Selective Absorption of Dietary Sphingoid Bases from the Intestine via Efflux by P-Glycoprotein in Rats

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Summary Various physiological functions of dietary sphingolipids, such as preventing inflammation and improving the skin barrier function, have been recently demonstrated. The sphingolipid most commonly used as a foodstuff is glucosylceramide from plant sources, which is composed of sphingoid bases that are distinctive from those found in mammals. Although the structure of sphingoid bases in higher plants is more complicated than the structure of those in mammals, the fate of dietary sphingolipids of plant origin is still not understood. In the present study, we investigated the absorption of 4,8-sphingadienine that originated from maize glucosylceramide in the rat intestine by using a lipid absorption assay of lymph collected from the thoracic duct. The cumulative recovery of 4,8-sphingadienine was lower than that of sphingosine. Verapamil, a P-glycoprotein inhibitor, significantly increased the absorption of 4,8-sphingadienine but did not affect the absorption of sphingosine. Plant-derived sphingoid bases were detected in the ceramide fraction of lymph fluid by using liquid chromatography-mass spectrometry analysis. These results indicate that 4,8-sphingadienine that originates from the glucosylceramide of higher plants is poorly absorbed in the intestine because of efflux by P-glycoprotein and can be incorporated into a ceramide moiety, at least in part, in intestinal endothelial cells.

Key Words sphingolipids, sphingoid bases, sphingadienine, intestinal absorption, P-glycoprotein

Sphingolipids are ubiquitous in all eukaryotic organisms and constitute a family of compounds that have a sphingoid base with an amide-linked fatty acid and a polar head group. Sphingolipids, having various structures of sphingoid bases, are ingested daily from foodstuffs because of the diverse structures of sphingoid bases that occur in nature. The most common sphingoid base of mammalian sphingolipids is sphingosine (trans-4-sphingenine, d18:14t). Smaller amounts of other sphingoid bases such as sphinganine (dihydrosphingosine, d18:0) and phytosphingosine (4-hydroxysphinganine, t18:0) are encountered frequently. In higher plants, the structures of sphingoid bases are more complicated than in mammals because they can be desaturated at the C8-position by D8-sphingolipid desaturase, yielding cis- and trans-isomers of D8-unsaturated sphingoid bases (d18:24t,8c(t)). Dietary sphingolipids from various sources have been reported to have beneficial effects such as preventing cancer (1–3), reducing inflammatory responses (4), and lowering levels of plasma lipids (5, 6). Dietary plant sphingolipids with sphingoid bases distinct from those found in mammals are able to prevent the formation of aberrant crypt foci in 1,2-dimethylhydrazine-treated mice (7, 8). In addition, the beneficial effects of dietary plant sphingolipids have been reported in patients with atopic eczema and in model mice of skin damage, although the compositions of sphingoid bases in the stratum corneum of mammals are quite different from those of dietary glucosylceramide derived from plant sources (9–11). Our previous findings suggest that the skin barrier-improving effect of dietary sphingolipids might be due to the activation of ceramide synthesis in the skin, rather than to the direct reutilization of dietary sphingolipids (12).

Dietary sphingolipids can be hydrolyzed to their components such as sphingoid bases, fatty acids, and the polar head group by intestinal enzymes and are then taken up by mucosal cells (13, 14). A large portion of the sphingosine absorbed by the intestine is metabolized to fatty acids, and a small part is resynthesized to complex sphingolipids (15, 16). We previously demon-
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Sodium taurocholate, sphingosine, and verapamil were purchased from Sigma Chemical Co. (St. Louis, MO). n-Phthalaldehyde (OPA) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Other chemicals and solvents were of reagent grade.

Glucosylceramides were prepared from maize by using a silica gel column after lipid extraction and saponification as described previously (17, 18). Trans-4,cis-8-sphingadienine (d18:2 4t,8c) was prepared from maize glucosylceramide after acid hydrolysis and was purified by using high-performance liquid chromatography (HPLC) as described previously (19). The purities of all the sphingoid bases prepared were >96% as determined by using HPLC.

Cannulation of the thoracic ducts of the rats. The surgeries and maintenance of the rats and all the other procedures were as described previously (18). Male Sprague-Dawley rats (9 wk old) were obtained from Japan SLC, Inc. (Hamamatsu, Japan) and were housed in stainless steel wire-mesh cages in a room maintained at 23 ± 1˚C with a 12-h light/dark cycle. The experimental or control group consisted of 5 rats. After acclimatization for 1 wk with MF Standard Rodent Chow (Oriental Yeast Co., Ltd., Tokyo, Japan) and distilled water (free access), the rats were anesthetized and a cannula (SP-55, Dural Plastics) was inserted in their left thoracic duct to collect lymphatic fluid and a catheter (SP-55, Dural Plastics) was inserted in their stomach. After sur-
collection of lymph for 2 h as a blank control, the rats were infused with 3 mL of a glucose-NaCl solution and the same solution was also infused. 30˚C until analyzed. The animal care procedures and methods were approved by the Animal Use Commit-
tide of Tohoku University (approval No. 22Noudou-5).

Lipid extraction. Lymphs were extracted from each sample by using chloroform-methanol (2:1, v/v). After evaporation of the collected chloroform phase, the residue was saponified with 0.4 mol/L KOH in methanol at 38˚C for 2 h to remove glycerolipids (20). The alkali-stable fraction was recovered with chloroform and then washed with water. The chloroform phase was evaporated to dryness, dissolved in chloroform-methanol (2:1, v/v), and then subjected to HPLC analysis for quantification of free sphingoid bases (free sphingoid base fraction). A portion of the alkali-stable fraction of each lymph extract was degraded with an aqueous methanolic solution of 1 mol/L HCl for 18 h at 70˚C (21). The free sphingoid bases liberated from the complex sphingolipids were then subjected to HPLC analysis for quantification of total sphingolipids (total sphingoid base fraction).

HPLC analyses. The OPA derivatives of the free forms of the sphingoid bases were analyzed by using a reverse-phase HPLC system equipped with a fluorescence detector (22). Each sample extract was dissolved in methanol and was then mixed with OPA reagent (methanol-OPA reagent, 4:1, v/v), which was freshly prepared daily by dissolving 5 mg of OPA and 50 μL of 2-mercaptoethanol in 0.1 mL of ethanol and then adjusting the volume to 10 mL with a borate buffer (pH adjusted to 10.5 with 1 M KOH). After the solution had been incubated for 30 min at room temperature, aliquots were injected into the HPLC system, which consisted of an LC-10AD pump and an RF-10AXL fluorescence detector (Shimadzu Co., Kyoto, Japan). Sphingoid bases were separated on a 4.6- × 250-mm TSK gel octadecylsilyl (ODS)-80Ts column (Toosho, Tokyo, Japan) and eluted by using a binary gradient consisting of acetonitrile (A) and water (B). The gradient profile was as follows: 0 to 15 min, 45% to 20% B linear; 15 to 30 min, 20% B; 30 to 40 min, 20% to 0% B linear; and 40 to 60 min, 0% B. The flow rate was 1.0 mL/min, and the column temperature was maintained at 40˚C. OPA derivatives were detected at an excitation wavelength of 334 nm and an emission wavelength of 440 nm. The sphingoid bases were quantified from their peak areas by comparison with standard curves. After the column eluent passed through the fluorescence detector, it was analyzed on a LCMS-2010EV mass spectrometer (Shimadzu Co.) equipped with an electrospray ionization interface for further identification of peak components (18).

Liquid chromatography tandem mass chromatography analyses. An HPLC system coupled to LCMS-IT-TOF
equipped with an electrospray ionization interface (Shi-
madzu Co.) was used. A TSK gel super ODS column
(2.0×50 mm, Tosoh) was eluted with methanol-water
(95 : 5, v/v) containing 5 mmol/L ammonium acetate at
a flow rate of 0.2 mL/min. The MS was operated under
the following conditions: probe voltage, 4.50 kV; CDL
temperature, 200˚C; block heater temperature, 200˚C;
nebulizer gas flow, 1.5 L/min; ion accumulation time,
100 m; MS range, m/z 500 to 700; MS2 range, m/z 200
to 300; and CID parameters, 60% energy and 60%
collision gas. For the structural analysis of ceramide,
[M+H−18]⁺ (loss of water) in the positive scan mode
was used for the tandem mass chromatography (MS/
MS) analysis to obtain the product ions. The typical
signal (m/z 262.3), which is characteristic for the
sphingadienine moiety, was observed as the product ion
by using the auto MS/MS detection mode. Pairs of the
structurally specific product ion of sphingadienine and
their precursor ions were used to identify the ceramide
molecules (18, 23, 24).

Fig. 1. Cumulative recovery of lymph fluid from the
thoracic ducts of rats. After infusion of test emulsions
containing 4t,8c-sphingadienine or sphingosine, lymph
flow was measured. Data are reported as mean±SE
(n = 5).

Fig. 2. HPLC chromatograms of free and total sphingoid base fractions extracted from rat lymph before and after adminis-
tration of 4t,8c-sphingadienine. The peaks corresponding to 4t,8c-sphingadienine are indicated by arrows.
Statistical analyses. Data are reported as mean±SE (n=5). Statistical analyses were performed by using one-way ANOVA or the Student t test.

RESULTS

No significant difference in lymph output was observed among the rats infused with any of the emulsions, confirming that the surgery and maintenance of the rats were appropriately performed (Fig. 1). To quantify the absorption of dietary sphingoid bases via the lymph, the OPA derivatives of the sphingoid bases in the lymph extracts were analyzed by using an HPLC system with a fluorescence detector (Fig. 2). After administration (0–1 h fraction), 4t,8c-sphingadienine clearly appeared in the alkali-stable fraction (free sphingoid base fraction). The peak ascribed to 4t,8c-sphingadienine was prominently increased in the lymph extract by hydrolysis with aqueous methanolic 1 mol/L HCl (total sphingoid base fraction). These results demonstrate that the intact form of 4t,8c-sphingadienine is absorbed from the digestive tract into the lymph and is mainly resynthesized into complex sphingolipids. In both the free and total sphingoid base fractions, the presence of verapamil increased the peak of 4t,8c-sphingadienine. A small peak at the same retention time as that of 4t,8c-sphingadienine, which might be due to the chow provided, was detected in lymph collected from −2 h to 0 h (i.e., before administration). The recovery of sphingoid bases after administration was corrected individually by using the baseline value.

The lymphatic recovery of 4t,8c-sphingadienine is shown in Fig. 3A and B. The cumulative recovery of sphingadienine from the total sphingoid base fraction, which contains hydrolysates of complex sphingolipids, was more abundant than that from the free form fraction in the lymph after administration. Verapamil significantly enhanced the recovery of 4t,8c-sphingadienine from the free form fraction until 4 h after administration. In the case of the total sphingoid base fraction, the cumulative recovery of 4t,8c-sphingadienine in the presence of verapamil was significantly higher than that in the absence of verapamil at 1 h after administration. The recovery of sphingosine in the lymph of rats infused with an emulsion containing triolein only was increased in a time-dependent manner, as chylomicrons contain endogenous sphingomyelin and ceramide (18, 24). Thus, the recovery of sphingosine after administration of sphingosine was corrected by using the mean value
of sphingosine in the rats infused with triolein. The lymphatic recovery of free and total sphingosine was higher in the sphingosine-treated rats than in the rats infused with 4t,8c-sphingadienine. However, no significant difference was found between the group treated with and that treated without verapamil. These results indicate that P-glycoprotein affects the intestinal absorption of 4t,8c-sphingadienine but not that of sphingosine.

To identify complex sphingolipids consisting of 4t,8c-sphingadienine in the lymph after administration of the free form of 4t,8c-sphingadienine, the alkali-stable fraction of the lymph extract (total sphingoid base fraction) was analyzed to obtain structural information by using LCMS-IT-TOF (Fig. 4). Sphingolipids containing sphingadienine showed a characteristic product ion at m/z 262.3. As the precursor ion of m/z 262.3, [M+H−18]+ ions at m/z 518.5, 544.5, and 546.5 were detected. According to these results, 3 types of ceramides consisting of 4,8-sphingadienine (i.e., N-palmitoyl-4,8-sphingadienine [C16:0-d18:2], N-oleoyl-4,8-sphingadienine [C18:1-d18:2], and N-stearoyl-4,8-sphingadienine [C18:0-d18:2]) were identified. Our results indicate that sphingadienine can be metabolized to ceramide in intestinal cells during absorption.

**DISCUSSION**

In the present study, we investigated the selective absorption of dietary sphingoid bases from the rat intestine via efflux by P-glycoprotein using verapamil, its specific inhibitor. We demonstrate that the intact form of 4t,8c-sphingadienine, one of the predominant structures of sphingoid bases in higher plants, can be absorbed into the lymph and synthesized to complex sphingolipids, including at least ceramide, by intestinal cells. The cumulative recovery of 4t,8c-sphingadienine is relatively lower than that of sphingosine. Verapamil significantly enhanced the recovery of 4t,8c-sphingadienine from the free and total sphingoid base fractions in the lymph. These results indicate that P-glycoprotein discharges 4t,8c-sphingadienine from enterocytes after absorption but not sphingosine.

Dietary sphingolipids would be hydrolyzed to free sphingoid bases in the digestive tract before absorption into the lymph (13–17). Alkaline sphingomyelinase is present in the intestinal mucosa (25–27), and the glycosylceramidase activity in the intestine is due to lactase-phlorizin hydrolase (28, 29). Neutral ceramidase encoded by *Asah2* is also present in the small intestine (30). Previously, we investigated the absorption of dietary glucosylceramide derived from maize into the rat intestine compared with that of glucosyl-N-palmitoylsphingosine and demonstrated that the intact form of sphingadienine was absorbed into the lymph by intestinal cells after administration of maize glucosylceramide. However, the recovery was lower than that of sphingosine after administration of glucosyl-N-palmitoylsphingosine (18). In addition, we reported that the uptake of sphingosine is significantly higher than that of other sphingoid bases (including 4,8-sphingadienine) in differentiated Caco-2 cells (a model for intestinal cells) and that P-glycoprotein probably contributes to this selective absorption of sphingosine (19). P-Glycoprotein, encoded by the MDR1 gene, transports various hydrophobic compounds, including drugs, natural products, toxicants, and peptides, and contributes to the barrier function of the gut (31). In this study, the recovery of 4t,8c-sphingadienine in the lymph after administration is enhanced by the presence of verapamil, a specific inhibitor of P-glycoprotein. Our observations thus support an important role for P-glycoprotein in the efflux of 4t,8c-sphingadienine, but not sphingosine, across the apical membranes of enterocytes after absorption,
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similar to the selective mechanism for the intestinal absorption of sterols. Dietary plant sterols are generally less absorptive than cholesterol because the ATP-binding cassette transporter (ABC)G5/ABCG8 preferentially excretes plant sterols in intestinal epithelial cells (32). The intestinal cells seem to act as a gatekeeper of the host defense to select utilizable molecules for the body from the diet.

In this study, ceramides consisting of sphingadienine were found in the lymph after administration of 4t,8c-sphingadienine. This result indicates that sphingadienine can be metabolized to ceramide in intestinal cells during absorption. As phosphorylation followed by dephosphorylation is required for the incorporation of exogenous sphingoid bases into complex sphingolipids (33, 34), 4t,8c-sphingadienene should be phosphorylated by sphingosine kinase after uptake by intestinal cells.

In conclusion, our results demonstrate that dietary sphingadienine derived from glucosylceramide originating from higher plants is transported out of intestinal cells by P-glycoprotein after absorption. Our findings provide an important and new insight into the mechanism for the selective intestinal absorption of dietary sphingolipids.

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