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Protective Effect of Citrate against Aβ-induced Neurotoxicity in PC12 Cells

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Abstract

Formation of β-amyloid (Aβ) fibrils has been identified as one of the major characteristics of Alzheimer's disease (AD). Inhibition of Aβ fibril formation in the CNS would be attractive therapeutic targets for the treatment of AD. Several small compounds that inhibit amyloid formation or amyloid neurotoxicity *in vitro* have been known. Citrate has surfactant function effect because of its molecular structure having high anionic charge density, in addition to the well-known antibacterial and antioxidant properties. Therefore, we hypothesized that citrate might have the inhibitory effect against Aβ fibril formation *in vitro* and have the protective effect against Aβ-induced neurotoxicity in PC12 cells. We examined the effect of citrate against the formation of Aβ fibrils by measuring the intensity of fluorescence in thioflavin-T (Th-T) assay of between $A\beta_{25-35}$ groups treated with citrate and the control with $A\beta_{25-35}$ alone. The neuroprotective effect of citrate against Aβ-induced toxicity in PC12 cells was investigated using the WST-1 assay. Fluorescence spectroscopy showed that citrate inhibited dose-dependently the formation of Aβ fibrils from β-amyloid peptides. The inhibition percentages of Aβ fibril formation by citrate (1, 2.5, and 5 mM) were 31%, 60%, and 68% at 7 days, respectively in thioflavin-T (Th-T) assay . WST-1 assay revealed that the toxic effect of $A\beta_{25-35}$ was reduced, in a dose-dependent manner to citrate. The percentages of neuroprotection by citrate (1, 2.5, and 5 mM) against Aβ-induced toxicity were 19%, 31%, and 34%, respectively. We report that citrate inhibits the formation of Aβ fibrils *in vitro* and has neuroprotective effect against Aβ-induced toxicity in PC12 cells. Neuroprotective effects of citrate against Aβ might be, to some extent, attributable to its inhibition of Aβ fibril formation. Although the mechanism of anti-amyloidogenic activity is not clear, the possible mechanism is that citrate might have two effects, salting-in and surfactant effects. These results suggest that citrate could be of potential therapeutic value in Alzheimer's disease.

Alzheimer's disease (AD) is the most common form of amyloidogenic diseases which are characterized by conformational changes that are followed by aggregation of proteins inside or outside cells. Amyloid-associated disorders include other human diseases such as Parkinson's disease, Huntington's disease, prion diseases, familial amyloidosis, and type II diabetes[1]. Over the years, evidence from biochemical, genetic, and pathologic investigations support the idea that amyloidogenesis is a pivotal and early event in AD pathogenesis[2]. The major neuropathological changes in the brains of AD patients include the accumulation of β-amyloid (Aβ) peptides in senile plaques, and the presence of hyperphosphorylated tau in neurofibrillary tangles. Aβ is generated by the sequential proteolytic processings of the type I membrane protein, termed amyloid precursor protein (APP), by β- and γ-secretases[3,4]. Aβ promotes pro-inflammatory responses and activates neurotoxic pathways, leading to dysfunction and death of brain cells[5]. Studies have demonstrated that when aggregated in fibrillar form, Aβ has neurotoxic effects in cell culture and *in vivo*[6-10]. Thus, finding molecules to inhibit the formation of Aβ fibrils could be of therapeutic value in AD.

The delineation of the molecular biology of AD has yielded a number of therapeutic targets whose suc-

cessful manipulation may result in disease modification[11-13]. Three general classes of therapies are anticipated: 1) antiamyloid agents, which affect primarily the production, accumulation, removal, or blockade of toxicity associated with the Aβ; 2) neuroprotective agents that reduce the injury associated with abnormal amyloid protein processing, including antioxidants, anti-inflammatory agents, tau-related therapies, myelin preservation agents, and nerve growth factors and promoters; and 3) neurorestorative approaches, including neurotrophic and nerve growth factor strategies, transplantations, and stem-cell related interventions[14,15]. Other therapeutic strategies such as cholinesterase inhibitors, muscarinic, glutamatergic or serotonergic agonists, anti-inflammatory drugs, estrogen-replacement therapy and anti-oxidant drugs are directed towards palliating existing cognitive symptoms or retarding the disease course. The advantage of an amyloid-related therapy for AD is that it is targeted at correcting or preventing the pathology believed to cause the disease; therefore, it might change the course of the disease or even prevent its onset. Preventing amyloid deposition will also allow evaluation of the actual role of amyloid in AD.

Small molecules have been shown to prevent Aβ fibrillogenesis or inhibit Aβ toxicity *in vitro*. Sulfonated dyes, such as Congo red, small sulfonated anions and benzofuran-based compounds have also been described as inhibitors of amyloid formation or neurotoxicity associated with amyloid fibrils[10,16,17]. In a similar way, melatonin, nicotine, estrogen and anthracycline 49-iodo-49-deoxydoxorubicin were identified as inhibitors of Aβ aggregation or Aβ neurotoxicity[18-21]. The usefulness of these small molecules as amyloid inhibitors is compromised by their lack of specificity and their unknown mechanism of action, which makes it difficult to improve them. In addition, many of these compounds are highly toxic.

β-Sheet breaker peptides (iAβ) were demonstrated to inhibit Aβ fibrillogenesis, disassemble pre-formed Aβ fibrils *in vitro* and to prevent neuronal death induced by Aβ fibrils in cell culture[22]. In addition, iAβ5 (LPFFD) significantly reduced Aβ deposition *in vivo* and completely blocked the formation of Aβ fibril in a rat brain model of amyloidosis[23].

A large number of studies indicate that oxidative injury may play a role in the development of AD. Many antioxidant compounds, such as vitamin E, NDGA, nicotine, vitamin A, and coenzyme Q10 have been shown to protect the brain from Aβ toxicity[24-28].

In addition, several surfactants have been found to delay or prevent fibril formation, possibly by binding to hydrophobic region that is necessary for self-assembly[29-32]. The cationic surfactant hexadecyl-N-me-

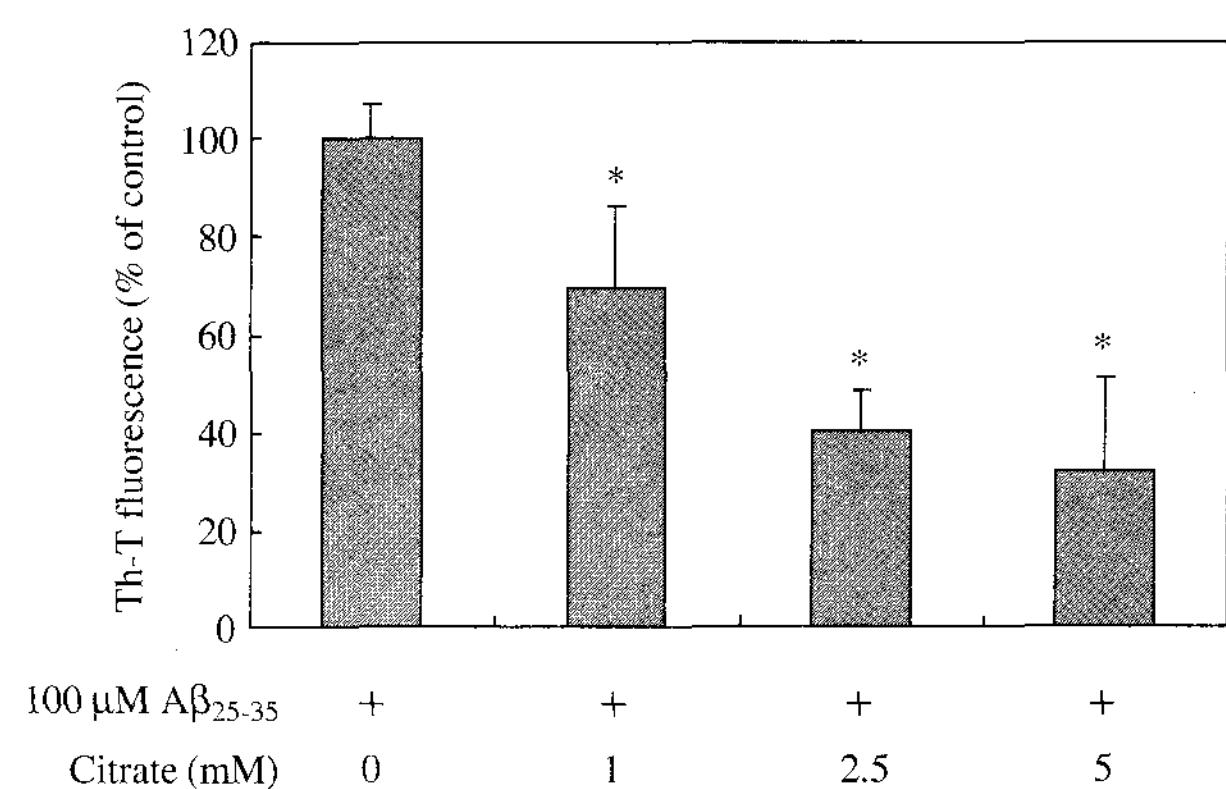**Figure 1.** The Effect of citrate on fibril formation of Aβ25-35 by Th-T fluorometric assay. Aβ25-35 at 100 μM was incubated with (1, 2.5, and 5 mM final concentration) citrate at 37°C for 7 days. The Th-T was mixed with each sample and fluorescence intensity was measured using a spectrofluorometer. The data are summarized as the mean ± S.E.M. of three experiments. Asterisk (*) indicates a significant ($P < 0.05$) difference between Aβ alone and citrate-treated Aβ group.

thylpiperidinium bromide was found in this way to be a specific inhibitor of Aβ aggregation[33].

Among the various groups of biomaterials having surfactant molecular structure, citrate is particularly interesting component having the high anionic charge density which may give Aβ more hydrophilicity.

In this study, we described an *in vitro* assay to search for an inhibitory effect of citrate on Aβ fibril formation by using fluorescence spectrophotometer (Thioflavin-T assay). We used the Aβ25-35 amino-acid peptide that preserves the properties of neurotoxicity and aggregation of the full-length sequence. Moreover, the Aβ25-35 fragment has the experimental advantage of rapidly aggregating upon solubilisation in aqueous buffers[34]. We also investigated the protective effect on PC12 cells from Aβ-induced toxicity by measuring WST-1 activity which is an indicator of the activity of living cells.

Fluorometric Studies of Effects of Citrate on the Formation of Aβ25-35 Fibril

The Aβ25-35 concentration 100 μM was chosen as working concentration as described previously[35]. Our results revealed that the inhibition of Aβ fibril formation is dependent on the concentration of citrate. At 7 days, the presence of citrate led to a significant decrease in the Aβ fibril formation as compared to Aβ25-35 peptide alone (Figure 1). The Th-T fluorescent assay, a valuable method for a real-time evaluation of formation of Aβ aggregation *in vitro*, was performed to verify the inhibition of Aβ fibril formation by citrate and revealed that citrate remarkably suppressed Aβ
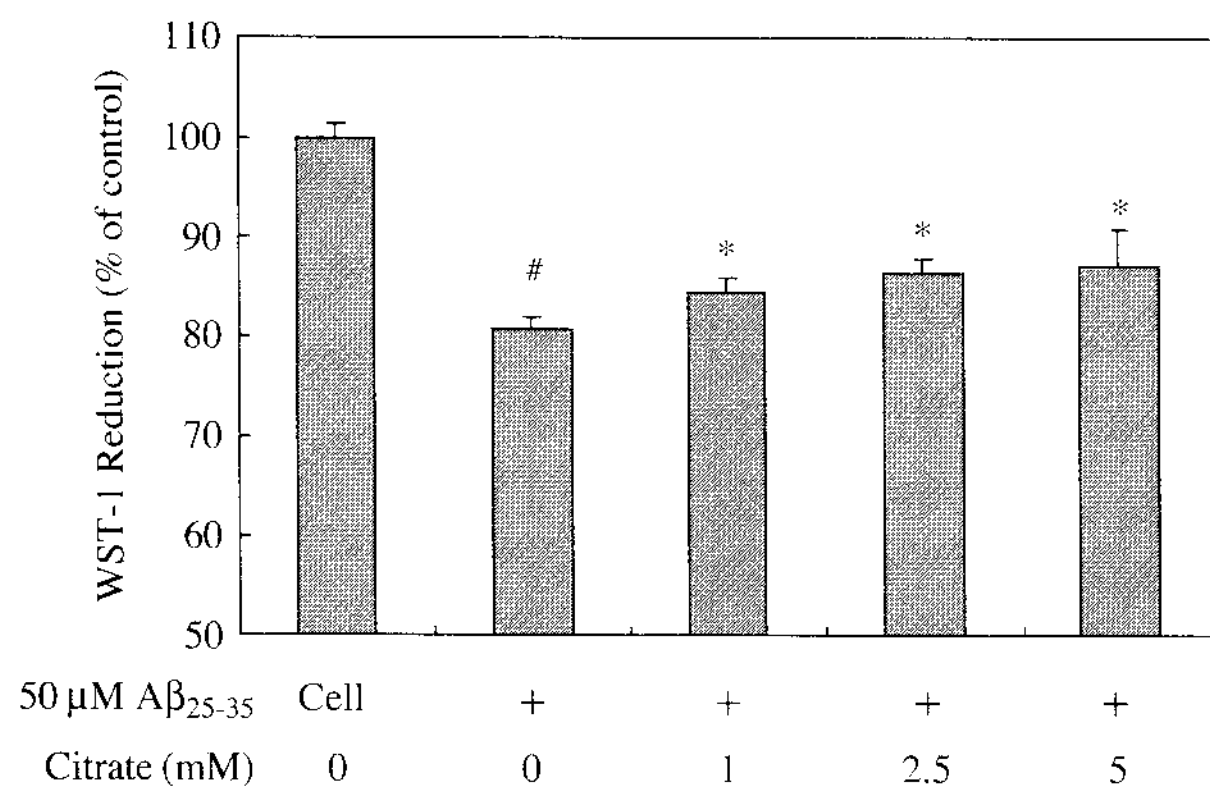
Figure 2. The effect of citrate on the viability of PC12 cell line. For 24 h, cells were incubated with 50 μM Aβ$_{25-35}$ preincubated in the 0, 1, 2.5, and 5 mM citrate at 37°C for 7 days. The cell viability was measured by WST-1 assay. The data are summarized as the mean ± S.E.M. of three experiments. Asterisk (*) indicates a significant ($P<0.05$) difference between Aβ alone and citrate-treated Aβ group and # $P<0.05$ compared Aβ alone with control (cell).

aggregation. Figure 1 showed that following 7-day incubation, 1, 2.5, and 5 mM citrate attenuated significantly Aβ$_{25-35}$ fibril formation in a dose-dependent manner. The percentages of Aβ fibril formation in the presence of citrate (1, 2.5, and 5 mM) were 69%, 40%, and 32% of Aβ$_{25-35}$ alone, respectively ($P<0.05$) .

Protective Effect of Citrate on Neurotoxicity in Aβ$_{25-35}$-treated Cells

The Aβ$_{25-35}$-induced cytoxicity was determined by the percentage of WST-1 reduction assay after culture for up to 24 h with increasing concentrations (1.0, 2.5 and 5.0 mM) of citrate (Figure 2). 50 μM Aβ$_{25-35}$ alone decreased about 20% cell viability in WST-1 values compared with the control (cell). The cytotoxic effects were attenuated by the co-treatment with citrate dose dependently ($P<0.05$) (Figure 2). The percentages of neuroprotection by citrate (1, 2.5, and 5 mM) were 19%, 31%, and 34%, respectively in which the gap of viability in between control and Aβ$_{25-35}$ alone is considered as 100%.

Discussion

The formation of insoluble Aβ deposits in the brain is a central event in the pathogenesis and a pathological hallmark of AD. Aβ has been shown to be toxic to neuronal cells in culture after an aging process of several days, which corresponds to the kinetics of *in vitro* amyloid fibril formation[9]. A number of therapeutic approaches to reducing the load of amyloid

plaque seem possible, focusing on βAPP production, βAPP processing, fibril formation by Aβ, or removal of Aβ from critical areas in the brain. A fruitful approach in the study of the relationship between the structure and fibrillogenicity of Aβ has been to alter the sequence of the peptide and to analyze the effect on amyloid formation. Replacing hydrophobic for hydrophilic residues in the internal Aβ hydrophobic region impairs fibril formation, suggesting that Aβ assembly is driven partially by hydrophobic interactions[36]. On the other hand, several reports indicate that the conformation adopted by Aβ peptides is crucial in amyloid formation. Barrow and Zagorski demonstrated that Aβ incubated at different pHs or in distinct solvents shows either mainly random coil/α-helical or β-sheet secondary structure. Under the conditions in which the peptide adopts the random coil/α-helical structure, it aggregates slowly, but when Aβ adopts the β-sheet conformation, it aggregates rapidly[37-39]. Therefore, it seems likely that a key event in amyloidogenesis is the conversion of the normal soluble Aβ conformer into the β-sheet-rich amyloidogenic intermediate. The emerging picture from studies with synthetic peptides is that Aβ amyloid formation is dependent on hydrophobic interactions among altered Aβ peptides that adopt an antiparallel β-sheet conformation.

Many of studies for inhibition of Aβ fibril formation have been done. Based on the fact that the major force driving Aβ aggregation is hydrophobicity, Murphy and colleagues have added charged residues to the ends of the recognition motif as a disrupting element[40]. Having shown that at least three lysines are required as an appropriate disrupting element, the compound (KLVFFKKKK) showed activity in altering fibril morphology and reducing cellular toxicity *in vitro* suggesting that the charged nature of the disrupting element is critical[41]. Another approach to produce peptide inhibitors have been reported by Findeis *et al.*[42,43]. Their strategy was to retain a peptide sequence that could bind to Aβ and add a bulky group, such as a steroid, at its terminus to hinder Aβ polymerization. The all-D-amino acid peptide cholyl-LVFFA-OH was shown to be a potent inhibitor of Aβ polymerisation, but was cleared up almost completely upon hepatic first pass, possibly because the cholyl group was recognised as an endogenous bile component. Several other molecules with different chemical groups for improved pharmacological properties of inhibition of Aβ fibril formation are under development.

A few reports have been focused on the interactions between Aβ and surfactant molecules. Three micelle systems, the negatively charged utilized sodium do-
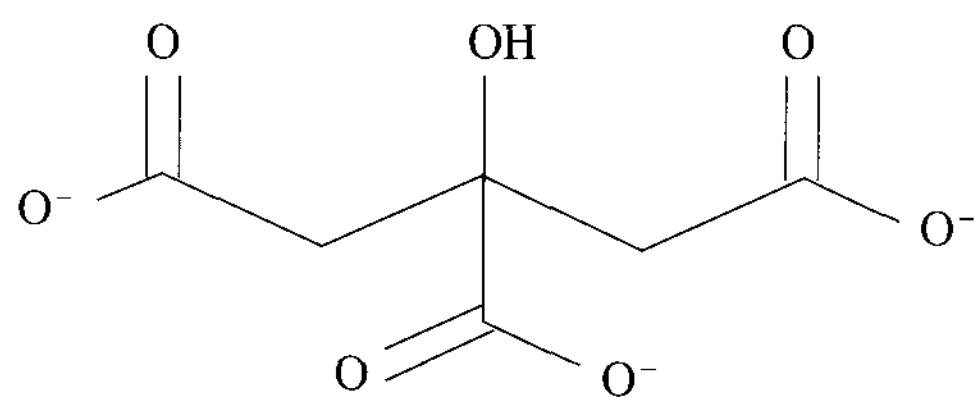
Figure 3. Molecular structure of three-valent citrate anion in physiological buffer solution (pH 7.4).

decyl sulfate, the neutral zwitterionic dodecylphosphochline, and the positively charged dodecyltrimethylammonium chloride, in heterogeneous amphiphilic environment have been utilized to interact with Aβ peptide fragments[31]. Their results showed that the promotion and the stabilization of α-helix secondary structure were highly correlated with the availability of the surfactant's charged surface, indicating the significant role of electrostatic forces in the interaction between Aβ and surfactants. Citrate is the low molecular weight organic acid carrying three valent anionic carboxyl groups (Figure 3). Therefore, in aqueous phase, citrate shows the high charge characteristics. It has been used as non-soap, oganic anionic surfactant. Furthermore, this is widely available and economical, and generally recognized as safe material[44,45]. Moreover, citrate, as the first Krebs cycle intermediate, has antioxidant properties due to its ability to chelate iron by forming inactive complexes at physiological range of concentrations[46].

Considering plasma citrate concentration ranges from 70 to 700 μM[47] our results demonstrated that citrate could have the anti-amyloidogenic and neuroprotective effects at supra-physiological range of concentrations while antioxidant effect of citrate was at physiological concentrations. This finding supports that the future therapeutic use of citrate might be considered.

The mechanisms that underlie the anti-amyloidogenic effect of citrate are still unclear, but as for the possible inhibitory mechanisms of citrate, we speculate that citrate might have two effects, salting-in and surfactant effects. The surfactant effect of citrate could provide a possible explanation for inhibition of fibril formation, based on the fact that the major force driving Aβ aggregation is hydrophobicity. Putative binding of citrate through hydrophobic backbone to a hydrophobic region of Aβ seems to block associations between Aβ molecules by encapsulating hydrophobic portions of Aβ and forming micelle, thereby increasing solubility and inhibiting fibril formation of Aβ. Wang *et al.* have indicated the anti-amyloidogenic activity of the amphiphilic surfactants, 1,2-dihexano-

yl-*sn*-glycero-3-phosphocholine (3-di-C6-PC) and 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine (di-C7-PC), in physiological condition[32]. Our experiments showed that citrate inhibited Aβ fibril formation at physiological pH. Inhibitory action of citrate on fibrillization of Aβ$_{25-35}$ in citrate-treatment conditions, was significant, being compared with Aβ$_{25-35}$ alone (Figure 1). Further verification of the exact mechanism of Aβ-surfactant interaction needs to be investigated. Another possible mechanism is salting-in (solubilizing) effect of citrate. The salt ions change the chemical potential of the macromolecule. According to the Hofmeister series, there are two species of anions, kosmotropes and chaotropes. The kosmotropes are strongly hydrated and have stabilizing and salting-out effects on proteins and macromolecules. On the other hand, chaotropes are known to destabilize folded proteins and give rise to salting-in or solubilizing behavior[48]. The effect of the anions could be explained on the basis of three direct interactions of the anions with the macromolecules. First, kosmotropic anions were found to polarize water molecules that were directly hydrogen bonded with the amide moieties of macromolecule. Second, both chaotropes and kosmotropes could increase the cost of hydrophobic hydration (i.e. raise the surface tension of the polymer/water interface). Third, chaotropic anions could bind directly to the side-chain amide moieties. The first and second of these interactions led to the salting-out (aggregation) of the polymer, whereas the third effect causes the polymer to salt-in[49]. It is possible that citrate, as a chaotropic anion, have exerted the solubilizing effect through the third effect. While citrate would be an important molecule for therapeutic strategy, the mechanism of anti-amyloidogenic activity is unknown so a molecular approach to these interactions is required. We suggest surfactant and salting-in effects as the possible mechanisms that underlie the anti-amyloidogenic effect of citrate. The neuroprotective effect of citrate against Aβ-induced toxicity *in vivo* needs to be investigated and the ability of Aβ fibril formation inhibitors to suppress the progression of Alzheimer's disease in humans needs to be evaluated.

Our findings have demonstrated that citrate inhibited Aβ fibril formation and reduced Aβ-induced toxicity. The concept of Aβ fibril formation inhibitors provides a potentially general strategy to treat amylodogenic diseases. A decrease in the occurrence of AD is a major treatment goal for patients, families, and society. We hope that citrate, as one of the inhibitors of Aβ fibril formation with antioxidant and neuroprotective effects, will prove to be useful therapeutic agents to prevent or retard amyloidogenesis in AD.

Materials and Methods

Synthetic Peptides and Citrate

Peptides $A\beta_{25-35}$ and citrate were purchased from Sigma (Saint Louis, MO, USA) and were used without further purification. T he sequence of $A\beta_{25-35}$ peptide is GSNKGAIIGLM, corresponding to residues of the human wild type sequence $A\beta_{1-40}$.

Preparation of Solutions

Stock solution of 1 mM was prepared by solubilizing the lyophilized $A\beta_{25-35}$ peptide by briefly vortexing in sterile water at 4°C, then by sonication for 1 min. The sterilized peptide stock solution was aliquoted and stored at -20°C. The citrate stock solution was neutralized to get pH 7.2 and filtered with 0.2 micromiter syringe filter. The citrate stock solution was aliquoted and stored at -20°C.

Thioflavin T Fluorescence (ThT) Assay of $A\beta_{25-35}$ Fibrillization

Experiments were carried out by using the reaction mixtures containing 80 μL phosphate buffer (10 mM final concentration) with 10 μL citrate (1, 2.5, and 5 mM final concentration) and 10 μL $A\beta_{25-35}$ (100 μM final concentration), pH 7.2. Moreover, one sample containing $A\beta_{25-35}$ alone and another containing several concentrations of citrate alone were used in parallel as control in the same experimental conditions. All reactions were carried out at 37°C for 7 days. The fluorescence intensities of Th-T dye of fibril formation in several experiments were measure by Perkin-Elmer LS 55 Fluorescence Spectrometer (Waltham, Massachusetts, USA) as described elsewhere[32]. 100 μL of Th-T dye solution (1 mM in phosphate buffered saline, pH 7.4, Sigma, Saint Louis, MO, USA) was added to 20 μL of the $A\beta_{25-35}$ reaction solutions. Then the fluorescence intensities of the reaction mixture were measured at the excitation and emission wavelengths of 450 and 485 nm, respectively. For clearly estimating of inhibitory effect of citrate on $A\beta_{25-35}$ fibrillization, the $A\beta_{25-35}$ absorption spectra of different concentrations of citrate were subtracted with the fluorescence spectra of citrate alone, respectively. At least three independent measurements were made for all cases. All results are presented with means and standard deviation.

PC12 Cell Cultures and Aβ-induced Toxicity

Rat PC12 cells were sustained in Dulbecco's modified Eagle's medium (Gibco, NY, USA) with 4.5 g/L D-glucose, 2 mM L-Glutamine and 110 mg/L sodium pyruvate supplemented with 10% fetal bovine serum (FBS) and antibiotic-antimycotic (10,000 u/mL penicillin G sodium, 10,000 μg/mL streptomycin sulfate and 25 μg/mL amphotericin B as Fungizone® in 0.85% saline) (Gibco, NY, USA) in a humidified 5% CO_2/ 95% O_2 atmosphere at 37°C. The culture medium was changed every two days. For WST-1 assay, the cells were plated at a density of 3×10^4 cells/well in 48-well plates and incubated for 24 h. At the end of incubation period, cells were treated with $A\beta_{25-35}$ (50 μM final concentration) preincubated in the absence or presence of citrate at 37°C for 7 days. Controls were not treated with both citrate and $A\beta_{25-35}$.

Measurement of Cell Viability

Cell viability was evaluated 24 h later using the 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1 reagent from Roche Diagnostics, Mannheim, Germany) assay, as described previously[50]. The measurement is based on the ability of viable cells to cleave tetrazolium salts by mitochondrial dehydrogenases. The optical density (OD) was determined at 450 nm using a microplate reader (Multiskan EX Thermo-Fisher scientific Waltham, MA, USA).

Statistical Analyses

The results shown represent the mean ± standard deviation from three experiments (n=3). Mann-Whitney's U-test was used to compare $A\beta_{25-35}$ alone with citrate-treated $A\beta_{25-35}$ groups or control (cell).

Acknowledgements

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