J Bioenerg Biomembr (2011) 43:399–407
DOI 10.1007/s10863-011-9362-7

Genistein inhibits mitochondrial-targeted oxidative damage induced by beta-amyloid peptide 25–35 in PC12 cells

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Received: 10 January 2011 / Accepted: 19 May 2011 / Published online: 6 July 2011

Abstract The antioxidative properties of genistein (Gen) have been demonstrated by our previous studies and others, but its potential mechanism was not very clear. Because of the key role of mitochondria in oxidant production, we wondered if mitochondria were one of Gen's neuroprotective targets. In the present study we investigated whether Gen has protective effects on mitochondria damaged by Aβ25-35. PC12 cells were pre-incubated with or without Gen for 2 h followed by the incubation with 20 μM Aβ25-35 for another 24 h before mitochondrial membrane fluidity (MMF), mitochondrial membrane potential (MMP) , and mitochondrial redox state were measured. The results showed that Gen alleviated the decrease of MMF induced by Aβ25-35, and maintained the MMP. Additionally, Gen promoted the mitochondrial antioxidative capability through increasing the GSH/GSSG ratio, GPx activity and MnSOD protein expression in mitochondria. Moreover, Gen reversed the changes of ChAT mRNA and AChE mRNA expression in cells induced by Aβ25-35. These results suggested that Gen can protect the mitochondrial membrane and maintain redox state in mitochondria damaged by Aβ25-35.

Keywords Genistein · Beta-amyloid peptide 25–35 · Mitochondria · PC12 cells

Yuan-Di Xi and Huan-Ling Yu contributed equally to this work.

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Introduction

It is well recognized that mitochondrial dysfunction is associated with neurodegenerative disease (Moreira et al. 2010). Many lines of evidence suggest that mitochondrial dysfunction is a hallmark of Alzheimer's disease (AD), and it is considered as an early event in AD pathology (Hauptmann et al. 2009; Reddy and Beal 2008). Aβ accumulates in mitochondria (Fernández-Vizarra et al. 2004; Lustbader et al. 2004) and mitochondrial structural abnormalities are observed (Hirai et al. 2001) in the brains of AD patients. Animal studies have demonstrated that extracellular Aβ causes oxidative stress in the brain of transgenic mice and mitochondrial malfunction (Manczak et al. 2006; Keil et al. 2004). *In vitro* studies indicate that Aβ leads to neurotoxicity by triggering the inhibition of neuronal mitochondrial protein import and consequent mitochondrial impairment (Sirk et al. 2007). Furthermore, it has been demonstrated that the mitochondrial membrane

is a critical target of Aβ-induced mitochondrial malfunction (Reddy and Beal 2008). Given the demonstrated neuroprotection of classic antioxidants such as Vitamin E (Yatin et al. 2000; Muñoz et al. 2005; Crouzin et al. 2010; Lee et al. 2010), lazaroids and hormones like estrogens (Behl 1999; Miranda et al. 2000) or melatonin (Zhou et al, 2008; Feng and Zhang 2004), antioxidant therapy was considered as a potentially effective way to prevent/ameliorate the neurotoxicity of Aβ (Miranda et al. 2000).

Functional food, especially the dietary consumption of antioxidant compounds has been the subject of much interest in protection against neurodegeneration. Soy isoflavone (SIF) is the major flavonoid found in the human diet (Song et al. 2007). A number of beneficial effects of SIF especially genistein (Gen), the main active component of SIF, has been shown on several diseases such as osteoporosis (Setchell and Lydeking-Olsen 2003; Bitto et al. 2009), cancer (Li et al. 2010), obesity (Kim et al. 2006; Kim et al 2005), and cardiovascular disease (Park et al. 2005; Si and Liu 2007). Recently, Gen has gained attention due to its potential health benefits in preventing neurodegeneration (Valles et al. 2010; Lee and Lee 2008; Wang et al. 2005). Our previous study has indicated that Gen could clear reactive oxygen species (ROS) partially and play an antioxidant role through the activation of redox-sensitive signaling pathways in PC12 cells treated by Aβ25-35 (Ma et al. 2010).

Although past studies have extensively explored its antioxidative (Ma et al. 2010), anti-inflammatory (Valles et al. 2010), and estrogenic effects (Zeng et al. 2004), there is little evidence regarding the protective effect of Gen on mitochondrial dysfunction induced by Aβ. Thus, we hypothesize that Aβ may damage the mitochondrial membrane and then disturb the mitochondrial redox system components resulting in the reduction of antioxidant capacity, subsequently affecting the cell function and ultimately lead to neuronal death. To test the hypothesis, we assessed the changes of MMF, MMP and mitochondrial redox state in PC12 cells pre-incubated with or without Gen followed by incubation with Aβ25-35 to find out the neuroprotective targets of Gen.

Materials and methods

Materials

PC12 cells were obtained from Peking Union Medical Center Laboratory, Beijing, China. Gen, Aβ25-35, poly-l-lysine, Rhodamine123 and DPH were purchased from Sigma-Aldrich. Aβ25-35 was dissolved in deionized distilled water at a concentration of 625 μM and kept at −20 °C until used. Cell culture media dulbecco's minimum essential medium (DMEM), horse serum, fetal bovine serum (FBS) and penicillin (10000 units/ml) and streptomycin (10000 μg/ml) (P/S) were purchased from Gibco. Manganese superoxide dismutase (MnSOD)and glutathione peroxidase (GPx) antibodies were from Santa Cruz Biotechnology.

Cell culture

PC12 cells were maintained in DMEM media supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin and 100 U/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO^2/95% air. Cells were seeded at an appropriate density (1×10^6 cells/cm^2) in poly-l-lysine-coated culture dishes. The culture medium was changed every other day.

Isolation of mitochondria

PC12 cells were pelleted by centrifugation and suspended in Mitochondrial Isolation Buffer (0.01 mol/L Tris-HCl, 0.0001 mol/L EDTA-2Na, 0.01 mol/L sucrose, 0.8%NaCl, pH adjusted to 7.4). Cells were ruptured by an Ultrasonic cracker (5 s each time, repeated four times with 5 s intervals), and centrifuged at 1,500 rpm for 10 min. The supernatant was collected and then centrifuged again at 10,000 rpm for 15 min. The precipitate is the mitochondria of PC12 cells.

Measurement of mitochondrial membrane fluidity

The measurement of mitochondrial membrane fluidity employed the fluorescence polarization technique (Liu et al. 2003) with minor modifications. DPH was used as a fluorescence probe. Cells were treated as described previously and the mitochondria of PC12 cells were suspended in 1 ml PBS. After that, 1 ml DPH (2×10^{-6} mol/L) was added to the mixtures, and a 30 min incubation was carried out at room temperature. Samples were analyzed by a fluorescence spectrophotometer (Infinite M200, TECAN, Switzerland). The excitation and emission wavelengths set at 362 nm and 430 nm, respectively. The temperature of the sample was maintained at 25 °C. Fluorescence anisotropy was calculated from the intensities of the light emitted parallel or perpendicular to the polarization plane of the incident light. I_{VV} is the fluorescence intensity when both the optical axes of the polarizer and the analyzer were vertical. I_{VH} is the fluorescence intensity when the optical axis was vertical and the analyzer was horizontal. I_{HV} is the fluorescence intensity when the optical axis was horizontal and the analyzer was vertical. I_{HH} is the fluorescence intensity when both the optical axis and the analyzer were vertical. η, the representation of the value of coefficient of

viscosity of the mitochondrial membrane, was calculated according to the formula:

(1) G (Correction factor for the instrument's polarization): $G = I_{HV}/I_{HH}$
(2) P (Degree of fluorescence polarization): $P = (I_{VV} - G \cdot I_{VH}) / (I_{VV}+G \cdot I_{VH})$
(3) γ (Anisotropy): $\gamma = (I_{VV} - I_{VH}) / (I_{VV}+2I_{VH}) = 2P/(3-P)$
(4) η (Viscosity of the mitochondrial membrane): $\eta = 2P/(0.42–0.92P)$

Measurement of mitochondrial membrane potential

Assessment of changes in MMP was conducted as previously described (Ma et al. 2010). Following Gen or CsA pretreatment 2 h before exposure to 20 μM Aβ25-35 for 24 h, the mitochondria of PC12 cells were incubated for 5 min with Rhodamine 123 at 37 °C. Fluorescence signals of cells were excited at 488 nm, emission monitored at 525 nm wavelength in a flow cytometer, corresponding to the fluorescence peak of the monomer and that of the aggregate. The value of fluorescence intensity was analyzed by the FCS Express Version 3.0 software (De Novo Software, Canada), and data were statistically analyzed.

Assay for mitochondrial GSH/GSSG ratio and GPx activity

The GSH/GSSG ratio and activity of GPx in mitochondria were measured using assay kits purchased from Biyuntian Biotechnology Company (Beijing, China). The GSH and GSSG levels were measured respectively, and the GSH/GSSG ratio was calculated. GPx levels in mitochondria were determined according to the guidelines of the kit.

Western blot analysis

Cells (1×10^7 cells) in different treatment groups were collected and washed with phosphate buffered saline (PBS). After centrifugation, sedimented cells were lysed by vigorous shaking in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl (pH 7.4), 1 mM DTT, and protease inhibitors) for 40 min at 4 °C, and then centrifuged again at 15,000 rpm for 20 min. The supernatant was separated and collected for protein analysis. The protein concentration was determined by using the BCA protein assay kit (Pierce Biotechnology, USA). Protein samples 50 μg were loaded and separated by a 10% or 12% SDS-acrylamide gel electrophoresis and transferred to polyvinylidene fluoride blots at the voltage of 60 V for 2 h. The membrane was blocked by fresh blocking buffer (Tris-buffered saline, containing 5% skim milk powder) at room temperature for 1 h. Immunoblots were performed with appropriate antibodies. Primary antibody (diluted in 1:1000 with TBST and 1% nonfat dry milk) for anti-MnSOD and anti-GPx (Abcam Biotechnology, USA) was incubated with membrane for 12 h at 4 °C. The proper secondary antibodies were incubated for 1 h at room temperature. The blots were washed again three times with TBST buffer and transferred proteins were visualized by an ECL reaction kit according to the manufacturer's instructions. Then FluorChem FC2 software (Alpha Innotech, USA) was used to analyze the gray value of the protein expression in each group.

Reverse transcriptase polymerase chain reaction

(Table 1) Total RNA was isolated from PC12 cells using Trizol (Invitrogen, Carlsbad, CA, USA) and reverse-transcribed using an RT kit (no. A3500; Applied Promega, Madison, WI, USA). mRNA encoding choline acetyltransterase (ChAT), acetylcholinesterase (AChE), and β-actin (used as an invariant control) were analyzed by RT-PCR. PCR was carried out as follows: denature at 94 °C for 5 min for the first cycle, and then cycles consisting of 94 °C for 30 s, annealing at 53 °C, 64 °C or 58 °C for 30 s, and extension at 72 °C for 30 s. Thirty-five cycles were performed for all the genes. Amplification products underwent electrophoresis on a 10% agarose gel and the relative quantity of mRNA was estimated by densitometry scanning with X-rays (Gel Doc XRTM; Bio-Rad, Hercules, CA, USA).

Statistical analysis

Data are presented as mean ± S.E. They were analyzed with the software SPSS 13.0 (SPSS Inc., Chicago, America) by one-way ANOVA as appropriate and $P < 0.05$ was considered to be significant.

Results

Mitochondrial membrane was damaged by Aβ25-35 and protected by Gen

All the experiments were performed under the conditions that PC12 cells were pre-incubated with or without Gen for 2 h followed by incubation with 20 μM Aβ25-35 for another 24 h, and DMEM was added in the blank control group correspondingly (Fig. 1).

MMF is one of physical properties of mitochondrial membranes. An abnormal MMF could directly reflect injuries to the mitochondrial membrane. To determine whether Gen protected the MMF damage caused by Aβ25-35, we measured the viscosity of the mitochondrial membrane. The MMF in the Aβ25-35 group decreased

Table 1 Primers used for RT-PCR

Name	Primer sequence	Annealing temperature [°C]
AChE	F: 5′-AAA CAT GCA GAA GAT GAG GAT-3′ R: 5′-GAC CAC TAT AGC AAG CAG GAA C-3′	53 °C
ChAT	F: 5′-GCC TGG TAT GCC TGG ATG GTC-3′ R: 5′-TGG AGG GCC ACC TGG ATG AAG-3′	64 °C
β-actin	F: 5′-TGG AAT CCT GTG GCA TCC ATG AAA C-3′ R: 5′-TAA AAC GCA GCT CAG TAA CAG TCC G-3′	58 °C

Forward (F) and reverse (R) primer sequences are shown in 5′to 3′orientation.

significantly ($P<0.05$) relative to the control group. However, in the Gen pretreatment groups, MMF exceeded significantly ($P<0.05$) that of the Aβ25-35 group. Dose-response relationships for the three Gen pretreatment groups are shown in Fig. 2.

We next examined the effect of Gen on MMP in the presence of Aβ25-35. Significant reduction ($P<0.05$) of MMP, compared with the control group, was detected after Aβ25-35 treatment. Gen pretreatment dramatically countered the inhibition of MMP induced by Aβ25-35 (Fig. 3). In order to elucidate whether the mPTP is the protective target of Gen, we used the known mPTP inhibitor CsA (23 nM). The MMP of Aβ25-35+CsA group is significantly higher than Aβ25-35 group. Compared with the CsA group, the MMP of Aβ25-35+CsA group was still lower. The groups pretreated with CsA plus Gen 50 μM or 100 μM could prevent the decrease of MMP induced by Aβ25-35.

Redox equilibrium status in mitochondria was disturbed by Aβ25-35 and maintained by Gen

In mitochondria, the redox status between GSH and GSSG is sensitive to oxidants (Santos et al. 2007). The present study showed that the GSH level and the GSH/GSSG ratio in the mitochondria of Aβ25-35-treated PC12 cells were lower ($P<0.05$) than that in the control group. Gen pretreatment at high concentrations (50 and 100 μM) for 2 h prior to addition of Aβ25-35 could lead to an apparent increase ($P<0.05$) in the GSH level and the GSH/GSSG ratio in contrast to incubation of Aβ25-35 alone (Fig. 4). In addition, Aβ25-35 down-regulated the activity of GPx (Fig. 5a) and the expression of MnSOD (Fig. 5c) in mitochondria, which could be reversed by Gen pretreatment, although no significant difference in protein expression of GPx (Fig. 5b) was detected in mitochondria.

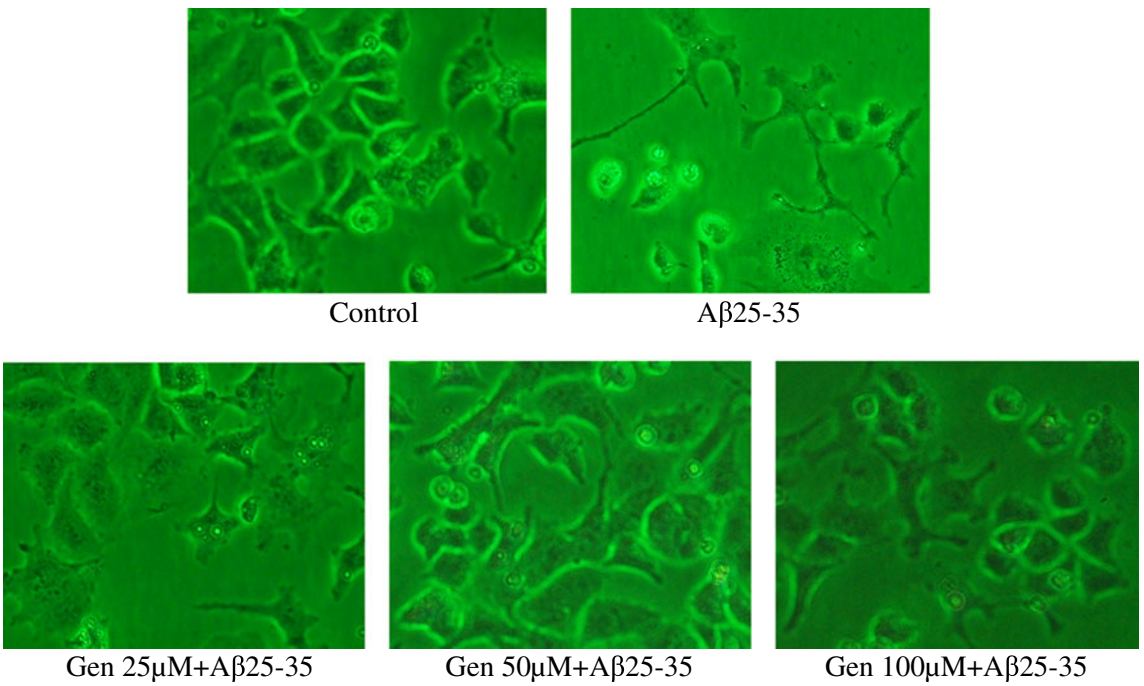

Fig. 1 The morphological shapes of PC12 cells. A microscope was used to observe the morphological shapes of PC12 cells relative to untreated cells. (Control group); cells exposed to 20 μM Aβ25-35 (Aβ25-35 group); cells exposed to 25 μM Gen 2 h before 20 μM Aβ25-35 was added (Gen 25 μM+Aβ25-35 group); cells exposed to 50 μM Gen 2 h before 20 μM Aβ25-35 was added (Gen 50 μM+Aβ25-35 group); cells exposed to 100 μM Gen 2 h before 20 μM Aβ25-35 was added (Gen 100 μM+Aβ25-35 group)

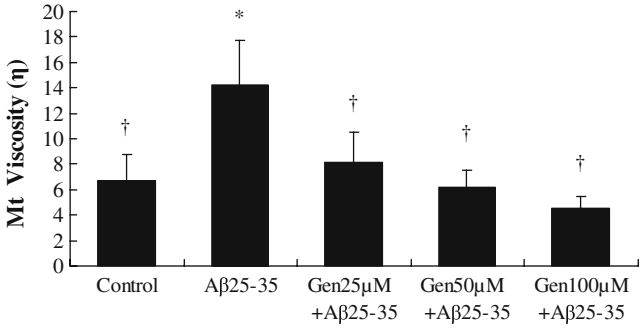

Fig. 2 Effects of Gen on mitochondrial membrane fluidity in PC12 cells damaged by Aβ25-35. The viscosity of the mitochondrial (Mt) membrane indicates the MMF was measured by fluorescence polarization in PC12 cells. Viscosity of the mitochondrial membrane of PC12 cells from untreated cells (Control group); cells exposed to 20 μM Aβ25-35 (Aβ25-35 group); cells exposed to 25 μM Gen 2 h before 20 μM Aβ25-35 was added (Gen 25 μM+Aβ25-35 group); cells exposed to 50 μM Gen 2 h before 20 μM Aβ25-35 was added (Gen 50 μM+Aβ25–35 group); cells exposed to 100 μM Gen 2 h before 20 μM Aβ25-35 was added (Gen 100 μM+Aβ25-35 group). All data were shown as mean ± SE. *$P<0.05$ compared with Control group; †$P<0.05$ compared with Aβ25-35 group

mRNA expression of AChE, ChAT was changed by Aβ25-35 and retained by Gen

Our data had confirmed that Aβ25-35 could induce damage to the mitochondrial membrane and an imbalance in the mitochondrial redox system. In view of this, to determine whether these injuries may be the causes for dysfunction of the Cholinergic system related to cognitive function, we measured AChE and ChAT mRNA expression in PC12 cells. The data showed that Aβ25-35 significantly up-regulated ($P<0.05$) the expression of AChE mRNA compared with the control group. Gen pretreatment could reverse the up-regulation effects induced by Aβ25-35 (Fig. 6a). On the contrary, Aβ25-35-treated PC12 cells had reduced expression of ChAT mRNA compared to the control group ($P<0.05$), and Gen pretreatment could significantly up-regulate the expression of ChAT mRNA (Fig. 6b).

Discussion

Mitochondria are the major organelle of essential energy production for cell activity, and have been suggested to be involved in many important physiological activities (Moreira et al. 2010; Robertson and Orrenius 2002). The mitochondrial respiratory chain is a major site of ROS production in the cell, and thus mitochondria are suggested as a prime target for oxidative damage (Trifunovic and Larsson 2008). In a physiological state, most mammalian cells keep a balance between the generation of ROS and its removal. However, when the mitochondria suffer from injuries this balance is likely broken resulting in oxidative stress that leads to cell death (Moreira et al. 2010; Hirai et al. 2001; Robertson and Orrenius 2002; Trifunovic and Larsson 2008; Sas et al. 2007). Numerous studies have demonstrated that Aβ can disrupt the redox balance in neurons and induce

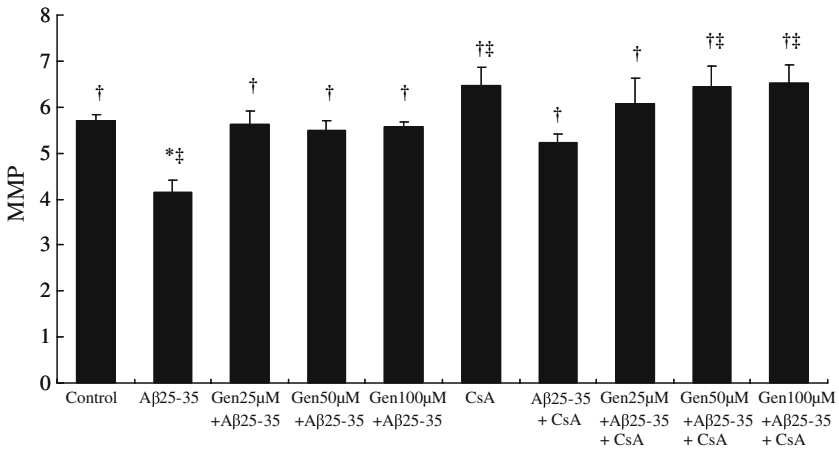

Fig. 3 Effects of Gen on the mitochondrial membrane potential of PC12 cells damaged by Aβ25-35. Fluorescence intensity of the mitochondria in PC12 cells was assayed by flow cytometry. Fluorescence intensity from untreated cells (Control group); cells exposed to 20 μM Aβ25-35 (Aβ25-35 group); cells exposed to 25 μM Gen 2 h before 20 μM Aβ25-35 was added (Gen 25 μM+Aβ25-35 group); cells exposed to 50 μM Gen 2 h before 20 μM Aβ25-35 was added (Gen 50 μM+Aβ25-35 group); cells exposed to 100 μM Gen 2 h before 20 μM Aβ25-35 was added (Gen 100 μM+Aβ25-35 group); cells exposed to 23nM CsA (CsA group); cells exposed to 23nM CsA 2 h before 20 μM Aβ25-35 was added (Aβ25-35+CsA group); cells exposed to 23nM CsA and 25 μM Gen 2 h before 20 μM Aβ25-35 was added (Gen 25 μM+Aβ25-35+CsA group); cells exposed to 23nM CsA and 50 μM Gen 2 h before 20 μM Aβ25-35 was added (Gen 50 μM+Aβ25-35+CsA group); cells exposed to 23nM CsA and 100 μM Gen 2 h before 20 μM Aβ25-35 was added (Gen 100 μM+Aβ25-35+CsA group). All data were shown as mean ± SE. *$P<0.05$ compared with Control group; †$P<0.05$ compared with Aβ25-35 group; ‡$P<0.05$ compared with Aβ25-35+CsA group

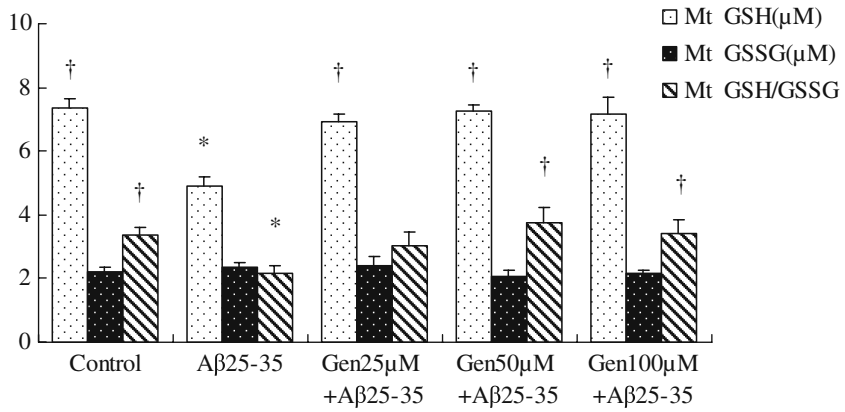

Fig. 4 Effects of Gen on GSH, GSSG and GSH/GSSG ratio in the mitochondria (Mt) of PC12 cells damaged by Aβ25-35. GSH, GSSG and GSH/GSSG ratio in the mitochondria from untreated cells (Control group); cells exposed to 20 μM Aβ25-35 (Aβ25-35 group); cells exposed to 25 μM Gen 2 h before 20 μM Aβ25-35 was added (Gen 25 μM +Aβ25-35 group); cells exposed to 50 μM Gen 2 h before 20 μM Aβ25-35 was added (Gen 50 μM+Aβ25-35 group); cells exposed to 100 μM Gen 2 h before 20 μM Aβ25-35 was added (Gen 100 μM+Aβ25-35 group). All data were shown as mean ± SE. *$P<0.05$ compared with the Control group; †$P<0.05$ compared with Aβ25–35 group

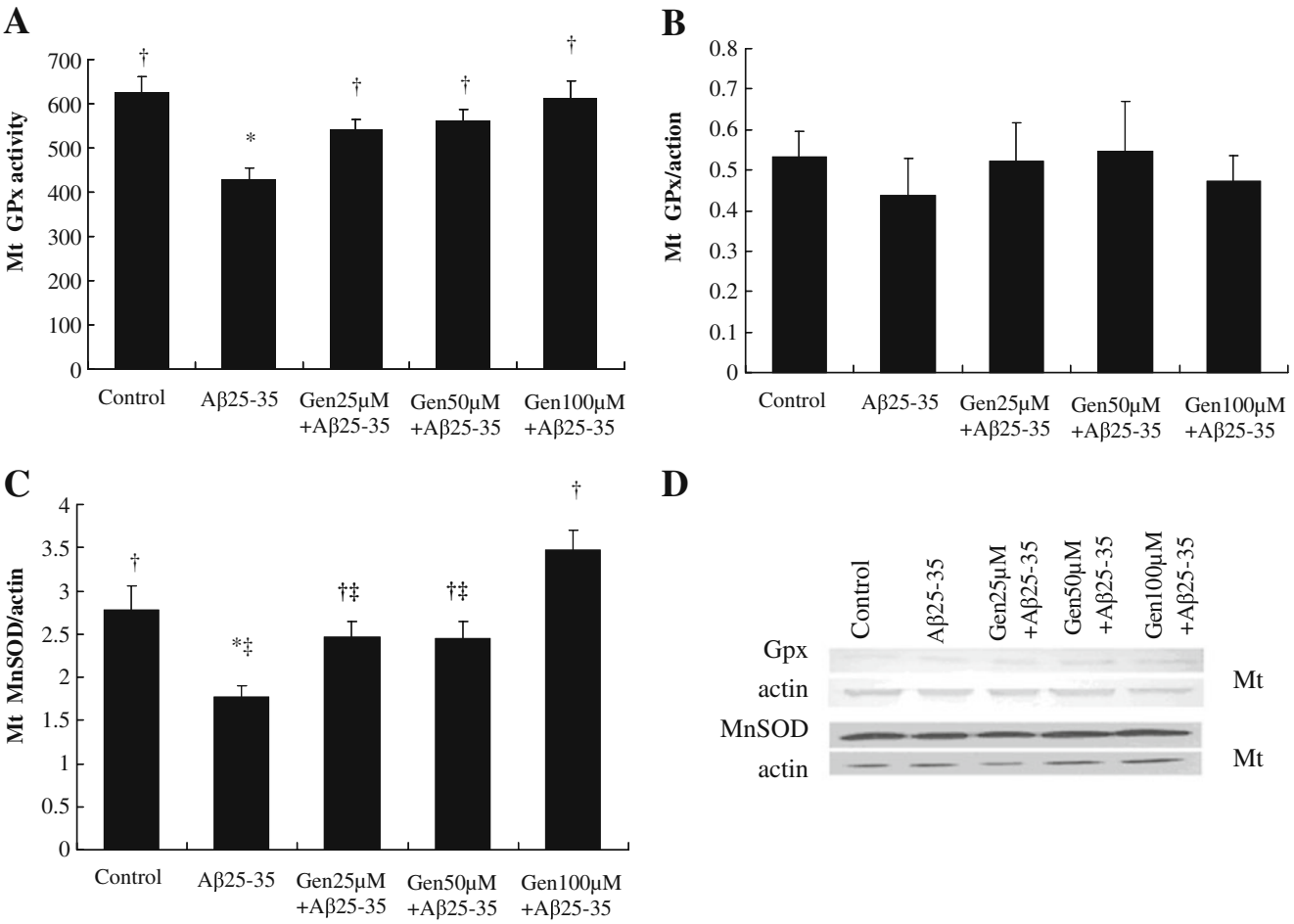

Fig. 5 Effects of Gen on GPx activity (**a**), GPx protein expressions (**b**), MnSOD protein expressions (**c**) and GPx, MnSOD protein electropherogram (**d**) in the mitochondria (Mt) of PC12 cells damaged by Aβ25–35. Activity of GPx, protein expressions of GPx and MnSOD in the mitochondria from untreated cells (Control group); cells exposed to 20 μM Aβ25–35 (Aβ25–35 group); cells exposed to 25 μM Gen 2 h before 20 μM Aβ25–35 was added (Gen 25 μM+Aβ25–35 group); cells exposed to 50 μM Gen 2 h before 20 μM Aβ25–35 was added (Gen 50 μM+Aβ25–35 group); cells exposed to 100 μM Gen 2 h before 20 μM Aβ25–35 was added (Gen 100 μM+Aβ25–35 group). All data were shown as mean ± SE. *$P<0.05$ compared with Control group; †$P<0.05$ compared with Aβ25–35 group; ‡$P<0.05$ compared with Gen 100 μM group

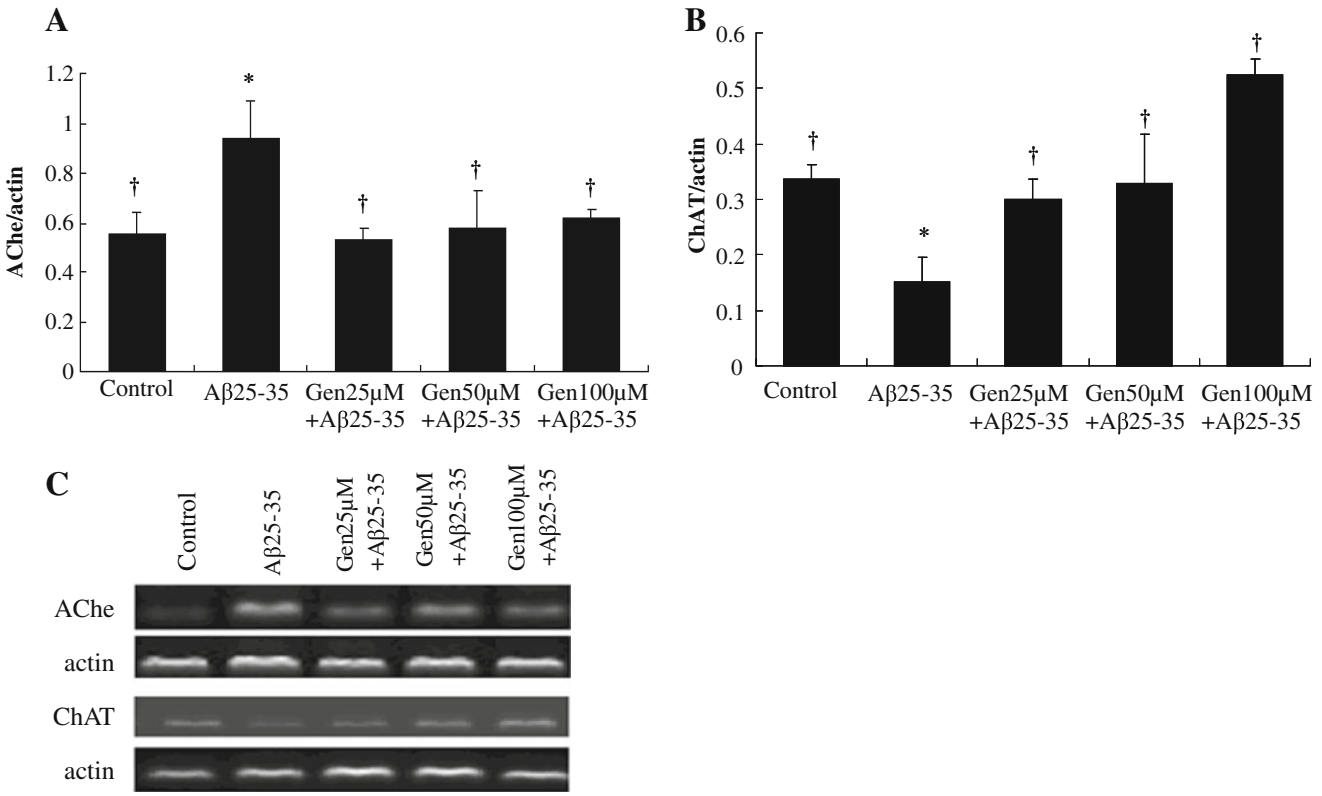

Fig. 6 Effects of Gen on the expression of AChE mRNA and ChAT mRNA in PC12 cells damaged by Aβ25-35. AChE mRNA and ChAT mRNA expressions in PC12 cells from untreated cells (Control group); cells exposed to 20 μM Aβ25–35 (Aβ25–35 group); cells exposed to 25 μM Gen 2 h before 20 μM Aβ25–35 was added (Gen 25 μM+Aβ25–35 group); cells exposed to 50 μM Gen 2 h before 20 μM Aβ25–35 was added (Gen 50 μM+Aβ25–35 group); cells exposed to 100 μM Gen 2 h before 20 μM Aβ25–35 was added (Gen 100 μM+Aβ25–35 group). All data were shown as mean ± SE. *: $P<0.05$ compared with Control group; †: $P<0.05$ compared with Aβ25–35 group

abnormality in mitochondrial structure and/or function in the brain (Reddy and Beal 2008; Fernández-Vizarra et al. 2004; Lustbader et al. 2004; Hirai et al. 2001; Sirk et al. 2007; Manczak et al. 2006; Keil et al. 2004). Both data of others and our previous studies have indicated that Gen as a dietary antioxidant plays a protective role in neurodegeneration (Valles et al. 2010; Lee and Lee 2008; Wang et al. 2005; Ma et al. 2010; Yu et al. 2009; Ma et al. 2009). Here, we found in PC12 cells that Gen can protect mitochondria from Aβ25-35 induced damage by maintaining MMF, MMP and redox balance.

Mitochondrial membrane fluidity (MMF) plays a vital role in regulating many physiological activities like glycometabolism and biological oxidations. Here, an alteration in MMF is always an early step leading to mitochondrial dysfunction (Hou et al. 2008; Aleardi et al. 2005). It has been reported that when mitochondria are directly exposed to Aβ mitochondrial membrane viscosity increases with a concomitant decrease in the efficiency of mitochondrial ATP production (Aleardi et al. 2005). Our present observations showed when PC12 cells are exposed to Aβ25-35 mitochondrial membrane viscosity is also dramatically increased. These data indicate that Aβ25-35 can lead to a change of MMF regardless of whether Aβ25-35 is given directly or indirectly to mitochondria. This membrane destabilizing property of Aβ can thus be regarded as the primary mechanism for mitochondrial toxicity in neurons. Compared with the Aβ-treated group, the viscosity of the mitochondrial membrane in Gen pretreated groups was significantly lower. This suggest that in PC12 cells Gen may help protect the MMF by diminishing damage to the mitochondrial membrane and therefore mitochondrial function. Earlier an interaction of flavonoids with the rat liver mitochondrial membrane resulting in a change in its fluidity was reported (Dorta et al. 2005). In contrast, we report here that Gen avoids decreasing the MMF induced by Aβ in PC12 cells.

Induction of a membrane permeability transition in mitochondria is a central event in the course of a variety of toxic and oxidative forms of cell injuries as well as apoptosis (Robertson and Orrenius 2002; Matsunaga et al. 2004). Opening of the mitochondrial permeability transition pore (mPTP), which is in response to some stimuli like ROS, Ca^{2+} overload or ATP depletion, has been shown to

induce loss of mitochondrial membrane potential (MMP), release of small solutes, Ca^{2+} and cytochrome c, cessation of ATP synthesis, and osmotic swelling (Bernardi and Petronilli 1996; Berman and Hastings 1999; Cassarino et al. 1999; Youn et al. 2002). It has been demonstrated by us (Ma et al. 2010; Yu et al. 2009) and others (Jin et al. 2010; Qiao et al. 2005) that Aβ could lead to the loss of MMP in neurons and isolated brain mitochondria of rats (Gao et al. 2009). Meanwhile our results also showed that Gen maintained the MMP of PC12 cells treated with Aβ25-35 (Ma et al. 2010). Moreover, other researchers have also reported that Gen prevents the opening of the mPTP induced by low potassium in cerebellar granule cells (Atlante et al. 2010). These data suggest that mPTP might be the target of Gen protecting neurons from the damage by Aβ25-35. Therefore, we used in this study CsA a known inhibitor of mPTP opening. The results indicated that Aβ25-35 might reopen the mPTP closed by CsA because the MMP in the Aβ25-35+CsA group was significantly lower than the CsA group alone. When the cells were pretreated with Gen, the MMP was significantly increased relative to the Aβ25-35+CsA group implying that mPTP might be one of the targets of Gen.

It has been suggested that MMP is affected by the imbalance between generation of free radicals and antioxidants like GSH, GPx (Whiteman et al. 2005). Therefore, we investigated the oxidative state in mitochondria after Gen treatment. The data showed that the GSH level and the GSH/GSSG ratio in mitochondria were dramatically decreased by Aβ25-35, while they were reversed by pretreatment with Gen. These results could be due to the increased GPx activity induced by Gen pretreatment. Moreover, the MnSOD protein expression was also enhanced by Gen and this might be another factor increasing mitochondrial antioxidative ability. Other researchers have shown that Aβ leads to oxidative stress and disturbs antioxidant defense system like GSH, GPx, MnSOD in cultured astrocytes (Jeong et al. 2005) and in brain tissue of rats (Kim et al. 2003). Gen protects the antioxidative system in regulation of antioxidative enzymes through its anti-oxidative function in liver of diabetic rats and in the kidney of ovariectomized rats (Lee 2006; Choi and Song 2009). Our previous results indicated that pretreatment of Gen restored the GSH level and the GSH/GSSG ratio which were decreased by Aβ25-35 in PC12 cells (Ma et al. 2010; Ma et al. 2009). Intracellular oxidative stress is also known to alter a cell's mitochondrial $\Delta\Psi m$ (Lee et al. 2011). Here, we focused on mitochondria of PC12 cells and found Gen maintained the mitochondrial redox system disordered by Aβ25-35 and then maintained MMP.

It has been shown that enhancement of AChE activity induced by Aβ25–35 is mediated by oxidative stress in cultured retinal cells, and this effect can be reversed by antioxidants such as α-tocopherol acetate and nitric oxide synthase inhibitors (Melo et al. 2003). Moreover, the abnormal expression of cholinergic enzymes is a consequence of a disturbance of calcium homeostasis (Melo et al. 2003; Sberna et al. 1997) which is related to the mitochondrial dysfunction. Based on the present results that Gen maintains MMP and redox system in mitochondria and PC12 cells (Ma et al. 2009, 2010), we speculate that the AChE and ChAT may be protected by Gen from the damage by Aβ25-35. As expected, the data obtained in our study show that Gen pretreatment significantly increases the mRNA expression of ChAT against the negative effects of Aβ25-35, and inversely depresses the mRNA expression of AChE which is increased by Aβ25-35 alone. Further research is needed to clarify the relationship between mitochondrial dysfunction and cholinergic dysfunction, and the mechanism of Gen to protect the central cholinergic system from Aβ induced dysfunction.

In conclusion, Aβ25-35 treatment, by disturbing the redox state of mitochondria in PC12 cells, leads to mitochondrial dysfunction through injuries in the mitochondrial membrane(s) and subsequent attenuation of antioxidative function(s). However, pretreatment with Gen can reverse the damage of the mitochondrial membrane and increase mitochondrial antioxidant function. Further studies are needed to explore the exact signalling pathway(s) by which Gen protects mitochondria and the cholinergic system from Aβ induced dysfunction.

Acknowledgments The authors appreciate the financial support provided by National Natural Science Foundation of China (No. 30972470 and 30771802), National High Technology Research and Development Program of China (No. 2010AA023003) and Funding Project for Academic Human Resources Development in Institutions of Higher Learning Under the Jurisdiction of Beijing Municipality (PHR 201006112).

Conflict of interest statement The authors declare that there are no conflicts of interest.

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