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Involvement of diacylglycerol produced by phospholipase D activation in Aβ-induced reduction of sAPPα secretion in SH-SY5Y neuroblastoma cells

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ABSTRACT

We previously reported that the thiol proteinase inhibitor, E-64-d, ameliorated amyloid β (Aβ)-induced reduction of soluble amyloid precursor protein α (sAPPα) secretion by reversing ceramide-induced protein kinase C down-regulation in SH-SY5Y neuroblastoma cells. In the present study, we showed that Aβ (1–42) peptide enhanced diacylglycerol (DAG) production by phospholipase D (PLD) activation in these cells. We subsequently examined whether PLD was involved in Aβ-induced reduction of sAPPα secretion and showed that 2 μM CAY10593, which selectively inhibits PLD2, ameliorated reduction of sAPPα secretion, whereas 50 nM CAY10593, which selectively inhibits PLD1, did not. Moreover, 50 μM propranolol, a phosphatidic acid phosphohydrolase inhibitor, also ameliorated Aβ-induced reduction of sAPPα secretion, suggesting that DAG may be responsible for Aβ-induced reduction of sAPPα. We subsequently examined whether DAG affects sAPPα secretion and showed that a DAG analog reduced sAPPα secretion in SH-SY5Y cells. In addition, DAG enhanced ceramide production by stimulating neutral sphingomyelinase (N-SMase) activity. We previously demonstrated that Aβ stimulates N-SMase activity in SH-SY5Y cells. Here, we showed that inhibition of PLD2 by 2 μM CAY10593 suppressed Aβ-induced N-SMase activation. Taken together, the results suggest that DAG produced through the PLD pathway is involved in Aβ-induced reduction of sAPPα secretion in SH-SY5Y cells.

1. Introduction

Alzheimer disease (AD) is a neurodegenerative disease characterized by amyloid β (Aβ) accumulation in the brain. The neuropathological features of AD include: senile plaques, neurofibrillary tangles, and lipid granule accumulation [1]. Elevation of soluble Aβ oligomers correlates with cognitive decline, which is consistent with the synaptotoxic properties [2,3]. Senile plaques comprise aggregated Aβ peptide. Aβ peptide is produced by sequential cleavage of amyloid precursor protein (APP) by β- and γ-secretases. In contrast, soluble amyloid precursor protein α (sAPPα), which is the product of α-secretase cleavage of APP, decreases Aβ production by directly decreasing APP association with β-secretase [4].

Oliveira et al. [5] recently reported that Aβ peptide (1–42) enhanced phospholipase D (PLD) activity in cultured neurons, and PLD2 is required for the synaptotoxic action of Aβ peptide. In addition, *pld2* ablation rescues memory deficits and confers synaptic protection in SwAPP, a transgenic AD mouse model, despite a significant Aβ load. The authors suggested that phosphatidic acid (PA) produced from phosphatidylcholine (PC) by the PLD pathway may be associated with AD pathogenesis [6].

We recently demonstrated that a thiol proteinase inhibitor, E-64-d, ameliorated Aβ-induced reduction of sAPPα secretion in carbachol-stimulated SH-SY5Y neuroblastoma cells [7]. In addition, E-64-d reverses ceramide-induced protein kinase C (PKC) down-regulation in these cells [7]. Sphingomyelin (SM) is hydrolyzed by both neutral sphingomyelinase (N-SMase) and acidic sphingomyelinase (A-SMase). SM is converted to ceramide, which is recognized as an important second messenger in cellular signal transduction [8]. Sphingolipid pathways are altered in AD [9]. We previously reported that Aβ peptide (1–40) activates N-SMase

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which induces ceramide production [7]. Diacylglycerol (DAG) is an A-SMase activator [10], and can be produced by phosphatidyl-inositol 4,5 bisphosphate (PIP_2)-specific phospholipase C (PLC), PC-specific PLC, and PLD enzymes [11]. PLD produces choline and PA, which are then converted to DAG by PA phosphohydrolase [11]. PLD was recently described as a critical factor for vesicle trafficking events, such as exocytosis in neurons [12] and endocrine cells [13]. In mammalian cells, at least two isoforms of PLD exist, PLD1 and PLD2. These isoforms differ in localization and their mechanisms of regulation [14].

In the present study, we demonstrate that a selective PLD2 inhibitor ameliorated Aβ-induced reduction of sAPPα secretion in SH-SY5Y cells. The PLD2 inhibitor prevented Aβ-induced N-SMase activation. Moreover, propranolol, which inhibits PA phosphohydrolase, reversed Aβ-induced reduction of sAPPα secretion. In addition, we present here that DAG analog reduced sAPPα secretion in SH-SY5Y cells. These results suggest that a DAG produced through the PLD pathway has an important role in the α-processing of APP in AD.

2. Materials and methods

2.1. Reagents

Aβ peptide (1–42), carbachol, and silica gel plates were purchased from Merck (Darmstadt, Germany). Aβ peptide was dissolved in dimethylsulfoxide, further diluted with Dulbecco's Minimum Essential Medium (DMEM), and incubated overnight at 37 °C before use. Retinoic acid, propranolol, and 1-oleoyl-2-acetyl-sn-glycerol (OAG) were obtained from Sigma Chemicals Co. (St. Louis, MO, USA). Anti-APPα antibody (6E10) was obtained from Covance Co. (Berkeley, CA, USA). Anti-mouse IgG AP-linked antibody was from Cell Signaling (Beverly, MA, USA). Fetal bovine serum (FBS) was obtained from Invitrogen (Carlsbad, CA, USA). [H^3] palmitic acid and [N-methyl-^{14}C] SM were purchased from Moravek Biochemicals (Brea, CA, USA). CAY10593, N-[2-[4-(5-chloro-2,3-dihydro-2-oxo-1H-benzimidazol-1-yl)-1-piperidinyl]-1-methylethyl]-2-naphthalenecarboxamide), was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). CAY10593 was dissolved in dimethylsulfoxide and further diluted with DMEM.

2.2. Cells and treatment protocols

SH-SY5Y neuroblastoma cells were obtained from the American Type Culture Collection (Manassas, VA, USA). SH-SY5Y cells were previously used for APP processing and in an AD study [15]. Cells were maintained in DMEM Ham's F-12 (Wako, Osaka, Japan) supplemented with 10% FBS, 1% penicillin and 1% streptomycin.

Cells (4×10^5) were seeded into six-well culture dishes (Becton Dickinson Labware, Franklin Lakes, NJ, USA) and cultured in DMEM supplemented with 10% FBS and antibiotics. On the next day, 10 μM retinoic acid was added and further cultured for 5 days to promote differentiation [16]. Cells were then cultured with DMEM supplemented with 1% FBS and 1 μM retinoic acid. On the next day, the cells were washed with medium and incubated with CAY10593 or propranolol for 30 min. After the cells were incubated with Aβ for 5.5 h, carbachol (1 mM), which stimulates muscarinic acetylcholine receptor-mediated signal transduction [17], was added and further incubated for 2 h.

2.3. Measurement of sAPPα production

sAPPα production was monitored by measuring secretion of the soluble products of APP cleaved by α-secretase. After the cells were treated with reagents, culture medium was harvested and concentrated by centrifugation using Viva Spin 2 (Sartorius, Gottingen, Germany). Concentrated supernatants (30 μg protein) were suspended in 20 μL of sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 0.1% bromphenol blue, and 5% mercaptoethanol), heated at 100 °C for 5 min, electrophoresed on 7.5% SDS/PAGE gels, and transferred to PVDF membranes (Millipore, Bedford, MA, USA) (200 mA, 1 h). The membranes were blocked overnight with 5% skim milk in 10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, and 0.1% Tween 20. The blocked membranes were incubated overnight at 4 °C with 1:1000 diluted 6E10 monoclonal antibody in blocking buffer. When 6E10 antibody was used,

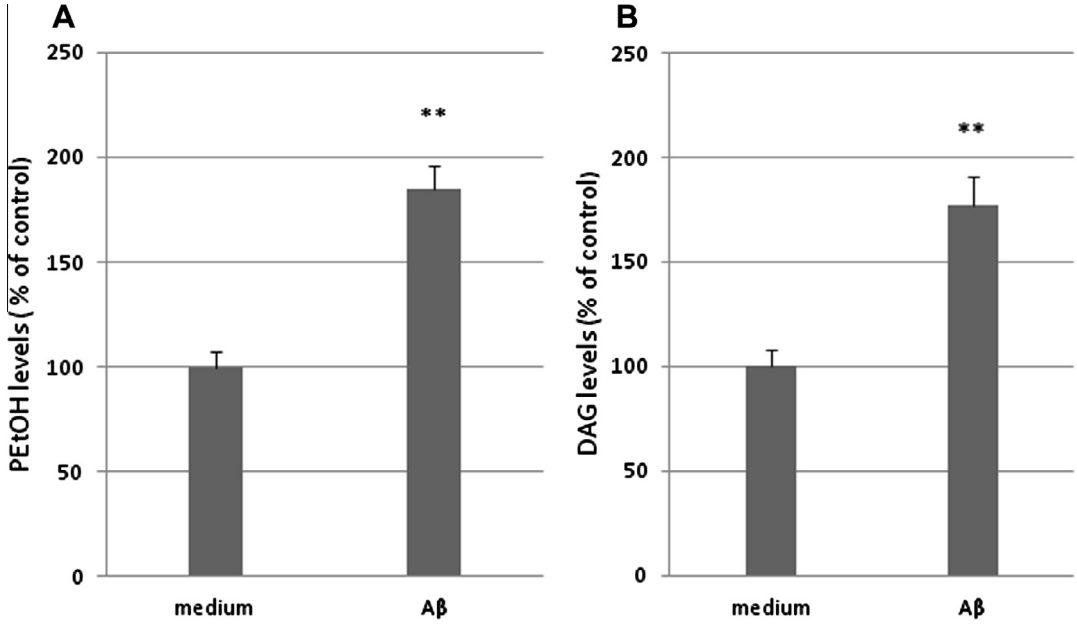

Fig. 1. Effect of Aβ on PLD activity in SH-SY5Y cells. Cells were labeled with [^{3}H] palmitic acid (1 μCi/ml) for 24 h at 37 °C. After the cells were washed with medium, they were incubated with 0.01 μM Aβ for 2.5 h, and PLD activity was examined. When PEtOH (A) was measured, the cells were incubated with Aβ in the presence of 0.5% ethanol. When DAG (B) was measured, the cells were incubated in the absence of ethanol. Lipids were separated by TLC and radioactive counts were determined as described in Materials and Methods. Values are presented as percentage changes (mean ± SE, $n = 5$) relative to the values for the cells with medium. $^{**}p < 0.01$.

sAPPα corresponded to 130 kDa and 120 kDa [7]. After washing, the membranes were incubated for 2 h at room temperature with anti-mouse IgG AP-linked secondary antibody (1:5000). The signals were detected by using CDP-Star Reagent (BioLabs, Ipswich, MA, USA) and analyzed using LAS-4000 (Fuji Film, Kanagawa, Japan) and Image Gause software ver. 3.0.

2.4. Assay for PLD activity

PLD activity was assayed according to the method of Schutze et al. [11]. In brief, cells cultured in the presence of 10 μM retinoic acid for 5 days in six-well culture dishes, as described for the measurement of sAPPα production, were labeled with [^{3}H] palmitic acid (1 μCi/mL) in DMEM supplemented with 1% FBS and 1 μM retinoic acid for 24 h. After the cells were washed with medium, Aβ peptide was added and incubated for 2.5 h at 37 °C. After the cells were washed with phosphate-buffered saline (PBS), lipids were extracted in the manner described by Bligh and Dyer [18]. When phosphatidylethanol (PEtOH) was examined, the cells were incubated with Aβ in the presence of 0.5% ethanol, which led to PEtOH production through a PLD-specific transphosphatidylation reaction. When DAG was measured, the cells were incubated with Aβ in the absence of ethanol. Phospholipids were separated by thin layer chromatography (TLC) in a solvent system containing chloroform:methanol:acetic acid:water (100:60:20:5). PEtOH was separated from PA and other phospholipids by TLC with a solvent system of ethyl acetate:acetic acid:water (110:20:110). DAG was separated by TLC with a solvent system of benzene:ethyl acetate (6.5:3.5). The individual lipids, identified by co-migration with commercial standards, were visualized in iodine vapor, and the silica gel was scraped to quantify radioactivity.

2.5. Assays for SMase activity

The micellar SMase assay using exogenous radiolabeled SM was performed by the method of Wiegmann et al. [19]. To measure N-SMase, cells in six-well culture dishes were treated with 100 ng/mL or 1000 ng/mL of OAG for 1.5 h. After washing with PBS, the cells were suspended in a buffer containing 20 mM HEPES(pH7.4), 10 mM $MgCl_2$, 2 mM EDTA, 5 mM DTT, 0.1 mM Na_3VO_4, 0.1 mM Na_2MoO_4, 30 mM p-nitrophenylphosphate, 10 mM β-glycerophosphate, 750 μM ATP, 1 mM phenylmethylsulfonylfluoride, 10 μM leupeptin, 10 μM pepstatin, and 0.2% TritonX-100. After incubation for 5 min at 4 °C, the cells were homogenized by repeated squeezing of cells through an 18-gauge needle. Nuclei and cell debrils were removed by low-speed centrifugation (800*g*). Protein (30 μg) was incubated for 2 h at 37 °C in a buffer (50 μL) containing 20 mM HEPES, 1 mM $MgCl_2$ (pH 7.4), and [N-methyl-^{14}C] SM

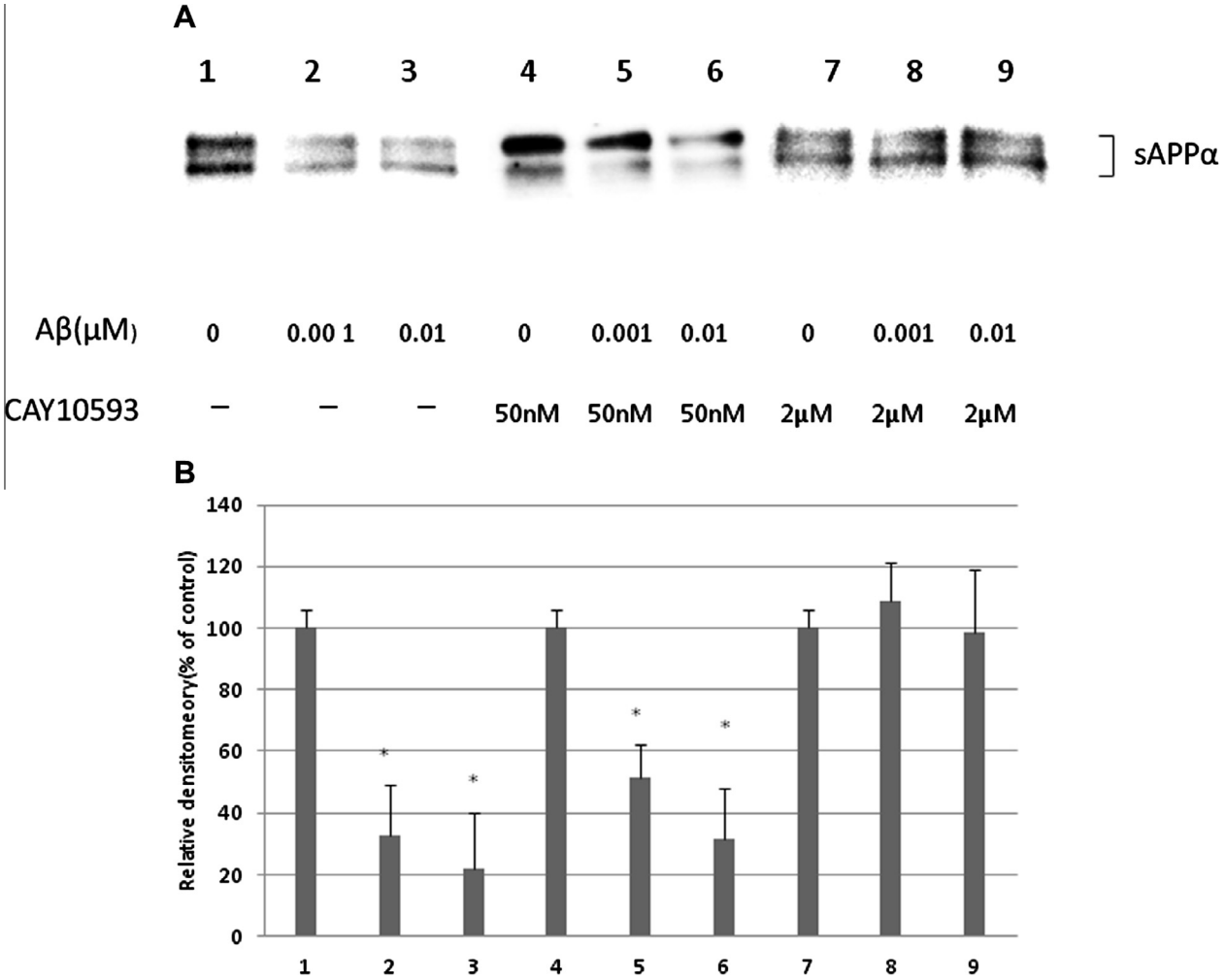

Fig. 2. Effect of CAY10593 on Aβ-induced reduction of sAPPα secretion in SH-SY5Y cells. (A) SH-SY5Y cells were pretreated with 50 nM or 2 μM CAY10593 for 30 min and incubated with Aβ (0.001 or 0.01 μM) for 5.5 h, followed by incubation with carbachol (1 mM) for 2 h. Secreted sAPPα was analyzed by Western blotting with the 6E10 antibody. Representative data are shown. (B) Quantitative densitometric analysis of the immunoreactive protein bands in (A). Values are shown as percentage changes (mean ± SE, $n = 5$) relative to the values for the cells without Aβ. $^*p < 0.05$.

(0.2 μCi/mL). The reaction was linear within this time frame, and the amount of [N-methyl-^{14}C] SM hydrolyzed did not exceed 10% of the total amount of radioactive SM added. Phosphorylcholine was then extracted with 800 μL of chloroform:methanol (2:1) and 250 μL of H_2O. Radioactive phosphorylcholine produced from [N-methyl-^{14}C] SM was identified by TLC and routinely determined in the aqueous phase by scintillation counting. Increments of radioactive phosphorylcholine were confirmed by TLC. To measure A-SMase, the cell pellet was resuspended in 200 μL of 0.2% Triton X-100 and incubated for 15 min at 4 °C. Cells were then homogenized and spun in a microfuge at 14,000 rpm. From the supernatant, protein (30 μg) was incubated for 2 h at 37 °C in a buffer (50 μL) containing 250 mM sodium acetate, 1 mM EDTA (pH 5.0), and [N-methyl-^{14}C] SM. The amount of radioactive phosphorylcholine produced was measured, as described for the N-SMase assay.

2.6. Assay for cellular ceramide and SM levels

Cellular ceramide and SM levels were examined according to the method previously described [7,20,21]. In brief, SH-SY5Y cells were labeled with [H^3] palmitic acid (1 μCi/mL) for 24 h at 37 °C, as described for the PLD assay. After the cells were washed with medium, they were incubated with 100 ng/mL or 1000 ng/mL of OAG for 1.5 h. After the cells were washed with PBS, the cells were precipitated in cold methanol (1 mL) containing 20 μg/mL each of SM and ceramide. Lipids were extracted [18] and separated by TLC in a solvent system containing chloroform:methanol:acetic acid:water (100:60:20:5) for examining SM, and chloroform: methanol:7 N ammonium hydroxide:water (170:30:1:1) for examining ceramide [10]. Sphingolipids were located by iodine vapor and the silica gel was scraped to quantify radioactivity.

2.7. Statistical analyses

Student's *t*-test was used to perform statistical analyses.

3. Results

3.1. Aβ enhanced DAG production by activation of PLD in SH-SY5Y cells

Oliveira et al. [5] reported that Aβ peptide (1–42) enhanced PLD activity in a pheochromocytoma PC12 cell line. We first examined whether Aβ peptide (1–42) could alter PLD activity in SH-SY5Y neuroblastoma cells. In the presence of primary alcohols, PLD catalyzes transphosphatidylation reactions, which results in the transfer of phospholipid phosphatidyl moieties to primary alcohols and phospatidylalcohol production [11]. Thus, we determined PLD activity by measuring PEtOH levels in the presence of 0.5% ethanol. When DAG was measured, cells were incubated with Aβ peptide in the absence of ethanol. As shown in Fig. 1 A, PEtOH production in cells treated with 0.01 μM Aβ in the presence of 0.5% ethanol for 2.5 h significantly increased related to that of control cells. DAG production also increased when cells were treated with 0.01 μM Aβ for 2.5 h (Fig. 1 B). These results indicated that Aβ enhanced PLD activity in SH-SY5Y cells.

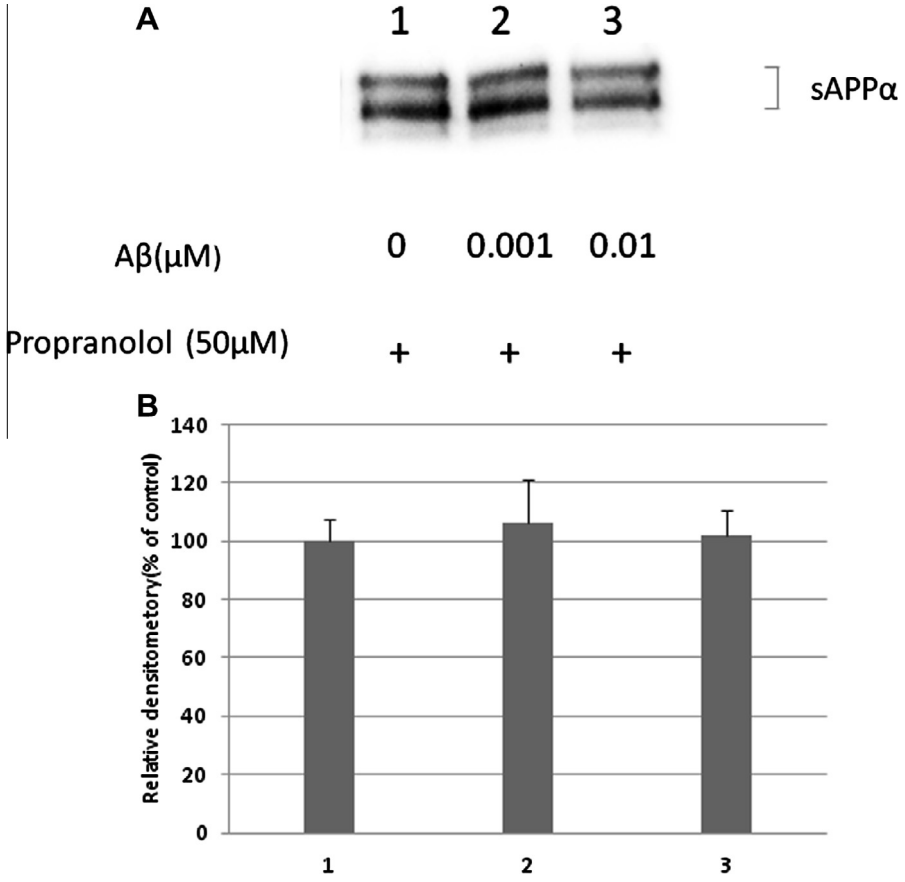

Fig. 3. Effect of propranolol on Aβ-induced reduction of sAPPα secretion in SH-SY5Y cells. (A) SH-SY5Y cells were pretreated with 50 μM propranolol for 30 min and incubated with Aβ (0.001 or 0.01 μM) for 5.5 h, followed by incubation with carbachol (1 mM) for 2 h. Secreted sAPPα was analyzed by Western blotting with the 6E10 antibody. Representative data are shown. (B) Quantitative densitometric analysis of the immunoreactive protein bands in (A). Values are shown as percentage changes (mean ± SE, $n = 5$) relative to the values for the cells without Aβ. $^*p < 0.05$.

3.2. A selective PLD2 inhibitor ameliorated Aβ-induced reduction of sAPPα secretion

We subsequently examined whether Aβ-induced activation of PLD could affect α-processing of APP in SH-SY5Y cells. We used a selective PLD inhibitor, CAY10593, N-[2-[4-(5-chloro-2,3-dihydro-2-oxo-1H-benzimidazol-1-yl)-1-piperidinyl]-1-methyl-ethyl]-2-naphthalenecarboxamide, in these experiments. The IC_{50} of CAY10593 for PLD1 was 46 nM *in vitro* and 11 nM in cells, and the IC_{50} for PLD2 was 933 nM *in vitro* and 1.8 μM in cells [22]. As shown in Fig. 2 (lane 1–3), both 0.001 μM and 0.01 μM Aβ significantly suppressed sAPPα secretion. Treatment of cells with 2 μM CAY10593 ameliorated Aβ-induced reduction of sAPPα secretion (Fig. 2, lane 7–9), whereas 50 nM CAY10593 did not (Fig. 2, lane 4–6). CAY10593 alone did not alter either sAPPα secretion or cell viability in these experimental conditions. These results suggest that PLD2 is associated with the Aβ-induced reduction of sAPPα secretion.

PLD is known to produce PA, which is converted to DAG through PA phosphohydrolase. We subsequently examined whether DAG was associated with α-processing of APP. Fig. 3 shows that 50 μM propranolol, which inhibits PA phosphohydrolase [23], ameliorated Aβ-induced reduction of sAPPα production. Propranolol at this concentration did not alter cell viability. These results suggest that DAG has an important role in α-processing of APP in SH-SY5Y cells.

3.3. DAG reduced sAPPα secretion by enhancing ceramide production

To determine whether DAG was associated with α-processing of APP, we examined the effect of OAG, a DAG analog, on sAPPα secretion in SH-SY5Y cells.

When SH-SY5Y cells were treated with 100 ng/mL or 1000 ng/mL OAG for 1.5 h, sAPPα production significantly decreased (Fig. 4). Because we previously reported that Aβ enhanced ceramide production [7], we examined whether OAG could promote ceramide production in SH-SY5Y cells. As shown in Table 1, treatment of cells with 100 ng/mL or 1000 ng/mL OAG for 1.5 h significantly enhanced ceramide production, whereas SM levels were reduced. These results suggest that OAG stimulated SM hydrolysis. We subsequently examined the effect of OAG on N-SMase and A-SMase activities. As shown in (Table 1)and 100 ng/mL or 1000 ng/mL OAG significantly stimulated N-SMase activity, whereas A-SMase activity was not altered in SH-SY5Y cells.

3.4. A PLD2 inhibitor abolished Aβ-induced activation of N-SMase

We previously demonstrated that Aβ peptide enhanced ceramide production by activating N-SMase activity [7]. We therefore examined whether a selective PLD inhibitor could affect Aβ-induced N-SMase activation in SH-SY5Y cells. As shown in Table 2, activation of N-SMase activity by 0.01 μM Aβ peptide was suppressed by treatment of cells with 2 μM CAY10593, whereas

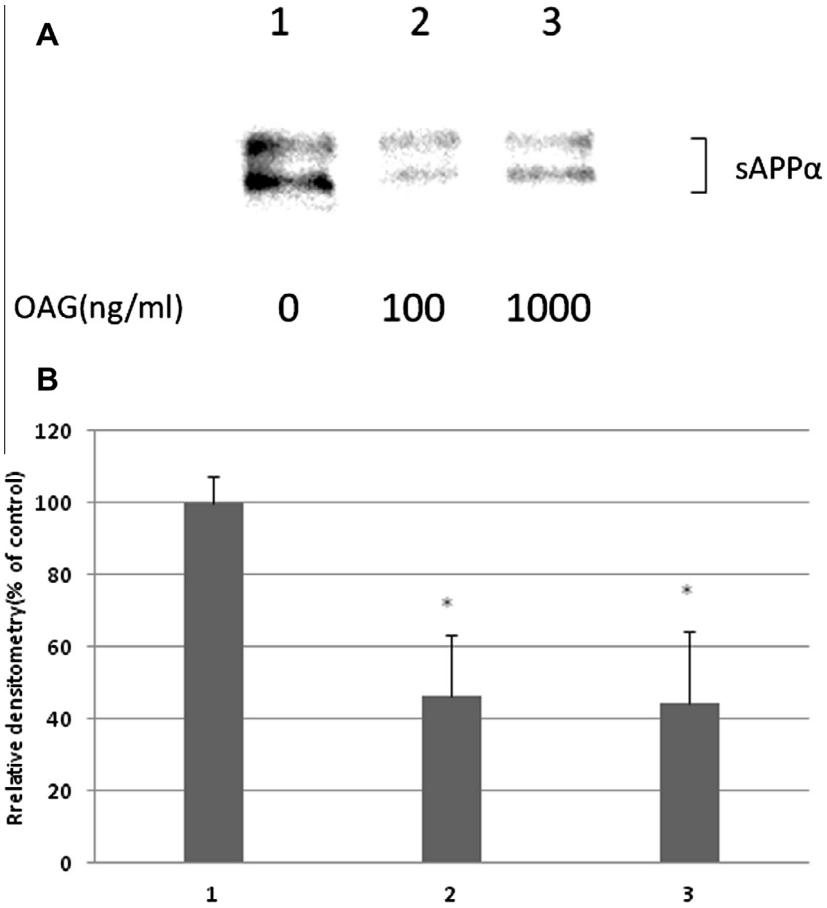

Fig. 4. Effect of a DAG analog on sAPPα secretion in SH-SY5Y cells. (A) SH-SY5Y cells were incubated with 100 ng/mL or 1000 ng/mL OAG for 5.5 h, followed by incubation with carbachol (1 mM) for 2 h. Secreted sAPPα was analyzed by Western blotting with the 6E10 antibody. Representative data are shown. (B) Quantitative densitometric analysis of the immunoreactive protein bands in (A). Values are shown as percentage changes (mean ± SE, $n = 5$) relative to the values for the cells without OAG. $^{*}p < 0.05$.

Table 1
Effect of a DAG analog on sphingolipid levels and SMase activities in SH-SY5Y cells.

Treatment	Ceramide	SM	N-SMase	A-SMase
Medium	100 ± 10.4	100 ± 3.9	100 ± 8.4	100 ± 3.8
OAG (100 ng/mL)	147.0 ± 15.9*	75.0 ± 9.0*	139.4 ± 14.6*	95.7 ± 12.9
OAG (1000 ng/mL)	159.1 ± 9.8*	59.5 ± 15.3*	150.2 ± 17.5*	102.2 ± 9.8

After cells were treated with OAG or medium for 1.5 h at 37 °C, sphingolipids (% of control) and SMase activities (% of control) were determined as described in Materials and Methods. The data are means ± SE of five experiments.
* $p < 0.05$, significant compared with medium.

Table 2
Effect of CAY10593 on SMase activities in SH-SY5Y cells.

Pretreatment	Treatment	N-SMase	A-SMase
Medium	Medium	100 ± 8.8	100 ± 4.2
Medium	Aβ (0.01 μM)	169.2 ± 14.3*	102.2 ± 7.6
CAY10593 (50 nM)	Aβ (0.01 μM)	184.1 ± 15.6*	96.6 ± 10.2
CAY10593 (2 μM)	Aβ (0.01 μM)	108.6 ± 12.6	98.3 ± 8.8

After cells were treated with CAY10593 or medium for 30 min at 37 °C, N-SMase and A-SMase activities (% of control) were determined as described in Materials and Methods. The data are means ± SE of five experiments.
* $p < 0.05$, significant compared with medium.

50 nM CAY10593 had no effect. In contrast, neither Aβ nor CAY10593 affected A-SMase activity (Table 2). These results suggest that PLD2 is involved in Aβ-induced activation of N-SMase.

4. Discussion

Lipids are important regulators of brain function and are implicated in neurodegenerative disorders including AD [24]. Several reports have suggested that phospholipid imbalance mediates key pathological process associated with AD [6]. Nitsch et al. [25] reported that AD patients had decreased levels of PC and phosphatidylethanolamine, suggesting that AD may be associated with increased membrane phospholipid degradation. Aβ primarily exerts its cytotoxic effects by perturbing cellular membranes through modulation of phospholipase activities.

PLD probably has a central role in membrane trafficking, cytoskeleton regulation, and signal transduction [26]. PLD hydrolyzes PC to PA, which is converted to DAG by PA phosphohydrolase [11]. PA also has important roles in membrane dynamics and signaling processes [27]. In our experiments, propranolol, which inhibits PA phosphohydrolase, reversed Aβ-induced reduction of sAPPα production. In addition, a DAG analog reduced sAPPα production and enhanced ceramide production by activating N-SMase in SH-SY5Y cells.

PLD1 and PLD2 are differentially localized in cells [14]. In a transgenic mouse model of AD, PLD2 had an important role in the synaptotoxic action of Aβ peptide which is related to AD pathogenesis [5]. We showed that CAY10593 ameliorated Aβ-induced reduction of sAPPα secretion at a concentration that inhibits PLD2 activity. Because major action sites of Aβ are cell surface, PLD2, which localizes at the plasma membrane, may be associated with Aβ action.

Ceramide is recognized as an important second messenger in intracellular signal transduction [8]. We reported that Aβ peptide enhanced ceramide production by activating N-SMase in SH-SY5Y cells [7]. In the present study, Aβ peptide (1–42), which is more cytotoxic than Aβ peptide (1–40) [5], stimulated PLD activity and enhanced DAG production in SH-SY5Y cells. In addition, we showed that DAG stimulates N-SMase activity and increased ceramide production in these cells, although the precise mechanism by which DAG activates N-SMase but not A-SMase remains unclear.

PKC is a Ca^{2+}-dependent serine–threonine protein kinase. PKC has pivotal roles in signal transduction that controls APP metabolism. PKC activators, such as phorbol myristate acetate, induce α-secretase-mediated APP cleavage [28]. PKC is proteolytically cleaved by calpain, a Ca^{2+}-dependent thiol proteinase [29], and calpain is involved in α-secretase processing of APP [30]. We reported that a potent calpain inhibitor, E-64-d, ameliorated Aβ-induced reduction of sAPPα secretion [7]. In addition, because we previously demonstrated that ceramide promoted calpain-mediated proteolysis of PKC-β [31], it is likely that increases in ceramide levels by Aβ peptide are linked to AD pathogenesis.

To the best of our knowledge, this is the first study to demonstrate that DAG produced through the PLD pathway was involved in Aβ-induced reduction of sAPPα secretion. Because PLD inhibitors prevent ceramide production, which induces PKC down-regulation, they may be effective for treatment of AD.

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