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Inhibition of β-amyloid$_{1-40}$ Peptide Aggregation and Neurotoxicity by Citrate

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The accumulation of β-amyloid (Aβ) aggregates is a characteristic of Alzheimer's disease (AD). Furthermore, these aggregates have neurotoxic effects on cells, and thus, molecules that inhibit Aβ aggregate formation could be valuable therapeutics for AD. It is well known that aggregation of Aβ depends on its hydrophobicity, and thus, in order to increase the hydrophilicity of Aβ, we considered using citrate, an anionic surfactant with three carboxylic acid groups. We hypothesized that citrate could reduce hydrophobicity and increase hydrophilicity of Aβ$_{1-40}$ molecules via hydrophilic/electrostatic interactions. We found that citrate significantly inhibited Aβ$_{1-40}$ aggregation and significantly protected SH-SY5Y cell line against Aβ$_{1-40}$ aggregates-induced neurotoxicity. In details, we examined the effects of citrate on Aβ$_{1-40}$ aggregation and on Aβ$_{1-40}$ aggregates-induced cytotoxicity, cell viability, and apoptosis. Th-T assays showed that citrate significantly inhibited Aβ$_{1-40}$ aggregation in a concentration-dependent manner (Th-T intensity: from 91.3% in 0.01 mM citrate to 82.1% in 1.0 mM citrate vs. 100.0% in Aβ$_{1-40}$ alone). In cytotoxicity and viability assays, citrate reduced the toxicity of Aβ$_{1-40}$ in a concentration-dependent manner, in which the cytotoxicity decreased from 107.5 to 102.3% as compared with Aβ$_{1-40}$ aggregates alone treated cells (127.3%) and the cell viability increased from 84.6 to 93.8% as compared with the Aβ$_{1-40}$ aggregates alone treated cells (65.3%). Furthermore, Hoechst 33342 staining showed that citrate (1.0 mM) suppressed Aβ$_{1-40}$ aggregates-induced apoptosis in the cells. This study suggests that citrate can inhibit Aβ$_{1-40}$ aggregation and protect neurons from the apoptotic effects of Aβ$_{1-40}$ aggregates. Accordingly, our findings suggest that citrate administration should be viewed as a novel neuroprotective strategy for AD.

Key Words: Citrate, Alzheimer's disease, β-amyloid, Aggregation, Apoptosis

INTRODUCTION

The accumulation of β-amyloid (Aβ) peptides in senile plaque represents a major pathological change in the AD brain. Aβ is generated by the sequential proteolytic processing of amyloid precursor protein by β- and γ-secretases, and is known to promote pro-inflammatory responses and to activate neurotoxic pathways (Selkoe, 2001; Sisodia and St George-Hyslop, 2002; Pereira et al., 2005). The main therapeutic target in AD is Aβ, and devised anti-Aβ agents are known to primarily affect the production and accumulation of Aβ and to block toxic Aβ forms (Seiffert et al., 2000; Scarpini et al., 2003; Citron, 2004). Neuroprotective agents represent another drug class, and include antioxidants and anti-inflammatory agents, and neurorestorative agents and procedures represent another, and include nerve growth factors, transplantation, and stem-cell related therapy (McConnell and Riggs, 2005; Cummings et al., 2007). It has been reported that soluble Aβ oligomers are more toxic than monomeric and fibrillar Aβ forms (Rochet and Lansbury, 2000). For this reason, molecules that can inhibit Aβ aggregates formation could be of therapeutic value. The Aβ molecules are amphiphilic peptides that possess a hydrophilic region (N-terminal) and a hydrophobic region (C-terminal), and can self-assemble to form aggregates with various morphologies, such as, dimers, oligomers, filaments, protofibrils, and fibrils (Koh and Yang, 1990; Pike et al., 1993; Howlett et al., 1995; Seilheimer et al., 1999; Watanabe et al., 2001). Furthermore, Aβ aggregations are driven by intermolecular hydrophobic and electrostatic interactions (Jarrett et al., 1993; Wood et al., 1996b; Lazo et al., 2005; Luhrs et al., 2005; Khandogin and Brooks, 2007).

Hence, many materials have been studied to modulate Aβ aggregate formation. Small molecules like Congo red and small sulfonated anions have been shown to prevent Aβ aggregation or inhibit Aβ-related toxicity (Lorenzo and

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ABBREVIATIONS: AD, Alzheimer's disease; Th-T, thioflavin-T; CMC, critical micelle concentration; HMP, hexadecyl-N-methyl-piperidinium.

Yankner, 1994; Kisilevsky et al., 1995). Melatonin, nicotine, and estrogen have also been found to inhibit Aβ aggregation in a similar way (Salomon et al., 1996; Mook-Jung et al., 1997; Pappolla et al., 1998). A small number of surfactants have been found to inhibit Aβ aggregation by binding to its hydrophobic region, which is necessary for its self-assembly (Lomakin et al., 1996; Marcinowski et al., 1998; Wang et al., 2005). In particular, one of the various types of surfactants, citrate is a particularly appealing surfactant (Brus, 2008) because of its small size and high anionic charge density. Using Aβ peptide fragment 25-35 (Aβ_{25-35}), we previously showed that citrate inhibits Aβ aggregation by fluorescence spectroscopy using thioflavin-T (Th-T) (Yang et al., 2008). Furthermore, Aβ_{25-35} peptides have the same aggregation and neurotoxic properties of full-length Aβ and have the experimental advantage of rapidly aggregating from solution (Pike et al., 1995). However, Aβ_{25-35} does not exist in the brain, which limits the clinical meaning results. Thus, in the present study, we used full length Aβ peptide (Aβ_{1-40}), which constitutes about 90% of the most abundant cleaved form of larger amyloid precursor protein (APP), and which has been shown to have a toxic effect in the AD brain (Cao et al., 2007).

In this study, we investigated the inhibitory effect of citrate on Aβ_{1-40} aggregation and Aβ_{1-40} aggregates-induced cell damage and death in SH-SY5Y cell line.

METHODS

Synthetic peptides and citrate

Aβ_{1-40} TFA was purchased from rPeptide (Athens, Georgia, USA) and citrate was purchased from Sigma (Saint Louis, MO, USA), both were used without further purification.

Preparation of solutions

The stock solution of 0.5 mg/ml Aβ_{1-40} peptide was prepared by solubilizing lyophilized Aβ_{1-40} peptide by briefly vortexing in sterilized 0.6 mM NaOH solution for 5 min at room temperature and sonicating for 1 min at 4°C. The peptide stock solution was aliquoted and stored at −20°C. Citrate stock solution was neutralized to pH 7.4 and filtered through a 0.2 μm syringe filter, and it too was aliquoted and stored at −20°C.

Thioflavin-T (Th-T) fluorescence assays of Aβ_{1-40} aggregation

Experiments were carried out at pH 7.4 using reaction mixtures containing 80 μl phosphate buffer (5 mM final concentration) with 10 μl citrate (0.01, 0.1, and 1.0 mM final concentration) and 10 μl Aβ_{1-40} (20 μM final concentration). In details, samples containing Aβ_{1-40} alone or Aβ_{1-40} plus different concentrations of citrate, were used. All reactions were carried out at 37°C for 6 days. The fluorescence intensities of Th-T dye, which represents the amount of Aβ_{1-40} aggregation, were measured using a PerkinElmer LS 55 Fluorescence Spectrometer (Waltham, Massachusetts, USA). 150 μl of 5 μM Th-T dye solution (in 50 mM sodium phosphate buffer, pH 6.0) was added to 20 μl of the Aβ_{1-40} reaction solutions. Fluorescence intensities of reaction mixtures were then measured at excitation and emission wavelengths of 450 and 490 nm, respectively.

SH-SY5Y cell culture and Aβ_{1-40}-induced toxicity

SH-SY5Y cell line was sustained in a 1 : 1 mixture of Dulbecco's modified Eagle's medium and Ham's 12 medium supplemented with 10% fetal bovine serum 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. Culture media were changed every two days. For WST-1 and LDH assays, the cell line was plated at a density of 1×10^4 cells/well in 96-well plates and incubated for 24 h. They were then treated for 3 days with the solutions of Aβ_{1-40} (20 μM final concentration) in the absence or presence of different concentrations of citrate preincubated at 37°C for 6 days.

Cell viability measurements

Cell viabilities were evaluated using WST-1 assays, which measure the combined activity of intramitochondrial and extramitochondrial dehydrogenases. Briefly, tetrazolium salts are cleaved by the dehydrogenases of viable cells to produce formazan which is detected spectrophotometrically. Dehydrogenase activities were assayed in collagen-coated 96 well-plates (density of 1×10^4 cells/well). The cells were exposed to the solutions of Aβ_{1-40} (20 μM) with/without various concentrations of citrate (0.01, 0.1 and 1.0 mM) preincubated for 6 days. After incubation for 3 days, media were removed and WST-1 reagent (diluted 1/10 with medium) was added. The cells were then incubated in a gassed atmosphere (5% CO_2) for 3 hours. After vibrating plates, the changes in absorbance were measured using a Biotek Synergy 2 Multi-Detection Microplate Reader (Vermont, USA) at wavelength 450 nm.

Results are expressed as percentages of WST-1 reduced by assuming that the absorbance of (no-drug treated) control cell was 100%.

Measurement of cell damage

SH-SY5Y cell membrane integrities (cell damage) in culture were determined using a colorimetric lactate dehydrogenase (LDH) assay (TOX-7; Sigma-Aldrich Co, MO, USA) according to the manufacturer's instructions. This assay measures membrane integrity as function of the amount of cytoplasmic LDH released into culture medium. Briefly, the assay mixture was prepared by mixing equal amounts of LDH assay substrate, cofactor, and dye solutions. Culture media were added to LDH assay mixtures and incubated for 30 min at room temperature in the dark; the color reaction was stopped by adding 1 M HCl. Absorbances were measured at 490 nm using a Biotek Synergy 2 Multi-Detection Microplate Reader (Vermont, USA). Background correction was performed at 650 nm.

Identification of apoptotic cells after Hoechst 33342 nuclei staining

The nuclear morphologies of cells were studied using Hoechst 33342 dye (a cell-permeable DNA dye). Cells with homogeneously stained nuclei were considered viable, whereas the presence of chromatin condensation and/or fragmentation was taken to indicate apoptosis (Gschwind and Huber, 1995; Lizard et al., 1995). Hoechst 33342 was

added to culture medium in a chamber slide (Lab-Tek Chamber Slide; NUNC) at a final concentration of 10 ug/ml and incubated for 1 h. The cells were then washed with phosphate buffered saline (PBS) containing 1% (w/v) paraformaldehyde, mounted on glass slides under coverslips using mounting solution (0.1 M citric acid : 0.2 M NaH_2PO_4 : glycerol=1 : 1 : 8), and stored in the dark for 30 min at 4°C. The cells were examined at 1,000×, and five random fields were chosen for each experimental condition. Hoechst 33342 stained images were obtained using a fluorescence photomicroscope (IX70; Olympus, Tokyo, Japan), with excitation centered at 350 nm and a 460 nm emission filter.

Statistical analyses

The results shown represent the means±standard deviations of three experiments. One-way analysis of variance (ANOVA) followed by Duncan's multiple-range test was used to determine whether means of treated groups were significantly different from that of (no-drug treated) control group or $A\beta_{1\text{-}40}$ aggregates alone treated group. In all cases, p values of <0.05 were deemed significant.

RESULTS

Inhibitory effect of citrate on the formation of $A\beta_{1\text{-}40}$ aggregates

In order to examine the concentration-dependency of the anti-aggregation effect of citrate on $A\beta_{1\text{-}40}$, the concentration of $A\beta_{1\text{-}40}$ was fixed at 20 μM and citrate was added at 0.01, 0.1, and 1.0 mM. After incubation for 6 days, $A\beta$ aggregation was found to have been suppressed by citrate in a concentration-dependent manner (91.3±1.7%, 87.5±4.8% and 82.1±2.7% (at 0.01, 0.1, and 1.0 mM of citrate, respectively) vs. 100±3.4% (control)) (Fig. 1). In particular, at citrate concentrations of 0.1 and 1.0 mM, $A\beta_{1\text{-}40}$ aggregation was significantly reduced ($p<0.05$).

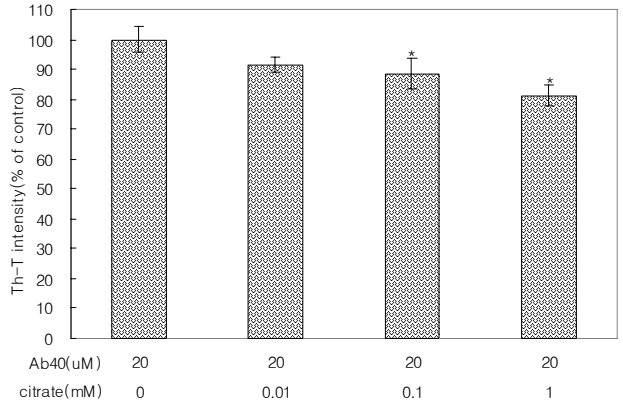

Fig. 1. The effect of citrate on aggregate formation of $A\beta_{1\text{-}40}$ was determined by Th-T fluorometric assays. $A\beta_{1\text{-}40}$ at 20 μM was incubated with citrate at 0.01, 0.1, or 1.0 mM at 37°C for 6 days. The fluorescence intensities of $A\beta_{1\text{-}40}$ aggregate formation were measured using a spectrofluorometer. Results are means±SDs of three experiments. The asterisk (*) indicates a significant ($p<0.05$) difference between treatments with $A\beta$ aggregates alone and $A\beta$ plus citrate.

Protective effect of citrate on cell viability in $A\beta_{1\text{-}40}$-treated cells

We analyzed the protective effect of citrate in the cells against the toxicity of exogenous $A\beta_{1\text{-}40}$. When SH-SY5Y cell line was exposed to 20 μM of $A\beta_{1\text{-}40}$ for 3 days, cell viability decreased as compared with that of (no-drug treated) control (65.3±5.2% vs. 100±3.6%). However, when cells were treated with the mixtures of $A\beta_{1\text{-}40}$ plus citrate (0.01, 0.1, and 1.0 mM) preincubated for 6 days, the cell viabilities improved to 84.6±3.4%, 90.8±5.4% and 93.4±3.6%, respectively (Fig. 2). As shown in Fig. 2, viabilities in the mixtures of $A\beta_{1\text{-}40}$ plus citrate treated groups were significantly greater than in $A\beta_{1\text{-}40}$ aggregates alone treated group, and citrate was found to have a concentration-dependent protective effect ($p<0.05$).

Inhibitory effect of citrate on cell damage induced with $A\beta_{1\text{-}40}$ aggregates

When exposed to 20 μM $A\beta_{1\text{-}40}$ aggregates alone for 3 days, SH-SY5Y cell line showed the approx. 30% increase in LDH release, which is indicative of cytotoxic damage. However, when the cells were treated with $A\beta_{1\text{-}40}$ plus citrate of 0.01, 0.1, or 1.0 mM final concentrations preincubated for 6 days, LDH releases of these were significantly reduced in a concentration-dependent manner as compared with $A\beta_{1\text{-}40}$ aggregates alone treated group (107.5±3.4%, 103.9±4.6% and 102.3±2.3%, respectively, vs. 127.3±6.7%) ($p<0.05$) (Fig. 3), showing that citrate protected SH-SY5Y cell line from $A\beta_{1\text{-}40}$ aggregates-induced cytotoxicity.

Inhibitory effect of citrate on apoptosis induced with $A\beta_{1\text{-}40}$ aggregates

To study the cytoprotective effect of citrate on apoptosis induced with $A\beta_{1\text{-}40}$ aggregates, nuclei of SH-SY5Y cells were stained with Hoechst 33342 for fluorescence microscopy. Hoechst 33342 is a DNA-binding dye that quantitatively stains the DNA of living cells. Briefly, the cells were treated with 20 μM of $A\beta_{1\text{-}40}$ aggregates coincubated with/

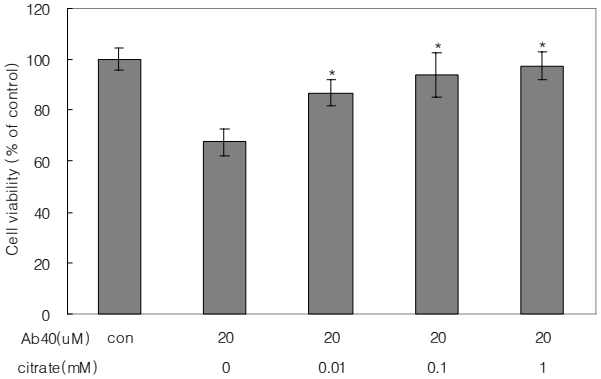

Fig. 2. The effect of citrate on the viability of SH-SY5Y cell line. The cells were treated for 3 days with the solution of 20 μM $A\beta_{1\text{-}40}$ with/without citrate preincubated at 37°C for 6 days. Results are means±SDs of three experiments. The asterisk (*) indicates a significant ($p<0.05$) difference between treatment with $A\beta$ alone and treatment with $A\beta$ plus citrate.

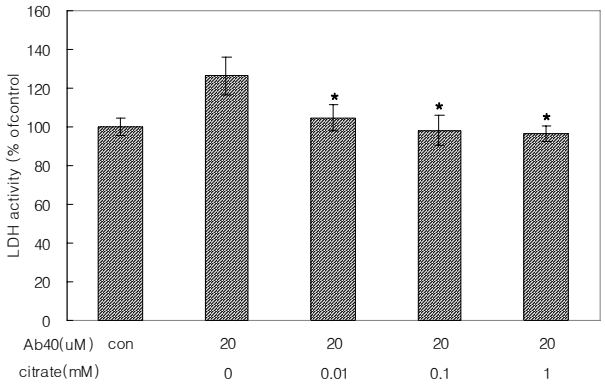

Fig. 3. The protective effect of citrate on $A\beta_{1\text{-}40}$ aggregates-induced neuronal damage of SH-SY5Y cell line. The cells were treated for 3 days with the solution of 20 μM $A\beta_{1\text{-}40}$ with/without citrate preincubated at 37°C for 6 days. Results are means±SDs of three experiments. The asterisk (*) indicates a significant ($p<0.05$) difference between treatment with $A\beta$ alone and treatment with $A\beta$ plus citrate.

without 1.0 mM citrate for 6 days. Microscopic images (Fig. 4) showed that the (no-drug treated) control group showed the large intact nuclei but that $A\beta_{1\text{-}40}$ aggregates alone treated cells displayed significant nuclear fragmentation and chromatin condensation (characteristic features of apoptosis). However, the cells treated with the mixtures of $A\beta_{1\text{-}40}$ plus citrate had larger nuclei and showed weaker staining than $A\beta_{1\text{-}40}$ aggregates alone treated group.

DISCUSSION

In this study to reduce $A\beta$ aggregation, we added citrate, an anionic surfactant (Brus, 2008), to $A\beta$ containing solutions and incubated for 6 days, and it was observed that $A\beta_{1\text{-}40}$ aggregation was significantly suppressed. Furthermore, $A\beta_{1\text{-}40}$ aggregates-induced cell damage was found to be significantly reduced and the viabilities of $A\beta_{1\text{-}40}$ aggregates-treated cells were significantly improved by citrate.

Several studies have indicated that $A\beta$ monomer must aggregate to polymeric or fibrillar form before it becomes

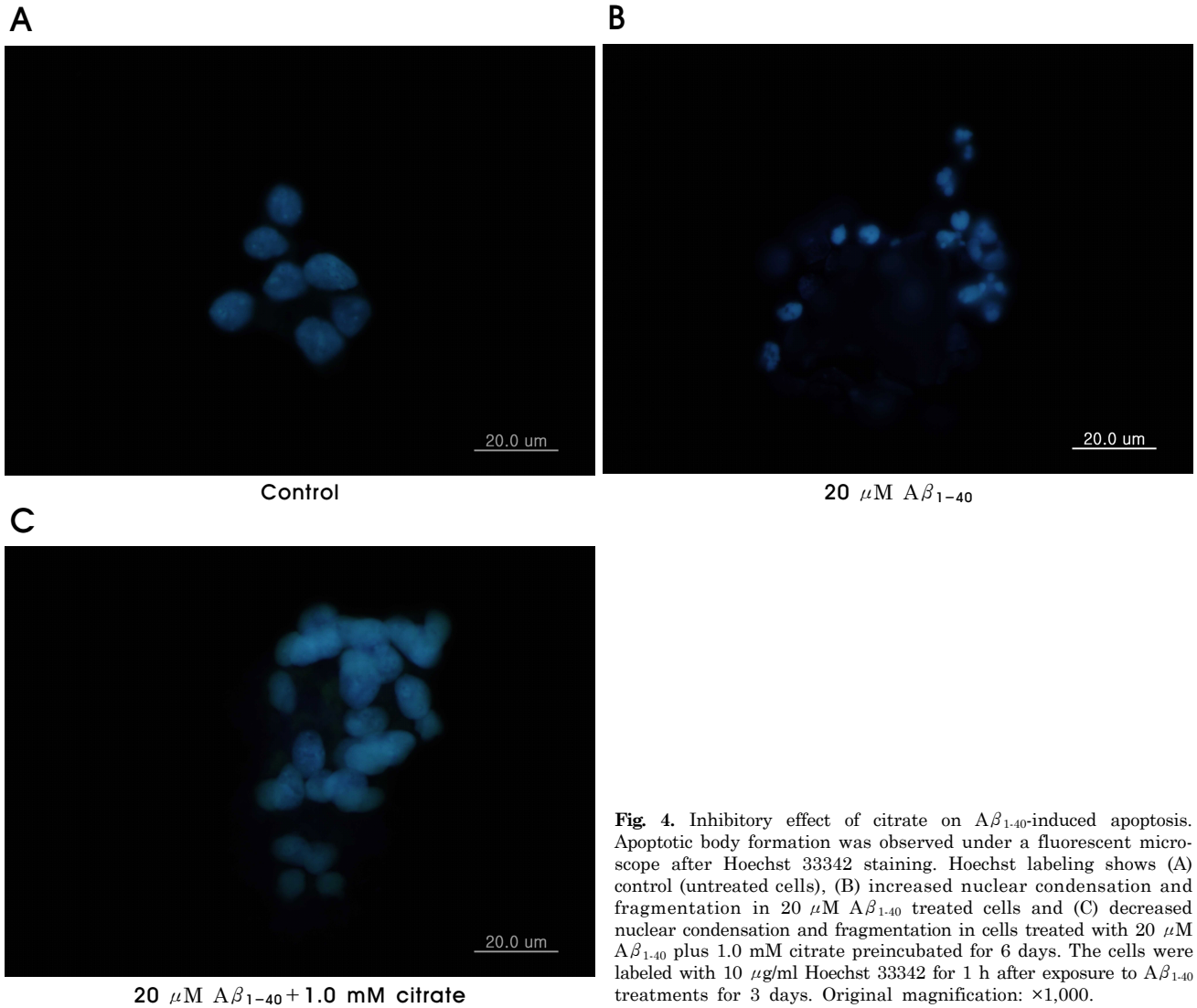

Fig. 4. Inhibitory effect of citrate on $A\beta_{1\text{-}40}$-induced apoptosis. Apoptotic body formation was observed under a fluorescent microscope after Hoechst 33342 staining. Hoechst labeling shows (A) control (untreated cells), (B) increased nuclear condensation and fragmentation in 20 μM $A\beta_{1\text{-}40}$ treated cells and (C) decreased nuclear condensation and fragmentation in cells treated with 20 μM $A\beta_{1\text{-}40}$ plus 1.0 mM citrate preincubated for 6 days. The cells were labeled with 10 μg/ml Hoechst 33342 for 1 h after exposure to $A\beta_{1\text{-}40}$ treatments for 3 days. Original magnification: ×1,000.

toxic to neurons in culture (Pike et al., 1991), and soluble Aβ oligomers are known to be more toxic than monomeric or fibrillar Aβ (Rochet and Lansbury, 2000).

Amyloid aggregation, is a complex process that can be affected by various extrinsic or environmental factors, such as, pH, peptide concentration, solvent hydrophobicity, temperature, ionic strength, and metal ions (Halverson et al., 1990; Barrow and Zagorski, 1991; Bush et al., 1994; Snyder, 1994; Sabate´ and Estelrich, 2005a). Hydrophobic and electrostatic interactions between Aβ molecules play pivotal roles in Aβ aggregation. According to Jarrett et al. (1993), the aggregation of Aβ peptides into amyloid fibrils is driven by hydrophobic interactions between highly apolar residues. Furthermore, it was found that replacing hydrophobic for hydrophilic residues in the internal hydrophobic region of Aβ can hinder aggregate formation, which suggested that Aβ aggregation is driven at least in part by hydrophobic interactions (Pike et al., 1995). In addition to hydrophobic forces, electrostatic interactions between residues also have a significant effect on the formation of Aβ aggregates (Lazo et al., 2005; Luhrs et al., 2005; Khandogin and Brooks, 2007). Several reports have concluded that positively charged Lys (28) within Aβ peptide plays a critical role in stabilizing the β-turn of Aβ, and thus, induces the growths of amyloid fibrils due intermolecular electrostatic interactions between negatively charged Glu (22) and Asp (23). In addition, at pH 7.4, both hydrophobic and electrostatic interactions are required to stabilize fibril structures during the aggregation of $A\beta_{1\text{-}40}$ (Wood et al., 1996b). Under physiological conditions, human $A\beta_{1\text{-}40}$ possesses several amino acid residues, namely, six cationic residues (three histidines, one arginine, and two lysines), six anionic residues (three aspartates, three glutamates) and 23 hydrophobic residues (three alanines, three phenylalanines, six glycines, six valines, two leucines, two isoleucines, and one methionine). Hence, human $A\beta_{1\text{-}40}$ is amphiphilic, meaning that possess both hydrophobic and hydrophilic groups, like surfactants (Soreghan et al., 1994; Ji et al., 1995; Bokvist et al., 2004). Many reagents have been developed to modulate the aggregation of Aβ peptide (Pallitto et al., 1999; Lowe et al., 2001; Nowick et al., 2002; Ban et al., 2004), and, surfactants belong to one important category of the reagents.

In terms of anionic surfactants, several studies have investigated on the roles of sodium dodesyl sulfate (SDS) on the Aβ fibrillogenesis. Rangachari et al. found over a narrow range of SDS concentrations that SDS accelerated aggregation (Rangachari et al., 2006). Jeng et al. (2006) found that SDS could inhibit fibril formation by $A\beta_{1\text{-}40}$ via the formation of a small peptide/SDS complex in aqueous solutions (Jeng et al., 2006), and this inhibitory effect is enhanced by SDS micelles when a larger SDS concentration is used.

Regarding cationic surfactants, Sabate´ and Estelrich in a study of interactions between $A\beta_{1\text{-}40}$ and three alkylammonium bromides that the surfactants promoted $A\beta_{1\text{-}40}$ aggregation below the critical micelle concentration (CMC), but that the presence of micelles delayed aggregation (Sabate´ and Estelrich, 2005b). Wood et al. (1996a) compared the effects of several surfactants on the aggregation of Aβ peptide and found that hexadecyl-*N*-methylpiperidinium (HMP) bromide selectively inhibited Aβ fibril formation (Wood et al., 1996a). Moreover, Sabate´ et al. (2003) and Wang et al. (2005) have demonstrated that cationic surfactants inhibit Aβ fibril formation in a concentration-dependent manner (Sabate´ et al., 2003; Sabate´ and Estelrich, 2005b; Wang et al., 2005).

Studies on the interaction(s) between surfactant molecules and Aβ species and their aggregates have shown that the promotion and the stabilization of the α-helix secondary structure are highly correlated with the availability of charge on the surfactant surface, which suggests that electrostatic forces play a substantial role in the interaction between Aβ species and the surrounding aqueous medium and its constituents (Wang et al., 2005). Therefore, in order to suppress the aggregation of $A\beta_{1\text{-}40}$, we selected citrate, an anionic surfactant which possesses three carboxylic acid groups (Brus, 2008).

Although the mechanisms responsible for the anti-aggregating effect of citrate have yet to be elucidated, we speculate that citrate inhibits Aβ aggregation by interacting with the amino acid residues of $A\beta_{1\text{-}40}$. According to this suggestion, the anionic carboxyl groups of citrate ion-pair with cationic residues of Aβ, and leave its anionic charges intact, which may hinder its aggregation. Moreover, the hydrophobic parts of citrate could coat the hydrophobic parts of Aβ and convert these regions of Aβ into charged areas. As a consequence, this disruption of the dispositions of surface charges and hydrophobic regions of Aβ molecules may in turn disrupt the aggregation mechanism.

Based on the above possibility, we examined whether $A\beta_{1\text{-}40}$ aggregation depends on the ratios of charges in the hydrophobic parts and hydrophilic parts of $A\beta_{1\text{-}40}$ and charge on citrate. Human $A\beta_{1\text{-}40}$ contains six cationic amino acids (three histidines, one arginine, and two lysines) and six anionic amino acids (three aspartates and three glutamates). In the experiment conducted, $A\beta_{1\text{-}40}$ (20 μM) contained 120 μM cationic and 120 μM anionic amino acid residues, and thus, citrate (10 μM) contained less anionic groups than would be required to neutralize the cationic residues of $A\beta_{1\text{-}40}$. We believe that one of three anionic groups of citrate binds to one cationic residues of $A\beta_{1\text{-}40}$, and thus, leaves two anionic groups exposed to medium. If this were the case, citrate would increase the number of charges carried by $A\beta_{1\text{-}40}$, improving the hydrophilicity and solubility of $A\beta_{1\text{-}40}$. When citrate is present at 100 μM, its anionic groups are three-times that required to neutralize $A\beta_{1\text{-}40}$ cationic residues. Thus, one third of the anionic groups of citrate could neutralize all $A\beta_{1\text{-}40}$ cationic residues, and the free citrate anionic groups would increase the net charge and hydrophilicity of $A\beta_{1\text{-}40}$. Hence, 100 μM citrate would be expected to increase the solubility of $A\beta_{1\text{-}40}$, and 100 μM citrate would be expected to inhibit $A\beta_{1\text{-}40}$ aggregation more than citrate at 10 μM, and similarly 1 mM citrate would be expected to have a greater effect. The observed concentration-dependent suppression of $A\beta_{1\text{-}40}$ aggregation by citrate suggests that citrate significantly reduced $A\beta_{1\text{-}40}$ aggregates-induced cytotoxicity and cell death via an apoptotic process in SH-SY5Y cells (Fig. 2, 4). Meanwhile, citrate has antioxidant activity (Puntel et al., 2007), which may partially contribute to inhibit $A\beta_{1\text{-}40}$ aggregates-induced cell damage and apoptosis because $A\beta_{1\text{-}40}$ aggregates-induced toxicities proceed via oxidative stress (Hensley et al., 1994).

Taken together, this study suggests that citrate has an inhibitory effect on the aggregation of $A\beta_{1\text{-}40}$, which can protect neurons from $A\beta_{1\text{-}40}$ aggregates-induced apoptosis. Our findings suggest that citrate should be viewed as a potential drug candidate for the treatment of AD.

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REFERENCES

Ban T, Hoshino M, Takahashi S, Hamada D, Hasegawa K, Naiki H, Goto Y. Direct observation of Abeta amyloid fibril growth and inhibition. *J Mol Biol* 344: 757−767, 2004.

Barrow CJ, Zagorski MG. Solution structures of beta peptide and its constituent fragments: relation to amyloid deposition. *Science* 253: 179−182, 1991.

Bokvist M, Lindström F, Watts A, Gröbner G. Two types of Alzheimer's beta-amyloid (1-40) peptide membrane interactions: aggregation preventing transmembrane anchoring versus accelerated surface fibril formation. *J Mol Biol* 335: 1039−1049, 2004.

Brus L. Noble metal nanocrystals: plasmon electron transfer photochemistry and single-molecule raman spectroscopy. *Acc Chem Res* 41: 1742−1749, 2008.

Bush AI, Pettingell WH, Multhaup G, d Paradis M, Vonsattel JP, Gusella JF, Beyreuther K, Masters CL, Tanzi RE. Rapid induction of Alzheimer Abeta amyloid formation by zinc. *Science* 265: 1464−1467, 1994.

Cao M, Han Y, Wang J, Wang Y. Modulation of fibrillogenesis of amyloid β (1-40) peptide with cationic gemini surfactant. *J Phys Chem B* 111: 13436−13443, 2007.

Citron M. Strategies for disease modification in Alzheimer's disease. *Nat Rev Neurosci* 5: 677−685, 2004.

Cummings JL, Doody R, Clark C. Disease-modifying therapies for Alzheimer disease: challenges to early intervention. *Neurology* 69: 1622−1634, 2007.

Gschwind M, Huber G. Apoptotic cell death induced by beta-amyloid 1-42 peptide is cell type dependent. *J Neurochem* 65: 292−300, 1995.

Halverson K, Fraser PE, Kirschner DA, Lansbury PT Jr. Molecular determinants of amyloid deposition in Alzheimer's disease: conformational studies of synthetic beta-protein fragments. *Biochemistry* 29: 2639−2644, 1990.

Hensley K, Carney JM, Mattson MP, Aksenova MV, Harris ME, Wu JF, Floyd RA, Butterfield DA. A model for β-amyloid aggregation and neurotoxicity based on free radical generation by the peptide: relevance to Alzheimer disease. *Proc Natl Acad Sci USA* 91: 3270−3274, 1994.

Howlett DR, Jennings KH, Lee DC, Clark MS, Brown F, Wetzel R, Wood SJ, Camilleri P, Roberts GW. Aggregation state and neurotoxic properties of Alzheimer beta-amyloid peptide. *Neurodegeneration* 4: 23−32, 1995.

Jarrett JT, Berger EP, Lansbury Jr. PT. The carboxy terminus of the β-amyloid protein is critical for the seeding of amyloid formation: implication for the pathogenesis of Alzheimer's disease. *Biochemistry* 32: 4693−4697, 1993.

Jeng US, Lin TL, Lin JM, Ho DL. Contrast variation SANS for the solution structure of the β-amyloid peptide$_{1\text{-}40}$ influenced by SDS surfactants. *Physica B* 385−386: 865−867, 2006.

Ji SR, Wu Y, Sui SF. Study of beta-amyloid peptide (Abeta40) insertion into phospholipid membranes using monolayer technique. *Biochemistry (Mosc)* 67: 1283−1288, 2002.

Khandogin J, Brooks III CL. Linking folding with aggregation in Alzheimer's beta-amyloid peptides. *Proc Natl Acad Sci USA* 104: 16880−16885, 2007.

Kisilevsky R, Lemieux LJ, Fraser PE, Kong X, Hultin PG, Szarek WA. Arresting amyloidosis in vivo using small-molecule anionic sulphonates or sulphates: implications for Alzheimer's disease. *Nat Med* 1: 143−148, 1995.

Koh JY, Yang LLY, Cotman CW. Beta-amyloid protein increases vulnerability of cultured neurons to excitotoxins. *Brain Res* 533: 315−320, 1990.

Lazo ND, Grant MA, Condron MC, Rigby AC, Teplow DB. On the nucleation of amyloid beta-protein monomer folding. *Protein Sci* 14: 1581−1596, 2005.

Lizard G, Fournel S, Genestier L, Dhedin N, Chaput C, Flacher M, Mutin M, Panaye G, Revillard JP. Kinetics of plasma membrane and mitochondrial alterations in cells undergoing apoptosis. *Cytometry* 21: 275−283, 1995.

Lomakin A, Chung DS, Benedek GB, Kirschner DA, Teplow DB. On the nucleation and growth of amyloid beta-protein fibrils: Detection of nuclei and quantitation of rate constants. *Proc Natl Acad Sci USA* 93: 1125−1129, 1996.

Lorenzo A, Yankner BA. Beta-amyloid neurotoxicity requires fibril formation and is inhibited by Congo red. *Proc Natl Acad Sci USA* 91: 12243−12247, 1994.

Lowe TL, Strzelec A, Kiessling LL, Murphy RM. Structure-function relationships for inhibitors of beta-amyloid toxicity containing the recognition sequence KLVFF. *Biochemistry* 40: 7882−7889, 2001.

Lührs T, Ritter C, Adrian M, Riek-Loher D, Bohrmann B, Dobeli H, Schubert D, Riek R. 3D structure of Alzheimer's amyloid-β (1-42) fibrils. *Proc Natl Acad Sci USA* 102: 17342−17347, 2005.

Marcinowski KJ, Shao H, Clancy EL, Zagorski MG. Solution structure model of residues 1-28 of the amyloid beta peptide when bound to micelles. *J Am Chem Soc* 120: 11082−11091, 1998.

McConnell S, Riggs J. Alzheimer's research: can it help save our nation's entitlement programs? *Alzheimer Dementia* 1: 84−86, 2005.

Mook-Jung I, Joo I, Sohn S, Kwon HJ, Huh K, Jung MW. Estrogen blocks neurotoxic effects of beta-amyloid (1-42) and induces neurite extension in B103 cells. *Neurosci Lett* 235: 101−104, 1997.

Nowick JS, Lam KS, Khasanova TV, Kemnitzer WE, Maitra S, Mee HT, Liu R. An unnatural amino acid that induces beta-sheet folding and interaction in peptides. *J Am Chem Soc* 124: 4972−4973, 2002.

Pallitto MM, Ghanta J, Heinzelman P, Kiessling LL, Murphy RM. Recognition sequence design for peptidyl modulators of beta-amyloid aggregation and toxicity. *Biochemistry* 38: 3570−3578, 1999.

Pappolla M, Bozner P, Soto C, Shao H, Robakis NK, Zagorski M, Frangione B, Ghiso J. Inhibition of Alzheimer beta-fibrillogenesis by melatonin. *J Biol Chem* 273: 7185−7188, 1998.

Pereira C, Agostinho P, Moreira PI, Cardoso SM, Oliveira CR. Alzheimer's disease-associated neurotoxic mechanisms and neuroprotective strategies. *Curr Drug Targets CNS Neurol Disord* 4: 383−403, 2005.

Pike CJ, burdick D, Walencewicz AJ, Glabe CG, Cotman CW. Neurodegeneration induced by β-amyloid peptides in vitro: the role of peptide assembly state. *J Neruosci* 13: 1676−1687, 1993.

Pike CJ, Walencewicz AJ, Glabe CG, Cotman CW. Aggregation related toxicity of synthetic beta-amyloid protein in hippocampal cultures, *Eur J Pharmacol* 207: 367−368, 1991.

Pike CJ, Walencewicz-Wasserman AJ, Kosmoski J, Cribbs DH, Glabe CG, Cotman CW. Structure-activity analyses of beta-amyloid peptides: contributions of the beta 25-35 region to aggregation and neurotoxicity. *J Neurochem* 64: 253−265, 1995.

Puntel RL, Roos DH, Grotto D, Garcia SC, Nogueira CW, Rocha JB. Antioxidant properties of Krebs cycle intermediates against malonate pro-oxidant activity in vitro: a comparative study using the colorimetric method and HPLC analysis to determine malondialdehyde in rat brain homogenates. *Life Sci* 81: 51−62, 2007.

Rangachari V, Reed DK, Moore BD, Rosenberry TL. Secondary structure and interfacial aggregation of amyloid-beta (1-40) on sodium dodecyl sulfate micelles. *Biochemistry* 45: 8639−8648, 2006.

Rochet JC Jr, Lansbury PT. Amyloid fibrillogenesis: themes and variations. *Curr Opin Struct Biol* 10: 60−68, 2000.

Sabate´ R, Gallardo M, Estelrich J. An autocatalytic reaction as a model for the kinetics of the aggregation of beta-amyloid. *Biopolymers* 71: 190−195, 2003.

Sabaté R, Estelrich J. Evidence of the existence of micelles in the fibrillogenesis of beta-amyloid peptide. *J Phys Chem B* 109: 11027−11032, 2005a.

Sabaté R, Estelrich J. Stimulatory and inhibitory effects of alkyl bromide surfactants on beta-amyloid fibrillogenesis. *Langmuir* 21: 6944−6949, 2005b.

Salomon AR, Marcinowski KJ, Friedland RW, Zagorski MG. Nicotine inhibits amyloid formation by the beta-peptide. *Biochemistry* 35: 13568−13578, 1996.

Scarpini E, Scheltens P, Feldman H. Treatment of Alzheimer's disease: current status and new perspectives. *Lancet Neurol* 2: 539−547, 2003.

Seiffert D, Bradley JD, Rominger CM, Rominger DH, Yang F, Meredith JE Jr, Wang Q, Roach AH, Thompson LA, Spitz SM, Higaki JN, Prakash SR, Combs AP, Copeland RA, Arneric SP, Hartig PR, Robertson DW, Cordell B, Stern AM, Olson RE, Zaczek R. Presenilin-1 and -2 are molecular targets for gamma-secretase inhibitors. *J Biol Chem* 275: 34086−34091, 2000.

Seilheimer B, Bohrmann B, Bondolfi L, Müller F, Stüber D, Döbeli H. The toxicity of the Alzheimer's beta-amyloid peptide correlates with a distinct fiber morphology. *J Struct Biol* 119: 59−71, 1997.

Selkoe DJ. Alzheimer's disease: genes, proteins, and therapy. *Physiol Rev* 81: 741−766, 2001.

Sisodia SS, St George-Hyslop PH. Gamma-Secretase, Notch, Abeta and Alzheimer's disease: where do the presenilins fit in? *Nat Rev Neurosci* 3: 281−290, 2002.

Snyder SW, Ladror US, Wade WS, Wang GT, Barrett LW, Matayoshi ED, Huffaker HJ, Krafft GA, Holzman TF. Amyloid-beta aggregation: selective inhibition of aggregation in mixtures of amyloid with different chain lengths. *Biophys J* 67: 1216−1228, 1994.

Soreghan B, Kosmoski J, Glabe C. Surfactant properties of Alzheimer's Abeta peptides and the mechanism of amyloid aggregation. *J Biol Chem* 269: 28551−28554, 1994.

Wang SS, Chen YT, Chou SW. Inhibition of amyloid fibril formation of beta-amyloid peptides via the amphiphilic surfactants. *Biochim Biophys Acta* 1741: 307−313, 2005.

Watanabe K, Segawa T, Nakamura K, Kodaka M, Konakahara T, Okuno H. Identification of the molecular-interaction site of amyloid beta peptide by using a fluorescence assay. *J Pept Res* 58: 342−346, 2001.

Wood SJ, MacKenzie L, Maleeff B, Hurle MR, Wetzel R. Selective inhibition of Abeta fibril formation. *J Biol Chem* 271: 4086−4092, 1996a.

Wood SJ, Maleeff B, Hart T, Wetzel R. Physical, morphological and functional differences between ph 5.8 and 7.4 aggregates of the Alzheimer's amyloid peptide Abeta. *J Mol Biol* 256: 870−877, 1996b.

Yang HD, Son IH, Lee SS, Park YH. Protective effect of citrate against Aβ-induced neurotoxicity in PC12 cells. *Mol Cell Toxicol* 4: 157−163, 2008.

Yankner BA. Mechanisms of neuronal degeneration in Alzheimer's disease. *Neuron* 16: 921−932, 1996.