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# Regulation of adenosine triphosphate-sensitive potassium channels suppresses the toxic effects of amyloid-beta peptide (25–35)<sup>☆</sup>

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## Abstract

In this study, we treated PC12 cells with 0–20 μM amyloid-β peptide (25–35) for 24 hours to induce cytotoxicity, and found that 5–20 μM amyloid-β peptide (25–35) decreased PC12 cell viability, but adenosine triphosphate-sensitive potassium channel activator diazoxide suppressed the decrease in PC12 cell viability induced by amyloid-β peptide (25–35). Diazoxide protected PC12 cells against amyloid-β peptide (25–35)-induced increases in mitochondrial membrane potential and intracellular reactive oxygen species levels. These protective effects were reversed by the selective mitochondrial adenosine triphosphate-sensitive potassium channel blocker 5-hydroxydecanoate. An inducible nitric oxide synthase inhibitor, Nω-nitro-L-arginine, also protected PC12 cells from amyloid-β peptide (25–35)-induced increases in both mitochondrial membrane potential and intracellular reactive oxygen species levels. However, the H<sub>2</sub>O<sub>2</sub>-degrading enzyme catalase could not reverse the amyloid-β peptide (25–35)-induced increase in intracellular reactive oxygen species. A 24-hour exposure to amyloid-β peptide (25–35) did not result in apoptosis or necrosis, suggesting that the increases in both mitochondrial membrane potential and reactive oxygen species levels preceded cell death. The data suggest that amyloid-β peptide (25–35) cytotoxicity is associated with adenosine triphosphate-sensitive potassium channels and nitric oxide. Regulation of adenosine triphosphate-sensitive potassium channels suppresses PC12 cell cytotoxicity induced by amyloid-β peptide (25–35).

## Key Words

neural regeneration; neurodegenerative diseases; amyloid-β peptide (25–35); PC12 cell; adenosine triphosphate-sensitive potassium channel; inducible nitric oxide synthase; mitochondrial membrane potential; reactive oxygen species; grant-supported paper; photographs-containing paper; neuroregeneration

## Research Highlights

- (1) Amyloid-β peptide (25–35) increases mitochondrial membrane potential and intracellular reactive oxygen species levels, resulting in PC12 cell damage.
- (2) Diazoxide increases the viability of PC12 cells damaged by amyloid-β peptide (25–35).
- (3) An inducible nitric oxide synthase inhibitor, Nω-nitro-L-arginine, protects PC12 cells from amyloid-β peptide (25–35)-induced neurotoxic effects.
- (4) Diazoxide and Nω-nitro-L-arginine protect PC12 cells from amyloid-β peptide (25–35)-induced increases in both mitochondrial membrane potential and intracellular reactive oxygen species levels.

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## Abbreviations

A $\beta$ , amyloid- $\beta$  peptide; K<sub>ATP</sub>, adenosine triphosphate-sensitive potassium; ATP, adenosine triphosphate; DCF, 2',7'-dichlorofluorescein; JC-1,5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide; DCFH-DA, 5, 6-carboxy-2',7'-dichlorofluorescein- diacetate

## INTRODUCTION

The role of the amyloid  $\beta$  peptide (A $\beta$ ) during neurodegeneration has become the focus of studies of the pathogenesis of Alzheimer's disease<sup>[1-2]</sup>. Recent discoveries have shown that A $\beta$  causes synaptic degeneration and cell apoptosis<sup>[3-4]</sup>. The A $\beta_{25-35}$  fragment has also been shown to be cytotoxic<sup>[5]</sup>. Furthermore, A $\beta$  might trigger mitochondrial dysfunction accompanied by the generation of free radicals, which in turn might impair ion-motive adenosine triphosphatase activity, resulting in membrane depolarization. Supporting this hypothesis, there is ample evidence for a central role of mitochondrial dysfunction in the pathogenesis of Alzheimer's disease<sup>[5-8]</sup>.

Adenosine triphosphate (ATP)-sensitive potassium (K<sub>ATP</sub>) channels link cell metabolism to membrane potential<sup>[9]</sup>. It has been shown that A $\beta$  can induce K<sub>ATP</sub> channel dysfunction<sup>[10]</sup>. Modulation of the K<sub>ATP</sub> channels expressed in neurons may exert a neuroprotective effect. Diazoxide, an activator of mitochondrial K<sub>ATP</sub> (mitoK<sub>ATP</sub>) channels, is becoming a promising protective agent. Potassium channel activators have been shown to protect cardiac myocytes against ischemic injury<sup>[11]</sup>. Diazoxide has also been shown to protect cultured neurons against toxicity induced by neurotoxins including glutamate, A $\beta$ , and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>)<sup>[12-13]</sup>. Additionally, the production of nitric oxide, which plays an important role in the regulation of mitochondrial function, is believed to be closely related to K<sub>ATP</sub> channel activity<sup>[14]</sup>. The formation of nitric oxide is catalyzed by inducible nitric oxide synthase<sup>[15]</sup>. In Alzheimer's disease brain cortex, inducible nitric oxide synthase expression levels are markedly increased<sup>[16]</sup>. Excess nitric oxide could impair neuronal function<sup>[17]</sup>. Therefore, regulation of inducible nitric oxide synthase activity may produce a neuroprotective effect. Despite the abundance of data, the precise mechanisms of these beneficial effects are still obscure, especially in the brain.

The present study was designed to determine whether the cytotoxic A $\beta_{25-35}$  fragment could change mitochondrial membrane potential and intracellular reactive oxygen species levels, and whether a K<sub>ATP</sub>

channel activator, diazoxide, and an inducible nitric oxide synthase inhibitor, N $\omega$ -nitro-L-arginine, could protect cells against A $\beta_{25-35}$  cytotoxicity.

## RESULTS

### Diazoxide counteracted the effected of A $\beta_{25-35}$ on PC12 cell viability

Cultured PC12 cells were treated with several concentrations of A $\beta_{25-35}$  and cellular viability was measured by assessing MTT reduction. At 5, 10 and 20  $\mu$ M, A $\beta_{25-35}$  significantly lowered cellular viability ( $P < 0.05$  or  $P < 0.01$ ). However, when cultured cells were pretreated with diazoxide (1 mM) for 1 hour and then exposed to A $\beta_{25-35}$  for 24 hours, their viability was significantly greater than that of cells exposed to A $\beta_{25-35}$  alone ( $P < 0.05$  or  $P < 0.01$ ). The protective effect of diazoxide was abolished by 5-hydroxydecanoate (500  $\mu$ M), a K<sub>ATP</sub> channel blocker ( $P < 0.05$  or  $P < 0.01$ ). The control reverse A $\beta_{35-25}$  had no effect on cellular viability (Figure 1).

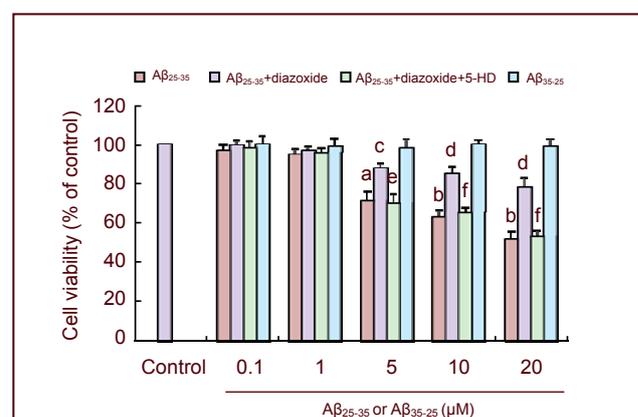


Figure 1 Diazoxide (a potassium channel activator) protected the cultured PC12 cells against amyloid- $\beta$  peptide (25–35) (A $\beta_{25-35}$ ) toxicity.

Cultured cells were treated with the indicated concentration of A $\beta_{25-35}$  or A $\beta_{35-25}$  for 24 hours. Cell viability ( $A_{570\text{ nm}}$ ) was detected by MTT reduction assay.

The data are expressed as mean  $\pm$  SEM from five independent experiments. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ , vs. control; <sup>c</sup> $P < 0.05$ , <sup>d</sup> $P < 0.01$ , vs. A $\beta_{25-35}$ ; <sup>e</sup> $P < 0.05$ , <sup>f</sup> $P < 0.01$ , vs. A $\beta_{25-35}$  + diazoxide (one-way analysis of variance, followed by two-tailed Student's *t*-test).

5-HD: 5-hydroxydecanoate.

### Diazoxide and N $\omega$ -nitro-L-arginine suppressed the increase in mitochondrial membrane potential induced by A $\beta_{25-35}$ in PC12 cells

5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1) produces two fluorescence emission peaks that reflect the existence of two physical forms of the dye. The monomer, which is the predominant form at low mitochondrial membrane potential, emits green fluorescence (emission maximum at 527 nm), and the so-called "J-aggregate", which is the predominant species at high mitochondrial membrane potential, emits orange-red fluorescence (emission maximum at 590 nm). Earlier observations suggested that the fluorescence intensity of the monomer is insensitive to changes in mitochondrial membrane potential<sup>[18]</sup>. Therefore, it was concluded that the lower the JC-1 590/527 nm fluorescence ratio is, the higher the mitochondrial membrane potential is (Figure 2A).

A $\beta_{25-35}$  (5  $\mu$ M) treatment for 24 hours caused a marked increase in mitochondrial membrane potential ( $P < 0.01$ ). However, addition of diazoxide rescued the membrane potential. 5-hydroxydecanoate abolished the protective effect of diazoxide, reverting the membrane potential to that of the A $\beta_{25-35}$ -treated cells, suggesting that diazoxide was responsible for the observed maintenance of mitochondrial membrane potential during A $\beta_{25-35}$  challenge.

When cultured PC12 cells were co-treated with A $\beta_{25-35}$  and inducible nitric oxide synthase inhibitor N $\omega$ -nitro-L-arginine for 24 hours, the JC-1 590/527 nm fluorescence ratio significantly increased compared with cells exposed to A $\beta_{25-35}$  alone ( $P < 0.01$ ), suggesting that the A $\beta_{25-35}$ -induced increase in mitochondrial membrane potential was prevented by N $\omega$ -nitro-L-arginine. The reverse control peptide A $\beta_{35-25}$  had no effect on mitochondrial membrane potential (Figure 2B).

### Diazoxide and N $\omega$ -nitro-L-arginine suppressed the increase in reactive oxygen species levels induced by A $\beta_{25-35}$ in PC12 cells

In control PC12 cells, flow cytometry indicated an average 2',7'-dichlorofluorescein (DCF) fluorescence intensity (527 nm) of  $600.6 \pm 85.2$ ; whereas in the A $\beta_{25-35}$  (5  $\mu$ M) group, the average DCF fluorescence intensity was  $1285.5 \pm 110.2$ . This showed that reactive oxygen species levels were significantly higher in A $\beta_{25-35}$ -treated cells than in controls ( $P < 0.05$ ), indicating oxidative stress. The A $\beta_{25-35}$ -induced increase in DCF fluorescence was counteracted by the addition of

N $\omega$ -nitro-L-arginine (average DCF fluorescence intensity  $700.6 \pm 101.7$ ), suggesting that nitric oxide was responsible for the A $\beta_{25-35}$ -induced production of intracellular reactive oxygen species.

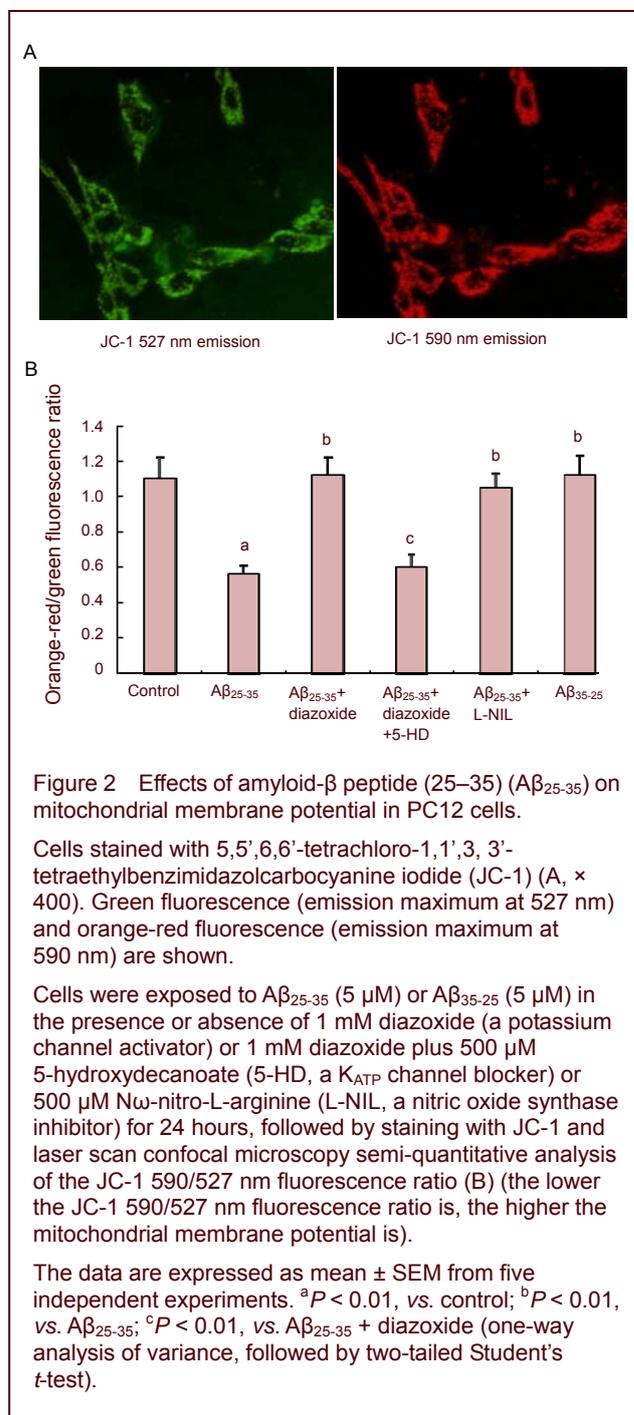


Figure 2 Effects of amyloid- $\beta$  peptide (25–35) (A $\beta_{25-35}$ ) on mitochondrial membrane potential in PC12 cells.

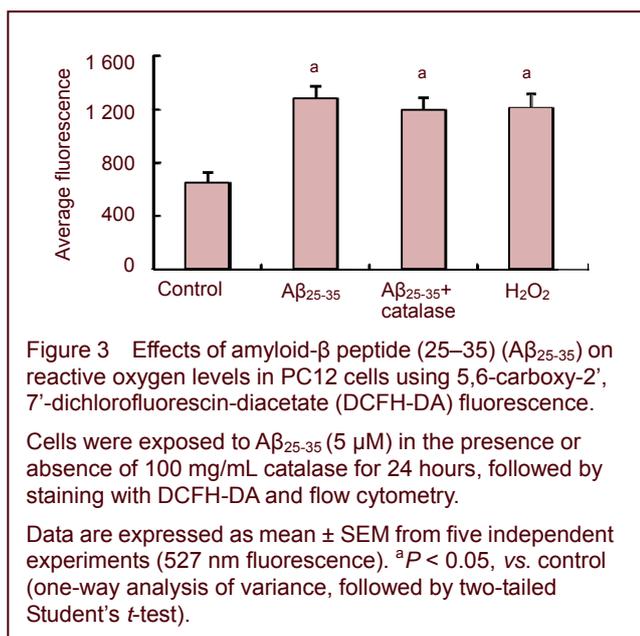
Cells stained with 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1) (A,  $\times 400$ ). Green fluorescence (emission maximum at 527 nm) and orange-red fluorescence (emission maximum at 590 nm) are shown.

Cells were exposed to A $\beta_{25-35}$  (5  $\mu$ M) or A $\beta_{35-25}$  (5  $\mu$ M) in the presence or absence of 1 mM diazoxide (a potassium channel activator) or 1 mM diazoxide plus 500  $\mu$ M 5-hydroxydecanoate (5-HD, a K<sub>ATP</sub> channel blocker) or 500  $\mu$ M N $\omega$ -nitro-L-arginine (L-NIL, a nitric oxide synthase inhibitor) for 24 hours, followed by staining with JC-1 and laser scan confocal microscopy semi-quantitative analysis of the JC-1 590/527 nm fluorescence ratio (B) (the lower the JC-1 590/527 nm fluorescence ratio is, the higher the mitochondrial membrane potential is).

The data are expressed as mean  $\pm$  SEM from five independent experiments. <sup>a</sup> $P < 0.01$ , vs. control; <sup>b</sup> $P < 0.01$ , vs. A $\beta_{25-35}$ ; <sup>c</sup> $P < 0.01$ , vs. A $\beta_{25-35}$  + diazoxide (one-way analysis of variance, followed by two-tailed Student's *t*-test).

The A $\beta_{25-35}$ -induced increase in DCF fluorescence was also counteracted by 1 mM diazoxide (average DCF fluorescence intensity  $750.5 \pm 99.3$ ), suggesting that diazoxide could protect cells from oxidative stress induced by A $\beta_{25-35}$  treatment. The protective effect of diazoxide was abolished by 5-hydroxydecanoate (500  $\mu$ M).

We also determined whether  $H_2O_2$  production was necessary for the  $A\beta_{25-35}$ -induced increase in reactive oxygen species levels. Incubating cells with  $H_2O_2$  increased DCF fluorescence compared with control cells ( $P < 0.05$ ). However, incubation with the  $H_2O_2$ -degrading enzyme catalase (100 mg/mL) did not reverse the  $A\beta_{25-35}$ -induced increase in DCF fluorescence intensity ( $P > 0.05$ ), suggesting intracellular  $H_2O_2$  levels did not play an important role in the  $A\beta_{25-35}$ -induced increase in reactive oxygen species levels (Figure 3).



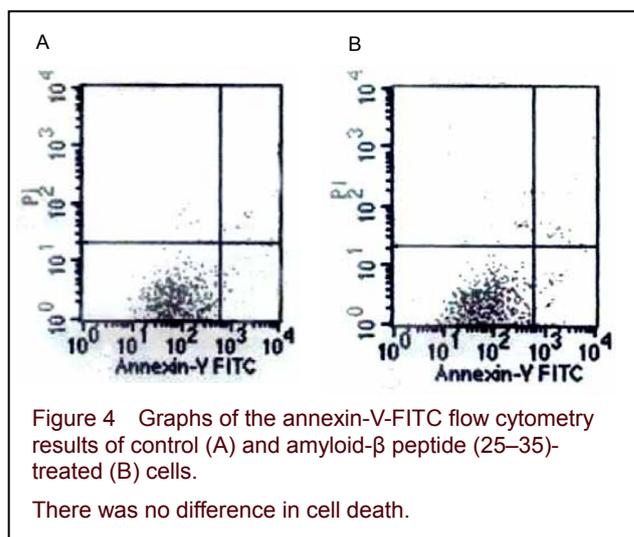
### Effect of $A\beta_{25-35}$ peptide on apoptosis and necrosis in PC12 cells

To study whether the  $A\beta_{25-35}$ -induced increases in mitochondrial membrane potential and reactive oxygen species levels were associated with apoptosis and necrosis, cells were exposed to  $A\beta_{25-35}$  (5  $\mu$ M) for 24 hours and then examined with annexin-V-FITC/ propidium iodide flow cytometry. The percentage of apoptotic and necrotic PC12 cells exposed to  $A\beta_{25-35}$  was  $6.4 \pm 1.8\%$  (Figure 4). There was no difference in cell death in  $A\beta_{25-35}$ -treated PC12 cells compared with the control.

## DISCUSSION

Previous studies confirmed that  $H_2O_2$  induces neuronal mitochondrial depolarization, and that simultaneous application of diazoxide and  $H_2O_2$  completely inhibits  $H_2O_2$ -induced mitochondrial membrane depolarization<sup>[11-13]</sup>. This inhibitory action of diazoxide is antagonized by the addition of 5-hydroxydecanoate (a  $mitoK_{ATP}$  channel blocker), suggesting that opening of the  $mitoK_{ATP}$  channel was responsible for the

maintenance of mitochondrial membrane potential during  $H_2O_2$  challenge<sup>[19]</sup>. The  $mitoK_{ATP}$  channel plays an important role in the regulation of mitochondrial membrane potential<sup>[20]</sup>.



This study determined that diazoxide protects cells from the neurotoxic effects of the  $A\beta_{25-35}$  fragment implicated in the molecular pathogenesis of Alzheimer's disease. An early indicator of toxicity is the inhibition of cellular MTT reduction to MTT formazan, a widely used assay for measuring cell viability<sup>[21-22]</sup>. We used this assay to investigate the toxicity of  $A\beta_{25-35}$  in PC12 cells. Our study indicated that  $A\beta_{25-35}$  induces a decrease in PC12 cell viability in a concentration- dependent manner, and this decrease can be significantly counteracted by diazoxide. This neuroprotective effect is attenuated by 5-hydroxydecanoate. These data indicate that the neuroprotective effect of diazoxide against  $A\beta_{25-35}$ -induced toxicity in PC12 cells is mediated through the  $mitoK_{ATP}$  channel.

Few studies have focused on the role of mitochondrial membrane integrity in the neuroprotection evoked by diazoxide. Measuring mitochondrial activity with the membrane potential-sensitive probe JC-1 revealed that exposure of PC12 cells to  $A\beta_{25-35}$  for 24 hours significantly increases mitochondrial membrane potential and strongly decreases the proportion of active mitochondria, and this effect can be significantly reversed by diazoxide. Potassium channel activators for  $K_{ATP}$  channels protected cardiac myocytes from ischemic injury<sup>[23]</sup> and neurons from oxidative injury induced by glutamate<sup>[12]</sup>. A possible mechanism for this protective effect is that diazoxide might activate  $K^+$  channels, decrease mitochondrial membrane potential and maintain normal physiological mitochondrial functions, protecting cells from amyloid  $\beta_{25-35}$  peptide-induced

intracellular oxidative stress.

Since oxidative stress is believed to be another important contributor to the neurodegenerative process in Alzheimer's disease<sup>[24]</sup>, we measured reactive oxygen species levels in PC12 cells treated with A $\beta$ <sub>25-35</sub> by flow cytometric analysis using the peroxide-sensitive fluorescence probe 5,6-carboxy-2',7'-dichlorofluorescein diacetate (DCFH-DA). Quantitative analysis revealed that, in cells exposed to A $\beta$ <sub>25-35</sub> for 24 hours, intracellular reactive oxygen species levels are significantly higher than in control cells. Diazoxide significantly inhibits the increase in reactive oxygen species levels induced by A $\beta$ <sub>25-35</sub> via interaction with mitochondria. Previous reports showed that mitochondrial deficits lead to oxidative stress<sup>[25-26]</sup>. Thus, a possible mechanism of cellular damage by A $\beta$ <sub>25-35</sub> involves an increase in reactive oxygen species and intracellular oxidative stress caused by impairment of the normal physiological function of K<sub>ATP</sub> channels in the mitochondria. Both intracellular reactive oxygen species and impaired mitochondria form a vicious cycle such that cells are subjected to increasing oxidative stress. Ozcan *et al*<sup>[27]</sup> found that potassium channel activators decreased the production of reactive oxygen species, such as superoxide and hydrogen peroxide. Therefore, we believe that diazoxide reverses the cytotoxic effect of A $\beta$ <sub>25-35</sub> by maintaining the conductance of K<sup>+</sup> channels and decreasing reactive oxygen species production.

Physiologically, normal plasma concentrations of nitric oxide play a critical role in signal transduction, memory and synaptic plasticity in the central nervous system, but excess nitric oxide synthesized by inducible nitric oxide synthase has been implicated in neuronal cytotoxicity<sup>[17]</sup>. In the present study, A $\beta$ <sub>25-35</sub> induced an increase in mitochondrial membrane potential, and the associated increase in intracellular reactive oxygen species levels was counteracted by the inducible nitric oxide synthase inhibitor Nw-nitro-L-arginine, suggesting that nitric oxide played a significant role in regulating intracellular reactive oxygen species production and the increase in mitochondrial membrane potential. Supporting our data, previous research showed that synthetic A $\beta$  elicited a marked and sustained induction of inducible nitric oxide synthase activity and the formation of nitric oxide metabolites in primary cultures of mixed rat neuronal and glial cells<sup>[28-29]</sup>. The generation of reactive oxygen species in response to A $\beta$ <sub>25-35</sub> treatment was mimicked by H<sub>2</sub>O<sub>2</sub>, which is converted to the highly toxic hydroxyl radical by the Fenton reaction. However, the H<sub>2</sub>O<sub>2</sub>-degrading enzyme catalase (a reactive oxygen

species inhibitor) could not reverse the A $\beta$ <sub>25-35</sub>-induced increase in intracellular reactive oxygen species, suggesting that intracellular H<sub>2</sub>O<sub>2</sub> does not play an important role in the A $\beta$ <sub>25-35</sub>-induced increases in reactive oxygen species levels. However, since the A $\beta$ <sub>25-35</sub>-induced elevation of intracellular reactive oxygen species levels was counteracted by an inducible nitric oxide synthase inhibitor, we suggest that nitric oxide plays an important role in this increase. A $\beta$ <sub>25-35</sub> activated inducible nitric oxide synthase activity and increased the formation of nitric oxide, resulting in increased intracellular reactive oxygen species levels and changes in mitochondrial membrane potential. In addition, increased intracellular reactive oxygen species levels might be responsible for nitric oxide-induced intracellular oxidative stress and cytotoxicity.

Flow cytometry found no difference in apoptosis and necrosis between the control and A $\beta$ <sub>25-35</sub>-treated cells, suggesting that cell death was preceded by the increase in mitochondrial membrane potential and in reactive oxygen species levels. These results are of significant importance for the understanding of Alzheimer's disease pathogenesis.

In summary, this study demonstrated for the first time that diazoxide and the inducible nitric oxide synthase inhibitor Nw-nitro-L-arginine exert their neuroprotective effects through inhibition of the increase of mitochondrial membrane potential and the associated increase in intracellular reactive oxygen species levels induced by A $\beta$ <sub>25-35</sub> application. A possible mechanism is that diazoxide might activate K<sup>+</sup> channels, decrease the mitochondrial membrane potential and maintain normal physiological mitochondrial functions. The effect of Nw-nitro-L-arginine indicates that nitric oxide plays an important role in regulating the production of reactive oxygen species and increasing mitochondrial membrane potential in response to A $\beta$ <sub>25-35</sub>. Increased mitochondrial membrane potential and intracellular reactive oxygen species levels might promote each other, eventually leading to cell death. Further studies are required to explore in more detail the mechanisms of the observed effects, which may offer a novel therapeutic strategy for neurodegenerative disorders such as Alzheimer's disease.

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## MATERIALS AND METHODS

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### Design

A comparative *in vitro* cell culture experiment.

### Time and setting

The experiments were performed at a laboratory in the Shanghai Jiao Tong University School of Medicine from December 2010 to June 2011.

### Materials

A $\beta_{25-35}$  and A $\beta_{35-25}$  purchased from Sigma (St. Louis, MO, USA) were dissolved in sterile distilled water at 500  $\mu$ M and incubated at 37°C for 4 days.

### Methods

#### PC12 cell culture and differentiation

Rat PC12 pheochromocytoma cells (Chinese Academy of Sciences, Shanghai, China) were cultured at 37°C in Dulbecco's modified Eagle's medium (Gibco, Carlsbad, CA, USA) supplemented with 5% horse serum (Gibco), 10% fetal bovine serum (Gibco), 2 mM L-glutamine (Sigma), 100 IU/mL penicillin (Sigma), and 100  $\mu$ g/mL streptomycin (Sigma) in a humidified atmosphere containing 5% CO<sub>2</sub> in air. Neuronal-like differentiation of PC12 cells was induced by 7-day treatment with neural growth factor (100 ng/mL) (Gibco) (Figure 5).



Figure 5 Identification of neuronal-like differentiation of PC12 cells (Axioplan 2 microscope,  $\times$  400).

PC12 cells with neuronal-like projections appeared after differentiation with nerve growth factor.

#### Grouping and intervention

Aggregated A $\beta_{25-35}$  (0.1, 1, 5, 10, 20  $\mu$ M) or A $\beta_{35-25}$  (0.1, 1, 5, 10, 20  $\mu$ M) were applied to the differentiated cells (at a density of  $6 \times 10^4$ /cm<sup>2</sup>) for 24 hours. Diazoxide (Sigma) was prepared as a stock solution of 50 mg/mL in dimethyl sulfoxide (final concentration 0.08%) and added to the media for a final concentration of 1 mM. Cells were preincubated for 1 hour with 1 mM diazoxide and then co-treated with A $\beta_{25-35}$  and diazoxide for 24 hours. The N $\omega$ -nitro-L-arginine (Roche, Basel, Switzerland) was dissolved in 0.2 mM PBS (pH 7.4) and added to the media for a final concentration of 500  $\mu$ M. Cells were co-incubated with N $\omega$ -nitro-L-arginine and A $\beta_{25-35}$  for 24 hours.

#### Cellular viability as detected by MTT

The viability of cultured cells exposed to various drugs for 24 hours was evaluated using the MTT colorimetric assay<sup>[30]</sup>. A total of 10  $\mu$ L MTT reagent (Sigma) was added to plated cells for 2 hours until a purple precipitate was visible. A total of 100  $\mu$ L detergent reagent was added at room temperature in the dark for 2 hours. MTT reduction was quantified at 570 nm using a microplate reader (Bio-Tek Instruments Inc., Winooski, VT, USA). The ratio of MTT reduction in samples treated with various drugs to the MTT reduction in control samples was used to determine cellular viability.

#### Determination of the mitochondrial membrane potential using JC-1 fluorescence

Mitochondrial membrane potential was quantified using the ratiometric probe JC-1<sup>[18]</sup>. In apoptotic cells, in which the mitochondrial membrane potential had collapsed, monomeric JC-1 remained in the cytosol and appeared green. Following exposure to 5  $\mu$ M A $\beta_{25-35}$  or A $\beta_{35-25}$  in the presence or absence of 1 mM diazoxide or 1 mM diazoxide plus 500  $\mu$ M 5-hydroxydecanoate (Sigma) or 500  $\mu$ M N $\omega$ -nitro-L-arginine for 24 hours, cells were incubated in culture medium-free serum containing 10  $\mu$ g/mL JC-1 (solubilized in N, N-dimethylformamide, 1% v/v; Molecular Probes Europe, Leiden, Netherlands) for 30 minutes at 37°C under 5% CO<sub>2</sub>, and then washed with 0.1 M PBS twice. Laser confocal microscopy (Leica, Wetzlar, Germany) semi-quantitative analysis of JC-1 fluorescence was used to determine the average intensity of green (527 nm) fluorescent monomers and J-aggregates with orange-red (590 nm) fluorescence. The ratio of fluorescence at 590 nm/fluorescence at 527 nm was used to monitor changes in mitochondrial membrane potential.

#### Measurement of reactive oxygen species by DCFH-DA fluorescence

The production of reactive oxygen species was estimated by flow cytometry using the oxidation-sensitive fluorescent probe DCFH-DA<sup>[31]</sup>, a cell-permeable dye that, once inside the cell, is cleaved by intracellular esterase into its non-fluorescent form DCFH. This form, which is no longer membrane permeable, may be further oxidized by H<sub>2</sub>O<sub>2</sub> or OH<sup>-</sup> to its fluorescent form, DCF. Following exposure to 5  $\mu$ M A $\beta_{25-35}$  or A $\beta_{35-25}$  in the presence or absence of 1 mM diazoxide or 1 mM diazoxide plus 500  $\mu$ M 5-hydroxydecanoate or 500  $\mu$ M N $\omega$ -nitro-L-arginine or 100 mg/mL catalase (Sigma) or 1  $\mu$ M H<sub>2</sub>O<sub>2</sub> (Sigma) for 24 hours, the cultured cells were incubated in serum-free culture medium containing 10  $\mu$ M DCFH-DA (Molecular Probes Europe) for 30 minutes at 37°C and were then washed with 0.2 M

PBS once. Cells were then trypsinized and washed, followed by flow cytometer detection using a FACS Calibur cytometry instrument (BD Biosciences, San Jose, CA, USA). Fluorescence emission from DCF (green) was detected at a wavelength of 527 nm.

### **Detection of apoptosis and necrosis using flow cytometry**

After exposure to 5  $\mu$ M A $\beta$ <sub>25-35</sub> or A $\beta$ <sub>35-25</sub> for 24 hours, free-floating cells pooled with cells detached by mild trypsinization were incubated in 100 mL annexin-V-FLUOS solution (Roche) containing 2 mL Annexin-V reagent (Roche) and 2 mL propidium iodide reagent (Roche) for 10–15 minutes at 20°C and then cooled so that the reaction was terminated. FITC-fluorescence was measured with a FACS Calibur flow cytometer at an excitation wavelength of 488 nm.

### **Statistical analysis**

All data are expressed as mean  $\pm$  SEM. Statistical analyses between controls and samples treated with various drugs were done by SPSS 10.0 software (SPSS, Chicago, IL, USA) using one-way analysis of variance, followed by two-tailed Student's *t*-test or multiple comparison test where appropriate. A value of *P* < 0.05 was considered statistically significant for all analyses.

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**Author contributions:** Min Kong was responsible for funding, conducting the experiments and writing the final version of the manuscript. Maowen Ba designed and evaluated the experiments. Hui Liang was responsible for data analysis. Peng Shao, Tianxia Yu and Ying Wang provided information and technological support. All authors approved the final version of the manuscript.

**Conflicts of interest:** None declared.

**Author statements:** The manuscript is original, has not been submitted to or is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application/funding source disputations.

## **REFERENCES**

- [1] Lehericy S, Hirsch EC, Cervera-Pirot P, et al. Heterogeneity and selectivity of the degeneration of cholinergic neurons in the basal forebrain of patients with Alzheimer's disease. *J Comp Neurol.* 1993;330(1):15-31.
- [2] Barnham KJ, Cappai R, Beyreuther K, et al. Delineating common molecular mechanisms in Alzheimer's and prion diseases. *Trends Biochem Sci.* 2006;31(8):465-472.
- [3] Abad MA, Enguita M, DeGregorio-Rocasolano N, et al. Neuronal pentraxin 1 contributes to the neuronal damage evoked by amyloid-beta and is overexpressed in dystrophic neurites in Alzheimer's brain. *J Neurosci.* 2006; 26(49):12735-12747.
- [4] Tickler AK, Wade JD, Separovic F. The role of Abeta peptides in Alzheimer's disease. *Protein Pept Lett.* 2005; 12(6):513-519.
- [5] Kaminsky YG, Marlatt MW, Smith MA, et al. Subcellular and metabolic examination of amyloid-beta peptides in Alzheimer disease pathogenesis: evidence for Abeta(25-35). *Exp Neurol.* 2010;221(1):26-37.
- [6] Gao X, Tang XC. Huperzine A attenuates mitochondrial dysfunction in beta-amyloid-treated PC12 cells by reducing oxygen free radicals accumulation and improving mitochondrial energy metabolism. *J Neurosci Res.* 2006;83(6):1048-1057.
- [7] Silva DF, Esteves AR, Oliveira CR, et al. Mitochondria: the common upstream driver of amyloid- $\beta$  and tau pathology in Alzheimer's disease. *Curr Alzheimer Res.* 2011;8(5): 563-572.
- [8] Maruszak A, Żekanowski C. Mitochondrial dysfunction and Alzheimer's disease. *Prog Neuropsychopharmacol Biol Psychiatry.* 2011;35(2):320-330.
- [9] Zawar C, Plant TD, Schirra C, et al. Cell-type specific expression of ATP-sensitive potassium channels in the rat hippocampus. *J Physiol.* 1999;514(Pt 2):327-341.
- [10] Ma G, Fu Q, Zhang Y, et al. Effects of Abeta1-42 on the subunits of KATP expression in cultured primary rat basal forebrain neurons. *Neurochem Res.* 2008;33(7):1419-1424.
- [11] Ala-Rämi A, Ylitalo KV, Hassinen IE. Ischaemic preconditioning and a mitochondrial KATP channel opener both produce cardioprotection accompanied by F1F0-ATPase inhibition in early ischaemia. *Basic Res Cardiol.* 2003;98(4):250-258.
- [12] Goodman Y, Mattson MP. K<sup>+</sup> channel openers protect hippocampal neurons against oxidative injury and amyloid b-peptide toxicity. *Brain Res.* 1996;706(2):328-332.
- [13] Hu LF, Wang S, Shi XR, et al. ATP-sensitive potassium channel opener iptakalim protected against the cytotoxicity of MPP<sup>+</sup> on SH-SY5Y cells by decreasing extracellular glutamate level. *J Neurochem.* 2005;94(6):1570-1579.
- [14] Lacza Z, Pankotai E, Busija DW. Mitochondrial nitric oxide synthase: current concepts and controversies. *Front Biosci.* 2009;14:4436-4443.
- [15] Blanco S, Molina FJ, Castro L, et al. Study of the nitric oxide system in the rat cerebellum during aging. *BMC Neurosci.* 2010;11:78.
- [16] Haas J, Storch-Hagenlocher B, Biessmann A, et al. Inducible nitric oxide synthase and argininosuccinate synthetase: co-induction in brain tissue of patients with Alzheimer's dementia and following stimulation with beta-amyloid 1-42 in vitro. *Neurosci Lett.* 2002;322(2):121-125.

- [17] Abramson SB, Amin AR, Clancy RM, et al. The role of nitric oxide in tissue destruction. *Best Pract Res Clin Rheumatol*. 2001;15(5):831-845.
- [18] Mathur A, Hong Y, Kemp BK, et al. Evaluation of fluorescent dyes for the detection of mitochondrial membrane potential changes in cultured cardiomyocytes. *Cardiovasc Res*. 2000;46(1):126-138.
- [19] Teshima Y, Akao M, Li RA, et al. Mitochondrial ATP-sensitive potassium channel activation protects cerebellar granule neurons from apoptosis induced by oxidative stress. *Stroke*. 2003;34(7):1796-1802.
- [20] Bednarczyk P. Potassium channels in brain mitochondria. *Acta Biochim Pol*. 2009;56(3):385-392.
- [21] Shearman MS, Ragan CI, Iversen LL. Inhibition of PC12 cell redox activity is a specific, early indicator of the mechanism of beta-amyloid-mediated cell death. *Proc Natl Acad Sci U S A*. 1994;91(4):1470-1474.
- [22] Liu Y, Schubert D. Cytotoxic amyloid peptides inhibit cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction by enhancing MTT formazan exocytosis. *J Neurochem*. 1997;69(6):2285-2293.
- [23] Han JS, Wang HS, Yan DM, et al. Myocardial ischemic and diazoxide preconditioning both increase PGC-1alpha and reduce mitochondrial damage. *Acta Cardiol*. 2010;65(6):639-644.
- [24] Dumont M, Beal MF. Neuroprotective strategies involving ROS in Alzheimer disease. *Free Radic Biol Med*. 2011;51(5):1014-1026.
- [25] Pagani L, Eckert A. Amyloid-Beta interaction with mitochondria. *Int J Alzheimers Dis*. 2011;2011:925050.
- [26] Chen JX, Yan SS. Role of mitochondrial amyloid-beta in Alzheimer's disease. *J Alzheimers Dis*. 2010;20( Suppl 2):S569-578.
- [27] Ozcan C, Bienengraeber M, Dzeja PP, et al. Potassium channel openers protect cardiac mitochondria by attenuating oxidant stress at reoxygenation. *Am J Physiol Heart Circ Physiol*. 2002;282(2):H531-539.
- [28] Ishii K, Muelhauser F, Liebl U, et al. Subacute NO generation induced by Alzheimer's beta-amyloid in the living brain: reversal by inhibition of the inducible NO synthase. *FASEB J*. 2000;14(11):1485-1489.
- [29] Tsai SJ, Liu WH, Yin MC. Trans fatty acids enhanced beta-amyloid induced oxidative stress in nerve growth factor differentiated PC12 cells. *Neurochem Res*. 2012;37(4):786-794.
- [30] Ahmadian S, Barar J, Saei AA, et al. Cellular toxicity of nanogenomedicine in MCF-7 cell line: MTT assay. *J Vis Exp*. 2009;(26). pii:1191.
- [31] Eruslanov E, Kusmartsev S. Identification of ROS using oxidized DCFDA and flow-cytometry. *Methods Mol Biol*. 2010;594:57-72.

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