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Activation of SIRT1 by curcumin blocks the neurotoxicity of amyloid-β_{25-35} in rat cortical neurons

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ABSTRACT

As one of the most important hallmarks of Alzheimer's disease (AD), β-amyloid (Aβ) plays important roles in inducing reactive oxygen species (ROS) generation, mitochondrial dysfunction and apoptotic cell death in neurons. Curcumin extracted from the yellow pigments spice plant turmeric shows multiplied bioactivities such as antioxidant and anti-apoptosis properties *in vitro* and *in vivo*. In the present study, the neuroprotective effect of curcumin against $A\beta_{25-35}$-induced cell death in cultured cortical neurons was investigated. We found that pretreatment of curcumin prevented the cultured cortical neurons from $A\beta_{25-35}$-induced cell toxicity. In addition, curcumin improved mitochondrial membrane potential ($\Delta\Psi m$), decreased ROS generation and inhibited apoptotic cell death in $A\beta_{25-35}$ treated neurons. Furthermore, we found that application of curcumin activated the expression of SIRT1 and subsequently decreased the expression of Bax in the presence of $A\beta_{25-35}$. The protective effect of curcumin was blocked by SIRT1 siRNA. Taken together, our results suggest that activation of SIRT1 is involved in the neuroprotective action of curcumin.

1. Introduction

Alzheimer's disease (AD) is the most progressive neurodegenerative disorder in the elderly accompanied with eventual impairment of the cognitive function. The pathological features of AD include deposition of massive β-amyloid (Aβ) in senile plaques, abnormal tau accumulation in neurofibrillary tangles and loss of synapses and neurons [1]. Aβ is thought to play a critical role in the development and progress of AD. Lots of evidences show that Aβ-induced neurotoxicity is associated with reactive oxygen species (ROS) generation, mitochondrial dysfunction and calcium homeostasis [2–4].

Curcumin (Fig. 1A), a low molecular weight polyphenol (MW: 368.39) extracted from the yellow pigments spice plant turmeric, has been implied multiply bioactivities [5]. Curcumin exerts antioxidative properties by inhibiting pro-apoptotic factors such as Bcl-2-associated X protein (Bax) and peroxisome proliferator-activated receptor-γ (PPAR-γ) [6,7]. Recently, a report suggests that curcumin scavenges free radicals and inhibited the formation of β-sheeted aggregation in cortical neurons [8]. Moreover, curcumin prevents the formation of Aβ aggregates and improves cognitive dysfunction in AD transgenic mice [9]. However, the underlying mechanisms of curcumin against Aβ-induced neurotoxicity are still not fully elucidated.

Silent information regulator 1 (SIRT1), a member of the sirtuin family, is a nicotinamide adenine dinucleotide (NAD)-dependent histone deacetylase. SIRT1 plays essential roles in regulating cellular functions and activities including stress responses, cell apoptosis and axonal degeneration [10–12]. SIRT1 activation by pretreatment of curcumin attenuates mitochondrial oxidative damage induced by myocardial ischemia reperfusion injury [13]. Regulation of SIRT1 is also involved in calorie restriction and aging [14,15]. A growing numbers of evidences show that SIRT1 may regulate metabolic process and apoptosis in AD, and loss of SIRT1 is closely associated with the Aβ accumulation in transgenic mice [16,17].

Based on above, we hypothesized that activation of SIRT1 participated the protection of curcumin against Aβ neurotoxicity. Here, we investigated the protective effects of curcumin against $A\beta_{25-35}$ toxicity in rat cortical neurons to elucidate the underlying mechanisms via activating SIRT1.

Abbreviations: Aβ, amyloid beta; AD, Alzheimer's disease; Bax, Bcl-2-associated X protein; $\Delta\Psi m$, mitochondrial membrane potential; MTT, methyl thiazolyl tetrazolium; ROS, reactive oxygen species; SIRT1, silent information regulator 1; TMRM, tetramethylrhodaminemethylester.

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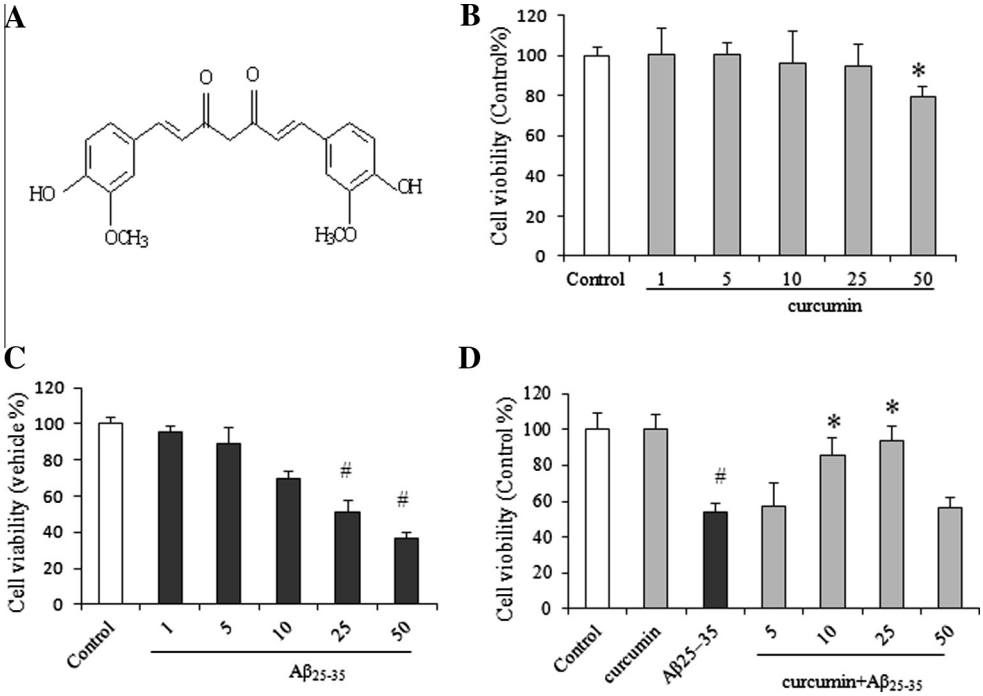

Fig. 1. Protective effects of curcumin against $A\beta_{25-35}$-induced cell death in cortical neurons. (A) Chemical structure of curcumin. (B) Neurons were treated with different concentrations (1, 5, 10, 25 and 50 μM) of curcumin for 24 h ($^{*}P < 0.01$ *vs* Control). (C) Neurons were treated with different concentrations (1, 5, 10, 25, 50 μM) of $A\beta_{25-35}$ for 24 h ($^{\#}P < 0.01$ *vs* Control). (D) Neurons were pretreated with different concentrations of curcumin (5–50 μM) for 2 h and then incubated in the presence/absence of $A\beta_{25-35}$ (25 μM) for 24 h ($^{\#}P < 0.01$ *vs* Control, $^{*}P < 0.01$ *vs* $A\beta_{25-35}$). $n = 6$.

2. Materials and methods

2.1. Materials

Curcumin (purity > 99%), $A\beta_{25-35}$, phenylmethanesulfonyl fluoride (PMSF), methyl thiazolyl tetrazolium (MTT), dimethylsulfoxide (DMSO) and poly-D-lysine were purchased from Sigma (USA). Dulbecco's modified Eagle's medium (DMEM), phosphatase inhibitor cocktail, Neurobasal A medium, B27 supplement and SIRT1 siRNA were obtained from Invitrogen (USA). Rabbit polyclonal primary antibodies cleaved caspase-3 and SIRT1 were purchased from Cell Signaling Technology (USA). Mouse anti-SIRT1 monoclonal antibody, rabbit anti-Bax polyclonal antibody and rabbit anti-β-actin monoclonal antibody were purchased from Santa Cruz Biotechnology (USA).

2.2. Primary cortical neurons cultures

Sprague–Dawley rats were supplied by the Experimental Animal Center of Xi'an Jiaotong University and approved by the Animal Care and Use Committee of Xi'an Jiaotong University. Cortices were dissected from Day 0 to 1 pups of *Sprague–Dawley* rat, dissociated with 0.05% trypsin, and triturated in ice-cold Neurobasal A medium. Cells were then centrifuged at 200×*g* for 5 min to remove debris. The resulting pellets were resuspended in culture medium (Neurobasal A medium with 2% B27 supplement, 0.5 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin) and plated onto poly-L-lysine coated culture plates with an appropriate density. Cultures were maintained at 37 °C in humidified air containing 5% CO_2. $A\beta_{25-35}$ or curcumin was added to the cultured medium of day 7 cultured neurons.

2.3. Cell treatments

$A\beta_{25-35}$ was dissolved in sterile deionized water to obtain a stock solution of 1 mM, kept at 37 °C for 72 h and then stored at 4 °C. Curcumin was dissolved in DMSO and the concentration of DMSO did not exceed 0.1%. Cultured neurons were pretreated with curcumin in different dose for 2 h. The groups were divided into Control group (non-treatment), $A\beta_{25-35}$ group ($A\beta_{25-35}$ treatment) and curcumin group (pretreatment of curcumin with $A\beta_{25-35}$). SIRT1-specific siRNA, and control siRNA were silencer select predesigned siRNAs from Life Technologies (Invitrogen) and experiments were performed according to manufacturer's instructions. Briefly, mouse primary cortical neurons were treated with 25 nm of either SIRT1 specific siRNA or control siRNA using lipofectamine RNA iMAX transfection reagent. 24 h later, the cells were treated with $A\beta_{25-35}$ for 24 h with or without pretreatment of curcumin and next experiments were performed after transfection.

2.4. MTT assay

After various treatments, cortical neurons viability was determined by MTT assay. 20 μL MTT solution in PBS (5 mg/mL stock solution) was added to each well at a final concentration of 0.5 mg/mL and the plates were incubated for an additional 4 h at 37 °C. Subsequently, the reaction was stopped by 150 μL DMSO/per well. Absorbance at 540 nm was measured using a Bio-Rad microplate reader. The results were presented as a percentage of control.

2.5. Cellular apoptosis analysis

Neurons were fixed in fresh 4% paraformaldehyde, 4% sucrose in PBS for 20 min at room temperature and permeabilized in 0.1% Triton X-100, 0.1% sodium citrate in PBS for 2 min on ice. Terminal deoxynucleotidyl transferase-biotin dUTP nick-end labeling (TUNEL) staining was performed using the in situ cell death detection kit I as described by the manufacturer (Roche). The coverslips were then washed once in distilled water for 5 min and mounted on glass slides to be observed under a fluorescence microscope.

2.6. Cellular ROS generation

To determine the levels of ROS in neurons, Amplex® Red reagent (Invitrogen) assay was employed according to the instruction. Hydrogen peroxide can be reacted with chemical substance, 10-acetyl-3, 7-dihydroxyphenoxazine, present in the Amplex® Red reagent along with horseradish perxidase (HRP) to produce a fluorescent oxidation product. The fluorescence signals were measured by fluorometer (Bio-rad).

2.7. Measurement of mitochondrial membrane potential ($\Delta\Psi m$) by TMRM assay

Cortical neurons were cultured in 8 well chamber slices. After various treatments, the medium was removed from plates, and 200 nm TMRM (Invitrogen) in phenol red-free medium was added. Plates were incubated for 30 min at 37 °C and gently washed 3 times with PBS. Fluorescent signals were captured using a fluorescence microscope (Olympus). The intensity of TMRM was analyzed with Image J software (NIH).

2.8. Western blot

Neurons after treatment were washed with cold PBS for 3 times and lysed in ice-cold cell lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1% Triton X-100, 100 μg/mL PMSF, pH 8.0) with phosphatase inhibitor cocktail. The mixture was then centrifuged at 12,000×g for 10 min at 4 °C and the supernatant was collected. The supernatant was treated with sample loading buffer and then by boiling for 5 min. Samples were separated on 8% or 12% Tris–glycine polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-rad, USA). The membranes were blocked with 5% non-fat milk in TBST (TBS containing 0.1% Tween-20) at RT for 1 h. The blots were incubated with primary antibody in blocking buffer at 4 °C overnight (anti-Bax, 1:500; anti-β-Actin, 1:1000; anti-SIRT1, 1:1000; anti-cleaved caspase-3, 1:1000). Membranes were washed 3 times with TBST and then incubated with the secondary antibody: peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG (1:5000, Invitrogen) for 1 h at RT. Membranes were washed 3 times again and then developed with Enhanced Chemiluminescence (ECL) (Pierce, USA) followed by apposition of the membrane to autoradiographic films (Kodda, Japan). The immunoblots were quantified by densitometric analysis.

2.9. Statistical analysis

All data obtained were expressed as means ± standard error (SE) from at least 3 independent experiments. Statistical significance was determined using one-way analysis of variance (ANOVA) followed by the Turkey–Kramer test for multiple comparisons when appropriate. A value $P < 0.05$ was considered statistically significant.

3. Results

3.1. Curcumin attenuated $A\beta_{25-35}$ -induced cell death in cortical neurons

After 7 days, cultured neurons were treated with curcumin at various doses for 24 h. The MTT assay showed that treatment of curcumin at 1, 5, 10 and 25 μM didn't affect cell viability, but treatment of 50 μM curcumin accessed about 15.4% reduction of cell viability compared with the control group (Fig. 1B, $P < 0.01$). To determine the toxicity of $A\beta_{25-35}$, cultured neurons were treated with $A\beta_{25-35}$ at various doses (1–50 μM) for 24 h. The MTT assay showed that $A\beta_{25-35}$ induced cortical neurons death in a dose-dependent manner. The cortical neurons treated with 25 μM $A\beta_{25-35}$ showed about 52.4% reduction of cell viability and cells were strongly insulted by 50 μM $A\beta_{25-35}$ with severe cell loss (Fig. 1C, $P < 0.01$). In hence, $A\beta_{25-35}$ at 25 μM was selected to establish AD cell model in next experiments. Pretreatment of 10 μM or 25 μM curcumin significantly increased cell viability compared with that in the $A\beta_{25-35}$ group (Fig. 1D, $P < 0.01$). Low dose of curcumin (5 μM) group or high dose of curcumin (50 μM) didn't show significant difference compared with $A\beta_{25-35}$ treatment alone.

3.2. Curcumin inhibited $A\beta_{25-35}$ -induced neuronal apoptosis

By employing TUNEL assay, the $A\beta_{25-35}$ group showed obviously higher apoptotic cells numbers. However, the numbers of apoptotic cells was decreased in the curcumin pretreatment group compared with the $A\beta_{25-35}$ group ($P < 0.01$). Few apoptotic cells were detected in the control group (Fig. 2A). To further confirm above results, we evaluated the expression of cleaved caspase-3 by Western blot (Fig. 2B). When exposed to 25 μM $A\beta_{25-35}$, the expression of cleaved caspase-3 was significantly increased in cortical neurons compared with the control group ($P < 0.01$). Pretreatment of curcumin at 10 μM or 25 μM reduced the expression of cleaved caspase-3 compared with that in the $A\beta_{25-35}$ group ($P < 0.01$) (Fig. 2C).

3.3. Curcumin protected cortical neurons against $A\beta_{25-35}$ induced ROS generation and mitochondrial membrane potential ($\Delta\Psi m$) collapse

To further investigate the possible mechanism of curcumin against Aβ toxicity, we examined ROS generation. Relative ROS levels were measured in the control group, Aβ group and curcumin group (Fig. 2D). $A\beta_{25-35}$ treated neurons showed remarkably increased ROS level, whereas pretreatment of curcumin at 10 μM or 25 μM dramatically decreased ROS level ($P < 0.01$). To determine the effect of curcumin on mitochondrial function, we measured mitochondrial membrane potential ($\Delta\Psi m$) by using TMRM staining. Upon $A\beta_{25-35}$ exposure, cortical neurons showed a statistically significant decreased $\Delta\Psi m$ compared with cells in the control group ($P < 0.01$). However, pretreatment of curcumin at 10 μM or 25 μM showed significantly increased $\Delta\Psi m$ compared with neurons treated with $A\beta_{25-35}$ alone (Fig. 2F, $P < 0.01$).

3.4. Curcumin increased the expression of SIRT1 and decreased the expression of Bax in $A\beta_{25-35}$ rich milieu

In addition, we examined the expression of SIRT1 and Bax by using Western blot. As shown in Fig. 3A and B, compared with the control group, $A\beta_{25-35}$ group showed significantly decreased expression of SIRT1 which attenuated by pretreatment of curcumin at 10 μM or 25 μM ($P < 0.01$). The expression of Bax was markedly increased by $A\beta_{25-35}$ compared with the control group, while such increase was obviously attenuated by pretreatment of curcumin at 10 μM or 25 μM (Fig. 3C and D, $P < 0.01$).

3.5. SIRT1 siRNA blocked the neuroprotection of curcumin in the presence of $A\beta_{25-35}$

Based on the changes of SIRT1 expression in neurons, we investigated the role of SIRT1 in the neuroprotection of curcumin by transfecting SIRT1 siRNA. Firstly, to confirm the effect of SIRT1 siRNA, we examined the expression of SIRT1 in neurons pretreated of curcumin followed by addition of $A\beta_{25-35}$. The results showed that SIRT1 siRNA blocked curcumin enhanced SIRT1 expression (Fig. 4A and B, $P < 0.01$). Secondly, the MTT assay results showed that although neurons loss in the $A\beta_{25-35}$ group was significantly attenuated by pretreatment of curcumin, such protective effect of

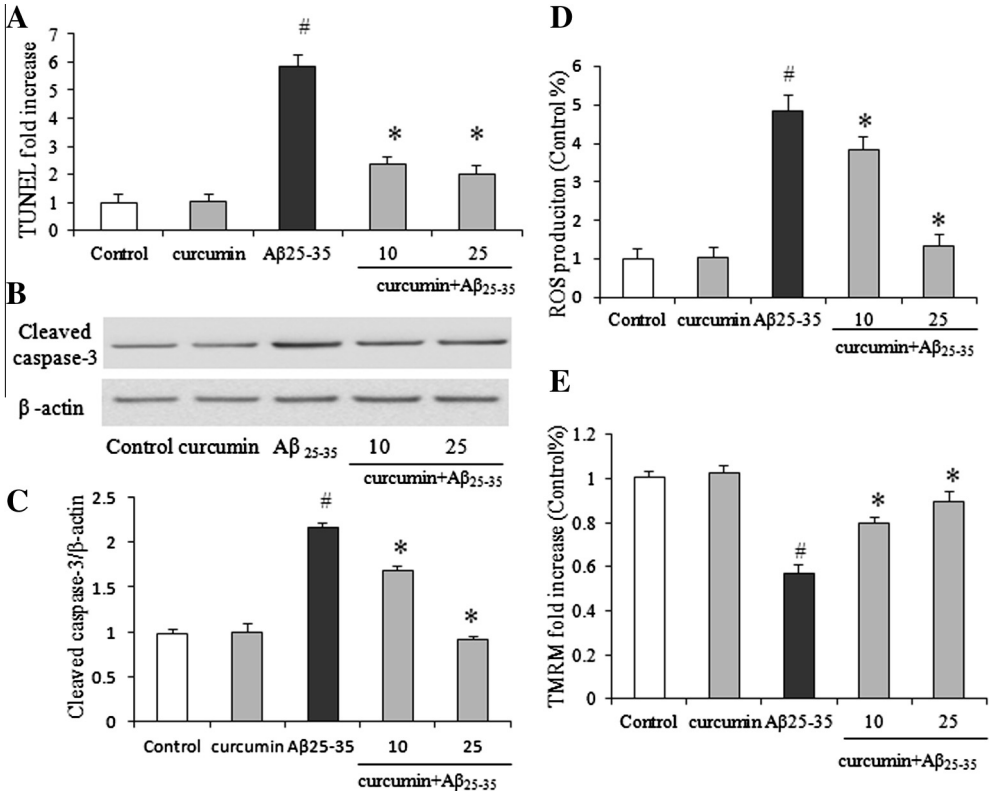

Fig. 2. Curcumin attenuated $A\beta_{25-35}$-induced apoptotic cell death, ROS generation and recovered mitochondrial membrane potential ($\Delta\Psi m$). (A) TUNEL assay showed that 25 μM $A\beta_{25-35}$ increased apoptosis compare with Control group ($^{\#}P < 0.01$ *vs* Control). Pretreatment of 10 or 25 μM curcumin significantly attenuated 25 μM $A\beta_{25-35}$-induced apoptosis ($^{*}P < 0.01$ *vs* $A\beta_{25-35}$). (B and C) 25 μM $A\beta_{25-35}$ increased the expression of cleaved caspase-3 compared with Control ($^{\#}P < 0.01$ *vs* Control). Pretreatment of 10 or 25 μM curcumin significantly decreased cleaved caspase-3 expression compared with $A\beta_{25-35}$ group ($^{*}P < 0.01$ *vs* $A\beta_{25-35}$). (D) Cellular ROS level. 25 μM $A\beta_{25-35}$ increased ROS generation compared with Control ($^{\#}P < 0.01$ *vs* Control). Pretreatment of 10 or 25 μM curcumin significantly attenuated 25 μM $A\beta_{25-35}$-induced ROS ($^{*}P < 0.01$ *vs* $A\beta_{25-35}$). (E) Mitochondrial membrane potential ($\Delta\Psi m$). 25 μM $A\beta_{25-35}$ decreased $\Delta\Psi m$ compared with Control ($^{\#}P < 0.01$ *vs* Control). Pretreatment of 10 or 25 μM curcumin significantly reversed $\Delta\Psi m$ ($^{*}P < 0.01$ *vs* $A\beta_{25-35}$). $n = 3$.

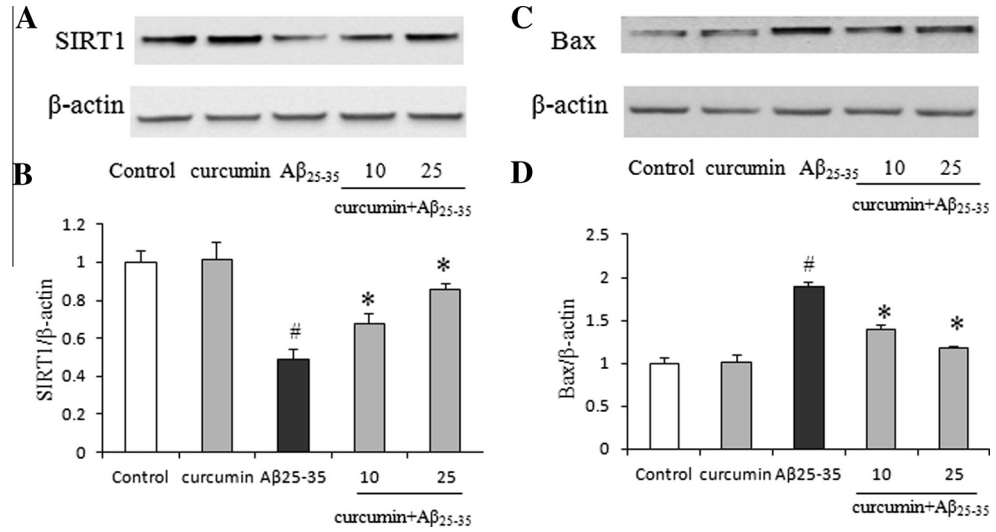

Fig. 3. SIRT1 and Bax expressions were determined by Western blot. (A and B) 25 μM $A\beta_{25-35}$ treatment significantly decreased expression of SIRT1 but markedly recovered by application of curcumin at 10 or 25 μM curcumin pretreatment. (C and D) Bax expression was increased in $A\beta_{25-35}$ treated neurons but attenuated with 10 or 25 μM curcumin pretreatment. $^{\#}P < 0.01$ *vs* Control, $^{*}P < 0.01$ *vs* $A\beta_{25-35}$, $n = 3$.

curcumin was blocked by the addition of SIRT1 siRNA (Fig. 4C, $P < 0.01$). Thirdly, TUNEL assay results showed that transfecting with SIRT1 siRNA weakened curcumin's protection against $A\beta_{25-35}$-induced apoptotic cells numbers (Fig. 4D, $P < 0.01$). In addition, in the presence of $A\beta_{25-35}$, neurons pretreated with curcumin showed significantly increased expression of cleaved caspase-3 in the SIRT1 siRNA group compared with the control siRNA group (Fig. 4E and F, $P < 0.01$). Compared with the control group, control siRNA had no effect on the cell viability as well as apoptotic cell death (data not shown).

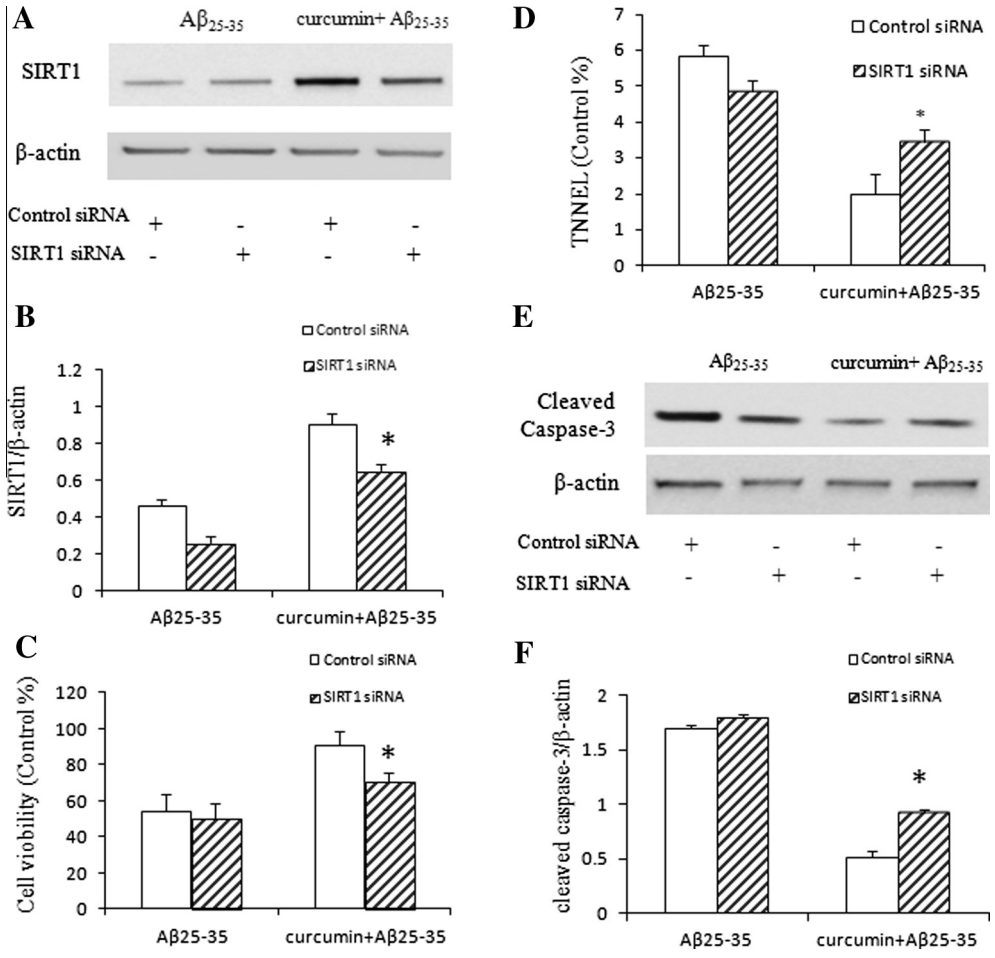

Fig. 4. SIRT1 siRNA attenuated curcumin's protection against $A\beta_{25-35}$ in neurons. (A and B) The expression of SIRT1 was detected by Western blot (*$P < 0.01$ *vs* $A\beta_{25-35}$, $n = 3$). (C) MTT assay tested cell viability. SIRT1 siRNA blocked the effect of curcumin to prevent loss of neurons (*$P < 0.01$ *vs* $A\beta_{25-35}$, $n = 3$). (D) TUNEL assay detected apoptosis. SIRT1 siRNA inhibited the protection of curcumin against $A\beta_{25-35}$-induced apoptosis in neurons (*$P < 0.01$ *vs* $A\beta_{25-35}$, $n = 3$). (E and F) The expression of cleaved caspase-3 was detected by Western blot. SIRT1 siRNA blocked curcumin decreased cleaved caspase-3 expression in the presence of $A\beta_{25-35}$ (*$P < 0.01$ *vs* $A\beta_{25-35}$, $n = 3$).

4. Discussion

The present study showed that curcumin significantly inhibited $A\beta_{25-35}$-induced neuronal loss and apoptosis in cultured rat cortical neurons. Pretreatment of curcumin decreased the generation of ROS and increased mitochondrial membrane potential ($\Delta\Psi m$) in Aβ rich milieu. Furthermore, the application of curcumin was able to attenuate the $A\beta_{25-35}$-induced down-regulation of SIRT1, which was blocked by SIRT1 siRNA. In addition, pretreatment of curcumin decreased the expression Bax enhanced by $A\beta_{25-35}$, which was blocked by SIRT1 siRNA either. Here, we reported for the first time that activation of SIRT1 is involved in the neuroprotective effects of curcumin against $A\beta_{25-35}$ toxicity.

Aβ has been implicated as one of the most important pathogenic hallmarks of Alzheimer's disease (AD). Lots of studies demonstrated that Aβ induced neurons loss *in vivo* and *in vitro* [18]. Curcumin possesses a variety of pharmacological and biological properties and potentially mediates neuronal protection [19]. Several studies indicate that application of curcumin protects neurons from Aβ-induced toxicity [20,21]. In the present study, application of $A\beta_{25-35}$ at various concentrations caused neuronal death with dose-dependent. Pretreatment of 10 or 25 μM curcumin significantly inhibited neuronal loss and apoptosis in rat cortical neurons treated with $A\beta_{25-35}$. In addition, we found that pretreatment of 10 or 25 μM curcumin reserved accumulation of ROS. Moreover, the decreased mitochondrial membrane potential ($\Delta\Psi m$) was rescued by curcumin in Aβ rich environment. These results imply that the neuronal protective role of curcumin in neurons injury induced by $A\beta_{25-35}$ may be associated with regulating cell energy metabolism and redox status.

SIRT1 is a member of the class III group of histone deacetylases. The deacetylase activity of SIRT1 is dependent on the ratio of NAD+ to NADH in the cell. In myocardial ischemia–reperfusion injury, the deacetylase activity of SIRT1 plays a key role in cardioprotection [22]. Many studies demonstrate that the activation of SIRT1 pathway is important for energy metabolism and neuronal survival [23]. Resveratrol, a SIRT1 activator, inhibits hypoxia-induced apoptosis via the SIRT1-FOXO1 pathway in H9c2 embryonic rat heart-derived cells [24]. In addition, a number of studies suggested that overexpression of SIRT1 is closely associated with preventing aging and Aβ accumulation [25,26]. A current report also suggests the expression of SIRT1 is lower in AD patients [27]. In this study, we found that the application of curcumin was able to attenuate the $A\beta_{25-35}$-induced down-regulation of SIRT1. Moreover, several evidences suggest that SIRT1 inhibits apoptotic cell death by deacetylating the DNA repair factor Ku70, which can sequester Bax away from mitochondria [28,29]. In the present study, we found that pretreatment of curcumin decreased the expression of Bax in $A\beta_{25-35}$-treated neurons. Therefore, down-regulation of SIRT1 and up-regulation of Bax might contribute to the onset of AD. To further confirm the effects of curcumin against Aβ toxicity are mediated by the SIRT1 pathway, we transfected neurons with

SIRT1 siRNA prior to curcumin and $A\beta_{25-35}$ treatment and then examined the cell viability and apoptosis. Our results showed that transfected SIRT1 siRNA blocked curcumin increased cell viability in the presence of $A\beta_{25-35}$. The curcumin decreased apoptosis was inhibited by SIRT1 siRNA. These results suggest that the SIRT1 pathway plays an important role in the neuroprotective properties of curcumin against Aβ toxicity in cultured cortical neurons.

In summary, curcumin protected cortical neurons from Aβ-induced neurotoxicity and cell apoptosis. It prevented the ROS generation and maintained the mitochondrial membrane potential. Particularly, $A\beta_{25-35}$ suppressed SIRT1 expression was significantly recovered by curcumin. Our data further demonstrated that the neuroprotective action of curcumin was attributed by activation of SIRT1. The present study provided new insights into the mechanism of the neuroprotective effects of curcumin.

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