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ORIGINAL RESEARCH

Reversal of Beta-Amyloid-Induced Neurotoxicity in PC12 Cells by Curcumin, the Important Role of ROS-Mediated Signaling and ERK Pathway

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Abstract Progressive accumulation of beta-amyloid (Aβ) will form the senile plaques and cause oxidative damage and neuronal cell death, which was accepted as the major pathological mechanism to the Alzheimer's disease (AD). Hence, inhibition of Aβ-induced oxidative damage and neuronal cell apoptosis by agents with potential antioxidant properties represents one of the most effective strategies in combating human AD. Curcumin (Cur) a natural extraction from curcuma longa has potential of pharmacological efficacy, including the benefit to antagonize Aβ-induced neurotoxicity. However, the molecular mechanism remains elusive. The present study evaluated the protective effect of Cur against Aβ-induced cytotoxicity and apoptosis in PC12 cells and investigated the underlying mechanism. The results showed that Cur markedly reduced Aβ-induced cytotoxicity by inhibition of mitochondria-mediated apoptosis through regulation of Bcl-2 family. The PARP cleavage, caspases activation, and ROS-mediated DNA damage induced by Aβ were all significantly blocked by Cur. Moreover, regulation of p38 MAPK and AKT pathways both contributed to this protective potency. Our findings suggested that Cur could effectively suppress Aβ-induced cytotoxicity and apoptosis by inhibition of ROS-mediated oxidative damage and regulation of ERK pathway, which validated its therapeutic potential in chemoprevention and chemotherapy of Aβ-induced neurotoxicity.

Keywords Beta-amyloid · Curcumin · Apoptosis · Neurotoxicity · Oxidative damage

Introduction

Alzheimer's disease (AD) is an age-related progressive chronic neurodegenerative diseases and the treatment of AD represents one of the most challenges in clinical neurology. Deposition of senile plaques, cognitive deterioration, the progressive loss of daily living abilities, and the other neuropsychiatric symptoms character its common behavioral disorders. Deposit of beta-amyloid (Aβ) leads to the formation of senile plaques, activation of oxidative damage to neuron and the connection between neuron and gliocyte (Huang and Jiang 2009; Selkoe 1998) and even to cause neuronal cell death, which are thought to be the main pathological mechanism of AD development. Mitochondria as the source of energy plays a key role in the regulation of cell apoptosis and redox balance, and the mitochondrial dysfunction may be a hallmark of Aβ-induced neuronal toxicity in AD (Pagani and Eckert 2011). Recently, studies show that mitochondrial dysfunction and subsequent energy deficiency might be a

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prominent and early event in AD (Eckert et al. 2010). Increasing evidences have confirmed that the process of Aβ-induced mitochondrial dysfunction involved mitochondrial biogenesis and induction of oxidative stress (Sheng et al. 2012). Powerful evidences have demonstrated the link between the ROS-mediated oxidative damage and neurodegenerative disease, including AD (Ho et al. 1998). Miranda et al. reported that overloaded Aβ could trigger cumulative oxidative stress, followed by the increase of reactive oxygen species (ROS) and then disrupt neuronal membrane lipids, proteins, nucleic acids, and mitochondrial respiration (Butterfield et al. 2006; Miranda et al. 2000; Onyango and Khan 2006). ROS mainly produced from cellular energy machinery-mitochondria may in turn act at different levels to impair mitochondrial function, activate caspase family protein, trigger the loss of mitochondrial membrane potential, and dysregulation of MAPKs and AKT pathways (Swerdlow 2007). Therefore, inhibition of mitochondrial dysfunction and ROS accumulation by novel antioxidant may be an effective way in inhibiting Aβ-induced cytotoxicity. However, the details of potential mechanism remain unclear.

Curcumin (Cur) is the principal curcuminoid of the popular South Asian spice turmeric, which is a member of the ginger family (Manolova et al. 2014). Cur is a bright-yellow color and may be used as a food coloring (food additive) (Esatbeyoglu et al. 2012). Studies showed that Cur displays multiple pharmacological properties, including antioxidant, anti-inflammatory, anti-angiogenic, and neuroprotective activities (Abe et al. 1999; Anand et al. 2007; Hatcher et al. 2008; Maier et al. 2013; Meng et al. 2014), which have powerful chemoprevention and chemotherapy potential against oxidative damage-mediated diseases. Evidences demonstrated that Cur exhibited novel neuroprotective effect against Aβ-induced oxidative damage and amyloid pathology in Alzheimer's transgenic mice. However, the specific detail of protective mechanism has not been well illuminated.

In this study, PC12 cells, a rat cell line derived from a pheochromocytoma cells, were employed to establish an in vitro model, and the Cur's protective effect and the underlying mechanism were evaluated. Our findings revealed that Aβ showed neurotoxicity by triggering ROS-mediated oxidative damage and apoptosis. However, Cur pre-treatment significantly attenuated Aβ-induced oxidative damage and apoptosis through inhibition of ROS generation and regulation of ERK pathway.

Materials and Methods

Chemicals

Cur, $A\beta_{25-35}$, propidium iodide (PI), JC-1 and DCFH-DA probes, glutathione (GSH), MTT, and other reagents were all purchased from Sigma. BCA assay kit was purchased from Beyotime Institute of Biotechnology. TUNEL-DAPI co-staining kit was purchased from Roche. DMEM medium, fetal bovine serum (FBS), and penicillin–streptomycin were purchased from Invitrogen. All solvents used were of high-performance liquid chromatography (HPLC) grade. The water used in this study was provided by a Milli-Q water purification system from Millipore. All antibodies and proteins inhibitors (LY294002, U0126, SB202190, and SP600125) used in this study were purchased from Cell Signaling Technology (Beverly, MA, USA).

Cell Culture and Drug Treatments

PC12 cells a rat cell line derived from a pheochromocytoma cells were grown in a mixture of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % FBS and penicillin(100 units/ml) and streptomycin (50 units/ml) at 37 °C under a humidified atmosphere of 5 % CO_2. The Aβ was dissolved in PBS and incubated in a 37 °C water for 3 days to induce aggregation. PC12 cells (10^4 cells/well) seeded in 96-well plate were pre-treated with Cur (12.5–200 μM) for 0, 1, 3, 6, or 12 h or/and co-treated with (2–40 μM) Aβ for another 24 h. After incubation, cell viability was detected by MTT assay.

Measurement of Cell Viability

PC12 cells viability were tested by MTT (3-[4,5-dimethylthiazol-3-yl]-2,5-diphenyltetrazolium bromide) assay. The method was performed as described before (Chen and Wong 2009). Briefly, succinate dehydrogenase, one of the most important enzymes for cellular metabolism, can transform the MTT to a purple formazan crystal in living cells. Therefore, when the cells were treated with Cur or Aβ or co-processing, MTT were added in wells with 20 μl/well (5 mg/ml), and incubated for 5 h at 37 C. Then, the medium were removed and replaced by 150 μl/well DMSO. Crystals were dissolved by DMSO and the intensity of the solvent was tested by microplate reader (Molecular Devices, USA) at 570 nm. The result reflects the growth condition of the PC12 cells after drug administration. Cell viability was expressed as % of control (as 100 %).

Analysis of Cell Apoptosis and Cell Cycle Distribution

Cell apoptosis and cell cycle were assayed by flow cytometry as reported before (Chen and Wong 2009). PC12 cells after pre-treatment with 20 μM Cur for 6 h were co-treated with 10 μM Aβ for 24 h. Cells after treatment were

obtained by centrifugation, washed with PBS, and fixed with 70 % ethanol at −20 °C overnight. Then cells were stained with PI buffer for 2 h at darkness, and the cell apoptosis was detected by flow cytometry. The sub-G1 peak representing hypodiploid DNA percentage means apoptosis in the cell cycle pattern. The cell cycle distribution was analyzed by Modfit LT 4.0 software.

TUNEL-DAPI Co-staining

DNA fragmentation results from apoptotic signaling cascades can be detected by Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). DAPI (4′,6′-diamidino-2-phenylindole) is a fluorescent stain method to identify the double-stranded DNA. PC12 cells were maintained with different drugs for 24 h in 6-well plates and then fixed with 4 % paraformaldehyde (PFA) for 1 h and washed with PBS. Triton X-100 (0.1 %) was used for changing the cells' permeability in PBS for 2 min. The cells were stained in TUNEL reaction mixture for 1 h and then counter-stained with DAPI (1 μg/ml) for 15 min under 37 °C atmosphere. The reaction mixture of TUNEL includes nucleotide mixture and terminal deoxynucleotidyl transferase (TdT). At last, the cells were washed with PBS and examined with fluorescence microscope (100×).

Measurement of Caspase Activities

Caspases activities were detected with specific substrates. Briefly, PC12 cells were pre-incubated with 20 μM Cur for 6 h and then co-treated with 10 μM Aβ for 24 h. Then, the cells were collected and the cell pellets were lysed in lysis buffer for total protein extraction. The total protein concentration was measured by BCA kit and 100 μg/well of total protein was added into 96-well plate, then the protein was incubated with specific caspase substrates (Ac-DEVD-AMC for caspase-3, and Ac-LEHD-AMC for caspase-9) for 2 h at 37 °C in dark. The activities of caspases were eventually detected by fluorescence microplate (Molecular Devices, USA) with the excitation and emission wavelengths of 380 and 440 nm, respectively.

Determination of ROS Accumulation

Intracellular accumulated ROS may oxidize the DCFH-DA without fluorescent into DCFH that belongs to dichlorofluorescein and eventually formed the fluorescent fluorescein. Thus, the generation of ROS in cells can be detected through DCF fluorescence assay (Chen and Wong 2008). ROS generation was usually accepted as an early event in regulating cell apoptosis, therefore, the ROS was detected in 2 h. Briefly, PC12 cells were labeled with 10 μM DCFH-DA at 37 °C for 15 min in dark. Then, the cells were collected, washed with PBS, and added into 96-well plate (10^5 cells/well). Then, cells were pre-treated with or without indicated concentration of Cur for 1 h and then co-treated with Aβ for 2 h. After incubation, the DCF fluorescence intensity representing the ROS level was detected using microplate system (Molecular Devices, USA) with the excitation and emission wavelengths set as 488 and 525, respectively. The time curve of ROS generation was also detected according to the supplementary information. The results were showed as percentages of control groups. The images of the labeled cells were observed through fluorescence microscopy (200×).

Evaluation of Mitochondrial Membrane Potential ($\Delta\psi m$)

Mitochondrial membrane potential ($\Delta\psi m$) was assessed by JC-1 assay. JC-1 exists as a multimer in mitochondria (red fluoresces) or a monomer in the cytosol (green fluoresces) in response to apoptotic stimuli, which depends on the polarity of the $\Delta\psi m$. The depolarization of $\Delta\psi m$ in response to apoptotic stimuli allows JC-1 to transfer from membrane into cytosol and then turns green as a monomer (Chen and Wong 2008). Briefly, PC12 cells seeded in 6-well plates were pre-treated with 20 μM Cur for 1 h and then co-treated with 10 μM Aβ for 2 h. Then, the cells were cultured with the JC-1 dye (5 μg/ml) for 30 min at 37 °C in darkness. After that, the cells were washed with PBS and then visualized by fluorescence microscopy (×200).

Western Blotting Analysis

Cells after treatment were collected and the cell pellets were lysed in lysis buffer for 1 h at 4 °C. Then the total protein was extracted and the concentration was quantified by BCA assay. The total protein was mixed with samples buffer, boiled for 5 min, and stored at −80 °C for subsequent SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Briefly, total protein (40 μg/lane) was loaded and separated in 10 % SDS-PAGE (110 V, 70 min). Then protein was transferred onto polyvinylidene difluoride membrane (PVDF) at 110 V for 75 min. The membranes were blocked with 5 % non-fat milk at room temperature for 2 h. Subsequently, the membranes were incubated with primary antibodies (1:1000) overnight and second antibodies (1:2000) for 2 h at room temperature, respectively. Then the target protein was scanned with an enhanced chemiluminescence reagent (ECL) under a Imaging System (ChemiDoc MP, Bio-Rad). β-actin plays a positive control.

Statistical Analysis

All the experiments were carried out in triplicate at least and repeated for three times. All the figures were expressed as the mean ± SD. Statistical analysis was performed using SPSS statistical package. Differences between two groups were evaluated by two-tailed Student's t test and the difference among three or more groups was assessed by one-way analysis of variance (ANOVA). Difference with $P < 0.05$ or $P < 0.01$ was thought to be statistically significant. Bars with different characters represent the statistical significance at $P < 0.05$.

Results

Cur Inhibits Aβ-Induced Cytotoxicity in PC12 Cells

To ascertain the appropriate dosages for combined treatment, the cytotoxicity of Cur and Aβ alone toward PC12 cells was primarily screened. As shown in Fig. 1a, b, Cur (0–100 μM) showed no toxicity to PC12 cells. Only slight toxicity was seen at 200 μM. However, Aβ treatment significantly inhibited PC12 cell viability in a dose-dependent manner, predicating the potential neurotoxicity. To evaluate the protective effect, the experimental schedules for time-course of Cur pre-treatment and differential protective effect were investigated. As shown in Fig. 1c, d, when PC12 cells were pre-treated with 20 μM Cur for 1, 3, 6, and 12 h and then co-treated with 10 μM Aβ for 24 h, Cur all significantly elevated the cell viability. Pre-treatment of Cur 6 h showed better protective effect than that of the other time-course (Fig. 1b, c). Moreover, pre-treatment showed better protective effect than that of co- and post-treatment (Figure S1). Herein, pre-treatment of Cur for 6 h was employed to explore the protective mechanism. As shown in Fig. 1e, pre-treatment of cells with indicated concentrations of Cur for 6 h dramatically inhibited Aβ-induced decrease in cell viability in a dose-dependent manner. For instance, cells treated with 10 μM Aβ showed obvious decrease in cell viability to 62.4 %. However, pre-treatment of cells with Cur (5, 10, and 20 μM) markedly up-regulated the cell viability from 62.4 % (Aβ) to 68.5, 76.3, and 93.1 %, respectively. Treatment with 40 μM Cur alone showed no toxicity to PC12 cells. Furthermore, improvement of cell morphology, such as weaken cell shrink, enhanced connection of cell-to-cell and cell number, further confirmed this protective effect. Taken together, these results clearly indicated that Cur had the potential to reduce Aβ-induced cytotoxicity in PC12 cells.

Cur Rescues Aβ-Induced Apoptosis in PC12 Cells

To elucidate the model of cell death caused by Aβ, PC12 cells after treatment were stained with PI, and the cell apoptosis was analyzed by flow cytometry. As shown in Fig. 2a, treatment with Aβ leaded to a significant increase in the proportion of apoptotic cells compared to that of control, as convinced by the increase of Sub-G1 peak. However, pre-treatment with Cur effectively blocked Aβ-induced apoptosis in PC12 cells. For instance, treatment with 10 μM Aβ for 24 h caused 36.2 % of cell apoptosis, but pre-treatment of 20 μM Cur for 6 h distinctly inhibited the apoptotic cells to 7.5 %. Cells treated with Cur alone showed no statistical differences in cell apoptosis compared to that of control cells. The result suggested that Cur inhibited Aβ-induced PC12 cell killing mainly by inhibiting Aβ-induced apoptosis.

Caspases a family of cysteine proteases play essential role in lunching cell apoptosis (Salvesen 2002). To further investigate the trait of Aβ-induced apoptosis, the activities of caspase-3, caspase-8, and caspase-9 were tested. As shown in Fig. 2b, exposure of cells to 10 μM Aβ for 24 h significantly activated caspase-3, caspase-8, and caspase-9 from 100 % (control) to 197.8, 121.3, and 158.6 %, respectively, indicating the activation of both extrinsic and intrinsic apoptosis pathways. Besides, activation of caspase-9, which thought to be related to the mitochondria-mediated intrinsic pathway, was activated more prominent than that of caspase-8, indicating that Aβ-mediated apoptosis was mainly triggered by mitochondria-mediated pathway. However, pre-treatment with Cur observably attenuated the activation of caspase-3, caspase-8, and caspase-9 to 124.7, 111.5, and 116.6 %, respectively.

To verify the underlying mechanisms for protective effect of Cur against Aβ-induced apoptosis, we analyzed whether PARP and caspases were involved in this process. As shown in Fig. 2c, exposure of PC12 cells to 10 μM Aβ for indicated time notably resulted in PARP cleavage and activation of caspase-3 and caspase-9 with a time-dependent manner. However, pre-treatment with 20 μM Cur rescued the PARP cleavage and caspases activation. Cur alone caused no changes in PARP cleavage and caspases activation. This protective effect of Cur on PC12 cells against Aβ-induced apoptosis was further convinced by TUNEL-DAPI assay. As shown in Fig. 2d, Aβ-treated cells showed apparent increased number of TUNEL-positive cells compared to that of control cells. In the presence of Cur, the number of TUNEL-positive cells was obviously inhibited in Aβ-treated PC12 cells. Taken together, these results all indicated that Cur rescued Aβ-induced apoptosis.

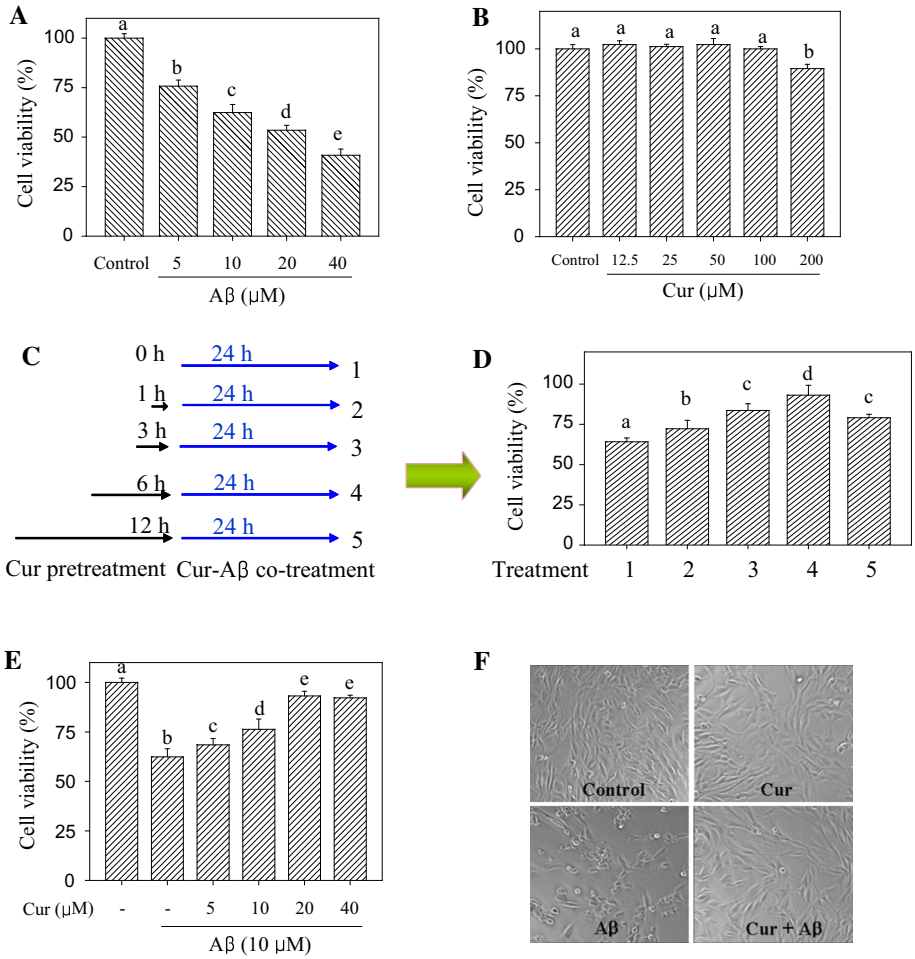

Fig. 1 Protective effects of Curcumin against Aβ-induced cytotoxicity in PC12 cells. Cytotoxicity of Aβ (**a**) and Cur(**b**) toward PC12 cells. Cells seeded in 96-well plate at concentration of 10^4 cell/well were pre-cultured for 24 h for attachment. Then, the cells were exposed to Curcumin for 24 h or Aβ for 24 h. After treatment, cell viability was examined by MTT assay. Experimental schedules (**c**) and differential protective effect (**d**). Cells were pre-treated with 20 μM Curcumin (0, 1, 3, 6, or 12 h) and co-incubated with 10 μM Aβ for 24 h. Cell viability was measured by MTT assay. **e** Curcumin inhibited Aβ-induced cell killing in PC12 cells. PC12 cells were co-treated with 10 μM Aβ for 24 h after Curcumin pre-treatment with indicated concentration. **f** Morphological observation. Cells after treatment were observed and photographed by inverted light microscopy (100×). All biological experiments were done triplicate, and all data and images were acquired from three independent tests. *Bars* with *different letters* are statistically different at $P < 0.05$ level

Cur Prevents PC12 Cells from Aβ-Induced Mitochondrial Dysfunction Through Modulating Bcl-2 Family

Mitochondria play a critical role in participating the intrinsic and extrinsic apoptosis pathways. Mitochondrial membrane potential ($\Delta\psi m$) controls the channel of the mitochondrial permeability transition pore (MPTP) and activation of caspase is associated with the loss of $\Delta\psi m$ in response to apoptotic stimuli (van Gurp et al. 2003). Hence, we vividly observed the change of $\Delta\psi m$ in Aβ-treated cells using JC-1 probe. As shown in Fig. 3a, cells incubated with Aβ alone showed notable shift of JC-1 fluorescence from red to green, implying the loss of $\Delta\psi m$. However, pre-treatment with Cur effectively prevented PC12 cells from Aβ-induced the loss of $\Delta\psi m$. The control cells and the cells treated with Cur alone exhibited no changes in $\Delta\psi m$. Moreover, Aβ treatment also caused PC12 cells the change of mitochondrial morphology. As shown in Fig. 3b, the control cells showed filamentous and extensively interconnected throughout the cytoplasm. Aβ treatment resulted in obvious mitochondrial fragmentation. However, Cur dramatically protected PC12 cells from Aβ-induced mitochondrial fragmentation. Taken together, these findings directly revealed that Cur blocked Aβ-induced mitochondrial dysfunction.

Bcl-2 family proteins play an important role in regulating $\Delta\psi m$ and initiating mitochondria-mediated apoptosis pathway through changing the mitochondrial permeability (Festjens et al. 2004). The balance between pro-apoptotic

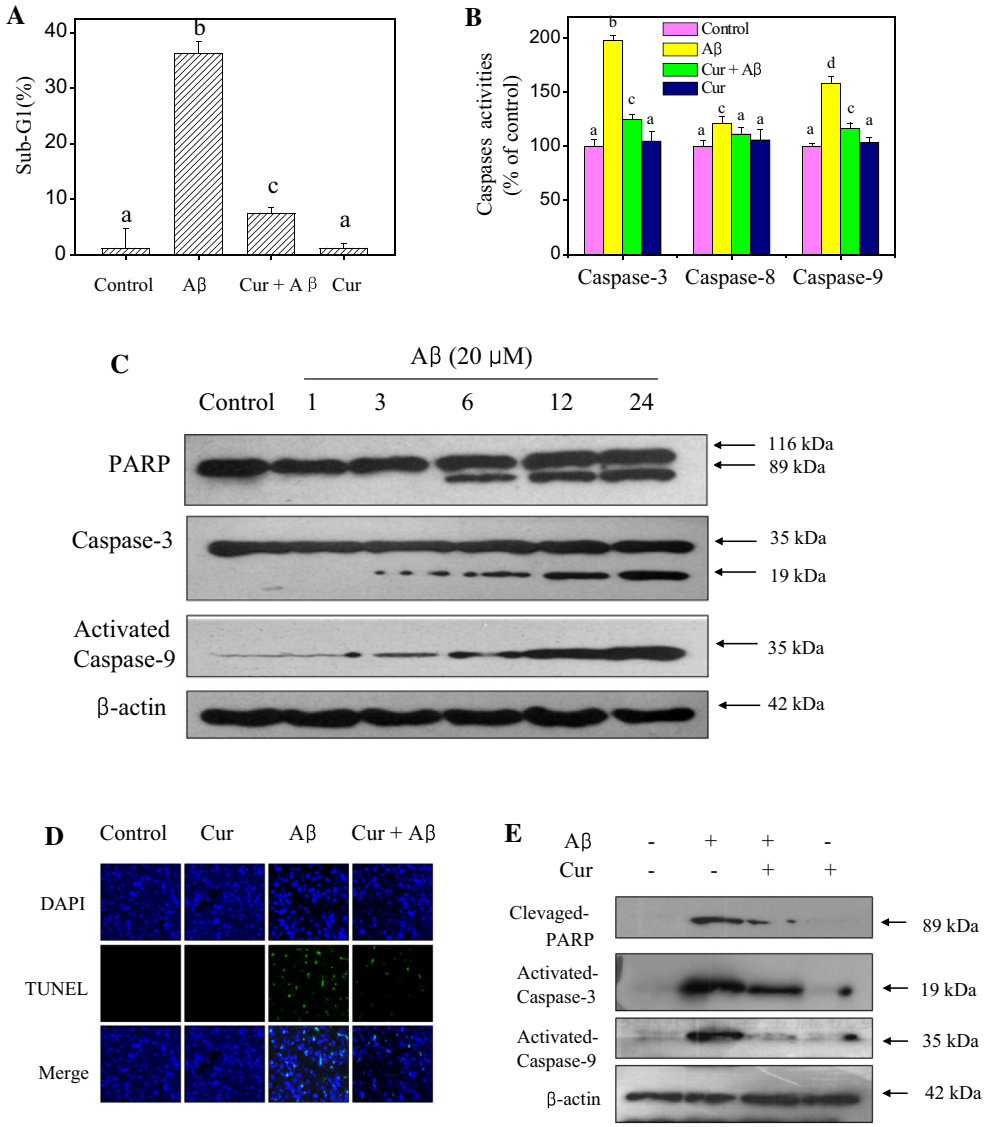

Fig. 2 Attenuation of Aβ-induced apoptosis by Curcumin in PC12 cells. **a** Curcumin suppressed Aβ-induced apoptosis in PC12 cells. Cells after pre-treatment with 20 μM Curcumin (6 h) or/and 10 μM Aβ (24 h) were collected, stained with PI solution and analyzed by flow cytometric analysis for detection of cell apoptosis and cell cycle distribution. **b** Defence of Curcumin against Aβ-induced caspase activation. Cells after treatment were collected, lysed, and total protein (100 μg/well) was employed to assay the caspase activation using specific substrate by fluorescence microplate reader. **c** Time-course of Aβ-induced apoptosis in PC12 cells. Cells after treatment were lysed and the total protein was used to examine the protein expression by Western blotting method. **d** TUNEL and DAPI co-staining. Cells after treatment were administrated with TUNEL and DAPI co-staining assay as described in section of methods. **e** Curcumin prevented PC12 cells from Aβ-induced the caspase activation and PARP cleavage. The protein expression was detected by Western blotting method. The analysis of β-actin confirmed as an equal protein loading in the extraction of protein. All biological experiments were done triplicate, and all data and images were acquired from three independent tests. *Bars* with *different characters* represent statistically different at $P < 0.05$ level

proteins (Bax, Bad, and Bid) and anti-apoptotic proteins (Bcl-2 and Bcl-xL) decides the fate of the cells (Festjens et al. 2004). As shown in Fig. 3d, Aβ treatment alone caused significant increase in Bad expression, but decrease the Bcl-xl expression. The time-course further confirmed Aβ-induced imbalance of Bcl-2 family (Fig. 3c). However, pre-treatment with Cur completely reversed Aβ-induced changes in Bcl-2 family expression. These results suggested that Cur prevented Aβ-induced mitochondrial dysfunction via modulating the expression of Bcl-2 family members.

Cur Suppresses Aβ-Induced DNA Damage by Inhibition of ROS Generation

ROS mainly from mitochondria plays an important role in induction of DNA damage and cell apoptosis. Various

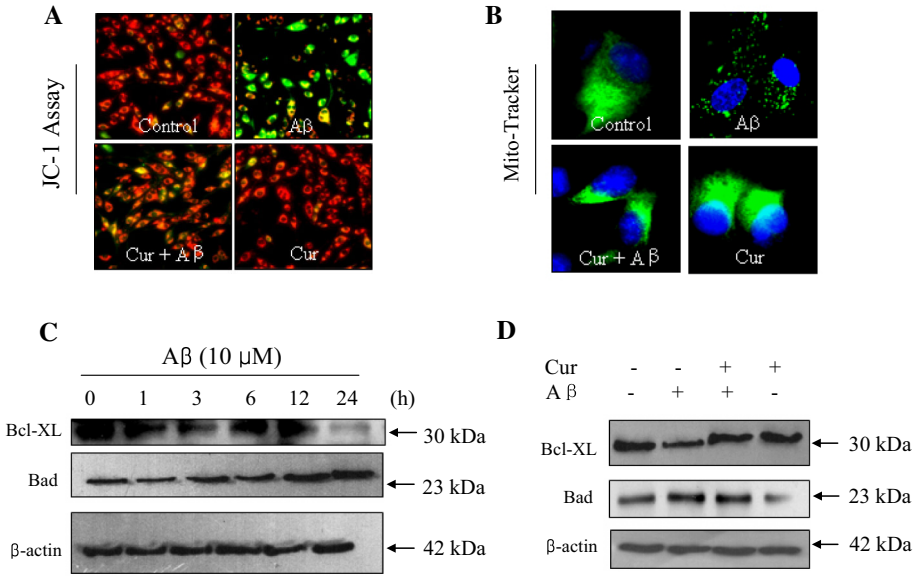

Fig. 3 Curcumin reversed Aβ-induced mitochondrial dysfunction through modulating Bcl-2 family proteins. **a** Curcumin alleviated Aβ-induced the depletion $\Delta\psi m$. PC12 cells were incubated with or without curcumin (20 μM) for 1 h then co-cultured in Aβ (10 μM) for 2 h. Then cells were stained with JC-1 probe and photographed by fluorescence microscope (100×). **b** curcumin prevented PC12 cells from Aβ-induced mitochondrial fragmentation. Cells were pre-treated with 20 μM Curcumin for 6 h and co-treated with 10 μM Aβ for 24 h. After treatment, the alternation of mitochondrial structure was detected by mito-tracker (mitochondria, *green*) and DAPI (nucleus, *blue*) co-staining under a fluorescent microscope (magnification, 400×). **c** Time-course effect of Aβ on Bcl-XL and Bad expression. Cells were treated with 10 μM Aβ for 0–24 h, and the protein expression was detected by Western blotting method. **d** Curcumin balanced the Bcl-2 family expression in Aβ-treated PC12 cells. Cells were pre-treated with 20 μM Curcumin for 6 h and co-treated with 10 μM Aβ for 24 h. The protein expression was detected by Western blotting method. All biological experiments were done triplicate, and all data and images were acquired from three independent tests

apoptotic stimulations can affect mitochondrial membrane permeability and result in the release of ROS. Accumulated studies suggest that ROS generation and subsequently the induction of oxidative damage were both involved in Aβ-induced neurotoxicity. Therefore, ROS generation in Aβ-treated cells was detected by DCFH-DA fluorescein-labeled dye. The results indicated that 10 μM Aβ (only 2 h treatment) caused significant ROS accumulation to 172 %, compared to that of control (Fig. 4c). The images observed by fluorescence microscope (Fig. 4a) further vividly confirmed the Aβ-induced ROS generation, as demonstrated by the bright green fluorescence. Furthermore, the time-course of ROS generation in 2 h further confirmed the Cur's real-time antioxidant potential (Figure S3). Moreover, Aβ treatment also caused notable mitochondrial superoxide generation in living cells, which was detected by MitoSOX-specific red dye (Fig. 4b). However, pre-treatment with Cur for 1 h acutely hindered Aβ-induced ROS and superoxide accumulation, respectively, which is similar to that of the positive control.

To investigate whether oxidative damage was activated by Aβ, several DNA damage markers (belonging to DNA damage signal axis) were detected with phosphorylated antibodies by Western blotting method (Sancar et al. 2004). As shown in Fig. 4d, notable increase of phosphorylated H_2A (Ser 139), phosphorylated p53 (Ser 15), and phosphorylated ATM (Ser 1981) were detected after Aβ treatment alone. However, Cur pre-treatment strongly repressed Aβ-induced DNA damage, as indicated by the weaken expression of phosphorylated H_2A (Ser 139), phosphorylated p53 (Ser 15), and phosphorylated ATM (Ser 1981). Taken together, the results above suggested that Aβ-mediated cytotoxicity in PC12 cells was in ROS-dependent manner, and Cur could effectively suppress Aβ-induced DNA damage through inhibition ROS overproduction.

Cur Blocks Aβ-Induced Activation of ERK and Suppression of AKT

MAPKs and PI3K/AKT family proteins play an important role in proliferation, differentiation, and survival of cells, including nerve cells (Pearson et al. 2001). To confirm whether MAPKs and PI3K pathways were involved in Aβ-induced cytotoxicity, the expression of total and phosphorylated AKT, JNK, ERK, and p38 were detected by western blotting using specific antibodies against the activated forms of kinase. Primarily, a detailed time- and dose-course effects of Aβ on the expression of phosphorylated of AKT, JNK, ERK, and p38 were performed. As shown in Fig. 5a, b, Aβ treatment slightly inhibited AKT phosphorylation, and phosphorylated ERK was detected as a sustained inactivation with time- and dose-dependent manner. However, p38

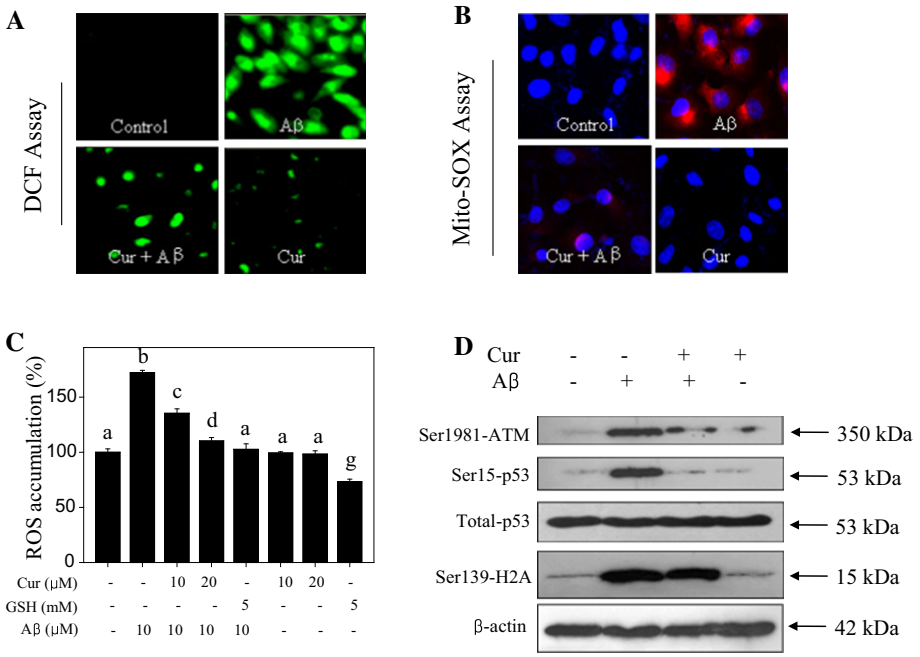

Fig. 4 Curcumin blocked Aβ-induced DNA damage through inhibition of ROS accumulation. PC12 cells resuspended in 10^7 cells/ml were labeled by DCFH-DA probe for 15 min, and the cells were washed and 100 μl/well of cells were added into 96-well plate, then the cells were incubated with Curcumin for 1 h and/or Aβ for 2 h. After incubation, the ROS generation in PC12 cells was detected by fluorescence microscope (**a**) and fluorescence microplate reader (**c**), respectively. **b** Curcumin inhibited Aβ-induced superoxide production. The superoxide in live cells was detected by MitoSOX-specific red dye which can target mitochondria superoxide (magnification, 200×). **d** Curcumin suppressed the activation of DNA damage signal axis in Aβ-treated cells. The protein expression was detected by Western blotting assay. All biological experiments were done triplicate, and all data and images were acquired from three independent tests. *Bars* with *different characters* represent statistically different at $P < 0.05$ level

and JNK, as two pro-apoptotic proteins, displayed a rapid elevation in the phosphorylated expression level with time- and dose-dependent manner. However, Cur pre-treatment significantly prevented Aβ-induced expression changes in MAPKs and PI3K/AKT members. Based on the role of MAPKs and PI3K/AKT pathway, specific inhibitors, SP600125 (JNK inhibitor), U0126 (ERK inhibitor), SB202190 (p38 inhibitor) and LY294002 (AKT inhibitor), were employed to evaluate Aβ-induced cytotoxicity and apoptosis in PC12 cells. As shown in Figure S2, addition of LY294002 and U0126 apparently augmented Aβ-induced cell cytotoxicity. However, pre-treatment with SP600125 and SB202190 dramatically hindered Aβ-induced decline in cell viability. These results all convinced that MAPKs and PI3K/AKT pathways both contributed to Aβ-induced cell cytotoxicity and apoptosis in PC12 cells. The possible signal pathway was proposed in Fig. 6.

Discussion

Mutations at the cleavage sites in amyloid precursor protein (APP) will result in overproduction of Aβ and the aggregation of Aβ to oligomers. Progressive deposition of Aβ in brain will form amyloid fibril and eventually constitute senile plaque, which is thought to be the major pathologic hallmarks of AD. Aβ-mediated neurotoxicity through triggering inflammation and oxidative damage has been accepted as the main cause of AD pathogenesis. Deposition of Aβ in brain will harm synapse, disturb neuronal communication, and lead to neurocyte apoptosis (Holmes et al. 2008; Riviere et al. 2007; Selkoe 1997; Selkoe 1998). Accumulated evidences proved that Aβ aggregation can affect mitochondrial membrane potential and trigger oxidative stress, accompanied by enhanced accumulation of free radicals (Pagani and Eckert 2011; Butterfield et al. 2006; Miranda et al. 2000; Onyango and Khan 2006). Therefore, inhibition of Aβ-mediated ROS generation and oxidative damage by potent antioxidants represents effective strategies in combating human AD. Cur has multiple pharmacological properties, including antioxidant, anti-inflammatory, and anti-angiogenic activities (Abe et al. 1999). Zhao et al. reported that Cur could block amyloid pathology on Alzheimer's transgenic mice (Zhao et al. 2012). Park et al. showed that Cur had the potential to reduce the generation of Aβ and avoid the progressing of AD through attenuating the level of calcium and phosphorylation of tau protein (Park et al. 2008). Rao

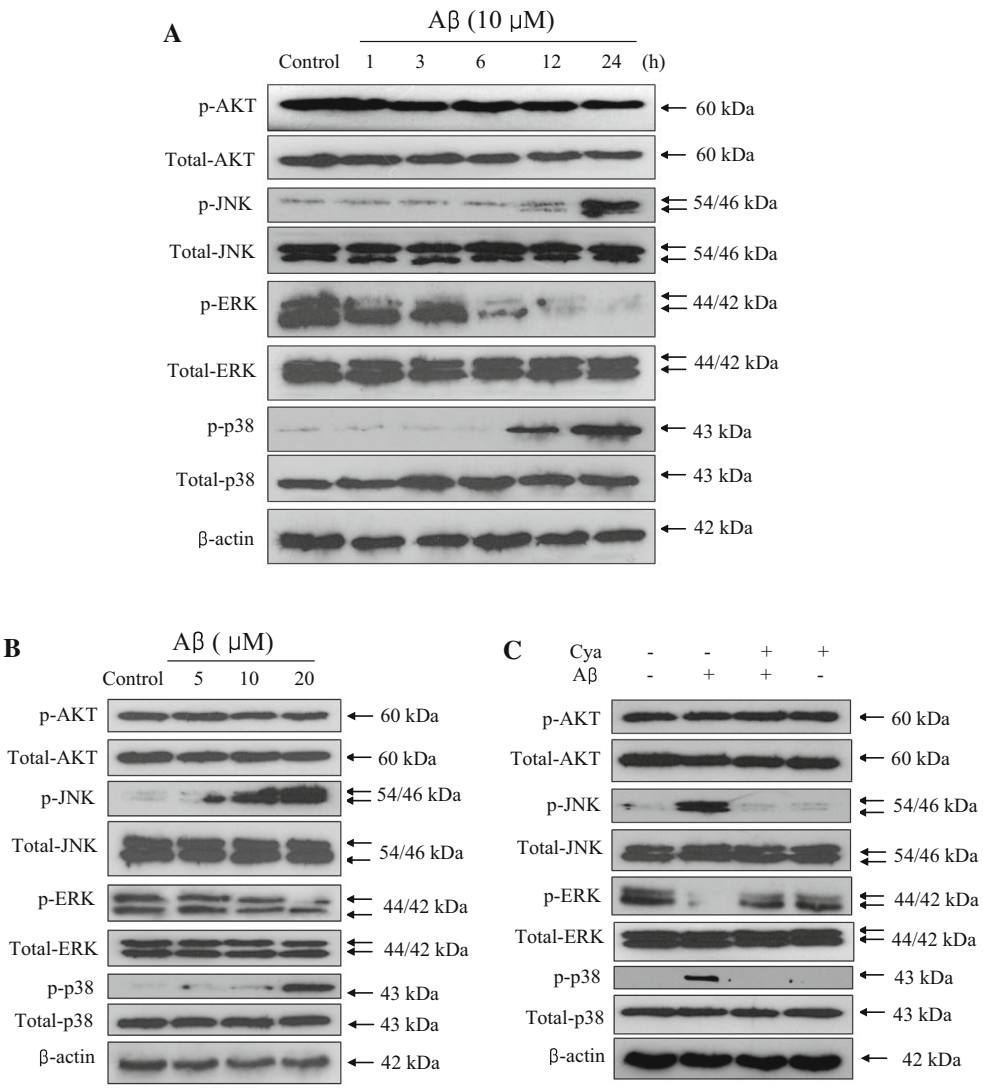

Fig. 5 Curcumin restored Aβ-induced the dysfunction of p38MAPK and AKT pathways. **a** Time-dependent effects of Aβ on p38MAPK and AKT pathways. Cells were treated with 10 μM Aβ for 0–24 h, and the protein expression was detected by Western blotting method. **b** Dose-dependent effects of Aβ on p38MAPK and AKT pathways. Cells were treated with 5, 10, and 20 μM Aβ for 24 h, and the protein expression was detected by Western blotting method. **c** Curcumin inhibited Aβ-induced dysregulation of p38MAPK and AKT pathways. Cells were pre-treated with 20 μM Curcumin for 6 h and co-treated with 10 μM Aβ for 24 h. Total and phosphorylated proteins were all detected by Western blotting method. All biological experiments were done triplicate, and all data and images were acquired from three independent tests

et al. summarized much evidences that Cur binding to Aβ could shift the equilibrium in the aggregation pathway by promoting the formation of non-toxic aggregates (Rao et al. 2015). But there is little reports about the details of the mechanism about the protection of Cur against Aβ-induced toxicity and apoptosis in PC12 cells, especially the molecular mechanism. Hence, PC12 cell line in this study was employed as ideal in vitro model (Greene and Tischler 1976) to investigate the protective effect of Cur against Aβ-mediated toxicity and apoptosis, and evaluated the underlying molecular mechanism.

Apoptosis, a programmed cell death, plays a key role in maintaining the homeostasis in brain. Death receptor-mediated extrinsic pathway and mitochondria-mediated intrinsic pathway are the two major apoptotic mechanisms. Caspases can be activated in response to apoptotic stimuli and active caspase-3 could inactivate PARP, and eventually lead to the occurrence of apoptotic cascade (Li et al. 1997; Nagata 1997; Bonegio and Lieberthal 2002; Isabelle et al. 2010). In present study, Aβ treatment dose-dependently activated the caspase-3, caspase-8, and caspase-9, indicating the activation of both death receptor-mediated pathway and mitochondria-mediated pathway. Quantitative proteins by Western blotting further verified Aβ-induced apoptosis. However, pre-treatment with Cur significantly blocked the process of apoptosis in Aβ-treated cells, as convinced by the reduction of caspases activation and PARP cleavage. These results indicated that Cur could suppress Aβ-induced cytotoxicity through inhibition of Aβ-mediated PC12 cells apoptosis.

Mitochondrial membrane potential ($\Delta\psi m$) plays a critical role in initiating mitochondria-mediated apoptosis pathway (Henry-Mowatt et al. 2004). Depletion of $\Delta\psi m$, followed by the release of apoptotic factors, such as cytochrome C, apoptosis inducing factor (AIF), and endonuclease G, all contribute to mitochondria-mediated

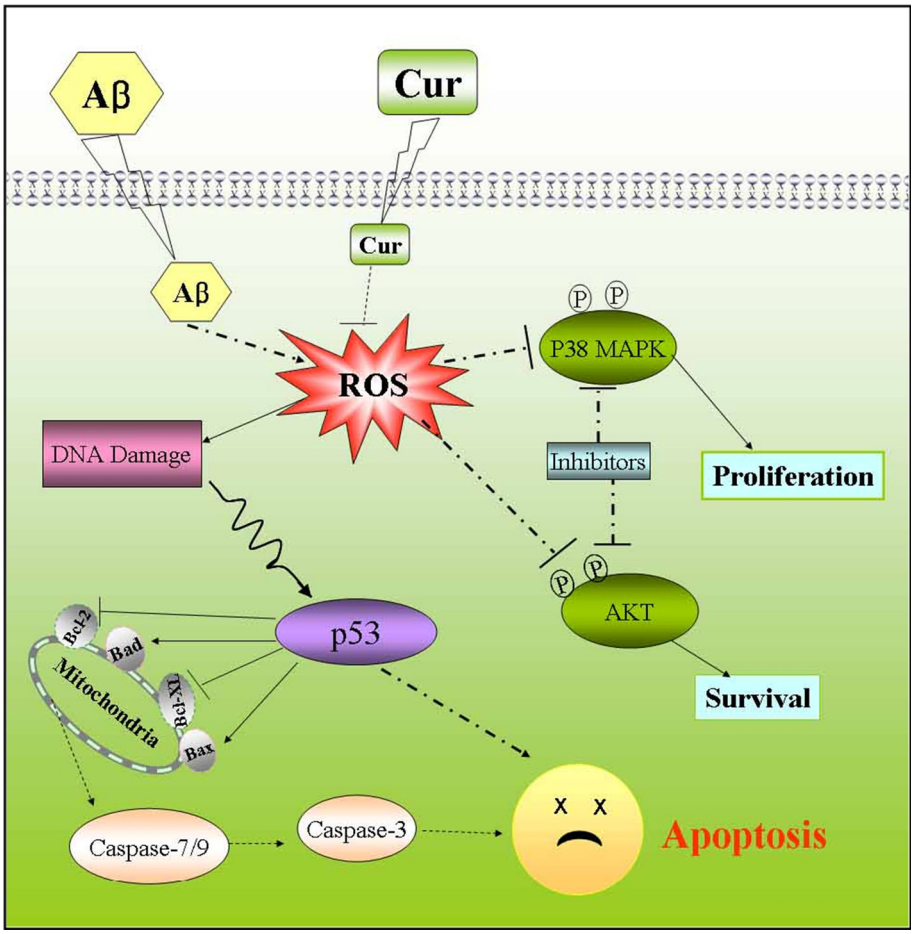

Fig. 6 Proposed signal pathway. Aβ triggered mitochondrial dysfunction via modulating Bcl-2 family, activated DNA damage-mediated p53 activation, disturbed the functions of MAPKs and AKT pathways, and ultimately induced PC12 cells apoptosis through ROS overproduction. However, Curcumin pre-treatment effectively suppressed Aβ-induced mitochondrial dysfunction, DNA damage-mediated p53 activation, and the dysfunction of MAPKs and AKT pathways, and eventually reversed Aβ-induced cytotoxicity and apoptosis in PC12 cells

apoptotic pathway (van Gurp et al. 2003). In this study, the disruption of $\Delta\psi m$ occurred rapidly when cells were exposed to Aβ, and pre-treatment with Cur reversed Aβ-induced the loss of $\Delta\psi m$, which was consist with the hypothesis that the disruption of $\Delta\psi m$ as an early cellular event acts as a key role in lunching the mitochondria-mediated apoptosis (Sancar et al. 2004).

Bcl-2 family proteins consisting of anti-apoptotic and pro-apoptotic proteins are tightly attached to the mitochondria-mediated pathway (Cory and Adams 2002; Festjens et al. 2004). Bcl-2 and Bcl-xL can prevent cytochrome C external flow through binding to the mitochondrial outer membrane. Bax can form membrane-integrated homo-oligomers to permeate the membrane and trigger the loss of $\Delta\psi m$, and eventually result in the release of apoptotic factors into cytoplasm (Chen et al. 2005; Glantz et al. 2006). Hence, the balance of pro- and anti-apoptotic factors ultimately decides the fate of cells. Our results revealed that Aβ-induced apoptosis involved the unbalance of Bcl-2 family proteins expression, whereas pre-treatment with Cur altered this tendency. These results implied that Cur blocked Aβ-induced the depletion of $\Delta\psi m$ by regulation of Bcl-2 family members.

ROS, including superoxide anion and peroxides (Dickinson and Chang 2011), play an important role in mediating cell signaling and maintaining cell homeostasis (Devasagayam et al. 2004). The balance of anti-antioxidant and pro-antioxidant system eventually decides the intracellular level of ROS. Besides that intracellular substances can induce the generation of ROS (Rada and Leto 2008), mitochondria generally represents the main source of ROS, and ROS accumulation in turn affects mitochondrial respiratory chain, redistribution of cytochrome C, and mitochondrial membrane permeability (Boya et al. 2003). Overexpression of ROS will lead to the accumulation of oxidative production of DNA, protein, and lipid and even trigger the signal of apoptosis and finally induce apoptosis (Lloyd et al. 1997; Dickinson and Chang 2011; Czarna and Jarmuszkiewicz 2006). Hence, ROS-mediated oxidative damage was accepted as the main pathogenesis in many human neurological diseases. In this study, we observed that Aβ treatment significantly activated DNA damage through inducing ROS generation, as convinced by the activation of DNA damage signal axis, implying that Aβ-induced cytotoxicity and apoptosis in PC12 cells were mainly achieved by triggering ROS-mediated DNA damage. However, pre-

treatment with Cur effectively attenuated Aβ-induced cytotoxicity and apoptosis by suppressing Aβ-mediated DNA damage through inhibition of ROS overproduction, indicating Cur as a novel scavenger of ROS (Pompella et al. 2003). Addition of reduced GSH, a positive ROS scavenger, further confirmed Cur's protective effect on combating Aβ-induced cytotoxicity and apoptosis.

Researchers have proved that ROS can regulate the MAPKs and AKT pathway, two kinases family members (Pearson et al. 2001). It is reported that ROS accumulation caused cell apoptosis by inactivating ERK and AKT, and activating p38 and JNK through affection of specific phosphorylation sites (Fan et al. 2013; Jo et al. 2005; Hilger et al. 2002). Increased evidences have certified that the protein kinase members can be activated in response to oxidative stress, UV irradiation, and osmotic shock (Boldt et al. 2002). Different cell types and species of stimulation can lead to specific function of MAPKs and AKT. In our present study, we detected that Aβ treatment dose and time dependently triggered the phosphorylation of JNK and p38, but activated ERK expression. Slight down-regulation of phosphorylated AKT was observed after Aβ treatment. The addition of specific inhibitors, SP600125 (JNK inhibitor), U0126 (ERK inhibitor), SB202190 (p38 inhibitor), and LY294002 (AKT inhibitor), further confirmed their roles in Aβ-induced cytotoxicity and apoptosis in PC12 cells. These results all suggested that MAPKs and AKT pathways both contributed to Aβ-induced cytotoxicity and apoptosis. As expected, pre-treatment with Cur effectively reversed this dysregulation of the two pathways. Based on above results, there is reason to speculate that Cur supplement restored antioxidant ability of intracellular antioxidase system and reversed Aβ-induced cytotoxicity and apoptosis through inhibiting ROS accumulation.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that there is no conflict of interest.

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