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Overexpression of Human Wild-Type Amyloid-β Protein Precursor Decreases the Iron Content and Increases the Oxidative Stress of Neuroblastoma SH-SY5Y Cells

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Abstract. The accumulation of amyloid-β protein precursor (AβPP) is related to the pathogenesis of Alzheimer's disease (AD); however, the underlying mechanism is still unclear. The abnormal interactions of AβPP with metal ions such as iron are implicated in the process of oxidative stress in AD brains. In this study, we found that the overexpression of wild-type human AβPP695 decreased the iron content and increased the oxidative stress in neuroblastoma SH-SY5Y cells. The catalase activity of stably transfected cells overexpressing wild-type AβPP695 (AβPP cells) was significantly lower than that of the control cells. Intracellular reactive oxygen species (ROS) generation and calcium levels significantly increased in AβPP cells compared to control cells. The mitochondrial membrane potential of AβPP cells was significantly lower than that of the control cells. Moreover, iron treatment decreased ROS and calcium levels and increased cell viability of AβPP cells. The iron deficiency in AβPP cells may contribute to the pathogenesis of AD.

Keywords: Alzheimer's disease, amyloid-β protein precursor, iron deficiency, oxidative stress

INTRODUCTION

The amyloid-β protein precursor (AβPP) is the precursor of the amyloid-β (Aβ) peptide, the main component of the senile plaque in Alzheimer's disease (AD). AβPP is a ubiquitously expressed type I transmembrane protein and well conserved across species, from APPL in Drosophila, APL-1 in *Caenorhabditis elegans*, and AβPP, APLP1, and APLP2 in mammals [1]. At least 10 isoforms of the protein derived by alternative splicing have been described. AβPP695, a major AβPP-spliced form, is expressed only in neurons. Most studies focused on mutated AβPP isoforms thereby neglecting the physiological impact of wild-type AβPP in AD [2]. Physiological and pathological roles of AβPP in neurons are not fully understood. Duplication of the AβPP gene has been reported to be associated with familial AD. AβPP overproduction leads to early-onset AD [3, 4]. Most individuals with Down syndrome show an early onset of AD, which potentially results from the presence of an extra

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copy of the AβPP gene on chromosome 21. Overexpression of AβPP in transgenic mice results in synaptic transmission and dendritic spine abnormalities [5, 6]. Transgenic adult mice with human wild-type AβPP displayed decreased neurogenesis in the hippocampal region and the learning-memory deficits [7, 8]. AβPP may be involved in iron homeostasis. The 5′ untranslated region (UTR) of AβPP mRNA possesses a functional iron-responsive element (IRE) stem loop with sequence homology to the IREs for ferritin and transferrin receptor (TfR) mRNA [9]. This IRE binds physiologically with iron regulatory protein 1 (IRP1), and not IRP2 [10]. Duce and colleagues found that AβPP possesses iron-export ferroxidase activity. AβPP catalytically oxidizes Fe^{2+} and loads Fe^{3+} into transferrin in HEK293T cells and in human cortical tissue. Stably transfected cells overexpressing wild-type AβPP695 loaded with $Tf(^{59}Fe)_2$ retained significantly less iron compared to cells transfected with empty vector. AβPP has a major interaction with ferroportin to facilitate iron export from certain cells including neurons [11].

To better investigate the biological function of AβPP, we established stably transfected human neuroblastoma SH-SY5Y cell strains overexpressing wild-type human AβPP695 (AβPP cells) or vector (control cells). The protein and mRNA levels of AβPP in AβPP cells are much more than those in control cells. No significant difference was observed between control and AβPP cells on the Aβ level in the condition media [12, 13].

METHODS

Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, Hepes, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Gibco BRL (Grand Island, NY, USA). 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) and ferric ammoniumcitrate (FAC) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fluo-3 acetoxymethyl ester (Fluo-3 AM) was purchased from Calbiochem (San Diego, CA, USA). All other chemicals made in China were analytical grade.

Cell cultures and transfection

Human neuroblastoma SH-SY5Y cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified, 5% CO_2 incubator. Stably transfected SH-SY5Y cells expressing human wild-type AβPP695 (AβPP cells) or empty vector (control cells). pCLNCXv.2 were made using Lipofectamine 2000 (Invitrogen). G418 (200 μg/ml) was added into the medium to maintain the genotypically stable cell strains.

Calcein loading

The cells were incubated with 0.5 μM calcein AM (Dojindo Laboratories, Japan) at 37°C for 10 min. Excess calcein AM on cell surface was removed by several washes with PBS. The fluorescence was measured with an F-4500 Fluorescent Spectrophotometer (Hitachi, Japan) (λex of 488 nm,λem of 517 nm at 37°C). The quenching of calcein fluorescence was continuously recorded in every half a second for 10 min.

Determination of metal contents using ICP-OES

The cells were collected and digested in 0.5 ml nitric acid. After the samples were heated at 100°C for 3 h, double distilled water was added to a final volume of 3 ml, respectively. The metal contents were determined using ICP-OES (5300DV, PerkinElmer) with auxiliary gas flow rate at 0.2 liter/min, nebulizer 0.8 liter/min, plasma 15 liter/min, and ICP RF power 1300 w. The standards were purchased from National Institute of Metrology, China.

Cellular catalase activity

The cells were lysed on ice for 20 min by lysis buffer (50 mM Tris-Cl, 150 mM NaCl, and 1% Triton X-100). The samples were then centrifuged at 6000 g for 20 min at 4°C and the catalase activity of the supernatant was measured using a kit from Nanjing Jiancheng according to the manufacturer's instructions.

Measurement of mitochondrial membrane potential

Mitochondrial membrane potential was measured by the incorporation of a mitochondria sensitive dye TMRE. The cells were changed to serum-free medium containing 200 nM TMRE and incubated for 15 min at 37°C. The cells were then collected and the fluorescence intensity was analyzed within 15 min by a spectrophotofluorimeter (Hitachi F-4500, 549 nm excitation and 574 nm emission).

Flow cytometry analysis

Late apoptosis of the cells was detected by flow cytometer using propidium iodide (PI). The cells were centrifuged (1200 g, 5 min) to remove the medium. After washed three times with PBS buffer, the cells were incubated with PI (final concentration 3.7 μM) for 30 min at 4°C in dark. Apoptosis was analyzed by FACSCalibur flow cytometry (Becton-Dickinson Immunocytometry System, San Jose, CA, USA).

Measurement of intracellular ROS

The level of intracellular ROS was monitored spectrofluorometrically using the oxidation sensitive (DCFH-DA, Sigma) fluorescent dyes. Oxidation of DCFH-DA by peroxides yielded the fluorescent derivative dichlorofluorescein (DCF). Cells were collected and incubated with 2 μM DCFH-DA (final concentration) for 20 min at 37°C. Loaded cells were washed three times and the fluorescence intensity of DCF was determined using a fluoroskan ascent with the excitation wavelength at 485 nm and the emission wavelength at 538 nm.

Intracellular calcium concentration $[Ca^{2+}]_i$ assay

The concentration of intracellular Ca^{2+} was measured using Fluo-3 AM. The cells were harvested, washed, and resuspended in a standard medium containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5.6 mM glucose, 1.5 mM $CaCl_2$, and 20 mM Hepes-Na (pH 7.4). 5 μM Fluo-3 AM (final concentration) was added to the cell suspension and incubated for 30 min at 37°C. After washing three times, cells were resuspended in the standard medium and transferred to a fluorometer cuvette. The fluorescence intensity of Fluo-3 was quantified using a fluorescence spectrophotometer (Hitachi F-4500) with excitation wavelength at 490 nm and emission wavelength at 526 nm. The concentration of intracellular $[Ca^{2+}]$ was calculated from the Fluo-3 fluoresce intensity using the formula:

$$[Ca^{2+}]_i = Kd(F - F_{min})/(F_{max} - F)(nmol/l).$$

Kd was assumed to remain constant between 10°C and 25°C and increased linearly up to 42°C at 25°C Kd = 400 nmol/l. The maximal Fluo-3 fluorescence intensity (F_{max}) was determined by adding 0.1% Triton X-100 and the minimal fluorescence intensity (F_{min}) was determined by quenching Fluo-3 fluorescence with 5 mM EGTA. F is the fluorescence intensity measured without adding Triton X-100 or EGTA.

Assessment of cell viability

Cell viability was measured in 96-well plates by quantitative colorimetric assay with MTT, showing the mitochondrial activity of living cells. Briefly, after 48 h FAC treatment, 500 μg/ml MTT (final concentration) was added into the medium, which continued to incubate at 37°C for 3 h. The MTT solution was removed and the colored formazan crystal was dissolved in dimethyl sulfoxide (DMSO). The absorbance at 595 nm was measured using a Bio-Rad 3350 microplate reader. Cell viability was expressed as the ratio of the signal obtained from treated cultures and control cultures.

Statistical analysis

All experiments were performed in triplicate unless otherwise indicated. Results are expressed as the means of three independent experiments. Data analysis was performed by one-way analysis of variance (ANOVA). Error bars represent standard deviation. A probability level of 95% ($p < 0.05$) was considered significantly different.

RESULTS

Overexpressed AβPP decreases intracellular labile iron level

The calcein fluorescence of the cells is shown in Fig. 1. Iron binding with calcein can quench its

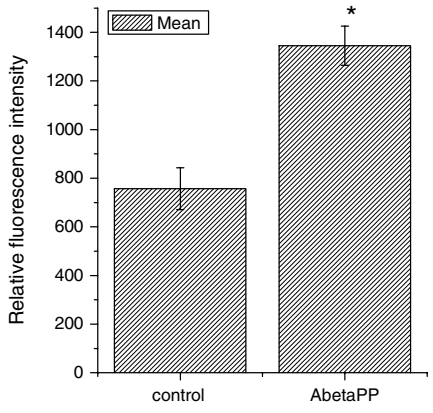

Fig. 1. The relative fluorescence intensity in the control cells (vector-only stably transfected cells) and AβPP cells (AβPP stably transfected cells). Changes in calcein fluorescence were examined for assessing the labile iron level in the cells. The results are expressed as means ± SEM, $n = 3$. Statistical analysis was done using ANOVA. $^*p < 0.05$ compared with the control cells.

fluorescence. The calcein fluorescence of the AβPP cells was higher than that of vector-only stably transfected cells (control cells), indicating that the intracellular labile iron level of the AβPP cells was lower than that of the control cells.

The metal contents in control and AβPP cells

We detected the total iron, calcium, copper, and zinc contents of the cells by the ICP-OES method (Fig. 2). The iron content in AβPP cells is significantly lower than that in the control cells. There are no significant differences between control and AβPP cells in calcium, copper, and zinc contents.

AβPP accumulation decreased the catalase activity and the mitochondrial membrane potential

To investigate the effect of iron homeostasis disruption on the cellular antioxidant levels, we detected the activity of catalase of the control and AβPP cell lyses. The activity of catalase of supernatant from AβPP cell was significantly weaker than that of the control cells (Fig. 3A). Mitochondrial membrane potential was expressed as the fluorescent intensity of TMRE. The fluorescent intensity of TMRE staining in AβPP cells was lower than that of control cells, representing a fall in the mitochondrial membrane potential (Fig. 3B).

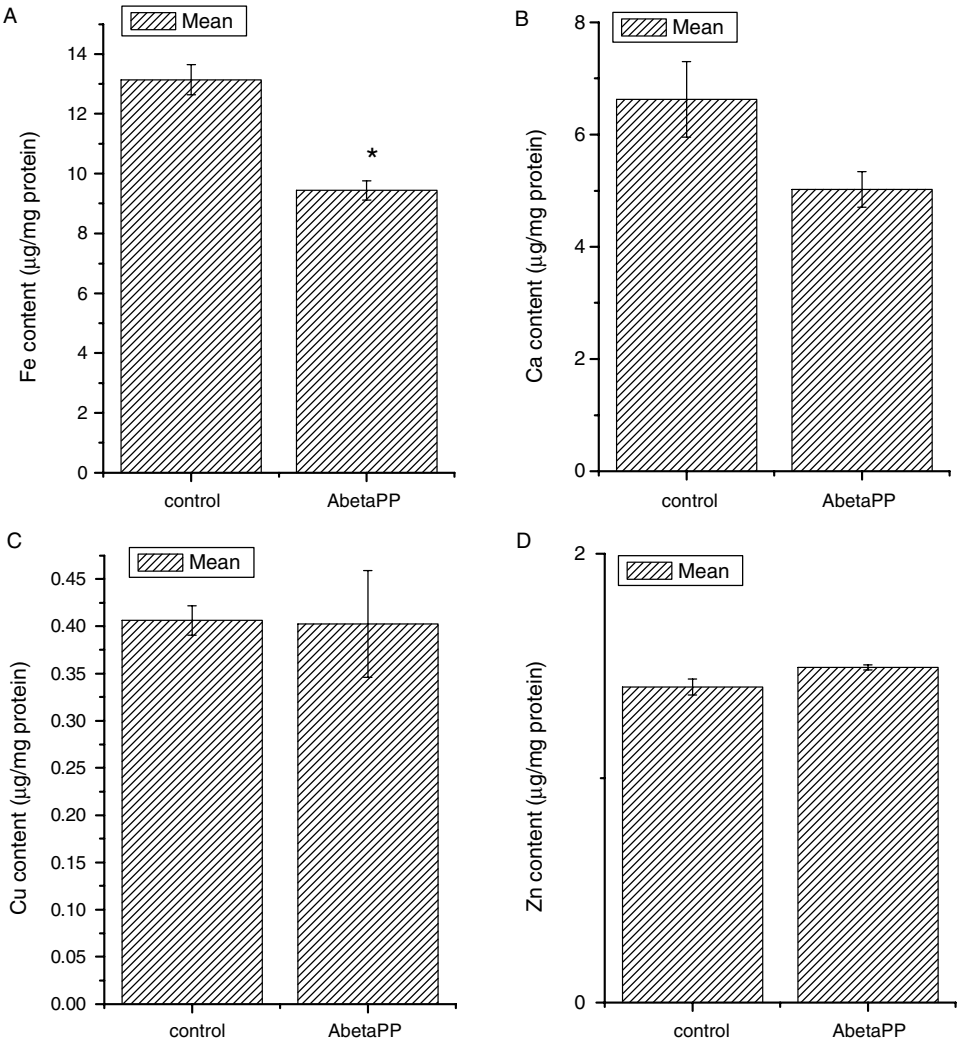

Fig. 2. Metal contents in control and AβPP cells determined by ICP-OES. A) Iron; B) Calcium; C) Copper; D) Zinc. The results are expressed as means ± SEM, $n = 3$. Statistical analysis was done using ANOVA. $^{*}p < 0.05$ compared with the control cells.

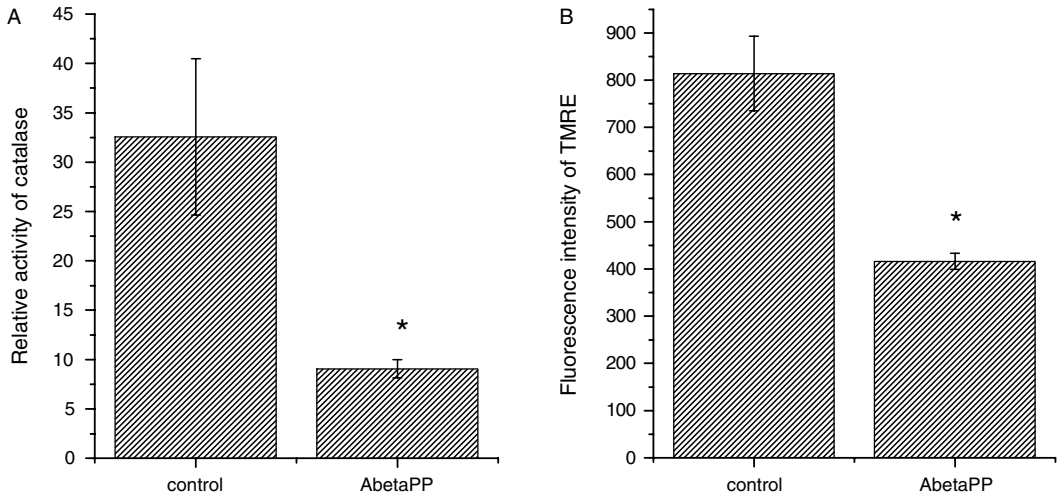

Fig. 3. A) The catalase activities in the control and AβPP cells. B) The control and AβPP cell mitochondrial membrane potential. The results are expressed as means ± SEM, $n = 3$. Statistical analysis was done using ANOVA. $^{*}p < 0.05$ compared with the control cells.

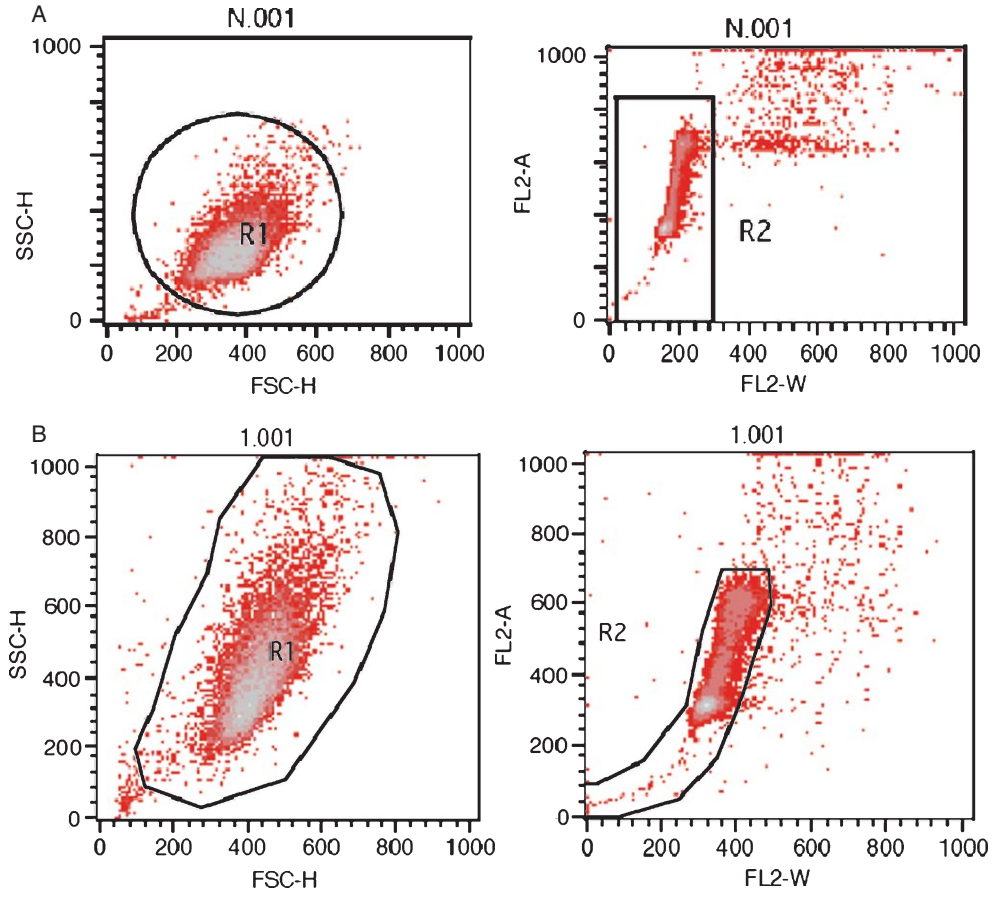

Fig. 4. Cell apoptosis detected by flow cytometry. A) Control cells; B) AβPP cells.

Apoptosis was not observed in control or AβPP cells

PI labeling followed by measurement of fluorescence intensity with flow cytometry was used to detect the apoptosis. The apoptosis percentages of control and AβPP cells were 0.35% and 1.24%, respectively. No obvious apoptosis was observed in AβPP cells (Fig. 4).

ROS generation in control and AβPP cells

The relative levels of ROS generation in control and AβPP cells was measured by the fluorescence method, and it was found that the ROS levels of AβPP cells significantly increased compared to control cells. When we added 50 μg/ml FAC to AβPP cells for 48 h, the ROS level decreased (Fig. 5).

Intracellular calcium levels in control and AβPP cells

To investigate the effects of overexpression of AβPP, we detected the $[Ca^{2+}]_i$ in control and AβPP cells. The $[Ca^{2+}]_i$ level in AβPP cells significantly increased compared to the control cells. When we added 50 μg/ml FAC to AβPP cells for 48 h, the calcium level decreased (Fig. 6).

Iron treatment increased the cell viability of AβPP cells

To investigate the effect of iron on cellular viability, we detected cell viability in control and AβPP cells with FAC (ferric ammonium citrate) treatment. The cell viability was expressed as an MTT conversion rate. We presumed both the viabilities of control cells

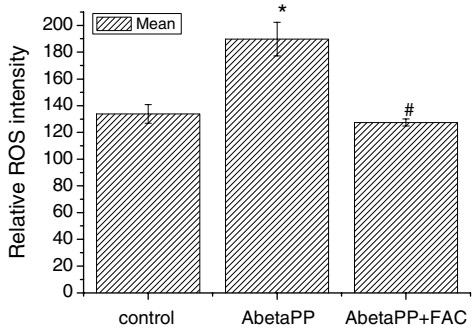

Fig. 5. ROS generation in control, AβPP, and FAC-treated AβPP cells measured using DCFH-DA. The results are expressed as means ± SEM, $n = 3$. Statistical analysis was done using ANOVA. $^{*}p < 0.05$ compared with the control cells. $^{\#}p < 0.05$ compared with the AβPP cells.

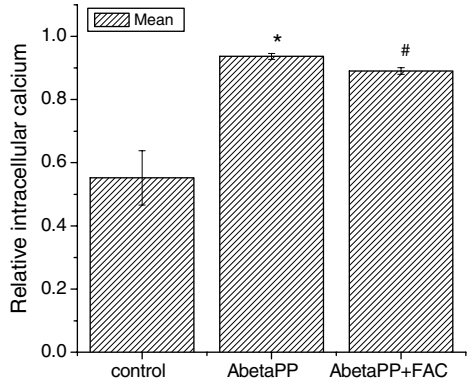

Fig. 6. Intracellular calcium levels in control, AβPP, and FAC-treated AβPP cells. The $[Ca^{2+}]_i$ was calculated from the fluorescence intensity of the probe Fluo-3. The results are expressed as means ± SEM, $n = 3$. Statistical analysis was done using ANOVA. $^{*}p < 0.05$ compared with the control cells. $^{\#}p < 0.05$ compared with the AβPP cells.

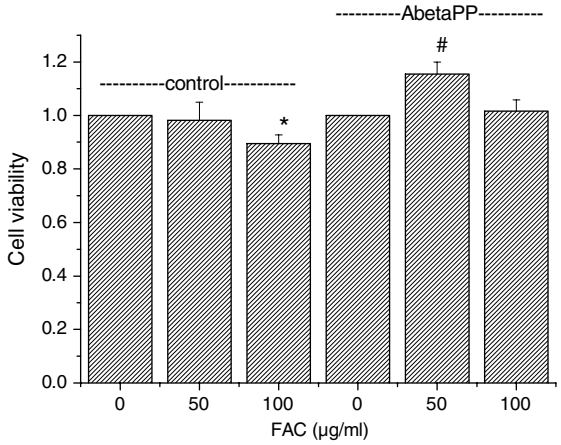

Fig. 7. The effects of FAC on the control and AβPP cell viability. The cells were treated with 0, 50, or 100 μg/ml FAC. Cell viability was measured by MTT method. $^{*}p < 0.05$ compared with control (0) cells. $^{\#}p < 0.05$ compared with AβPP (0) cells. The results are expressed as means ± SEM, $n = 8$. Statistical analysis was done using ANOVA.

and AβPP cells are 1.0. Cell viability increased in the presence of 50 μg/ml FAC in AβPP cells, while no significant difference was seen in control cells by the same treatment. When we added 100 μg/ml FAC, control cell viability decreased and AβPP cell viability has no change (Fig. 7).

DISCUSSION

In order to better resemble the *in vivo* overgenerated AβPP-induced neurotoxicity, a cell system overexpressing wild-type AβPP was used. AβPP cells

behave as control cells, except their growth rates are slower.

In the present study, we found that the content of iron in AβPP cells was lower than that in the control cells. Iron is an essential component of many enzymes. Catalase contains iron as cofactor and catalyzes the breakdown of hydrogen peroxide (H_2O_2) to water and oxygen. The catalase activity was lower in AβPP cells than in control cells, while the ROS level and Ca^{2+} concentration increased and the mitochondrial membrane potential decreased in AβPP cells. Accumulated data demonstrated that cellular insults resulting from free radicals may be a major contributor to the neurotoxicity of AD. Free radicals peroxidize membrane lipid and oxidize proteins, resulting in damage of the plasma membrane and crosslinking of cytoskeletal proteins [14]. Multiple lines of evidence have suggested that abnormalities in calcium homeostasis might be involved in the pathophysiology of AD [15]. Several mechanisms are responsible for the elevation of intracellular calcium, one of which is the overproduction of ROS [16]. An elevation of $[Ca^{2+}]_i$ evoked by H_2O_2 has been shown in a variety of cell types. This H_2O_2-induced $[Ca^{2+}]_i$ rise has been attributed to mobilization from intracellular stores, to influx across plasma membrane, or to both mechanisms. Calcium release evoked by H_2O_2 from intracellular stores might be induced by either inhibition of the sarcoplasmic reticulum calcium ATPase (SERCA) pump and (or) by activation of calcium release channels [17]. Mitochondrial dysfunction, which is closely related to intracellular calcium overload and excessive free radicals, is an important cause of AD. We found that mitochondrial free Ca^{2+} levels in AβPP cells were higher than those of the control cells [18]. Pathobiology of AD is associated with mitochondrial dysfunction and decreased energy metabolism is one of the earliest detectable defects in AD. AβPP localizes to both the plasma membrane and the mitochondria [19].

Iron is the most abundant redox active metal in humans and plays a critical role in tissue oxygenation and in virtually all energy-dependent developmental processes. Iron is required to support the brain's high respiratory rate as well as for myelination, gene expression, and neurotransmitters synthesis [20]. In this study, we found that AβPP cells were in the iron deficiency status and we basically confirmed the observation of Duce et al. [11]. Iron treatment decreased ROS and calcium level and increased viability of AβPP cells. In Down syndrome, there is an extra copy of the AβPP gene on chromosome 21 and AβPP is overgenerated. Prevalence of iron deficiency/iron deficiency anemia in children with Down syndrome was comparable with that in the general pediatric population [21]. Children with Down syndrome are at risk for anemia and iron deficiency similar to the general population [22]. Heme is a major functional form of iron in the cell. The metabolic consequences resulting from heme deficiency seem similar to dysfunctional neurons in patients with AD. Heme deficiency or dysregulation may be an important and preventable component of the neurodegenerative process [23]. A new study shows that in older persons without dementia, lower hemoglobin levels are associated with an increased hazard for developing AD and more rapid cognitive decline [24].

The early cognitive impairment and neuropathological alteration in transgenic mice overexpressing wild-type human AβPP were not related to Aβ levels [25]. Different studies have shown cognitive deficits in the absence of Aβ peptide and cognitive improvement without changes in Aβ pathology [26]. In our cell models, there is no significant difference in the $Aβ_{1-40}$ level in media between control and AβPP cells [13].

In summary, we found the neuronal cell iron homeostasis loss by AβPP accumulation in cellular models of AD. This study provides important insights into AβPP biological function and its potential implications for neuronal dysfunction in early stages of AD and suggests that overexpressed AβPP and iron deficiency may play an important role in the pathology of AD.

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Authors' disclosures available online (http://www.j-alz.com/disclosures/view.php?id=1184).

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