

Neuroprotective effects of ginseng pectin through the activation of ERK/MAPK and Akt survival signaling pathways

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Abstract. In this study, we investigated the neuroprotective activities of ginseng pectin (GP) against hydrogen peroxide (H₂O₂)-induced neuronal toxicity in different neuronal cells. GP selectively attenuated H₂O₂-induced damage up to 26% in primary cortical neuron cells and human glioblastoma U87 cells. Following H₂O₂ exposure, DAPI staining and neuron-specific β -tubulin antibody probing indicated that GP maintained cell integrity and decreased nuclei condensation. Data from western blot analysis revealed that pre-treatment with GP increased the phosphorylation of both the extracellular signal-regulated kinases 1 and 2 (ERK1/2) and Akt in cortical neuron cells. However, the phosphorylation of ERK1/2 was increased, but that of Akt was decreased in U87 cells. These results suggest that the protective effects of GP against H₂O₂-induced apoptosis may be due to the activation of the phosphorylation of ERK1/2 and Akt; however, the mechanisms involved differ depending on the cell line. This neuroprotective property indicates that GP could serve as a potential therapeutic agent for neurodegenerative diseases.

Introduction

Oxidative stress is a prominent factor of cell death in glutamate- or hydrogen peroxide (H₂O₂)-induced cytotoxicity and neurodegenerative diseases, such as Alzheimer's disease (AD), atherosclerosis, Parkinson's disease and stroke (1,2). Oxidative stress-induced damage is mediated by reactive oxygen species (ROS), including H₂O₂, superoxide and hydroxyl radicals, which are generated as products of normal and aberrant metabolic processes that utilize molecular oxygen. ROS attacks proteins, deoxyribonucleic acids and lipid membranes, thereby disrupting cellular function and integrity (3,4). H₂O₂ induces

apoptosis in many different cell types, and this effect can be blocked by the addition of anti-oxidants (5-7).

Mitogen-activated protein kinases (MAPKs) respond to extracellular stimuli, and regulate various cellular activities, such as differentiation, proliferation, survival and apoptosis. Extracellular signal-regulated kinases 1 and 2 (ERK1/2) are known as classical MAP kinases, which were activated in response to stress signals. A previous study revealed that abnormal levels of phosphorylated ERK1/2/MAPK in the brains of AD patients are associated with oxidative stress (8). Alternatively, the modulation of signaling through the serine/threonine kinase, Akt/protein kinase B (PKB), one of the main downstream effectors of phosphatidylinositol 3-kinase (PI-3K) and a pivotal kinase in neuronal survival, may also play an important role (9-11).

Panax ginseng C.A. Meyer (ginseng) is a medicinal plant with a wide range of therapeutic benefits and has many active chemical components, such as ginsenosides, ginseng polysaccharides and ginseng peptides (12,13). Ginsenoside Rb1 is the main neuroprotective molecule of ginseng. It facilitates cholinergic function and increases synaptophysin levels in the hippocampus, potentiates the nerve growth factor (NGF)-mediated neurite outgrowth (14-16), reduces oxidative stress caused by hydrogen peroxide (17), and promotes neurite length and number after exposure to glutamate or hydrogen peroxide (18).

Ginseng polysaccharides contain starch-like glucans and pectins. Ginseng pectins (GPs) are the main active compounds with various bioactivities. They impair cell migration through decreasing cell adhesion and cell spreading (19,20); have anti-fatigue activity via reducing glucose and glutathione peroxidase and increase creatine phosphokinase, lactic dehydrogenase and malondialdehyde levels (21); inhibit human colon cancer HT-29 cell proliferation accompanied by the activation of caspase-3; and induce cell cycle arrest in the G2/M phase (22). In this study, we report the neuroprotective effects of GP against H₂O₂-induced damage and the possible mechanisms involved. The results may be useful for the development of drugs from GP to treat neurodegenerative diseases.

Materials and methods

Materials. Ginsenoside Rb1 (>98% pure) was purchased from the Chinese Inspection and Identification Institute of

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Biological Products and Medicine (Beijing, China). Neuro 2A (mouse neuroblastoma) cells, U87 (human glioblastoma) cells and SH-SY5Y (human neuroblastoma) cells were all obtained from the American Type Culture Collection (ATCC); 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was from Promega (G3581); the mouse monoclonal, Tuj, antibody was from Neuromics (MO15013); and monoclonal anti- γ -tubulin antibody was from Sigma (T5326). p44/42 MAP kinase (3A7) mouse monoclonal antibody (9107), phospho-p44/42 MAPK (Thr202/Tyr204) antibody (9101S), Akt antibody (9272) and phospho-Akt (Thr308) antibody (9275S) were all from Cell Signaling Technology. All other reagents were of analytical grade or better.

Preparation of ginseng pectins. Ginseng polysaccharides were dissolved in water and digested with α -amylase (E.C. 3.2.1.1) at 37°C for 16 h. The enzyme was inactivated by boiling for 10 min before being removed by centrifugation. To remove proteins, the resulting supernatant was further treated with Sevag reagent (n-butanol:chloroform 1:4), and then precipitated with 75% ethanol to gain the GP. After dialysis against distilled water followed by lyophilization, pure GPs were obtained.

Cell culture. Neuro 2A, U87 and SH-SY5Y cells were obtained from the ATCC. Neuro 2A cells were cultured in DMEM medium, and the others in RPMI-1640 medium. All media were supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μ g/ml streptomycin and 10 mM HEPES at 37°C in a humidified atmosphere of 5% CO₂.

Preparation of mouse cortical neuron cells. Pregnant animals were cared and handled in accordance with the guidelines of Northeast Normal University for the use of laboratory animals. Pregnant C57BL6/J mice (15th day of gestation) were sacrificed with sodium pentobarbital (80 mg/kg), the uteri were dissected and the embryos were carefully removed and transferred to a culture dish with harvest medium (Hank's medium with 1% HEPES buffer and 1% antibiotics). The brains were dissected, the cortex was excised and primary cultures were prepared as follows: briefly, the cortex was removed from the hemisphere and minced into small pieces. Cells were disaggregated in Hank's medium supplemented with 2.5% trypsin and 1% DNase and incubated for 15 min at 37°C. After centrifugation at 100 rpm for 5 min, the cell pellet was resuspended with plating medium (MEM medium with 2.5% NaHCO₃, 1% pyruvic acid, 20% glucose, 10% FBS and 1% antibiotics). Cell density was adjusted to 4x10⁴ cells/cm² in 65-mm dishes coated with 40 μ g/ml of poly-D-lysine. The following day, the maintaining medium (neurobasal medium with 2% B-27 and 5% L-glutamine) was replaced with plating medium and cells were cultured for 5-7 days.

Oxidative stress assay. Stock solution of ginsenoside Rb1 (100 μ M) and GP (5 mg/ml) in medium was prepared. Cells were pre-treated either with Rb1 at final concentration of 0, 20, 40 and 80 μ M, or GP at 0.0, 0.01, 0.05 and 0.5 mg/ml for 1 h at 37°C in a humidified atmosphere of 5% CO₂. Removing Rb1 or GP, cells were exposed to 100 μ M of H₂O₂ for 30 min at 37°C. After rinsing off H₂O₂, the cells were maintained in

the same concentration of Rb1 or GP for 24, 48, 72 and 96 h. Control cells were kept in parallel with medium alone. The cell viability was determined by MTS assay. MTS reagent (100 μ l) was added to each well and cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ for 30 min. The OD₄₉₀ and OD₆₉₀ values were measured by an automated spectrophotometric plate reader. The value of OD₄₉₀ subtracted from OD₆₉₀ represented the cell survival rate.

Morphology assay. On the 5th day of cortical neuron cell culture, cells were fixed with 4% paraformaldehyde/PBS (pH 7.0) at room temperature for 20 min. They were then washed with PBS and 100 mM NH₄Cl/PBS. The cells were permeabilized with 0.025% saponin/PBS at room temperature for 10 min, with no shaking. Incubated monoclonal Tuj antibody (neuron-specific class III β -tubulin) was diluted with blocking buffer (2%BSA/5%FBS/PBS-filtered saponin at 2:1) at 1:100 at room temperature for 60 min. The cells were then washed with PBS. The cells were incubated with FITC-goat anti mouse IgG (1:100), at room temperature for 30 min, in the dark. The cells were washed with PBS again. The slides were mounted with 2.5 μ l of vecta shield and sealed in nail polish.

ERK/MAPK and Akt signaling pathway. For the determination of protein phosphorylation after GP or Rb1 treatment, cells were scratched off the plate in cold PBS and then lysed in lysis buffer [150 mM NaCl, 1% TX-100, 1 mM EDTA, 1 mM PMSF, 1 mM Na₃VO₄, protease inhibitor cocktail (Roche) and Tris-Cl; pH 7.4]. After centrifugation at 14,000 rpm for 5 min, the supernatant was recovered and mixed with SDS-PAGE loading buffer. The separated proteins on the gel were transferred onto a nitrocellulose membrane and blocked in 5% non-fat milk. Then, the membrane was incubated with primary antibodies [monoclonal anti- β -tubulin antibody 1:1,000; p44/42 MAP kinase (3A7) mouse monoclonal antibody 1:100; phospho-p44/42 MAPK 1:100; Akt antibody 1:100, phospho-Akt (Thr308) antibody 1:100] followed by HRP-conjugated secondary antibodies. Protein bands were revealed by the ECL protein detection system (GE Healthcare).

Statistical analysis. The results were expressed as the means \pm SD. Statistical analysis of the data was performed using one-way ANOVA (SPSS Statistics 17.0). Differences were considered significant when p<0.01.

Results

Ginseng pectins preparation. GP were prepared and purified as previously described (19,23). Total ginseng polysaccharides were isolated from *Panax ginseng* C.A. Meyer, deproteinized using the Sevag method and digested using α -amylase. Prepared GP contained 96.1% (w/w) sugar and 45.1% (w/w) uronic acid. GP consisted of 44.3% galacturonic acid, 20.1% galactose, 14.9% glucose, 14.1% arabinose, 3.1% rhamnose, 1.9% glucuronic acid and 1.5% mannose.

Effects of hydrogen peroxide on different neuronal cells. The oxidative stress effects of hydrogen peroxide on different neuronal cells was evaluated by the MTS method. The cells were incubated with optimal medium for 48 h, the medium was

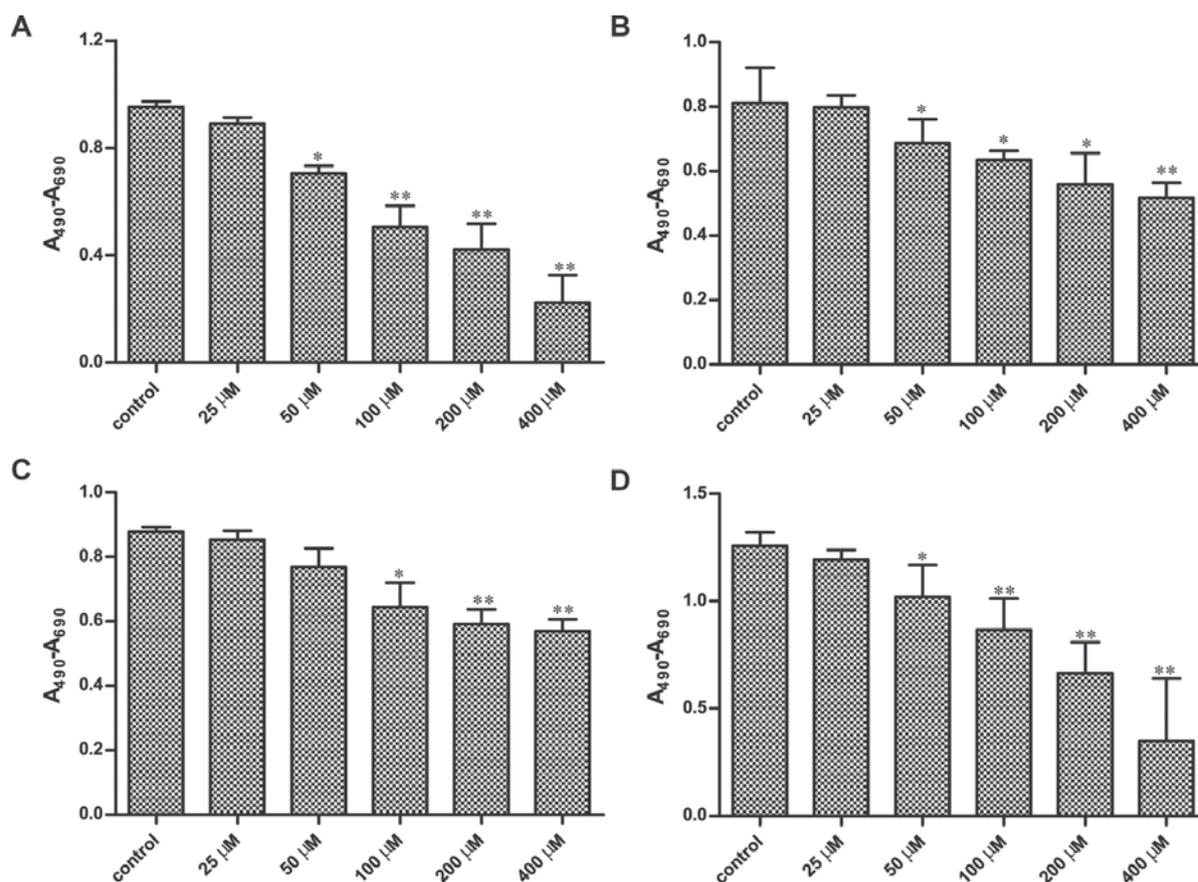


Figure 1. Effects of hydrogen peroxide (H₂O₂) on different neuronal cells. H₂O₂ activity was measured by MTS assay. (A) Cortical neuron cells, (B) Neuro 2A cells, (C) SH-SY5Y cells and (D) U87 cells, at 30 min after exposure to various concentrations of H₂O₂ (25–400 μM). In the control group, cells were incubated with medium alone. Each bar represents the means ± SD of 3 experiments. *p < 0.01, **p < 0.001.

discarded and then cells were treated with different concentrations of hydrogen peroxide for 30 min. The effect of hydrogen peroxide on the cells is shown in Fig. 1. Exposing neuronal cells to hydrogen peroxide for 30 min caused the cell viability to decrease in a dose-dependent manner. The cortical neuron cells and U87 cells were more sensitive to oxidative injury, the survival percentages with 400 μM H₂O₂ treatment were merely 24 and 28% (Fig. 1A and D), and the medium effect was at 100 μM. However, the survival percentage of Neuro 2A cells and SH-SY5Y cells with 400 μM of H₂O₂ treatment was 64 and 65%, respectively (Fig. 1B and C). Therefore, in the neuronal protective activity assay, the protective effects of both ginsenoside Rb1 and GPs on the neuronal cells were tested with treatment of 100 μM H₂O₂ in cortical neuron and U87 cells, but 400 μM H₂O₂ in Neuro 2A and SH-SY5Y cells for 30 min.

GP protects neuronal cells from hydrogen peroxide-induced cell death. The neuronal protective effect of GP against hydrogen peroxide-induced cell death was examined in different neuronal cells. The cells were pre-treated with either 40 μM of Rb1 or different concentrations of GP (0.01, 0.05 and 0.5 mg/ml) for 24 h. GPs and Rb1 were found to protect neuronal cells from hydrogen peroxide-induced cell death, but this effect was not related to concentration (Fig. 2). GP (0.05 mg/ml), which increased cell viability after exposure to hydrogen peroxide, did not affect cell proliferation compared

to the control cells. The exposure of the cortical neuron cells for 30 min to 100 μM of hydrogen peroxide caused a 40% decrease in cell viability. However, there was only a 14% decrease in cell viability in the cells pre-treated with 0.05 mg/ml of GP, meaning that GP recovered the cell viability by 26%. The protective effect of GP was similar to that of the positive control, Rb1, in the cortical neuron cells (Fig. 2A). After exposure to 400 μM of hydrogen peroxide, Neuro 2A and SH-SY5Y cell viabilities showed a significant decrease, 34 and 34%, respectively. However, pre-treatment with 0.05 mg/ml of GP resulted in a decrease of 31 and 26%, suggesting that GP has little protective effect on hydrogen peroxide-induced toxicity in these two cell lines (Fig. 2B and C). As for the U87 neuroblastoma cells, 0.05 mg/ml of GP dominantly attenuated cell viability by 25% after 100 μM of hydrogen peroxide-induced cell injury (Fig. 2D).

GP protects cortical neuron cell neurites from degeneration. Maintaining the integrity of neurites was an important index of neuroprotective effects. After treatment with 100 μM of H₂O₂ for 30 min, the morphology of the cortical neuron cells was absolutely destroyed. The neurites were fragmented into many small pieces and the nuclei had shrink to one third of the untreated cell nuclei. Under treatment with 40 μM of Rb1 or 0.05 mg/ml of GP, cell morphologies were similar to the control cells, as cell neurites were long with rich branches and nuclei were large. However, after exposure to H₂O₂, a

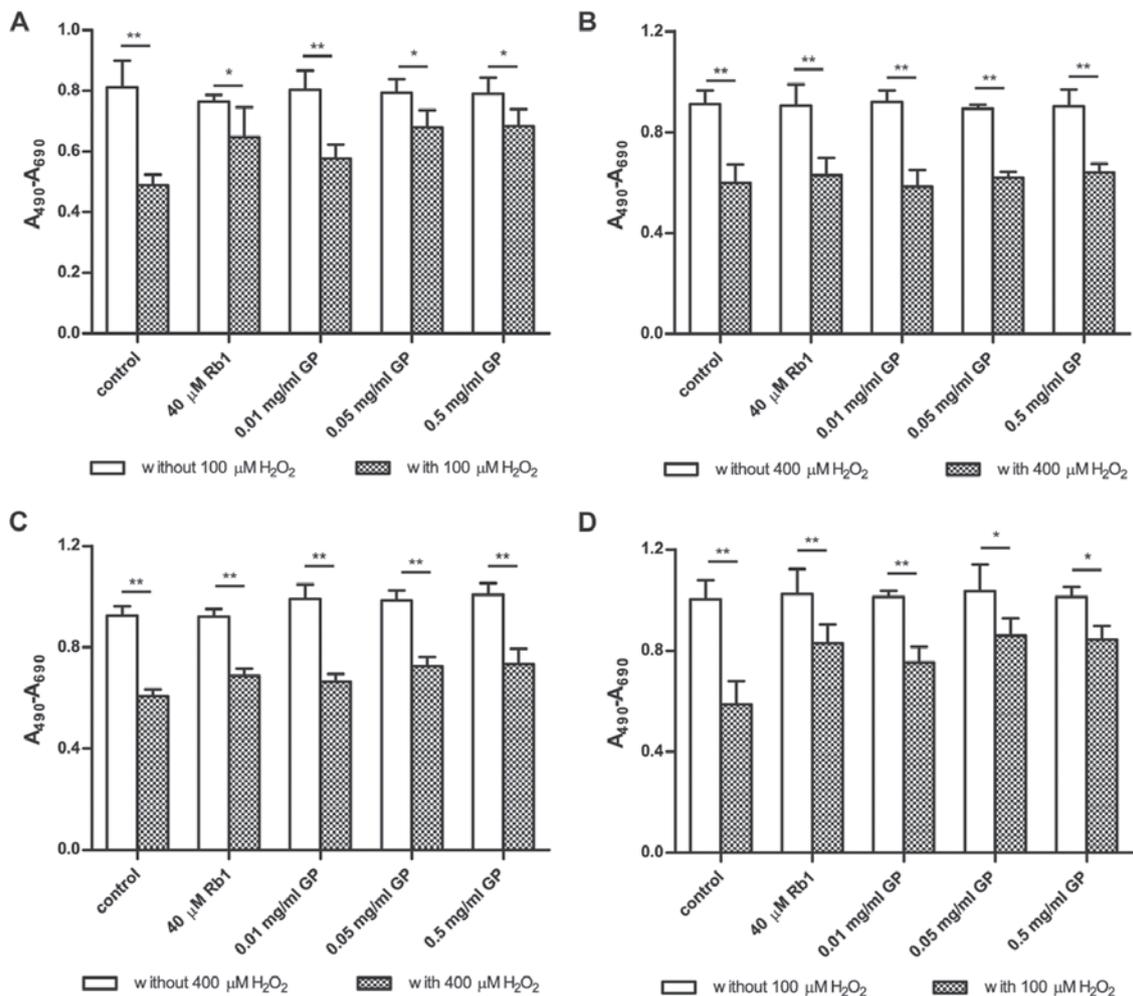


Figure 2. Effects of Rb1 and ginseng pectin (GP) on the protection against hydrogen peroxide (H_2O_2)-induced toxicity in neuronal cells. Cells were incubated either with 40 μM of Rb1 or with 0.01, 0.05 and 0.5 mg/ml of GP for 24 h, then exposed to 100 or 400 μM of H_2O_2 for 30 min. After washing off the H_2O_2 , cell viability was determined by MTS assay. Data are the means \pm SD of 3 experiments. (A) GP significantly protected cortical neuron cells from oxidative-induced toxicity, which was similar to the effect of Rb1. (B) GP and Rb1 seemed to have little protective effect on Neuro 2A cells against oxidative-induced toxicity. (C) The neuroprotective effect of GP on SH-SY5Y cells resembled that in Neuro 2A cells. (D) GP also had neuroprotective effects in U87 cells. * $p < 0.01$, ** $p < 0.001$.

few changes in cell morphology appeared, including certain breaks in the neurite structure. Cell morphology alteration showed that both Rb1 and GP protected cells from hydrogen peroxide-induced cell toxicity through their neuroprotective effects on neurite integrity.

GP neuroprotective effect occurs through the activation of ERK/MAPK and Akt survival signaling pathways. The effects of Rb1 and GP on ERK/MAPK and Akt signaling pathways were examined. After serum starvation overnight, the cells were treated with 40 μM of Rb1 or 0.05 mg/ml of GP for 0 and 30 min, then scrapped, lysed and subjected to western blot analysis using phosphorylated Akt (p-Akt) (S473), total-Akt, p-ERK1/2 and total-ERK antibodies. As shown in Fig. 4, there were some differences in protein phosphorylation status between Rb1 and GP treatment. In cortical neuron cells, both GP and Rb1 significantly increased p-ERK and p-Akt levels after 30 min of treatment compared to the untreated control. In the U87 cells, GP and Rb1 significantly increased p-Akt levels, but decreased p-ERK levels compared to the untreated control. However, in Neuro 2A and SH-SY5Y cells, treatment

with GP and Rb1 did not alter the phosphorylated levels of Akt and ERK.

Discussion

The neuronprotective effects of ginsenosides on the central nervous system have been reported in the literature. In this study, we report for the first time that GP selectively protects certain neuronal cell lines from hydrogen peroxide-induced toxicity. Before neuronal protective assay, we confirmed that treating cells with H_2O_2 resulted in cell viability loss in a dose-dependent manner (Fig. 1). Pre-treatment with GP significantly attenuated H_2O_2 -induced cell damage in cortical neuron and U87 cells (Fig. 2). The neuroprotective activity was similar to that of Rb1. When exposed to H_2O_2 after pre-treatment with GP or Rb1, cell morphologies under GP treatment were healthier than those under Rb1 treatment, especially the neurite length (Fig. 3). The results indicate that GP acts as a new neurotrophin.

The activation of the phosphorylation of MAPKs and Akt takes place in response to a variety of stresses, including

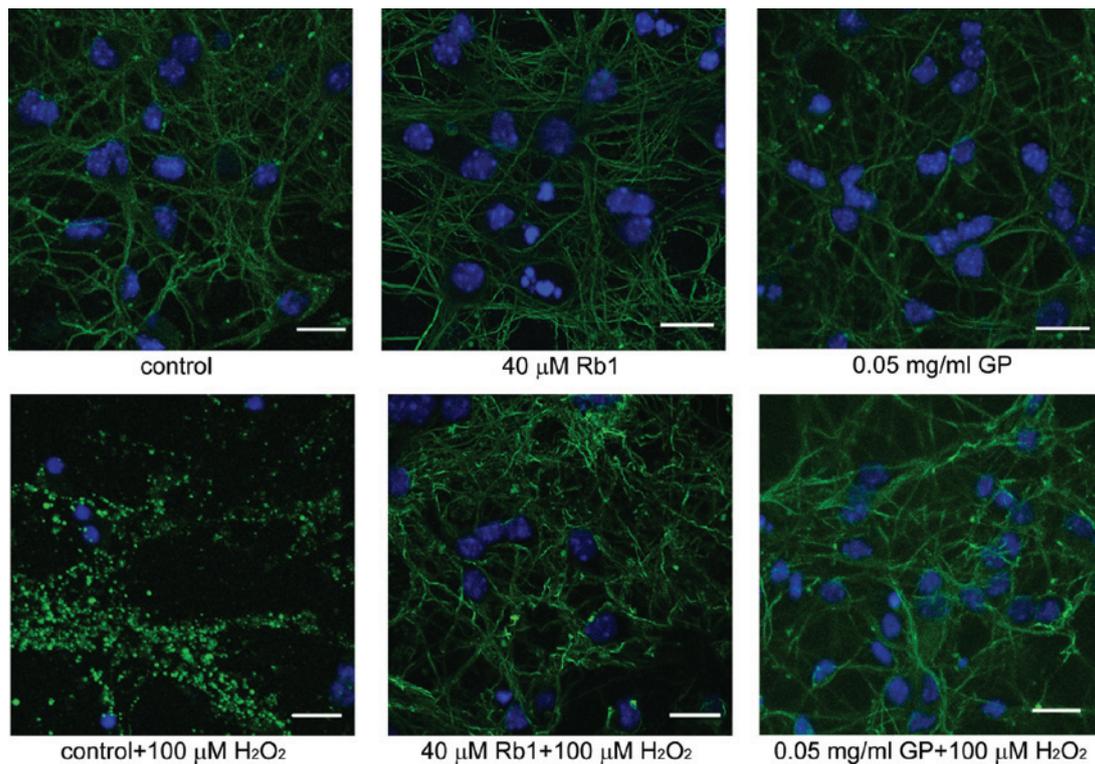


Figure 3. Neuroprotective effects of ginseng pectin (GP) against hydrogen peroxide (H_2O_2)-induced toxicity. Cortical neuron cells were pre-treated with $40 \mu M$ of Rb1 or 0.05 mg/ml of GP for 24 h (the control cells were not pre-treated), then exposed to $100 \mu M$ of H_2O_2 for 30 min. Cells were processed for immunofluorescent imaging using DAPI for nucleus (blue) and monoclonal Tuj antibody for tubulin in neurites (green). Representative images were from 3 experiments under each condition. Bar, $20 \mu m$.

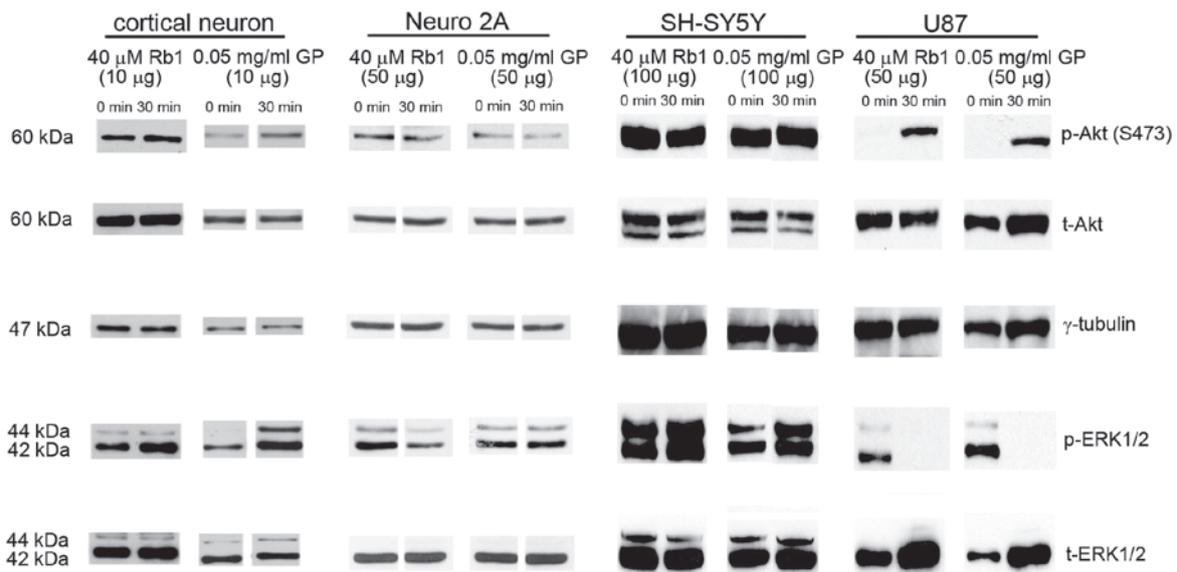


Figure 4. Rb1 and ginseng pectin (GP) activated ERK/MAPK and Akt signaling pathway in neuronal cells. After incubation for 36 h and serum starvation for 12 h, neuronal cells were exposed to $40 \mu M$ of Rb1 or 0.05 mg/ml of GP for 30 min. The cells were lysed with lysis buffer, applied to western blotting with phosphorylated Akt (S473), total-Akt, γ -tubulin, phosphorylated ERK1/2 and total-ERK1/2 antibodies.

oxidative stress and anoxia (24). Activation of ERK/MAPK and Akt signaling pathways have been found to correlate with the ischemia protective model (25). Therefore, in this study, the pro-survival ERK/MAPK and Akt signaling pathways in different neuronal cells were examined (Fig. 4). The results showed that p-ERK1/2 and p-Akt were not altered by GP or

Rb1 treatment in Neuro 2A and SH-SY5Y cells, combined with the neuroprotective assay results suggesting that GP or Rb1 may modulate the phosphorylation of ERK/MAPK and Akt pathways to protect the H_2O_2 -induced Neuro 2A and SH-SY5Y cell damage. Besides, p-ERK1/2 and p-Akt were upregulated significantly under GP or Rb1 treatment in cortical

neuron cells, upregulated p-Akt and suppressed p-ERK1/2 in U87 cell. Those neuroprotective assay results accord with the hypothesis that GP protect neuronal cells from H₂O₂-induced toxicities via pro-survival ERK/MAPK and Akt pathways.

In previous studies, ginsenoside Rb1 has been shown to protect neuronal cells against hydrogen peroxide-induced cell damage possibly by scavenging free radicals, inhibiting the production of nitric oxide, preventing lipid peroxidation and avoiding decrease in SOD activity. Rb1 has also been shown to promote neurite growth by increasing the expression of the nerve growth factor (14,15,17). Our experimental results showed that GP and Rb1 protected neuronal cells against oxidative stress via the activation of p-ERK1/2 and p-Akt. However, the action of GP on modulating the survival signaling pathways mediated by the nerve growth factor remains to be elucidated.

Panax ginseng C.A. Meyer has been used to treat many diseases and replenish vital functions for thousands of years. In this study, GP showed similar protective effects on neuronal cells against H₂O₂-induced oxidative stress via regulating the pro-survival ERK/MAPK and Akt pathways. Moreover, GP preserved the structural integrity of neurons, suggesting that it may be a new neurotrophin. In conclusion, GP appears to be anti-oxidant without side-effects, which may eventually lead to further development of therapeutics for neurodegenerative diseases.

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