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·Original Article·

Reactive oxidative species enhance amyloid toxicity in APP/PS1 mouse neurons

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Abstract: Objective To investigate whether intracellular amyloid β (iAβ) induces toxicity in wild type (WT) and APP/PS1 mice, a mouse model of Alzheimer's disease. **Methods** Different forms of Aβ aggregates were microinjected into cultured WT or APP/PS1 mouse hippocampal neurons. TUNEL staining was performed to examine neuronal cell death. Reactive oxidative species (ROS) were measured by MitoSOX™ Red mitochondrial superoxide indicator. **Results** Crude, monomer and protofibril Aβ induced more toxicity in APP/PS1 neurons than in WT neurons. ROS are involved in mediating the vulnerability of APP/PS1 neurons to iAβ toxicity. **Conclusion** Oxidative stress may mediate cell death induced by iAβ in neurons.

Keywords: amyloid; reactive oxidative species; APP/PS1; aggregation; toxicity.

1 Introduction

Alzheimer's disease (AD) has three major pathological features: neuronal/synaptic loss, extracellular senile plaques and intracellular neurofibrillary tangles. Plaques are formed from amyloid β (Aβ) peptides, which are produced from amyloid precursor protein (APP) through β- and γ-cleavage. One major component of γ-secretase is presenilin-1 protein (PS1). The APP_{swe}/PS1 double transgenic mouse is a widely used model of AD, with a combination of the Swedish mutation of APP_{swe} and deletion of exon 9 of *PS1*[1]. In these transgenic mice, intracellular Aβ peptide (iAβ) accumulation precedes the formation of neurofibrillary tangles[2].

The accumulation of iAβ has been reported in various systems. $iA\beta_{1-42}$ accumulation has been found in the pyramidal neurons of the hippocampus and the entorhinal cortex in mild cognitive impairment and early AD brains[3-9], and occurs earlier than plaque formation[3,4,6,7,10]. iAβ accumulation has also been reported in cell culture systems[11,12] and in *APP* mutant mice that exhibit synaptic loss prior to the presence of extracellular Aβ (eAβ)[13,14]. In addition, microinjection of $A\beta_{1-42}$ into primary human neurons induces dramatic cell death through activation of p53, Bax and caspase-6[15,16]. $iA\beta_{1-42}$ also induces changes in the electrophysiological properties of primary human neurons[17] while androgen[18], estrogen[18], galanin[19] and morphine[20] protect against such toxicity.

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Like many other amyloidogenous proteins, Aβ undergoes oligomerization and fibrillation under physiological situations[21]. Recent studies have demonstrated that soluble Aβ oligomers are toxic[22,23], and further form trimers and tetramers that disrupt normal synaptic function[24] prior to synaptic loss[24]. Aβ oligomers also inhibit long-term potentiation and enhance long-term depression[25].

The present study investigated whether iAβ causes toxicity in primary neurons by delivering Aβ into the cytosol of both-wild type (WT) and APP/PS1 mouse neurons.

2 Materials and methods

2.1 Cell culture Primary neurons were cultured from newborn WT C57BL/6J or $APP_{swe}/PS1_{\Delta E9}$ mouse hippocampus. The procedures were approved by and followed the regulations of the Peking University Animal Care and Use Committee. Briefly, fresh mouse hippocampal tissue was dissociated with 0.25% trypsin (Invitrogen, Carlsbad, CA), which was then inactivated by 10% decomplemented fetal bovine serum (FBS; HyClone, Logan, UT). The mixture was triturated with a pipette, filtered through 70-μm sterilized filters, and centrifuged. The pellets were washed once with phosphate buffered saline (PBS) and once with Dulbecco's modified Eagle's medium (DMEM) containing 0.225% sodium bicarbonate, 1 mmol/L sodium pyruvate, 2 mmol/L *L*-glutamine, 0.1% dextrose, 1× antibiotic Pen-Strep (all from Invitrogen) with 5% FBS. Cells were then plated onto poly-*L*-lysine (Sigma, St. Louis, MO)-coated plates or glass coverslips at a density of 1×10^5 cells/mL. Neurons were cultured at 37°C in phenol red-free DMEM containing 5% FBS and with 5% circulating CO_2. Cytarabine (10 μmol/L) was added into the culture medium 24 h after plating, to inhibit cell division. The medium was changed every 48 h. Experiments were conducted on day 7 of culture.

2.2 Separation of Aβ protofibrils and monomers Size exclusion chromatography (SEC) fractionation was carried out using an ÄKTA Explorer FPLC (GE Healthcare, Uppsala, Sweden) placed inside a 4°C chamber. SEC columns were thoroughly equilibrated with SEC running buffer (10 mmol/L Tris-HCl, pH 7.4) prior to Aβ injections. Crude $Aβ_{1-42}$ solution was prepared as previously described[26,27]. Briefly, 1 mg lyophilized $Aβ_{1-42}$ was dissolved in 50 μL 100% anhydrous DMSO in a 1.5 mL sterile microtube. Then, 800 μL high purity water was immediately added, and the pH adjusted to 7.6 by adding 10 μL Tris-base solution (2 mol/L, pH 7.6). The solution was always freshly prepared and used immediately. After centrifugation (16 000 g, 4°C, 10 min), the supernatant was fractionated on a Superdex 75 column. The fractions eluting in the void volume were combined and labeled as protofibrils, whereas the fractions eluting under the 11–13 mL peak were combined and labeled as monomers. The protofibril and monomer fractions were used immediately for toxicity studies.

2.3 Microinjection Thin-walled borosilicate glass capillaries (outer diameter, 1.0 mm; inner diameter, 0.5 mm) with microfilament (MTW100F-4, World Precision Instruments, Sarasota, FL) were pulled with a Flaming/Brown micropipette puller (P-97, Sutter, Novato, CA) to obtain injection needles with a tip diameter of ~0.5 μm. Microinjection into the cytosol of each cell was performed using a microinjector (FemtoJet) and micromanipulator (both from Eppendorf, Hamburg, Germany). Neurons were injected with 25 fL/shot at an injection pressure of 100 hPa, a compensation pressure of 50 hPa, and an injection time of 0.1 s. The solutions were injected at the indicated concentrations with 100 μg/mL dextran Texas Red (DTR, molecular weight: 3 000; Molecular Probes, Eugene, OR) as a fluorescent marker to identify the injected cells. Approximately 90% of the injected neurons survived the injections for at least 16 days[28].

2.4 Measurement of neuronal cell death After treatment, cells were fixed in fresh 4% paraformaldehyde and 4% sucrose in PBS for 20 min at room temperature and permeabilized in 0.1% Triton X-100 and 0.1% sodium citrate in PBS for 2 min on ice. Terminal deoxynucleotidyl transferase-biotin dUTP nick-end labeling (TUNEL) staining was performed using *in situ* cell death detection kit I (Roche, Quebec, Canada) according to the manufacturer's instructions. The coverslips were then washed once in distilled water for 5 min, mounted onto glass slides, and observed under a Zeiss LSM-510 inverted confocal micro-

scope (Carl Zeiss, Oberkochen, Germany). The percentage of cell death was calculated as the ratio of the number of TUNEL-positive cells to the total 100 cells in one count. The average of 5 counts was calculated as the percentage of neuronal cell death in a certain treatment.

2.5 Immunocytochemistry After treatments, cells were fixed with 4% paraformaldehyde and 4% sucrose in PBS for 20 min at room temperature and permeabilized in PBS-Triton at 4°C, blocked in 10% donkey serum at room temperature, and then incubated with rabbit anti-MAP2 antibody (1:1 000, Abcam, Cambridge, UK) at 4°C for 24 h. Cy3-conjugated donkey anti-rabbit antibody was applied as the secondary antibody. The nuclei were then stained with DAPI (1 μg/mL, Sigma) for 15 min in the dark. The coverslips were mounted with Immunon^{TM} mounting medium (Shandon, Pittsburgh, PA) onto glass slides. Cells were observed under a fluorescence microscope (BH2-RFCA, Olympus, Tokyo, Japan) with a digital camera (Olympus DP70 Digital Microscope camera).

2.6 Reactive oxidative species (ROS) measurement H_2O_2 (100 μmol/L; Beijing Chemicals Co., Beijing, China) and N-acetyl-*L*-cysteine (NAC; Sigma, 10 μmol/L), a reduced glutathione provider and a direct scavenger of ROS, were applied to WT and APP/PS1 neuron culture media respectively. MitoSOX™ Red mitochondrial superoxide indicator (M36008, Molecular Probes, Eugene, OR) was used to measure ROS as described by the manufacturer. The relative ROS levels were measured by counting the ROS-positive cells in 1 000 cells and standardized with WT at the first time point as 1 (100%). The nuclei were then stained by DAPI (1 μg/mL) for 15 min in dark.

2.7 Statistical analysis Data are presented as mean ± SEM, and were analyzed using one-way analysis of variance (ANOVA). Sheffé's test was applied *post hoc* for the significant differences shown by ANOVAs. P <0.05 was considered statistically significant.

3 Results

3.1 iAβ induced more toxicity in APP/PS1 mouse neurons than in WT Crude, monomer and protofibril $A\beta_{1\text{-}42}$ were microinjected into the cytosol of cultured hippocampal neurons from WT and APP/PS1 mice. Given the injection volume was 25 fL/shot and the Aβ fractions were injected at 10 nmol/L, the amount of Aβ delivered into each neuron was roughly 1 500 molecules[29]. The intracellular and extracellular $A\beta_{1\text{-}42}$ were measured as 20.36 and 7.50 pmol/L for WT, 19.76 and 10.68 pmol/L for APP/PS1 neurons respectively (unpublished data). Therefore, the Aβ injected into the cytosol was far less than the endogenous Aβ produced in the cells. In WT mouse neurons, compared with $A\beta_{1\text{-}42}$ monomers, crude and protofibril $A\beta_{1\text{-}42}$ induced a much higher level of toxicity as indicated by the higher percentages of cell death at 24 h after injection (Fig. 1). Monomeric $A\beta_{1\text{-}42}$ did not induce significant cell death compared with the control (DTR injection alone) (Fig. 1). Moreover, in APP/PS1 neurons, injection of the different forms of $A\beta_{1\text{-}42}$ all induced greater degrees of cell death than in WT mouse neurons. Even monomeric $A\beta_{1\text{-}42}$, which was not toxic in WT neurons, caused significant cell death (Fig. 1). These results suggested that APP/PS1 neurons were more vulnerable to $A\beta_{1\text{-}42}$ toxicity.

3.2 ROS were involved in the vulnerability of APP/PS1 mouse neurons to Aβ toxicity Since previous evidence

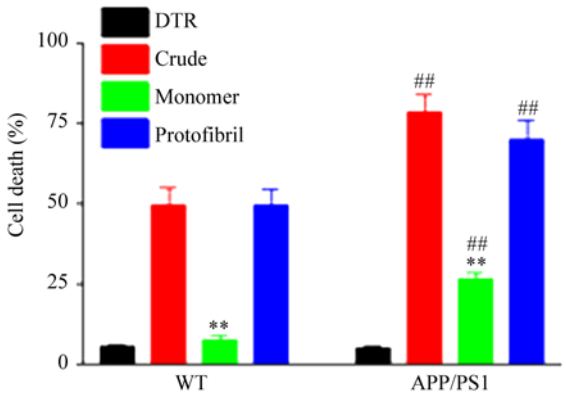

Fig. 1. Different forms of Aβ aggregates induced toxicity in wild-type (WT) and APP/PS1 mouse neurons, as assessed by TUNEL staining at 24 h after injection. Primary hippocampal neurons received injections of crude $A\beta_{1\text{-}42}$ + fluorescent marker (DTR), monomeric $A\beta_{1\text{-}42}$ + DTR, protofibril $A\beta_{1\text{-}42}$ + DTR, or DTR alone. Data are presented as mean ± SEM (n = 200 cells/preparation, each experiment repeated in 3 preparations). $^{**}P$ <0.01 compared with crude group; $^{\#\#}P$ <0.01 compared with WT mice with the corresponding injection.

has shown that Aβ interacts with mitochondria[30], here we investigated whether ROS were involved in the high vulnerability of APP/PS1 mouse neurons to iAβ toxicity. In our culture system, ~90% of the cells were neurons[31]. This was confirmed by staining with the neuronal marker MAP2 in WT (Fig. 2A–C) and APP/PS1 (Fig. 2D–F) neurons. Relative ROS levels were higher in APP/PS1 neurons (Fig. 2I and 2J) than in WT neurons (Fig. 2G and 2H) with no Aβ application, suggesting that at the resting state, APP/PS1 neurons produced more ROS than did the WT neurons. We also found that ROS production increased dramatically from 7 days of culture, and this lasted for at least 16 days (Fig. 2K).

When WT mouse neurons (7 days in culture) were treated with H_2O_2 and then microinjected with crude, monomer or protofibril $A\beta_{1\text{-}42}$, all had higher percentages

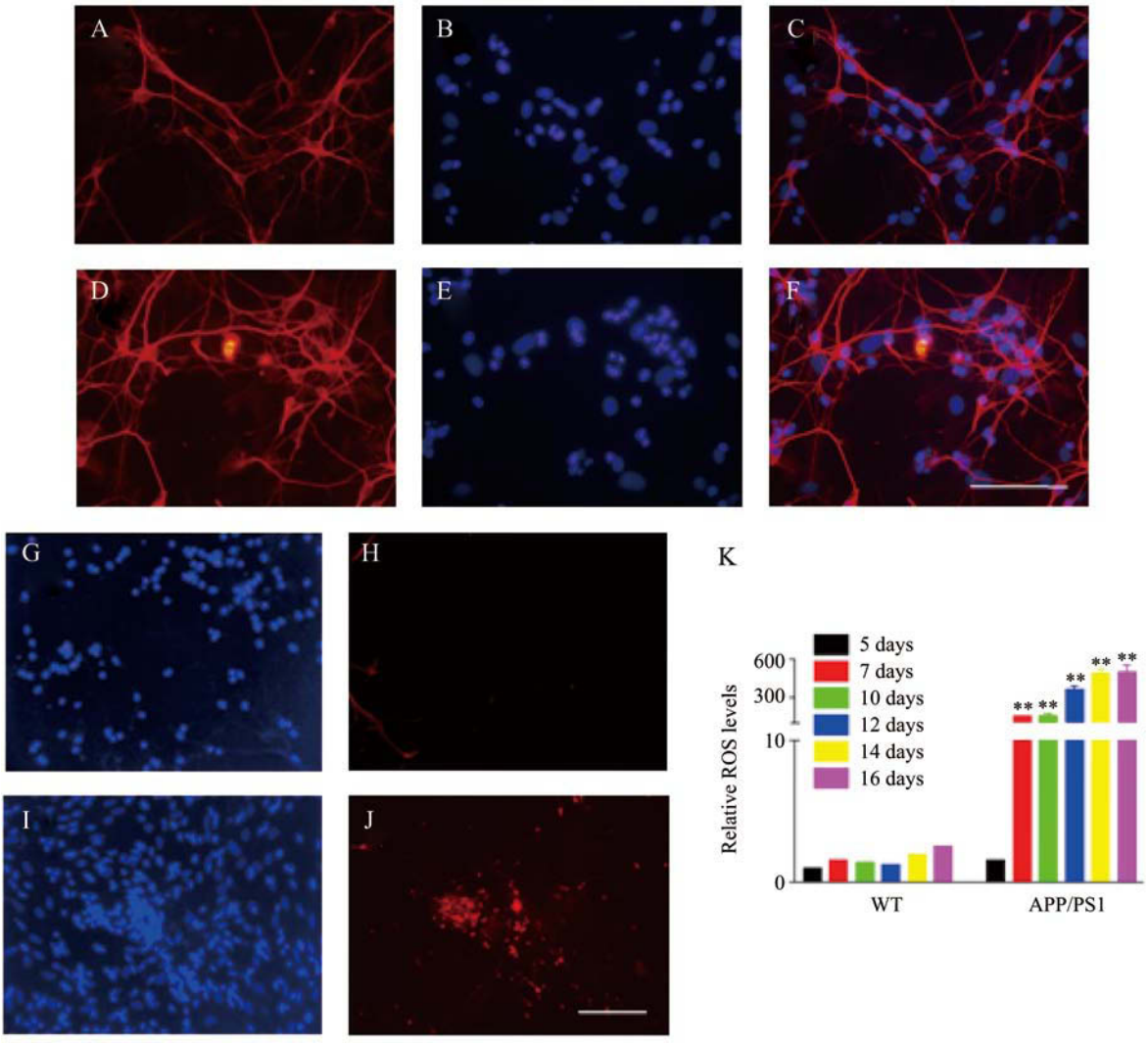

Fig. 2. ROS levels in wild-type (WT) and APP/PS1 mouse hippocampal neurons. A: Neuronal marker MAP2 staining of primary WT cells. B: DAPI staining indicated the cell population in WT cultures. C: A and B merged. D: MAP2 staining of primary APP/PS1 neurons. E: DAPI staining indicated the cell population in APP/PS1 cultures. F: D and E merged. Scale bar, 50 μm. G: DAPI staining indicated the cell population in WT cultures. H: ROS level in primary WT neurons stained with MitoSOX™ Red mitochondrial superoxide indicator. I: DAPI staining indicated the cell population in APP/PS1 cultures. J: ROS level in primary APP/PS1 neurons stained with MitoSOX™ Red. Scale bar, 100 μm. K: Quantification of relative ROS levels in WT and APP/PS1 neurons with time. Data are presented as mean ± SEM (n = 1 000 cells/preparation, each experiment repeated in 3 preparations). **P <0.01 compared with WT group.

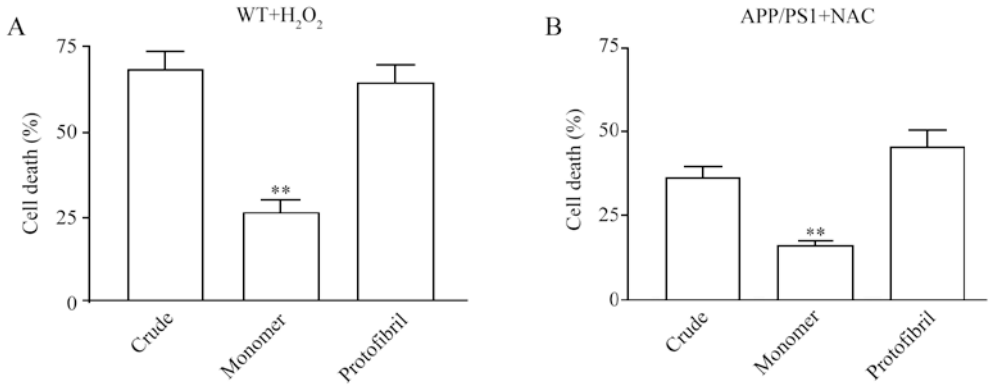

Fig. 3. ROS were involved in the vulnerability to Aβ toxicity. TUNEL staining of wild-type (WT) and APP/PS1 hippocampal neurons at 24 h after Aβ injection. A: WT mouse neurons were treated with H_2O_2 and injected with Aβ. B: APP/PS1 neurons were treated with N-acetyl-*L*-cysteine (NAC) and injected with Aβ. Data are presented as mean ± SEM (*n* = 100 cells/preparation, each experiment repeated in 3 preparations). ***P* <0.01 compared with crude group.

of cell death 24 h after injection, compared with their WT counterparts with no H_2O_2 treatment (Fig. 3A). This indicated that oxidative stress increases the vulnerability of WT neurons to Aβ stimulation. In contrast, when APP/PS1 neurons (7 days in culture) were treated with NAC to reduce ROS, and then injected with Aβ, the toxicity of all three forms of Aβ decreased almost to the levels in WT neurons (Fig. 3B), confirming that ROS play an important role in mediating iAβ toxicity in APP/PS1 neurons.

4 Discussion

The present study showed that the crude and protofibril fractions of Aβ were toxic whereas monomeric Aβ barely induced toxicity in both WT and APP/PS1 mouse neurons. The mechanisms of amyloid fibril formation have been suggested as "templating and nucleation models", "linear colloid-like assembly of spherical oligomers", and "domain-swapping"[21]. The mechanisms of Aβ oligomer toxicity have been suggested to be associated with calcium dysregulation[25], inflammation[24], potassium efflux[24] and interaction with membrane lipid rafts[32] and microglia[25]. In our study, the APP/PS1 neurons were more vulnerable to iAβ toxicity. However, with this double transgenic line, we still cannot attribute this phenotype to the APP_{swe} or the $PS1_{\Delta E9}$ mutation. Further investigation using single transgenic lines is required to clarify this issue.

There is evidence that the neurotoxicity induced by iAβ is partly due to the formation of ROS, leading to oxidative stress[33]. In our experiments, after 7 days of culture, ROS increased remarkably in APP/PS1 neurons compared with that in WT neurons. This difference continued to 16 days, suggesting that the production of ROS is relatively difficult to stop once it starts. Previous work has shown that endocytosed Aβ, through binding to specific potential receptors, such as the receptor for advanced glycation end products and the class A scavenger-receptor, leads to increased ROS production[34-36]. On the other hand, oxidative stress can influence Aβ formation by interacting with APP[37], and also indirectly by modulating the activity and levels of key enzymes such as β- and γ-secretases[34,38,39]. Therefore, ROS may trigger a positive feedback mechanism for iAβ toxicity.

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