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Sphingomyelin Regulates the Activity of Secretory Phospholipase A_2 in the Plasma Membrane

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ABSTRACT

We examined the effect of the cellular sphingolipid level on the release of arachidonic acid (AA) and the activity of secretory phospholipase A_2 ($sPLA_2$) using two Chinese hamster ovary (CHO)-K1 cell mutants, LY-B and LY-A cells, deficient in sphingolipid synthesis. In LY-B cells, deficiency of sphingolipids enhanced the release of AA induced by bee venom $sPLA_2$-III or human $sPLA_2$-V. These alterations were reversed by replenishment of exogenous sphingomyelin (SM). In LY-A cells, deficiency of SM increased the release of AA induced by $sPLA_2$. In CHO-K1 cells, decrease and increase of SM level in the plasma membrane by pharmacological methods increased and inhibited the release of AA, respectively. SM inhibited the activity of $sPLA_2$ in vitro. Niemann-Pick disease type C (NPC) is a lysosomal storage disorder caused by mutation of either the NPC1 or NPC2 gene, and is characterized by accumulation of cholesterol and sphingolipids including SM in late endosomes/lysosomes. Increased levels of AA and $sPLA_2$ activity are involved in various neurodegenerative diseases. In CHO cells lacking NPC1 (A101 cells), SM level was lower in the plasma membrane, while it was higher in late endosomes/lysosomes. The release of AA induced by $sPLA_2$ was increased in A101 cells than that in parental cells (JP17 cells), which was attenuated by adding exogenous SM. In addition, $sPLA_2$-III-induced cytotoxicity in A101 cells was much higher than that in JP17 cells. These results suggest that SM in the plasma membrane plays important roles in regulating $sPLA_2$ activity and the enzyme-induced cytotoxicity in A101 cells. J. Cell. Biochem. 116: 1898–1907, 2015.

KEY WORDS: GROUP III SECRETORY PHOSPHOLIPASE A_2; GROUP V SECRETORY PHOSPHOLIPASE A_2; SPHINGOMYELIN; CHINESE HAMSTER OVARY CELLS; LY-A; LY-B; NIEMANN-PICK DISEASE TYPE C

INTRODUCTION

Phospholipase A_2 (PLA_2) catalyzes the hydrolysis of the sn-2 position of membrane glycerophospholipids to produce lysophospholipids and free fatty acids including arachidonic acid (AA). PLA_2 has been established to play roles in physiological and pathological events such as the production of proinflammatory lipid mediators and cell toxicity [Murakami et al., 2011]. Mammalian cells have structurally diverse forms of PLA_2, including secretory PLA_2 ($sPLA_2$), Ca^{2+}-dependent cytosolic PLA_2, and Ca^{2+}-independent PLA_2. The $sPLA_2$ family, for which 11 enzymes have been identified in mammals, represents a group of structurally related, disulfide-rich, Ca^{2+}-dependent, low-molecular-weight enzymes with a catalytic histidine [Murakami and Lambeau, 2013]. AA and its metabolites have been established to show cytotoxicity and be involved in various diseases such as neurological disorders and liver and myocardial injuries [Farooqui et al., 2006; Yagami et al., 2014]. Previously, we reported that exogenously added AA and AA released endogenously by Ca^{2+}-dependent cytosolic PLA_2 and by other PLA_2s caused cell death in L929 fibrosarcoma cells [Kurosawa et al., 2009; Shimizu et al., 2009] and CHO cells [Nakamura et al., 2011]. Several $sPLA_2$ such as human $sPLA_2$-IB and -IIA, and $sPLA_2$-III from bee venom have been shown to cause cell death including apoptosis in neuronal cells [Yagami et al., 2002,2003; DeCoster, 2003]. In

Abbreviations: PLA_2, phospholipase A_2; AA, arachidonic acid; $sPLA_2$, secretory PLA_2; SM, sphingomyelin; CHO, Chinese hamster ovary; NPC, Niemann-Pick disease type C; PAPC, 1-palmitoyl-2-[^{14}C]-arachidonyl phosphatidylcholine; bSMase, Bacillus cereus sphingomyelinase; PS, phosphatidylserine; PE, phosphatidylethanolamine; LDH, lactate dehydrogenase; HSPG, heparan sulfate proteoglycan; ROS, reactive oxygen species.

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addition, $sPLA_2$ have been implicated in various biological events, such as eicosanoid production [Murakami et al., 2001], lipoprotein metabolism [Tietge et al., 2000], anti-bacterial defense [Koduri et al., 2002], and lung surfactant hydrolysis [Chabot et al., 2003]. However, the regulatory mechanisms of $sPLA_2$ activity in cells have not been well established.

Sphingolipids are a class of lipids containing a backbone of sphingoid bases. Not only do sphingolipids play a structural role in the cellular membrane, but they have also been implicated in various significant cell signaling pathways and physiological processes [Hannun and Obeid, 2008]. There are some reports that the activity of $sPLA_2$ is modulated by sphingolipids such as ceramide and sphingomyelin (SM). It has also been reported that ceramide increases the activities of $sPLA_2$-IIA [Koumanov et al., 2002], $sPLA_2$-V [Gesquiere et al., 2002], and $sPLA_2$-X [Singh and Subbaiah, 2007], while SM inhibits those activities [Koumanov et al., 1997; Gesquiere et al., 2002; Singh and Subbaiah, 2007]. In these previous studies, the mechanisms regulating $sPLA_2$ activity were investigated by exogenous addition of sphingolipids or in vitro analysis, so little is known about the role of endogenous sphingolipids in those regulatory functions.

Niemann-Pick disease type C (NPC) is a lysosomal storage disorder caused by mutation of either the NPC1 or NPC2 gene, and is characterized by accumulation of cholesterol and sphingolipids including SM, glucosylceramide, and gangliosides in late endosomes/lysosomes [Carstea et al., 1997; Naureckiene et al., 2000]. Increased levels of AA and $sPLA_2$ activity are involved in various neurodegenerative diseases, and SM modulates $sPLA_2$ activity as described above. Thus, we speculate that changes in SM levels may regulate cytotoxicity via $sPLA_2$-mediated AA release in NPC cells. Here, we examined alterations of $sPLA_2$-dependent AA release in sphingolipid-deficient cells, namely, the Chinese hamster ovary (CHO)-K1-derived mutant cell lines LY-B and LY-A. The LY-B strain has a defect in the LCB1 subunit of serine palmitoyltransferase and is therefore incapable of de novo synthesis of any sphingolipid species [Hanada et al., 1998]. The LY-A strain has a missense mutation in the ceramide transfer protein CERT and is defective in the de novo synthesis of SM [Hanada et al., 2003]. We found that cellular levels of sphingolipids, especially SM level in the plasma membrane, regulate bee venom $sPLA_2$-III- and human $sPLA_2$-V-dependent AA release. In addition, this study showed for the first time that $sPLA_2$ is involved in the cell toxicity in NPC model cells.

MATERIALS AND METHODS

MATERIALS

[5,6,8,9,11,12,14,15-^{3}H]AA (215 Ci/mmol, 7.96 TBq/mmol) was purchased from Amersham (Buckinghamshire, UK); 1-palmitoyl-2-[^{14}C]-arachidonyl phosphatidylcholine (PAPC, 48 mCi/mmol, 1776 MBq/mmol) from Perkin Elmer (Boston, MA); SM (from bovine brain; mixture of various SM species), D-*erythro*-sphingosine, desipramine, and *Bacillus cereus* sphingomyelinase (bSMase) from Sigma (St. Louis, MO); bee venom $sPLA_2$-III, human $sPLA_2$-V, thioetheramide-phosphorylcholine (TEAPC), and U18666A from Cayman (Ann Arbor, MI); Nutridoma-SP from Roche (Basel, Swizerland); bovine phosphatidylserine (PS) from Matreya (Pleasant Gap, PA); 1-palmitoyl-2-oleoyl-phosphatidylethanolamine (PE) from Avanti Polar Lipids (Alabaster, AL); and GW4869 from Calbiochem (La Jolla, CA). Several reagents including U18666A and sphingosine were dissolved in DMSO, and TEAPC and SM were dissolved in ethanol and ethanol:dodecan (98:2), respectively. GW4869 was routinely stored at −30 °C as a 1.5 mM stock suspension in DMSO. Immediately before use, this suspension was solubilized by the addition of 0.25% methane sulfonic acid (2.5 μL of 5% methane sulfonic acid in sterile distilled H_2O was added to 47.5 μL of GW4869 stock solution). The suspension was mixed and heated at 37 °C until clear.

CELL CULTURES AND CELL TOXICITY

The CHO-K1-derived mutant cell lines, LY-A and LY-B, and their complementary derivatives, LY-A/hCERT and LY-B/cLCB1, were established in Dr. K. Hanada's laboratory. A CHO-K1 cell line lacking NPC1 (A101) and its parental cell line (JP17) were kindly provided by Dr. H. Ninomiya. Ham's F-12 medium supplemented with 10% FBS, 100 U/mL penicillin G sodium, and 100 μg/mL streptomycin sulfate was used as a normal culture medium (Normal medium). Nutridoma medium (F-12 medium containing 1% Nutridoma-SP and 0.1% FBS) was used as a sphingolipid-deficient culture medium. All CHO cells were maintained in Normal medium at 37 °C and 5% CO_2. Cell toxicity was estimated by the lactate dehydrogenase (LDH) leakage method according to the manufacturer's instructions (Roche, Basel, Switzerland).

AA RELEASE ASSAY

The cells (1×10^4 cells) were seeded on 24-well plates in Normal medium. For the depletion of sphingolipids, the medium was removed, and the cells were cultured in Nutridoma medium for 30 h. The cells were then labeled overnight with 33 nCi [^{3}H]AA in Ham's F12 medium containing 0.1% fatty acid-free BSA. For the accumulation of cellular SM, the cells were cultured in Normal medium containing SMase inhibitors for 30 h, and then labeled overnight with [^{3}H]AA in Normal medium containing SMase inhibitors. Washed cells were stimulated with $sPLA_2$ at 37 °C. The radioactivity of supernatants and cell lysates (in 1% Triton X-100) was measured by liquid scintillation counting. The amount of radioactivity released into the supernatant is expressed as a percentage of the total amount of incorporated radioactivity.

$sPLA_2$ ASSAY

$sPLA_2$ activity was measured using mixed micelles each containing labeled PAPC, SM, PS, or PE. The mixed lipids in the solvent were dried under nitrogen. A solution of 0.00125% Triton X-100 was added and the lipids were vortexed vigorously for 2 min. Several micelles of 2 μM labeled PAPC containing phospholipids, SM, PS, or PE, at a molar ratio of 1:1, were prepared by sonication for 5 min in a water bath. When the micelle was generated separately, every lipid sample was sonicated separately. The assay buffer contained 100 mM HEPES (pH7.4), 1 mg/mL BSA, and 4 mM $CaCl_2$. The reaction (50 μL of the lipid micelle solution and 175 μL of the assay buffer) was started by the addition of $sPLA_2$ (25 μL), and the reaction mixture was incubated at 37 °C for 30 min. The reaction was

terminated with Dole's reagent, and free fatty acid was recovered as described previously [Nakamura et al., 2010].

LIPID EXTRACTION AND TLC

Cells were rinsed three times with PBS buffer. Lipids were extracted by the Bligh and Dyer method [Bligh and Dyer, 1959]. The organic phase was dried under nitrogen. Dried samples were dissolved in 10 μL of chloroform:methanol (1:1) and analyzed on Silica Gel 60 TLC plates (Merck) using chloroform:methanol:water (65:25:4). The plates were dried and sprayed with 47% sulfuric acid. They were then heated at 150 °C on a hot plate and imaged using Fuji Film LAS1000. Quantitative analysis of TLC plates was performed using the ImageJ software.

LYSENIN STAINING

Cells cultured onto glass-bottomed dishes were washed with PBS and fixed with 3.7% formaldehyde in PBS for 20 min at room temperature. After incubation with 0.5% saponin in PBS for 15 min at room temperature, the cells were blocked with 2% BSA in PBS for 1 h. Then, they were incubated with 1 μg/mL lysenin with 0.2% BSA in PBS for 1 h, washed with PBS, and incubated with anti-lysenin antiserum diluted 1:250 with 0.2% BSA in PBS for 1 h, followed by incubation with Alexa Fluor 488 goat anti-rabbit IgG for 1 h. Fluorescence images were obtained using a Fluoview FV500 confocal laser scanning microscope with a 60×1.00 water immersion objective (Olympus, Tokyo).

STATISTICS

Data are the means ± S.E.M. for three or more independent experiments. In the case of multiple comparisons, the significance of differences was determined using a one-way analysis of variance with Tukey's test. For pairwise comparisons, Student's two-tailed t-test was used. P values <0.05 were considered to be significant.

RESULTS

SM AFFECTS THE $sPLA_2$-DEPENDENT AA RELEASE IN CELLS

Strain LY-B, a CHO-K1 cell mutant defective in the LCB1 subunit of serine palmitoyltransferase, is unable to synthesize any sphingolipid species de novo. As shown in Figure 1A, when cells were cultured in sphingolipid-deficient medium (Nutridoma medium) for 30 h and then in Ham's F-12 medium containing 0.1% BSA for 18 h, the SM level was ~30% of the level in knock-in cells that expressed hamster LCB1 (LY-B/cLCB1 strain), as previously reported [Nakamura et al., 2010]. In addition, when LY-B cells were cultured in Normal medium instead of Nutridoma medium, the SM level was ~85% of the level in LY-B/cLCB1 cells. We confirmed that the cultivation of these cells in sphingolipid-deficient culture conditions did not cause cytotoxicity during the test period (data not shown). Using this culture system, we determined whether the reduction in the cellular sphingolipid level affected the release of AA from cells. The release of AA induced by bee venom $sPLA_2$-III or human $sPLA_2$-V in LY-B cells cultured in Nutridoma medium was 2.1-fold or 1.9-fold of the release in LY-B/cLCB1 cells, respectively (Fig. 1B). When cells were cultured in Normal medium containing 10% FBS, there was no appreciable difference in the release of AA induced by $sPLA_2$ between LY-B and LY-B/cLCB1 cells. These results indicate that sphingolipid deficiency enhances the release of AA induced by $sPLA_2$-III or $sPLA_2$-V in LY-B cells.

It has been reported that the addition of D-*erythro*-sphingosine restored the amounts of SM in LY-B cells to wild-type levels without affecting other lipids such as ceramide and glucosylceramide [Fukasawa et al., 2000]. As shown in Figure 1C, the amount of SM in LY-B cells was restored to the LY-B/cLCB1 level by supplementation of the culture medium with 1 μM D-*erythro*-sphingosine or 10 μM SM. Under these conditions, the enhanced release of AA by $sPLA_2$ from LY-B cells was suppressed to the LY-B/cLCB1 level (Fig. 1D).

To examine the effect of cellular SM content on the release of AA induced by $sPLA_2$-III or $sPLA_2$-V, we used a CHO-K1 cell mutant, strain LY-A, defective in the de novo synthesis of SM because of a mutation in the ceramide transfer protein CERT, as well as LY-A cells stably transformed with human CERT cDNA (LY-A/hCERT strain). Indeed, when cells were cultured in Nutridoma medium for 30 h and then in Ham's F-12 medium containing 0.1% BSA for 18 h, the SM level was lower in LY-A cells than in LY-A/hCERT cells (Fig. 1E). Using this culture system, we determined whether a reduction in the cellular SM level affected the release of AA in response to $sPLA_2$. As shown in Figure 1F, the amount of AA released by $sPLA_2$ was significantly greater from LY-A cells than from LY-A/hCERT cells. These results suggest that SM has an inhibitory effect on $sPLA_2$-dependent AA release in cells.

SM IN THE PLASMA MEMBRANE AFFECTS THE ACTIVITIES OF $SPLA_2$ IN CHO-K1 CELLS

We next attempted to alter the level of SM in the plasma membrane where $sPLA_2$ hydrolyzes phospholipids. SMase catalyzes the hydrolysis of SM to ceramide, and there are three major classes of SMase, namely, acid, neutral, and alkaline, which are categorized according to the pH optima of their activities [Hannun and Obeid, 2008]. Treatment of CHO cells for 48 h with GW4869, an inhibitor of neutral SMase, a plasma membrane-associated enzyme, significantly increased the cellular SM level (Fig. 2A), while treatment of cells for 30 min with bSMase, a neutral SMase, significantly reduced the cellular SM level. To determine whether or not the SM level in the plasma membrane was altered by treatment with GW4869 or bSMase, cells were incubated with the SM-binding probe lysenin, followed by anti-lysenin antibody. The immunofluorescence images in Figure 2B show higher plasma membrane levels in GW4869-treated cells, but lower levels in bSMase-treated cells, compared with those of control cells. Under these conditions, we determined whether the alteration of SM level in the plasma membrane affected the release of AA in response to $sPLA_2$-III or $sPLA_2$-V. As shown in Figure 2C, the release of AA induced by $sPLA_2$ was significantly decreased or increased by treatment with GW4869 or bSMase, respectively.

A tricyclic antidepressant, desipramine, has been shown to induce degradation of acid SMase that is localized in the endosome/lysosome, resulting in the accumulation of cellular SM [Kölzer et al., 2004]. As shown in Figure 3A, cellular SM level was increased by

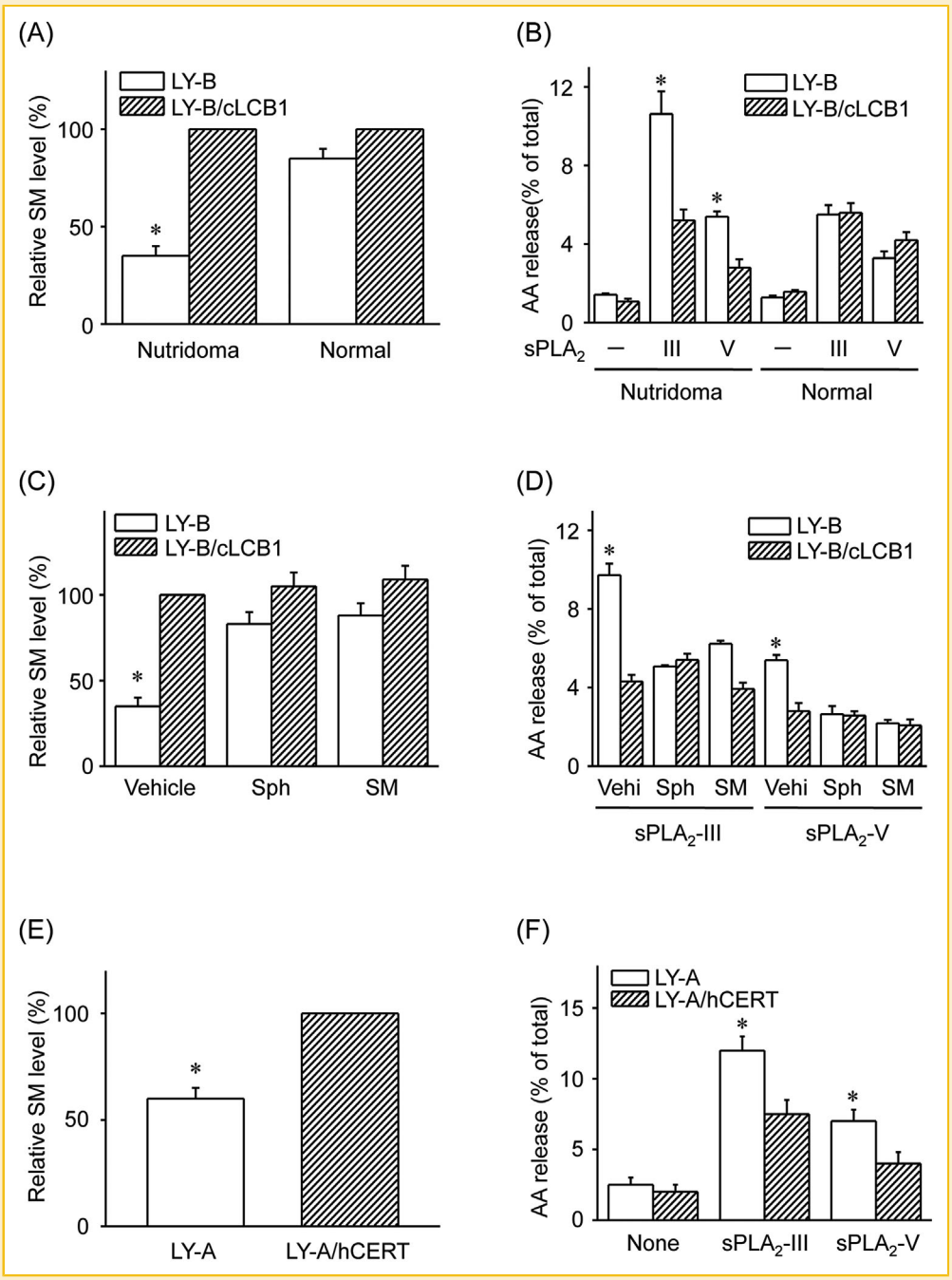

Fig. 1. Enhancement of $sPLA_2$-stimulated AA release in sphingolipid-deficient cells. (A and B) Cells were cultured in Normal or Nutridoma medium at 37 °C for 30 h. They were then incubated for 18 h in Ham's F-12 medium containing 0.1% BSA. (A) Lipids were extracted from washed cells and the amounts of SM were quantified after separation with TLC. (B) The [^{3}H]AA-labeled cells were stimulated with 1 μg/mL $sPLA_2$-III or 0.2 μg/mL $sPLA_2$-V for 30 min at 37 °C. (C and D) Cells were cultured in Nutridoma medium with or without 1 μM D-*erythro*-sphingosine (Sph) or 10 μM SM at 37 °C for 30 h, followed by Ham's F-12 medium with the respective reagents for 18 h. (C) The amounts of SM were quantified. (D) AA releases for 30 min were examined from the [^{3}H]AA-labeled cells with $sPLA_2$-III or $sPLA_2$-V. The data shown are the means ± S.E.M. of three experiments. *$P < 0.05$, significantly different from the values in LY-B/cLCB1 cells. (E) The amounts of SM in LY-A and LY-A/hCERT cells were quantified. (F) [^{3}H]AA-labeled cells were prepared as above, and then stimulated with $sPLA_2$-III or $sPLA_2$-V. The data shown are the means ± S.E.M. of three experiments. *$P < 0.05$, significantly different from the values in LY-A/hCERT cells.

treatment with desipramine for 48 h. Interestingly, treatment with desipramine caused the accumulation of SM in endosome/lysosome, while the level of SM in the plasma membrane was decreased (Fig. 3B). Under these conditions, the release of AA induced by $sPLA_2$ was significantly increased (Fig. 3C). Replenishment of exogenous SM in desipramine-treated cells rescued the reduced SM level in the plasma membrane, which inhibited the enhancement of AA release by treatment with $sPLA_2$.

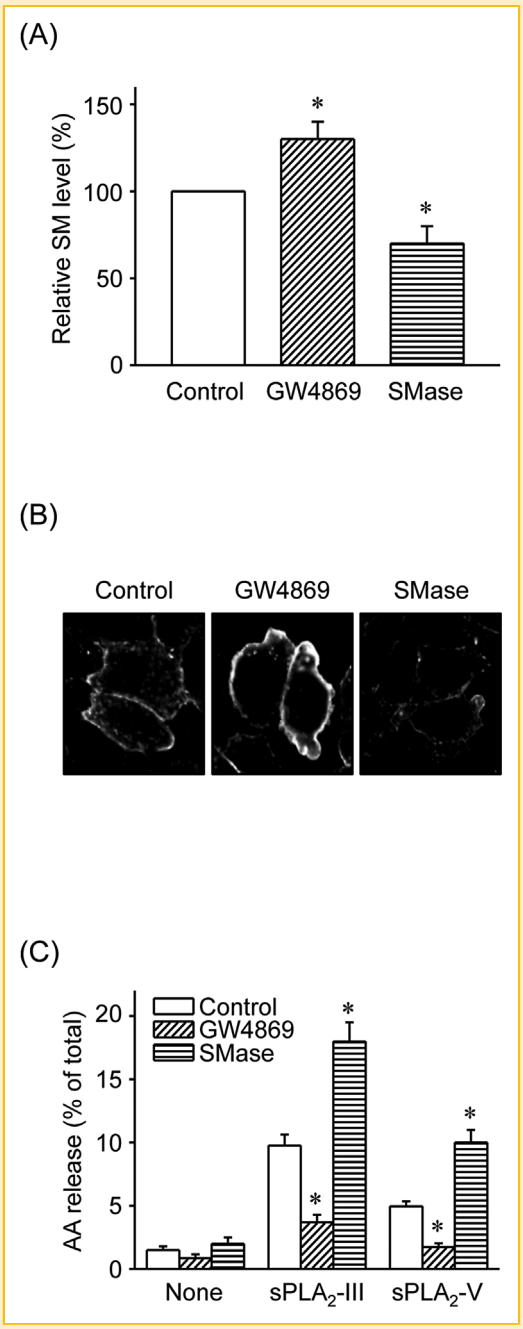

Fig. 2. SM level affects $sPLA_2$ activity in cells. CHO-K1 cells were incubated for 48 h or 30 min in Normal medium with 10 μM GW4869 or 25 mU/mL bSMase, respectively. (A) The amounts of SM were quantified. (B) The amounts and localization of SM were detected by immunofluorescence using lysenin and an anti-lysenin antibody. Similar representative results were obtained in three independent experiments. (C) GW4869-treated cells were labeled with [^{3}H]AA for 18 h at the last stage of the treatment. The [^{3}H]AA-labeled cells were treated with or without bSMase for 30 min at the last stage of the labeling. The washed cells were stimulated with 1 μg/mL $sPLA_2$-III or 0.2 μg/mL $sPLA_2$-V for 30 min at 37 °C. The data in A and C are the means ± S.E.M. of three experiments. *$P < 0.05$, significantly different from the control.

SM INHIBITS THE ACTIVITY OF $SPLA_2$ IN VITRO

To examine whether SM directly reduces the enzymatic activity of $sPLA_2$, micelles containing PAPC and SM were prepared and tested for activity in vitro. The activities of $sPLA_2$-III and $sPLA_2$-V in the presence of PAPC micelles alone were about 1,200 dpm and 800 dpm, respectively (Fig. 4A). The $sPLA_2$ activity in the presence of micelles, which combined PAPC with SM but not with PS or PE at a molar ratio of 1:1, attenuated $sPLA_2$ activity. The SM micelles generated separately from PAPC micelles did not reduce $sPLA_2$ activity (Fig. 4B).

$sPLA_2$ INDUCES AA RELEASE IN A101 CELLS

NPC is an inherited neurodegenerative lipid storage disorder caused by mutations in the NPC1 or NPC2 gene [Carstea et al., 1997; Naureckiene et al., 2000]. It is known that NPC1 deficiency leads to the accumulation of SM in late endosome/lysosome, the same as cholesterol, while reducing the SM level in the plasma membrane [Koivusalo et al., 2007]. TLC analyzes of cellular lipids revealed an increased level of SM (1.4-fold) in A101 cells, a CHO-K1 cell mutant defective in the NPC1 gene, compared with that in its parental JP17 cells (Fig. 5A). In addition, lysenin staining of A101 cells showed a weak fluorescent signal in the plasma membrane relative to that of JP17 cells (Fig. 5B). To examine the effect of NPC1 deficiency on the activity of $sPLA_2$, we measured the release of AA from JP17 and A101 cells. As shown in Figure 5C, when both cells were treated with $sPLA_2$-III or $sPLA_2$-V for 30 min, the release of AA from A101 cells was 1.5- or 1.3-fold, respectively, of that from JP17 cells. To determine whether the enhancement of $sPLA_2$-dependent AA release in A101 cells is due to reduction of the SM level in the plasma membrane, exogenous SM was added to A101 cells. Replenishment of SM in A101 cells rescued the level of SM in the plasma membrane, which significantly decreased $sPLA_2$-induced AA release (Fig. 5B and C). U18666A is a cholesterol transport-inhibiting agent that is used widely to mimic NPC [Liscum and Faust, 1989]. When JP17 cells treated with U18666A for 48 h were stimulated with $sPLA_2$ for 30 min, the release of AA was much greater than that from vehicle-treated cells (Fig. 5D). We next examined whether or not endogenous $sPLA_2$-dependent AA release is increased in A101 cells. Incubation of both cells without stimuli increased the release of AA in a time-dependent manner for up to 6 h, and the release at 3 h and 6 h in A101 cells was about 1.5-fold that in JP17 cells (Fig. 5E). The increased release of AA from A101 cells was attenuated by treatment with TEAPC, an inhibitor of $sPLA_2$ (Fig. 5F). These results indicate that (1) release of AA induced by both endogenous $sPLA_2$ activity and exogenously added $sPLA_2$ was increased in A101 cells, and (2) reduction of SM level in the plasma membrane was involved in the enhanced release of AA.

$sPLA_2$ INDUCES CYTOTOXICITY IN A101 CELLS

We reported previously that AA metabolism via cytosolic PLA_2 induced cytotoxicity in A101 cells [Nakamura et al., 2012]. Therefore, we next examined whether treatment with $sPLA_2$ induced cytotoxicity in A101 cells. When JP17 and A101 cells were treated with $sPLA_2$-III for 8 h, LDH leakage from A101 cells was 2-fold that from JP17 cells (Fig. 6). In addition, when JP17 and A101 cells were pre-treated with SM, $sPLA_2$-III-induced LDH leakage from A101

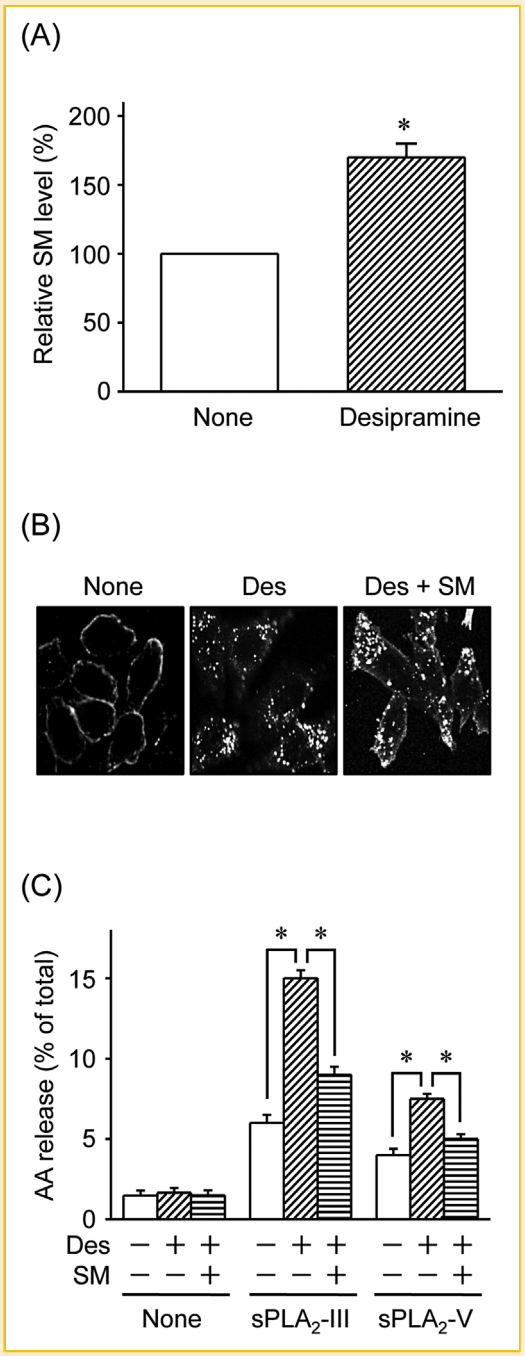

Fig. 3. SM level in the plasma membrane regulates $sPLA_2$ activity. (A) CHO-K1 cells were incubated for 48 h in Normal medium with or without 30 μM desipramine (Des). (A) The amounts of SM were quantified. (B) The cells were then treated with or without 10 μM SM for 1 h, and the amounts and localization of SM were detected by immunofluorescence using lysenin and an anti-lysenin antibody. Similar representative results were obtained in three independent experiments. (C) The cells were labeled with [^{3}H]AA for 18 h at the last stage of culture, and then treated with or without 10 μM SM for 1 h. The labeled and washed cells were stimulated with 1 μg/mL $sPLA_2$-III or 0.2 μg/mL $sPLA_2$-V for 30 min at 37 °C. The data in A and C are the means ± S.E.M. of three experiments. *$P < 0.05$, significantly different between compared groups.

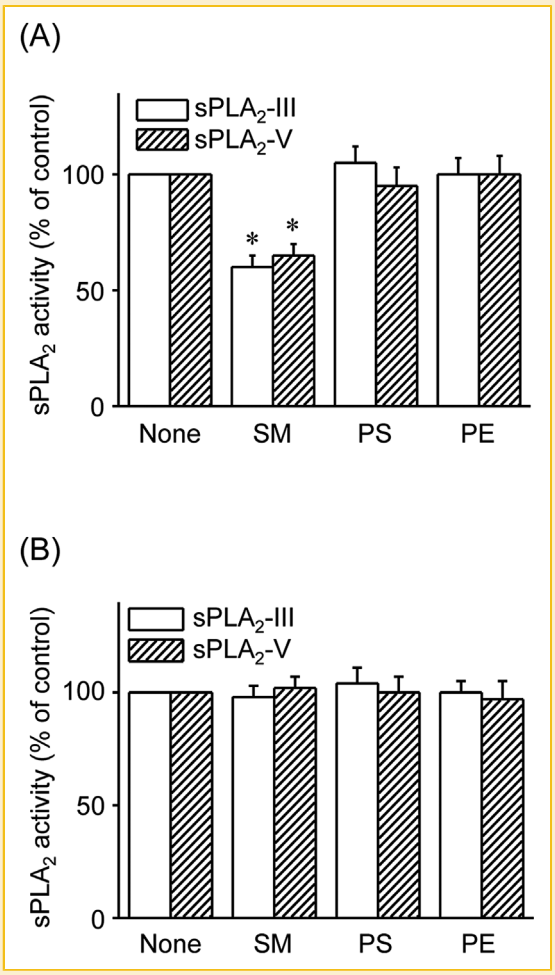

Fig. 4. SM reduces the activity of $sPLA_2$ in vitro. The $sPLA_2$ activity was measured as described in Materials and Methods. (A) Several micelles of 2 μM labeled PAPC containing phospholipids, SM, PS, or PE, at a molar ratio of 1:1, were prepared by sonication. (B) The micelles containing 2 μM labeled PAPC, 2 μM SM, 2 μM PS, and 2 μM PE were generated separately by sonication. Then, 0.1 μg/mL $sPLA_2$-III or 0.2 μg/mL $sPLA_2$-V was added, followed by incubation for 30 min at 37 °C. The data are the mean ± S.E.M. of three experiments. *$P < 0.05$, significantly different from the control.

cells was inhibited, and there was no appreciable difference in its leakage between the two types of cell. These results suggest that reduction of the SM level in the plasma membrane induces $sPLA_2$-dependent cytotoxicity in A101 cells.

DISCUSSION

In this study, we investigated the effects of alteration of cellular sphingolipids on $sPLA_2$-dependent AA release. In LY-B cells, deficiency of sphingolipids enhanced $sPLA_2$-dependent AA release (Fig. 1B). This alteration was restored by cultivation in serum-containing medium or by adding sphingolipids such as sphingosine and SM, indicating that the cellular sphingolipid level is a critical modulator of $sPLA_2$-dependent AA release (Fig. 1D). We also showed

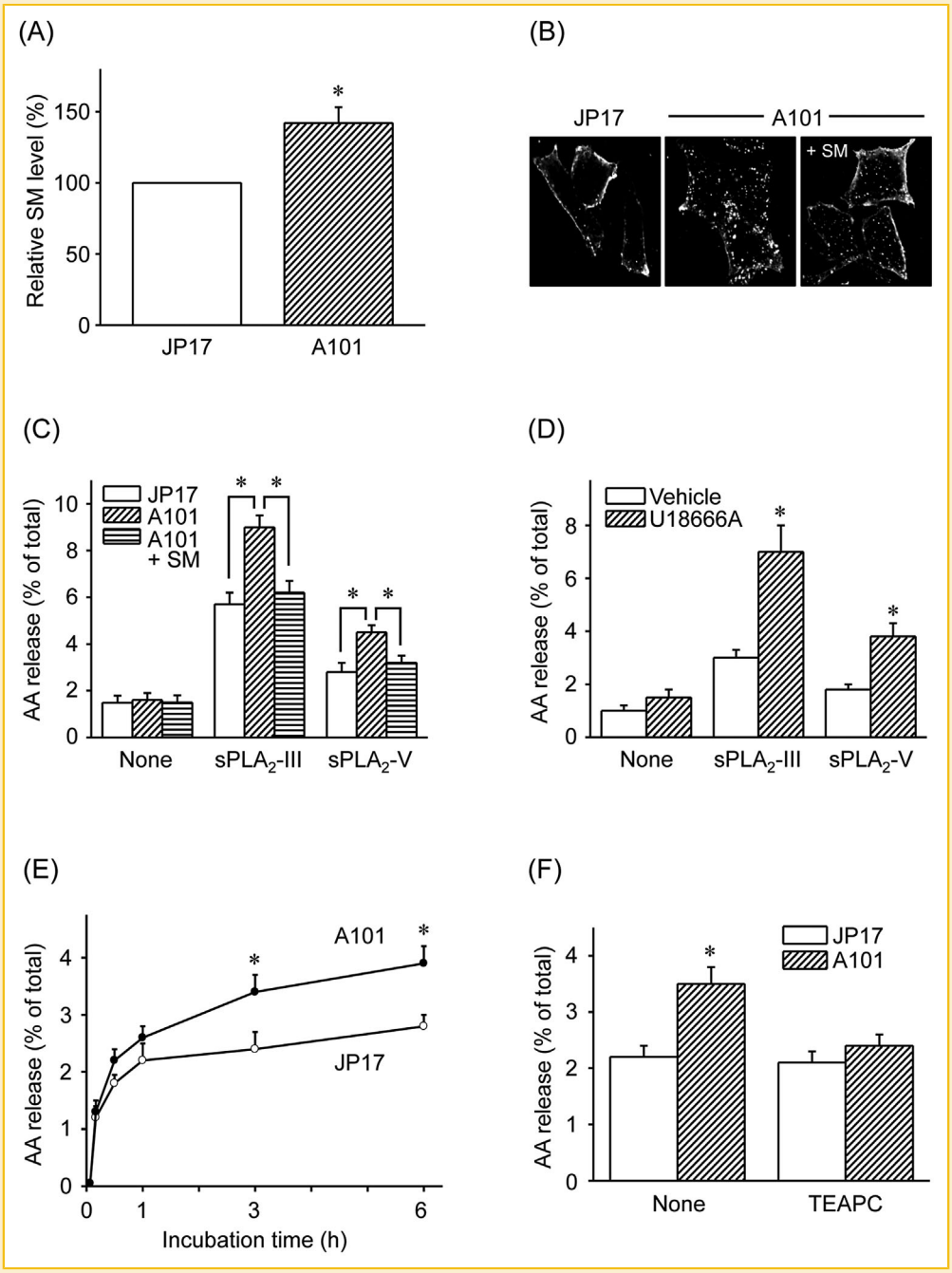

Fig. 5. Enhancement of $sPLA_2$-dependent AA release in A101 cells. (A) The amounts of SM were quantified in A101 and JP17 cells. (B) A101 cells were treated with or without 10 μM SM for 1 h. The cells were treated with lysenin and an anti-lysenin antibody for the detection of SM by immunofluorescence. (C) [^{3}H]AA-labeled cells were further treated with 10 μM SM for 1 h at the final stage of the labeling. (D) JP17 cells were treated with 5 μM U18666A for 30 h, and then further labeled with [^{3}H]AA for 18 h with 5 μM U18666A . In (C and D), the labeled and washed cells were stimulated with 1 μg/mL $sPLA_2$-III or 0.2 μg/mL $sPLA_2$-V for 30 min at 37 °C. (E) [^{3}H]AA-labeled cells were incubated for the indicated periods. (F) [^{3}H]AA-labeled cells were treated with 30 μM TEAPC for 30 min at the final stage of the labeling. The labeled and washed cells were incubated for 3 h with or without TEAPC for measurement of AA release. In B, similar representative results were obtained in three independent experiments. The data in other panels are the means ± S.E.M. of 3–4 experiments. *$P < 0.05$, significantly different between compared groups.

that, in LY-A cells having a lower level of SM, the amount of AA released by $sPLA_2$ was much greater than that released from LY-A/hCERT cells (Fig. 1F). It has been reported that the treatment of cells with exogenous SMase to remove SM enhances $sPLA_2$-stimulated AA release [Gesquiere et al., 2002; Koumanov et al., 2002; Singh and Subbaiah, 2007], as we also showed in Figure 2C. However, those experiments were not able to rule out the possibility that the enhancement of AA release by $sPLA_2$ after SMase treatment was due to the accumulation of ceramide. In fact, incorporation of ceramide into phosphatidylcholine micelles enhances the hydrolysis of AA by

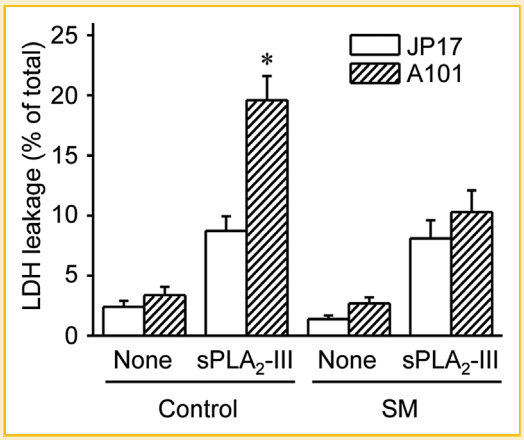

Fig. 6. $sPLA_2$-III induces cytotoxicity in A101 cells. Cells pretreated with or without 10 μM SM for 1 h were treated with $sPLA_2$-III for 8 h, and the LDH leakage was measured. The data shown are the mean ± S.E.M. of three experiments. *$P < 0.05$, significantly different from JP17 cells.

$sPLA_2$-IIA, $sPLA_2$-V, and $sPLA_2$-X in vitro [Koumanov et al., 2002; Gesquiere et al., 2002; Singh and Subbaiah, 2007]. To resolve the problem, we used LY-A cells because the content of ceramide in LY-A cells is almost same as in wilt-type cells [Fukasawa et al., 2000]. We demonstrated that the reduction of cellular SM content without any increase in ceramide level enhances $sPLA_2$-stimulated AA release in LY-A cells. Thus, we conclude that SM plays an important role in the regulation of $sPLA_2$-dependent AA release.

The liberation of AA from membrane phospholipids by $sPLA_2$ is known to occur mainly at the plasma membrane, where SM is predominantly located. In the present study, alteration of the SM level in the plasma membrane, but not in endosomes/lysosomes, affected the release of AA induced by $sPLA_2$-III and $sPLA_2$-V, indicating that the SM level in the plasma membrane is critical for those activities. Some $sPLA_2$, including $sPLA_2$-V, are sorted into the caveolin-rich vesicular domains through binding to glypican, a glycosylphosphatidylinositol-anchored heparan sulfate proteoglycan (HSPG) [Murakami et al., 2001]. After appropriate sorting, $sPLA_2$-V is internalized into the lumen side of the perinuclear membrane and releases AA. In the present study, although treatment with desipramine induced $sPLA_2$V-dependent AA release, it seemed like desipramine treatment did not affect the SM level in the perinuclear membrane, as judged by lysenin staining (Fig. 3B). $sPLA_2$-V can also act on the plasma membrane to release AA independently of HSPG [Han et al., 1999; Murakami et al., 2001]. The hydrolysis of the plasma membrane and that of the perinuclear membrane by $sPLA_2$-V occur sequentially, and plasma membrane hydrolysis by $sPLA_2$-V appears to be required for the initiation of HSPG-dependent internalization of the enzyme [Kim et al., 2002; Munoz et al., 2003]. Thus, SM in the plasma membrane may play important roles in regulating the $sPLA_2$-V-dependent AA release in the plasma membrane and subsequently the internalization of the enzyme.

Various investigators have demonstrated that SM inhibits several phospholipases. Dawson et al. reported that SM inhibited diacylglycerol-stimulated intracellular phospholipases [Dawson et al., 1985]. Subbaiah and Liu reported that lecithin-cholesterol acyltransferase, a specialized phospholipase A responsible for the esterification of cholesterol in plasma, was inhibited by SM [Subbaiah and Liu, 1993]. The activities of lipoprotein lipase and $sPLA_2$ have also been shown to be inhibited by SM in vitro [Koumanov et al., 1997; Gesquiere et al., 2002; Singh et al., 2007]. However, the exact mechanisms for the inhibition of phospholipases are not clear in most cases. We reported previously that SM inhibited $cPLA_2\alpha$ activity due to disturbance of the binding of $cPLA_2\alpha$ to glycerophospholipids, but not due to direct binding between SM and $cPLA_2\alpha$ [Nakamura et al., 2010]. The present study also found that the incorporation of SM into PAPC micelles induced the inhibition of $sPLA_2$ activities in vitro (Fig. 4A), while the SM micelles generated separately from PAPC micelles did not affect the activities of $sPLA_2$ (Fig. 4B). Also, free SM did not inhibit PAPC degradation by the enzyme (data not shown). Thus, it does not seem to be the case that SM inhibits $sPLA_2$ by binding to them. Because SM is a non-hydrolyzable structural analog of phosphatidylcholine, it may competitively inhibit $sPLA_2$. In addition, because SM decreases the fluidity of membranes and increases packing density in the hydrophobic core [Scarlata et al., 1996], it may decrease $sPLA_2$ binding. However, we are not able to rule out the possibility of direct binding of $sPLA_2$ with SM. More precise analysis of the interaction between $sPLA_2$ and SM should help to clarify the mechanisms of inhibition of $sPLA_2$ by SM.

SM is known to have the structural diversity and biological function of distinct species [Peter, 2013]. Lipidomic studies of human fibroblasts revealed that their SM fraction contained at least 18 different molecular species, with d18:1/16:0 SM being the dominant species, followed by markedly lower amounts of d18:1/24:1 and d18:1/24:0 [Valsecchi et al., 2007]. The other molecular species were present only in trace amounts and included additionally 14:0, 17:0, 18:0, and 22:0 *N*-linked acyl chains. The d18:1 long chain base is most prominent, but d18:0 and d18:2 long chain bases are also present. Some additional long chain bases present in trace amounts only [Valsecchi et al., 2007]. In the present study, we used the bovine brain SM which is contained several SM species. The effect of distinct SM species on $sPLA_2$ activity should be studied in future.

Activity of $sPLA_2$ including $sPLA_2$-V has been established to be regulated by various factors, such as Ca^{2+} ions, oxidative conditions, and proteins including their receptors and/or binding proteins [Murakami et al., 2011]. In addition, $sPLA_2$-III from bee venom and $sPLA_2$-V have been shown to be proteins particularly involved in eliciting AA release from intact cellular membranes, probably because of their high capacity to bind phosphatidylcholine that is abundant on the outer plasma membrane. In the present study, the inhibitory effects of SM on the degradation by $sPLA_2$ were similar between in the presence of 4 mM and 20 mM Ca^{2+} (data not shown). Thus, inhibitory effect of SM on $sPLA_2$ activity may not due to Ca^{2+}-related events.

NPC is a neurodegenerative lipid storage disorder caused by mutations in the NPC1 or NPC2 gene [Carstea et al., 1997; Naureckiene et al., 2000]. Loss of function of either protein results in the accumulation of cholesterol in late endosomes/lysosomes. NPC1 protein is a key molecule in cholesterol trafficking from late endosome to the Golgi apparatus and the endoplasmic reticulum. In

NPC cells, SM is also accumulated in late endosomes/lysosomes, the same as cholesterol. SM internalized from the plasma membrane is recycled from endosome to the plasma membrane or targeted to lysosomes for degradation by acid SMase. It has been reported that NPC1 may also be involved in the recycling of SM from late endosome to the plasma membrane [Koivusalo et al., 2007]. Accordingly, NPC1 deficiency might lead to the accumulation of SM in the endosomal compartment, while reducing the SM level in the plasma membrane (Fig. 5A). In addition, an increase in the $sPLA_2$-dependent AA release from A101 cells was inhibited by the replenishment of exogenous SM, indicating that the enhancement of AA release from A101 cells compared with that from JP17 cells was due to reduction of the SM level in the plasma membrane in A101 cells (Fig. 5B). Since several $sPLA_2$ are reported to be released to extracellular space [Murakami et al., 2011], the enhanced release of AA in A101 cells may be due to the activity of released $sPLA_2$ in the medium. The mechanism causing neuronal injury in NPC has been unclear. In the present study, we showed that $sPLA_2$-III had cell toxicity and the effect of $sPLA_2$-III-induced cytotoxicity in A101 cells was much greater than that in JP17 cells (Fig. 6). Although the mechanism of cytotoxicity induced by $sPLA_2$-III is not clear in this study, $sPLA_2$-III from bee venom has been shown to cause neuronal apoptosis in rat cortical neurons [DeCoster, 2003]. In addition, it has been reported that $sPLA_2$-IB and $sPLA_2$-IIA induce neuronal apoptosis in rat cortical neurons through the excess influx of Ca^{2+} into neurons via L-type voltage-dependent Ca^{2+} channels [Yagami et al., 2002, 2003]. Moreover, the generation of reactive oxygen species (ROS) by NADPH oxidase is involved in $sPLA_2$-IIA-induced Ca^{2+} influx and apoptosis. ROS play a role in the progression of NPC, and treatment of NPC mice with anti-oxidants led to a 12–23% increase in life expectancy and neuromuscular function [Bascunan-Castillo et al., 2004]. We reported previously that AA metabolism via $cPLA_2\alpha$ induced cytotoxicity and ROS formation in A101 cells [Nakamura et al., 2012]. Thus, it may be linked to $sPLA_2$-, $cPLA_2\alpha$-, and ROS-induced neuronal injury in NPC. Lysophospholipids, counterparts of fatty acids formed by hydrolysis of phospholipids by $sPLA_2$, have been established to act as bioactive molecules and to be metabolized to lysophosphatidic acid, a stimulator of G protein-coupled receptors [Murakami et al., 2011; Quach et al., 2014], thus possible roles of the molecules on cytotoxicity in NPC cells remained to be solved.

In summary, we showed that SM in the plasma membrane regulated $sPLA_2$ inhibitory activities. In addition, we showed that reduction of the SM level in the plasma membrane enhanced $sPLA_2$-III-induced cytotoxicity in NPC model cells. These findings may provide new insight into not only the regulatory mechanisms of $sPLA_2$ by SM but also the pathogenesis of NPC.

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