

# Secretory phospholipase A<sub>2</sub> type III enhances $\alpha$ -secretase-dependent amyloid precursor protein processing through alterations in membrane fluidity

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**Abstract** In the non-amyloidogenic pathway, amyloid precursor protein (APP) is cleaved by  $\alpha$ -secretases to produce  $\alpha$ -secretase-cleaved soluble APP (sAPP <sub>$\alpha$</sub> ) with neuroprotective and neurotrophic properties; therefore, enhancing the non-amyloidogenic pathway has been suggested as a potential pharmacological approach for the treatment of Alzheimer's disease. Here, we demonstrate the effects of type III secretory phospholipase A<sub>2</sub> (sPLA<sub>2</sub>-III) on sAPP <sub>$\alpha$</sub>  secretion. Exposing differentiated neuronal cells (SH-SY5Y cells and primary rat neurons) to sPLA<sub>2</sub>-III for 24 h increased sAPP <sub>$\alpha$</sub>  secretion and decreased levels of A $\beta$ <sub>1–42</sub> in SH-SY5Y cells, and these changes were accompanied by increased membrane fluidity. We further tested whether sPLA<sub>2</sub>-III-enhanced sAPP <sub>$\alpha$</sub>  release is due in part to the production of its hydrolyzed products, including arachidonic acid (AA), palmitic acid (PA), and lysophosphatidylcholine (LPC). Addition of AA but neither PA nor LPC mimicked sPLA<sub>2</sub>-III-induced increases in sAPP <sub>$\alpha$</sub>  secretion and membrane fluidity. Treatment with sPLA<sub>2</sub>-III and AA increased accumulation of APP at the cell surface but did not alter total expressions of APP,  $\alpha$ -secretases, and  $\beta$ -site APP cleaving enzyme. Taken together, these results support the hypothesis that sPLA<sub>2</sub>-III enhances sAPP <sub>$\alpha$</sub>  secretion through its action to increase membrane fluidity and recruitment of APP at the cell surface.—Yang, X., W. Sheng, Y. He, J. Cui, M. A. Haidekker, G. Y. Sun, and J. C-M. Lee. Secretory phospholipase A<sub>2</sub> type III enhances  $\alpha$ -secretase-dependent amyloid precursor protein processing through alterations in membrane fluidity. *J. Lipid Res.* 2010. 51: 957–966.

**Supplementary key words** arachidonic acid • amyloid- $\beta$  peptide • Alzheimer's disease

The senile plaque composed of neurotoxic amyloid- $\beta$  peptide (A $\beta$ ) is a pathologic characteristic of Alzheimer's disease (AD) (1–6). In the amyloidogenic pathway, A $\beta$  is derived from a proteolytic process of amyloid precursor protein (APP), in which APP is cleaved sequentially by  $\beta$ - and  $\gamma$ -secretases (7). Alternatively, the non-amyloidogenic pathway is mediated by  $\alpha$ -secretase, which cleaves between amino acids 16 and 17 within the A $\beta$  domain. This secretase is a member of the ADAM (a disintegrin and metalloprotease) family and produces a soluble fragment of APP generally regarded as  $\alpha$ -secretase-cleaved soluble APP (sAPP <sub>$\alpha$</sub> ) (8, 9). Due to the neurotrophic and neuroprotective properties of sAPP <sub>$\alpha$</sub>  (10), increasing the APP processing by  $\alpha$ -secretase has been suggested as a new strategy for the treatment of AD (11).

APP is a transmembrane protein, and recent studies show that APP processing can be affected by the local membrane environment. The activity of  $\beta$ -site APP cleaving enzyme (BACE) to produce neurotoxic A $\beta$  is favorable in lipid rafts, which are highly ordered membrane microdomains enriched in cholesterol, sphingolipids, and saturated phospholipids (12–17). On the other hand, cleavage of APP by  $\alpha$ -secretases is known to occur mainly in nonraft domains (18). Therefore, APP processing can be altered

Abbreviations: AA, arachidonic acid; A $\beta$ , amyloid- $\beta$  peptide; AD, Alzheimer's disease; ADAM, a disintegrin and metalloprotease; APP, amyloid precursor protein; BACE,  $\beta$ -site APP cleaving enzyme; BCA, bicinchoninic acid; cPLA<sub>2</sub>, cytosolic phospholipase A<sub>2</sub>; DHA, docosahexaenoic acid; DRM, detergent-resistant membrane; FACS, fluorescence-activated cell sorting; FCVJ, farnesyl-(2-carboxy-2-cyanovinyl)-julolidine; FRAP, fluorescence recovery after photobleaching; iPLA<sub>2</sub>, calcium-independent phospholipase A<sub>2</sub>; LPC, lysophosphatidylcholine; M $\beta$ CD, methyl- $\beta$ -cyclodextrin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NMDA, N-methyl D-aspartate; PA, palmitic acid; PLA<sub>2</sub>, phospholipase A<sub>2</sub>; RA, all-*trans* retinoic acid; sAPP <sub>$\alpha$</sub> ,  $\alpha$ -secretase-cleaved soluble APP; sPLA<sub>2</sub>-III, secretory phospholipase A<sub>2</sub> type III.

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by manipulating membrane lipid composition, such as cholesterol and sphingolipids removals (19–22).

Phospholipases A<sub>2</sub> (PLA<sub>2</sub>s) are ubiquitous enzymes responsible for maintenance of phospholipid homeostasis in cell membranes. Aberrant PLA<sub>2</sub> activity has been implicated in neurodegenerative diseases, including AD, Parkinson's disease, ischemia, spinal cord trauma, and head injury (23–26). Among many types of secretory PLA<sub>2</sub>s, secretory phospholipase A<sub>2</sub> type III (sPLA<sub>2</sub>-III) has been found to express in human neuronal cells and contribute to neuronal differentiation (27). sPLA<sub>2</sub>-III from bee venom is highly homologous to the enzymatic-active central s-domain of human sPLA<sub>2</sub>-III (28). This protein has been reported to alter cellular membrane properties (29). In this study, we investigate whether sPLA<sub>2</sub>-III alters sAPP<sub>α</sub> in differentiated neuronal cells, including SH-SY5Y cells and primary rat neurons, and Aβ secretion. In addition, we also examine the effects of its hydrolyzed products, i.e., arachidonic acid (AA), palmitic acid (PA), and lysophosphatidylcholine (LPC), on sAPP<sub>α</sub> secretion, membrane fluidity, recruitment of APP to the cell surface, as well as the expressions of α-secretases and BACE.

## MATERIALS AND METHODS

### Chemicals and reagents

DMEM with high glucose, DMEM/F12 medium (1:1), Ham's F-12 medium, FBS, penicillin, and streptomycin (pen/strep), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Invitrogen (Carlsbad, CA). Neurobasal medium, B27, and trypsin-EDTA were obtained from Gibco (Carlsbad, CA). Bee venom sPLA<sub>2</sub>-III was from Cayman Chemical (Ann Arbor, MI). AA, LPC, PA, phorbol 12-myristate 13-acetate (PMA), DMSO, all-*trans* retinoic acid, and poly-L-lysine were from Sigma-Aldrich (St. Louis, MO). Farnesyl-(2-carboxy-2-cyanovinyl)-julolidine (FCVJ) was from Dr. Haidekker's Laboratory (University of Georgia) (30).

### Cell culture

Human neuroblastoma SH-SY5Y cells ( $1.0 \times 10^5$  cells/well) were seeded into 12-well plates or  $1.0 \times 10^6$  cells/dish into 60 mm dishes and were cultured in DMEM/F12 medium (1:1) containing 10% FBS. For differentiation, SH-SY5Y cells were exposed to 10 μM all-*trans* retinoic acid for 6 days with change of fresh culture medium every 2 days.

Primary cortical neurons were prepared from embryonic day 17 Sprague-Dawley rats as described previously (31) with slightly modification. In brief, cortical neurons were enzymatically dissociated (0.05% trypsin with EDTA) and dispersed into a single-cell suspension with pasture pipette and seeded onto glass growth chambers and 6-well dishes coated with 50 mg/l poly-L-lysine. The cells were maintained in neural basal medium with 2% B27, 2 mM glutamine, and 1% pen/strep for 7 days before experiments. All cells were maintained at 37°C in a 5% CO<sub>2</sub> humidified incubator.

### Cell viability by MTT test

Cell viability was determined by MTT reduction. Briefly, differentiated SH-SY5Y cells or primary neurons cultured in 12-well plates were treated with different compounds, e.g., sPLA<sub>2</sub>-III, AA, LPC, and PA. After treatment, medium was removed and 1 ml of

MTT reagent (0.5 mg/ml) in DMEM was added into each well. Cells were incubated for 4 h at 37°C, and after dissolving formazan crystals with DMSO, absorption at 540 nm was measured.

### Characterization of membrane fluidity by fluorescence microscopy of FCVJ-labeled cells

A fluorescent molecular rotor, FCVJ was used to measure the relative membrane fluidity in SH-SY5Y cells. FCVJ was designed to be a more membrane-compatible fluorescent molecular rotor (32) with the quantum yield strongly dependent on the local free volume. A higher fluorescent intensity of FCVJ reflects the intramolecular rotational motions being restricted by a smaller local free volume, indicating a more viscous membrane. Previously, we verified the application of FCVJ for measuring membrane viscosity by comparing the results obtained using FCVJ with those from the technique of fluorescence recovery after photobleaching (30). In this study, we adapted the protocol from Haidekker et al. (32) to fluorescently label cells with FCVJ. Briefly, after undergoing different treatment protocols, e.g., sPLA<sub>2</sub>-III, AA, PA, and LPC, SH-SY5Y cells or primary neurons were washed with PBS and incubated in DMEM containing 20% FBS and 1 μM FCVJ for 20 min. Excess FCVJ was removed by washing cells with PBS three times. Fluorescence intensity measurements were performed at room temperature using a Nikon TE-2000 U fluorescence microscope with an oil immersion 60× objective lens. Images were acquired using a CCD camera controlled by a computer running MetaVue imaging software (Universal Imaging, PA). The fluorescence intensities of FCVJ per cell were measured. Background subtraction was done for all images prior to data analysis.

### Western blot analysis of sAPP<sub>α</sub> released from SH-SY5Y cells and primary neurons

After treating cells with sPLA<sub>2</sub>-III or lipid metabolites for 24 h, culture medium was collected and the same volume of the cell lysate from each sample was used for Western blot analysis using β-actin as internal standard. The culture medium was centrifuged at 12,000 *g* for 5 min to remove cell debris, and the same volume of medium from each sample (e.g., 40 μl) was diluted with Laemmli buffer, boiled for 5 min, subjected to electrophoresis in 7.5% SDS-polyacrylamide gels, and transferred to nitrocellulose membranes. Membranes were blocked for 1 h with 5% (w/v) nonfat dry milk in TBS containing 0.1% (v/v) Tween 20 (TBST) and incubated overnight at 4°C in 3% (w/v) BSA with 0.02% (w/v) sodium azide in TBST with a 6E10 monoclonal antibody (1:1,000 dilution; Millipore, Billerica, MA) that recognizes residues 1–17 of the Aβ domain of human sAPP<sub>α</sub> or with a rodent specific polyclonal antibody (1:1,000 dilution; Covance, Dedham, MA). Membranes were washed three times during a 15 min period with TBST and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG antibody (1:2,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) in 5% (w/v) nonfat dry milk in TBST at room temperature for 1 h. After washing with TBST for three times, the membrane was subjected to SuperSignal West Pico Chemiluminescent detection reagents from Pierce (Rockford, IL) to visualize bands. The protein bands detected on X-ray film were quantified using a computer-driven scanner and Quantity One software (Bio-Rad).

### Western blot analysis of APP, ADAM9, ADAM10, ADAM17, and BACE1 in SH-SY5Y cells

After treatments, the protein concentration of the cell lysate was determined by BCA protein assay kit (Pierce Biotechnology) according to the manufacturer's instruction. Equivalent amounts of protein from each sample (e.g., 30 μg) was diluted with Laemmli buffer, boiled for 5 min, subjected to electrophoresis in 7.5%

SDS-polyacrylamide gels, and transferred to nitrocellulose membranes. Membranes were blocked for 1 h with 5% (w/v) nonfat dry milk in TBST and incubated overnight at 4°C in 3% (w/v) BSA with 0.02% (w/v) sodium azide in TBST with 6E10 monoclonal antibody, anti-ADAM9 antibody (1:1,000 dilution; Abcam, Cambridge, MA), anti-ADAM10 antibody (1:1,000 dilution; Millipore), anti-ADAM17 antibody (1:1,000 dilution; Santa Cruz Biotechnology) or anti-BACE1 antibody (1:1,000 dilution; Sigma-Aldrich). Membranes were washed three times during a 15 min period with TBST and incubated with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG antibody (1:2,000 dilution; Santa Cruz Biotechnology) in 5% (w/v) nonfat dry milk in TBST at room temperature for 1 h. After washing with TBST for three times, the membrane was subjected to SuperSignal West Pico Chemiluminescent detection reagents from Pierce to visualize bands. The protein bands detected on X-ray film were quantified using a computer-driven scanner and Quantity One software (Bio-Rad).

### Immunofluorescent staining and assessment of APP at the cell surface of SH-SY5Y cells

SH-SY5Y cells were plated onto cover slips. After differentiation and treatments, cells were fixed in PBS containing 4% paraformaldehyde without prior permeabilization with detergent. After washing three times with PBS, nonspecific binding of antibodies was blocked by 5% goat serum for 1 h at room temperature. Cells were then incubated overnight at 4°C in 3% goat serum with anti-APP mouse antibody (1:200 dilution; Assay Designs, Ann Arbor, MI) that recognizes the N terminus of APP. The cover slips were washed with PBS and incubated for 1 h at room temperature with FITC-labeled goat anti-mouse secondary antibody (1:400) and washed with PBS. Cover slips were then mounted, and fluorescent intensity measurements were performed at room temperature using the Nikon TE-2000 U fluorescence microscope and oil immersion 60× objective lens. Images were acquired using a CCD camera controlled by a computer running MetaVue imaging software (Universal Imaging). The fluorescent intensities per cell area were measured. Background subtraction was done for all images prior to data analysis.

### Fluorescence-activated cell sorting analysis of APP at cell surface of SH-SY5Y cells

After differentiation and treatments, SH-SY5Y cells were detached with nonenzymatic cell dissociation solution (Gibco, Carlsbad, CA). The cells were fixed in PBS containing 4% paraformaldehyde without permeabilization. After washing three times with PBS, nonspecific binding of antibodies was blocked by 5% goat serum for 1 h at room temperature. The cells were then incubated for 2 h at room temperature in 3% goat serum with anti-APP mouse antibody (1:200 dilution; Assay Designs) that recognizes the N terminus of APP. After washing with PBS, cells were then incubated for 1 h at room temperature with FITC-labeled goat anti-mouse secondary antibody (1:400) and washed with PBS. Background fluorescence intensity was assessed in the absence of primary antibody. All measurements were performed on a FACS-can flow cytometry system (BD Biosciences, San Jose, CA) equipped with an argon laser. The excitation wavelength was 488 nm and emission intensity was detected with a FITC 525/30 nm filter set. A total of 10,000 cells were analyzed from each sample. Curves were generated with CellQuest software (BD Biosciences), and the median values of intensity were measured for data analysis.

### Quantification of secreted A $\beta_{1-42}$

After treatments, culture medium was collected, supplemented with protease inhibitor cocktail, and centrifuged at 12,000 *g* for 5 min at 4°C to remove cell debris.

An aliquot (100  $\mu$ l) of supernatant was used for A $\beta_{1-42}$  quantification using an ELISA kit (Invitrogen) following the manufacturer's recommendation. According to the instruction manual, substances including A $\beta_{1-12}$ , A $\beta_{1-20}$ , A $\beta_{12-28}$ , A $\beta_{22-35}$ , A $\beta_{1-40}$ , A $\beta_{1-43}$ , A $\beta_{42-1}$ , and APP have no cross-reactivity. The minimum detectable dose of A $\beta_{1-42}$  is <1.0 pg/ml. The level of A $\beta_{1-42}$  in each sample was measured in duplicates and expressed in pg/ml.

### Statistical analysis

Data are presented as mean  $\pm$  SD from at least three independent experiments. Comparison between two groups was made with a Student's *t*-test. Comparisons of more than two groups were made with one-way ANOVA, followed by Bonferroni's post hoc tests. Values of *P* < 0.05 are considered to be statistically significant.

## RESULTS

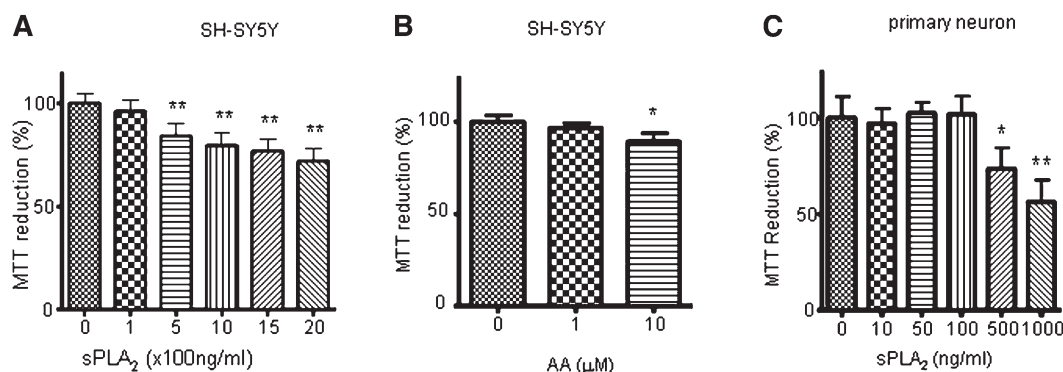
### Exogenous sPLA<sub>2</sub>-III and AA increased sAPP $_{\alpha}$ secretion in neuronal cells

sPLA<sub>2</sub>-III hydrolyzes sn-2 fatty acids of phospholipids in cell membranes, resulting in release of PUFAs and lysophospholipids. To test whether fatty acids or lysophospholipids are responsible for the increase in sAPP $_{\alpha}$  secretion and alteration of membrane fluidity, we used AA and LPC as representative polyunsaturated fatty acids and lysophospholipids, respectively. For a negative control, PA, a saturated fatty acid and not likely a hydrolyzed product of sPLA<sub>2</sub>-III, was also applied.

Since sPLA<sub>2</sub>-III from bee venom is highly homologous to the sPLA<sub>2</sub>-III in human (28), sPLA<sub>2</sub>-III from bee venom was used to investigate the effect of sPLA<sub>2</sub>-III on sAPP $_{\alpha}$  secretion in neuronal cells in relation to membrane fluidity. We first examined the viability of SH-SY5Y cells and primary rat neurons in response to different doses of sPLA<sub>2</sub>-III using the MTT test. As shown in Fig. 1, there is a dose-dependent decrease in cell viability upon exposing SH-SY5Y cells and primary neurons to sPLA<sub>2</sub>-III for 24 h. Based on these results, subsequent studies used 100 and 500 ng/ml of sPLA<sub>2</sub>-III for treating SH-SY5Y cells and 50 and 100 ng for treating primary neurons. Similar approaches were applied to determine the concentrations of AA (Fig. 1B), PA, and LPC (data not shown) for this study. In this study, 1 and 10  $\mu$ M of AA (Fig. 1B), 10 and 100  $\mu$ M of PA, and 1 and 10  $\mu$ M of LPC were used.

Western blot analysis showed that sPLA<sub>2</sub>-III and AA increased sAPP $_{\alpha}$  secretion in SH-SY5Y cells in a dose-dependent manner (Fig. 2A). Since it has been reported that PMA, a protein kinase C agonist, increases sAPP $_{\alpha}$  secretion (33–35), treatment with PMA (10 nM) was used as a positive control. However, PA and LPC did not alter sAPP $_{\alpha}$  secretion (Fig. 2A). The increase in sAPP $_{\alpha}$  secretion induced by sPLA<sub>2</sub>-III and AA was not due to the change of APP content in cells as shown in Fig. 2B; exposing cells to sPLA<sub>2</sub>-III, AA, LPC, and PA for 24 h did not alter total APP expression in SH-SY5Y cells (Fig. 2B). Consistent with the results from SH-SY5Y cells, sPLA<sub>2</sub>-III also increased sAPP $_{\alpha}$  secretion in primary rat neurons (Fig. 2C).



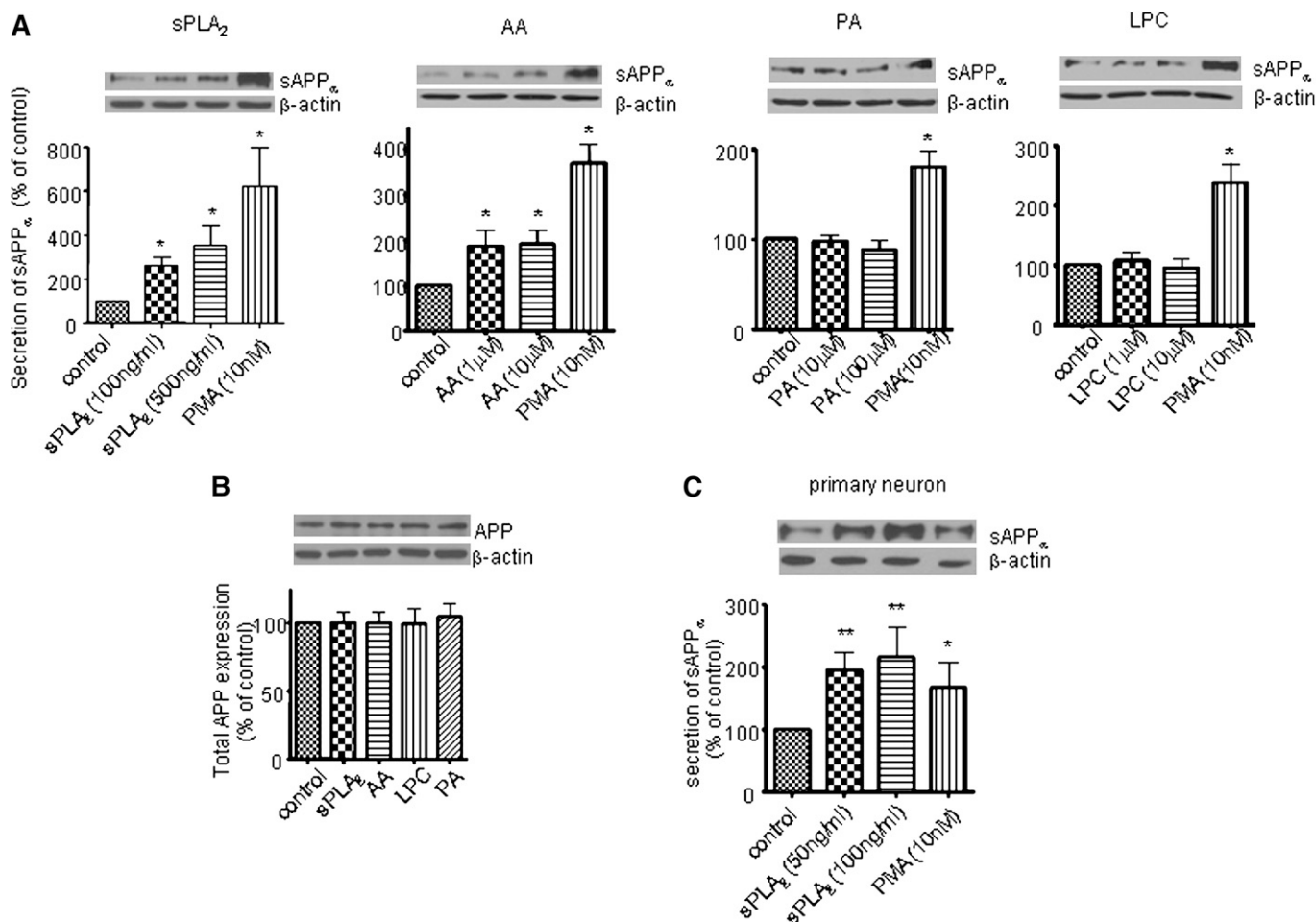


**Fig. 1.** sPLA<sub>2</sub>-III and AA on the viability of neuronal cells. MTT test was applied to examine the viability of cells. MTT reduction was determined by absorption at wavelength of 540 nm for differentiated SH-SY5Y cells in medium with 1% FBS treated with sPLA<sub>2</sub>-III (A) and AA (B) for 24 h and for primary rat neurons treated with sPLA<sub>2</sub>-III (C) for 24 h. Data are expressed as percentages of control and mean  $\pm$  SD from at least three independent experiments (\*  $P < 0.05$ ; \*\* $P < 0.01$ ).

### sPLA<sub>2</sub>-III and AA increased membrane fluidity

To study the effects of sPLA<sub>2</sub>-III and its hydrolyzed products on membrane fluidity, we applied a fluorescent molecular rotor, FCVJ. As explained in Materials and

Methods, FCVJ integrated into a highly fluidized membrane exhibits lower quantum yield, as reflected by a lower fluorescent intensity. To validate the application of this technique for the measurement of membrane

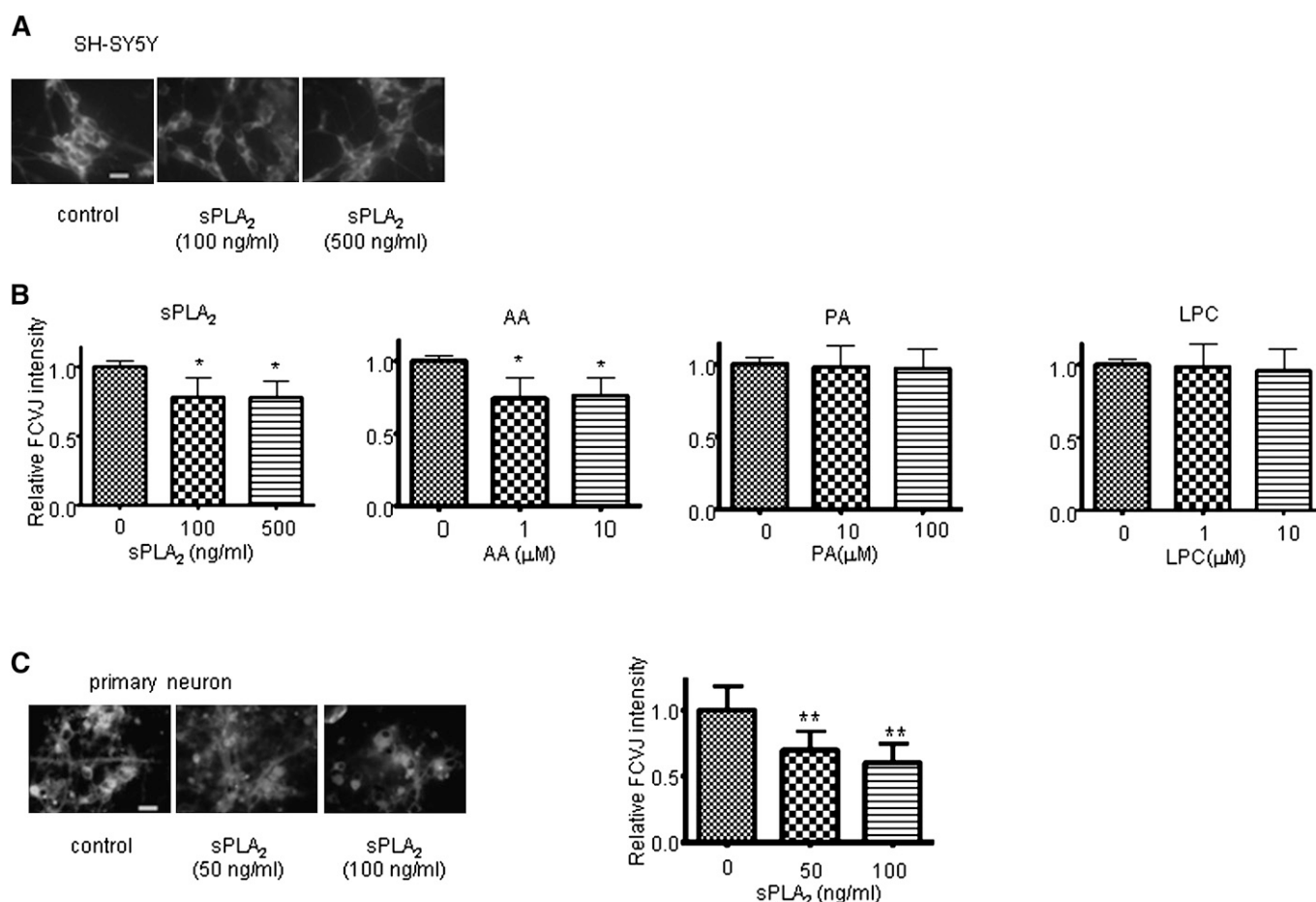


**Fig. 2.** sPLA<sub>2</sub>-III, AA, PA, and LPC on sAPP<sub>α</sub> secretion and total APP expression in neuronal cells. A: Western blot analysis of sAPP<sub>α</sub> shows that sPLA<sub>2</sub>-III and AA increased sAPP<sub>α</sub> secretion to medium from SH-SY5Y cells, but PA and LPC did not. PMA treatment known to increase sAPP<sub>α</sub> secretion in cells was used as a positive control. B: Western blot analysis of total APP shows that sPLA<sub>2</sub>-III, AA, LPC, and PA did not alter the total APP expressions in SH-SY5Y cells. C: sPLA<sub>2</sub>-III increased sAPP<sub>α</sub> secretion to medium from primary neurons. Data are expressed as percentages of control and mean  $\pm$  SD from at least three independent experiments (\*  $P < 0.05$ ; \*\* $P < 0.01$ ).

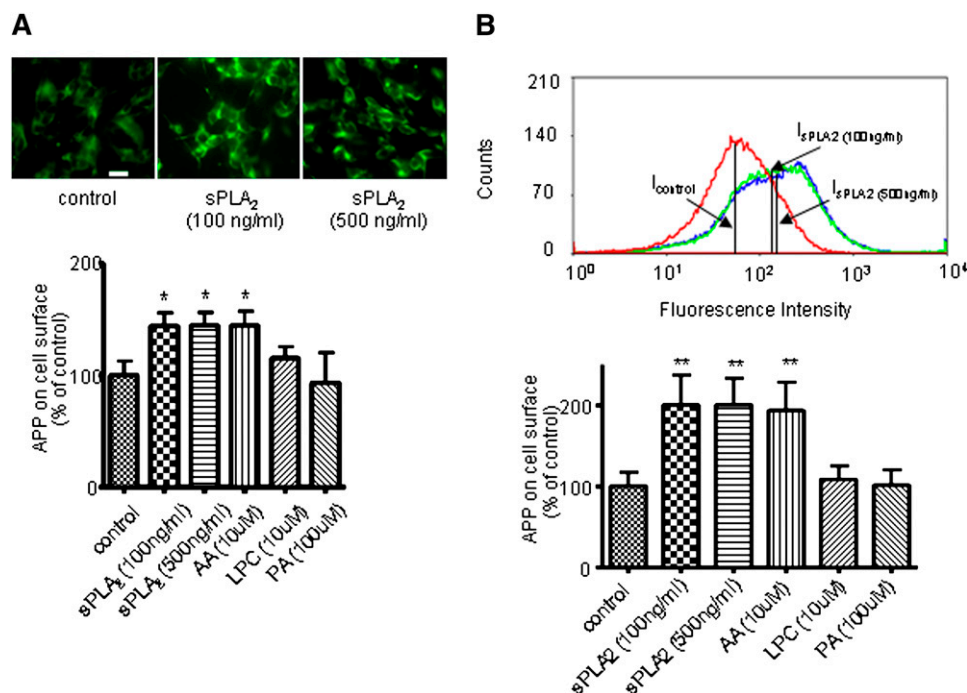
fluidity in neuronal cells, we exposed cells to ethanol, a compound known to increase membrane fluidity, and measured the fluorescent intensity of FCVJ integrated in cell membranes. Consistent with the notion that ethanol makes phospholipid bilayer membranes become more fluidized, ethanol caused a decrease in fluorescent intensity of FCVJ in SH-SY5Y cell and primary rat neuron membranes (data not shown). After treatment with sPLA<sub>2</sub>-III and AA, cells exhibited a lower fluorescent intensity of FCVJ compared with control (Fig. 3A, B), indicating that sPLA<sub>2</sub>-III and AA increased membrane fluidity in SH-SY5Y cells. These results are in agreement with the ability for sPLA<sub>2</sub>-III to increase membrane fluidity. However, PA and LPC were not capable of increasing membrane fluidity (Fig. 3B). Consistent with the results from SH-SY5Y cells, sPLA<sub>2</sub>-III was capable of increasing membrane fluidity in primary rat neurons. Together with the results for sAPP<sub>α</sub> secretion, these data suggest that sPLA<sub>2</sub>-III and AA increased sAPP<sub>α</sub> through their actions to increase membrane fluidity.

### sPLA<sub>2</sub>-III and AA increased APP at the cell surface of SH-SY5Y cells

There is strong evidence suggesting that the amyloidogenic pathway to generate Aβ occurs preferentially in the intracellular compartments, whereas the non-amyloidogenic pathway for production of sAPP<sub>α</sub> preferentially occurs at the plasma membranes (22, 36–42). Based on results of our studies, it is reasonable to hypothesize that sPLA<sub>2</sub>-III and AA alter APP metabolism, resulting in an increase of APP at the cell surface of SH-SY5Y cells. To test this hypothesis, we fluorescently labeled the extracellular domain of APP without invoking the procedure for membrane permeabilization. Immunofluorescence microscopy of APP at the cell surface showed that sPLA<sub>2</sub>-III enhanced the labeling of APP at the cell surface (Fig. 4A). Quantitative measurement of the fluorescent intensity indicated that both sPLA<sub>2</sub>-III and AA increased APP at the membrane surface by ~50%, whereas LPC and PA did not cause any significant changes compared with control (Fig. 4A). Consistent results were also obtained using the technique of fluorescence-activated cell sorting (Fig. 4B).



**Fig. 3.** Effects of sPLA<sub>2</sub>-III, AA, PA, and LPC on neuronal cell membrane fluidity. **A:** Representative images of SH-SY5Y cells fluorescently labeled with FCVJ. Bar = 20 μm. **B:** sPLA<sub>2</sub>-III and AA increased membrane fluidity in SH-SY5Y cells, as indicated by a decreased in the fluorescent intensity of FCVJ-labeled cells. Treatments with PA and LPC did not affect membrane fluidity of cells. **C, Left:** Representative images of primary neurons fluorescently labeled with FCVJ. Bar = 20 μm. **C, Right:** sPLA<sub>2</sub>-III increased membrane fluidity in primary neurons, as indicated by a decreased in the fluorescent intensity of FCVJ-labeled cells. Data are expressed as mean ± SD from at least three independent experiments (\* *P* < 0.05; \*\* *P* < 0.01).



**Fig. 4.** Effects of sPLA<sub>2</sub>-III, AA, PA, and LPC on APP accumulation at the cell surface of SH-SY5Y cells. Immunofluorescence microscopy of APP at the cell surface of SH-SY5Y cells was applied. Cells treated with sPLA<sub>2</sub>-III, AA, LPC, and PA for 24 h were labeled with anti-APP antibody and FITC-conjugated secondary antibody without the cell permeabilization procedure. Representative images of fluorescently labeled SH-SY5Y cells without treatment (A, top left) and with sPLA<sub>2</sub>-III treatment (A, top, middle, and right). Bar = 20 μm. sPLA<sub>2</sub>-III and AA increased the APP accumulation at the cell surface, but LPC and PA did not (A, bottom). Representative curves of fluorescence-activated cell sorting after sPLA<sub>2</sub>-III treatment (B, top). The median values of curves were used to perform data analysis. sPLA<sub>2</sub>-III and AA increased the APP accumulation at the cell surface, but LPC and PA did not (B, bottom). Data are expressed as percentages of control and mean ± SD from at least three independent experiments (\**P* < 0.05).

#### sPLA<sub>2</sub>-III, AA, PA, and LPC did not alter expressions of total α-secretases in SH-SY5Y cells

Incubation of sPLA<sub>2</sub>-III and its hydrolytic products for 24 h may change α-secretase expression for release of sAPP<sub>α</sub>. Western blot analysis showed that exposure of sPLA<sub>2</sub>-III and its hydrolytic products to SH-SY5Y cells for 24 h did not alter the expressions of different isoforms of α-secretases, including ADAM9, ADAM10, and ADAM17 (**Fig. 5**). These results show that APP (**Fig. 2B**) and α-secretases expressions were not factors in the increase in sAPP<sub>α</sub> secretion in SH-SY5Y cells induced by sPLA<sub>2</sub>-III and AA.

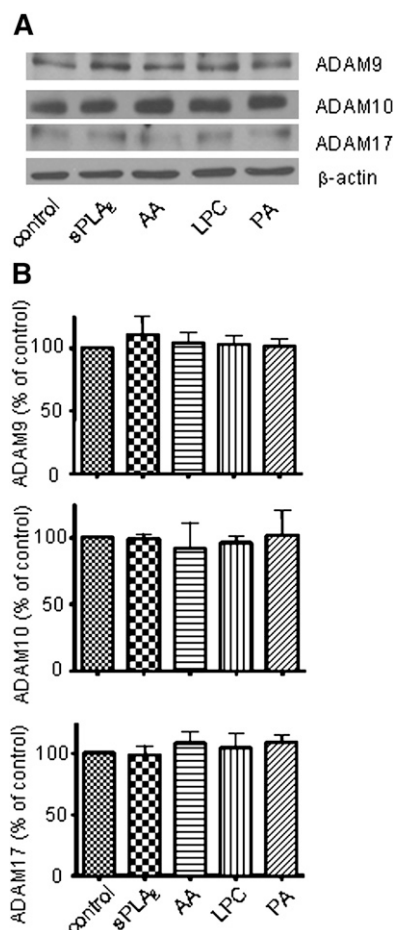
#### sPLA<sub>2</sub>-III decreased secretion of Aβ<sub>1-42</sub> in SH-SY5Y cells

Since sPLA<sub>2</sub>-III and AA increase the secretion of sAPP<sub>α</sub>, they may decrease the secretion of Aβ<sub>1-42</sub> in SH-SY5Y cells and primary rat neurons. **Figure 6** shows that ELISA measurement of Aβ<sub>1-42</sub> secreted from SH-SY5Y cells was decreased upon treatment of sPLA<sub>2</sub>-III for 24 h. Consistent results were obtained from primary rat neurons (data not shown). On the other hand, AA, LPC, and PA did not induce a significant change in Aβ<sub>1-42</sub> (data not shown). The decreased in secretion of Aβ<sub>1-42</sub> was not due to the change in BACE since sPLA<sub>2</sub>-III and its hydrolyzed products did not alter the expression of BACE1 in SH-SY5Y cells (**Fig. 7**).

#### DISCUSSION

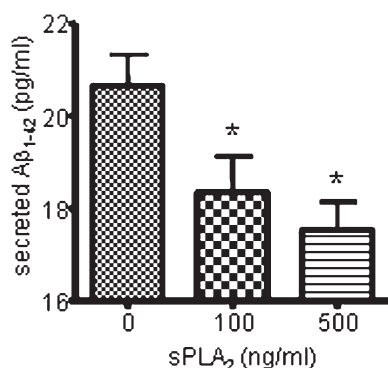
This study demonstrated for the first time the ability of exogenous sPLA<sub>2</sub>-III to cause the increase in sAPP<sub>α</sub> release from differentiated SH-SY5Y cells and primary neurons. This study further unveiled a special sensitivity of low concentration of AA in mediating the non-amyloidogenic pathway of APP processing and attributed its effect to an increase in membrane fluidity but not protein synthesis.

There is strong evidence that PLA<sub>2</sub>, including the group IV cPLA<sub>2</sub> and group IIA sPLA<sub>2</sub>, participate in the pathogenesis of AD. Previous studies demonstrated an increase in mRNA expression and immunoreactivity of cPLA<sub>2</sub> (43–45) and sPLA<sub>2</sub>-IIA (26) in AD brains. We have also reported that both cPLA<sub>2</sub> and calcium-independent PLA<sub>2</sub> are key enzymes mediating Aβ-induced mitochondrial dysfunction in primary rat astrocytes (46). The role of cPLA<sub>2</sub> in ameliorating cognitive deficits in an AD mouse model was recently demonstrated using cPLA<sub>2</sub> deficient mice cross with APP transgenic mice (47). Most recently, sPLA<sub>2</sub>-III has been reported to express in neuronal cells such as peripheral neuronal fibers, spinal dorsal root ganglia neurons, and cerebellar Purkinje cells, and the expression of sPLA<sub>2</sub>-III in these cells has been suggested to contribute to neuronal differentiation and neuronal outgrowth (27). Human sPLA<sub>2</sub>-III comprises unique N- and C-terminal domains and a central domain, the S domain. The mature

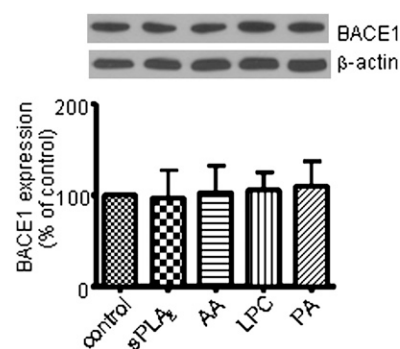


**Fig. 5.** Effects of sPLA<sub>2</sub>-III, AA, PA, and LPC on the expressions of  $\alpha$ -secretases in SH-SY5Y cells. Western blot analysis (A) of  $\alpha$ -secretases shows that sPLA<sub>2</sub>-III, AA, PA, and LPC did not alter the expressions of different isoforms of  $\alpha$ -secretases, including ADAM9 (B, top), ADAM10 (B, middle), and ADAM17 (B, bottom). Data are expressed as percentages of control and mean  $\pm$  SD from at least three independent experiments.

form of human sPLA<sub>2</sub>-III contains only the S domain, which is sufficient for enzymatic function (28, 48, 49). Bee venom sPLA<sub>2</sub>-III has high homology with the S domain of human sPLA<sub>2</sub>-III (28). In fact, neurotoxicity of sPLA<sub>2</sub>-III



**Fig. 6.** sPLA<sub>2</sub>-III decreases A $\beta_{1-42}$  release from SH-SY5Y cells. Release of A $\beta_{1-42}$  from SH-SY5Y cells was decreased with an increasing dose of sPLA<sub>2</sub>-III. Data are expressed as mean  $\pm$  SD from three independent experiments (\* $P$  < 0.05).



**Fig. 7.** Effects of sPLA<sub>2</sub>-III, AA, PA, and LPC on the expressions of BACE1 in SH-SY5Y cells. Western blot analysis shows that sPLA<sub>2</sub>-III, AA, PA, and LPC did not alter the expressions of BACE1 in SH-SY5Y cells. Data are expressed as percentages of control and mean  $\pm$  SD from at least three independent experiments.

has been attributed to its binding to N-type receptors (50). In earlier studies, sPLA<sub>2</sub>-III was shown to induce cell death in primary neuronal cultures through the ability for AA to modulate N-methyl D-aspartate receptors and/or calcium channels and subsequently potentiate glutamate-induced calcium influx (51–54).

APP processing to generate A $\beta$  is known to depend on cholesterol-enriched lipid rafts (12, 16). A model of membrane compartmentation has been suggested for APP present in two cellular pools, one associated with the cholesterol-enriched lipid rafts where A $\beta$  is generated and another outside of rafts (i.e., non-raft domains) where  $\alpha$ -cleavage occurs (16). Nevertheless, lowering cholesterol by treatment with statins, compounds that inhibit cholesterol synthesis pathway, was found to reduce (16, 20, 55) or enhance A $\beta$  generation, depending on the condition of the study (56). An epidemiological study indicated that lowering cholesterol is associated with reduced risk for AD (57, 58). One possible explanation for the controversial results is that moderate reduction in cholesterol is associated with a disorganization of detergent-resistant membranes or lipid rafts and allowing more BACE to contact APP, resulting in increased A $\beta$  generation, whereas a strong reduction of cholesterol inhibits the activities of BACE and  $\gamma$ -secretase, resulting in a decrease in A $\beta$  generation (12). Consistent with the membrane compartmentation model, treatment with either methyl- $\beta$ -cyclodextrin or lovastatin to reduce cellular cholesterol resulted in increase in membrane fluidity and an increase in nonamyloidogenic cleavage by  $\alpha$ -secretase to produce sAPP $\alpha$  (21). Interestingly, substitution of cholesterol by the steroid 4-cholesten-3-one induces minor change in membrane fluidity and reduces sAPP $\alpha$  secretion, whereas substitution of cholesterol by lanosterol increases membrane fluidity and sAPP $\alpha$  secretion (21). These results suggest reversible effects of cholesterol on the  $\alpha$ -secretase activity depending on membrane fluidity (21). These results also suggest that other pharmacological agents capable of altering membrane fluidity can modulate sAPP $\alpha$  secretion. In this study, we demonstrated that sPLA<sub>2</sub>-III and its hydrolyzed product AA increased sAPP $\alpha$  secretion and membrane fluidity in SH-SY5Y cells. Our data are consistent with those from



others that sPLA<sub>2</sub>-III increased fluidity of hepatic membranes (59) and that AA resulted in increased fluidity of membranes in cultured human umbilical vein, cerebral endothelial cells (60, 61), and hippocampal neurons in vivo (62). Another hydrolyzed product of PLA<sub>2</sub>, docosa-hexaenoic acid, has also been demonstrated to increase membrane fluidity and sAPP<sub>α</sub> secretion in HEK cells and in neuronal SH-SY5Y-overexpressing APP cells (63). In addition, it has been reported that nonspecific PLA<sub>2</sub> inhibitor partially suppressed muscarinic receptor-stimulated increase in sAPP<sub>α</sub> secretion in SH-SY5Y (64).

APP is a transmembrane protein and its internalization from the plasma membrane is regulated by key regulators of endocytosis, such as Rab5, and this process has been found to enhance APP cleavage by β-secretase leading to increased Aβ levels (65). Many studies support the notion that Aβ production occurs in endosomes (22, 38–42). APP lacking its cytoplasmic internalization motif can accumulate at the plasma membrane and undergo cleavage by α-secretase (36, 37). Alternatively, APP internalization can be reduced by lowering cholesterol, which leads to increase in membrane fluidity, APP accumulation at the cell surface, and increased sAPP<sub>α</sub> secretion (21). Increased sAPP<sub>α</sub> secretion by benzyl alcohol (C<sub>6</sub>H<sub>5</sub>OH) has also been shown to be associated with increased membrane fluidity, reduced CTF C99, and elevated CTF C83 levels, indicating enhanced α-secretase cleavage of APP, while decreased sAPP<sub>α</sub> secretion by Pluronic F68 is associated with decreased membrane fluidity, elevated CTF C99, and reduced CTF C83 levels, indicating enhanced β-secretase cleavage of APP (66). Similar to these studies, our studies show that sPLA<sub>2</sub>-III and AA increase in membrane fluidity, APP recruitment to the cell surface, and sAPP<sub>α</sub> secretion. Taken together, our data and those from Kojro et al. (21) and Peters et al. (66) support the notion that increasing membrane fluidity, in general, leads to increased APP recruitment to the cell surface and favoring process by α-secretase leading to sAPP<sub>α</sub> secretion (Table 1).

Numerous studies have been reported to provide evidence that sAPP<sub>α</sub> possesses both neurotrophic and neuroprotective effects. For example, sAPP<sub>α</sub> was shown to induce neurite outgrowth in cultured fibroblasts (67,

68), PC cells (69), human neuroblastoma cells (70), and cortical and hippocampal neurons (71–73). Neuroprotective effects of sAPP<sub>α</sub> have been shown to increase cortical synaptogenesis (74) and counteract oxidative impairment (75) and hypoglycemia-induced cytotoxicity (76). In addition to the neurotrophic and neuroprotective of sAPP<sub>α</sub>, there is evidence that α-secretase cleavage of APP competes and precludes the BACE cleavage, the primary step for production of neurotoxic Aβ (36, 37). We also found that ~3- to 4-fold enhanced sAPP<sub>α</sub> secretion in SH-SY5Y cells induced by sPLA<sub>2</sub>-III led to a decrease in Aβ<sub>1-42</sub> generation (Fig. 6). However, ~2-fold enhanced sAPP<sub>α</sub> secretion induced by AA did not lead to an observable decrease in Aβ production. In fact, other in vitro studies also showed that reduced secretion of sAPP<sub>α</sub> did not result in corresponding increase in Aβ production, and decreased Aβ production did not result in corresponding increase in secretion of sAPP<sub>α</sub> (77). Certainly, more systematic studies will be required to further understand this discrepancy.

Increasing production of sAPP<sub>α</sub> has been suggested as a potential therapeutic strategy for AD treatment. In this study, we provide evidence that sPLA<sub>2</sub>-III and its hydrolyzed product, AA, increase sAPP<sub>α</sub> secretion through their effects on membrane fluidity in SH-SY5Y cells. More studies are needed to examine if sAPP<sub>α</sub> plays a role in sPLA<sub>2</sub>-III-promoted neuronal outgrowth and differentiation. **FIG**

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TABLE 1. Summary of the effects of sPLA<sub>2</sub>-III, AA, PA, LPC, MβCD, benzyl alcohol (C<sub>6</sub>H<sub>5</sub>OH), and Pluronic F68 (PF68) on membrane fluidity, accumulation of APP at the cell surface, and secretion of sAPP<sub>α</sub> and Aβ

| Cell Treatment                   | Membrane Fluidity | APP at Cell Surface | Secretion of sAPP <sub>α</sub> | Aβ               |
|----------------------------------|-------------------|---------------------|--------------------------------|------------------|
| sPLA <sub>2</sub> -III           | ↑                 | ↑                   | ↑                              | ↓                |
| AA                               | ↑                 | ↑                   | ↑                              | NC               |
| LPC                              | NC                | NC                  | NC                             | NC               |
| PA                               | NC                | NC                  | NC                             | NC               |
| MβCD                             | ↑ <sup>a</sup>    | ↑ <sup>a</sup>      | ↑ <sup>a</sup>                 | ↓ <sup>a,b</sup> |
| C <sub>6</sub> H <sub>5</sub> OH | ↑ <sup>c</sup>    | NA                  | ↑ <sup>c</sup>                 | ↓ <sup>c</sup>   |
| PF68                             | ↓ <sup>c</sup>    | NA                  | ↓ <sup>c</sup>                 | ↑ <sup>c</sup>   |

↑ Denotes increase, ↓ denotes decrease, and NC denotes no change. MβCD, methyl-β-cyclodextrin.

<sup>a</sup> Results obtained from Kojro et al. (21).

<sup>b</sup> Aβ<sub>1-40</sub>.

<sup>c</sup> Results obtained from Peters et al. (66).



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