Ginsenoside Rg1 exerts a protective effect against $A\beta_{25-35}$-induced toxicity in primary cultured rat cortical neurons through the NF-κB/NO pathway

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Received August 27, 2015; Accepted February 11, 2016

DOI: 10.3892/ijmm.2016.2485

Abstract. Ginsenoside Rg1 (Rg1) is a multipotent triterpene saponin extracted from ginseng, and has been proven to act as a nootropic agent against various types of neurological damage. The present study was designed to investigate the neuroprotective effect and the underlying mechanisms of Rg1 on apoptosis induced by β-amyloid peptide 25-35 ($A\beta_{25-35}$) in primary cultured cortical neurons. The primary neurons were preincubated with 20 μM Rg1 for 24 h and exposed to 10 μM $A\beta_{25-35}$ for 72 h. In the present study, we found that Rg1 prevented nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) nuclear translocation and IκB-α phosphorylation in primary cultured cortical neurons after $A\beta_{25-35}$ exposure by scavenging excess reactive oxygen species (ROS); ROS was measured using DCFDA and examined using a fluorescence microscope. In addition, Rg1 successfully suppressed $A\beta_{25-35}$-inducible nitric oxide synthase (iNOS) expression and nitric oxide (NO) production in a NF-κB-dependent manner; the suppression of NO was clearly illustrated by the NO production assay. Pretreatment of the cells with Rg1 elevated the proportion of Bcl-2/Bax, lessened the release of cytochrome *c* from mitochondria into cytoplasm and then blocked mitochondrial apoptotic cascades after $A\beta_{25-35}$ insult by lowering NO generation. Taken together, our data demonstrate that Rg1 rescues primary cultured cortical neurons from $A\beta_{25-35}$-induced cell apoptosis through the downregulation of the NF-κB/NO signaling pathway.

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Abbreviations: AD, Alzheimer's disease; $A\beta_{25-35}$, amyloid β-peptide 25-35; carboxy-DCFDA, 5-(and-6)-carboxy-2',7'dichlorofluorescein diacetate; DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; H_2O_2, hydrogen peroxide; iNOS, inducible nitric oxide synthase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor κ-light-chain-enhancer of activated B cells; NO, nitric oxide; PDTC, ammonium pyrrolidinedithiocarbamate; Rg1, ginsenoside Rg1; ROS, reactive oxygen species; SMT, s-methylisothiourea hemisulfate salt; tempol, 4-hydroxy-2,2,6,6-tetramethyl-piperidine 1-oxyl

Key words: ginsenoside Rg1, amyloid β-peptide, neuroprotection, nuclear factor κ-light-chain-enhancer of activated B cells, inducible nitric oxide synthase, apoptosis

Introduction

Alzheimer's disease (AD), which has a high prevalence in the elderly, is a progressive and fatal neurodegenerative disease characterized by loss of synapses and neurons, amyloid plaque deposition, neurofibrillary tangle aggregation and neuroinflammation; previous research has confirmed that the accumulation of intracellular β-amyloid (Aβ) is an early event in the development of AD (1,2). Evidence from *in vivo* and *in vitro* experiments have demonstrated that Aβ induces the inflammatory response, oxidative stress and neuronal apoptosis, resulting in loss of neurons, particularly in the cerebral cortex and hippocampal cortex (3,4). Despite considerable progress that has been made in exploring the complicated underlying mechanisms of AD, there is still no effective treatment which is able to reverse, prevent or even halt the development of AD (5).

Ginseng, the root of *Panax ginseng* C.A. Meyer, has been used extensively as a drug in traditional Chinese medicine for over 2,000 years. Currently, more than 40 kinds of ginsenoside constituents have been extracted from ginseng. Among them, ginsenoside Rg1 (Rg1) is considered an important constituent which exerts important pharmacological effects and has also been proven to potentiate prominent neuroprotective properties both *in vivo* and *in vitro* (6-9). Our previous study has reported that the steroid receptor-dependent anti-protein tyrosine nitration pathway plays a vital role in the protection of Rg1 against Aβ-induced toxicity (7). However, the molecular mechanism of how Rg1 suppresses nitric oxide (NO) production and nitrotyrosine formation remains ambiguous, and needs to be studied further.

Nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) is a protein complex which is in charge of DNA transcription, cytokine secretion and cell survival (10). NF-κB is present in almost all cell types in the nervous system. Incorrect modulation of NF-κB has been connected to inflammation, cancer, neurodegenerative diseases and improper immune development (11-13). Autopsy results have demonstrated that the NF-κB activity in neurons and astrocytes near amyloid plaques was abnormally increased in the brains of patients with AD (14-16), suggesting that Aβ plays a critical role in the activation of NF-κB in AD. Furthermore, it has been shown that Rg1 downmodulated LPS-induced proinflammatory cytokines release, and prevented NF-κB nuclear translocation and DNA binding activity in RAW264.7 and A549 cells (17). In PC12 cells, Rg1 has been shown to prevent the cell lesions caused by hydrogen peroxide (H_2O_2) solution attack via modulation of the NF-κB and MAPK signaling pathways (18). Therefore, we hypothesized that NF-κB serves as an important pharmacological target in the neuroprotective mechanisms of Rg1.

In the present study, we used an *in vitro* model of aggregated $A\beta_{25-35}$-induced AD to investigate the potential cellular and molecular mechanisms by which Rg1 counteracts mitochondrial dysfunction and $A\beta_{25-35}$-mediated apoptosis in primary cultured rat cortical neurons.

Materials and methods

Reagents and antibodies. Rg1 was purchased from the National Institute for the Control of Pharmaceutical and Biological Products, with more than 99% purity (China). β-amyloid peptide 25-35 ($A\beta_{25-35}$), s-methylisothiourea hemisulfate (SMT) salt, ammonium pyrrolidine dithiocarbamate (PDTC), H_2O_2, dimethyl sulfoxide (DMSO), poly-D-lysine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (tempol), and 5-(and-6)-carboxy-2',7'-dichlorofluorescein diacetate (carboxy-DCFDA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, B27, and neurobasal medium were all purchased from Gibco-BRL (Burlington, ON, Canada). Primary antibodies against NF-κB (p65) (sc-372), IκB-α (sc-371), phosphorylated (p-)IκB-α (sc-8404), lamin B (sc-6217), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (sc-47724), inducible nitric oxide synthase (iNOS) (sc-651), Bcl-2 (sc-7382), Bax (sc-7480) were all purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Primary antibodies against cytochrome *c* (#4272), cytochrome *c* oxidase (COX IV) (#11967), pro-caspase-9 (#9508), cleaved caspase-3 (#9661), and pro-caspase-3 (#9662) were all purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Secondary antibodies against mouse IgG (HRP-conjugated) (BA1051), rabbit IgG (HRP-conjugated; BA1054), DyLight 488-conjugated (BA1127), goat IgG (HRP-conjugated) (BA1060) were all from Boster, Inc. (Wuhan, China).

Cell culture. Primary cultured cortical neurons were prepared from embryonic day (D17-18) Sprague Dawley (SD) rat fetuses as previously described (7). Briefly, pregnant rats were anesthetized with halothane and sacrificed by cervical dislocation. All of the fetal rats were seperated from the maternal body. The brain cortex of the fetuses was then dissected in D-Hank's buffer (137 mmol/l NaCl, 5.4 mmol/l KCl, 0.4 mmol/l KH_2PO_4, 0.34 mmol/l $Na_2HPO_4 \cdot 7H_2O$, 10 mmol/l glucose and 10 mmol/l Hepes) containing 0.125% trypsin. After incubation at 37˚C for 8-10 min, cortical tissues were dissociated by passing through a series of fire-polished constricted Pasteur pipettes. Approximately $5x10^5$ cells/ml were seeded onto poly-D-lysine (10 μg/ml) coated 96-well or 6-well plates. Cells were routinely cultured in Neurobasal medium supplemented with 2% B27, 10 U/ml penicillin, 10 U/ml streptomycin and 0.5 mmol/l glutamine at 37˚C in an atmosphere with 5% CO_2 and observed by inverted phase-contrast microscopy. Neuronal cultures were maintained for 7 days *in vitro* before the various chemical treatments. After identification with a phase contrast microscope and immunocytochemical analysis for neuronal markers, the primary neuronal cultures were found to be more than 90% pure. All animals were purchased from the Laboratory Animal Center of Zhejiang University. The experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee of Zhejiang University (Hangzhou, China).

Immunocytochemical analysis. After treatments, cells were rinsed with ice-cold PBS buffer solution and immediately fixed with 100% methanol (-2˚C) for 15 min. Fixed cells were washed with PBS, blocked with 10% normal fetal serum for 1 h, and then incubated at 4˚overnight with primary antibodies. After this, the cells were washed three times in PBS solution for 5 min each. Cells were then incubated with fluorescent secondary antibodies (1:400). The cultures were then incubated in 2 mg/ml DAPI solution in PBS for 1 min to label the nuclei, and immunoreactivity was visualized by microscopic examination carried out using a Leica inverted microscope equipped for fluorescence analysis (Leica Microsystems, Wetzlar, Germany).

Experimental treatment of cultures. $A\beta_{25-35}$ was dissolved in sterile distilled water at a concentration of 0.5 mg/ml as a stock solution. The stock solution was incubated at 37˚C for 72 h for the aggregation of $A\beta_{25-35}$ and then stored at 4˚C. After the medium was refreshed, the cortical neuronal cultures at day 7 were preincubated with the ROS scavenger tempol (10 μM), iNOS-selective inhibitor SMT (100 μM), NF-κB-selective inhibitor PDTC (1 μM) or Rg1 (20 μM), for 36 h, followed by exposure to 10 μM aggregated $A\beta_{25-35}$ for 72 h. All the chemicals were dissolved in DMSO. Control rats were treated with the same volume of vehicle (DMSO) as the experimental groups; the final concentration was 0.1%, which had no toxic effect on cell viability.

Exposure to H_2O_2. A total of 30% H_2O_2 solution (Sigma-Aldrich) was diluted with sterile distilled water to 0.15 mol/l as a working solution. As a positive control to release ROS, H_2O_2 solution was added to the culture medium at a final concentration of 150 μM for 24 h.

Cell viability assay. The viability of cell cultures was detected by MTT assay, as previously described, (19) after $A\beta_{25-35}$ treatment for 72 h. Primary neurons were incubated with MTT stock solution (0.5 mg/ml) for 3 h at 37˚C, washed in PBS three times and then shaken for at least 20 min at room temperature to

dissolve the formazane crystals in DMSO. The absorbance was measured using a microplate reader at 570 nm (DTX880; Beckman Coulter, Inc., La Brea, CA, USA). The cell viability of the control was defined as 100%. Assays were repeated in three independent culture preparations, each performed in triplicate.

Measurement of intracellular reactive oxygen species (ROS) generation. The production of intracellular ROS was evaluated in primary neurons with an oxidation sensitive fluorescent dye, carboxy-DCFDA. The intracellular ROS oxidize non-fluorescent intracellular DCFDA into the highly fluorescent dichlorofluorescein. An increase in the green fluorescence intensity was used to quantify the generation of intracellular ROS. After carboxy-DCFDA was added at a final concentration of 15 μM to culture medium for 30 min at 37˚C, neurons were photographed with a fluorescence microscope. The fluorescence intensity was measured by a microplate reader (DTX880; Beckman Coulter, Inc.) with an argon laser with 488 and 525 nm bandpass filters.

NO production assay. After primary neuronal cells were exposed to $A\beta_{25-35}$ for 24 h, the supernatant was collected from plates and NO production was determined spectrophotometrically using a Griess assay reagent kit (Jiancheng Bioengineering Institute, Nanjing, China) (20). Briefly, 100 μl supernatant was mixed with 100 μl Griess reagent (0.1% N naphthyl ethylethylenediamine dihydrochloride in 5% phosphoric acid and 1% sulfanilamide were mixed according to the proportion of 1:1). The absorbance was then detected spectrophotometrically at 550 nm using the microplate reader. Nitrite production in the control group was defined as a value of 1.0.

Western blot analysis. After the various treatments, medium was removed and cells were harvested at 4˚C. Total proteins were obtained by cell lysis (Tris-HCl 50 mM, pH 7.5, NaCl 150 mM, EGTA 20 mM, 1% Triton X-100, 0.5% sodium deoxycholate, DTT 1 mM, NaF 20 mM, sodium vandate 1 mM, PMSF 1 mM, leupeptin 10 μg/ml and aprotinin 30 μg/ml) as previously described (19). To detect the cytosolic release of cytochrome *c*, the untreated and drug-treated cells were harvested by centrifugation at 1,000 x g for 5 min at 4˚C. Cell pellets were washed once with ice-cold PBS and resuspended with 5 volumes of EGTA (1 mM), DTT (1 mM), HEPES-KOH (pH 7.5, 20 mM), KCl (10 mM), $MgCl_2$ (1.5 mM), PMSF (0.1 mM), sucrose (250 mM), and EDTA (1 mM). Cells were homogenized and centrifuged at 750 x g for 10 min at 4˚C. The sediments were the fraction of cytoplasmic protein and were lysed in lysis buffer. Supernatants were further centrifuged at 100,000 x g for 15 min at 4˚C. The obtained supernatants were the fraction of mitochondrial protein and were lysed in lysis buffer as well. The concentration of protein was determined by a modified Lowry assay (DC Protein assay; Bio-Rad Laboratories, Inc., Hercules, CA, USA). SDS-PAGE and western blot analysis were operated under standard protocols. GAPDH was used as a housekeeping protein for cytosolic fraction and total protein. COX IV was used as a housekeeping protein for mitochondrial fraction.

For nucleoprotein extraction, the nuclear protein was extracted with a ReadyPrep™ Protein Extraction kit (Cytoplasmic/Nuclear) (#163-2089, Bio-Rad Laboratories, Inc.). Briefly, for each 0.05 ml packed cells, 0.5 ml ice-cold cytoplasmic protein extraction buffer was added. This was followed by vortexing to suspend the cell pellet, and the cells were then incubated on ice for 30 min to lyse the cells without damaging the nuclei. The cell lysate was centrifuged at 1000 x g for 10 min at 4˚C. The supernatant contained the cytoplasmic proteins and the pellet in the tube contained nuclei. The nuclei was suspended in 0.5 ml protein solubilization buffer. The tube was vortexed to solubilize the nuclear proteins and centrifuged at maximum speed (12-16,000 x g) for 15-20 min at room temperature. The clarified supernatant was the nuclear protein fraction.

Statistical analysis. All data are expressed as the means ± standard deviation of at least three independent experiments. Student's t-test was examined for statistical analysis and a P-value <0.05 was considered to indicate a statistically significant difference.

Results

Rg1 inhibits $A\beta_{25-35}$-induced neuronal cell death by decreasing ROS accumulation. Primary rat cortical neuronal cultures were preincubated with Rg1 or different inhibitors for 24 h, followed by exposure to $A\beta_{25-35}$ 10 μM for 72 h. We reported previously that Rg1 attenuated neural injury induced by 10 μM $A\beta_{25-35}$ in a dose-dependent manner, and the 20-μM dose which was shown to be the optimal concentration for Rg1 was thus used in subsequent experiments (7). The viability of neuronal cultures, established by MTT metabolism, decreased by nearly 35% in $A\beta_{25-35}$-treated neurons compared to non-treated controls. Tempol, a superoxide scavenger, was used as the antioxidative positive control. Upon pretreatment with Rg1 and tempol, MTT metabolism rose from 65.6±4.3% of the model group to 86.0±2.8% and 87.9±2.5% in the presence of 20 μM Rg1 and 10 μM tempol, respectively (Fig. 1A). We subsequently evaluated changes in ROS production following $A\beta_{25-35}$ exposure using the fluorescent dye carboxy-DCFDA. After exposure to $A\beta_{25-35}$ for 24 h, the fluorescence intensity of ROS was significantly augmented (2.11±0.16-fold) (Fig. 1B) compared with the control. Both Rg1 and tempol reduced $A\beta_{25-35}$-triggered ROS release almost to the baseline level. These data suggest that Rg1 inhibited $A\beta_{25-35}$-induced neuronal death, at least in part through the ROS-scavenging pathway.

Rg1 compromises Aβ-triggered NF-κB activation through its antioxidative effects. NF-κB is regarded as a reduction/oxidation (redox)-sensitive factor. Under healthy physiological conditions, as a passive form, NF-κB combines with its inhibitor IκB-α to form a cytoplasmic complex. When suffering from stress such as that caused by ROS and Aβ, however, IκB-α is phosphorylated and degraded, subsequently freeing NF-κB to translocate to the nucleus and stimulate the expression of target genes (21,22). To confirm whether Aβ-induced NF-κB activation is oxidative stress-dependent, we investigated NF-κB nuclear translocation in primary cultured neurons. As shown in Fig. 2A, immunofluorescence imaging demonstrated that the level of NF-κB (p65) nuclear translocation significantly increased after $A\beta_{25-35}$ treatment for 24 h, and tempol pretreatment counteracted this effect. The modulating effects of Rg1 and tempol on the expression of NF-κB and its depressor IκB-α

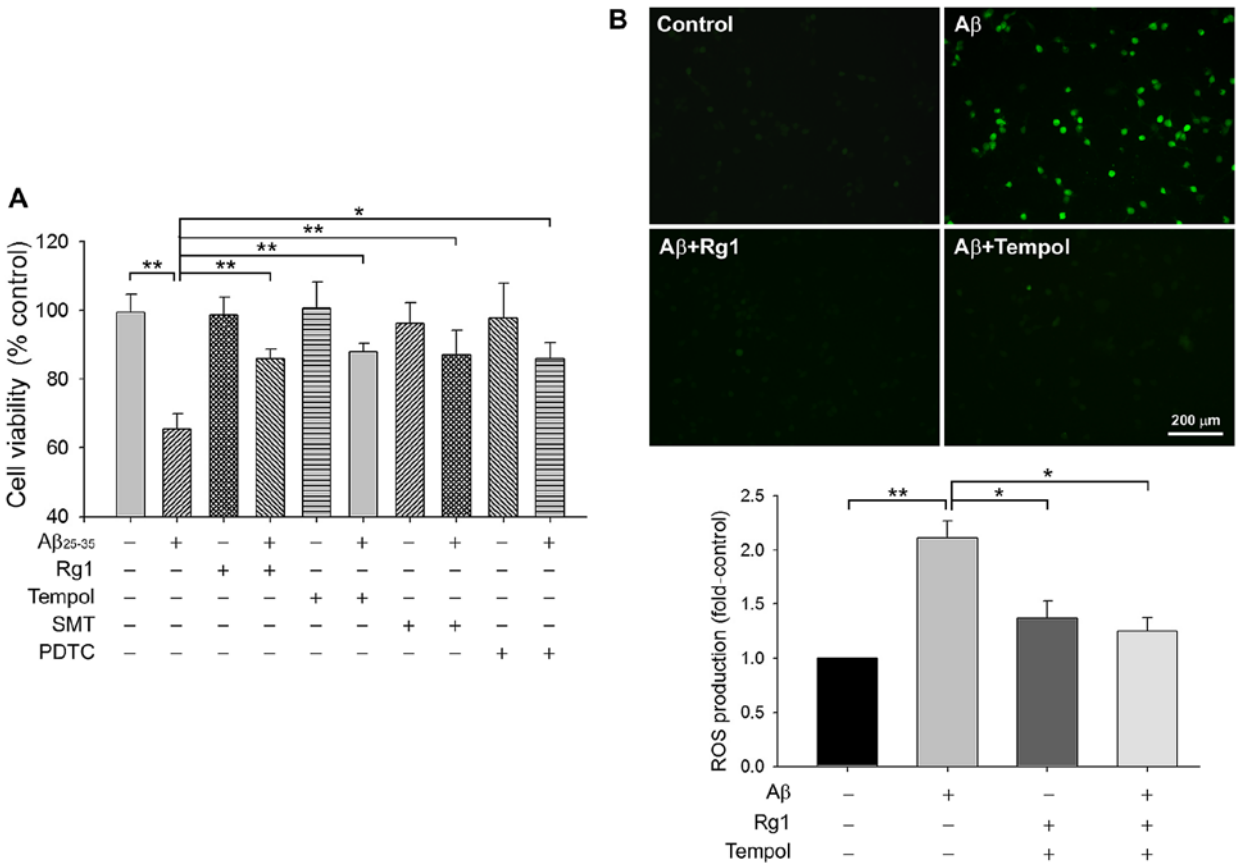

Figure 1. Effects of ginsenoside Rg1 (Rg1) on the scavenging of reactive oxygen species (ROS) in primary cultured neurons after amyloid β-peptide 25-35 ($A\beta_{25-35}$) insult. Neuronal cells were maintained with 20 μM Rg1 for 24 h, and then exposed to $A\beta_{25-35}$ (10 μM) for another 24 h. (A) Cell viability was measured by MTT assay. (B) ROS level was assessed with the fluorescence dye carboxy-DCFDA after $A\beta_{25-35}$ treatment (scale bar, 200 μM). The degree of green fluorescence was quantificationally detected using a microplate reader. Data are represented as the means ± standard deviation of three independent experiments. The statistical significance was defined with the Student's t-test as $^{*}P<0.05$, $^{**}P<0.01$.

were further tested by western blot analysis of the cytoplasmic and nuclear fractions. Consistent with the immunofluorescence imaging results, $A\beta_{25-35}$ markedly enhanced the NF-κB level in the nuclear fractions of primary neurons, while the level was decreased in cytoplasmic fractions ($P<0.05$ and $P<0.01$, respectively). Tempol pretreatment decreased $A\beta_{25-35}$-induced NF-κB activation, while H_2O_2 exposure aggravated it (Fig. 2B). As an antioxidant, Rg1 also exerted a positive effect in terms of the suppression NF-κB nuclear translocation, and stabilized accumulated IκB-α stimulated by $A\beta_{25-35}$ through restraint of IκB-α phosphorylation (Fig. 2C and D). Our results indicated that Rg1 decreased $A\beta_{25-35}$-stimulated NF-κB activation by 'mopping up' redundant cellular ROS in primary neurons.

Rg1 represses Aβ-induced NO production in an NF-κB-dependent manner. Since NO, ROS and endoplasmic reticulum (ER) stress have been shown to be involved in $A\beta_{25-35}$ insult, we detected NO production using Griess reagent in primary cortical neuronal cells. As shown in Fig. 3A, $A\beta_{25-35}$ incubation for 24 h caused marked upregulation in NO synthesis (1.62±0.08-fold vs. control, $P<0.01$), whereas Rg1 significantly decreased $A\beta_{25-35}$-mediated NO generation to 1.16±0.10-fold. Notably, the NF-κB inhibitor PDTC also decreased NO production, as well as iNOS-specific inhibitor SMT, suggesting that iNOS is responsible for $A\beta_{25-35}$-induced NO release. We further explored the expression of iNOS in primary cortical neurons and found that the level of iNOS was markedly elevated after $A\beta_{25-35}$ treatment. Both Rg1 and PDTC exerted distinctly negative effects on the expression of iNOS, suggesting that NF-κB is a pivotal regulatory factor which is targeted by Rg1 in precluding $A\beta_{25-35}$-mediated NO production (Fig. 3B). In addition, as shown in Fig. 1A, both PDTC and SMT treatment reduced the cell death caused by $A\beta_{25-35}$ injury, suggesting that the NF-κB-mediated decrease in NO production is cardinal to the protective effect exerted by Rg1 in cases of $A\beta_{25-35}$-induced neurotoxicity.

Rg1 hampers $A\beta_{25-35}$-induced mitochondrial apoptotic cascades by decreasing NO production. The ratio of Bcl-2/Bax expression is generally recognized to be the controller of mitochondria permeability, which modulates the release of cytochrome *c* from the mitochondria to cytoplasm during mitochondrion-mediated apoptotic cascades (23). Compared with the control, $A\beta_{25-35}$ exposure diminished the level of Bcl-2, while raised the level of Bax, resulting in a low proportion of Bcl-2/Bax. Rg1 exerted neuroprotective effects and elevated the ratio of Bcl-2/Bax, as did SMT (Fig. 4A and B). We subsequently prepared both the cytosolic and mitochondria-rich fractions of primary neurons, in order to verify the subcellular distribution of cytochrome *c*. In the $A\beta_{25-35}$-exposed group, the expression of cytochrome *c* in the cytosol increased, whereas mitochondrial cytochrome *c*

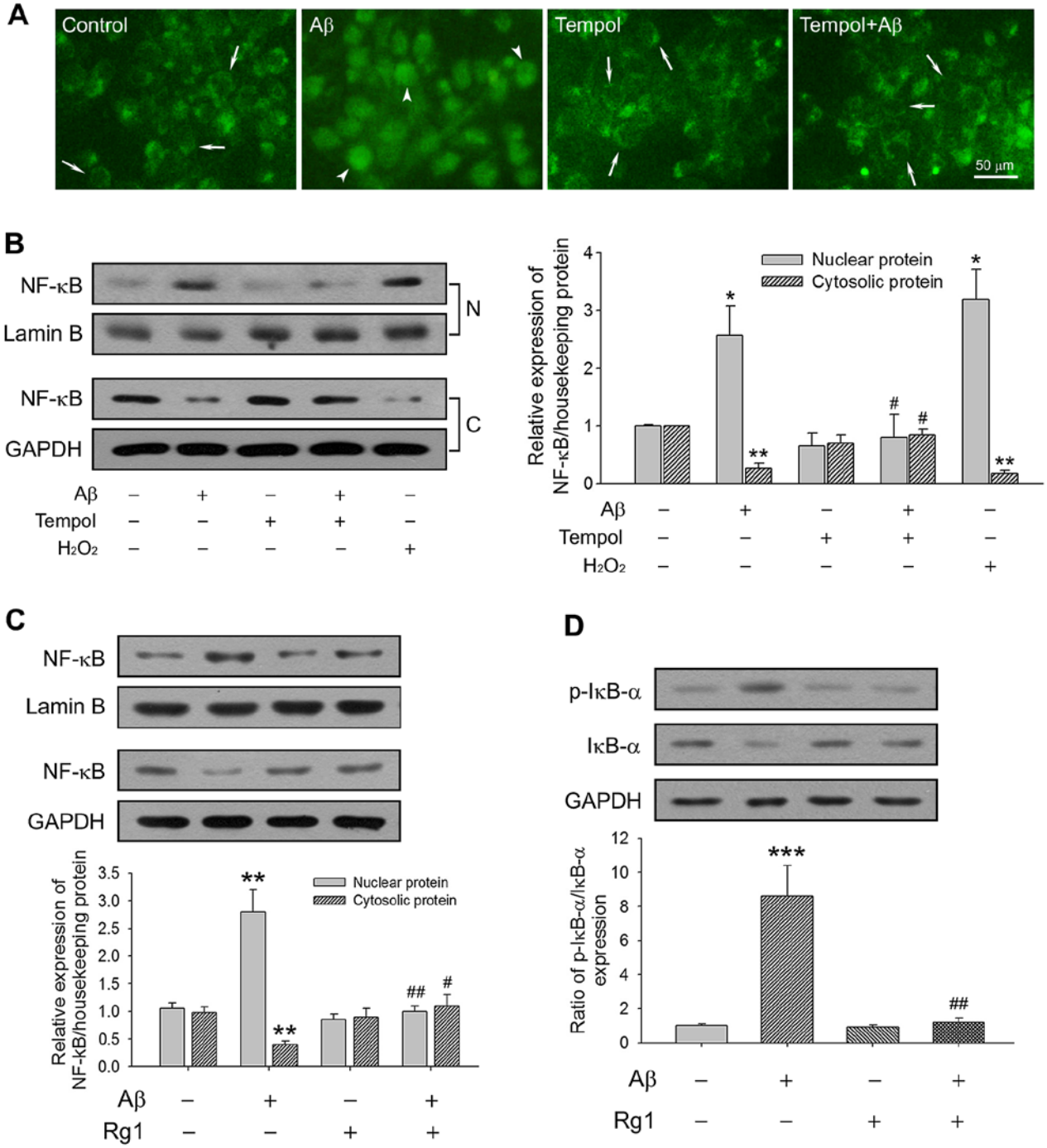

Figure 2. Ginsenoside Rg1 (Rg1) attenuates amyloid β-peptide 25-35 ($A\beta_{25-35}$)-induced nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) activation. Primary cultured neurons were preincubated with 20 μM Rg1 for 24 h, and subsequently treated with 10 μM $A\beta_{25-35}$ for another 24 h. (A) The fluorescence images indicated NF-κB translocation to the nucleus after $A\beta_{25-35}$ treatment (as shown by arrows). Scale bar, 50 μM. (B and C) After stimulation with $A\beta_{25-35}$ and 150 μM hydrogen peroxide (H_2O_2) solution for 24 h, the nucleoprotein was extracted using a nuclear and cytoplasmic protein extraction kit. NF-κB expression was detected by western blot analysis. Lamin B was used as a housekeeping protein for the nuclear protein and GAPDH was used for cytoplasmic protein. (D) Phosphorylation of IκB-α was detected by western blot analysis. Data are represented as the means ± standard deviation of three independent experiments. The statistical significance was defined using Student's t-test as $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. control group, $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. model group. N, nuclear fractions; C, cytoplasmic fractions.

content decreased. By contrast, endogenous cytochrome *c* expression in the control and Rg1-treated group was detected to be mostly in mitochondria (Fig. 4C). The decrease in NO production by SMT also potentiated the inhibition of cytochrome *c* release from the mitochondria (Fig. 4D). The full-length pro-form of caspase-9 and caspase-3 were used to assess caspase-9 and caspase-3 activation through their cleavage, indicated here by the loss of signal for the pro-form. Both Rg1 and SMT markedly affected $A\beta_{25-35}$-induced caspase activation (Fig. 4E and F), indicating that Rg1 blocked $A\beta_{25-35}$-induced mitochondrion-mediated apoptosis through suppression of NO generation.

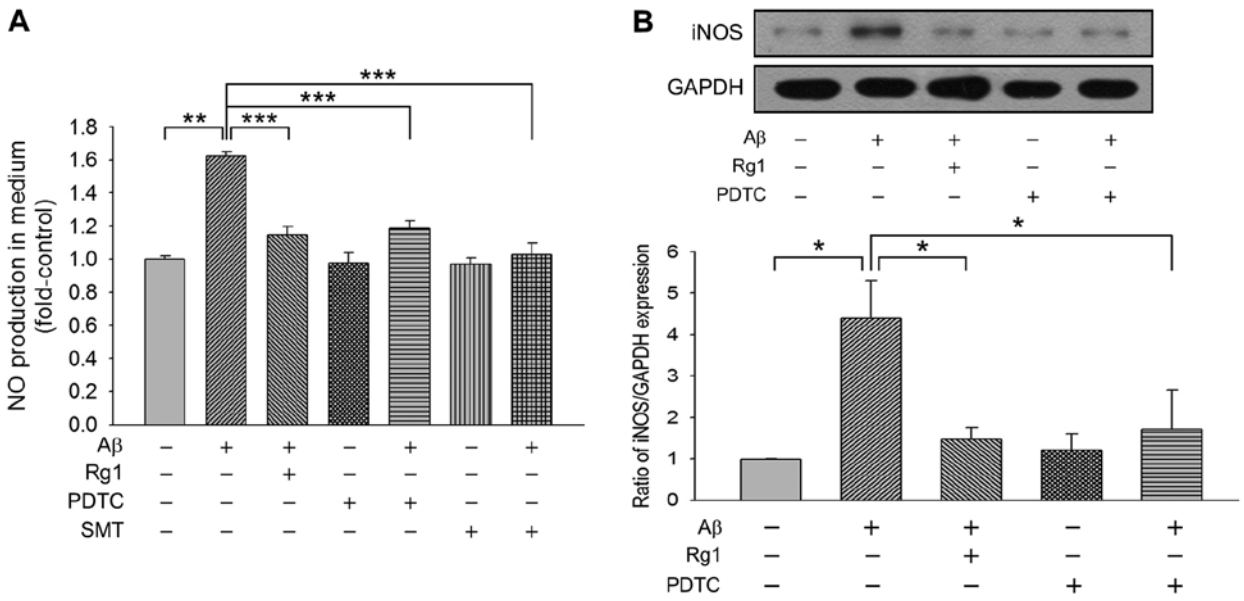

Figure 3. Ginsenoside Rg1 (Rg1) represses β-amyloid (Aβ)-induced nitric oxide (NO) production in a nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB)-dependent manner. Primary cultured neurons were pretreated with Rg1 (20 μM), ammonium pyrrolidine dithiocarbamate (PDTC) 1 μM or s-methylisothiourea hemisulfate salt (SMT) 100 μM for 24 h, followed by exposure with Aβ (10 μM) for another 24 h. (A) NO production was detected by Griess reagent. PDTC is an inhibitor for NF-κB and SMT is an inhibitor for inducible nitric oxide synthase (iNOS). (B) Expression of iNOS was determined by western blot analysis. In the absence of Rg1, $A\beta_{25-35}$ treatment caused an increase in the expression of iNOS and the generation of NO. Preincubation with Rg1 attenuated these effects, as well as PDTC. Data are represented as the means ± standard deviation of three independent experiments. The statistic significance was defined using the Student's t-test as $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$.

Discussion

Previous studies have suggested that Rg1 exerts neuroprotective properties *in vitro* and *in vivo* (7,24-26), but the underlying mechanisms are yet been fully understood. In the present study, we indicated that Rg1 protects primary cultured rat cortical neuronal cells from $A\beta_{25-35}$-ignited apoptosis via the NF-κB/NO signaling pathway.

NF-κB, a typical redox-sensitive factor, plays a central role in the regulation of transcription, inflammation, oxidative stress, and apoptosis (27). Much evidence has shown that inflammation is relevant to AD. The deposition of Aβ has been revealed to activate neuroinflammatory responses by triggering the expression of inflammatory cytokines, chemokines and mediators through the NF-κB and mitogen-activated protein kinase (MAPK) signaling pathways (28). Moreover, it has previously been reported that the level of NF-κB (p65) is markedly elevated in the brains of patients with AD, and NF-κB (p65) activation further leads to upregulated β-secretase cleavage and Aβ deposition (29), suggesting that NF-κB plays a pivotal role in AD pathological processes. In the present study, we found that both Aβ and H_2O_2 triggered the activation of NF-κB (Fig. 2). Furthermore, Rg1 was also proven to exert neuroprotective effects (Fig. 1). Thus, it is reasonable to suggest that Rg1 reduced Aβ-induced NF-κB activation by upregulating intracellular antioxidation.

NO, an important cell signaling molecule, is generated from the amino acid L-arginine by NOSs, and it has been suggested that iNOS is the main source of pathological NO generation (30,31). However, it has also been demonstrated that Aβ exposure increases the level of iNOS and the production of NO (32), but the signaling cascades involved in Aβ stimulation and NO generation remain ambiguous. Certain studies have documented close interaction between NO and NF-κB, but the modes of action between the two in different cell types were quite different. It has been reported that NO is the pivotal controller of the signaling pathways, facilitating IL-1 to NF-κB activation in chondrocytes (33). On the other hand, the level of iNOS is dependent on the activation of NF-κB in microglial cells (34). Futhermore, it has also been reported that iNOS gene expression directly blocked the phosphorylation and the subsequent degradation of IκB-α, and NO inhibits cytokine-induced NF-κB activation in rat vascular smooth muscle cells (35). In the present study, we demonstrated that Rg1 and the NF-κB inhibitor PDTC considerably reduced iNOS expression and NO generation, which were stimulated by $A\beta_{25-35}$ (Fig. 3), suggesting that the decline of NO synthesis through inactivation of NF-κB is one of the most important mechanisms of Rg1 in the defense of primary neurons against $A\beta_{25-35}$.

Mitochondria play a major role in apoptosis triggered by many stimuli, which is relevant to various neurodegenerative disorders, such as AD, Parkinson's disease (PD) and Huntington's disease (HD) (36). Neuronal exposure to Aβ impairs mitochondrial dynamics and function; loss of mitochondrial membrane potential ($\Delta\Psi m$) in primary neurons was observed after incubation with $A\beta_{25-35}$ (7). This was a hallmark of mitochondrial dysfunction and may subsequently stimulate mitochondrial apoptotic signaling (37). The Bcl-2 family is made up of pro- and anti-apoptotic members. As an antiapoptotic protein, Bcl-2 has the ability to suppress the release of cytochrome *c* from mitochondria, to hinder apoptosis, whereas Bax is a proapoptotic protein and acts as a promoter of apoptosis. As a result, the proportion of Bcl-2/Bax serves as a kind of an on-off switch, regulating cytochrome *c* release from mitochondria and the downstream mitochon-

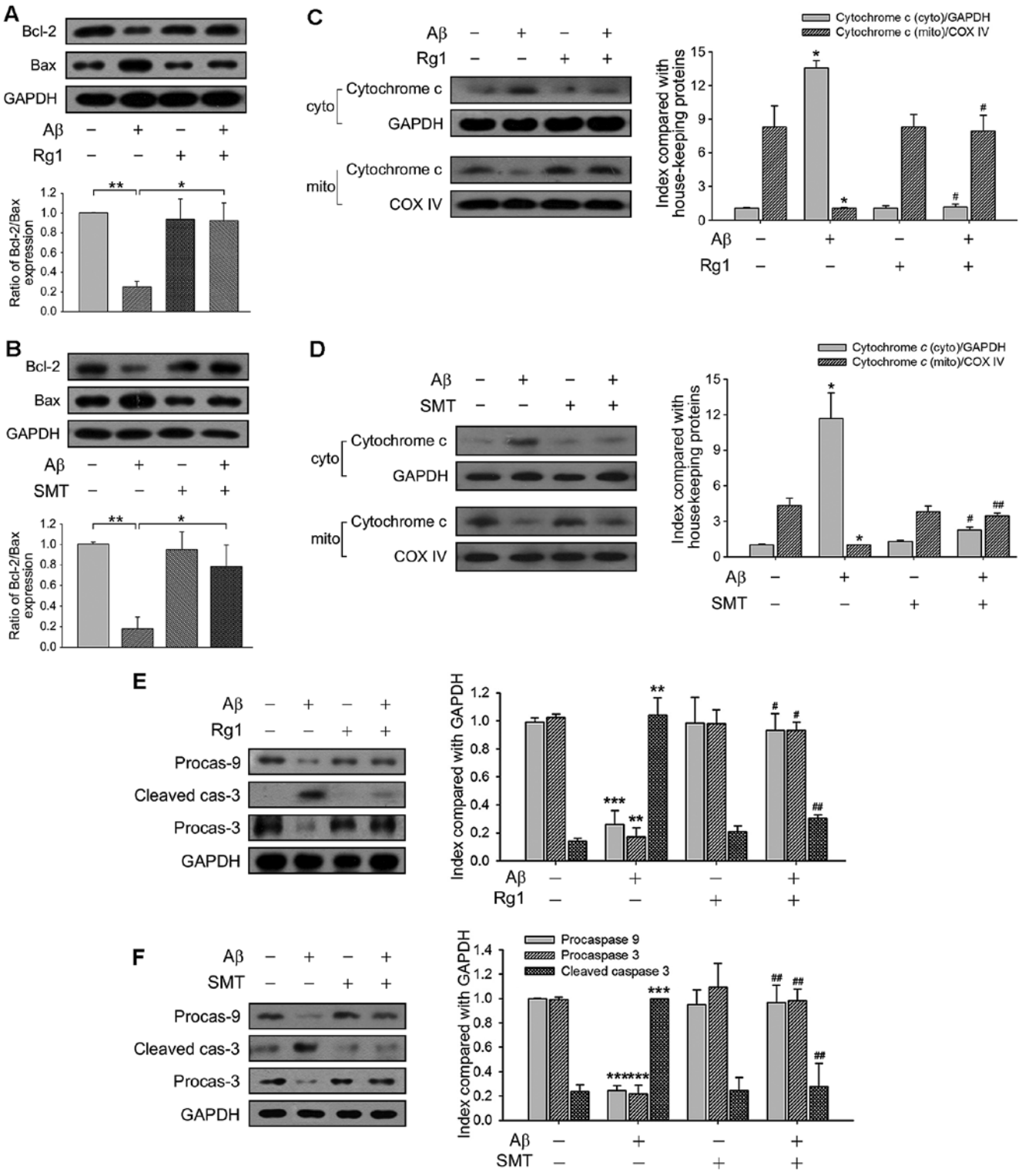

Figure 4. Changes in mitochondrion-dependent proapoptotic protein expression in primary neuronal cells after β-amyloid peptide 25-35 (Aβ25-35) exposure. Neuron cultures were preincubated with ginsenoside Rg1 (Rg1) or s-methylisothiourea hemisulfate salt (SMT) for 24 h, followed by exposure to $A\beta_{25-35}$ 10 μM. (A and B) Impact of Rg1 or SMT on the expression of Bcl-2 family proteins in $A\beta_{25-35}$ injury model. Results are shown as the proportion of Bcl-2/Bax. Protein loading was normalized to GAPDH. (C and D) The cytosolic release of cytochrome *c* in primary neurons after $A\beta_{25-35}$ insult. To examine cytochrome *c* in the cytosolic fraction, primary neurons were treated as shown above. Cytosolic constituents were extracted and resolved by SDS-PAGE. Results were normalized to housekeeping proteins (GAPDH or COX IV). (E and F) The expression of mitochondrial-mediated caspase cascades in primary cortical neurons after $A\beta_{25-35}$ exposure. Data are represented as the means ± standard deviation of three independent experiments. The statistic significance was defined with Student's t-test as: *P<0.05, **P<0.01, ***P<0.001 vs. control, $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. model. Procas, procaspase; cas, caspase.

drial apoptotic pathway (38). The findings of our present study demonstrated that Rg1 increased the ratio of Bcl-2/Bax and decreased the following release of cytochrome *c* from the mitochondrion in primary cultured neurons after $A\beta_{25-35}$ exposure, while the effects were imitated by iNOS inhibitor SMT (Fig. 4). Thus, the reduction of NO generation likely

contributed to the antiapoptotic activities of Rg1 in primary cultured neuronal cells after $A\beta_{25-35}$ exposure.

In conclusion, our study demonstrated that Rg1 exerts neuroprotective effects on primary cultured rat cortical neuronal cells against $A\beta_{25-35}$ injury by interfering with mitochondrial apoptotic pathways via downregulation of the NF-κB/NO signaling pathway.

Acknowledgements

The present study was supported by the National Natural Sciences Foundation of China (no. 81402907), the Zhejiang Provincial Natural Science Foundation of China (no. LQ14H310002) and the Zhejiang Medical Technology Program (no. 201474924).

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