radioisotope can be detected at high sensitivity, the technique is not readily applied to the structural identification of metabolites in the body. Furthermore, there are restrictions in terms of safety issues.

Acetic acid bacteria are Gram-negative obligate aerobes that are used to ferment traditional food products, such as vinegar. Because acetic acid bacteria oxidize ethanol into

acetic acid and are tolerant to the high osmotic stress and low pH caused by acetic acid, they have characteristic membrane lipids. The membrane contains alkali-stable lipids that are found in only a few bacterial species (18). Sphingolipids of acetic acid bacteria are components of alkali-stable lipids and have sphinganine as the sphingoid base and 2-hydroxypalmitoyl-sphinganine (dihydroceramide) as the main compound (18). Acetic acid bacteria can efficiently utilize acetic acid in the culture medium as a carbon source (19). Specifically, acetic acid is metabolized to acetyl-CoA and acyl-CoA and can be used as a source of fatty acid synthesis. Labeled acetic acid is thought to be efficiently incorporated into membrane lipids derived from fatty acids (**Fig. 1**). Sphingolipids labeled with a stable isotope can be detected by mass spectrometry. Data derived from such experiments may be used to evaluate not only the incorporation of sphingolipids into tissues but also their conversion (metabolism) in tissues after oral ingestion.

Therefore, we aimed to synthesize dihydroceramide labeled with a stable isotope by culturing acetic acid bacteria with ^{13}C-labeled acetic acid. Then we studied the incorporation and metabolic conversion of orally administered, labeled dihydroceramide in various tissues, such as skin epidermis, liver, skeletal muscle, and brain (synapse membranes) of mice.

MATERIALS AND METHODS

Strain, medium, and culture conditions

We isolated the acetic acid bacterial strain *Acetobacter malorum* NCI1683 (S24), which contains a large amount of dihydroceramide, from fermented milk (20). *Acetobacter malorum* NCI1683 was cultured on YPG medium containing 2% yeast extract, 0.15% polypeptone, and 3% glucose (pH 6.5). A starter culture in 50 ml medium was grown and used to seed a 5 l jar fermenter (CLN-5000B, Mitsuwa Frontech Corp., Osaka, Japan) containing 3.3 l medium. The culture was grown for 24 h at 28°C with 1.0 vol/vol/

Fig. 1. Predicted metabolic pathway from [1-13C]acetic acid to [13C]dihydroceramide (2-hydroxypalmitoyl-sphinganine) in the biosynthesis of [13C]dihydroceramide by acetic acid bacteria. Acetic acid bacteria accumulate highly pure dihydroceramide as the main sphingolipid compound.

min aeration and agitation at 500 rpm. All the culture was used to inoculate a 500 l fermentation tank (Hokusetsu Kakouki Corp., Osaka, Japan) containing 330 l medium supplemented with 0.25% [1-13C]acetic acid and cultivated for 28 h at 28°C with 1.5 vol/vol/min aeration and agitation at 120 rpm. The bacterial pellet was separated, and the medium was washed with purified water by continuous centrifugation using a disk separator (SJ30F, Mitsubishi Kakoki Kaisha Ltd., Kanagawa, Japan). The pellet was lyophilized, and the dried cells were used in the lipid extraction described below. A bacterial pellet cultivated on nonlabeled acetic acid was also used for the preparation of nonlabeled lipid components.

Extraction and purification of acetic acid bacterial lipids

Extraction of total lipids from acetic acid bacteria and weak alkaline treatment were performed according to a previous report (20). Briefly, solvent (chloroform:methanol:water at 1:1:0.8, v/v/v) was added to the dried cells, and the mixture was incubated at room temperature with occasional shaking. Partitioning of the mixture was performed by adding 1.25 vol of chloroform and water. The lower layer was collected as total lipids. The total lipids were treated with 0.4 M KOH in methanol to decompose glyceryl phospholipids. The alkaline-treated lipids were obtained from the reaction mixture by Folch's method (21). The lipids were fractionated by silica gel column chromatography (PSQ-60B, Fuji Silysia Chemical Ltd., Aichi, Japan). Dihydroceramide was eluted with chloroform:methanol at 96:4 (v/v). The dihydroceramide fraction was applied to a silica gel thin layer plate (Silica Gel 60, 20 × 20 cm, Merck Ltd. Japan, Tokyo, Japan) and developed with chloroform:methanol (96:4, v/v). The lipid spots on the plate were then visualized with 50% H_2SO_4. The spot of purified dihydroceramide was confirmed to be a single molecular species and identical to the standard, with a purity of over 98%.

Animal treatment and tissue preparation

Male C57BL/6 mice (Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan) were maintained until they were six months old with free access to a solid commercial diet (CRF-1, Charles River Laboratories Japan Inc., Kanagawa, Japan) and water under 12 h light/dark conditions. ^{13}C-labeled dihydroceramide was dispersed in 6 mM sodium taurocholate solution and administered through a stomach tube for 12 days (20 mg/mouse/day). After administration, the liver, skin epidermis, skeletal muscle, and brain were extracted. The synapse membrane (synaptosome) fraction was prepared from the brain tissue by Ficoll discontinuous gradient centrifugation according to a previous report (22). Handling of the experimental animals was performed according to the "Guiding Principles for the Care and Use of Laboratory Animals" and the ethical treatment of laboratory animals at the Central Research Institute of the Mizkan Group Corporation (Aichi, Japan).

Extraction, hydrolysis, and fractionation of total sphingoid base in tissue lipids

Tissues were homogenized using a Potter homogenizer, and the total lipids were extracted from the tissue homogenates with chloroform:methanol (2:1, v/v) with occasional shaking. The dried total lipid was hydrolyzed using concentrated HCl:methanol:water at 8.6:9.4:100 (v/v) by incubation at 70°C for 18 h. The hydrolyzed sample was dried and applied for silica gel column chromatography. The total sphingoid base fraction was collected by elution with chloroform:methanol (1:1, v/v).

Analysis of the dihydroceramide and sphingoid base

For the preparation of trimethylsilyl (TMS) derivatives, 25 µl anhydrous pyridine and 2.5 µl bis(trimethylsilyl)trifluoroacetamide
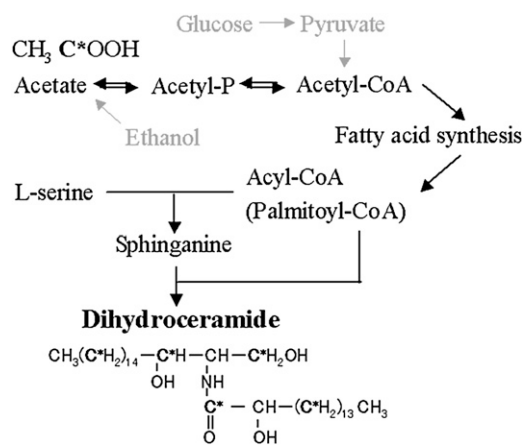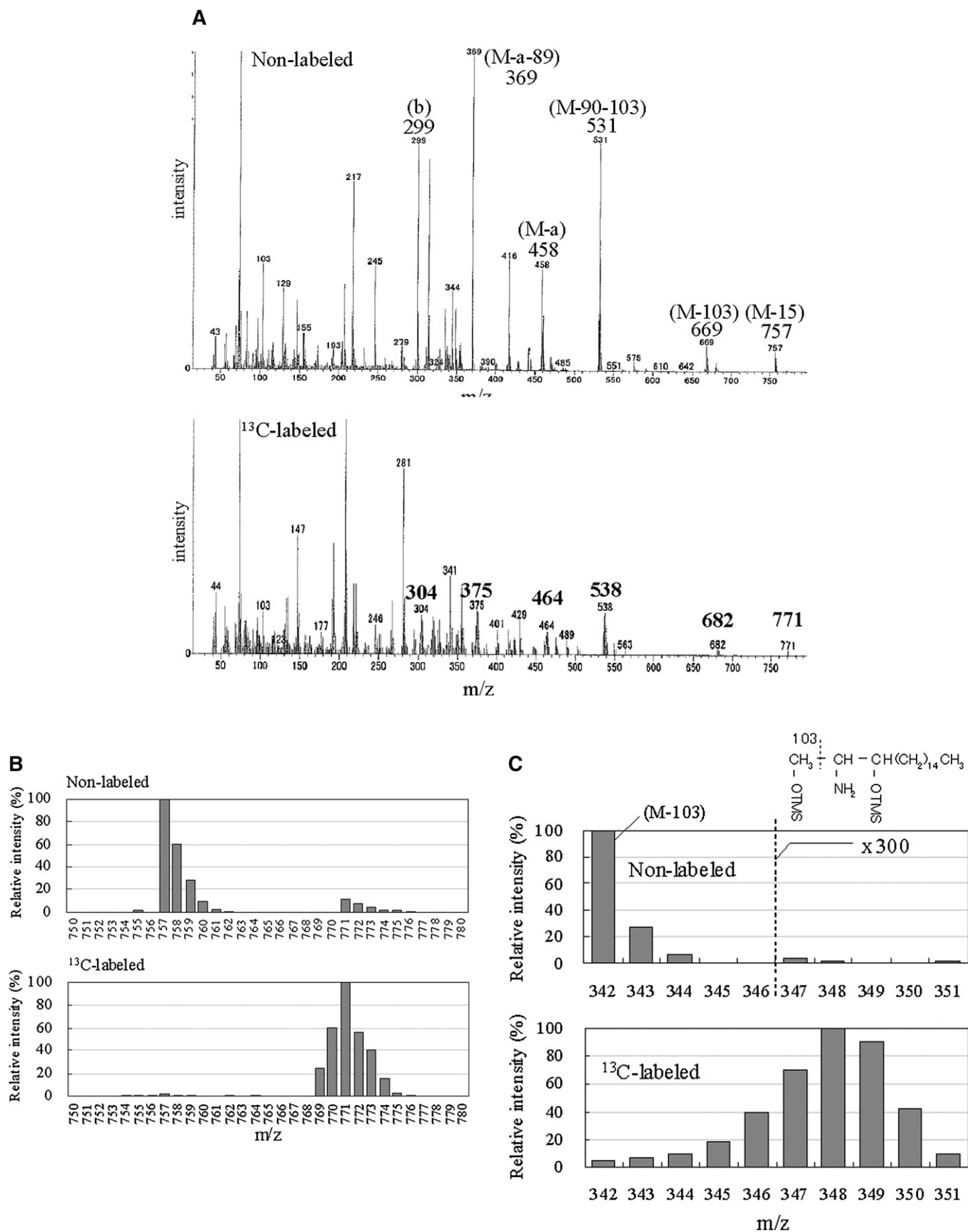
Fig. 2. Analysis of the trimethylsilyl (TMS) derivatives of [^{13}C]dihydroceramide and the sphingoid base by GC/MS. A: Mass spectrum of nonlabeled and ^{13}C-labeled dihydroceramide analyzed by total ion monitoring (m/z 0–800). B: Relative intensities of nonlabeled and ^{13}C-labeled dihydroceramide (m/z 750–780). C: Relative intensities of the sphingoid base (sphinganine) derived from hydrolyzed nonlabeled and ^{13}C-labeled dihydroceramide (m/z 342–351) analyzed in selected ion monitoring mode.

(BSTFA) were added to total sphingoid base fraction that corresponds to 50–100 mg wet tissue weight. The sample was incubated at 60°C for 5 min and then at room temperature for 10 min. Then 5 µl of the sample was injected into a GC/MS (QP-5050, Shimadzu Corp., Kyoto, Japan) equipped with a BPX5 GC capillary column (30.0 m × 250 µm × 0.25 µm, SGE, Melbourne, Australia). The oven temperature was raised from 190°C to 350°C by 10°C/min and maintained for 10 min for the analysis of dihydroceramide. The sphingoid base was analyzed by raising the oven temperature from 190°C to 260°C by 2°C/min and maintaining the temperature for 10 min. Samples were detected under 70 eV electron ionization. D-erythro-dihydrosphingosine (sphinganine) and D-erythro-sphingosine (sphingosine) were used as standards (Sigma-Aldrich Japan Co., Tokyo, Japan). The intensities of m/z 342–351 for sphinganine and m/z 340–349 for sphingosine were detected using selected ion monitoring mode, and relative intensities of the ions were calculated against m/z 342 and 340 (both derived from nonlabeled compounds), respectively. The replacement ratio of the pools of sphingosine and/or sphinganine in the tissues by ^{13}C-labeled sphingoids were calculated from the relative intensities of the standard mixture, which contained nonlabeled and [^{13}C]sphingoid base at 1:1 (w/w). The average ratios of the [^{13}C]sphingoid base concentration to relative intensities of m/z 347–349 for sphinganine and 345–347 for sphingosine were used for the coefficient of the calculation.

RESULTS

Analysis of dihydroceramide labeled by acetic acid bacteria

^{13}C-labeled dihydroceramide was purified from *Acetobacter malorum* NCI1683 (S24). The yield of ^{13}C-labeled dihydroceramide was 5.0 g from 400 g dry cell weight. In the mass spectrum of the TMS derivative of dihydroceramide, the ion of (M-15)+, corresponding to the molecular ion, was shifted from m/z 757 for nonlabeled dihydroceramide to m/z 769–774 for the ^{13}C-labeled species (**Fig. 2A**, B). The fragment ions derived from 2-hydroxypalmitate [fragment ion (b) in Fig. 2A] also shifted by 5 mass units in ^{13}C-labeled dihydroceramide. In the spectrum of the TMS derivative of sphinganine that was generated by the hydrolysis of ^{13}C-labeled dihydroceramide, the fragment of m/z 342 (M-103) for the nonlabeled compound was shifted to m/z 346–350 (Fig. 2C). These analyses by GC/MS show that dihydroceramide labeled with the ^{13}C-stable isotope can easily be prepared in relatively large amounts by the culture of acetic acid bacteria with ^{13}C-labeled acetic acid.

Analysis of sphingoid base in tissue of mice administered with ^{13}C-labeled dihydroceramide

After 12-day administration of ^{13}C-labeled dihydroceramide to mice, the total sphingoid base fractions were prepared from the skin epidermis, skeletal muscle, liver, and brain synaptosomes of the animals. TMS derivatives of the sphingoids were subjected to GC/MS analyses to detect incorporated ^{13}C-labeled sphingoids in the tissues (**Fig. 3**). The partial spectrum patterns of sphinganine (m/z 342–351 derived from the fragment ion shown in Fig. 2C) in the epidermis, liver, skeletal muscle, and synapse membrane of a representative mouse are shown in **Fig. 4A**. In the skin epidermis and liver, the spectrum patterns spe-

cific to ^{13}C-labeled sphinganine (i.e., high relative intensities of m/z 347–350) were clearly detected in all mice (n = 5). Identical patterns of the labeled sphinganine were also found in the skeletal muscle and synapse membrane of all mice, although the intensities were lower than for epidermis and liver. These results indicate that sphinganine derived from orally administered [^{13}C]dihydroceramide was incorporated into each tissue tested. Intriguingly, a series of fragment ions derived from ^{13}C-labeled sphingosine was detected in liver (Fig. 4B). This result implies that orally administered [^{13}C]sphinganine was converted to sphingosine in the liver. In other tissues, the specific pattern of labeled sphingosine was not found. **Table 1** shows the estimated replacement ratios of the total pools of sphinganine and sphingosine in each tissue by [^{13}C]sphinganine and [^{13}C]sphingosine. Sphinganine obtained from skin epidermis, liver, skeletal muscle, and synaptic plasma membrane was replaced at about 4.5, 4.0, 1.0, and 0.3%, respectively, by [^{13}C]sphinganine. Moreover, the ratio of [^{13}C]sphingosine in liver was about 1.0% to total sphingosine.

DISCUSSION

In this study, we cultivated acetic acid bacteria with [^{13}C]acetic acid. The labeled acetic acid was efficiently incorporated into the lipid membrane of the bacteria. Dihydroceramide was labeled with ^{13}C as indicated by GC/MS analysis, which detected a shift in the fragment ion from m/z 757 to m/z 769–774, in which both sphinganine and 2-hydroxypalmitate were labeled.

In animal cell cultures, the activity of ceramide synthesis has been analyzed by measuring the incorporation of radiolabeled acetic acid into ceramide (23). Acetic acid bacteria can tolerate high acetic acid concentrations that exceed 5% in the culture medium and can assimilate acetic acid as a carbon source (19). An amount of 0.25% [^{13}C]acetic acid in the culture broth was preferentially used as a
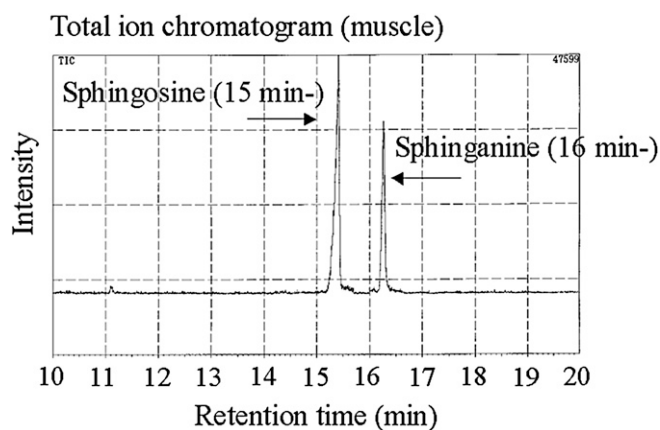

Fig. 3. Total ion chromatogram of the total sphingoid base fraction from murine muscle in GC/MS analysis. The total lipid of the tissue was hydrolyzed, applied for silica gel column chromatography. Eluate with chloroform:methanol (1:1, v/v) was collected and the trimethylsilyl (TMS) derivative of the total sphingoid base fraction was injected into GC/MS.
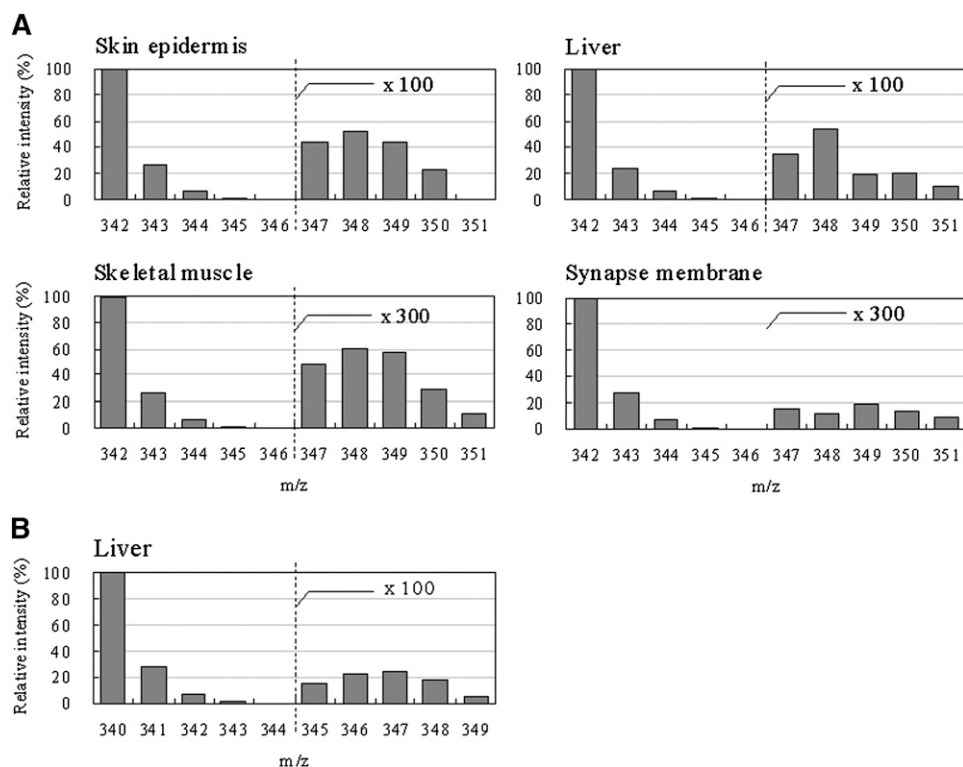
Fig. 4. Total sphingoid base fractions of the tissues from [^{13}C]dihydroceramide-administered mice. Trimethylsilyl (TMS) derivative of the sphingoid base was analyzed by GC/MS in selected ion monitoring mode. A: Relative intensities of sphinganine in epidermis, liver, skeletal muscle, and synapse membrane of the representative mouse (m/z 342–351). B: Relative intensities of sphingosine in the liver of the representative mouse (m/z 340–349).

precursor of acyl-CoA, although the medium in this study also contained 3% glucose as an alternative carbon source. Thus, ^{13}C incorporation into dihydroceramide was not 100% efficient, but it was sufficient for the distinction between labeled and nonlabeled dihydroceramide in GC/MS analysis. Chemical synthesis of ceramide could generate a mixture of R- and S-isomers, but ceramide synthesized by fermentation is thought to contain only biological (2S, 3R)-ceramide (24). Dihydroceramide is a saturated molecule, synthesized by acetic acid bacteria from serine and palmitoyl-CoA (Fig. 1). In eukaryotes, dihydroceramide is converted to ceramide, which has a double bond in the molecule. Sphingolipids, such as glycosphingolipids and sphingomyelin, are synthesized from ceramide by addition of oligosaccharides or phosphocholine to the 1- hydroxy group of the sphingoid base. Thus, dihydroceramide can also be used as a substrate for the enzymatic synthesis of various sphingolipids. In this study, the intact molecule or metabolites in the tissues could not be examined because of a lack of detection sensitivity. However, analysis using LC/MS/MS should be able to detect [^{13}C]dihydroceramide or its derivatives at remarkably high sensitivity. Therefore, ^{13}C-labeled dihydroceramide prepared from acetic acid bacteria can be a useful tool for studying sphingolipid metabolism or the markers of various diseases caused by abnormal sphingolipid metabolism.

The purified [^{13}C]dihydroceramide was administered orally to mice, and the total sphingoid base fractions in the tissues were analyzed to confirm incorporation into tissues and metabolism of sphingoids after ingestion. ^{13}C-labeled sphinganine was detected in the skin epidermis, liver, skeletal muscle, and synapse membrane of the brain. In addition to labeled sphinganine, ^{13}C-labeled sphingosine was detected in liver tissue. These results indicate that ingested dihydroceramide can be incorporated into each tissue and metabolized to a different sphingolipid in the liver.

TABLE 1. Distribution of ^{13}C-labeled sphinganine and sphingosine in total pools of each sphingoid base

Tissue	[^{13}C]sphinganine (%)[a]		[^{13}C]sphingosine (%)[a]	
	Mean	SE	Mean	SE
Skin epidermis (n = 5)	4.422	0.787	trace	
Liver (n = 5)	4.113	0.397	1.051	0.153
Skeletal muscle (n = 4)	0.994	0.158	trace	
Synapse membrane (n = 5)	0.292	0.048	trace	

Replacement ratios by [^{13}C]sphinganine and [^{13}C]sphingosine in each tissue were calculated.
[a]Values were corrected by the intensities of non-labeled sphinganine and sphingosine standard.

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In a human squamous carcinoma cell line or epidermal keratinocytes, ceramide and sphingosine at 5–10 μM are reported to promote the expression of proteins, such as involucrin and transglutaminase, which are involved in barrier function of the stratum corneum (25, 26). With regard to brain function, in vitro studies using hippocampal neuronal cells or cerebellar Purkinje cells showed that 1–20 μM ceramide can promote dendrite and axon elongation and survival of cells (8, 27–29) as well as inhibit cytotoxicity from reactive oxygen or amyloid-β peptide (30). Gangliosides, composed of sialic acid and oligosaccharides conjugated to ceramide, are also reported to act on in vitro neurons like neurotrophic factors at concentrations in the nanomolar range (31, 32). Schmelz et al. reported that dietary ceramide in mice is hydrolyzed to a sphingoid base and fatty acids by ceramidase in the intestinal tract and then absorbed in the small intestine (33). Intact ceramide can also be absorbed in the intestine (11). Nilsson showed that radiolabeled sphinganine administered orally to rats could be absorbed and metabolized much better than sphingosine in the intestinal tract. A part of these sphingoid bases could be incorporated into ceramide and sphingomyelin in the mucosal cells (15). The results of this study and those reports indicate that sphinganine decomposed from dihydroceramide or intact dihydroceramide could be absorbed in the intestine of mice at higher efficiency than other sphingoid base structures. Absorbed sphinganine, which can be reconstructed to complex sphingolipids, or intact dihydroceramide might then exert various physiological functions in peripheral tissues as discussed earlier. To evaluate whether ingested dihydroceramide or its derivatives are involved in various physiological functions, it is necessary to conduct quantitative determination and identification of complex sphingolipid structures derived from ingested ^{13}C-labeled dihydroceramide. Such studies are currently underway in our laboratory.

In conclusion, this study describes the preparation of various sphingolipids labeled with stable isotopes and the development of a novel methodology to analyze the metabolism and nutritional importance of sphingolipids.

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