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N-oleoyl-D-*erythro*-sphingosine-based Analysis of Ceramide by High Performance Liquid Chromatography and Its Application to Determination in Diverse Biological Samples

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Abstract

Ceramide is involved in cell death as a lipid mediator of stress responses. In this study, we developed an improved method of ceramide quantification based on added synthetic ceramide and thin layer chromatography (TLC) separation, and applied to biological samples. Lipids were extracted from samples spiked with N-oleoyl-D-*erythro*-sphingosine (C_{17} ceramide) as an internal standard. Ceramide was resolved by TLC, complexed with fatty-acid-free bovine serum albumin (BSA), and deacylated by ceramidase (CDase). The released sphingosine was derivatized with o-phthalaldehyde (OPA) and measured by high performance liquid chromatography (HPLC). The limit of detection for ceramide was about 1-2 pmol and the lower limit of quantification was 5 pmol. Ceramide recovery was approximately 86-93%. Ceramide concentrations were determined in biological samples including cultured cells, mouse tissues, and mouse and human plasma. TLC separation of ceramide provides HPLC chromatogram with a clean background without any interfering peaks and the enhanced solubility of ceramide by BSA-ceramide complex leads to the increased deacylation of ceramide. The use of an internal standard for the determination of ceramide concentration in these samples provides an accurate and reproducible analytical method, and this method can be applicable to diverse biological samples.

Keywords: Ceramide, HPLC, Cell death, ICR mice, LLC-PK1 cells

Ceramide is involved in the regulation of cell death[1] and acts as a lipid mediator of cellular stress responses[2]. The ceramide level in cells is up-regulated by various types of stress conditions including ionizing radiation[3], serum deprivation[4,5] and anti-cancer drugs[6]. There are two potential pathways for intracellular ceramide formation: *de novo* biosynthesis via the condensation of serine and palmitoyl-CoA, and the breakdown of sphingomyelin via sphingomyelinase.

Many analytical methods for ceramide quantification in biological samples have been published. In the radiolabelled method, ceramide is phosphorylated into [^{32}P]ceramide 1-phosphate by *E. coli* diacylglycerol (DAG) kinase and [γ-^{32}P]ATP, and the amount of ceramide is calculated by measuring the radioactivity on a thin-layer chromatography (TLC) plate and compared to a standard curve[7]. Although this is a sensitive method that is used by most researchers, its disadvantages include the use of radioisotopes and the lack of an internal standard.

An alternative method involves the use of mass spectrometry for the quantitative analysis of ceramides following extraction of all lipids[8,9]. Although each class of ceramide can be quantified with high sensitivity, this method requires the use of expensive equipment that is not available in most laboratories. Another method uses HPLC analysis of ceramide following lipid extraction and ceramide deacylation with CDase[10]. Although detection with this method is sensitive and the procedure is simple, the absence of purification step and an incomplete solubility of ceramide in deacylation reaction, and different extraction efficiencies due to no internal standard may cause variation for the quantification of ceramide.

Here we present an improved method with high specificity and good reproducibility for determining the amount of ceramide and dihydroceramide in samples, using TLC separation, enzymatic deacylation and HPLC analysis. It is applicable to a variety of biological samples, the use of an internal standard makes it reproducible and TLC separation may pro-

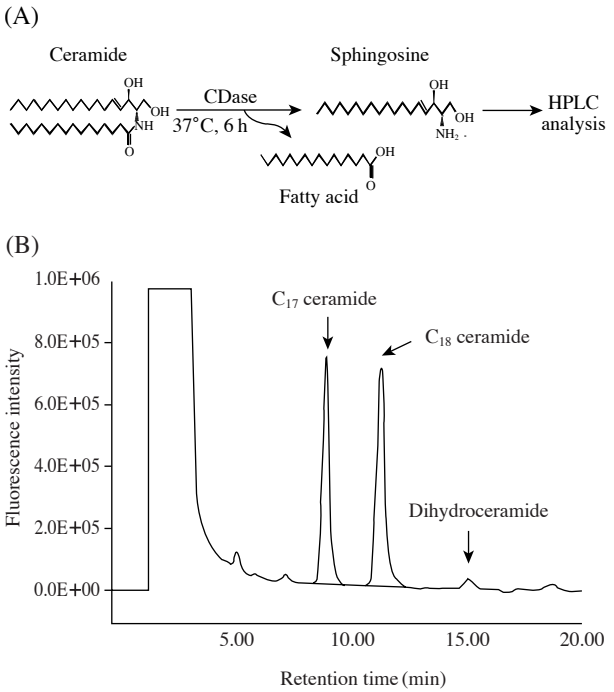

Figure 1. The procedure for ceramide quantification. (A) Following TLC separation ceramides were deacylated by neutral ceramidase (CDase), and the released sphingosine was derivatized with OPA fluorescence dye and analyzed by HPLC. (B) The retention times for C_{17} and C_{18} ceramide standards were 9.0 and 11.3 min, respectively, on HPLC chromatogram. Dihydroceramide was detected at 15.2 min.

vide HPLC chromatogram with clean background without any interference.

TLC Separation of Ceramide from Lipid Extract and HPLC Analysis

Ceramide was specifically deacylated by CDase, and sphingosine was released and analyzed by HPLC following OPA derivatization (Figure 1A). Peaks of synthetic C_{17} ceramide and naturally occurring C_{18} ceramide on the HPLC chromatogram were shown at the retention times of 9.0 and 11.3 min, respectively (Figure 1B). The dihydroceramide peak on the HPLC chromatogram had a retention time of 15.2 min although it was not added to CDase reaction, indicating that the natural ceramide standard purified from porcine brain may contain the trace amounts of dihydroceramide. C_{17} ceramide as an internal standard for the determination of endogenous ceramide concentration was added to the biological samples and lipids were extracted. Ceramide was separated from other sphingolipids by TLC. For the determination of ceramide contents in the samples, a TLC plate loaded with the C_{17} ceramide standard in the end lanes and

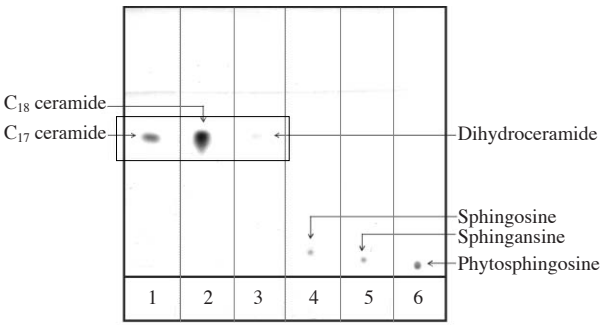

Figure 2. TLC chromatogram of sphingolipids. Sphingolipids on the TLC chromatogram include C_{17} and C_{18} ceramides, dihydroceramide, sphingosine, sphinganine and phytosphingosine. Sphingolipid standards were spotted on the TLC plate, and developed in diisopropylether/methanol/29% NH_4OH (40 : 10 : 1, v/v/v) and visualized with 10% sulfuric acid.

samples in the inside lanes was developed with a mixture of diisopropylether/methanol/29% NH_4OH (40 : 10 : 1, v/v/v). The ceramide standard lanes were cut from the TLC plate and the ceramide standard band was visualized with sulfuric acid reagent. Ceramide and dihydroceramide appeared as the same band as the C_{17} ceramide standard on the TLC plate with an R_f value of 0.54 indicating that both ceramide and dihydroceramide could be simultaneously quantified. Possible contamination of the lipid extract with sphingosine and sphinganine was excluded by the TLC separation. The R_f values of sphingosine, sphinganine and phytosphingosine are 0.09, 0.06 and 0.04, respectively (Figure 2). Therefore, sphingoid bases did not interfere with the ceramide analysis. Separation of ceramide from lipid extract by TLC provides HPLC chromatogram with clean background (Figure 3). The chromatogram showed many non-specific peaks without ceramide peaks when the lipid extract from human plasma was analyzed by HPLC following ceramide deacylation without TLC separation (Figure 3A). Thus, ceramide purification from the lipid extract of human plasma by TLC led to the good separation of ceramides on the HPLC chromatogram (Figure 3B). Spiking the biological samples with C_{17} ceramide as an internal standard overcame the variation which could be caused by the multistep procedure including lipid extraction, TLC separation, enzymatic deacylation, and HPLC quantification.

Optimization of Enzymatic Deacylation and Reproducibility of Ceramide Quantification

CDase reaction was increased in the BSA concentration-dependent manner ranging from 0 to 15% in the reaction buffer (Figure 4). Ceramide deacyla-

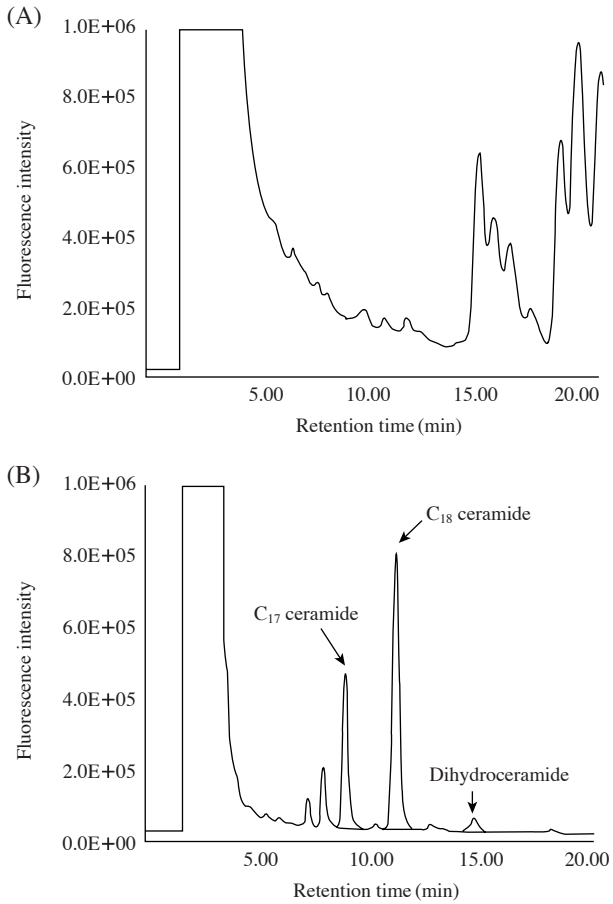

Figure 3. HPLC profiles of ceramides from human plasma. Blood samples were obtained from healthy volunteers and plasma was prepared by centrifugation at 3,000 × g for 10 min. Lipids were extracted from 50 μL of plasma. (A) Ceramides were deacylated by CDase and analyzed by HPLC. (B) Ceramides were separated from lipid extracts by TLC, deacylated and quantified.

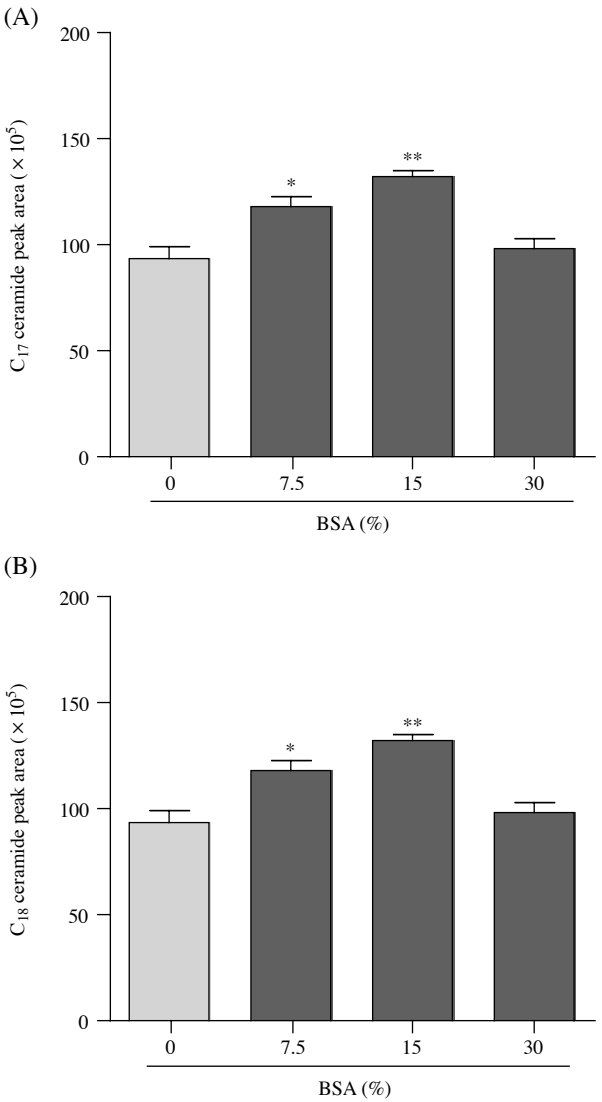

Figure 4. Effect of BSA on ceramide deacylation. (A) C_{17} and (B) C_{18} ceramide standards were hydrolyzed by CDase in a reaction buffer at concentrations of 0, 7.5, 15 and 30% fatty-acid-free BSA for 6 hrs. Sphingosines released from ceramides were analyzed by HPLC. Values are expressed as the mean ± S.D. (n=3-5). Difference with $*P<0.05$ and $**P<0.01$ were defined as statistically significant between untreated and treated samples.

tion by CDase at 15% BSA was maximal. Deacylation of C_{17} and C_{18} ceramides was dependent on the incubation time until 6 hrs (Figure 5A). Thus, the incubation time for ceramide deacylation with 150 μ units of CDase was set at 6 hrs. Deacylation of C_{17} and C_{18} ceramides was concentration-dependent at the range of 5-1,000 pmol with linearity of 0.9982 and 0.9968, respectively, between ceramide concentration and peak area (Figure 5B). Thus, the quantitative analysis of ceramide in human plasma was performed by comparison of the peak area on the chromatogram, and calculated based on 200 pmol of added C_{17} ceramide as the internal standard. Although the ceramide band on the TLC plate was visually detected at the level of nmol level after spraying with sulfuric acid and heating the plate, the analytical method using HPLC equipped with a fluorescence detector lowered the detection limit to the 1-2 pmol range, indicating that the method used in this study was very sensitive. The sensitivity was achieved by the OPA-fluorescence derivatization of either sphingosine or sphinganine. Ceramide recovery was measured by addition of the ceramide standard to human plasma. The level of ceramide showed a linear relationship between the added and the measured amounts, and the recoveries

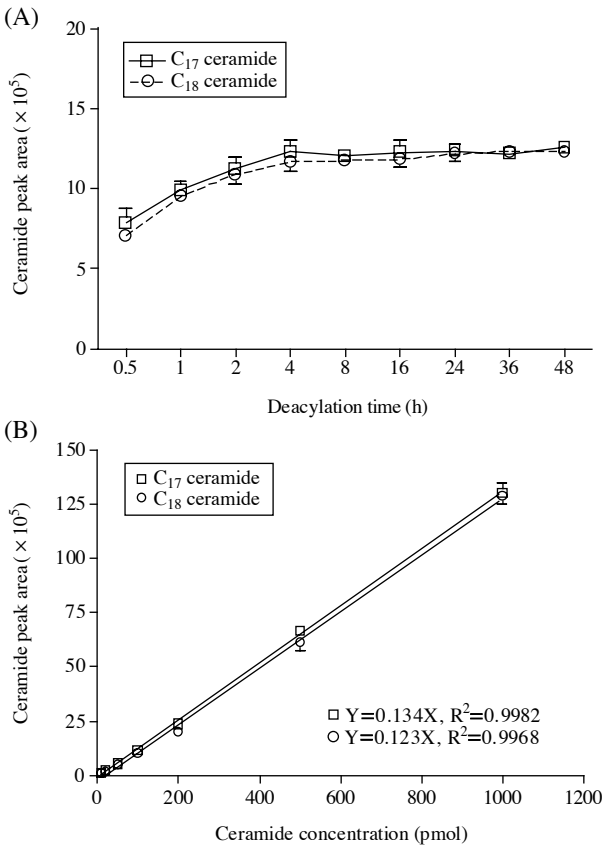

Figure 5. Optimal conditions for ceramide deacylation by CDase. (A) The time-dependent release of sphingosine and sphinganine from ceramide standards. C_{17} and C_{18} ceramides (200 pmol each) were added to the reaction buffer and incubated at 37°C for varying periods of time. The released sphingosine and sphinganine were analyzed by HPLC. (B) Linearity between ceramide concentration and fluorescence peak area at the concentrations of 5-1,000 pmol. The CDase deacylation of C_{17} and C_{18} ceramides was continued for 6 hrs. Values are expressed as the mean±S.D. (n=3-5).

Table 1. Recovery of ceramide in human plasma.

Added ceramide solution (μL)[a]	Ceramide		
	Measured (μM)[b]	Expressed (μM)	Recovery (%)
0	5.01		
3	5.94	6.81	87
6	8.09	8.61	93
9	9.56	10.41	91
15	12.61	14.01	90
30	19.83	23.01	86

[a]Methanolic solution with 30 μM natural ceramide
[b]Values are the means of triplicate measurements

Table 2. Levels of ceramide and dihydroceramide in cultured cells, plasma and tissues.

Sources	Ceramides[a]	Dihydroceramides
Cell type		
HeLa	3.54±0.18	0.20±0.02
VSMC	3.08±0.15	0.13±0.02
LLC-PK1	1.94±0.08	0.12±0.01
Plasma		
Human	5.01±0.25	0.21±0.11
ICR mice	4.68±0.21	0.22±0.02
Tissues (ICR mice)		
Brain	4.18±0.17	0.50±0.26
Intestine	2.20±0.10	0.19±0.07
Testis	2.07±0.03	0.32±0.05
Lung	1.53±0.06	ND[b]
Kidney	1.35±0.06	0.07±0.02
Eye	1.21±0.05	ND
Spleen	0.82±0.05	ND
Heart	0.75±0.02	ND
Liver	0.72±0.03	ND
Muscle	0.61±0.01	ND

[a]Values are in nmol/mg protein for cultured cells and tissues and μM for plasma and are means±SDs; n=3
[b]ND, not detected

were in the range of 86 to 93% (Table 1).

Quantification of Ceramide and Dihydroceramide in Human Plasma and Mouse Tissues

Ceramide concentrations were determined in 10-200 μL of human plasma with the addition of 200 pmol C_{17} ceramide. Although ceramide quantification was possible with 10 μL human plasma, the optimum range for ceramide determination appeared to be 50-150 μL. The concentration of endogenous ceramide in healthy human plasma is approximately 5 μM (Table 2). The levels of total ceramide in ICR mouse tissues varied from 0.8 to 4.0 nmol per mg protein (Table 2). Brain and intestine had the highest levels of ceramide and liver, spleen, muscle, and heart had the lowest levels. Since ceramide is known as a signaling molecule for cell death, the ceramide levels in animal tissues may be related to the organ's sensitivity to toxicity.

Close Relationship between the Elevated Level of Ceramide Content and Cell Death in LLC-PK1 Cells

There was a relationship between released LDH activity and ceramide content in LLC-PK1 cells. Released LDH activity, an indicator of cytotoxicity, was increased in a concentration-dependent manner by etoposide at 10, 20 and 50 μM in the absence of FBS (Figure 6A). Under the same conditions, total

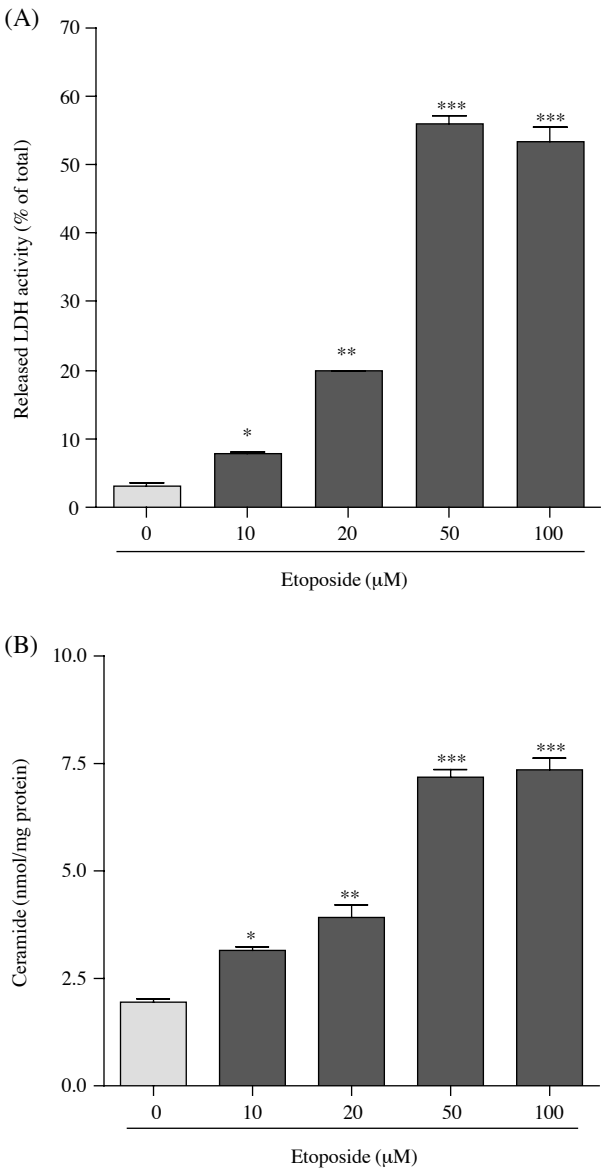

Figure 6. The relationship between ceramide levels and etoposide-induced cell death in LLC-PK1 cells. The cultured cells were grown to 70% confluency and deprived of FBS with the simultaneous addition of various concentrations of etoposide (10, 20, 50, 100 μM) for 24 hrs. The cell pellets were harvested, and the released-LDH assay (A) and ceramide analysis (B) were performed following the determination of total protein content. Values are expressed as the mean ± S.D. (n=3-5). Differences between untreated and treated samples with $*P<0.05$, $**P<0.01$ and $**P<0.001$ were defined as statistically significant.

ceramide increased in a concentration-dependent manner up to 50 μM (Figure 6B). In the absence of FBS, the ceramide concentration in LLC-PK1 cells increased about 3.8-fold, from 1.9 nmol to 7.2 nmol per mg protein, with 50 μM etoposide treatment. However, ISP-1, an inhibitor of serine palmitoyltransferase in the *de novo* sphingolipid biosynthesis pathway, partially protected the cells from the serum-starvation-induced cell death (Figure 7A). This result suggests that cell death under serum deprivation may occur via sphingolipid metabolism. Prolonged serum deprivation elevated the intracellular ceramide concentration by 2.2-fold, from 2.0 nmol in the presence of FBS to 4.4 nmol in the absence of FBS (Figure 7B). ISP-1 partially reduced the elevated ceramide level by 63%, indicating that the activation of *de novo* ceramide biosynthesis may contribute to serum-deprivation-induced cell death in LLC-PK1 cells.

Discussion

Ceramide is involved in cell death[1] as a lipid mediator of stress responses[2] and has been implicated in human diseases such as Alzheimer's disease[11,12] and cancer[6,13]. Therefore, several methods have been developed for ceramide quantification, including the radioactive DAG kinase assay[7,14,15] and mass spectrometry method[8,9]. However, these methods are dependent on either radioisotopes or expensive instruments. Mass spectrometry analysis provides quantification of molecular species of ceramides with high sensitivity. However, the sensitivity can be dependent on the mode of ionization and may vary with the molecular species. A recent method for ceramide quantification utilizes HPLC, following CDase deacylation and naphthalene-2, 3-dicarboxyaldehyde fluorescence derivatization[10]. Although this method appears to be simple and sensitive, variations due to different extraction efficiencies remain. Another method utilizes microwave-assisted deacylation of ceramide, glucosylceramide, and ceramide trihexoside to measure lysosphinogolipids by HPLC[16,17]. However, the use of sphinganine as an internal standard in the microwave deacylation method is not appropriate for ceramide quantification because small amounts of sphinganine exist in biological samples.

Our procedure includes the TLC separation of ceramide, hydrolysis of ceramide to sphingosine using *Pseudomonas* CDase[18], OPA fluorescence derivatization, and HPLC analysis (Figure 1B, 3B). In addition, spiking with C_{17} ceramide as an internal standard compensates for variations in the extraction of endogenous ceramide, the degree of enzymatic hydrolysis, and differences in sensitivity. With this method, the analytical detection limit for ceramide is shown to be 1-2 pmol and the optimal amount of sample is either cell lysate equivalent to 100 μg total protein or 50-

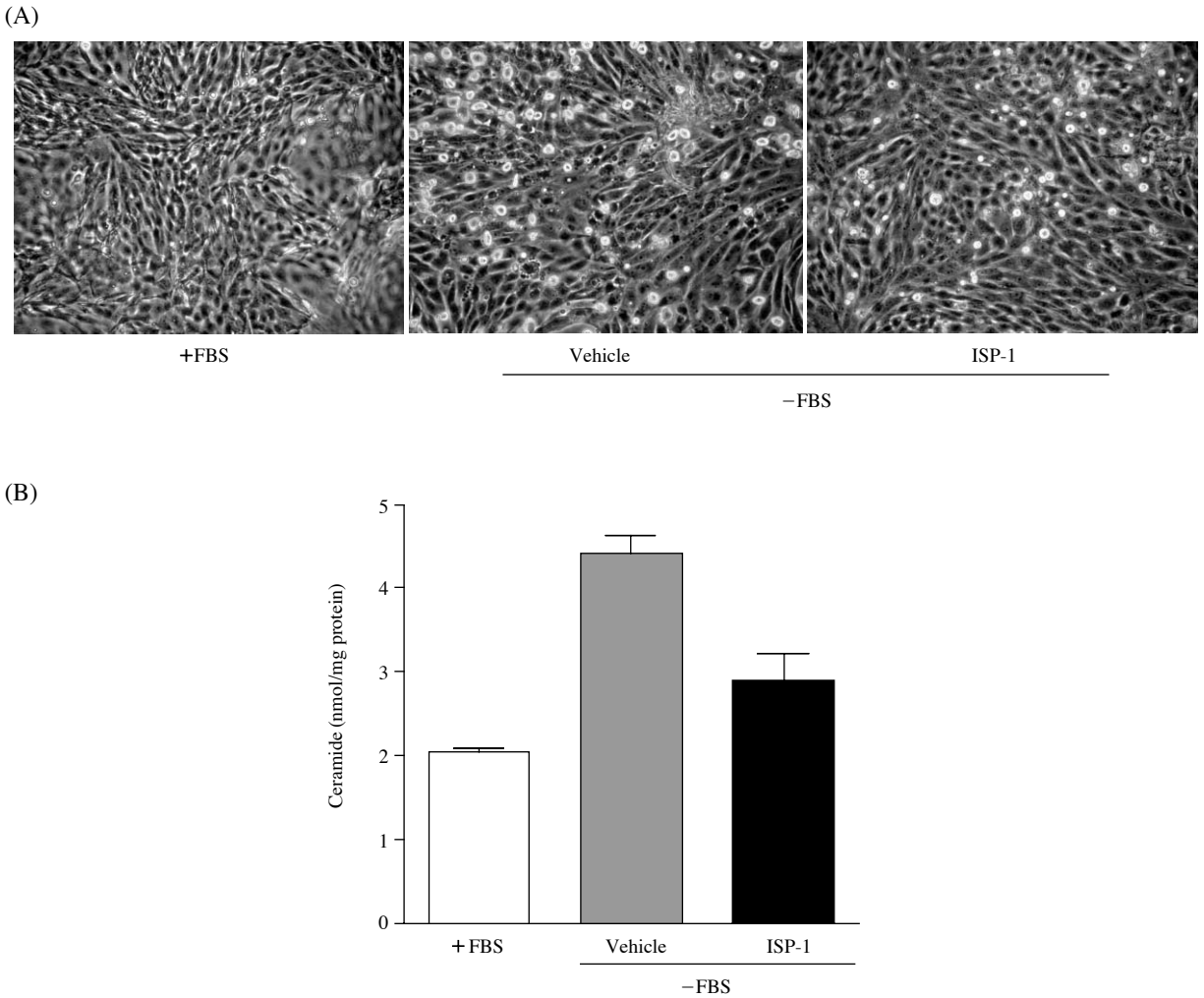

Figure 7. Protection of LLC-PK1 cells from serum-deprivation-induced cell death by ISP-1 inhibition of ceramide biosynthesis. LLC-PK1 cells were grown to 50% confluency, deprived of FBS with the simultaneous addition of 1 μM ISP-1, a serine palmitoyltransferase inhibitor of the *de novo* sphingolipid biosynthetic pathway and incubated for 48 hrs. Cell morphology was observed visually (A) and ceramide concentrations were determined by HPLC analysis (B). Values are expressed as the mean ± S.D. (n=3-5). White arrowheads indicate the formation of characteristic dome in LLC-PK1 cells.

100 μL of human plasma (data not shown). The spiked C_{17} ceramide standard in the samples enables the actual amount of endogenous ceramide to be measured. This HPLC method is specific and more reproducible than previously published methods for the measurement of ceramide content. The introduction of a proper internal standard (C_{17} ceramide) into biological samples improves the precision of the quantitative analysis of endogenously occurring C_{18} ceramide and dihydroceramide. This greatly enhances the reproducibility of the results, which vary in other methods of ceramide quantification. One method for ceramide quantification employs *E. coli* DAG kinase and [γ-^{32}P] ATP to convert ceramide into [^{32}P]ceramide 1-phosphate[7,14]. However the 14-day half-life of ^{32}P decreases the experimental flexibility and provides only a relative comparison of the radioactivity among samples.

In our method, possible contamination by sphingoid bases in ceramide is excluded by TLC separation. The efficient solvent system for TLC development allows the ceramide band to move far from the sphingosine and sphinganine bands. The R_f values for ceramide, sphingosine, and sphinganine are 0.54, 0.09, and 0.06, respectively (Figure 2). To verify that ceramide was not contaminated with sphingoid bases,

ceramide was measured following the extraction procedure with and without deacylation (data not shown). Contaminating sphingoid bases were not detected in any of the biological samples tested. Although OPA fluorescence detection is known to be specific for the amino group of analytes, the HPLC chromatogram shows many nonspecific peaks which interfere with the true sphingolipid peaks without the procedure of TLC purification (Figure 3A). Ceramide separated by TLC from crude lipid extract can be quantified without any interference of unknown peaks (Figure 3B), indicating that ceramides need to be purified for deacylation and HPLC analysis. Ceramide deacylation by CDase occurred efficiently by fatty-acid-free BSA in enzymatic reaction buffer. The solubility of ceramide appeared to be enhanced by the complex with BSA, resulting in the increased amount of ceramides on the HPLC analysis (Figure 4). Ceramide analysis using more than 200 μL of human plasma could not be performed in polypropylene 1.5-mL tubes. Large-scale analyses using glass tubes would be inconvenient and time-consuming, and might limit the number of samples. The increased sensitivity of our method allows small-scale analysis and can be applied to a large numbers of samples. This analytical method is able to measure ceramides from a diversity of biological samples including cultured cells, mouse tissues, and mouse and human plasma (Table 2), and may be applicable to the use of ceramide as a biomarker for toxicity (Figure 6, 7) as well as for several human diseases[19-22].

In this improved HPLC method, TLC separation of ceramide provides HPLC chromatogram with a clean background without any interfering peaks and the enhanced solubility of ceramide by BSA-complex leads to the increased deacylation. The use of an internal standard for the determination of ceramide concentration in samples provides an accurate and reproducible analytical method, and this method can be applicable to diverse biological samples including cultured cells, mouse tissues, and mouse and human plasma.

Methods

Materials

ISP-1 (myriocin) and D-*erythro*-sphingosine were purchased from Biomol Research, Inc. (Plymouth Meeting, PA). N-oleoyl-D-*erythro*-sphingosine (C_{17} ceramide) was from Avanti Polar Lipids, Inc. (Alabaster, AL). Pyridine and diisopropylether were from Sigma (St. Louis, MO). Fetal bovine serum (FBS) and culture medium for cell cultures were obtained from Invitrogen (Gaithersburg, MD). HPLC-grade methanol was purchased from Merck (Darmstadt, Germany). *o*-Phthalaldehyde (OPA) was obtained from Molecular Probes, Inc. (Eugene, OR). Other reagents were of the highest purity available.

Cell Culture

Pig kidney epithelial (LLC-PK1) cells obtained from ATCC (Richmond, VA) were seeded at a density of 5×10^5 cells in flat-bottomed 6-well dishes and cultured in DMEM/F12 medium supplemented with 1.2 g/L sodium bicarbonate, 100 U/mL penicillin-streptomycin, and 5% (v/v) FBS at 37°C in a humidified 5% CO_2 atmosphere. LLC-PK1 cells were grown to 50% confluence, deprived of FBS with the simultaneous addition of 1 μM ISP-1, a serine palmitoyltransferase inhibitor of the *de novo* sphingolipid biosynthetic pathway, and incubated for 48 hrs. Cells were harvested with a rubber policeman. Pellets of rat aortic vascular smooth muscle cells (VSMC) and HeLa cells were obtained from professor WY Park (Chungbuk National University, Korea).

Animals

The protocol was approved by the Ethical Committee of the College of Pharmacy at Chungbuk National University. ICR mice (20 g body weight and 5-6 weeks-of-age) were obtained from Daehan Biolink (Eumsung, Korea), and acclimated for 1 week in the animal facility (22°C, 12 hrs light/dark cycle). Blood was collected and organs were removed for sphingolipid analysis.

Human Plasma

Blood samples were obtained from healthy volunteers. Informed consent was obtained according to the Declaration of Helsinki before any blood was sampled. Plasma was prepared by centrifugation at 3,000 × g for 10 min and stored at −20°C.

Lipid Extraction

Biological samples for lipid extraction were pellets of cultured cells and mouse tissues of 100 μg protein content and 50 μL of plasma from mice and humans. Total lipid was extracted with 1 mL ethanol at 37°C for 1 hr following the addition of C_{17} ceramide as the internal standard. The extract was centrifuged at 15,000 × g for 10 min. The supernatant was dried in a Speed-Vac concentrator (Vision Scientific Co., Daejeon, Korea).

Thin Layer Chromatography (TLC)

The dry residue of the lipid extract was dissolved in 30 μL of chloroform/methanol (1 : 2, v/v) and spotted

on a high-performance thin-layer chromatography silica-gel plate (Merck, Darmstadt, Germany). The plate was developed in diisopropylether/methanol/ 29% NH_4OH (40 : 10 : 1, v/v/v). Ceramide standard lanes were cut from the sample lanes of the TLC plate and visualized by dipping the plate in 10% sulfuric acid and drying at 150°C. The areas in the sample lane with the same R_f values as the visualized band of C_{17} ceramide standard were scraped off, and both ceramide and dihydroceramide were eluted with 1 mL methanol. The eluates were transferred to polypropylene 1.5-mL tubes and dried in a Speed-Vac concentrator.

Enzymatic Deacylation

The ceramide residue was mixed with reaction buffer containing 25 mM Tris-HCl buffer, pH 7.5, 1% sodium cholate, 15% fatty-acid-free BSA, and 150 μU CDase. Ceramide and dihydroceramide were deacylated into sphingosine and sphinganine, respectively, by CDase at 37°C for 6 hrs. BSA in the reaction buffer was precipitated by adding ethanol and removed by centrifugation, and the supernatant was dried.

HPLC Analysis

The sphingolipid extracts were dissolved in 120 μL methanol, mixed with 15 μL OPA reagent (50 mg OPA, 1 mL ethanol, 200 μL β-mercaptoethanol, and 50 mL 3% (w/v) boric acid buffer, pH 10.5), and incubated at room temperature for 30 min for derivatization. The HPLC analysis was performed using a Shimadzu (Tokyo, Japan) Model LC-10AT pump, a SIL-$10A_{XL}$ autosampler system, and an analytical Radial-Pak cartridge (Waters Associates, Inc., Milford, MA) packed with a Nova-Pak C_{18} reversed-phase column (4 μm, 100 mm × 8 mm). The isocratic mobile phase composition of methanol/distilled water/triethylamine (92 : 8 : 0.1, v/v/v) and a flow rate of 1.0 mL/min were accurately controlled by the HPLC system controller (Shimadzu SCL-10A). The Shimadzu RF-10_{XL} fluorescence detector was set at an excitation wavelength of 340 nm and an emission wavelength of 455 nm. The resulting data and chromatographic profiles were evaluated using Borwin system manager software (JMBS, France).

Protein Assay

The total protein content of samples was determined in order to normalize the results. The lysates from the cell pellets and tissues were solubilized with 0.2 N NaOH, mixed with PIERCE BCA reagents (Rockford, IL), and incubated for 30 min. The protein content was quantified with a Molecular Devices ELISA reader (Sunnyvale, CA) at 562 nm based on the BSA standard curve.

Statistics

All values were expressed as the means ± standard deviations (SDs). Differences between untreated and treated samples were analyzed statistically by the unpaired Student's *t*-test for single comparisons. Differences with $*P<0.05$, $**P<0.01$ and $***P<0.001$ were defined as statistically significant.

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