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The thiol proteinase inhibitor E-64-d ameliorates amyloid-β-induced reduction of sAPPα secretion by reversing ceramide-induced protein kinase C down-regulation in SH-SY5Y neuroblastoma cells

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ABSTRACT

In Alzheimer's disease (AD), enhancing α-secretase processing of amyloid precursor protein (APP) is an important pathway to decrease neurotoxic amyloid β (Aβ) secretion. The α-secretase is reported to be regulated by protein kinase C (PKC) and various endogenous proteins or cell surface receptors. In this report, we first examined whether Aβ reduces α-secretase activity, and showed that Aβ peptide 1–40 (0.001 and 0.01 μM) reduced the secretion of soluble amyloid precursor protein α (sAPPα) in carbachol-stimulated SH-SY5Y neuroblastoma cells. E-64-d (3 μM), which is a potent calpain inhibitor that prevents PKC degradation, ameliorated the Aβ-induced reduction of sAPPα secretion. In addition, we observed that Aβ significantly enhanced ceramide production by activating neutral sphingomyelinase. The cell-permeable ceramide analog, C_2-ceramide (1 μg/mL), also reduced sAPPα secretion, and in addition, E-64-d eliminated the observed decrease of sAPPα secretion. C_2-ceramide induced down-regulation of PKC-α, -β_1, and -β_2 isozymes in SH-SY5Y cells. These findings suggest that ceramide may play an important role in sAPPα processing by modulating PKC activity.

1. Introduction

Alzheimer's disease (AD) is characterized by the accumulation of aggregated amyloid-β (Aβ). It is well known that the release of Aβ peptide from the amyloid precursor protein (APP) requires cleavage by β- and γ-secretases. In contrast, soluble APPα (sAPPα), which is the product of APP by α-secretase cleavage, has neuroprotective effect [1]. Because both α- and β- secretases compete for the same substrate (APP), sAPPα generation was reported to decrease Aβ production by directly decreasing APP association with β- secretase [2].

It has been reported that expression of protein kinase C (PKC), which is a Ca^{2+}-dependent serine-threonine protein kinase, was attenuated in an AD brain [3]. Bryostatin significantly activates PKC in AD mouse brain and increases and decreases sAPPα and Aβ, respectively [4]. PKC comprises a family of several isozymes, and Nelson et al. [5] reported that activation of PKC-ε reduced Aβ levels. Favit et al. [6] showed that nanomolar concentration of Aβ (1–40) peptide degrades PKC-α and -γ in normal fibroblasts, and that a selective proteasome inhibitor prevented Aβ-mediated PKC degradation. However, the precise mechanism by which Aβ affects PKC remains unclear.

PKC is known to be proteolytically cleaved by calpain, which is a Ca^{2+}-dependent thiol proteinase [7]. There are several reports suggesting that calpain is involved in the α-secretase processing of APP [8–10], however, the mechanism for this has not been completely elucidated. We previously reported that a potent calpain inhibitor, E-64-d, prevents the abnormal proteolysis of PKC in Chediak–Higashi syndrome, a primary immunodeficiency syndrome characterized by oculocutaneous albinism, deficient bactericidal activity of neutrophils, and deficient natural killer cell activity [11]. We presented that in this disorder, abnormally increased ceramide led to calpain-mediated degradation of PKC [12,13]. In addition, we demonstrated that ceramide promotes calpain-mediated proteolysis of PKC-β in polymorphonuclear leukocytes [14]. Ceramide has recently been reported to be involved in APP processing [15].

In the present study, we report that E-64-d when administered that inhibits calpain, restored the Aβ-induced decrease of sAPPα in SH-SY5Y neuroblastoma cells. In addition, we demonstrate that Aβ enhances ceramide production, and ceramide induces calpain-mediated degradation of PKC-α, -β_1, and -β_2. Our findings suggest

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that calpain inhibitors may be effective in enhancing the secretion of neuroprotective sAPPα.

2. Materials and methods

2.1. Reagents

E-64-d was obtained from Peptide Institute Inc. (Osaka, Japan). E-64-d was dissolved in dimethylsulfoxide (DMSO) and diluted with phosphate-buffered saline. Carbachol and Aβ peptide (1–40) were purchased from Merck (Darmstadt, Germany). Aβ was dissolved in DMSO, further diluted with Dulbecco's Minimum Essential Medium (DMEM), and incubated overnight at 37 °C before use. Retinoic acid and C_2-ceramide (N-acetylsphingosine) were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Anti-APPα antibody (6E10) was obtained from Covance Co. (Berkeley, CA, USA). Anti-PKC isozymes antibodies were all purchased from Santa Cruz Co. (Santa Cruz, CA, USA) and anti-mouse IgG AP-linked antibody was from Cell Signaling (Beverly, MA, USA). Fetal bovine serum (FBS) was from Invitrogen (Carshad, CA, USA). [H^3] palmitic acid and [N-methyl-^{14}C] sphingomyelin (SM) were purchased from Moravek Biochemicals Inc. (Brea, CA, USA) and American Radiolabeled Chemicals Inc. (St. Louis, MO, USA), respectively.

2.2. Cells and treatment protocols

SH-SY5Y neuroblastoma cells were obtained from American Type Culture Collection (Manassas, VA, USA). SH-SY5Y cells have been used for APP processing and AD study [16]. 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 (Falcon) and cultured in DMEM supplemented with 10% FBS and antibiotics. On the next day, 10 μM of retinoic acid was added and the cells were cultured for 5 days. To promote differentiation, cells were subsequently cultured with DMEM supplemented with 1% FBS and 1 μM of retinoic acid [17]. The following day, confluent cells were washed with medium and incubated with E-64-d for 30 min. Furthermore, the cells were incubated with Aβ or C_2-ceramide in the same medium for 5.5 h, and carbachol (1 mM) that stimulates muscarinic acetylcholine receptor-mediated signal transduction [18] was added before further incubation for 2 h.

2.3. Measurement of sAPPα production and PKC isozyme analysis

sAPPα production was monitored by measuring the secretion of the soluble products of APP cleaved by α-secretase. After the cells were treated with reagents, culture media were harvested, and concentrated by centrifugation using Viva Spin 2 (Sartorius, Gottingen, Germany). At the same time, cells in plates were lysed using Cell Culture Lysis 5 × Reagent (Promega, Madison, WI, USA) according to the manufacturer's protocol. Halt Protease Inhibitor Set (Thermo Scientific, Rockford, IL, USA) was also added and used for the detection of PKC isozymes.

Concentrated supernatants (30 μg protein) were resuspended in 20 μl of sample buffer [62.5 mM Tris–HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 10% glycerol, 0.1% bromphenol blue and 5% mercaptoethanol], heated at 100 °C for 5 min, electrophoresed on 7.5% SDS/polyacrylamide gels, and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA) (200 mA, 1 h). 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. Blocked membranes were incubated overnight at 4 °C with 1:1000 diluted 6E10 monoclonal antibody in a blocking buffer. Because SH-SY5Y cells express different isoforms of APP, sAPPα corresponded to 130 kDa and 120 kDa [16]. After washing, membranes were incubated for 2 h at room temperature with anti-mouse IgG AP-linked secondary antibody (1:5000). Signals were detected using CDP-Star Reagent (BioLabs, Ipswich, MA, USA) and analyzed using LAS-4000 (Fuji Film, Kanagawa, Japan) and Image Gause software ver. 3.0. The procedure used for the production of sAPPα was employed for the detection of PKC isozymes using monoclonal antibodies.

2.4. Assay for cellular ceramide levels

Cellular ceramide levels were examined by method previously described [12,13].

Cells were radiolabeled by the addition of [H^3] palmitic acid (1 μCi/mL) to the culture medium for 24 h. The cells were washed with the medium and incubated with E-64-d or medium for 30 min. Furthermore, the cells were treated with Aβ or medium for 5.5 h. The medium was removed, and the cells were precipitated in cold methanol (1 mL) containing 20 μg/mL of ceramide and SM. Lipids were extracted and separated using sequential thin layer chromatography (TLC). Sphingolipids were detected by iodine vapor and the silica gel was scraped to qualify radioactivity.

2.5. Assays for SMase activity

The micellar SMase assay using exogenous radiolabeled SM was performed as previously described [17]. To measure N-SMase, cells in six-well plates were incubated with E-64-d or medium for 30 min. The cells were then treated with Aβ or medium for 5.5 h. Cell pellets were dissolved in a buffer containing 20 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 2 mM EDTA, 5 m MDTT, 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 (PMSF), 10 μM leupeptin, 10 μM pepstatin, 0.2% Triton X-100. After incubation for 5 min at 4 °C, cells were homogenized by repeated passage of cells through an 18-gauge needle. Nuclei and cell debrils were removed using 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 (0.2 μCi/mL). The reaction was linear within this time frame, and the amount of [^{14}C] SM hydrolyzed did not exceed 10% of the total amount of radioactive SM added. Phosphorylcholine was subsequently extracted with 800 μl of chloroform: methanol (2:1) and 250 μl of H_2O. Radioactive phosphorylcholine produced from [^{14}C] SM was identified by TLC and routinely determined in the aqueous phase by scintillation counting. Increment of radioactive phosphorylcholine was confirmed using TLC. To measure A-SMase, cell pellets were resuspended in 200 μl of 0.2% Triton X-100 and incubated for 15 min at 4 °C. Cells were subsequently homogenized and spun in a microfuge at 14,000 rpm. From the supernatant, 30 μg protein 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 [^{14}C] SM (0.2 μCi/mL). Furthermore, the amount of radioactive phosphorylcholine produced was measured as described for the N-SMase assays.

2.6. Statistics

Statistic analysis was performed with Student's *t*-test.

4. Results

4.1. E-64-d restores the Aβ-induced reduction of sAPPα secretion in SH-SY5Y cells

We first examined sAPPα secretion in carbachol-stimulated SH-SY5Y cells following the addition of Aβ peptide to the culture.

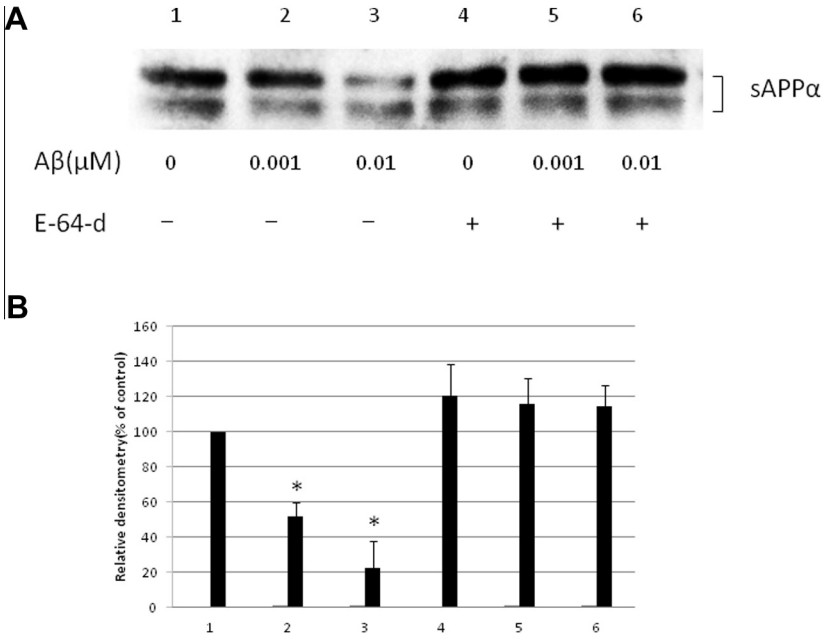

Fig. 1. Effect of E-64-d on sAPPα secretion from Aβ-treated SH-SY5Y cells. (A) SH-SY5Y cells were pretreated with or without E-64-d (3 μM) and incubated with Aβ (0.001 or 0.01 μM) for 5.5 h, followed by incubation with carbachol (1 mM) for 2 h. Secreted sAPPα was analyzed using Western blotting with the 6E10 antibody. The representative data are shown. (B) Quantitative densitometric analysis of the immunoreactive protein bands in (A). Numbers in the figure correspond to those in (A). Values are shown as percentage changes (mean ± SE, $n = 5$) compared with the data from cells without E-64-d and Aβ. $^{*}p < 0.05$.

Table 1
Sphingolipid levels in SH-SY5Y cells.

Addition	Ceramide (cpm/dish)		Sphingomyelin (cpm/dish)	
	Medium	E-64-d	Medium	E-64-d
Medium	349.8 ± 82.6	340.8 ± 68.4	4677.3 ± 244.1	4588.8 ± 302.2
Aβ (0.001 μM)	575.3 ± 48.2*	552.8 ± 50.4*	3342.8 ± 328.5*	3395.4 ± 287.6*
Aβ (0.01 μM)	673.8 ± 52.1*	683.2 ± 72.7*	2064.3 ± 412.8*	2234.6 ± 388.4*

After cells were labeled with [^{3}H] palmitic acid for 24 h, the cells were incubated with E-64-d (3 μM) or medium for 30 min, followed by treatment with Aβ for 5.5 h. Sphingolipids were extracted and separated using TLC as described in materials and methods. The data are means ± SE of five experiments.
* $p < 0.05$, significant when compared with the data from cells without E-64-d and Aβ.

Table 2
SMase activity in SH-SY5Y cells.

Addition	N-SMase (cpm/dish)		A-SMase (cpm/dish)	
	Medium	E-64-d	Medium	E-64-d
Medium	295.5 ± 38.7	300.6 ± 52.2	4589.2 ± 44.8	4385.6 ± 70.4
Aβ (0.001 μM)	388.7 ± 48.9*	406.4 ± 54.4*	4460.7 ± 209.7	4486.4 ± 96.2
Aβ (0.01 μM)	486.2 ± 74.6*	474.8 ± 62.4*	4646.3 ± 163.2	4422.8 ± 186.6

After cells were incubated with E-64-d (3 μM) or medium for 30 min, the cells were then treated with Aβ for 5.5 h. N-SMase and A-SMase activities were measured as described in materials and methods. The data are means ± SE of five experiments.
* $p < 0.05$, significant when compared with the data from cells without E-64-d and Aβ.

As shown in Fig. 1A, 5.5 h incubation with Aβ (0.001 or 0.01 μM) reduced sAPPα secretion. When cells were pretreated with 3 μM of E-64-d, a potent inhibitor of calpain, the Aβ-mediated suppression of sAPPα secretion was eliminated.

As shown in Fig. 1B, the relative densitometry values of secreted sAPPα when 0.001 and 0.01 μM of Aβ was added, were 51.7% and 22.3%, respectively, as compared with the control (without Aβ and E-64-d) ($p < 0.05$). When cells were pretreated with 3 μM of E-64-d, the relative densitometry values of sAPPα when 0, 0.001, and 0.01 μM of Aβ was added, were 120.5%, 115.3%, and 114.3%, respectively, as compared with the data from cells without Aβ and E-64-d. E-64-d (3 μM) alone did not significantly alter sAPPα secretion.

4.2. Aβ enhances ceramide production in SH-SY5Y cells

Furthermore, we examined whether Aβ affects the production of ceramide in SH-SY5Y cells. As shown in Table 1, the ceramide levels significantly increased when cells were incubated with 0.001 or 0.01 μM of Aβ for 5.5 h. Because ceramide is known to be produced by SM hydrolysis, we measured SM levels in the cells. SM levels were significantly reduced when cells were incubated

with 0.001 or 0.01 μM of Aβ. Preincubation of cells with E-64-d (3 μM) affected neither ceramide levels nor SM levels.

4.3. Aβ enhances N-SMase activity in SH-SY5Y cells

We measured both N-SMase and A-SMase activities because SM levels were decreased by the addition of Aβ in SH-SY5Y cells. As shown in Table 2, when cells were incubated with 0.001 or 0.01 μM of Aβ for 5.5 h, N-SMase activity was significantly increased. In contrast, Aβ did not alter A-SMase activity. Preincubation of cells with E-64-d (3 μM) affected neither N-SMase nor A-SMase activities.

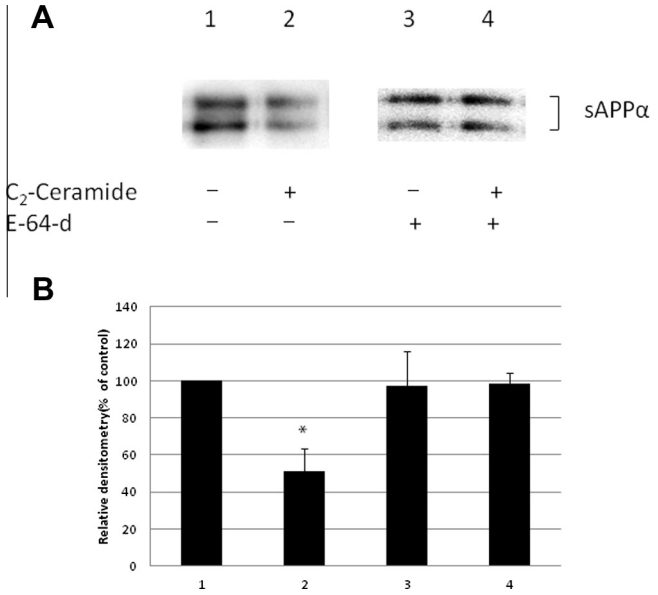

Fig. 2. Effect of E-64-d on sAPPα secretion from C_2-ceramide-treated SH-SY5Y cells. (A) SH-SY5Y cells were pretreated with or without E-64-d (3 μM) and incubated with C_2-ceramide (1 μg/mL) for 5.5 h, followed by incubation with carbachol (1 mM) for 2 h. Secreted sAPPα was analyzed using Western blotting with the 6E10 antibody. The representative data are shown. (B) Quantitative densitometric analysis of the immunoreactive protein bands in (A). Numbers in the figure correspond to those in (A). Values are shown as percentage changes (mean ± SE, $n = 5$) compared with the data from cells without E-64-d and C_2-ceramide. $^*p < 0.05$.

4.4. Cell-permeable ceramide analog suppressed sAPPα secretion in SH-SY5Y cells

We subsequently examined the effect of a cell-permeable ceramide analog (C_2-ceramide) on sAPPα secretion in carbachol-stimulated cells. We observed that incubation of cells with C_2-ceramide (1 μg/mL) for 5.5 h decreased sAPPα secretion (Fig. 2A). In addition, we show that pretreatment of cells with 3 μM of E-64-d eliminated the suppression of sAPPα secretion induced by C_2-ceramide.

The relative densitometry of sAPPα following the addition of C_2-ceramide was 51.4% (Fig. 2B). The decrease was significant as compared with the data from cells without Aβ ($p < 0.05$). When cells were pretreated with 3 μM of E-64-d, the relative densitometry values of sAPPα with or without C_2-ceramide were 98.6% and 97.2%, respectively.

4.5. C_2-ceramide down-regulates PKC-α, -β_1, and -β_2 in SH-SY5Y cells

We examined the PKC isozymes that were affected by C_2-ceramide treatment. In SH-SY5Y cells, PKC-α, -β_1, -β_2, -δ, and -ε were identified, whereas PKC-γ and other isozymes were not detected. As shown in Fig. 3A, incubation of cells with C_2-ceramide (1 μg/mL) for 5.5 h significantly down-regulated PKC-α, -β_1, and -β_2, whereas PKC-δ and -ε were not affected. In addition, pretreatment of cells with 3 μM of E-64-d reversed the down-regulation of PKC isozymes. The relative densitometry values of PKC isozymes (compared with the corresponding control data from cells without C_2-ceramide) are presented in Fig. 3B. E-64-d alone did not significantly affect the relative densitometry value of PKC isozyme (data not shown).

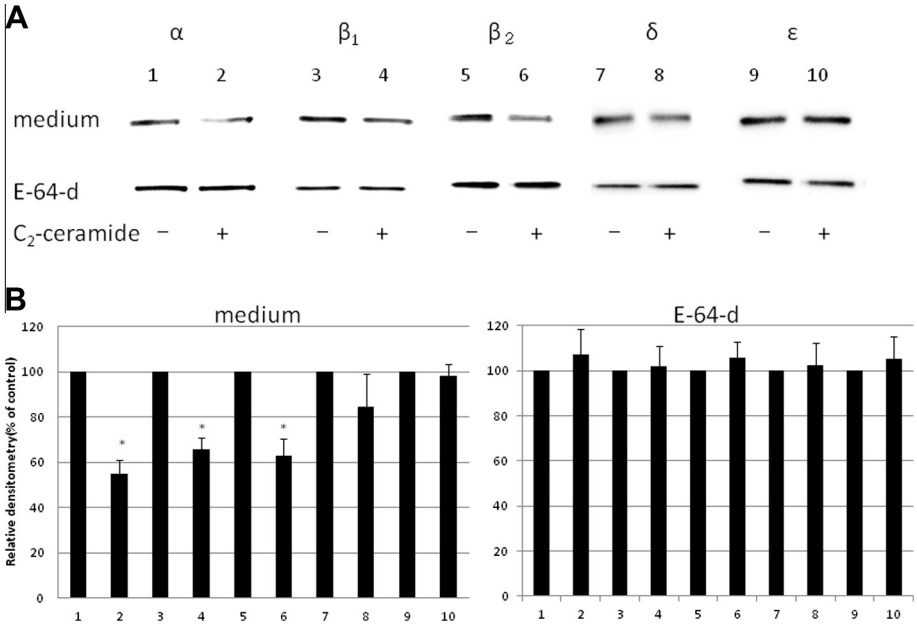

Fig. 3. Effects of C_2-ceramide and E-64-d on PKC isozymes detected from SH-SY5Y cells. (A) SH-SY5Y cells were pretreated with or without E-64-d (3 μM) and incubated with C_2-ceramide (1 μg/mL) for 5.5 h, followed by incubation with carbachol (1 mM) for 2 h. PKC isozymes were analyzed using Western blotting with PKC isozyme specific antibodies. The representative data are shown. (B) Quantitative densitometric analysis of the immunoreactive protein bands in (A). Numbers in the figure correspond to those in (A). Values are shown as percentage changes (mean ± SE, $n = 5$) compared with the corresponding control data from cells without C_2-ceramide; pretreated with medium (left panel), or 3 μM of E-64-d (right panel). $^*p < 0.05$.

5. Discussion

Several previous reports have demonstrated that PKC appears to play an important role in signal transduction that controls APP metabolism. The PKC activator, phorbol myristate acetate (PMA), was observed to induce α-secretase-mediated APP cleavage [1]. Involvement of specific PKC isozymes in APP processing has been reported. It is known that PKCs exist in at least 11 isozymes, including conventional PKCs, atypical PKCs, and novel PKCs. Shrott et al. [19] demonstrated that bryostatin-1 significantly increased the amount of sAPPα and reduced Aβ peptide levels in AD mice, probably by activating PKC-δ and -ε. In SH-SY5Y cells, it was reported that down-regulation of PKC-α and -ε by use of antisense cDNA impairs sAPPα production induced by PMA stimulation [20,21]. It was also reported that attenuation of PKC-α leads to impaired sAPP secretion [22]. In addition, PKC-β_1 [23] and -β_2 [24] may be involved in APP processing by modulating intracellular Aβ levels. In the guinea pig cortex, PKC-α and -β_1 are suggested to be key regulators of α-secretory APP processing [23]. Nelson et al. [5] demonstrated that PKC-ε may be involved in activating of sAPP production and suppressing Aβ production by using a specific inhibitor of PKC-ε. PKC-ε does not affect α-secretase, however, it promotes Aβ degradation by affecting endothelin-converting enzyme [5]. The discrepancy regarding the effect of various PKC isozymes on sAPPα processing may be because of the differences in cell types or experimental conditions.

In the present study, we report that Aβ-induced down-regulation of PKC activity suppressed sAPPα secretion. Several PKC isozymes, including PKC-α, -β_1, -β_2 may be involved in sAPPα processing and secretion in SH-SY5Y cells. However, the differential roles of PKC isozymes in sAPPα secretion are not known.

Ceramide has been recognized as an important second messenger in intracellular signal transduction. We previously demonstrated that ceramide promotes calpain-mediated proteolysis of PKC-β in PMA-stimulated murine polymorphonuclear cells [14]. We also presented that ceramide directly activates PKC-β and promotes its autophosphorylation. In this study, it is possible that C_2-ceramide affected PKC-α, -β_1, and -β_2 in SH-SY5Y cells by a similar mechanism, because all these isozymes were classified as conventional PKCs. However, the actual mechanism remains to be resolved.

It is known that N-SMase and A-SMase are activated independently by cytoplasmic factors [25]. In this study, we demonstrated that the addition of Aβ peptide enhanced ceramide production and N-SMase activity in SH-SY5Y cells, however, we cannot explain why N-SMase is enhanced by Aβ. Further studies on the mechanism are required. Li et al. [15] reported that synthetic ceramide analogs modulate APP processing, and Mielke et al. [26] stated that sphingolipid pathways are altered in AD. Our findings support the hypothesis that ceramide plays an important role in sAPPα processing in AD.

Favit et al. [6] reported that Aβ degrades PKC-α and -γ in normal and AD fibroblasts. They also presented that selective proteasome inhibitor prevented Aβ-induced degradation of PKC. E-64-d is a thiol proteinase inhibitor, and a membrane-permeable type of E-64-c [12]. The Ki of E-64-c for calpain is 0.96 μM. In this study, it was considered that the improvement of reduced sAPPα secretion by Aβ or C_2-ceramide was because of calpain inhibition.

To the best of our knowledge, ours is the first study to demonstrate that Aβ increases ceramide production by the activation of N-SMase and causes calpain-mediated down-regulation of PKC-α, -β_1, and -β_2 in SH-SY5Y cells. We also showed that a potent calpain inhibitor reversed the observed decrease in sAPPα secretion. Medeiros et al. [27] recently reported that the novel calpain inhibitor A-705253 attenuated cognitive impairment and synaptic dysfunction in a dose-dependent manner in 3xTgAD mice. Our findings also suggest that calpain inhibition increases neuroprotective sAPPα and reduces Aβ production. Taken together, the existing evidence indicates that calpain inhibitors may be effective for the treatment of AD.

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