

Astragaloside IV attenuates the H₂O₂-induced apoptosis of neuronal cells by inhibiting α -synuclein expression via the p38 MAPK pathway

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Abstract. An oxidative stress insult is one of the principal causes of Parkinson's disease. Astragaloside IV (AS-IV), a constituent extracted from *Astragalus membranaceus*, has been demonstrated to exert antioxidant effects. However, the mechanisms responsible for the antioxidant properties and neuroprotective effects of AS-IV remain unclear. In this study, we examined the protective effects of AS-IV against the apoptosis of human neuronal cells (SH-SY5Y cells) induced by hydrogen peroxide (H₂O₂). The results revealed that AS-IV pre-treatment attenuated the H₂O₂-induced loss of SH-SY5Y cells in a dose-dependent manner; AS-IV exerted significant protective effects by decreasing the apoptotic ratio and attenuating reactive oxygen species overproduction in H₂O₂-exposed SH-SY5Y cells. By means of immunofluorescence staining, AS-IV was found to decrease the expression of α -synuclein and to increase the expression of tyrosine hydroxylase (TH) in the cells, which had been increased and decreased, respectively by H₂O₂. As shown by western blot analysis, the protective effects of AS-IV against SH-SY5Y cell injury induced by H₂O₂ were also mediated via the downregulation of the ratio of Bax/Bcl-2. We found that the neuroprotective effects of AS-IV were associated with the inhibition of the expression of the α -synuclein via the p38 mitogen-activated protein kinase (MAPK) signalling pathway. On the whole, our results suggest that AS-IV exerts protective effects against neurodegenerative diseases by targeting α -synuclein or TH.

Introduction

Parkinson's disease (PD) is an age-related neurodegenerative disorder that is characterised by hypokinesia (1). An oxidative stress insult is one of the principal causes that is associated with the profound loss of dopamine (DA)-producing neurons in the substantia nigra pars compacta (2,3). Even though the mecha-

nisms underlying neuronal damage remain unclear, apoptosis or oxidative stress, as crucial contributors to the pathogenesis of PD, have been reported (4-8). Oxidative stress-induced cell lesions are usually induced by hydrogen peroxide (H₂O₂), hydroxyl radicals and superoxide, which are known as reactive oxygen species (ROS) and are generated through metabolic processes in cells. The oxidative stress *in vitro* model has been proposed by using H₂O₂ as an inducer when it is added to the cell culture medium (9).

The Bcl-2 family, as regulatory proteins, are vital apoptosis-related factors in H₂O₂-induced oxidative stress *in vitro* models. Among this family, Bcl-2 is an anti-apoptotic protein and forms a heterodimer with Bax, an apoptotic activator, to control the fate of cells (10,11). The ratio of Bax to Bcl-2 protein is a common indicator with which to determine whether a cell is alive or is dead via apoptosis. In addition, the extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun kinases (JNKs) and p38 kinases have been reported to participate in the regulation of apoptosis. ERK1/2, p38 and JNK are activated when a cell is injured by oxidative stress or a pro-inflammatory environment (12,13). Abnormal levels of phosphorylated JNK, p38 and ERK1/2 in the brains of patients with Alzheimer's disease (AD) are associated with oxidative stress (14). Consequently, the illumination of the biochemical processes surrounding H₂O₂-mediated neuronal apoptosis may aid in the understanding of the pathogenesis of neurodegenerative diseases and in discovering new drug targets for the treatment of diseases.

α -synuclein, a protein highly expressed in the human brain, is localised in the inner membrane of the mitochondria. α -synuclein not only dose-dependently inhibits complex I activity of the mitochondrial respiratory chain, but also aggregates to form insoluble fibrils in PD characterised by Lewy bodies (15). Tyrosine hydroxylase (TH) catalyses the synthesis of catecholamines in the rate-limiting step. Alterations in TH activity may be involved in PD. TH may help to produce H₂O₂ and other ROS in pathological conditions. Nevertheless, TH is also a possible target for the damaging alterations induced by ROS or may be a target for radical-mediated injury (16). It has been proposed that the abnormal expression of TH induced by oxidative damage leads to a reduction in DA levels, which is associated with the degeneration of dopaminergic neurons in PD (17). Therefore, α -synuclein and TH may be novel drug targets.

In recent years, natural substances extracted from plants have attracted increasing attention due to their unique biolo-

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gical activities, such as neuroprotective potential that can protect cells from oxidative damage. A number of Chinese herbal effects have been evaluated and have been shown to exert beneficial effects in various models related to PD (18,19), suggesting that herbs, as drug candidates, have a bright future in the treatment of PD. Astragaloside IV (AS-IV), an ingredient extracted from *Astragalus membranaceus*, is frequently used as a food additive and in herbal medicine. AS-IV is included in some efficacious medicinal prescriptions and as a supplement in various health foods (20). AS-IV has been shown to exert anti-hypertensive (21), positive inotropic (22), anti-inflammatory (23) and anti-infarction effects (24). Despite the use of *Astragalus membranaceus* as a traditional therapy for degenerative diseases in China, few scientific studies investigating the antioxidant mechanism of AS-IV in neurons have been reported to date, at least to the best of our knowledge. Moreover, further research is required in order to fully examine the effects of the antioxidant activity of AS-IV.

Thus, the aims of the present study were to evaluate the neuroprotective effects of AS-IV *in vitro* using SH-SY5Y cells exposed to H_2O_2 and to discover novel targets of AS-IV. Our findings demonstrate that AS-IV protects the cells from oxidative damage by downregulating the Bax/Bcl-2 ratio. The effects of AS-IV were also mediated via the downregulation of the expression of α -synuclein and the increase in TH expression via the p38 signalling pathway. To the very best of our knowledge, this is a fundamental new discovery of the mechanisms through which AS-IV protects neuronal cells from damage.

Materials and methods

Chemicals and reagents. AS-IV (Fig. 1), with a purity >98%, was obtained from the Nanjing Zelang Medical Technology Co., Ltd. (Nanjing, China). Bovine serum albumin (BSA), 3-(4,5-dimethylthiazol-2-yl)-2,5-dephenyl-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), 2,7'-dichlorofluorescein diacetate (DCFH-DA), vitamin C (Vit C) and H_2O_2 were all purchased from Sigma-Aldrich (St. Louis, MO, USA). The Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit was obtained from KeyGen Biotech Co., Ltd. (Nanjing, China). Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium/F12 (DMEM/F12) were both purchased from Gibco (Grand Island, NY, USA). Anti- α -synuclein antibody (#ab138501) was purchased from Epitomics (Burlingame, CA, USA). Anti- β -actin (#3700), anti-Bcl-2 (#15071) and anti-Bax (#5023) monoclonal antibodies were all purchased from Cell Signalling Technology, Inc. (Beverly, MA, USA). Anti-p38 mitogen-activated protein kinase (MAPK; sc-7972), anti-p-p38 (sc-17852-R), anti-p-JNK (sc-293136), anti-ERK1/2 (sc-514302), anti-p-ERK1/2 (sc-16981-R) and anti-TH (sc-7847) monoclonal antibodies were all purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). All of the other reagents were of analytical grade.

Cell culture. The SH-SY5Y cell line (#CRL-2266, ATCC, Rockville, MD, USA) was cultured in DMEM supplemented with penicillin (final concentration, 100 U/ml), streptomycin (final concentration, 0.1 mg/ml) and 10% (v/v) FBS in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. The SH-SY5Y cells were plated in 6-well plates for assays and

96-well plates (for MTT assay) at a density of approximately 1×10^4 cells/well. After 24 h, the cells were treated with various concentrations of AS-IV (50–200 μ mol/l containing 0.1% DMSO) for 24 h and were then exposed to the same fresh medium containing 300 μ mol/l H_2O_2 for 4 h.

Determination of cell viability. By means of MTT assay, we first assessed the drug treatment toxicity. The cells (8,000 cells/well) were plated in 96-well microplates and grown in DMEM for 24 h and exposed to various concentrations of H_2O_2 (100, 200, 300 and 400 μ mol/l).

The effects of various concentrations of AS-IV on cell viability were analysed following treatment for 24 h. The medium was then replaced by 200 μ l of fresh medium plus 10 μ l of the MTT solution (1 mg/ml). The microplates were incubated at 37°C in 5% CO_2 for 4 h. The precipitated formazan was dissolved in 100 μ l DMSO. The optical density of the samples was measured at 492 nm. The untreated SH-SY5Y cells were used as controls. For pre-treatment with vitamin C (Vit C), the SH-SY5Y cells were treated with 200 mg/l Vit C (A8100; Solarbio, Beijing, China) for 1 h and then exposed to H_2O_2 for 4 h.

Cell morphological observation. Following exposure to 300 μ mol/l H_2O_2 , the SH-SY5Y cells were observed under a phase-contrast microscope (TE2000-U; Nikon, Tokyo, Japan). Moreover, the SH-SY5Y cells were observed under a microscope following exposure to H_2O_2 and DAPI staining.

Detection of apoptotic cells by flow cytometry. The cell apoptotic rate following exposure to H_2O_2 was assayed by flow cytometry using the Annexin V-FITC/propidium iodide (PI) double-labelling method. The SH-SY5Y cells (1×10^5 cells/ml) were seeded in 60-mm dishes and treated with AS-IV and H_2O_2 (300 μ mol/l). The cells were trypsinised and collected by centrifugation at 800 rpm for 5 min. An Annexin V-FITC apoptosis detection kit was used to double-stain the cells according to the manufacturer's instructions. The samples were analysed using a FACSVantage SE flow cytometer (BD Biosciences, San Jose, CA, USA).

Measurement of intracellular ROS levels. The SH-SY5Y cells (4×10^4 cells/ml) were seeded in 6-well plates for 48 h. The cells were treated with AS-IV for 24 h prior to exposure to H_2O_2 (300 μ mol/l) for 4 h. Following stimulation, the cells were incubated with 10 μ mol/l DCFH-DA, which was oxidised to the highly fluorescent compound, DCF, at 37°C for 30 min and, the cells were then washed 3 times with phosphate-buffered saline (PBS). The fluorescence intensity, expressed as the intracellular ROS, was measured with a FACSVantage SE flow cytometer.

Immunofluorescence staining. The immunofluorescence staining technique was performed as follows: briefly, the cells, on coverslips, were fixed with a 4% paraformaldehyde solution for 20 min and were permeabilised with 0.5% Triton X-100 in PBS for 15 min, and the background was blocked with 5% BSA in PBS for 1 h before staining with primary and secondary antibodies. Primary antibodies to α -synuclein (1:100; #ab138501; rabbit anti- α -synuclein monoclonal antibody; Epitomics) and TH (1:100; sc-7847; goat anti-TH monoclonal antibody; Santa Cruz Biotechnology, Inc.)

were diluted by 0.5% Triton X-100 and incubated with the cells for 90 min at 37°C. A secondary FITC-conjugated goat-anti-rabbit IgG (1:100; sc-2012; Santa Cruz Biotechnology, Inc.) and a TRITC-conjugated mouse-anti-goat IgG (sc-516243) were diluted in 0.5% Triton X-100 and were incubated with the cells for 60 min at room temperature. The cells were then stained with DAPI for 3 min. The slides were rinsed with PBS after each step. The slides were mounted with glycerol and stored for detection.

Western blot analysis. The SH-SY5Y cells (2×10^6 cells/well) were seeded and treated with 10 $\mu\text{mol/l}$ SB203580 (S8307; Sigma-Aldrich) for 1 h or various concentrations of AS-IV for 24 h prior to exposure to H_2O_2 (300 $\mu\text{mol/l}$) for 4 h. The treated cells were collected and were resuspended in a lysis buffer (RIPA buffer with protease inhibitor cocktail) at 4°C for 2 h. The lysate was centrifuged at 12,000 rpm for 15 min at 4°C, and the supernatant was then collected. The Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and a UV spectrophotometer were used to equalize the protein loading. Equal amounts of protein (40 μg) were subjected to 12% SDS-PAGE and were transferred onto polyvinylidene difluoride membranes. Blocking buffer [5% (v/v) non-fat dry-milk in TBS containing 0.1% Tween-20 (TBST), pH 7.5] was used to treat the membranes for 1 h at room temperature. The membranes were then incubated with primary antibodies to Bcl-2 (1:1,000), Bax (1:1,000), α -synuclein (1:1,000), TH (1:1,000), p38 (1:1,000), p-p38 (1:1,000), ERK1/2 (1:1,000), p-ERK1/2 (1:1,000), p-JNK (1:1,000) and β -actin (1:1,000) overnight at 4°C and were then incubated with HRP-conjugated rabbit or mouse IgG secondary antibodies (1:1,000) for 1 h. The secondary antibodies HRP-conjugated goat-anti-rabbit IgG (sc-2004), HRP-conjugated goat-anti-mouse IgG (sc-2005) and HRP-conjugated mouse anti-goat IgG (sc-2354) were obtained from Santa Cruz Biotechnology, Inc. The proteins were detected by an enhanced chemiluminescence (ECL) solution (Amersham Biosciences, Buckinghamshire, UK), and densitometric analysis was performed with the use of a PDI ImageWare System (Bio-Rad Laboratories, Inc.).

Statistical analysis. The results were analysed using a one-way analysis of variance (one-way ANOVA) followed by the Tukey's test to examine the effects of the different drug concentrations. The data are expressed as the means \pm SEM. A Dunnett's test and an ANOVA followed by a Bonferroni correction were performed to determine the statistical significance. A P-value < 0.05 was considered to indicate a statistically significant difference.

Results

Protective effects of AS-IV against H_2O_2 -induced SH-SY5Y cell damage. We initially examined the viability of the SH-SY5Y cells, which was examined by MTT assay, after incubating the cells for 2, 4 and 6 h with H_2O_2 in the absence or presence of AS-IV for 24 h. As shown in Fig. 2A, exposure to H_2O_2 induced a gradual reduction in cell viability in a time- and dose-dependent manner. The cell viability was approximately 57% of the control value at 4 h following H_2O_2 exposure (300 $\mu\text{mol/l}$). Therefore, for all the subsequent

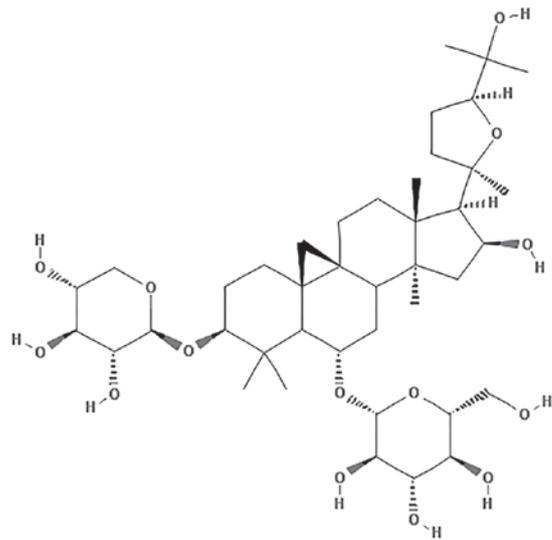


Figure 1. Chemical structure of astragaloside IV (AS-IV). Molecular formula, $\text{C}_{41}\text{H}_{68}\text{O}_{14}$; molecular weight, 784.9702 g/mol.

experiments, a concentration of H_2O_2 at 300 $\mu\text{mol/l}$ was used for the cell damage model.

As shown in Fig. 2B, cell viability was not affected by AS-IV at a concentration of up to 200 $\mu\text{mol/l}$. After the SH-SY5Y cells were treated with AS-IV (50–200 $\mu\text{mol/l}$) for 24 h and then exposed to H_2O_2 (300 $\mu\text{mol/l}$) for 4 h, cell viability was increased with the increasing concentrations of AS-IV compared with the control group. As shown in Fig. 2C, cell viability following exposure to H_2O_2 for only 4 h was 59% of the control value. However, treatment with AS-IV (50, 100 and 200 $\mu\text{mol/l}$) for 24 h prior to exposure to H_2O_2 increased cell viability to 70, 71 and 75%, respectively, which was similar to the activity of Vit C, a drug that was used as a positive control. Evidently, AS-IV was effective in protecting the SH-SY5Y cells against H_2O_2 -induced injury.

We also assessed the cell morphological changes using a phase-contrast microscope. Exposure to 300 $\mu\text{mol/l}$ H_2O_2 for 4 h clearly induced the aggregation and shrinkage of cell bodies and reduced the number of SH-SY5Y cells. However, treatment with AS-IV (200 $\mu\text{mol/l}$) prior to H_2O_2 exposure significantly prevented the morphological manifestations of cell damage (Fig. 2D, upper panel). DAPI staining also revealed that nuclear fragmentation and nuclear DNA condensation occurred following exposure to 300 $\mu\text{mol/l}$ H_2O_2 ; however, pre-treatment with AS-IV inhibited these apoptotic features, exerting effects similar to those of Vit C at 200 mg/l (Fig. 2D, bottom panel). These results suggested that AS-IV exerted an anti-apoptotic effect in SH-SY5Y cells exposed to H_2O_2 .

AS-IV inhibits the H_2O_2 -induced apoptosis of SH-SY5Y cells.

The inhibitory effects of AS-IV on the apoptosis of SH-SY5Y cells induced by H_2O_2 were assessed by flow cytometry. As shown in Fig. 3A, the percentage of apoptotic cells increased to 33.2% following exposure to 300 $\mu\text{mol/l}$ H_2O_2 for 4 h. However, treatment with AS-IV (50, 100 and 200 mg/l) for 24 h prior to H_2O_2 exposure prevented apoptosis in a concentration-dependent manner, and the rate of apoptosis

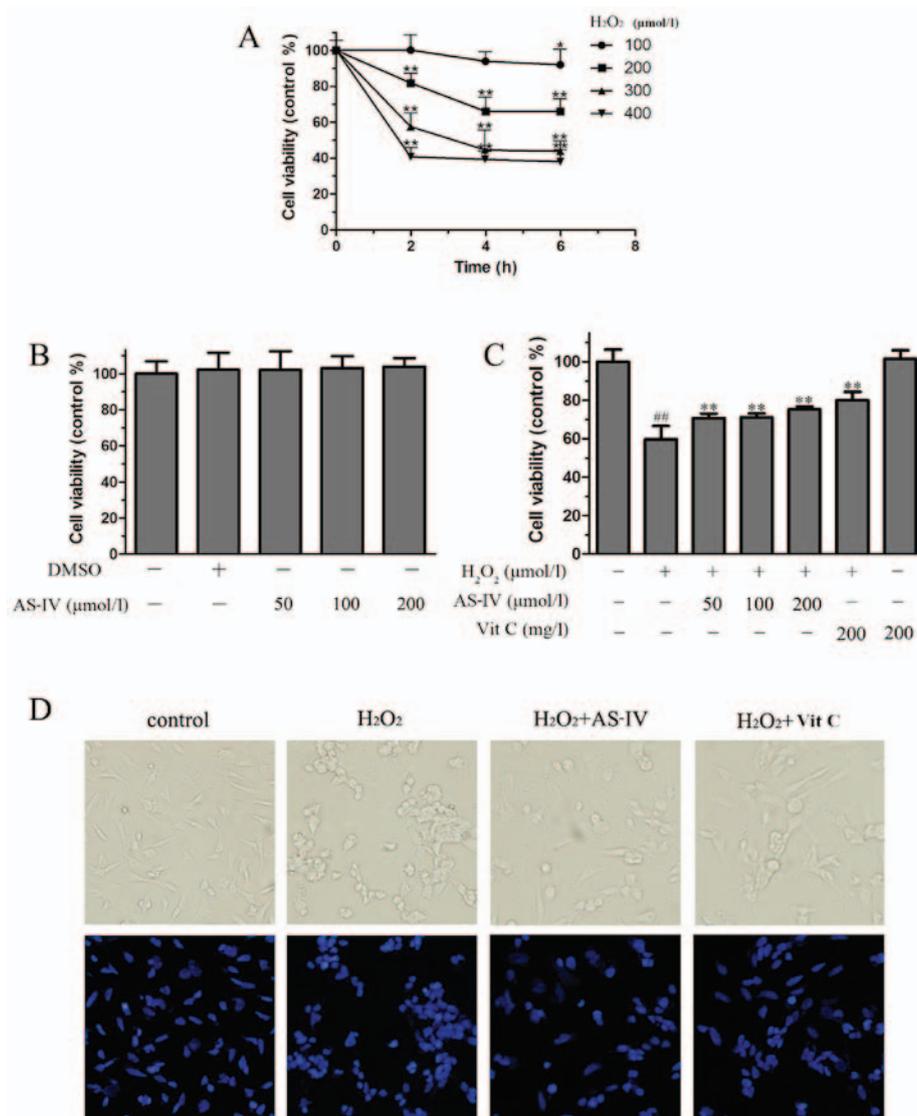


Figure 2. Effect of astragaloside IV (AS-IV) on the viability of cells exposed to hydrogen peroxide (H_2O_2) in a time- and concentration-dependent manner. The viability of the cells was determined by MTT assay after incubating the cells for 2, 4 and 6 h with H_2O_2 for 24 h. (A) Cell viability at 4 h after H_2O_2 exposure (300 $\mu\text{mol/l}$). (B) Cell viability group in the absence or (C) presence of H_2O_2 exposure. (D) DAPI staining showing nuclear fragmentation and nuclear DNA condensation following exposure to 300 $\mu\text{mol/l}$ H_2O_2 , AS-IV and vitamin C (Vit C) at 200 mg/l. Medium with 0.1% DMSO was used as a standard to normalise the results. The data are the means \pm SEM (n=3). ##P<0.01 vs. control (untreated cells); **P<0.05 vs. H_2O_2 (300 $\mu\text{mol/l}$).

decreased to 28.9, 22.6 and 14.8%, respectively. AS-IV at 200 mg/l exerted similar effects to those of Vit C. Moreover, incubation with AS-IV alone for 24 h had no effects on the cell apoptotic ratio of the SH-SY5Y cells (data not shown).

AS-IV inhibits ROS production in SH-SY5Y cells induced by H_2O_2 . Intracellular ROS plays a crucial role in oxidative stress-induced cell damage; thus, the effects of AS-IV on H_2O_2 -induced ROS production in SH-SY5Y cells were assessed by flow cytometry. As shown in Fig. 3B, the cells with fluorescence induced by 300 $\mu\text{mol/l}$ H_2O_2 for 4 h displayed a 6.5-fold increase greater than that of the control group (P<0.05). By contrast, treatment with AS-IV for 24 h prior to exposure to H_2O_2 suppressed the production of DCFH fluorescence (for ROS production) in a concentration-dependent manner. The inhibitory effects of AS-IV at 200 $\mu\text{mol/l}$ were significant (53.8% compared to the H_2O_2 -exposed group not treated with AS-IV (P<0.05) and were similar to the effects of Vit C at 200 mg/l.

AS-IV decreases the H_2O_2 -induced increase in the Bax/Bcl-2 ratio in SH-SY5Y cells. The levels of Bax and Bcl-2 were determined by western blot analysis as Bcl-2 family members are involved in apoptosis. As shown in Fig. 4A, compared with the control, Bax protein expression was upregulated in the H_2O_2 -exposed group, while AS-IV treatment downregulated Bax expression. The levels of Bcl-2 decreased in the H_2O_2 group and increased following AS-IV pre-treatment. The Bax/Bcl-2 ratio was increased 4.2-fold of the normal control group upon exposure to H_2O_2 , whereas this increase was attenuated in the cells pre-treated with 50–200 $\mu\text{mol/l}$ AS-IV.

AS-IV decreases the expression of α -synuclein and increases the expression of TH in SH-SY5Y cells. α -synuclein is a major component of Lewy bodies that plays an important role in H_2O_2 -induced apoptosis in neurons (25). Thus, to determine whether the observed neuroprotective effects of AS-IV were associated with the expression of α -synuclein, we performed

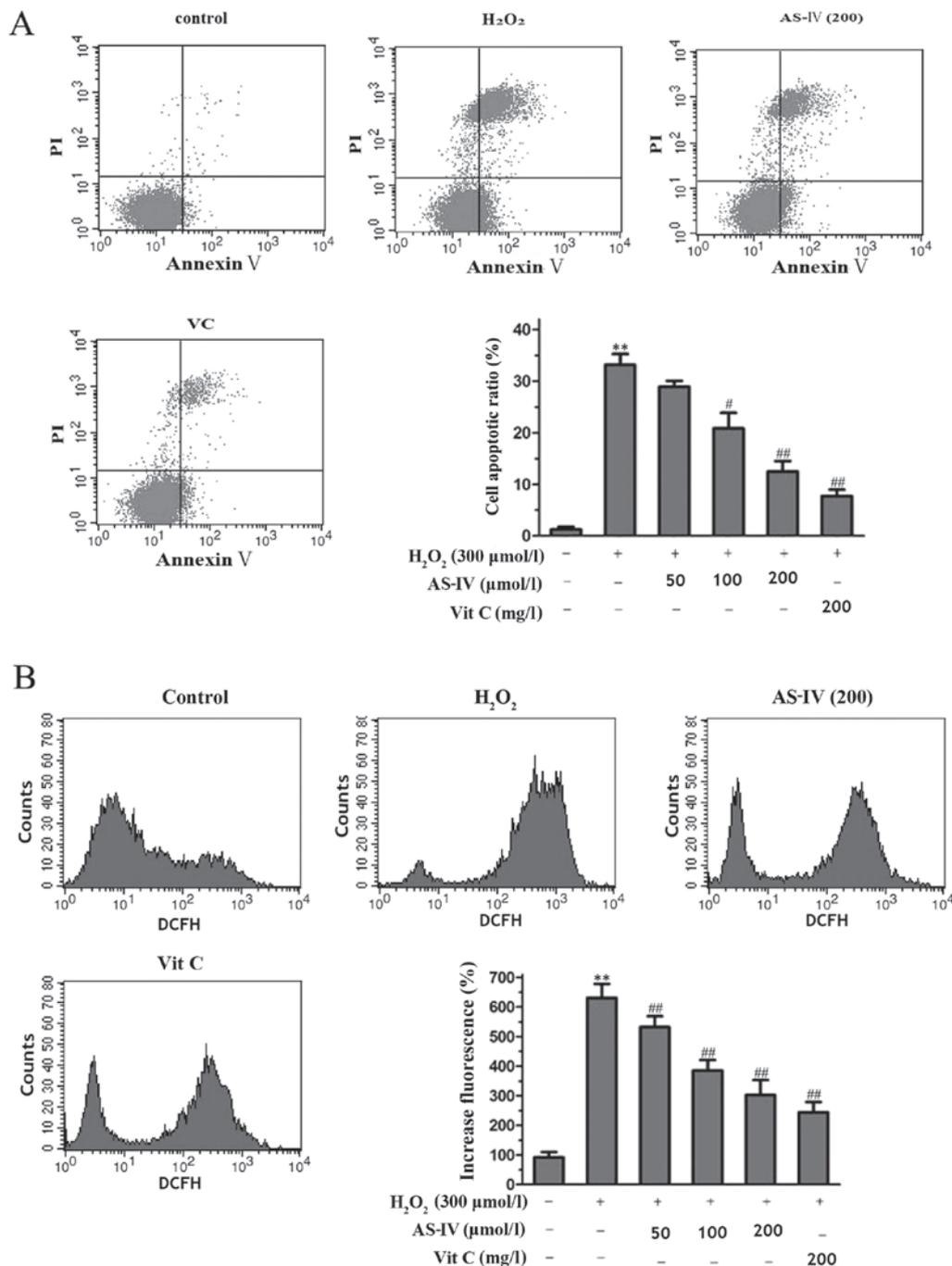


Figure 3. Astragaloside IV (AS-IV) inhibits hydrogen peroxide (H₂O₂)-induced apoptosis and attenuates reactive oxygen species (ROS) production stimulated by H₂O₂ (300 μmol/l). (A) The results of flow cytometry show that pre-treatment with AS-IV (50-200 mg/l) for 24 h prior to H₂O₂ exposure decreased apoptosis and (B) suppressed the production of DCFH fluorescence (for ROS production) in concentration-dependent manner. The medium was used as a standard to normalise the results. The data are shown as the means ± SEM (n=3). **P<0.01 vs. control (untreated cells); #P<0.05 vs. H₂O₂ (300 μmol/l); ##P<0.01 vs. H₂O₂ (300 μmol/l).

western blot analysis using a monoclonal rabbit antibody against α-synuclein. As shown in Fig. 4B, when the cells were exposed to H₂O₂, the expression of α-synuclein significantly increased 2.25-fold compared with the control group. By contrast, pre-treatment with AS-IV (50, 100 and 200 μmol/l) markedly decreased the expression of α-synuclein by 22.2, 27.1 and 42.2%, respectively, compared with the H₂O₂-exposed cells. To further confirm this effect, we performed a double immunofluorescence assay using a monoclonal rabbit antibody against α-synuclein. As shown in Fig. 4C, the results were consistent with those of western blot analysis. These

results suggest that AS-IV inhibited the overexpression of α-synuclein induced by H₂O₂. Consequently, we hypothesized that the neuroprotective effects of AS-IV against oxidative stress-induced damage are mediated by the decrease in the expression of α-synuclein.

Previous studies have shown that α-synuclein, implicated in the pathogenesis of PD, is involved in the regulation of DA metabolism, possibly by downregulating the expression of TH (26). TH catalyses the rate-limiting step in the biosynthesis of the catecholamines, DA, norepinephrine and epinephrine (27). Therefore, TH expression was also evaluated by double

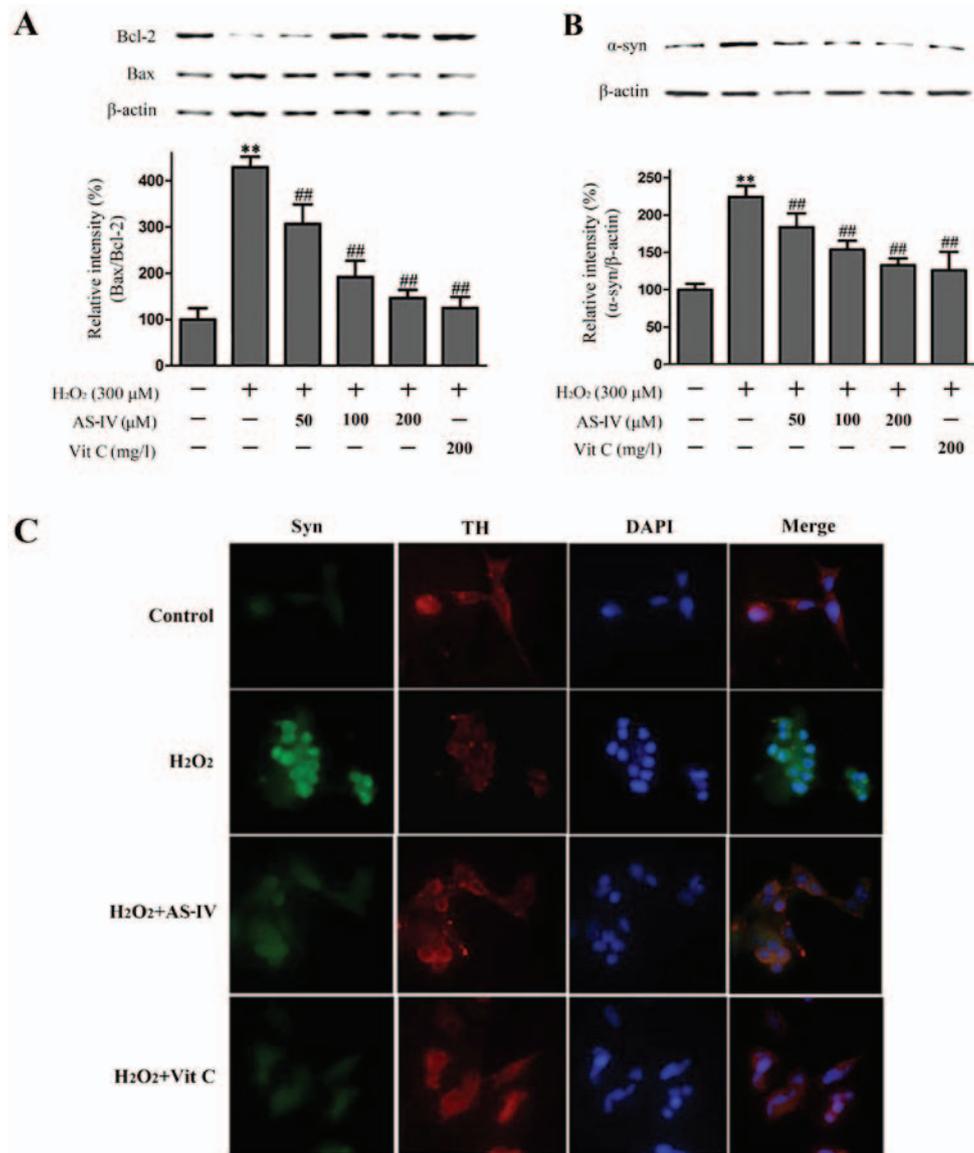


Figure 4. Astragaloside IV (AS-IV) decreases the hydrogen peroxide (H₂O₂)-induced increase in the Bax/Bcl-2 ratio, decreases the expression of α -synuclein (α -syn) and increases the expression of tyrosine hydroxylase (TH) in SH-SY5Y cells. The expression of Bax and Bcl-2 was determined by western blot analysis. (A) Bax/Bcl-2 ratio following exposure to H₂O₂ and pre-treatment with 50–200 μ mol/l of AS-IV. (B) Western blot analysis and (C) a double immunofluorescence assay showing the expression of α -syn and TH in SH-SY5Y cells exposed to H₂O₂. The data are the means \pm SEM (n=3). **P<0.01 vs. control (untreated cells); ##P<0.05 vs. H₂O₂ (300 μ mol/l).

immunofluorescence assay. As shown in Fig. 4C, pre-treatment with AS-IV prevented the H₂O₂-induced downregulation of TH levels in the SH-SY5Y cells. The results indicated that AS-IV treatment inhibited H₂O₂-induced α -synuclein upregulation.

AS-IV attenuates the H₂O₂-induced phosphorylation of p38 in SH-SY5Y cells. To clarify the mechanisms underlying the neuroprotective effects of AS-IV, we evaluated the expression of p-p38, p-JNK and p-ERK to determine whether the protective effects of AS-IV are mediated via the MAPK signalling pathway. Our results revealed that the levels of phosphorylated p38 increased (Fig. 5A); however, no effect was observed on the levels of phosphorylated ERK1/2 (Fig. 5B) and phosphorylated JNK (Fig. 5C) in the H₂O₂-exposed cells. By contrast, AS-IV (200 μ mol/l) pre-treatment markedly decreased the levels of phosphorylated p38 compared with those in the untreated cells exposed to H₂O₂ (Fig. 5A).

To elucidate whether H₂O₂ stimulates α -synuclein expression via the p38 MAPK signalling pathway, a chemical inhibitor targeting p38 kinase (SB203580) was used. The SH-SY5Y cells were pre-incubated with SB203580 (10 μ mol/l) for 1 h and were then exposed to H₂O₂ (300 μ mol/l) for 4 h, and the levels of α -synuclein were determined. As shown in Fig. 6A, AS-IV (200 μ mol/l) pre-treatment markedly decreased the expression of α -synuclein 0.73-fold compared with the untreated cells exposed to H₂O₂.

Of note, when the H₂O₂-exposed SH-SY5Y cells were incubated with SB203580 alone, the expression of α -synuclein was attenuated compared with the H₂O₂ group, and incubation of the cells with SB203580, H₂O₂ and AS-IV (200 μ mol/l) together also decreased the expression of α -synuclein. We found identical results in the double immunofluorescence analysis, as shown in Fig. 6B. Thus, these results indicated that AS-IV protected the SH-SY5Y cells from oxidative

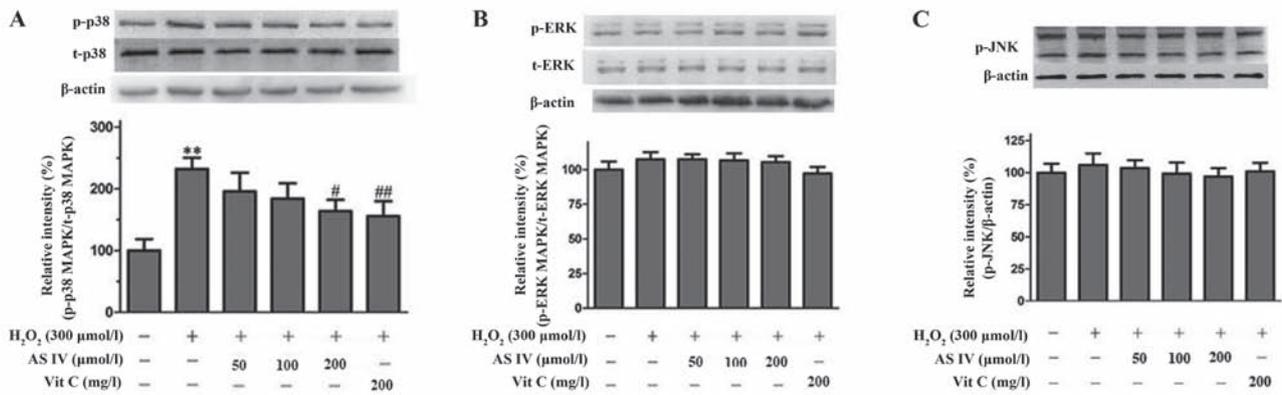


Figure 5. Astragaloside IV (AS-IV) decreases the hydrogen peroxide (H₂O₂)-induced phosphorylation of p38, but has no effect on the phosphorylation of c-Jun kinase (JNK) or extracellular signal-regulated kinase (ERK). Western blot analysis showing the levels of (A) phosphorylated p38, (B) phosphorylated ERK1/2 and (C) phosphorylated JNK in the H₂O₂-exposed groups. (A) Effects of AS-IV (200 μmol/l) pre-treatment on the levels of phosphorylated p38 compared with those of the H₂O₂ group. The data are the means ± SEM (n=3). **P<0.01 vs. control (untreated cells); #P<0.05 vs. H₂O₂ (300 μmol/l); ##P<0.01 vs. H₂O₂ (300 μmol/l).

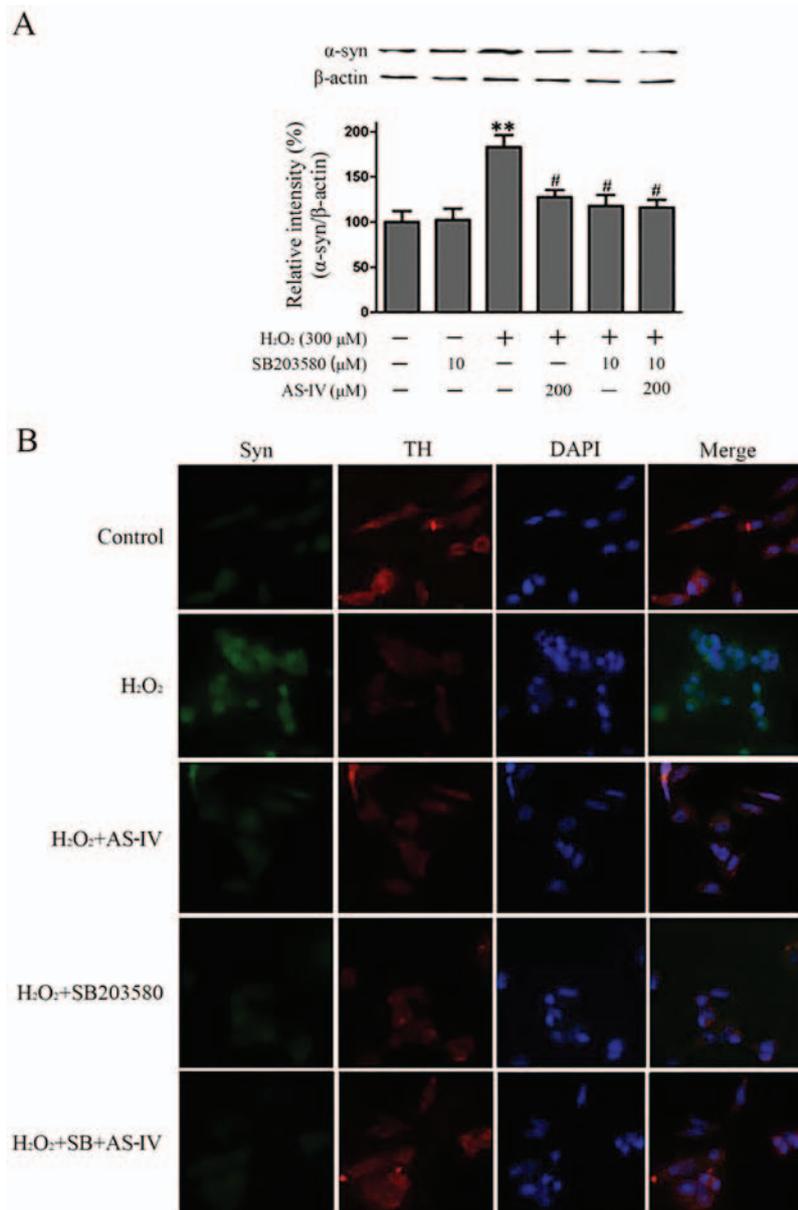


Figure 6. Chemical inhibitor targeting p38 kinase (SB203580) inhibits the expression of α-synuclein (α-syn) and increases the expression of tyrosine hydroxylase (TH) in SH-SY5Y cells. (A) Incubation with SB203580 in the SH-SY5Y cells decreased the expression of α-syn compared with the hydrogen peroxide (H₂O₂) group as shown by western blot analysis. (B) Results of the double immunofluorescence assay in SH-SY5Y cells. The data are the means ± SEM (n=3). **P<0.01 vs. control (untreated cells); #P<0.05 vs. H₂O₂ (300 μmol/l).

stress-induced damage and that these effects were mediated via the decrease in the expression of α -synuclein through the p38 MAPK signalling pathway.

Discussion

PD is a neurodegenerative disorder resulting from the gradual and progressive loss of dopaminergic neurons in the substantia nigra. Accumulating evidence suggests that a pathological mechanism for this is that oxidative stress is implicated in different neurodegenerative diseases, such as PD and AD (28-32). H_2O_2 , as an oxidative stress inducer, is widely used *in vitro* models (4,33). The human neuroblastoma cell line, SH-SY5Y, is extensively used as a cell model for researching neuronal cell death induced by H_2O_2 (34,35). In this study, H_2O_2 -induced oxidative stress in SH-SY5Y cells was used to examine H_2O_2 -induced neurotoxicity and the effects of AS-IV.

Although the exact mechanisms of oxidative stress are not completely clear, the use of antioxidant agents as a method of neuroprotection may be a potential treatment strategy for neurodegenerative diseases (36). AS-IV, as a major active constituent of *Astragalus membranaceus*, exerts multipotent effects under pathophysiological conditions. As previously demonstrated, AS-IV not only protected primary DA neurons from 6-OHDA-induced neurotoxicity and neurodegeneration, but also promoted dopaminergic neurite outgrowth (37). In our study, we demonstrated that AS-IV exerted protective effects against the H_2O_2 -induced loss of cell viability. Similarly, the results of DAPI staining suggested that AS-IV prevented H_2O_2 -induced morphological changes associated with apoptosis in the SH-SY5Y cells.

It is well known that ROS can injure the cardinal cellular components, such as proteins, DNA and lipids, resulting in subsequent cell death by necrosis or apoptosis. To provide further evidence, we examined the effects of AS-IV on cells by flow cytometry. When the SH-SY5Y cells were pre-treated with AS-IV for 24 h and were then cultured with 300 μ mol/l H_2O_2 for 4 h, the percentage of apoptotic cells decreased and the production of ROS was also reduced compared with the cells exposed to H_2O_2 alone. Therefore, the protective effects of AS-IV may contribute to the antioxidant activity. However, the balance and maintenance of the intracellular redox state is largely dependent on the existence of glutathione, particularly the ratio of glutathione (GSH) to oxidized glutathione (GSSG). An increased GSSG-to-GSH ratio is an important indicator of oxidative stress. Some studies have analysed the status of GSH in SH-SY5Y cells induced by H_2O_2 (38-40). In this study, we wished to determine the mechanisms involved in the protective effects of AS-IV against oxidative stress. We focused on α -synuclein, a very important factor, and thus the levels and the status of GSH in the SH-SY5Y cells were not analysed in our study. α -synuclein is a 14-kDa soluble, intrinsically unfolded protein that is expressed in all neurons (41,42). The overexpression of α -synuclein may be associated with the selective degeneration and toxicity of dopaminergic neurons (43). TH, as a key enzyme, plays a critical role in the differentiation and survival of dopaminergic neurons. In this study, we found that AS-IV inhibited the overexpression of α -synuclein induced by H_2O_2 and increased the levels of TH. Our results provide new evidence that AS-IV protects cells from H_2O_2 -induced injury and is associated with the expression

of α -synuclein and TH. Cell survival depends mostly on the balance between the anti- and pro-apoptotic proteins of the Bcl-2 family in the early phases of the apoptotic cascade. Any changes in the balance between the anti- and pro-apoptotic proteins will affect cell death. Bax (pro-apoptotic group) and Bcl-2 (anti-apoptotic group) play a vital role in the apoptotic pathway. The ratio of Bax/Bcl-2 is a better predictor of apoptosis than the absolute expression of either Bcl-2 or Bax alone (44). Our results revealed that the expression of these Bcl-2 family members was influenced profoundly by H_2O_2 . Treatment with AS-IV upregulated the expression of anti-apoptotic Bcl-2 and reduced the expression of pro-apoptotic Bax. Thereby, AS-IV decreased the H_2O_2 -induced increase in the Bax/Bcl-2 ratio in SH-SY5Y cells.

ROS play a key role as a second messenger in signal transduction cascades and regulate signalling pathways. The intracellular redox state may lead to the activation of MAPKs (45). H_2O_2 , as an oxidative stress inducer, may activate ERK and p38 proteins in cells, which may be closely related to MAPK signalling (46,47). In our experiments, treatment with AS-IV notably inhibited the H_2O_2 -induced increase in phosphorylated p38 levels. We further found that the protective effects of AS-IV were associated with the expression of α -synuclein and TH through the p38 MAPK signalling pathway using chemical inhibitors targeting p38 kinase (SB203580).

In conclusion, the results of this study demonstrated that AS-IV decreased H_2O_2 -induced cell damage, prevented cell morphologic changes, and decreased ROS production and the apoptotic rate. Furthermore, the neuroprotective effects of AS-IV against the H_2O_2 -induced apoptosis of SH-SY5Y cells were associated with the downregulation of the Bax/Bcl-2 ratio, decreased levels of α -synuclein and increased levels of TH via the p38 MAPK signalling pathway. Our study may provide a novel therapeutic strategy for PD, although further research into the neuroprotective mechanisms of AS-IV is warranted.

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