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# Zinc Inhibits Amyloid β Production from Alzheimer's Amyloid Precursor Protein in SH-SY5Y Cells

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**Zinc released from excited glutamatergic neurons accelerates amyloid β (Aβ) aggregation, underscoring the therapeutic potential of zinc chelation for the treatment of Alzheimer's disease (AD). Zinc can also alter Aβ concentration by affecting its degradation. In order to elucidate the possible role of zinc influx in secretase-processed Aβ production, SH-SY5Y cells stably expressing amyloid precursor protein (APP) were treated with pyrrolidine dithiocarbamate (PDTC), a zinc ionophore, and the resultant changes in APP processing were examined. PDTC decreased Aβ40 and Aβ42 concentrations in culture media bathing APP-expressing SH-SY5Y cells. Measuring the levels of a series of C-terminal APP fragments generated by enzymatic cutting at different APP-cleavage sites showed that both β- and α-cleavage of APP were inhibited by zinc influx. PDTC also interfered with the maturation of APP. PDTC, however, paradoxically increased the intracellular levels of Aβ40. These results indicate that inhibition of secretase-mediated APP cleavage accounts -at least in part- for zinc inhibition of Aβ secretion.**

**Key Words: Zinc, Amyloid beta, Amyloid precursor protein, Pyrrolidine dithiocarbamate**

## INTRODUCTION

Alzheimer's disease (AD) is the most common cause of dementia. The pathologic hallmarks of AD are neurofibrillary tangles and senile plaques, the main components of which are tau (a microtubule-associated protein) and amyloid beta peptide (Aβ) respectively (Glenner and Wong, 1984; Kosik et al., 1986; Selkoe, 1999). Aβ is produced by the sequential cleavage of amyloid precursor protein (APP) by β- and γ-secretases. Alternatively, APP can be cleaved by α-secretase at a position between the β- and γ-cleavage points, resulting in the production of non-amyloidogenic sAPPα (Vassar and Citron, 2000). APP can also be subject to posttranslational modifications including glycosylation, sulfation, and phosphorylation. The immature form of APP is *N*-glycosylated and the mature form is *N*- and *O*-glycosylated (Weidemann et al., 1989). This maturation is considered to be a prerequisite for Aβ production and leads to an increase in molecular mass of 19∼22 kilodaltons (kD) (Weidemann et al., 1989; Pahlsson et al., 1992; Tomita et al., 1998). Any factors that accelerate Aβ aggregation can be precipitating factors in the development of AD, including enhanced production of Aβ, a decreased Aβ clearance rate and the promotion of Aβ precipitation (Bush et al., 1994).

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Zinc is concentrated in presynaptic vesicles containing the neurotransmitter glutamate and co-released into the synaptic cleft (Frederickson, 1989). The local concentration of zinc spikes to hundreds of micromoles upon excitotoxic events including ischemia, seizure, and trauma (Frederickson, 1989). The synaptic zinc released in excitotoxic conditions translocates into postsynaptic neurons, thereby causing neuronal damage (Choi and Koh, 1998).

The role of zinc in the pathogenesis of AD has been suggested by many studies. Bush et al. (1984) reported that zinc precipitates Aβ peptide (Bush et al., 1994). Clioquinol, which chelates zinc and copper ions, decreases Aβ accumulation and enhances memory functions in transgenic mouse models of AD (Cherny et al., 2001). *ZnT3*(−/−) mice that fail to accumulate zinc in glutamate-containing synaptic vesicles show a markedly reduced number of senile plaques compared with *ZnT3*(+/+) mice (Lee et al., 2002). These reports have focused on zinc-mediated acceleration of Aβ aggregation in the extracellular space. On the other hand, it has also been reported that zinc can modulate the extracellular levels of Aβ. Zinc activation of matrix metalloproteases (MMPs) has been suggested as one of the underlying mechanisms for accelerated clearance of secreted monomeric Aβ, as MMPs contribute to Aβ destruction (White et al., 2006). However, whether APP processing can be regulated by zinc as well as the effect of zinc on intracellular Aβ levels have yet to be tested. In this study, we

**ABBREVIATIONS:** Aβ, amyloid beta; AD, Alzheimer's disease; APP, amyloid precursor protein; CTF, C-terminal fragment; PDTC, pyrrolidine dithiocarbamate; FBS, fetal bovine serum; PBS, phosphate buffered saline.

show that pyrrolidine dithiocarbamate (PDTC), a zinc ionophore, inhibits secretase-executed APP processing pathways and causes intracellular A$\beta$ accumulation.

## METHODS

### *Construction of plasmids*

The coding region of human APP695 in cDNA prepared from HEK293 cells was amplified by PCR using the following primers: 5'-CCCAAGCTTGCCACCATGCTGCCCGGTTTGG-3' and 5'-GCTCTAGACTAGTTCTGCATCTGCTCAAAG-3'. The amplified DNA fragment was digested, subcloned into pcDNA3 (Invitrogen, Carlsbad, CA, USA) at *HindIII/XbaI* sites and named pcDNA3-hAPP695. The Swedish mutant form (K670N/M671L) pcDNA3-hAPP695swe, a two base pair transversion (GA to TC) of hAPP695, was made by site-directed mutagenesis using the primers 5'-AGGAGATCTCTGAAGTGAATCTGGATGCAGAATTCCGACA-3' and 5'-TGTCGGAATTCTGCATCCAGATTCACTTCAGAGATCT CCT-3' (the nucleotides underlined were changed).

### *Cell culture*

Cell culture media, fetal bovine serum (FBS), and antibiotics were purchased from Invitrogen (Carlsbad, CA, USA). SH-SY5Y cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, 100-U/ml penicillin, and 100-$\mu$g/ml streptomycin at 37°C in 5% $CO_2$/95% air. The SH-SY5Y cells were lipotransfected with pcDNA3-hAPP695 or pcDNA3-hAPP695swe and 500-$\mu$g/ml geneticin was used for three weeks to select stable clones, named SH-SY5Y-wt and SH-SY5Y-swe respectively. The polyclonal stable cell lines were maintained with 250-$\mu$g/ml geneticin.

### *Sandwich ELISA*

For the measurement of secreted A$\beta$40 and A$\beta$42 as well as intracellular A$\beta$40, SY5Y-wt or SY5Y-swe cells were plated on 60-mm culture dishes at 70% confluence. After 24 h of incubation, culture media was changed with 2 ml of fresh media and incubated for 4 h either with or without drugs. In case of serum deprivation, DMEM was supplemented with 0.2-mg/ml bovine serum albumin (BSA) (Sigma, St. Louis, MO, USA). The conditioned media was supplemented with 5-mM EDTA (ethylenediaminetetraacetate) and 1-mM AEBSF (4-(2-aminoethyl)-benzenesulfonyl fluoride) and briefly centrifuged for removal of cell debris. Cells were collected by scraping and lysed with phosphate buffered saline (PBS) containing 1% Triton X-100, Complete protease inhibitor cocktail (Roche Applied Sciences, Indianapolis, IN, USA), and 10-$\mu$M DAPT (*N*-[*N*-(3,5-difluorophenacetyl)-*L*-alanyl]-*S*-phenylglycine *t*-butyl ester), a $\gamma$-secretase inhibitor (EMB Chemicals, San Diego, CA, USA). The levels of A$\beta$40 and A$\beta$42 in the conditioned media and cell lysates were measured by sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instruction (IBL, Takasaki-Shi, Japan).

### *Immunoprecipitation and immunoblotting*

For the immunoblot assay of A$\beta$40, SH-SY5Y-wt cells were washed twice with PBS containing 1.8-mM $Ca^{2+}$ and incubated in DMEM supplemented with 0.2-mg/ml BSA − the concentration of albumin in human cerebrospinal fluid − for 4 h. The secreted proteins from SH-SY5Y-wt cells in the conditioned media were precipitated by adding up to 10% (w/v) trichloroacetic acid (TCA) and centrifuging at 2,500 g for 1 h at 4°C. The protein pellets were washed twice with 100% acetone and dried. The proteins were dissolved in TNT lysis buffer (50-mM Tris pH 8.0, 100-mM NaCl, 1% Triton X-100, Complete protease inhibitor cocktail) and immunoprecipitated with A$\beta$40 antibody (Invitrogen, Carlsbad, CA, USA). The immunoprecipitated proteins were electrophoresed in 16.5% Tris-Tricine gels before being transferred to a nitrocellulose membrane (Whatman, Florham Park, NJ, USA) which was then boiled in PBS for 5 min for fixation before being probed with the same antibody.

To measure C99 and C83, carboxy-terminal fragments of APP cleaved by $\beta$- and $\alpha$-secretase respectively, cell lysates supplemented with 10-$\mu$M DAPT were immunoprecipitated with rat anti-APP C-terminus antibody. This antibody was raised against the 561~676 residue of human APP and separated in 16.5% Tris-Tricine gel for immunoblotting with rabbit anti-APP C-terminus antibody (IBL, Takasaki-Shi, Japan).

For sAPP$\alpha$ and full length APP (holoAPP), proteins in culture media or cell lysates were separated by 7% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. The membrane was probed with monoclonal anti-A$\beta$(1~17) antibody 6E10 (Sigma, St. Louis, MO, USA) for sAPP$\alpha$ and with rabbit anti-APP C-terminus antibody (IBL, Takasaki-Shi, Japan) for holoAPP.

## RESULTS

### *Effect of PDTC on secreted A$\beta$40 and A$\beta$42 levels in culture media*

To investigate the effects of zinc influx on A$\beta$ levels in SH-SY5Y-wt conditioned culture media, PDTC was administered as a zinc ionophore into media containing 10% FBS (Kim et al., 1999a; Kim et al., 1999b). Sandwich ELISA for A$\beta$40 showed that the treatment of 0-, 20-, 50-, and 100-$\mu$M PDTC for 4 h decreased A$\beta$40 levels in a dose-dependent manner (Fig. 1A). At 100 $\mu$M, PDTC reduced A$\beta$40 concentration in the culture media by 29±7.2%. The concentration of A$\beta$42, one of the two most common isoforms of A$\beta$, also decreased to a level similar to the level of A$\beta$40 (Fig. 1B).

### *Mechanism of PDTC action*

To confirm the zinc-ionophore action of PDTC, media was pretreated with EDTA for 30 min before PDTC treatment. EDTA, which sequesters divalent and trivalent metal ions, restored A$\beta$40 reduction by PDTC, indicating that the PDTC action was due to a metal ionophore (Fig. 2A). PDTC is not only a zinc ionophore, but also a copper ionophore in certain conditions (Meyer et al., 1993; Chinery et al., 1997; Verhaegh et al., 1997; Iseki et al., 2000). To determine which metal is involved in the PDTC reduction of A$\beta$, the serum-depleted culture media bathing SH-SY5Y-wt cells was supplemented with zinc sulfate and copper sulfate, 1.5 $\mu$M each, and PDTC was co-administered. Neither PDTC single

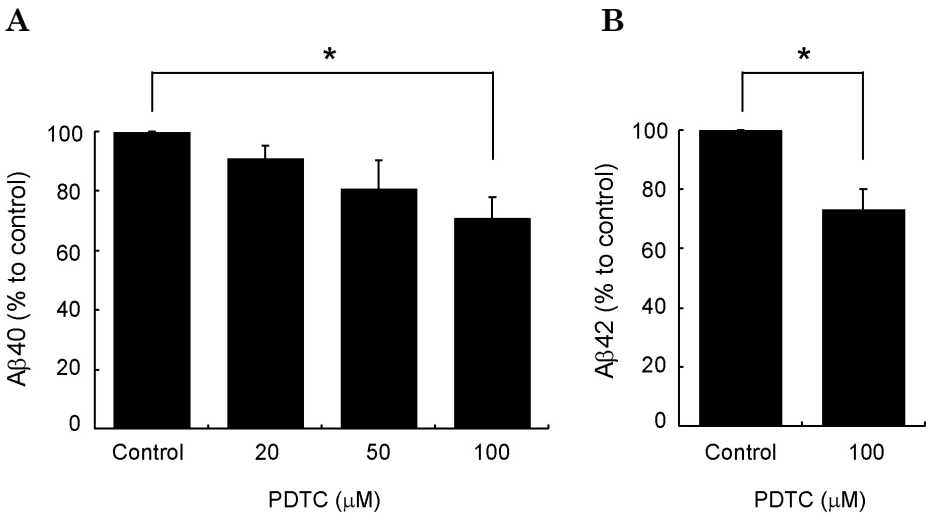


**Fig. 1.** PDTC decreases Aβ levels in the conditioned media. PDTC was added to SH-SY5Y-wt cells at the concentration indicated for 4 h. Aβ40 (A) and Aβ42 (B) in the conditioned media were quantified by sandwich ELISA. Bars represent the means± S.D. of three independent experiments. Control represents no drug treatment. Statistical significance was represented as *($p < 0.05$).

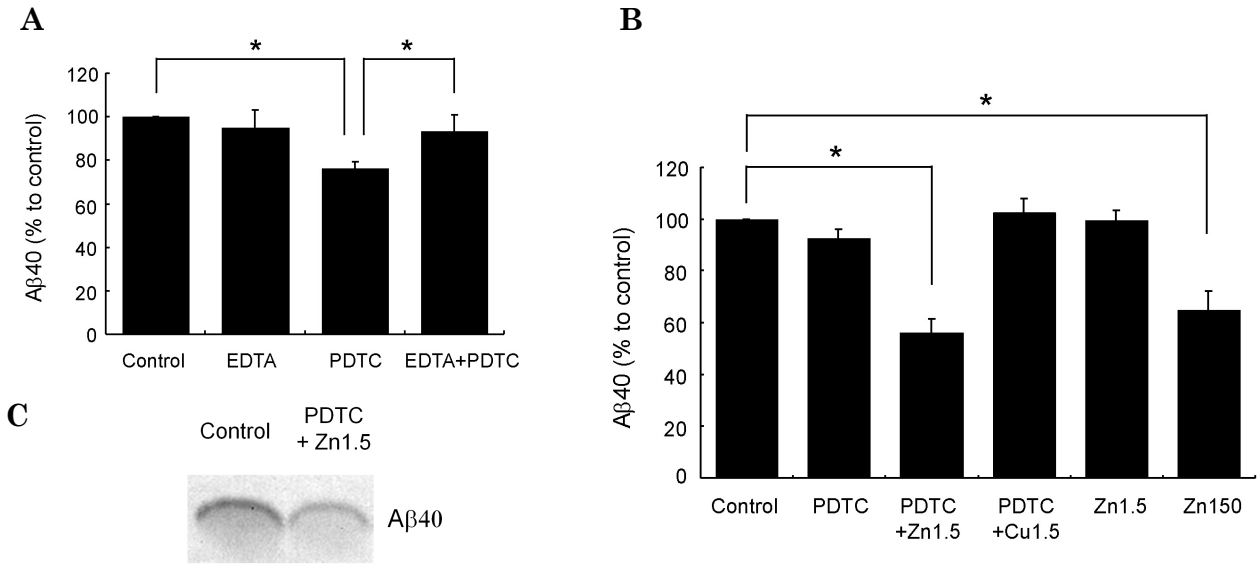


**Fig. 2.** Zinc is required for PDTC action. Aβ40 levels in the conditioned media from SH-SY5Y-wt cells were measured by sandwich ELISA (A and B) or by TCA precipitation followed by combined immunoprecipitation and immunoblot assays with Aβ40 antibody (C). (A) SH-SY5Y-wt cells were incubated with or without 100-μM PDTC and/or 10-μM EDTA for 4 h. (B) Serum-deprived SH-SY5Y-wt cells were treated as follows: control, 100-μM PDTC, 100-μM PDTC with 1.5-μM $ZnSO_4$ or 1.5-μM $CuSO_4$, 1.5-μM $ZnSO_4$, and 150-μM $ZnSO_4$ for 4 h. (C) The serum-free conditioned media from SH-SY5Y-wt cells treated with or without PDTC plus 1.5-μM zinc were concentrated by TCA precipitation for Aβ40 assay (see methods). Bars represent the mean±S.D. of three different experiments. Control represents no drug treatment. Statistical significance was represented as *($p < 0.05$).

treatment nor copper treatment with PDTC decreased Aβ 40 levels. In contrast, PDTC-zinc co-treatment induced robust reduction in Aβ40 levels (Fig. 2B). This result was supported by an additional experiment: proteins in the culture media were precipitated with TCA and subjected to combined immunoprecipitation and immunoblot assays for Aβ40 (Fig. 2C), demonstrating that PDTC plus 1.5-μM zinc decreased the band intensity representing Aβ40. Furthermore, treatment of 150-μM zinc sulfate alone decreased Aβ40 levels in the media (Fig. 2B). These results indicate that PDTC-facilitated zinc influx into SH-SY5Y cells reduced secreted Aβ40 levels in the culture media.

### *Effects of PDTC on the levels of APP metabolites*

To understand the underlying mechanisms of zinc reduction in intracellular Aβ40 levels, changes in concentration of APP metabolites produced by distinct secretase-mediated cleavage of APP were analyzed after treatment with PDTC. The amino-terminal fragment generated by α-cleavage, sAPPα, was assayed by immunoblot using the anti-Aβ (1～17) antibody. C-terminal fragment (CTF) α (C83) and

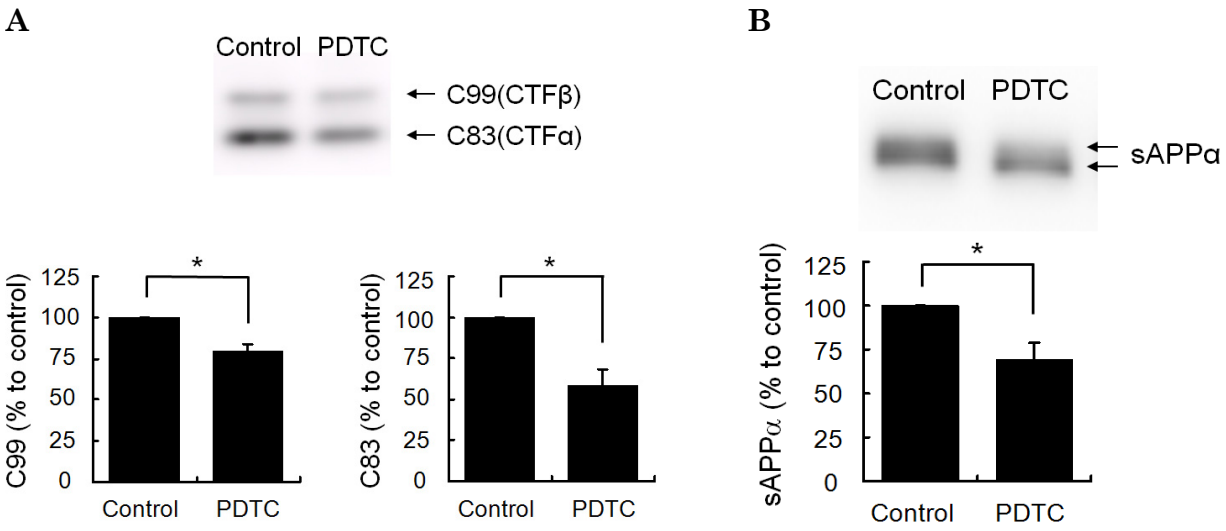


**Fig. 3.** APP processing by $\beta$- and $\alpha$-secretase is inhibited by PDTC. The cell lysates (A) or conditioned media (B) of SH-SY5Y-wt cells treated with or without 100-$\mu$M PDTC were analyzed by immunoblot assay (B) or combined immunoprecipitation-immunoblot assay (A) (see methods). The amounts of C99 and C83 detected in immunoblot assays with APP C-terminus antibodies were semi-quantified with densitometry. A monoclonal 6E10 antibody (against a.a. 1~17 of human A$\beta$) was used for the sAPP$\alpha$ assay. Bars represent the means±S.D. of two or three independent experiments. Control represents no drug treatment. Mature APP is represented as mAPP; immature APP, imAPP. Statistical significance was represented as *($p<0.05$).

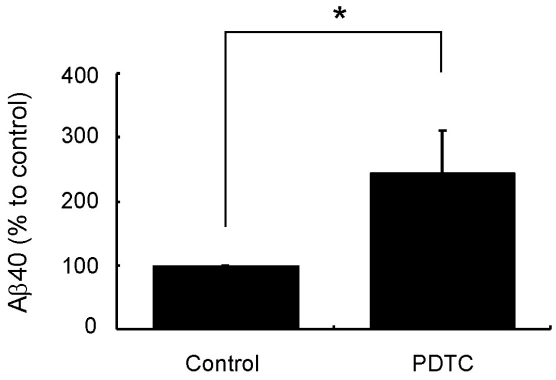


**Fig. 4.** PDTC increases intracellular A$\beta$40. SH-SY5Y-swe cells were incubated with or without 100-$\mu$M PDTC for 4 h and then scraped, pelleted and lysed. A$\beta$40 in the cell lysates was assayed by sandwich ELISA. Bars represent the mean±S.D. of three independent experiments. Control represents no drug treatment. Statistical significance was represented as *($p<0.05$).

CTF$\beta$ (C99), a longer CTF form generated by $\beta$-cleavage, were enriched by immunoprecipitation with rat anti-APP C-terminus antibody and resolved by SDS-PAGE for detection with rabbit anti-APP C-terminus antibody. PDTC decreased the levels of both C99 and C83 in cell lysates and also the level of sAPP$\alpha$ in the media (Fig. 3). These results suggested that zinc influx inhibited secretase-mediated A$\beta$40 processing. Next, PDTC-induced changes in intracellular A$\beta$40 levels in cell lysates were examined. We used SH-SY5Y-swe instead of SH-SY5Y-wt cells because the A$\beta$ level in SH-SY5Y-wt cells was too low to be detected. APP is also more susceptible to $\beta$-secretase in SH-SY5Y-swe cells. Interestingly, the A$\beta$40 level in the SH-SY5Y-swe cell lysates, which was assayed by sandwich ELISA, increased following PDTC treatment (Fig. 4).

### *Inhibition of APP maturation by PDTC*

Intriguingly, the electrophoretic mobility of sAPP$\alpha$ was also changed by PDTC (Fig. 3B), suggesting a potential PDTC effect on posttranslational modifications of APP. Thus, gel mobility of full-length APP (holoAPP) was examined following PDTC treatment. APP is known to be detected as multiple bands on immunoblotting when labeled with an APP C-terminus antibody. The immature form has been reported as 91~106 kD and the mature form as 20 kD larger (Weidemann et al., 1989; Caporaso et al., 1992; Pahlsson et al., 1992). Tomita et al. (1998) reported immature APP as two bands, results consistent with our findings (Fig. 5A). PDTC did not affect the level of holoAPP in the cell lysates (Fig. 5A, B). It did, however, inhibit APP maturation, as demonstrated by the decrease in the mature APP to immature APP ratio (Fig. 5C). An actin immunoblotting was used as a loading control.

## DISCUSSION

In this study, we have shown for the first time that PDTC-facilitated influx of zinc into cells stably expressing hAPP695 inhibits not only APP processing by both $\alpha$- and $\beta$-secretase but also APP maturation. An intracellular increase in zinc concentration is required for APP processing because although low concentrations of zinc did not induce APP processing suppression, co-treatment with PDTC – a zinc ionophore – efficiently suppressed $\alpha$- and $\beta$-secretase-dependent APP cleavage. This inhibitory effect of intracellular zinc on amyloid production is in striking contrast to the seemingly opposite role of extracellular zinc in precipitating A$\beta$ aggregation, a process which leads to the development of amyloid plaques. Until now, relatively little was known about the role of intracellular zinc in A$\beta$ pathology. To date, the only report available found that zinc

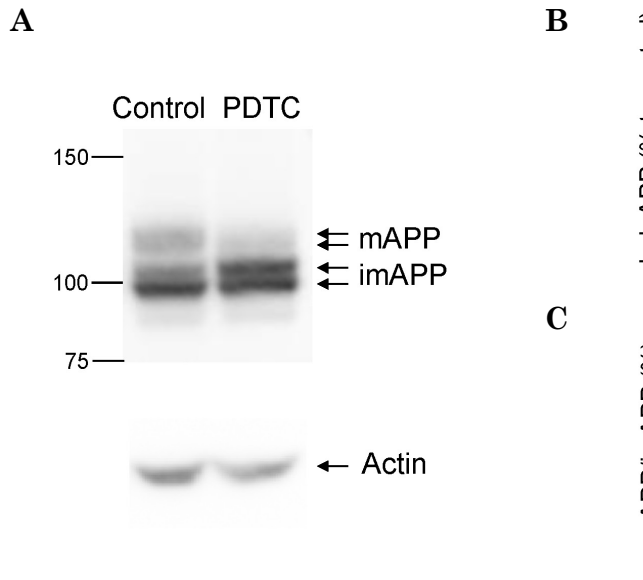


**Fig. 5.** Maturation of APP is inhibited by PDTC. Cell lysates of SH-SY5Y-wt cells treated with or without 100-$\mu$M PDTC for 4 h were separated in 7% SDS-PAGE. (A) APP was immunoblotted with APP C-terminus antibody. (B) Quantitative analysis of the holoAPP bands (mature form+immature form). Amount of holoAPP was normalized to actin levels. (C) The maturation degree of APP was shown by the percentage of mAPP to imAPP. Bars represent the mean±S.D. of three independent experiments. Control represents no drug treatment. Statistical significance was represented as *($p<0.05$).

recruited into the intracellular space by the metal-chelator clioquinol culminates in a reduction of A$\beta$ levels by activation of MMPs capable of cleaving A$\beta$ at multiple sites (White et al., 2006). Clioquinol, like PDTC, increases the intracellular levels of copper and zinc, important biometals in the development of AD (Caragounis et al., 2007). The proposed modes of action of these two compounds differ in terms of their proposed recruited-metal targets. Our study, however, still supports the general hypotheses that intracellular biometal concentration is important for regulating the secretion of A$\beta$ *in vitro* and that metal-ligands might have therapeutic potential for AD *in vivo*.

Glycosylation of APP potentially plays an important role in the secretion of A$\beta$ and other APP derivatives. Glycosylation inhibitors diminish sAPP$\alpha$ secretion in Chinese hamster ovary (CHO) cells expressing hAPP695-wt (Pahlsson and Spitalnik, 1996). Protein kinase A (PKA) inhibitors decrease mature APP and lead to an accumulation of immature APP, processes associated with reduced A$\beta$ production (Su et al., 2003). Tomita et al. demonstrated that the aberration of *O*-glycosylation of APP by certain mutations leads to a reduction of C83 in cell lysates and the suppression of A$\beta$ secretion (Tomita et al., 1998). Zinc inhibition of APP maturation may also account for the decrease in A$\beta$ secretion because $\alpha$-, $\beta$-, and $\gamma$-secretase cleavage of APP occurs only after *O*-glycosylation of APP. Moreover, it is likely that zinc affects certain earlier processes in the APP secretion pathway rather than affecting the secretases themselves, because zinc did not show any selective action on either $\alpha$- or $\beta$-cleavage of APP.

Another important finding of this study is that PDTC-facilitated zinc influx causes an accumulation of intracellular A$\beta$ in SH-SY5Y cells. In astrocytes of patients with Down syndrome, secretion of sAPP$\alpha$ and A$\beta$ are decreased, but intracellular A$\beta$ accumulates, which might be due to mitochondrial dysfunction in Down syndrome (Busciglio et al., 2002). Reynolds and his colleagues also demonstrated that zinc could be a mitochondrial toxin in neurodegeneration (Dineley et al., 2003). Considering these reports, mitochondrial damage caused by PDTC-facilitated influx of zinc might lead to accumulation of A$\beta$ in SH-SY5Y cells. As described previously, an increase in intracellular zinc can activate MMPs and this might cause degradation of intracellular A$\beta$. On the other hand, intracellular free zinc might interact directly with A$\beta$ as well, thereby affecting A$\beta$ stability. The net effect of zinc-A$\beta$ interactions in our study is the accumulation of A$\beta$ in SH-SY5Y cells, suggesting that, at least at intracellular sites, zinc activation of MMPs does not play a major role in determining A$\beta$ levels. The reason for the discrepancy in the effect of zinc-MMP interaction might be due to different experimental conditions such as the presence of 10% FBS which has much anti-protease activity or different cell systems we used.

In this report, zinc influx reduces neurotoxic A$\beta$ secretion. This begs the complicated question of whether zinc is a protective factor for Alzheimer's disease. Some reports have shown that the zinc content in the brains of patients with AD is somewhat decreased (Wenstrup et al., 1990; Corrigan et al., 1993). Zinc is also one of the important biometals that promotes the extracellular formation of amyloid plaque. Thus, limited by the current paucity of evidence, it is difficult to predict the role of zinc in protection from or promotion of amyloid pathology. However, the accumulation of intracellular A$\beta$ by zinc influx might still contribute to the development of AD.

Oligomerization of A$\beta$ begins intracellularly (Walsh et al., 2000), suggesting that zinc-induced A$\beta$ accumulation in cells might accelerate its oligomer formation. Synaptic dysfunction and neuronal death are produced by A$\beta$ oligomers (Lambert et al., 1998; Walsh et al., 2002), which become a core for further oligomerization and aggregation. Although elevated levels of intracellular zinc are beneficial in decreasing the secretion of A$\beta$, accumulated A$\beta$ (or possibly A$\beta$ oligomerized by zinc) might be released once zinc concentration returns to the basal level, resulting in aggregation of A$\beta$. Moreover, the secreted form of APP, sAPP$\alpha$, has neuroprotective and memory enhancing effects (Furukawa et al., 1996; Meziane et al., 1998), leading to the postulation that zinc influx is detrimental to AD patients due to the suppression of sAPP$\alpha$ secretion. It remains to be determined

whether zinc influx can precipitate A$\beta$ oligomerization and which zinc-provoked events are most relevant and important in AD pathogenesis.

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