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ORIGINAL ARTICLE

Association of Neuroprotective Effect of Di-*O*-Demethylcurcumin on $A\beta_{25–35}$-Induced Neurotoxicity with Suppression of NF-κB and Activation of Nrf2

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Abstract Amyloid-β peptides (Aβ), a major component of senile plaques, play an important role in the development and progression of Alzheimer's disease. Several lines of evidence have demonstrated that Aβ-induced neuronal death is mediated by oxidative stress. The present study aimed to evaluate the potential involvement of di-*O*-demethylcurcumin, an analog of curcuminoid, on Aβ-induced neurotoxicity in culture neuroblastoma cells (SK-N-SH cells) through the activation of nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway and the suppression of nuclear factor-κB (NF-κB) signaling pathway and their downstream targets. The results showed that pretreatment with di-*O*-demethylcurcumin elevated cell viability and decreased the level of reactive oxygen species. Moreover, treatment with di-*O*-demethylcurcumin promoted the translocation of Nrf2 protein from the cytoplasm to the nucleus, increased the expression of Nrf2-ARE pathway-related downstream proteins including heme oxygenase (HO-1), NAD(P)H:quinone oxidoreductase 1 and glutamate-cysteine ligase catalytic subunit, and increased the activity of superoxide dismutase enzymes. On the other hand, di-*O*-demethylcurcumin suppressed the degradation of IκBα, translocation of the p65 subunit of NF-κB from cytoplasm to nucleus and thereby, attenuated the expression of inducible nitric oxide synthase protein and nitric oxide production. Taken together, these results suggest that neuroinflammatory effect of di-*O*-demethylcurcumin might potentially be due to inhibit NF-κB and activate Nrf2 signaling pathways induced by $A\beta_{25–35}$.

Keywords Alzheimer's disease · Amyloid-β peptides · Di-*O*-demethylcurcumin · Nuclear factor-κB · Nuclear factor erythroid 2-related factor 2 · Oxidative stress

Introduction

Alzheimer's disease (AD) is a progressive and irreversible neurodegenerative disease, which is the most common type of dementia in elderly people. The pathological hallmarks of AD include massive accumulation of beta-amyloid (Aβ) in senile plaques and abnormal tau filaments in neurofibrillary tangles (NFT) within neurons (Selkoe 2004). Recent studies have suggested that Aβ plays an important role in the pathogenesis of AD (Selkoe 2001). Aβ is a peptide fragment derived from sequential proteolysis of amyloid precursor protein (APP) through cleavage by β-secretase and γ-secretase (Hardy and Higgins 1992). Aβ accumulation has been causatively implicated in the neuronal dysfunction and neuronal loss that underlie the clinical manifestations of AD (Christen 2000). Accumulating evidence suggests that oxidative stress and inflammation are the major mechanisms of Aβ-induced neurotoxicity. Several lines of researches have suggested that Aβ induced the production of pro-inflammatory cytokines

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through nuclear factor κ-B (NF-κB) signaling pathway (Garcia-Ospina et al. 2003; Guglielmotto et al. 2012), thereby upregulating the related inflammatory cytokines including interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α), inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) (Diaz et al. 2014; Lin et al. 2013; Xu et al. 2014). These pro-inflammatory molecules contribute to neuronal damage and accelerate the progression of neurodegenerative diseases. On the other hand, neuronal cells have neuroprotective mechanism to protect from oxidative stress (Jhang et al. 2014; Johnson et al. 2008; Meng et al. 2014). The transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) is a key protein that controls the redox state of the cell under oxidative stress. Under basal conditions, Nrf2 localizes in the cytoplasm which is associated with the actin-binding protein, Kelch-like ECH associating protein 1 (Keap1), and degraded by the ubiquitin–proteasome pathway. Under several stimuli including oxidative stress, Nrf2 translocates into nucleus for transactivating the antioxidant response element (ARE) in the promoter of antioxidant genes, such as heme oxygenase (HO-1), glutamate-cysteine ligase catalytic (γ-GCLC), quinone oxidoreductase 1 (NQO-1), and superoxide dismutase (SOD) (Lou et al. 2014). The Nrf2-ARE signaling pathway is considered to be a protective molecular mechanism in several pathological processes, especially in oxidative stress (Habas et al. 2013). Therefore, inhibition of Aβ-mediated oxidative cell death might be one of the promising strategies to prevent AD.

Di-*O*-demethylcurcumin is a modified analog of curcumin which is a major constituent of curcuminoids isolated from turmeric (*Curcuma longa* L.). Curcumin has been shown to exhibit anticarcinogenic, antioxidant, anti-inflammatory, and cytoprotective properties (Bandgar et al. 2014; Belviranli et al. 2013; Rath et al. 2013). Recently, our study reported that di-*O*-demethylcurcumin exerts neuroprotective effects in glial cells by attenuating lipopolysaccharide (LPS)-induced inflammation in microglial cells (Tocharus et al. 2012). It demonstrates a potent anti-inflammatory activity greater than that of the parent curcumin. However, the ability of di-*O*-demethylcurcumin to attenuate neurotoxicity in SK-N-SH cells activated by $A\beta_{25-35}$ has not yet been studied. Hence, in this study, we investigated the underlying mechanisms of di-*O*-demethylcurcumin against $A\beta_{25-35}$-induced neurotoxicity in SK-N-SH cells mediated by NF-κB and Nrf2 signaling pathways.

Materials and Methods

Materials

Minimum essential media (MEM), fetal bovine serum (FBS), penicillin, and streptomycin were purchased from GIBCO-BRL (Gaithersburg, MD, USA). The following antibodies were used in this study: anti-Nrf2, anti-NQO-1, anti-γ-GCLC and anti-HO-1 (Abcam, Cambridge, UK), anti-iNOS, anti-IκBα, anti-p65 subunit, anti-β-actin, anti-lamin B1, anti-mouse IgG peroxidase-conjugated secondary antibody, and anti-rabbit IgG peroxidase-conjugated secondary antibody (Millipore, Bedford, MA, USA). EnzyChrome TH SOD assay kit was purchased from Bioassay (BioAssay, CA, USA). $A\beta_{25-35}$ and other results were purchased from Sigma (St. Louis, MO, USA).

Cell Culture

Human neuroblastoma SK-N-SH cell line was obtained from the American Type Culture Collection (ATCC, Mannassas, VA, USA). The SK-N-SH cells were cultured in MEM supplemented with 10 % FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 95 % air, and 5 % CO_2 in incubator. The cells were passaged by trypsinization every 2–3 days.

Preparation of Di-*O*-Demethylcurcumin

Curcumin (850 mg, 2.30 mmol), obtained from *Curcuma longa* as described previously (Changtam et al. 2010), was dissolved in dry CH_2Cl_2 (90 ml); the mixture was stirred at 0 °C for 5 min, and BBr_3 (1 ml) was slowly added. Then, the reaction mixture was stirred at 0 °C for 30 min and more BBr_3 (1 ml) was slowly added. After stirring at 0 °C for 1 h, water (200 ml) was added and the mixture was extracted with EtOAc. The combined organic phase was washed with water, and dried over anhydrous Na_2SO_4, and the solvent was removed under vacuum. The crude products were separated by column chromatography using CH_2Cl_2–MeOH (10:1) as the eluting solvent to yield mono-*O*-demethylcurcumin 240 mg (29 %) and di-*O*-demethylcurcumin 450 mg (57 %). The spectroscopic (^{1}H NMR and mass spectra) data were found to be consistent with the reported values (Venkateswarlu et al. 2005).

Evaluation of Cell Viability

Cell viability was determined using the MTS assay. The SK-N-SH cells were plated at a density of 2×10^4 cells/well into 96-well plates. The cells were pretreated with di-*O*-demethylcurcumin at the indicated concentrations (1, 4, or 8 μM) for 2 h, and this was followed by the addition of 10 μM $A\beta_{25-35}$ for 24 h. Presto Blue reagent was added to each well, and the wells were incubated further for 1 h at 37 °C in 5 % CO_2. The absorbance was measured using a microplate reader (Bio-Tek, Instruments, Winoaski, VT,

USA) at the wavelength 540 nm. The background absorbance was measured at 600 nm and subtracted.

Determination of Intracellular Reactive Oxygen Species (ROS)

The extent of intracellular ROS was measured as 2′,7′-dichlorofluorescein (DCFH) oxidation assay. The cells at the density of 2×10^4 cells/well were plated in 96-well plates and then incubated for 24 h at 37 °C in CO_2 incubator. The cells were pretreated with di-*O*-demethylcurcumin (2, 4 or 8 μM), 20 μM JSH 23, or 20 μM ochratoxin A (OTA) for 2 h, and then treated with 10 μM $A\beta_{25-35}$ for 24 h. The medium was removed, and 2′,7′-dichlorofluorescein-diacetate (DCFH-DA) solutions were added for 25 min. The absorbance was measured using a fluorescence microplate reader (DTX800, Beckman Coulter, Austria) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

Western Blot Analysis

The cells were plated at a density of 2×10^5 cells/ml in 60 mm culture dish at 37 °C for 24 h. The cells were then pretreated with di-*O*-demethylcurcumin at concentrations of 2, 4, or 8 μM for 2 h, followed by treatment in the presence or absence of 10 μM Aβ for 24 h. Then, all the cells were collected and resuspended in the lysis buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.5 mM DTT, 1.5 mM $MgCl_2$, 0.5 mM PMSF, and 1 % protease inhibitors, at 4 °C for 15 min. The homogenate was centrifuged at 13,000 rpm for 30 min. The supernatant (cytosolic fraction) was collected. The nuclear pellets were resuspended in a lysis buffer containing 20 mM HEPES (pH 7.9), 1 mM EDTA, 1 mM EGTA, 400 mM NaCl, 0.5 mM PMSF, and 1 % protease inhibitors. The homogenate was centrifuged at 13,000 rpm for 5 min. The quantities of proteins in each extract were measured using the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Protein extracts (50 micrograms) were separated in 10–15 % sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred from gel to polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore, Bedford, MA, USA). The membrane was incubated with 5 % skimmed milk in Tris-buffered saline/Tween (TBST) for 2 h and then incubated with primary antibodies, anti-Nrf2 (1:1000), anti-NQO-1 (1:2000), anti-γGCLC (1:1000), anti-HO-1 (1:500), anti-iNOS (1:1000), anti-IκBα (1:500), and anti-p65 (1:500), overnight at 4 °C. The blots were then incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies. The blots incubated with Immobilon Western (Millipore, MA, USA) and finally exposed to X-ray films. The densitometric measurements were determined using the Image-J® software.

Measurement of SOD Activity

The SOD activity was measured using the nitroblue tetrazolium method, which utilized a tetrazolium salt to quantify the superoxide radicals generated by xanthine oxidase and hypoxanthine, according to the instruction given in the SOD assay kit (Cayman Chemicals, Ann Arbor, MI). Briefly, cells were plated in 96-well plates at 1×10^6 cells/plate and allowed to attach for 24 h. The cells were then pretreated with di-*O*-demethylcurcumin at concentrations of 2, 4, or 8 μM for 2 h, followed by treatment in the presence or absence of 10 μM Aβ for 24 h. The cells were lysed in a lysis buffer and centrifuged at 12,000 rpm at 4 °C for 5 min. Thereafter, the supernatant was collected and mixed with tetrazolium salt as manufacturer's direction for detection of superoxide radicals. The enzyme activity was read at 440 nm using a microplate reader (Bio-Tek, Instruments, Winoaski, VT, USA).

Measurement of Nitrite Production

The level of nitric oxide was measured as nitrite. The SK-N-SH cells were plated at the density of 2×10^4 cells/well in a 96-well plate. Pretreatment with di-*O*-demethylcurcumin for 2 h was followed by 10 μM $A\beta_{25-35}$ for 24 h. After incubation, 100 μl of the supernatant of each samples was mixed with an equal volume of Griess reagent (a mixture of 1 % sulphanilamide in 5 % phosphoric acid and 0.1 % *N*-1-naphthyl-ethylenediamine dihydrochloride) for 10 min. The absorbance was measured at 540 nm using a microplate reader (Bio-Tek, Instruments, Winoaski, VT, USA), using sodium nitrite ($NaNO_2$) as a standard curve.

Immunocytochemistry

The SK-N-SH cells were plated at the density of 2×10^4 cells/well in a 6-well plate on poly-D-lysine coated coverslips. The cells were pretreated with di-*O*-demethylcurcumin for 2 h and then exposed to 10 μM $A\beta_{25-35}$ for 24 h. After that, the cells were fixed in 4 % paraformaldehyde for 20 min and permeabilized with 0.5 % triton X-100 for 15 min. Thereafter, the cells were blocked with 5 % BSA for 1 h, and then incubated with anti-p65 subunit (1:500) and anti-Nrf2 (1:500) monoclonal antibody at 4 °C overnight. The goat anti-mouse IgG FITC-conjugated antibody for anti-p65 and the goat anti-rabbit IgG Alexa-fluor-conjugated antibody for anti-Nrf2 were added at room temperature for 2 h. The cells were then washed with PBS and the nuclease counterstained with 10 μg/ml of DAPI in the

dark for 10 min. The cells were then visualized under an inverted microscope (Nikon Eclipse, TE2000-U, Japan).

Data Analysis

All data are presented as the mean ± SD of three independent experiments. Statistical difference was performed using One-Way Analysis of Variance (ANOVA) followed by Post Hoc Dunnett's test to compare the significance between the individual groups. A value of $p < 0.05$ was considered statistically significant.

Results

Di-*O*-Demethylcurcumin Improved Cell Viability Against $A\beta_{25–35}$ in SK-N-SH Cells

SK-N-SH cells were treated with 1, 10, or 100 μM of $A\beta_{25–35}$ and 2, 4, or 8 μM of di-*O*- demethylcurcumin for 24 h. The values of cells viability were determined using the MTS assay. Exposure to 1, 10, or 100 $A\beta_{25–35}$ for 24 h significantly decreased the cell viability (Fig. 1a). The cytotoxic effect was significantly observed in the cells treated with $A\beta_{25–35}$ at concentrations of 1, 10, or 100 μM for 24 h. Taking this result into consideration, $A\beta_{25–35}$ at a concentration of 10 μM was subsequently used to examine and conduct the study further. It was observed that the di-*O*-demethylcurcumin (2, 4, or 8 μM) treated cells did not alter the cell viability (Fig. 1b). Pretreatment with di-*O*-demethylcurcumin for 2 h, followed by 10 μM $A\beta_{25–35}$ for 24 h significantly increased the cell viability in a concentration-dependent manner. The values of viability of cells were 84.3 ± 1.1 % ($p < 0.001$), 88.9 ± 1.3 % ($p < 0.001$), and 93.2 ± 2.3 % ($p < 0.001$) when the cells were pretreated with 2, 4, or 8 μM of di-*O*- demethylcurcumin, respectively (Fig. 1c).

Di-*O*-Demethylcurcumin Attenuates $A\beta_{25–35}$-Induced ROS Production

Pretreatment of cells with di-*O*-demethylcurcumin at the concentrations of 2, 4, or 8 μM for 2 h, followed by 10 μM $A\beta_{25–35}$ for 24 h, significantly prevented $A\beta_{25–35}$-induced ROS generation when compared to the group treated with $A\beta_{25–35}$ alone (Fig. 2d). The levels of ROS production were 214.3 ± 8.5 % ($p < 0.001$), 199.8 ± 8.2 % ($p < 0.001$), and 152.2 ± 13.7 % ($p < 0.001$) when the cells were pretreated with 2, 4, or 8 μM of di-*O*- demethylcurcumin, respectively (Fig. 2d). However, treatment with di-*O*-demethylcurcumin alone did not affect intracellular ROS production when compared to the control group.

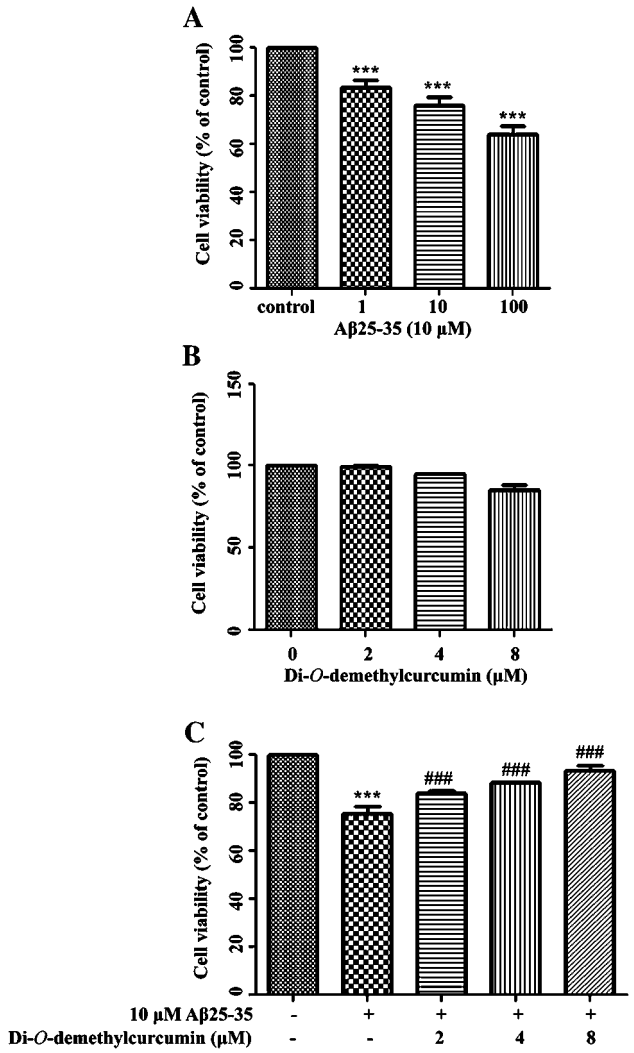

Fig. 1 The effect of di-*O*-demethylcurcumin on cell viability in SK-N-SH cells induced by $A\beta_{25–35}$. **a** The SK-N-SH cells were treated with various concentrations of $A\beta_{25–35}$ (1, 10, or 100 μM) for 24 h. **b** The cells were treated with various concentrations of di-*O*-demethylcurcumin (2, 4, or 8 μM) for 24 h. **c** The cells were treated with various concentrations of di-*O*-demethylcurcumin (2, 4, or 8 μM) for 2 h before being treated with 10 μM $A\beta_{25–35}$ for 24 h. The cell viability was assessed using the MTS assay. The results are presented as the mean ± SD of three independent experiments. ***$p < 0.001$ when compared with the control. $^{###}p < 0.001$ when compared with the $A\beta_{25–35}$ group

Di-*O*-Demethylcurcumin Attenuates $A\beta_{25–35}$-Induced iNOS Expression, NO Production

To determine whether di-*O*-demethylcurcumin has anti-inflammatory effects in $A\beta_{25–35}$-treated SK-N-SH cells, we investigated the production of NO is measured as nitrite in response to di-*O*-demethylcurcumin using the Griess reagent assay. Treatment of the SK-N-SH cells with $A\beta_{25–35}$ significantly increased the NO production, compared with the untreated control group (Fig. 2c). Pretreatment with di-*O*-

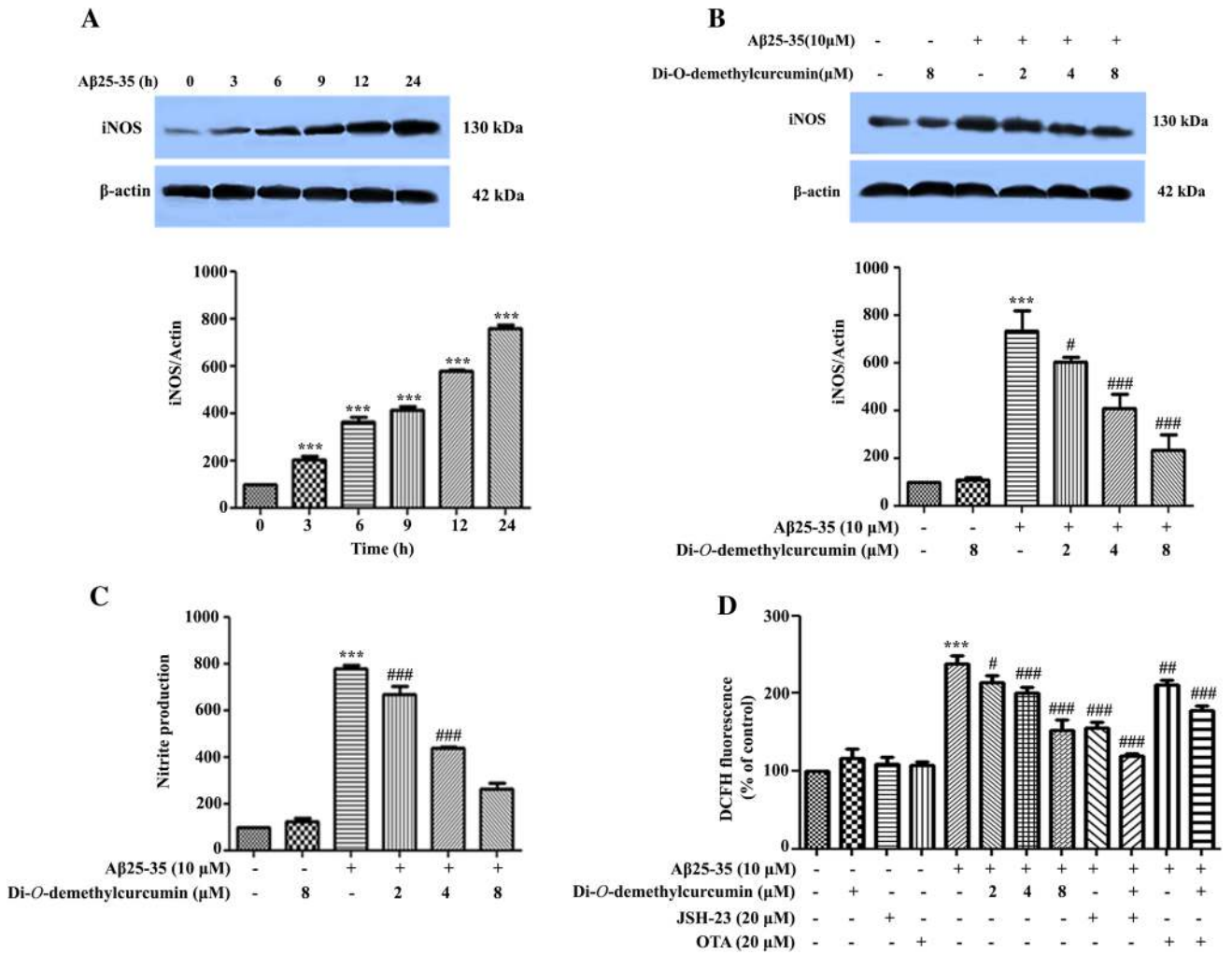

Fig. 2 The effect of di-*O*-demethylcurcumin on the iNOS protein expression and the inflammatory mediator NO production. **a** The cells were exposed to 10 μM $A\beta_{25–35}$ as time indicated 0, 3, 6, 9, 12, and 24 h. The cytosolic fractions were immunoblotted for the iNOS protein. **b** The cells were pretreated with di-*O*-demethylcurcumin (2, 4, or 8 μM) for 2 h prior treatments with 10 μM $A\beta_{25–35}$ for 24 h before being harvested. The expression of the iNOS protein was determined using the Western blot analysis. **c** The effect of di-*O*-demethylcurcumin on $A\beta_{25–35}$-induced NO production in the SK-N-SH cells. **d** The effect of di-*O*-demethylcurcumin on $A\beta_{25–35}$-induced ROS level in the SK-N-SH cells. The data presented are the mean ± SD. ***$p < 0.001$ when compared with the control group; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ when compared with $A\beta_{25–35}$

demethylcurcumin at a concentration of 2, 4, or 8 μM significantly attenuated $A\beta_{25–35}$-induced NO production in a dose-dependent manner [669.2 ± 35.3 % ($p < 0.001$), 436.5 ± 9.3 %, ($p < 0.001$) and 261.9 ± 28.8 % ($p < 0.001$), respectively]. To improve the molecular mechanisms underlying the expression of NO in SK-N-SH cells, we determined the expression of iNOS protein by Western blotting (Fig. 2). Results revealed that treatment with 10 μM of $A\beta_{25–35}$ increased the iNOS protein expression in a time-dependent manner, with the maximum level at 24 h (Fig. 2a). However, treatment with di-*O*-demethylcurcumin significantly inhibited $A\beta_{25–35}$-induced iNOS protein expression in a concentration-dependent manner, whereas di-*O*-demethylcurcumin alone was observed to have no effect (Fig. 2b). These data indicate that di-*O*-demethylcurcumin suppresses the production of the pro-inflammatory mediator NO mediated by inhibiting the iNOS expression in $A\beta_{25–35}$-stimulated SK-N-SH cells.

Di-*O*-Demethylcurcumin Inhibits NF-κB Nuclear Translocation

NF-κB regulates the expression of several pro-inflammatory cytokines, including ROS, NO; we next determined the effects of di-*O*-demethylcurcumin on the NF-κB activation after exposure to $A\beta_{25–35}$. The expression of IκBα was investigated using the Western blot analysis. The degradation of IκBα in the SK-N-SH cells was found to

have significantly increased after the treatment with 10 μM of $A\beta_{25–35}$. Di-*O*-demethylcurcumin had significantly inhibited the $A\beta_{25–35}$-induced IκBα degradation in the SK-N-SH cells. To determine NF-κB translocation, the cells were exposed to $A\beta_{25–35}$ treatment for different time intervals (3, 6, 9, 12, and 24 h). Our results indicate that the NF-κB migration into the nucleus in the $A\beta_{25–35}$-treated cells increased in a time-dependent manner, with the maximum increase being observed at 24 h (Fig. 3a). The pretreatment of cells with di-*O*-demethylcurcumin prior to $A\beta_{25–35}$ markedly decreased the expression of p65 in the SK-N-SH cells in a concentration-dependent manner, and retarded the retention of p65 in the cytoplasm (Fig. 3b). To further confirm whether di-*O*-demethylcurcumin inhibits ROS mediated by NF-κB signaling, we pretreated the SK-N-SH cells with NF-κB inhibitor, JSH 23 followed by 10 μM $A\beta_{25–35}$ for 24 h, significantly inhibited $A\beta_{25–35}$-induced ROS production. Similarly, $A\beta_{25–35}$-induced ROS was significantly attenuated by treatment with di-*O*-demethylcurcumin (Fig. 2d). These data suggest that di-*O*-demethylcurcumin can suppress Aβ-induced ROS production mediated by NF-κB signaling pathway.

Di-*O*-Demethylcurcumin Protects $A\beta_{25–35}$-Induced Neuroinflammation by Activating Nrf2 Translocation

We next examined whether di-*O*-demethylcurcumin could stimulate the activity of the Nrf2 pathway in SK-N-SH cells. As shown in Fig. 4, treatment with $A\beta_{25–35}$ of the SK-N-SH cells for 0, 3, 6, 9, 12, and 24 h decreased the level of the nuclear Nrf2 protein in a time-dependent manner, whereas the cytosolic Nrf2 protein level was found to significantly increase in a time-dependent manner, with the maximum level being observed at 24 h (Fig. 4a). Pretreatment with di-*O*-demethylcurcumin at concentrations of 2, 4, or 8 μM for 2 h, followed by exposure by 10 μM of $A\beta_{25–35}$ for 24 h, markedly increased the Nrf2 protein expression in the nucleus, an increase which was directly proportional to the decrease in the cytoplasm (Fig. 4b). These results reveal that di-*O*-demethylcurcumin-treated cells increased the expression of the Nrf2 protein in the nucleus, whereas there was no Nrf2 protein expression in the nucleus in the case of the $A\beta_{25–35}$-treated cells.

Di-*O*-Demethylcurcumin Protects $A\beta_{25–35}$-Induced Neuroinflammation by Activating Nrf2 Translocation

We next investigated whether di-*O*-demethylcurcumin could stimulate Nrf2 mechanism, with its transcriptional activity being related to phase II antioxidant enzyme genes such as HO-1, NQO-1, and γ-GCLC which are responsible for scavenging ROS. To further confirm whether di-*O*-demethylcurcumin attenuated ROS mediated by activating Nrf2 signaling, we pretreated the SK-N-SH cells with Nrf2 inhibitor, ochratoxin A (OTA), followed by 10 μM $A\beta_{25–35}$ for 24 h. OTA treatment promoted the production of ROS in SK-N-SH cells resembling to $A\beta_{25–35}$ treated alone. In the presence of OTA, the levels of ROS in SK-N-SH cells treated with di-*O*-demethylcurcumin significantly increased ROS level compared to the control group (Fig. 2d). These data support the role of di-*O*-demethylcurcumin on suppression $A\beta_{25–35}$-mediated ROS production in SK-N-SH cells via Nrf2 signaling pathway. We further examined the expression of the phase II antioxidant-associated proteins, including HO-1, NQO-1, and γ-GCLC. Treatment with 10 μM of $A\beta_{25–35}$ of the SK-N-SH cells for 0, 3, 6, 9, 12, and 24 h significantly decreased the expression of HO-1, NQO-1, and γ-GCLC in a time-dependent manner, with the maximum level being observed at 24 h (Fig. 5a). Pretreatment with di-*O*-demethylcurcumin at the concentrations of 2, 4, or 8 μM for 2 h, followed by exposure to 10 μM of $A\beta_{25–35}$ for 24 h, significantly increased the expression of HO-1, NQO-1, and γ-GCLC in a concentration-dependent manner (Fig. 5b). Furthermore, the activity of SOD, the antioxidant enzyme, was investigated under the same conditions as in the previous experiment. The $A\beta_{25–35}$ treatment alone significantly decreased SOD activity in the SK-N-SH cells. Pretreatment with di-*O*-demethylcurcumin significantly increased the SOD activity in a concentration-dependent manner (Fig. 5c). It was observed, however, that di-*O*-demethylcurcumin treatment alone did not show any effect in the SOD activity.

Discussion

Chronic inflammation associated with oxidative stress in the brain is a major cause of AD. Several lines of evidence have demonstrated that oxidative stress and inflammation are the responding effects of a variety of neurotoxins such as LPS, homocysteine (Hcy), 6-hydroxydopamine (6-OHDA) (Jakel et al. 2007; Sudduth et al. 2013; Vasconcelos et al. 2014) including $A\beta_{25–35}$ (Qi et al. 2013). Several studies have reported that Aβ plays an important role in the pathogenesis of AD (Behl et al. 1994; Joshi et al. 2014). The neurotoxic effects of Aβ are, at least, in part mediated by the generation of ROS and reactive nitrogen species (RNS) such as NO and these effects subsequently activate pro-inflammatory processes (Guo et al. 2013). Thus, the removal of excess ROS may be effective in preventing oxidative cell death. Therefore, in our study, we investigated whether di-*O*-demethylcurcumin, a synthetic analog of curcuminoid, possesses neuroprotective effect in Aβ-induced neurotoxicity. Our data demonstrated that

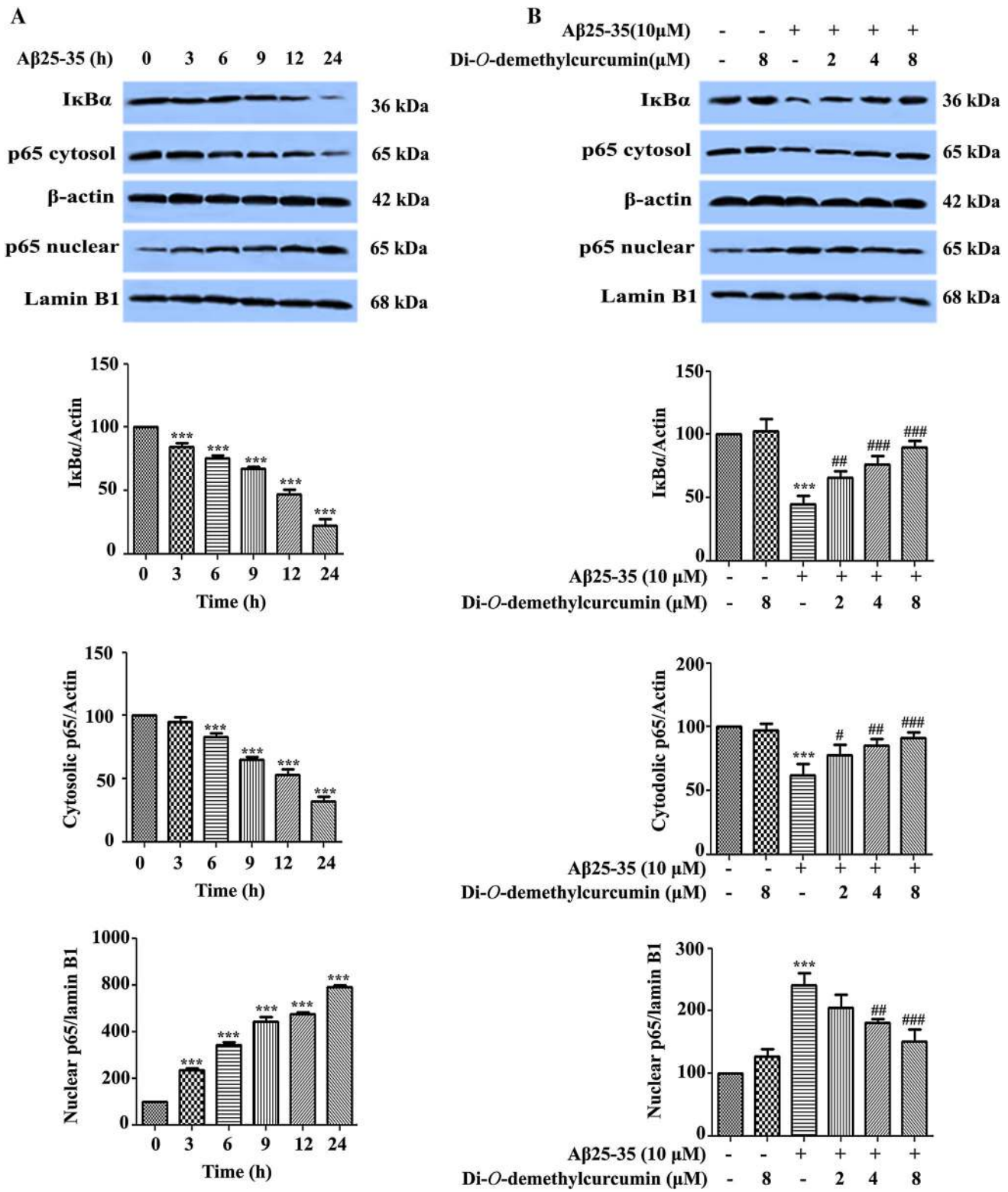

Fig. 3 The effect of di-*O*-demethylcurcumin on $A\beta_{25-35}$-induced NF-κB pathway activation in SK-N-SH cells. **a** The cells were treated with 10 μM $A\beta_{25-35}$ as time indicated 0, 3, 6, 9, 12, and 24 h. Protein from cytosol and nuclear extracts was used in the Western blotting analysis with antibodies for Iκβ-α and p65 proteins. **b** The cells were pretreated with di-*O*-demethylcurcumin before being exposed to 10 μM $A\beta_{25-35}$ for 2 h with or without di-*O*-demethylcurcumin. The data are expressed as the mean ± SD of three independent experiments. ***$p < 0.001$ when compared with the control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, ###$p < 0.001$, when compared with the $A\beta_{25-35}$ group

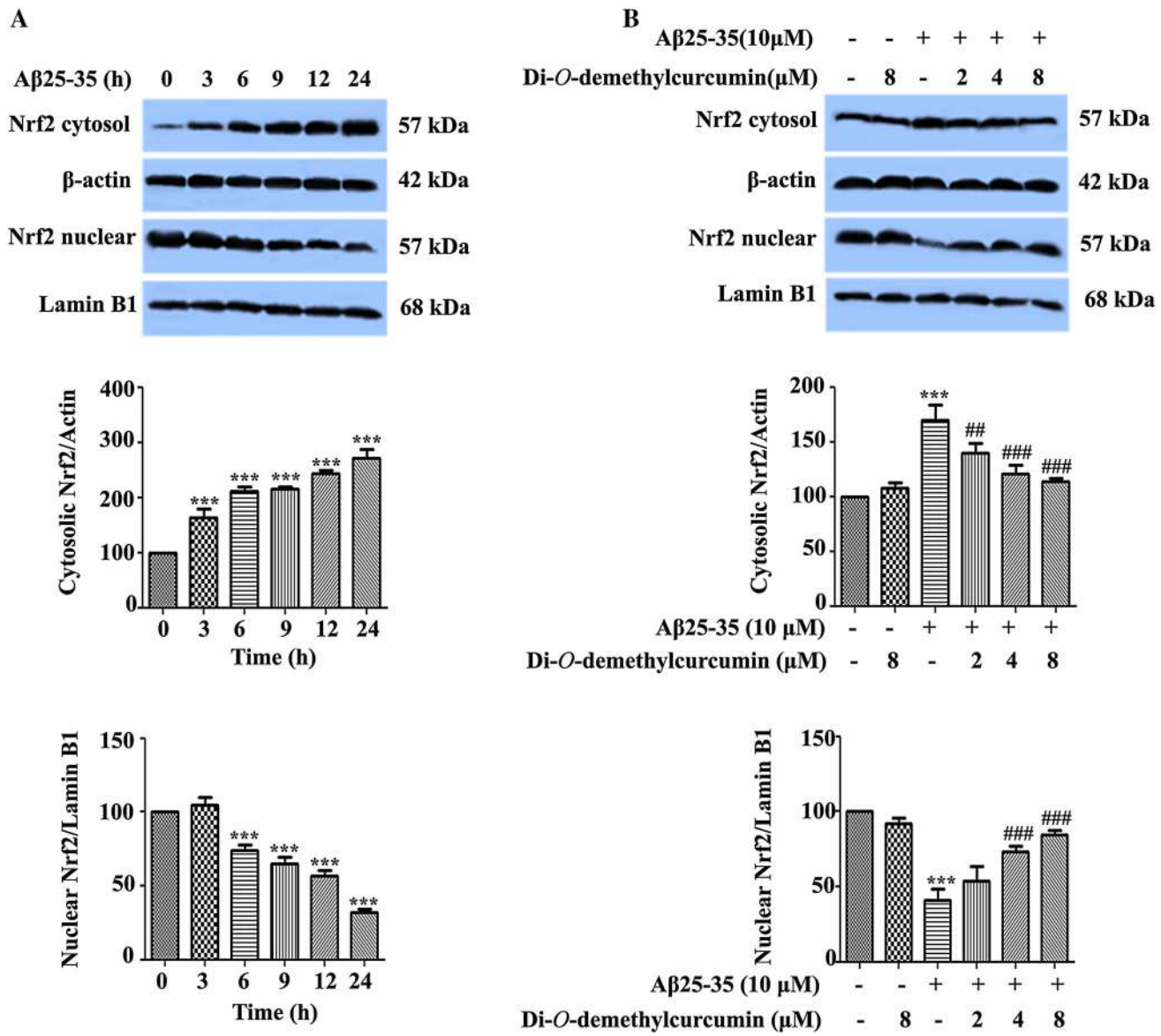

Fig. 4 The effects of di-*O*-demethylcurcumin on the expression of the Nrf2 protein in SK-N-SH cells treated with $A\beta_{25–35}$. **a** The cells were treated with 10 μM $A\beta_{25–35}$ as time indicated 0, 3, 6, 9, 12, and 24 h. Protein from cytosol and nuclear extracts was used in the Western blotting analysis with antibodies for Nrf2. **b** The cells were pretreated with di-*O*-demethylcurcumin before being exposed to 10 μM $A\beta_{25–35}$. The data are expressed as mean ± SD of three independent experiments. $^{***}p < 0.001$, when compared with the control. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, compared with the $A\beta_{25–35}$ group

treatment of cells with $A\beta_{25–35}$ results in cell death, which was observed to decrease in the presence of di-*O*-demethylcurcumin. This result showed that di-*O*-demethylcurcumin did significantly protect the SK-N-SH cells from $A\beta_{25–35}$-induced neurotoxicity. We also investigated the effect of di-*O*-demethylcurcumin on $A\beta_{25–35}$-induced cytotoxicity mediated by oxidative stress. It has been reported that $A\beta_{25–35}$ induces oxidative damage in neuronal cells by the overproduction of ROS and the attenuation of the activity of the antioxidant defense system including GSH, catalase, and SOD (Zhou et al. 2014). In the present study, it was observed that $A\beta_{25–35}$ treatment led to intracellular accumulation of ROS, and NO in SK-N-SH cells. The di-*O*-demethylcurcumin treatment before the $A\beta_{25–35}$ stimulation was found to have resulted in significant down-regulation of the expression of iNOS involved in the NO synthesis as well as ROS. It is well known that oxidative stress and inflammation are the responding effects of $A\beta_{25–35}$ and that are closely implicated with NF-κB signaling pathway. NF-κB signaling pathway plays a key role in mediating inflammation-related development of neurodegenerative disorders, through the induction of pro-

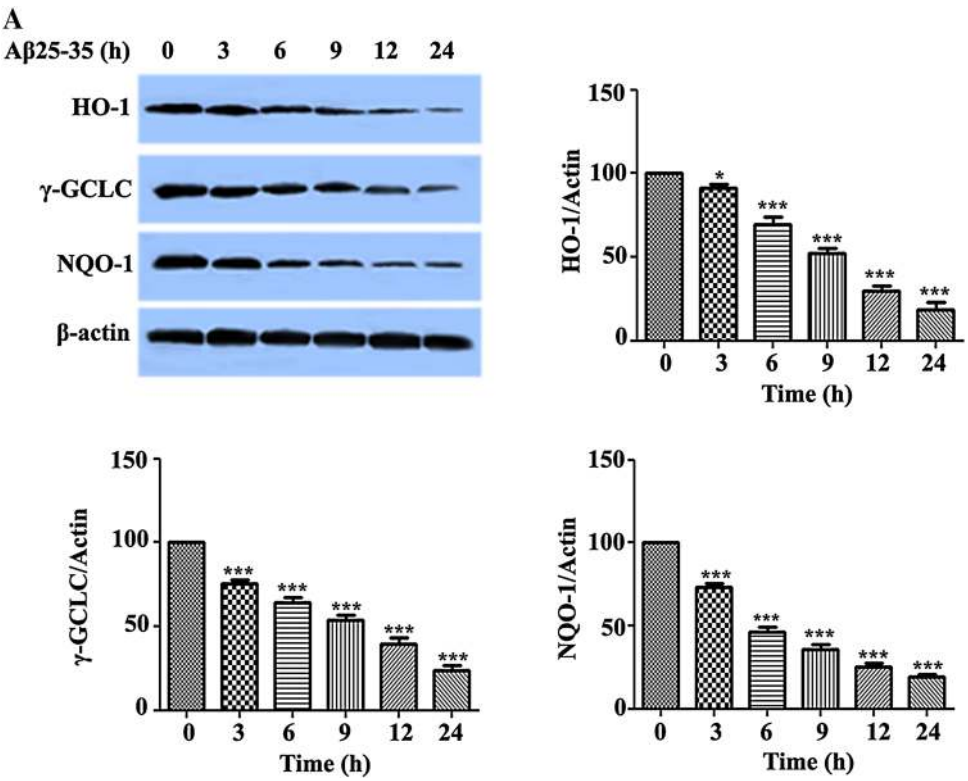

Fig. 5 The effect of $A\beta_{25–35}$ on the expression of HO-1, NQO-1, and γ-GCLC proteins in SK-N-SH cells. **a** The cells were treated with 10 μM $A\beta_{25–35}$ as time indicated 0, 3, 6, 9, 12, and 24 h. Protein was used in the Western blotting analysis with antibodies for HO-1, NQO-1, and γ-GCLC. **b** The cells were pretreated with di-*O*-demethylcurcumin (2, 4, or 8 μM) for 2 h before being exposed to 10 μM $A\beta_{25–35}$ for 24 h. Protein was used in the Western blotting analysis with antibodies for HO-1, NQO-1, and γ-GCLC. **c** SOD activity was determined using the SOD commercial assay kit. $^{*}p < 0.05$, $^{***}p < 0.001$ when compared with the control. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, when compared with the $A\beta_{25–35}$ group

inflammatory cytokines such as iNOS, IL-1β, TNF-α, and COX-2 (Perry 2004). The overproduction of pro-inflammatory cytokines damages neuronal cells and declines memory and cognitive function in AD. NF-κB is known to be activated by the phosphorylation and subsequent degradation of its inhibitor IκB through selective ubiquitination which results in the activation of NF-κB (Gilmore 2006). Activated NF-κB or release from IκBα will translocate itself from cytosol into the nucleus and bind at the promoter regions of pro-inflammatory molecules. Several studies have considered that the antagonistic effects of natural compounds on NO production are due to the suppression of NF-κB activation by the degradation of IκBα via phosphorylation. Therefore, we examined the effects of di-*O*-demethylcurcumin on IκBα degradation, and NF-κB p65 translocation. Following treatment with di-O-demethylcurcumin, the translocation of NF-κB p65 into the nucleus was suppressed in $A\beta_{25–35}$-induced SK-N-SH cells. The expression of IκBα, the upstream factors associated with NF-κB signaling, was also increased and consequently diminished NO production by suppressing the expression of the iNOS protein. Inhibition of NF-κB by JSH 23 also diminished the level of ROS in $A\beta_{25–35}$-induced SK-N-SH cells similar to di-O-demethylcurcumin-treated cells. This finding suggests that di-*O*-demethylcurcumin inhibits the activation of NF-κB, a transcription factor that regulates the expression of genes involved in inflammatory response in $A\beta_{25–35}$-stimulated SK-N-SH cells. Altogether, our data suggest that di-*O*-demethylcurcumin protects neuroinflammation induced by $A\beta_{25–35}$ through its suppression of NO production by reducing the iNOS protein expression mediated by transcriptional mechanism modulation and the activation of the NF-κB signaling pathway. Oxidative stress is a disturbance in the oxidant–antioxidant balance which generates ROS, causing cell death in AD (Klein and Ackerman 2003; Wu et al. 2014). ROS plays a significant role in causing cell stress and related cell death, and, along with superoxide anion (O_2^-), hydroxyl radical ($OH^{\bullet}$), and hydrogen peroxide (H_2O_2), causes the disturbance of the antioxidant mechanism, especially Nrf2 signaling which acts as a regulator of antioxidant enzymes. In order to find out whether the protection of di-*O*-demethylcurcuminin $A\beta_{25–35}$-induced neuroinflammation is associated with maintaining the redox balance, we investigated the activities of SOD. Antioxidant enzymes such as catalase and SOD can eliminate the level of the ROS generated by the activation of Nrf2 signaling (Kang et al. 2005). Our result showed that pretreatment with di-*O*-demethylcurcumin can significantly increase the activity of SOD and thereby decrease the level of ROS. These results imply that di-*O*-demethylcurcumin is able to attenuate $A\beta_{25–35}$-induced oxidative damage by controlling the balance of the redox system in the body. To further investigate the mechanism of di-*O*-

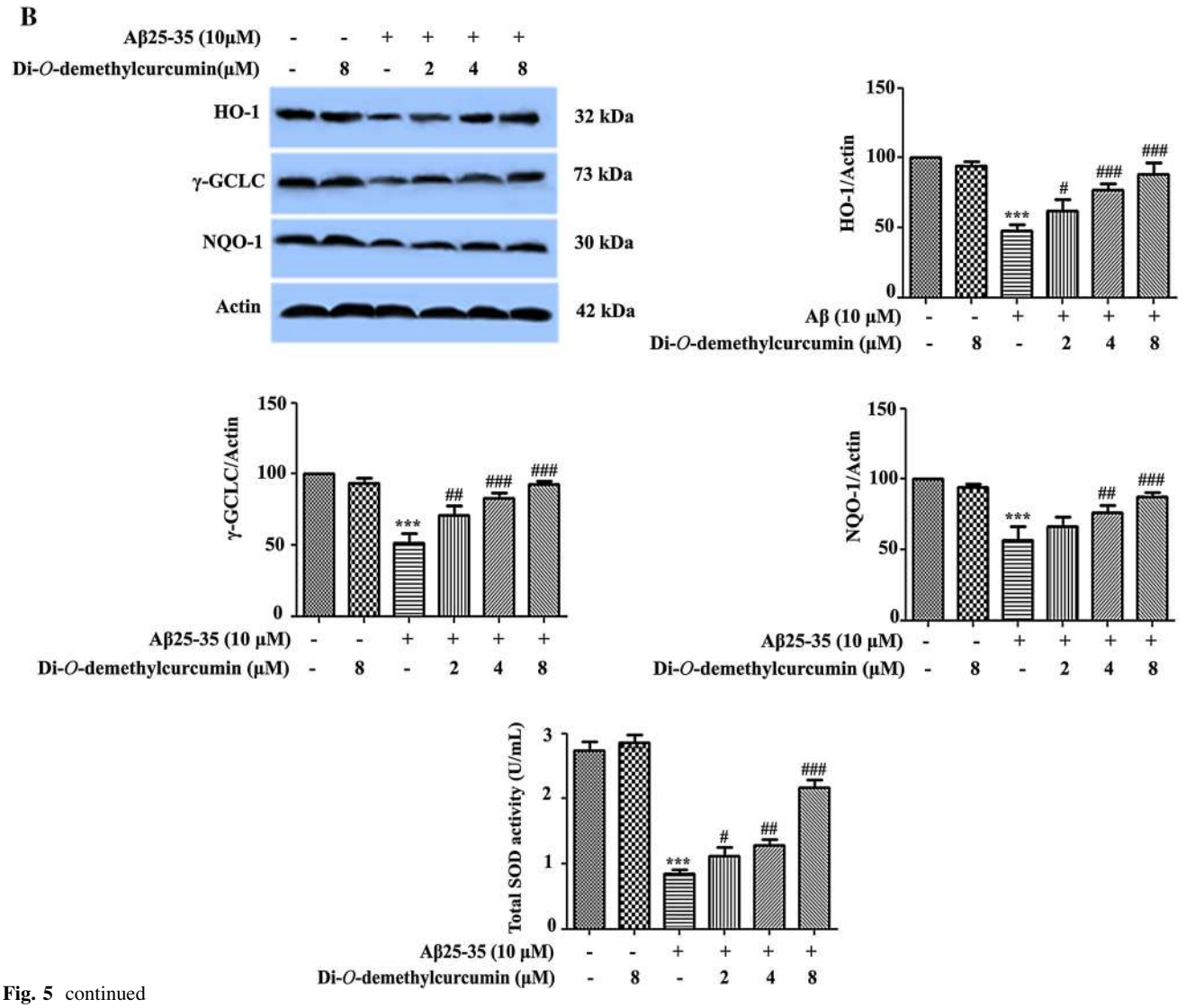

Fig. 5 continued

demethylcurcumin in maintaining the redox homeostasis and playing a role in antioxidation, redox-sensitive signaling pathways were explored in the present study. HO-1, γ-GCLC, NQO-1, and its upstream regulator Nrf2 signaling pathway were examined in the SK-N-SH cells exposed to $A\beta_{25–35}$. Nrf2, a nuclear transcription factor, plays a central role in the transcriptional regulation of several antioxidants, which regulates the cellular antioxidant response to ROS and also plays a role in modulating acute inflammatory responses. Upon stimulation, Nrf2 dissociates from its cytosolic inhibitor Keap-1, translocates into the nucleus, and binds to the antioxidant response element (ARE) promoter region. The binding of Nrf2 to ARE induces the production of antioxidant enzyme and the phase II detoxifying genes such as HO-1, γ-GCLC, and NQO-1, resulting in a cytoprotective response (Johnson et al. 2008; Nguyen et al. 2003). Recent reports revealed that Aβ treatment causes a significant decrease in the expression of Nrf2 and its downstream factors in neuronal cells. Our present study is in agreement with the findings of the earlier studies which demonstrated that $A\beta_{25–35}$ reduced Nrf2 translocation to nucleus and down-regulated the expression of HO-1, γ-GCLC, and NQO-1 in SK-N-SH cells and reducing antioxidant enzyme activity. Therefore, we next examined whether di-*O*-demethylcurcumin induced expression of HO-1, γ-GCLC, and NQO-1 via Nrf2 translocation in $A\beta_{25–35}$-induced SK-N-SH cells. Our results demonstrated that pretreatment with di-*O*-demethylcurcumin elevated the protein expression and activity in the detoxifying phase II proteins including HO-1, γ-GCLC, NQO-1, and SOD as well as increased the Nrf2 translocation in parallel with the decrease of inflammatory mediator such as NO. SOD is, primarily, an antioxidant enzyme that scavenges oxygen free radicals,

occurring widely during cellular metabolism or cytotoxic exposure (Vanuprasad et al. 2013). The increasing activities of SOD in the SK-N-SH cells brought about by di-*O*-demethylcurcumin prior to the $A\beta_{25-35}$ treatment, lead to a decrease in ROS, oxidative stress, inflammation, and death in the SK-N-SH cells. Inhibition of Nrf2 by OTA, di-*O*-demethylcurcumin failed to decrease the level of ROS compared to di-*O*-demethylcurcumin-treated cells. The cytoprotective effect of di-*O*-demethylcurcumin against $A\beta_{25-35}$-induced oxidative damage seemed to be mediated by up-regulation of antioxidant enzymes through Nrf2 activation. Di-*O*-demethylcurcumin has been considered as an indirect antioxidant because of its ability solely to induce many cytoprotective antioxidant enzymes through the Nrf2-ARE pathway. This finding is consistent with the results of previous studies which demonstrated molecular mechanisms related to various antioxidants as being involved in the Nrf2 signaling pathway by promoting the translocation of Nrf2 from the cytoplasm into the nucleus and sequentially binds to ARE (Li and Kong 2009; Kelly and Scarpulla 2004). The Nrf2 signaling pathway plays a protectant role in intracerebral hemorrhage, traumatic brain injury, and cerebral ischemia models (Chen et al. 2011; Cheng et al. 2013; Yang et al. 2009). The important role of Nrf2 is in modulating the activity of the antioxidant enzyme in the scavenging of ROS before they lead to stress and inflammation. It is possible that di-*O*-demethylcurcumin plays a role in redox modulation; it may also play a role in the potential crosstalk between NF-κB and Nrf2.

In conclusion, it can be stated that our present study demonstrated that di-*O*-demethylcurcumin attenuated $A\beta_{25-35}$-induced neurotoxicity in SK-N-SH cells. The mechanism underlying the activity of di-*O*-demethylcurcumin is mediated by the up-regulation of the antioxidant enzymes; HO-1, NQO-1, and γ-GCLC through Nrf2 activation, leading to reduction in the $A\beta_{25-35}$-induced oxidative damage. In addition, di-*O*-demethylcurcumin was also observed to suppress the NF-κB signaling pathways, thereby reducing the NO production in the SK-N-SH cells. Taken together, our findings indicate that di-*O*-demethylcurcumin attenuates the production of the pro-inflammatory mediator NO in $A\beta_{25-35}$-stimulated SK-N-SH cells by suppressing the NF-κB signaling pathways and inducing the Nrf2-mediated HO-1, NQO-1, and γ-GCLC expression. These findings provide first-hand evidence that di-*O*-demethylcurcumin possesses potential neuroprotective activities in vitro.

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