The relationship between the metabolism of sphingomyelin species and the hemolysis of sheep erythrocytes induced by *Clostridium perfringens* α-toxin[S]

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Abstract ***Clostridium perfringens*** **α-toxin induces the hemolysis of sheep erythrocytes by activating the metabolism of sphingomyelin (SM) via a GTP binding protein in membranes. α-Toxin stimulated the formation of 15-*N*-nervonoyl sphingosine ($C_{24:1}$-ceramide), which was identified by positive ion fast atom bombardment-MS and ^{1}H-NMR spectroscopy. $C_{24:1}$-ceramide stimulated the toxin-induced hemolysis of saponin-pretreated sheep erythrocytes and increased the production of sphingosine 1-phosphate (S1P) in the cells, but *N*-lignoceroyl sphingosine did not. These events elicited by the toxin in the presence of $C_{24:1}$-ceramide were significantly attenuated by treatment with dihydrosphingosine, a sphingosine kinase inhibitor. TLC showed that the level of $C_{24:1}$-ceramide was highest among the ceramides with an unsaturated bond in the fatty acyl chain in the detergent-resistant membranes (DRMs). The toxin specifically bound to DRMs rich in cholesterol, resulting in the hydrolysis of *N*-nervonoic sphingomyelin ($C_{24:1}$-SM) in DRMs. Treatment of the cells with pertussis toxin (PT) inhibited the α-toxin-induced formation of $C_{24:1}$-ceramide from $C_{24:1}$-SM in DRMs and hemolysis, indicating that endogenous sphingomyelinase, which hydrolyzes $C_{24:1}$-SM to $C_{24:1}$-ceramide, is controlled by PT-sensitive GTP binding protein in membranes. These results show that the toxin-induced metabolism of $C_{24:1}$-SM to S1P in DRMs plays an important role in the toxin-induced hemolysis of sheep erythrocytes.**—Oda, M., T. Matsuno, R. Shiihara, S. Ochi, R. Yamauchi, Y. Saito, H. Imagawa, M. Nagahama, M. Nishizawa, and J. Sakurai. **The relationship between the metabolism of sphingomyelin species and the hemolysis of sheep erythrocytes induced by *Clostridium perfringens* α-toxin. *J. Lipid Res.* 2008.** 49: **1039–1047.**

Supplementary key words $C_{24:1}$-sphingomyelin • LC-MS/MS • detergent-soluble fractions

Clostridium perfringens α-toxin is an important agent of gas gangrene (1–4). The toxin, which exhibits phospholipase C and sphingomyelinase (SMase) activities, causes hemolysis, necrosis, and death. It induces the hemolysis of various erythrocytes (5, 6). We reported that α-toxin induces the hemolysis of rabbit erythrocytes and the generation of superoxide anion in rabbit neutrophils through the activation of endogenous phospholipase C via a pertussis toxin (PT)-sensitive GTP binding protein, Gi (7, 8). We also reported that the toxin induces the hemolysis of sheep erythrocytes through the activation of endogenous SMase via Gi (9).

The activation of the sphingomyelin (SM) cycle, analogous to the glycerophospholipid cycles, has been recognized as a key event in the signal transduction cascade involved in cellular proliferation, differentiation, and apoptosis (10). Ceramide causes the arrest of cell growth and apoptosis (11, 12). Sphingosine was found to be a potent inhibitor of protein kinase C (13) and to inhibit cell growth and induce apoptosis (11). Sphingosine 1-phosphate (S1P) has been reported to promote cell growth and inhibit apoptosis, in contrast with ceramides (14). Therefore, it appears that the dynamic balance among the intracellular levels of ceramide, sphingosine, and S1P is important in determining whether a cell survives or dies. The variation of SM molecular species is attributed to the

Abbreviations: $C_{16:0}$-ceramide, *N*-palmitoyl sphingosine (*d*18:1/16:0); $C_{24:0}$-ceramide, *N*-lignoceroyl sphingosine (*d*18:1/24:0); $C_{24:1}$-ceramide, 15-*N*-nervonoyl sphingosine (*d*18:1/24:1); $C_{24:2}$-ceramide, 15,18-*N*-tetracosadienoyl sphingosine (*d*18:1/24:2); $C_{24:1}$-SM, *N*-nervonoic sphingomyelin; DGK, 1,2-diacylglycerol kinase; DHS, dihydrosphingosine; DRM, detergent-resistant membrane; FAB, fast atom bombardment; MβCD, methyl-β-cyclodextrin; *N*-OE, *N*-oleoylethanolamine; PT, pertussis toxin; S1P, sphingosine 1-phosphate; SM, sphingomyelin; SMase, sphingomyelinase.

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[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of three figures.

Manuscript received 19 December 2007 and in revised form 4 February 2008.

Published, JLR Papers in Press, February 8, 2008.
DOI 10.1194/jlr.M700587-JLR200

degree of saturation, the length of fatty acyl chains, and the (α)-hydroxylation of the *N*-linked fatty acids. The generation of C_{16}-ceramide induced by acidic SMase contributed to the tumor necrosis factor-α-induced apoptosis of hepatocytes (15). An increase in the long-term accumulation of C_{16}-ceramide was also seen during Fas-induced apoptosis, and the initial increase in C_{16}-ceramide levels closely paralleled the decrease in mitochondrial mass during Fas- or radiation-induced apoptosis (16). Kroesen et al. (17) reported that the B-cell receptor-induced formation of C_{16}-ceramide results in proteasomal activation and degradation of the X-linked inhibitor of protein and the subsequent activation of effector caspases, demonstrating an important cell biological mechanism through which C_{16}-ceramide may be involved in the progression of B-cell receptor triggering-induced apoptosis. The mechanism underlying these actions is one key to understanding the relationship between the metabolism of molecular species of ceramide and biological reactions. However, the relationship remains poorly understood.

In the present study, to clarify whether the metabolism of a particular species of SM is involved in the α-toxin-induced hemolysis of sheep erythrocytes, we investigated the relationship between the metabolism of SM molecular species in detergent-resistant membranes (DRMs) and hemolysis induced by the toxin.

MATERIALS AND METHODS

Materials

Sheep erythrocytes were purchased from Nippon Bio-Test Laboratories, Inc. (Tokyo, Japan). Cardiolipin, methyl-β-cyclodextrin (MβCD) from bovine heart, and ATP were from Sigma Chemical Co. (St. Louis, MO). Hexanoic anhydride was obtained from Sigma-Aldrich Chemical Co. (Milwaukee, WI). Sphingosine from bovine brain, dihydrosphingosine (DHS), and S1P were from Calbiochem-Novabiochem Co. (San Diego, CA). Leupeptin and pepstatin were obtained from Chemicon International, Inc. (Temecula, CA). 1-*O*-*n*-Octyl-β-D-glucopyranoside was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). [γ-^{32}P]ATP (4,500 Ci/mmol) was supplied by ICN Biochemicals, Inc. (Irvine, CA). *N*-Lignoceroyl sphingosine ($C_{24:0}$-ceramide) and 15-*N*-nervonoyl sphingosine ($C_{24:1}$-ceramide) were purchased from Avanti Polar Lipids, Inc. All other drugs were of analytical grade.

Synthesis of *N*-tetracosadienoyl sphingosine

After the conversion of (15*Z*,18*Z*)-tetracosa-15,18-dienoic acid into acid chloride by a reaction with $SOCl_2$, condensation of the carboxylic acid with sphingosine was accomplished using Et_3N in CH_2Cl_2 at 0°C for 15 min to give 15,18-*N*-tetracosadienoyl sphingosine ($C_{24:2}$-ceramide) in 75% yield.

Preparation of sheep erythrocytes

Sheep erythrocytes were suspended in 0.02 M Tris-HCl buffer (pH 7.5) containing 0.9% NaCl (TBS) and centrifuged at 1,100 *g* for 3 min. The erythrocytes were washed by centrifugation three times. The number of erythrocytes was determined with a cell counter (Celltac; Nihon Kohden Co., Tokyo, Japan).

Determination of hemolytic activity

α-Toxin was incubated with sheep erythrocytes (12×10^{10} cells/ml) in TBS containing 0.025% gelatin (GTBS) at 37°C for 30 min, and then the cells were chilled at 4°C. The hemolysis of the erythrocytes was measured as described previously (9). Hemolysis was expressed as a percentage of the amount of hemoglobin released from 0.1 ml of erythrocytes suspended in 0.4 ml of 0.4% NaCl.

Treatment of sheep erythrocytes with saponin

Treatment of sheep erythrocytes with saponin was performed as described previously (9).

Determination of S1P

The amount of S1P in sheep erythrocytes was measured as described previously (9).

SM extraction

The extraction of polar lipids in sheep erythrocytes was performed by the method of Bligh and Dyer (18). Phospholipids in the extract were fractionated by HPLC (Jasco, Tokyo, Japan) with a normal-phase column (YMC-Pack SIL column, S-5). The elution was carried out at a flow rate of 0.2 ml/min with the following solvent system: acetonitrile-methanol-85% phosphoric acid (100:40:0.4), and the SM fraction was collected.

HPLC-MS/MS analysis

Mass spectrometry was carried out using a Bruker-Daltonics HCT-plus ion-trap mass spectrometer equipped with an ESI ion source, a Hystar data-analysis system, and an Alliance 2695 HPLC apparatus. Molecular species of SM were fractionated on a 3 μm, 2.0 mm × 150 mm Cadenza CD-C18 column (Imtakt, Kyoto, Japan). Elution was carried out at a flow rate of 0.2 ml/min with a solvent system composed of 0.1% formic acid and 0.1% ammonia in methanol-distilled water (1:1, v/v) (solvent A) and 0.1% formic acid and 0.1% ammonia in methanol-acetonitrile (1:2, v/v) (solvent B). The gradient elution program was as follows: 0/0, 10/10, 20/50, 30/100, 120/100, 125/0 (min/solvent B%). Methanol was also used to wash the column for 10 min. This was followed by an equilibration procedure with solvent A for 15 min and then with solvent A for 10 min. The fragmentation voltage used for collision-induced dissociation to obtain MS2, MS3, and MS4 was 40–60%. Mass spectra were acquired over an *m/z* range of 100–1,000. As an internal standard, synthetic C_2-ceramide (*d*18:1/2:0) was used. A 50 mM stock solution of internal standard in 5 mM ammonium acetate in methanol was prepared and stored at −20°C. Serial dilutions were prepared from the stock solution and used to make calibration curves.

Determination of ceramide molecular species

An erythrocyte suspension (1×10^{11} cells/ml) treated with 100 μM *N*-oleoylethanolamine (*N*-OE) was incubated with or without α-toxin in a total volume of 0.5 ml of TBS containing 3 mM $CaCl_2$ at 37°C for 30 min. The reaction was terminated by the addition of 1.8 ml of chloroform-methanol (1:2, v/v). The lipids were extracted by the method of Bligh and Dyer (18) except that 0.2 M KCl and 5 mM EDTA were used instead of water. Ceramide content was determined by measuring the amount of [^{32}P]phosphorylated ceramide converted by *Escherichia coli* 1,2-diacylglycerol kinase (DGK) in the presence of [γ-^{32}P]ATP. Molecular species of phosphorylated ceramide were separated by reverse-phase TLC with chloroform-methanol-acetic acid-formic acid (10:85:5:0.5, v/v). Labeled lipids on the plate were visualized with a Bio-Imaging Analyzer FLA-2000 (Fujifilm).

Extraction and determination of ceramides

Sheep erythrocytes (5×10^{11} cells/ml) were incubated with α-toxin in GTBS at 37°C for 60 min, and then the reaction was stopped by $CHCl_3$/MeOH (2:1). The lipids extracted by the method of Bligh and Dyer (18), except for the use of 0.2 M KCl and 5 mM EDTA instead of water, were subjected to Iatrobeads column chromatography (Iatron Laboratories, Inc.). The lipids were eluted with $CHCl_3$ and then with acetone. The acetone fraction containing various ceramides was fractionated by HPLC (Cosmosil 5SL-2 column; Nacalai, Kyoto, Japan) with a solvent of ethylacetone-hexane (9:1, v/v). All fractions were phosphorylated with DGK from *E. coli* and developed by reverse-phase TLC with chloroform-methanol-acetic acid-hormic acid (10:85:5:0.5, v/v). The fraction including each ceramide was analyzed by positive ion fast atom bombardment (FAB)-MS, collision-induced dissociation spectroscopy, and LC-MS/MS. All mass spectra were acquired with a JMS-AX double-focusing mass spectrometer (JEOL Ltd., Tokyo, Japan). Positive ion FAB-MS was performed using only the first spectrometer. The spectra were measured under the following conditions: xenon atom beam, 5 kV; ion source accelerating potential, 10 kV; matrix, *m*-nitrobenzyl alcohol + NaCl. The conditions for LC-MS/MS were as described above.

Iodiation of α-toxin

^{125}I-labeled α-toxin was prepared according to the method of Bolton and Hunter (19). α-Toxin (25 μg) was incubated with 250 μCi of ^{125}I-labeled Bolton-Hunter reagent (2,000 Ci/mmol; GE Healthcare UK, Ltd.). The labeled α-toxin retained >90% of its original hemolytic activity.

Sucrose gradient fractionation

Separation of the DRMs was carried out by flotation-centrifugation on a sucrose gradient (20). Sheep erythrocytes were incubated in GTBS containing 20 ng/ml α-toxin at 37°C for various periods of time. The cells were washed with TBS and treated with 0.5% Triton X-100 for 30 min at 4°C in TBS containing the protease inhibitor mixture and sonicated in 30 s pulses using a tip-type sonicator. The lysates were adjusted to 40% sucrose (w/v), overlaid with 2.4 ml of 36% sucrose and 1.2 ml of 5% sucrose in TBS, centrifuged at 45,000 rpm (250,000 *g*) for 18 h at 4°C in an SW55 rotor (Beckman Instruments, Palo Alto, CA), and fractionated from the top (0.4 ml each, a total of 10 fractions). The aliquots were subjected to SDS-PAGE and autoradiography.

Preparation of sheep erythrocytes treated with MβCD and MβCD-cholesterol complex

To remove cholesterol, sheep erythrocytes were incubated for 1 h at 37°C in the presence or absence of 10 mM MβCD in TBS and then washed with TBS. For the cholesterol repletion experiment (21), 40 mg of cholesterol was coated on the walls of a glass tube and sonicated for 15 h in the presence of 5 ml of 50 mM MβCD in TBS. The treated solution (50 mM MβCD/cholesterol) was filtered and added back to cholesterol-depleted erythrocytes by incubation for 2 h at 37°C with the MβCD/cholesterol. Cholesterol levels were assayed spectrophotometrically using a diagnostic kit (Cholesterol C-test; Wako Pure Chemical, Osaka, Japan).

Immunoblot analysis of DRM marker proteins

Aliquots of the flotation sucrose gradient fractions were heated in 2% SDS-sample buffer at 100°C for 3 min. The samples were electrophoresed on an SDS-PAGE gel, followed by transfer to a polyvinylidene difluoride membrane. The membrane was blocked with TBS containing 2% Tween 20 and 5% skim milk and incubated with a primary antibody such as anti-Giα (1:1,000), anti-flotilinin1 (1:1,000), or anti neutral SMase (1:1,000) in TBS containing 1% skim milk, then with a horseradish peroxidase-conjugated secondary antibody, and finally with an enhanced chemiluminescence analysis kit (GE Healthcare UK, Ltd.).

Statistical analysis

All values are expressed as means ± SEM. Student's unpaired *t*-test and one-way ANOVA were used for statistical analyses. $P < 0.05$ was considered statistically significant.

RESULTS

Identification of molecular species of SM in sheep erythrocytes

To determine the molecular species of SM in sheep erythrocytes, SM was extracted from the cells according to a modified version of Bligh and Dyer's method (17) and isolated from the extract using HPLC. Fatty acids in the SM were analyzed using ESI-MS/MS. The collision-induced dissociation of SM in positive ion electrospray generates a characteristic product ion of *m/z* 184, corresponding to the phosphorylcholine ion, and other less abundant ions (22–24). Because ions of *m/z* 264 appear in all of them, it may be assumed that these ions correspond to the base of sphingosine with the loss of two molecules of water. Eighteen protonated molecular species of SM at least were detected from membranes of sheep erythrocytes (**Table 1**). The relative value of each molecular species is shown in Table 1. The predominant forms of SM were *d*18:1/16:0, *d*18:1/24:0, *d*18:1/24:1 (N-9), and *d*18:1/24:2 (N-6, N-9) (Table 1). Koumanov et al. (25) reported that much SM is composed of nervonoic acid ($C_{24:1}$), the most

TABLE 1. Relative amounts of molecular species of SM in sheep erythrocyte membranes

Species	Ghosts	Detergent-Resistant Membranes
*d*18:1/14:0	0.7 ± 0.1	0.3 ± 0.1
*d*18:1/15:0	1.2 ± 0.3	0.2 ± 0.1
*d*18:1/16:0	18.5 ± 0.3	74.4 ± 0.1
*d*18:1/16:1	2.1 ± 0.6	0.3 ± 0.1
*d*18:1/17:0	3.2 ± 0.2	0.2 ± 0.1
*d*18:1/18:0	1.6 ± 0.3	4.9 ± 0.4
*d*18:1/18:1	1.2 ± 0.3	3.4 ± 0.5
*d*18:1/19:0	0.9 ± 0.2	0.2 ± 0.1
*d*18:1/20:0	0.5 ± 0.2	0.1 ± 0.1
*d*18:1/22:0	1.3 ± 0.3	0.3 ± 0.2
*d*18:1/23:0	2.1 ± 0.3	0.2 ± 0.1
*d*18:1/23:1	1.3 ± 0.3	0.1 ± 0.1
*d*18:1/24:0	8.3 ± 0.8	1.3 ± 0.11
*d*18:1/24:1	49.2 ± 2.3	13.7 ± 0.12
*d*18:1/24:2	5.6 ± 0.7	2.3 ± 0.13
*d*18:1/25:0	2.3 ± 0.2	0.3 ± 0.1
*d*18:1/25:1	1.6 ± 0.3	0.1 ± 0.1
*d*18:1/26:1	1.4 ± 0.1	0.2 ± 0.1
Total	100	100
Saturated	38.5	79.9
Unsaturated	61.5	20.1

SM, sphingomyelin. The relative values of various molecular species of SM were calculated for the total SM component. Values represent means ± SEM for five to six experiments.

abundant unsaturated fatty acid of membranes in sheep and goat erythrocytes. Our results agree with their findings in that SM composed of nervonoic acid is present in the largest quantity in sheep erythrocytes.

The detection of molecular species of ceramide in sheep erythrocytes treated with α-toxin

We reported that α-toxin stimulates the metabolism of SM in membranes of sheep erythrocytes through endogenous SMase via Giα (9). To determine the molecular species of ceramide formed in the erythrocytes treated with α-toxin, sheep erythrocytes pretreated with *N*-OE, a ceramidase inhibitor, were incubated with 20 ng/ml α-toxin in GTBS at 37°C for 30 min. The lipids extracted with a modified version of Bligh and Dyer's method (17) were phosphorylated by DGK from *E. coli*, and the phosphorylated lipids were developed by reverse-phase TLC as described in Materials and Methods. Four phosphorylated-spots (lipid-A, lipid-B, lipid-C, and lipid-D) were detected from sheep erythrocytes treated with α-toxin (**Fig. 1**). Furthermore, PT inhibited the formation of lipid-B, -C, and -D in the cells treated with α-toxin in a dose-dependent manner but had little effect on the formation of lipid-A (Fig. 1). These lipids were analyzed by positive ion FAB-MS and LC-MS/MS. The positive ion FAB mass spectra of lipid-A, -B, -C, and -D revealed single ($M + Na^+$) ion peaks at *m/z* 561, 673, 671, and 669, respectively. The collision-induced dissociation spectra of fragment ions produced by FAB-MS and LC-MS/MS indicated the frame of sphingosine and the length of the fatty acyl chain (see supplementary Fig. I). We revealed that lipid-A, -B, -C, and -D were *N*-palmitoyl-sphingosine ($C_{16:0}$-ceramide), $C_{24:0}$-ceramide, $C_{24:1}$-ceramide (N-9), and $C_{24:2}$-ceramide (N-6, 9), respectively (see supplementary Fig. I). The mobility of phosphorylated lipid-A, -B, -C, and -D on TLC corresponded to that of phosphorylated $C_{16:0}$-ceramide, $C_{24:0}$-ceramide, $C_{24:1}$-ceramide, and $C_{24:2}$-ceramide, respectively.

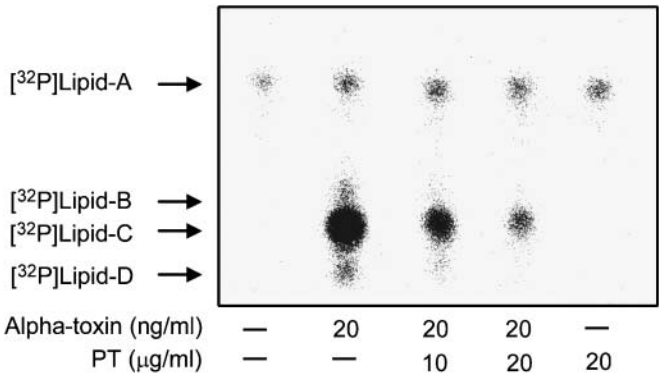

Fig. 1. Identification of molecular species of ceramide formed by treatment of sheep erythrocytes with α-toxin. Sheep erythrocytes (1×10^{11} cells/ml) were preincubated with various concentrations of pertussis toxin (PT) in GTBS (see Materials and Methods) at 37°C for 120 min. After the incubation, the erythrocytes were washed and incubated with α-toxin at 37°C for 30 min. The lipids containing ceramides extracted by a modified Bligh and Dyer method (17) were phosphorylated by 1,2-diacylglycerol kinase and separated by reverse-phase TLC.

Effects of $C_{24:0}$-, $C_{24:1}$-, and $C_{24:2}$-ceramide on hemolysis induced by α-toxin

To determine the effect of $C_{24:1}$-ceramide on the hemolysis and the formation of S1P induced by α-toxin, the events induced by $C_{24:1}$-ceramide were compared with those induced by $C_{24:0}$- and $C_{24:2}$-ceramides. Sheep erythrocytes were pretreated with saponin, a permeabilized reagent (26), in the presence of 2 mM ATP, incubated with ceramides suspended in saline containing 0.7% BSA at 37°C for 30 min, and then incubated with a subhemolytic dose of α-toxin in the presence of $C_{24:0}$-, $C_{24:1}$-, or $C_{24:2}$-ceramide at 37°C for 30 min. The level of $C_{24:0}$- and $C_{24:2}$-ceramides in the cell was the same as that of $C_{24:1}$-ceramide. Addition of $C_{24:1}$- and $C_{24:2}$-ceramides stimulated the toxin-induced hemolysis and formation of S1P in a dose-dependent manner but did not lyse the saponin-treated cells in the absence of the toxin (**Fig. 2A, B**). On the other hand, $C_{24:0}$-ceramide had no effect on the hemolysis and formation of S1P induced by α-toxin (Fig. 2A, B). Furthermore, the effect of $C_{24:1}$- and $C_{24:2}$-ceramides was significantly attenuated by treatment with DHS (Fig. 2A, B). Therefore, it appears that the metabolism of ceramides with unsaturated bonds in the fatty acyl chain, especially $C_{24:1}$-ceramide, to S1P is related to the toxin-induced hemolysis.

Relationship between α-toxin-induced hemolysis and the DRMs

It has been reported that bacterial toxins such as *C. perfringens* β-toxin (27), perfringolysin O (20), and Shiga toxin (28) specifically bind to DRMs, which are implicated in signal transduction. Furthermore, DRMs are reported to contain a high proportion of SM and cholesterol. Using LC-MS/MS, at least six protonated molecular species of SM were detected in DRMs of sheep erythrocytes (Table 1). The predominant species were $C_{16:0}$-SM(~74%) and *N*-nervonoic sphingomyelin ($C_{24:1}$-SM; ~14%) (Table 1). To analyze the binding of α-toxin to erythrocyte membranes, sheep erythrocytes were incubated with a ^{125}I-labeled variant toxin (^{125}I-H148G), which does not have the hemolytic and enzymatic activities of phospholipase C and SMase, at 37°C for 15 min and then treated with 1% Triton X-100 at 4°C. The Triton X-100-insoluble components were fractionated by sucrose density gradient centrifugation. The fractions were subjected to SDS-PAGE and Western blotting. When the DRM markers in the fractions obtained by sucrose density gradient centrifugation were analyzed using anti-flotillin-1 and anti-Giα, they were only detected in the low density fractions (fractions 3–5), and the fractions contained a much higher proportion of cholesterol (**Fig. 3**). As shown in Fig. 3, fractions 3–5 (low density fractions) contained an α-toxin of ~43 kDa. Furthermore, endogenous SMase in sheep erythrocytes was also detected in the low density fractions. These results suggests that the toxin specifically binds to the DRMs of sheep erythrocytes.

Effect of MβCD on α-toxin-induced hemolysis

It has been reported (29) that MβCD selectivity encapsulates cholesterol in membranes but does not deplete

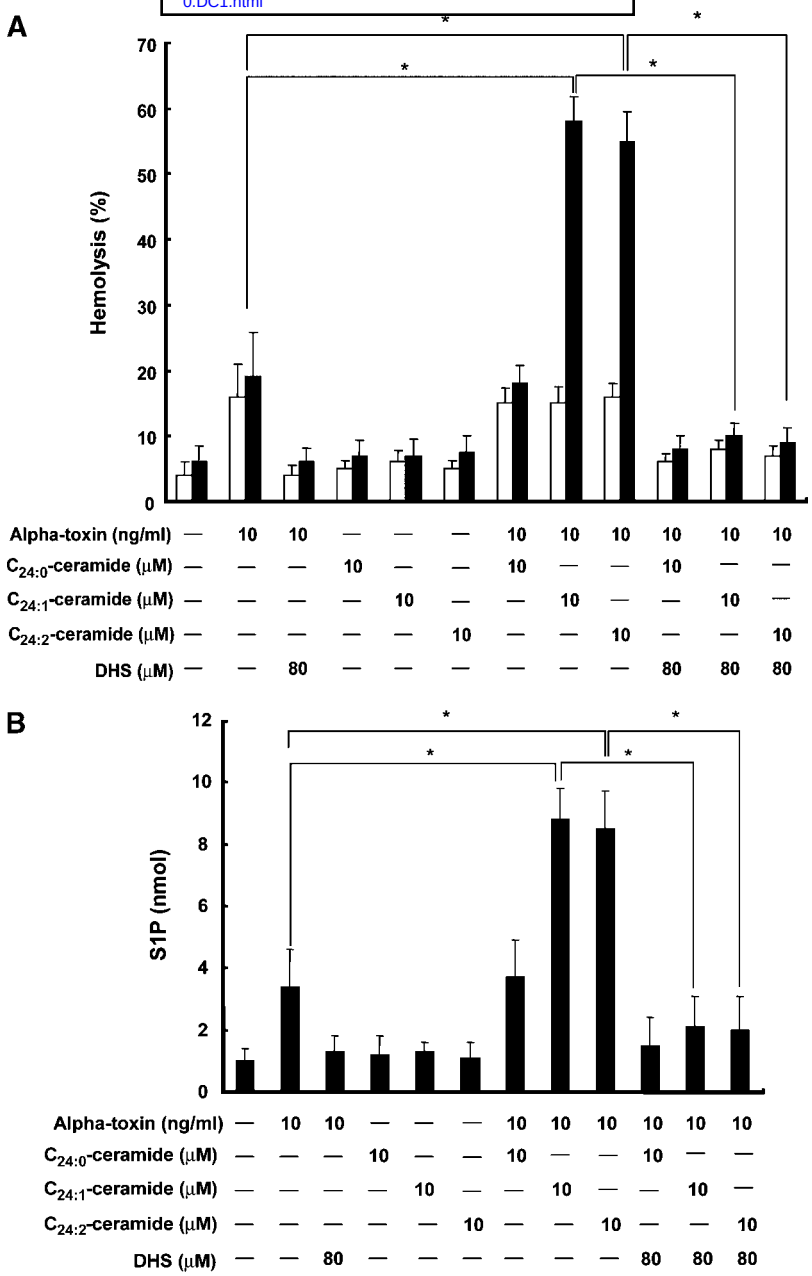

Fig. 2. Effects of *N*-lignoceroyl sphingosine ($C_{24:0}$-ceramide), 15-*N*-nervonoyl sphingosine ($C_{24:1}$-ceramide), and 15,18-*N*-tetracosadienoyl sphingosine ($C_{24:2}$-ceramide) on hemolysis and the formation of sphingosine 1-phosphate (S1P) induced by α-toxin in saponin-treated sheep erythrocytes. A: Saponin-pretreated (closed columns) or untreated (open columns) sheep erythrocytes (1×10^{11} cells/ml) were incubated with various species of ceramide (10 μM) in GTBS at 37°C for 30 min. After the incubation, the erythrocytes were washed and incubated with or without 80 μM dihydrosphingosine (DHS) at 37°C for 30 min. The treated erythrocytes were incubated with or without α-toxin (10 ng/ml) at 37°C for 30 min, followed by chilling at 4°C for 10 min. Hemolysis was measured as described in Materials and Methods. Values represent means ± SEM for five to six experiments. * $P < 0.01$. B: Saponin-pretreated sheep erythrocytes (1×10^{11} cells/ml) were incubated with various species of ceramide at 10 μM and 10 μCi/ml [γ-^{32}P]ATP in GTBS at 37°C for 30 min. After the incubation, the erythrocytes were washed and incubated with or without 80 μM DHS at 37°C for 30 min. The treated erythrocytes were incubated with or without α-toxin (10 ng/ml) at 37°C for 30 min. Labeled S1P was measured as described in Materials and Methods. Values represent means ± SEM for five to six experiments. * $P < 0.01$.

other lipids at concentrations of <10 mM. The effect of MβCD on toxin-induced hemolysis and the binding of the toxin to the erythrocytes was investigated. When the erythrocytes were incubated with 10 mM MβCD at 37°C for 60 min, the cholesterol content decreased to ~35% of that in untreated cells, and no hemolysis was observed. Incubation of the MβCD-treated sheep erythrocytes with the toxin caused a drastic decrease in the binding of the

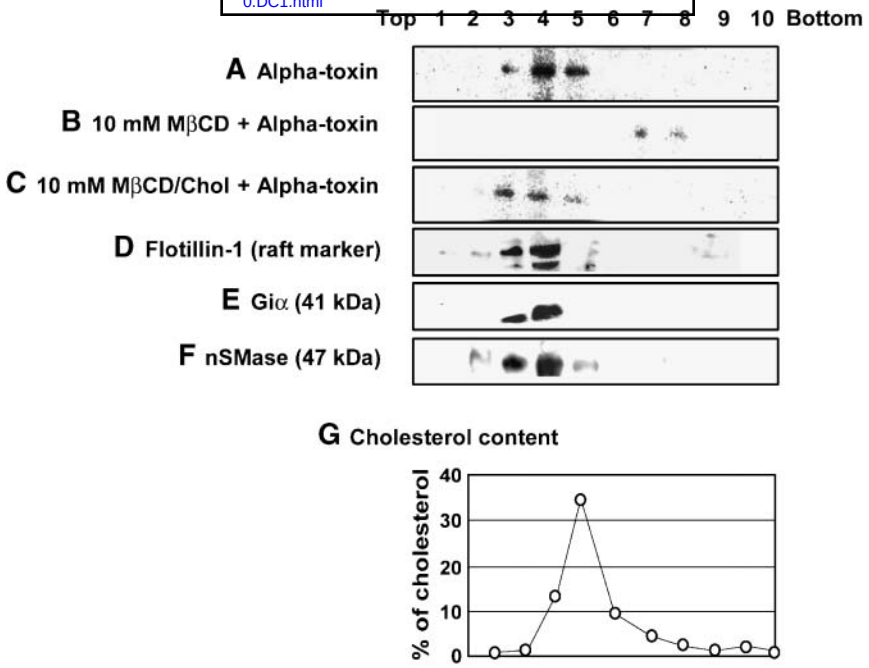

Fig. 3. Sucrose density gradient analysis of α-toxin-bound erythrocytes. Sheep erythrocytes (1×10^{11} cells/ml), untreated (A) or treated with 10 mM methyl-β-cyclodextrin (MβCD) (B), and the MβCD-pretreated cells treated with 10 mM MβCD/cholesterol (C) were incubated with ^{125}I-H148G (500 ng/ml) in GTBS at 37°C for 30 min and then fractionated by sucrose gradient ultracentrifugation. Fractions were collected from the top and subjected to SDS-PAGE, followed by autoradiography. Aliquots of the fractions were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. After the transfer, the blots were treated with anti-flotillin1 (D), anti-GTP binding protein Giα (E), and anti-nSMase (F). Peroxidase-conjugated secondary antibody bound to the membrane was detected by enhanced chemiluminescence as described in Materials and Methods. The amount of cholesterol in the fractions was determined as described in Materials and Methods (G). A typical result from one of five experiments is shown.

toxin and the hemolysis induced by the toxin (Figs. 3, 4). When the cells pretreated with 10 mM MβCD were incubated with 10 mM MβCD-cholesterol complex at 37°C for 120 min, the cholesterol level in the cells was restored by the back-addition of cholesterol (data not shown). Incubation of the MβCD-treated cells with 10 mM MβCD-cholesterol complex recovered to ~40% the sensitivity of the cells to the toxin (**Fig. 4**). Furthermore, the inhibitory effect of MβCD on the binding of ^{125}I-H148G to DRMs also recovered significantly in the MβCD-cholesterol complex-treated cells (Fig. 3). However, cholesterol itself did not inhibit the toxin-induced hemolysis of intact sheep erythrocytes (data not shown).

Effect of PT on the formation of ceramide induced by α-toxin in DRMs

We examined whether the toxin-induced formation of $C_{24:1}$-ceramide from SM occurs specifically in DRMs. Sheep erythrocytes treated with *N*-OE, a ceramidase inhibitor, were preincubated with or without 20 μg/ml PT at 37°C for 120 min and then incubated with or without α-toxin in GTBS at 37°C for 30 min. As shown in **Fig. 5**, $C_{24:1}$-ceramide was specifically detected in the DRMs of the cells treated with α-toxin. However, pretreatment of the cells with PT inhibited the α-toxin-induced formation of $C_{24:1}$-ceramide in the DRMs.

DISCUSSION

We have reported that endogenous SMase activated by α-toxin in sheep erythrocytes plays an important role in hemolysis (9). The present study demonstrates that α-toxin hydrolyzes the unsaturated SM, especially $C_{24:1}$-SM, by activating endogenous SMase through Gi in the DRMs

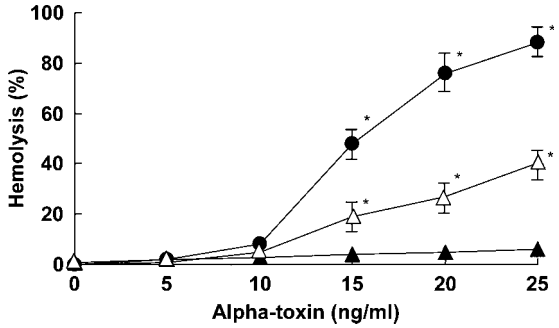

Fig. 4. Effect of MβCD on α-toxin-induced hemolysis. Sheep erythrocytes (1×10^{11} cells/ml), treated with 10 mM MβCD (closed triangles) or untreated (closed circles), and the MβCD-pretreated erythrocytes treated with 10 mM MβCD/cholesterol (open triangles) were incubated with various concentrations of α-toxin at 37°C for 30 min, followed by chilling at 4°C for 10 min. Values represent means ± SEM for five to six experiments. * $P < 0.02$.

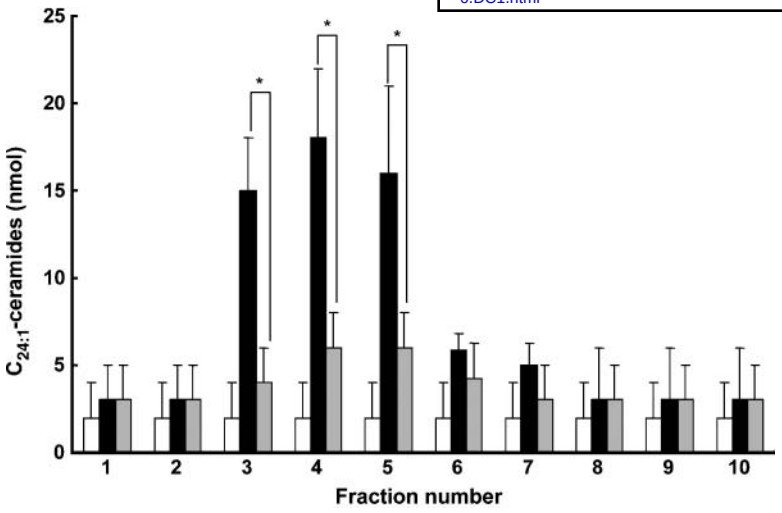

Fig. 5. Effect of PT on the formation of ceramide induced by α-toxin in detergent-resistant membranes. Sheep erythrocytes (5×10^{11} cells/ml) were preincubated with or without 20 μg/ml PT at 37°C for 120 min and then incubated with or without 50 ng/ml α-toxin in GTBS at 37°C for 30 min. The treated erythrocytes were suspended with Triton X-100 and separated by sucrose density ultracentrifugation. The level of $C_{24:1}$-ceramide in each fraction was measured as described in Materials and Methods. Open columns, without α-toxin; closed columns, treatment with α-toxin; shaded columns, treatment with PT and α-toxin. Values represent means ± SEM for five to six experiments. * $P < 0.01$.

of sheep erythrocytes and that the toxin-induced hemolysis of sheep erythrocytes is linked to the metabolism of $C_{24:1}$-ceramide (**Fig. 6**).

The toxin bound almost exclusively to the DRMs of sheep erythrocytes. MβCD reduced the binding of the toxin as well as the hemolysis induced by the toxin. Furthermore, incubation of the MβCD-treated cells with the MβCD-cholesterol complex recovered the cholesterol level in membranes, the binding of the toxin to DRMs, and the hemolysis induced by α-toxin. However, the hemolysis induced by the toxin was not inhibited by cholesterol. Several studies have reported that disruption or depletion of membrane-associated cholesterol induced by MβCD results in major changes in the distribution, function, and integrity of DRM-associated membrane components (27, 29). Therefore, it appears that the inhibitory effect on the toxin's action could be attributable to changes in the properties of DRMs that occur when cholesterol is removed from DRMs by MβCD. In the DRM fraction of sheep erythrocytes, $C_{16:0}$- and $C_{24:1}$-SM were especially abundant, and $C_{24:0}$- and $C_{24:2}$-SM were also detected. $C_{24:1}$-ceramide was specifically produced in the DRMs of the toxin-treated erythrocytes. The DRMs in which many different components are assembled in a coordinated manner to carry out signaling are called a "signaling platform." Immunoblot analysis with Gi- and nSMase-specific antibodies revealed the presence of these proteins in the DRMs of sheep erythrocytes. These proteins served as the signal transduction machinery in the early stages of the hemolysis caused by the toxin. Therefore, it is thought

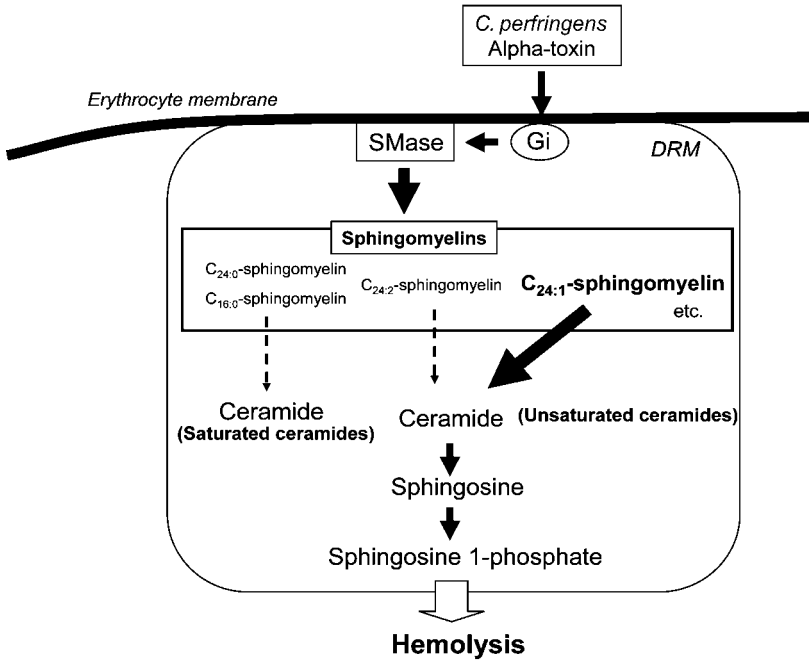

Fig. 6. Schematic representation of hemolysis induced by α-toxin of sheep erythrocytes. DRM, detergent-resistant membrane; SMase, sphingomyelinase.

that a signaling platform in the toxin-induced hemolysis exists in the DRMs of sheep erythrocytes.

α-Toxin specifically stimulated the hydrolysis of $C_{24:1}$-SM to $C_{24:1}$-cermide in DRMs, despite the much higher proportion of $C_{16:0}$-ceramide. $C_{16:0}$- and $C_{24:1}$-SM were especially abundant in the DMRs (~75% vs. 13.7% $C_{24:1}$-SM, as shown in Table 1). However, as shown in Fig. 5, treatment of the cells with α-toxin resulted in the formation of $C_{24:1}$-ceramide in the DRMs, but not $C_{16:0}$-ceramide. Therefore, it appears that α-toxin activates endogenous SMase specific for $C_{24:1}$-SM. On the other hand, $C_{18:1}$-, $C_{24:1}$-, and $C_{24:2}$-ceramides were rapidly metabolized to sphingosine and S1P in the erythrocytes treated with α-toxin, but $C_{16:0}$-, $C_{18:0}$-, and $C_{24:0}$-ceramides were not (see supplementary Figs. II, III). These results suggest that SM species and their metabolites in the acyl chain are specifically metabolized to sphingosine in the cells treated with the toxin, implying that $C_{24:1}$-SM containing an unsaturated bond is mainly metabolized in DRMs of the cells treated with the toxin. Furthermore, treatment of the erythrocytes with PT inhibited the conversion of $C_{24:1}$-SM to $C_{24:1}$-ceramide and hemolysis induced by α-toxin. Therefore, it appears that the hemolysis of sheep erythrocytes treated with the toxin is dependent on the formation of $C_{24:1}$-ceramide through the activation of endogenous SMase via Gi in DRMs. The hemolysis and formation of S1P induced by α-toxin was enhanced by the addition of $C_{24:1}$- and $C_{24:2}$-ceramide, but not $C_{24:0}$-ceramide. The difference between the molecular species of these ceramides is the degree of saturation in the fatty acid side chain. Therefore, it is thought that the unsaturated bond of the fatty acid side chain in these ceramide molecules is important for the effect of ceramide in sheep erythrocytes treated with the toxin.

We have reported that sphingosine is rapidly metabolized to S1P by the activated sphingosine kinase in sheep erythrocytes treated with the toxin (9). Mao et al. (30) reported that overexpression of mouse alkaline ceramidase decreased the cellular levels of $C_{24:1}$-ceramide and caused an increase in the levels of S1P. Because $C_{24:0}$-, $C_{24:1}$-, and $C_{24:2}$-ceramides have the common frame of sphingosine, it is possible that sphingosines generated from these ceramides are similarly converted into S1P in the toxin-treated erythrocytes. However, exogenous $C_{24:1}$- and $C_{24:2}$-ceramide potentiated the toxin-induced formation of S1P in saponin-permeabilized erythrocytes, but $C_{24:0}$-ceramide did not. Furthermore, the unsaturated ceramides had no effect on the saponin-treated cells in the absence of the toxin. These observations suggest that $C_{24:1}$- and $C_{24:2}$-ceramides were metabolized to S1P by the toxin-activated ceramidase, which selectively recognizes unsaturated ceramides as a substrate, and/or sphingosine kinase in sheep erythrocytes.

The metabolism of $C_{24:1}$- and $C_{24:2}$-ceramides was abrogated by treatment with DHS. We reported that hemolysis induced by the toxin of sheep erythrocytes is closely related to the formation of S1P from SM (9). Therefore, it appears that the formation of S1P from these unsaturated ceramides is linked to the toxin-induced hemolysis.

The addition of $C_{24:1}$- and $C_{24:2}$-ceramides had an equal effect on the toxin-induced hemolysis and formation of S1P. $C_{24:1}$-SM, which is the source of $C_{24:1}$-ceramide, was the predominant molecular species of SM in sheep erythrocytes. When sheep erythrocytes were treated with the toxin, $C_{24:1}$-ceramide was predominantly generated in the cells, but little $C_{24:2}$-, $C_{24:0}$-, or $C_{16:0}$-ceramide was formed. These results suggest that the metabolism of $C_{24:1}$-SM activated by the toxin is a key step in the hemolysis of sheep erythrocytes (Fig. 6).

In conclusion, the toxin-induced activation of the metabolism of $C_{24:1}$-SM through Gi in DRMs is closely involved in hemolysis. This result may present a new therapeutic approach to blocking systemic hemolysis in type A *C. perfringens*-infected animals. Our observation provides new insights into the role of the metabolism of molecular species of SM in various biological activities and suggests that the turnover of erythrocytes is dependent on the metabolism of SM composed of unsaturated fatty acyl chains such as $C_{24:1}$-SM in erythrocyte membranes.

The authors thank Keiko Kobayashi, Mituaki Kodama, Hideaki Hioki, Takahiro Imagawa, Hiroki Yoshioka, Atsushi Todaka, Masaya Takahashi, Daisuke Higo, and Jouji Seta for technical assistance. This research was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan; by the Open Research Center Fund for Promotion; and by the Mutual Aid Corporation for Private Schools of Japan.

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