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Original Article

Ginsenoside Rg1 prevents MPP^+-induced apoptosis of SHSY5Y cells via the inhibition of a Bax-mediated mitochondrial pathway and by suppressing oxidative stress

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Abstract: Neuroprotective therapies to rescue dopamine neurons have been proposed for Parkinson's disease. In the present study, we investigated the neuroprotective effect and the mechanisms underlying the neuroprotection of ginsenoside Rg1, one of the biologically active ingredients of ginseng, on MPP^+-induced apoptosis in human neuroblastoma (SHSY5Y) cells. Our results indicated that Rg1 can effectively decrease the expression of MPP^+-induced upregulation of Bax increase the expression of Bcl-2 and inhibit the activation of caspase-3. Interestingly, Rg1 can protect SHSY5Y cells from MPP^+-induced high ROS level and inhibit the release of cytochrome c. Together these results suggested that ginsenoside Rg1 may attenuate MPP^+-induced apoptotic cell death through suppression of the Bax-mediated mitochondrial signaling pathway and intracellular oxidative stress, and that it may rescue or protect dopamine neurons in PD.

Keywords: PD, Rg1, protective role

Introduction

Parkinson's disease (PD) is a progressive motor disorder of the central nervous system, and has severely affected elderly health [1]. Numerous studies indicated that oxidative stress is involved in the pathogenesis of Parkinson's disease. Dopamine can be oxidized to generate semiquinones, quinones, oxygen radicals and other reactive oxygen species, which are toxic and ultimately contribute to the inhibition of mitochondrial respiration and causes lipid per-oxidation, and may play an important role in neuronal cell death [2-4]. Indeed, dopamine has been shown to be toxic to cell cultures in vitro, and the administration of dopamine directly into the brain can result in cell death [5]. However, the dopamine-induced cell death was shown to be attenuated by some antioxidants, such as glutathione and N-acetylcysteine [6].

Apoptosis is a genetically determined mechanism of programmed cell death that can be triggered by various internal and external stimuli. During the development of nervous system, apoptosis is normal, but its reemergence appears to contribute to neurodegenerative disorders in the PD, and delayed encephalopathy after acute carbon monoxide poisoning [7-9]. Also, several studies suggested that oxidative stress, caused by imbalanced cellular production and elimination of reactive oxygen species, leads to neuronal apoptosis and necrosis [10, 11]. Therefore, it is essential to discover compounds that can antagonize the deleterious action of reactive oxygen species and act as antioxidants to protect neurons from apoptosis for the treatment of Parkinson's disease.

Ginsenoside Rg1 is a steroidal saponin of high abundance in ginseng. Ginsenosides are the most important active constituents identified in

all species of ginseng [12]. There are two major classes of ginsenosides, namely, the derivatives of either protopanaxatriol (Rg1, Rg2, Re, and Rf) or protopanaxadiol (Rb1, Rb2, Rc, and Rd). They possess four trans-ring rigid steroid skeleton with a modified side-chain at C20, whereas estradiol has no side-chain [13]. A previous study found that ginsenoside Rg1 played a major role in modulating neurotransmission and prevented scopolamine-induced memory deficits by increasing the cholinergic activity [14]. Recently, studies have also shown that ginsenoside Rg1 has potential neurotrophic and neuroprotective effects [15]. Studies also indicated that Rg1 could attenuate apoptosis in cultured cells [16]. However, the molecular events involved in the neuroprotective processes remain unknown. In this study, we investigated the effects of ginsenoside Rg1 on MPP^+-induced apoptosis in SHSY5Y cells in terms of suppression of intracellular oxidative stress and mitochondrial signal pathway.

Materials and methods

Reagents

Ginsenoside Rg1 (purity >99%) was obtained from the National Institute for Drug and Bio product Inspection. Ginsenoside Rg1 was dissolved in DMSO with the concentration of 1 mM (stock). MPP^+ was dissolved in sterile ddH_2O with the concentration of 1 M (stock). MPP^+, DMSO, Hoechst 33258, penicillin and streptomycin were purchased from Sigma Ltd., Co. BSA, MTT, DMEM and trypsin were purchased from Invitrogen. Triton X-100 was purchased from Xi'an Wolsen Bio-technology Co., Ltd. Primary antibodies included rabbit monoclonal antibodies to rat cytochrome c, inducible nitric oxide synthase (iNOS), and cleaved caspase-3 were from BD biotech Ltd., Co.

Cell culture and transfection

The SHSY5Y cell line was obtained from American Type Culture Collection (ATCC; Manassas, VA USA) and was maintained in Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum and 100 U/ml of penicillin-streptomycin. All cell lines were cultured in a humidified incubator in the atmosphere with 5% CO_2 at 37°C. To interfere the endogenous Bax protein expression, Bax-specific oligonucleotide was synthesized according to the description in literature [17]. The synthesized sequence was 5'-TGCTCCCCGGACCC GTCCATC-3'. Bax-siRNA (10 mM) was added into cultured cells as per the protocol of Lipofectamine TM 2000 manufacturer.

MTT assay

For the detection of cell survival rate, approximately 1×10^5 cells/well cells in log phase were plated into 96-well microtitre plates and incubated in DMEM medium containing 10% FBS. At various times following the treatment, MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide) (500 μg/m) was added to each well and incubated at 37°C for 4 h, and 200 μl DMSO was added into each well. The absorbance at 492 nm wavelength was detected by multifunctional enzyme-labeled instrument (Thermo Fisher).

Hoechst 33258 staining

The cells were then transferred to a 24-well plate at 1×10^3 cells/well and left to adhere and grow. The culture medium was discarded and the cells were washed with 1×PBS three times for 5 min each time. Then the cells were fixed in 4% paraformaldehyde at room temperature for 20 min and washed again with 1×PBS three times for 5 min each time. After treatment with 0.5% Triton X-100 for 15 min, the cells were washed with 1×PBS three times for 5 min each time. The cells were stained with Hoechst 33258 for 10 min in the dark and washed with 1×PBS three times for 5 min each time. The cover was sealed with water-based sealing agent. The nuclear morphology changes were observed under the fluorescence microscope.

Apoptosis detection by flow cytometry

The SHSY5Y cells were transferred to a 100 ml culture flask. After the cell adhesion, the cells were pretreated with Rg1 or MPP^+. When the cells grew to 80% confluence (about 1×10^6), trypsin was added for digestion. Digestion was terminated by adding serum containing complete medium. Then flow cytometry was performed to detect cell apoptosis according to the instruction of PE-Texas RED-A/FITC.

Western blot

Cells were lysed and protein assays were performed by BCA kits (Thermo, Waltham, MA, USA). The protein samples were analyzed by

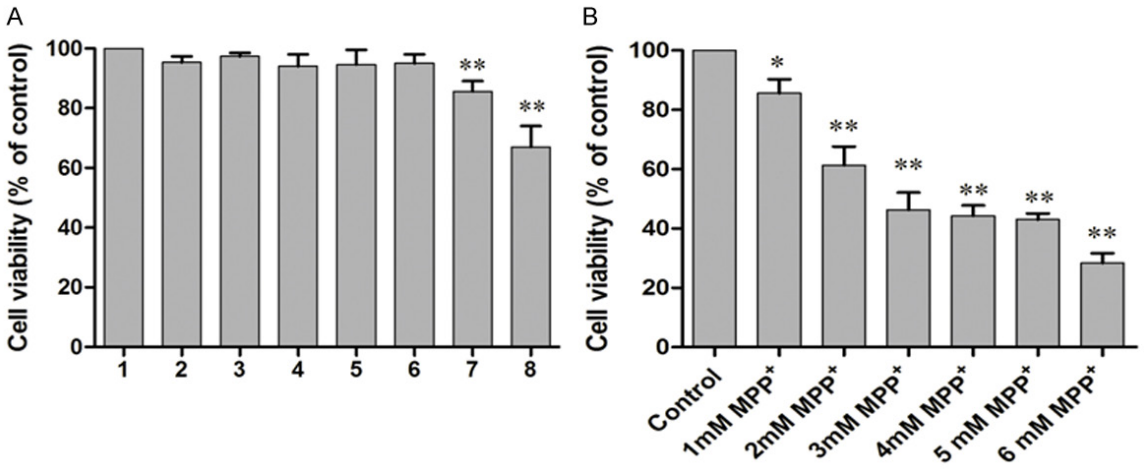

Figure 1. Effect of different concentrations of ginsenoside Rg1 and MPP^+ on cell viability in SHSY5Y cells. A. MTT assay was used to detect ginsenosid Rg1 on cell viability. B. MTT assay was used to detect MPP^+ on cell viability. Values are expressed as mean ± SEM, n=3. **P<0.01 as compared to control cells.

12% SDS-PAGE and transferred to nitrocellulose membranes at 100 V for 2 h. The membranes were blocked with 5% BSA for 1 h. The blocked membranes were incubated with rabbit anti-TH polyclonal antibodies (1:1000) and rabbit anti-cytochrome c polyclonal antibodies (1:4000). Then 1:5000 HRP-conjugated goat anti-rabbit secondary antibodies were added and the membranes were incubated at room temperature for 1 h. Then corresponding antibodies were added for different groups: mouse anti-Bax monoclonal antibody (1:1000), mouse anti-Bcl-2 monoclonal antibody (1:1000), mouse anti-caspase-3 monoclonal antibody (1:1000), and rabbit anti-β-actin polyclonal antibody (1:4000) for the control group. After that the membranes were washed with TBST three times for 10 min and subjected to ECL detection reagents. β-actin was used as internal control.

ROS activity detection

The cells in experimental groups were further cultured for 24 h. The culture medium was discarded, and the cells were digested with trypsin, counted and collected. After centrifugation, the cells were resuspended in 1 ml complete medium. DCFH-DA with the final concentration of 10 μmol/L was added, and the cells were incubated in a water bath with 37°C in the dark for 60 min. The cells were centrifuged at 800 rpm for 5 min with supernatant discarded, and the cells were washed with 1 ml PBS (pH 7.4) once. The cells were centrifuged again at 800 rpm for 5 min with supernatant discarded and re-suspended in 1 ml PBS. The fluorescence intensity was detected using a fluorescent luminance meter (Ex: 488 nm/Em: 530 nm). ROS activity was represented by average fluorescence intensity measured for every 10^5 cells.

Statistical analysis

All data are presented as mean ± SD and analyzed by SPSS19.0 software. One-way analysis of variance (ANOVA) and unpaired Student's t-test were used to determine the statistical significance of differences. P<0.05 was considered to be statistically significant.

Results

Cytotoxicity assay of Rg1 on SHSY5Y cells

The cytotoxicity of Rg1 and MPP^+ on SHSY5Y cells was detected by the MTT assay. The cells were cultured in a 96-well plate at 1×10^5 cells/well, with 0-200 μM Rg1 or 0~6 mM MPP^+ added, incubating for 24 hr before the MTT assay. The results showed that 1-50 μM Rg1 had no obvious cytotoxicity on SHSY5Y cells, while higher concentration of Rg1 (100-200 μM) exhibited cytotoxic effects. However, the cytotoxicity was caused by the DMSO in the Rg1 solution with higher concentration (**Figure 1A**). Thus, subsequent experiments were performed using the safe concentration of 25-50 μM. In addition, the data showed that the treat-

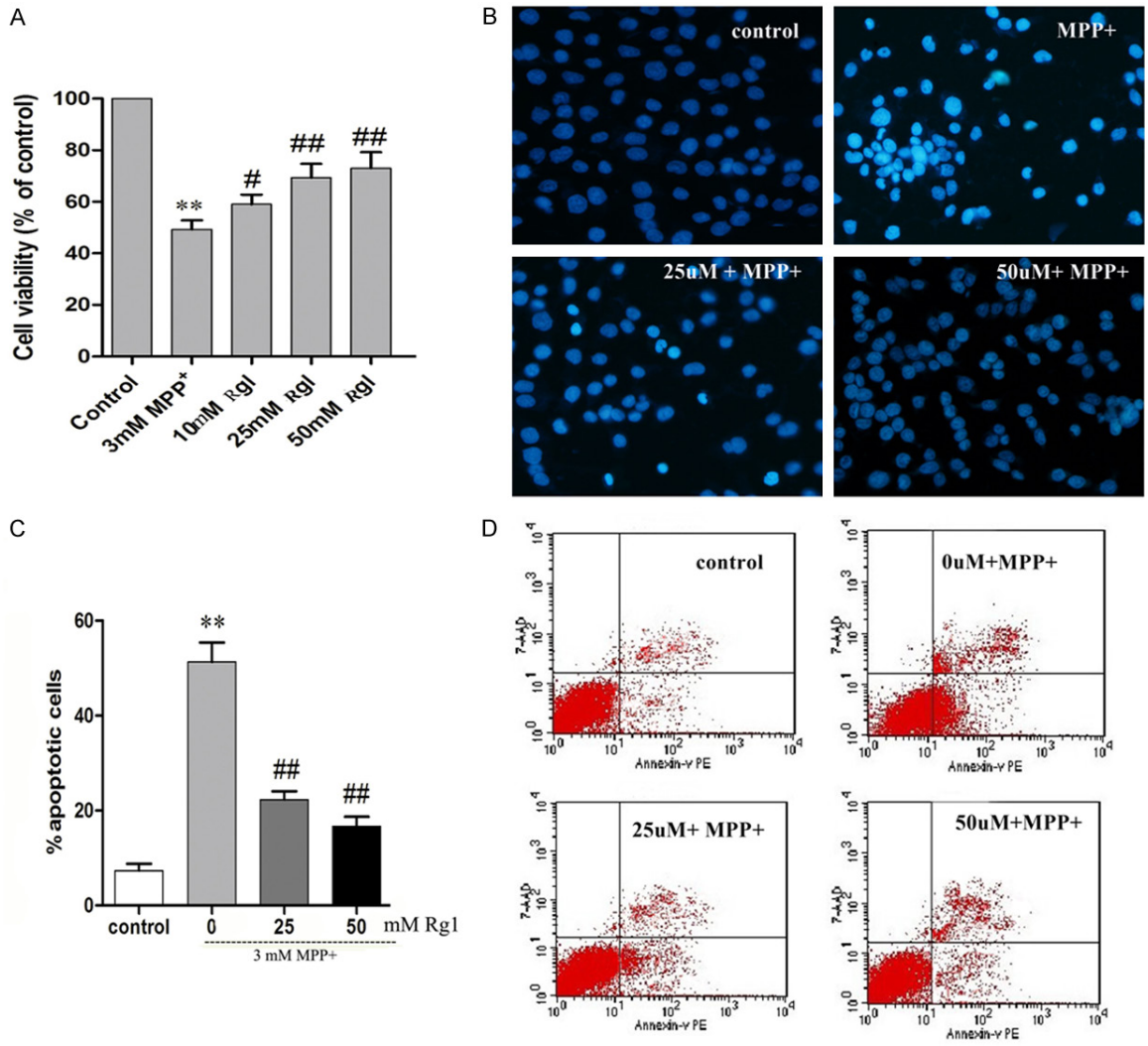

Figure 2. Protective effect of Rg1 on MPP+-induced cell impairment in SHSY5Y cell. A. MTT assay was performed to determine the protective role of Rg1 after the MPP+ treatment. B. Hoechst 33258 staining in SHSY5Y cells after treatment with MPP+. Living cells were observed with normal blue nuclei, while apoptotic cells were observed with blue nuclei. C. Flow cytometry assay detected Rg1 on the apoptosis of SHSY5Y cell. D. Typical profiles of apoptosis detected by flow cytometry. Values are presented as mean ± SEM, n=3. **P<0.01 compared to control group. #P<0.05 and ##P<0.01 compared to model group.

ment with 3 mM MPP+ for 24 hours induced the 50% inhibition of cell activity, and therefore, this concentration was used for the further studies (**Figure 1B**).

The protection effect of ginsenoside Rg1 on SHSY5Y cell impairment induced by MPP+

To determine whether ginsenoside Rg1 has protective effect on dopaminergic neuron impairment, 3 mM MPP+ was used to induce SHSY5Y cell death to mimic an *in vitro* Parkinson's disease (PD) model with or without ginsenoside Rg1 pretreatment (25, 50 μM) for 2 hours. Results of the MTT assay indicated that the cell viability in ginsenoside Rg1 pretreated groups was significantly increased (P<0.05) in a concentration-dependent manner compared to the untreated group (**Figure 2A**).

Hoechst 33258 staining also showed that ginsenoside Rg1 protected dopaminergic neuron from apoptosis. After 3 mM MPP+ treatment, the cell number was decreased, and nucleus became pyknosis, karyorrhexis and karyolysis, indicating increased apoptosis compared to untreated cells. Pretreatment with ginsenoside Rg1 significantly attenuated the morphonucle-

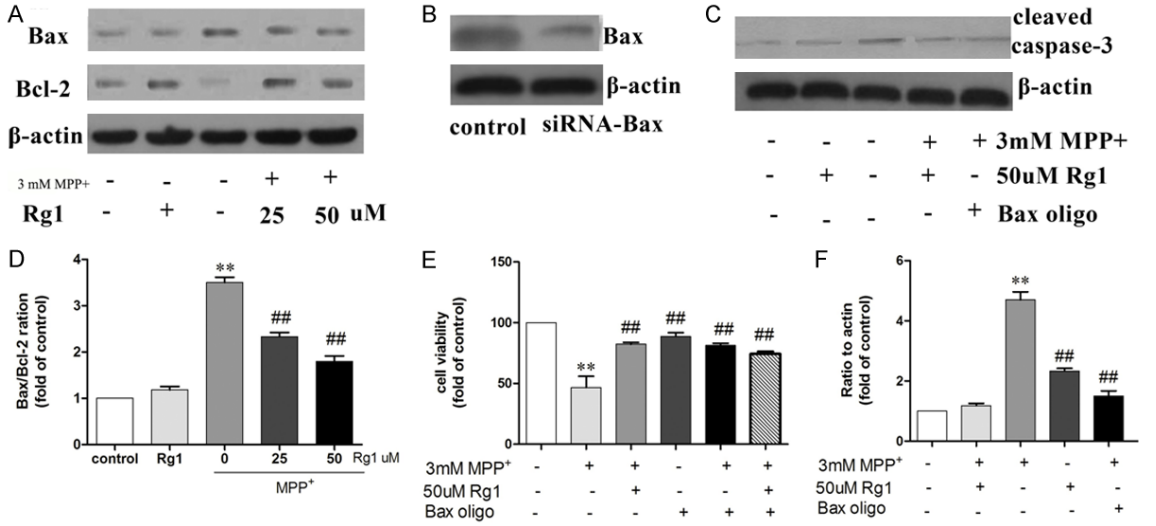

Figure 3. Rg1 effectively inhibit apoptosis induced by MPP+ treatment. (A) Representative micrographs of Bax and Bcl-2 expression by western blot analysis. (B) Western blot assay verified siRNA-Bax effectively decreased the expression of Bax in SHSY5Y cells. (C) Western blotting analysis of expression of cleaved caspase-3 in SHSY5Y cells after siRNA-Bax treatment or Rg1 treatment at different concentration. (D) Statistical data of (A). Rg1 can decrease the ration of Bax to Bcl-2 after MPP+ treatment. (E) After siRNA-Bax transfection, MTT assay verified the role of Bax in the Rg1 protective effect on MPP+ induced apoptosis. (F) Statistical data of (C). Values are presented as mean ± SEM, n=3. **P<0.01 compared to control group. #P<0.05 and ##P<0.01 compared to model group.

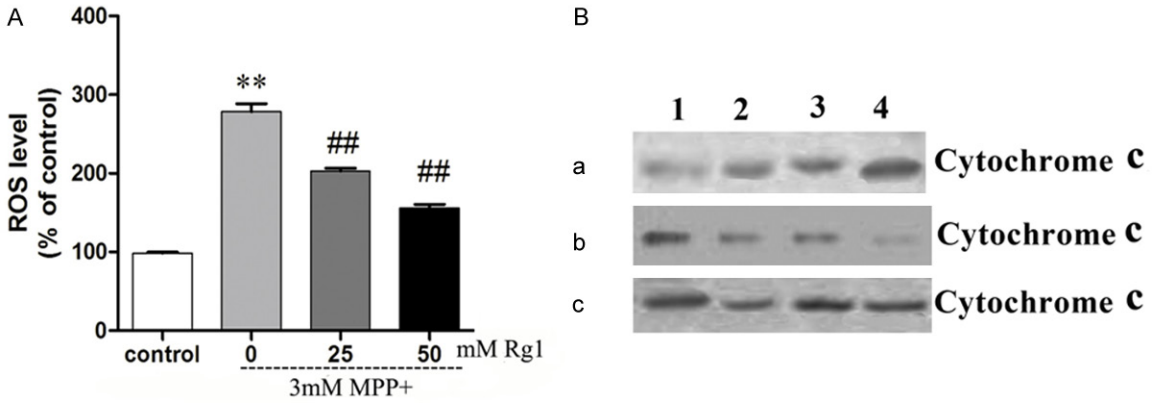

Figure 4. MPP+ treatment induced cytochrome c release and decreasing of ROS level. A. ROS level decreased as the increasing of Rg1 concentration. B. MPP+ treatment induced cytochrome c release and the effects of ginsenoside Rg1. SHSY5Y cells were treated with (a) 1-3 mM dopamine for 24 h or (b) were pretreated with 0-50 μM ginsenoside Rg1 or (c) with 10 μM cyclosporin A or 0.1 mM Ac-DEVD-CHO or aminoguanidine for 24 h. Next, they were cultured with 3 mM MPP+ for 24 h. (a) 1: normal; 2, 3, 4: MPP+ 1, 2 and 3 mM, respectively. (b) 1: MPP+, 3 mM; 2, 3, 4: Rg1 5, 25, 50 μM and MPP+ 3 mM, respectively. (c) 1: MPP+, 3 mM; 2, 3, 4: cyclosporin A, Ac-DEVDCHO, or aminoguanidine and MPP+, 3 mM, respectively.

ar changes induced by MPP+ compared to the untreated group. Also, high dosage of Rg1 showed a better protective effect than lower dosage of Rg1 (**Figure 2B**). Flow cytometry results showed that ginsenoside Rg1 reduced the apoptosis of neurons induced by MPP+, indicating the anti-apoptotic effects of Rg1 (**Figure 2C** and **2D**). These findings suggest that ginsenoside Rg1 can enhance the stability of the neuronal cell cycle.

Effect of Rg1 on Bax and Bcl-2 expression in SHSY5Y cells

To investigate the neuroprotective molecular mechanism of Rg1, we examined the change of

Bax and Bcl-2 expression in SHSY5Y cells induced by MPP^+ in vitro PD model. Western blot results showed that the treatment of MPP^+ induced a significant increase in the level of Bax, but decrease in the level of Bcl-2 as shown in **Figure 3A**. This increase of Bax and the decrease of Bcl-2 were inhibited significantly with the treatment of Rg1, while insignificant differences were observed between Rg1 alone treated group and the control group. The ratio of Bax/Bcl-2 expression level was statistically analyzed, as shown in **Figure 3D**. The ratio of Bax/Bcl-2 in the model group was increased by approximately 4 times compared with control group ($P<0.01$), however, treatments of 25 μM and 50 μM Rg1 made Bax/Bcl-2 ratio decline significantly compared with model group ($P< 0.01$), and the degree of decrease in high-dose group was more pronounced.

Bax-siRNA synthesized to silence endogenous Bax expression in SHSY5Y cells. **Figure 3B** showed that the interference efficiency of Bax-siRNA by Western blot was at least up to 50%. As shown in **Figure 3D**, Bax siRNA interfered with the expression of endogenous Bax, the cell survival rate was increased, which was consistent with the Rg1 effect. Meanwhile, under the combined action of Bax siRNA and Rg1, cell survival rate had no significant improvement, and there is no synergy. We also investigated the effect of Rg1 on caspase-3, which is the downstream signaling molecule of Bax. **Figure 3C** showed that treatment of MPP^+ significantly enhanced the level of cleaved caspase-3 in SHSY5Y cells ($P<0.01$). However, the expression of caspase-3 was significantly decreased after the treatment of 50 μM of Rg1 ($P<0.01$). In addition, we employed Bax-siRNA to interfere with the expression of Bax once again, found that Bax can inhibit the MPP^+-induced activation of caspase-3 in SHSY5Y cells similar as the effect of Rg1.

Rg1 can effectively decrease MPP^+-induced high ROS level and the release of cytochrome c in SHSY5Y cells

To investigate the molecular mechanisms of neuroprotective effect of astragaloside and discuss whether astragaloside could inhibit the generation of ROS, we detected the activity of ROS induced by MPP^+ in SHSY5Y cells. As shown in **Figure 4A**, MPP^+ treatment resulted in a significant increase of ROS levels in SHSY5Y cells, which was significantly inhibited after the treatment of 25 μM and 50 μM Rg1. The suppression effect of the Rg1 on the ROS level was more notable in the high-dose group than low-dose group.

To address whether or not the release of cytochrome c contributes to MPP^+-induced apoptosis, cytosolic fractions were isolated from the lysates of cultured SHSY5Y cells after treated with 1-3 mM MPP^+ for 24 h. Western blot analysis revealed the accumulation of cytosolic cytochrome c as the increasing of MPP^+ concentration. However, pretreatment with ginsenoside Rg1 effectively decreased the release of cytochrome c into the cytosol (**Figure 4B**). Importantly, preincubation with 10 AM cyclosporin A, a mitochondrial permeability transition pore inhibitor, decreased the release of cytochrome c from the mitochondria, whereas the pretreatment with 0.1 mM aminoguanidine did not affect cytochrome c release (**Figure 4C**). These results suggested that the dopamine-induced generation of reactive oxygen species was a trigger rather than a consequence of cytochrome c release and caspase-3 activation in MPP^+-induced apoptosis.

Discussion

The selective neurotoxin MPP^+ has been widely used to create animal models of PD in vitro and in vivo [18]. MPP^+ causes selective destruction of the nigrostriatal dopaminergic pathway, which is similar to that observed in PD, and inhibits mitochondrial NADH-linked electron transport at complex I, resulting in the loss of ATP production and subsequent cell death [19]. Although the neurotoxin MPP^+ induces apoptosis in several neuronal cell types, the exact mechanism of toxicity remains unknown. In this study, we elucidate the mechanism of MPP^+-induced cell death and the protective effects of Rg1 against MPP^+ toxicity in human neuroblastoma SHSY5Y cells. Our present study clearly showed that exposure to MPP^+ resulted in a decrease in cell viability, and that cell death involved Bax-dependent mitochondrial signaling, a finding consistent with previous studies of other groups [20, 21].

Mitochondria are the unique organelles consuming oxygen, producing ATP, generating oxygen radicals, and mediating the mobilization of

calcium [22]. Mitochondrial functions are controlled by the Bcl-2 family proteins, which are subdivided into two functional groups, an anti-apoptotic group, including Bcl-2 and Bcl-xL, and a pro-apoptotic group, including Bax and Bak [23]. Mitochondrial involvement in apoptosis includes two crucial events: the onset of multiple parameters of mitochondrial malfunction [24], and the release of cytochrome c from the intermembrane space of the mitochondria into the cytoplasm [25]. Activation of caspase-3 is an important step in the execution of apoptosis and its inhibition blocks apoptotic cell deat h [26]. Cytosolic cytochrome c has previously been demonstrated to bind to apoptosis protease activating factor-1 and to subsequently trigger the sequential activation of caspase-9 and caspase-3 [27]. In this study, we showed that MPP^+ treatment resulted in cytosolic accumulation of cytochrome c and in caspase-3 activation.

Oxidative species produced from dopamine might be a trigger of and could in turn affect the release of cytochrome c from the mitochondria and the subsequent activation of caspase-3. Furthermore, in agreement with a previous finding [28], cytochrome c release was an essential event in mitochondria triggered apoptosis, leading to caspase activation, and the generation of reactive oxygen species or NO, but also might be an upstream event in the process of dopamine-induced apoptosis.

Although the exact mechanism of dopamine-induced apoptosis is unclear, antioxidants have been proved to be effective in preventing dopamine-induced apoptosis [6]. Antioxidant molecules such as N-acetyl-L cysteine, vitamin E and catalase appear to protect cells from oxidative insults and the resultant induction of apoptotic cell death [29, 30]. Ginsenoside, such as Rg1, represents the major active ingredient of ginseng, which has been shown to exert a variety of biomedical effects, such as anti-apoptotic activity [16]. In this study, Rg1 was shown to attenuate dopamine-induced apoptosis in a dose-dependent manner. These results provide the first direct evidence that ginsenoside Rg1 could protect neuronal cells from dopamine neurotoxicity through an antioxidative mechanism, and that ginsenoside Rg1 might act as a free radical scavenger.

In this study, the mechanism by which ginsenoside Rg1 protected SHSY5Y cells from MPP^+-induced apoptosis was further investigated. Results showed that ginsenoside Rg1 might attenuate the MPP^+-induced elevation of reactive oxygen species generation and subsequently protect mitochondria from reactive oxygen species-induced injury and reduced cytochrome c release, and eventually inhibit the activation of caspase-3. Thus, the target of ginsenoside Rg1 for protecting SHSY5Y cells from MPP^+-induced apoptosis in SHSY5Y cells may be the mitochondria.

In summary, present study indicated that dopamine is capable of promoting neuronal cell death via the inhibition of a Bax-mediated mitochondrial pathway through an oxidative mechanism. Ginsenoside Rg1, an extract of ginseng, could interrupt the MPP^+-induced elevation of reactive oxygen species generation and attenuate the neurotoxicity of MPP^+, which might offer a potential means to rescue or protect dopamine neurons in Parkinson's disease.

In conclusion, the neuroprotective mechanism of ginsenosides was studied and analyzed in SHSY5Y cells model. The treatment group of ginsenosides can significantly inhibit the expression of Bax in SHSY5Y cells induced by MPP^+, while increasing the expression of Bcl-2 and inhibiting the activation of caspase-3 induced by MPP^+. The interference of Bax-siRNA against the endogenous Bax expression indicated that ginsenosides exhibited a neuroprotective effect via the Bax pathway. Finally, the results of the detected ROS levels showed that ginsenosides had obvious inhibitory effect on excessive ROS produced from SHSY5Y cells induced by MPP^+.

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Disclosure of conflict of interest

None.

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