Protective Effects of Ginsenoside Rd on PC12 Cells against Hydrogen Peroxide

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Oxidative stress-induced cell damage has been implicated in a variety of neurodegenerative disorders. In the current study, we investigated the protective role of ginsenoside Rd against the cytotoxicity induced by exposure to hydrogen peroxide (H₂O₂) and the underlying mechanism in the PC12 cell line. The protective effects of ginsenoside Rd (1, 10 μM) on H₂O₂-induced cytotoxicity may be ascribed to its antioxidative properties by reducing the intracellular reactive oxygen species level; decreasing malondialdehyde production, a common index of lipid peroxidation; and enhancing the antioxidant enzymatic activities of superoxide dismutase and glutathione peroxidase. Additionally, ginsenoside Rd could stabilize the mitochondrial membrane potential after H₂O₂ exposure. These findings suggested that ginsenoside Rd may be considered a potential antioxidant agent and should encourage further research in neurodegenerative diseases to explore the potential neuroprotective effects of ginsenoside Rd.

Key words ginsenoside Rd; neuroprotection; oxidative stress; PC12 cell

Ginseng, the root of Panax ginseng C. A. Meyer (Araliaceae), has been used as a tonic to treat a wide variety of disorders in China for millennia. Extensive studies have confirmed that the molecular components responsible for the pharmacologic effects of ginseng are ginseng saponins, namely ginsenosides. Currently, over 30 ginsenosides have been identified and isolated from ginseng. Dammar-24(25)-ene-3β,12β,20(S)-triol-(20-O-β-D-glucopyranosyl)-3-O-β-D-glucopyranosyl-(1→2)-β-D-glucopyranoside (ginsenoside Rd, Fig. 1) is one of the main active components of ginsenosides. It has been demonstrated to have a number of pharmacologic actions such as inhibiting Ca²⁺ influx through receptor- and store-operated Ca²⁺ channels, enhancing astrocyte differentiation from neural stem cells, and significantly reducing the 3-nitropropionic acid-induced motor impairment and cell loss in the striatum. Ginsenoside Rd is also one of the major ingredients in the total saponins from Panax notoginseng and its content in the total notoginseng saponins is 4.07%, making it inexpensive for practical use.

Oxidative stress-induced cell damage has long been implicated both in the physiologic process of aging and in a variety of neurodegenerative diseases such as Alzheimer’s disease, Parkinson’s disease, and amyotrophic lateral sclerosis. It is mediated by reactive oxygen species (ROS), including free radicals such as superoxide ions (O₂⁻) and hydroxyl radical (·OH) as well as non-free radical species such as hydrogen peroxide (H₂O₂), which are generated as byproducts of normal and aberrant metabolic processes that utilize molecular oxygen. ROS cause oxidative damage to various biological macromolecules including DNA, lipids, and proteins, thereby altering several signaling pathways that ultimately promote cellular damage and death. There is evidence indicating that ginsenoside Rd exerts antioxidant effects in kidney injury models and in senescence-accelerated mice. In the central nervous system, ginsenoside Rd was reported to be effective in decreasing ROS formation in cultured astrocytes, but its antioxidant properties in neuron-like cells have not been established in the literature. Therefore we examined the protective role and mechanism of ginsenoside Rd against oxidative stress induced by H₂O₂ in cultured PC12 cells.

MATERIALS AND METHODS

Materials Ginsenoside Rd with a purity of 98% was obtained from Tai-He Biopharmaceutical Co., Ltd. (Guangzhou, China). Ginsenoside Rd stock solutions were prepared in saline containing 10% 1,3-propanediol (v/v). Propidium iodide (PI), Hoechst 33342, α-tocopherol, rhodamine 123 (Rh 123), and 2,7-dichlorofluorescein diacetate (DCFH-DA) were purchased from Sigma-Aldrich Inc. (St. Louis, MO, U.S.A.). The commercial kits for the detection of lactate dehydrogenase (LDH), malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GPX) were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All other reagents were from commercial suppliers and of standard biochemical quality.

Cell Culture Rat PC12 pheochromocytoma cells were kindly provided by the PLA Institute of Neurosciences,

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Fourth Military Medical University. PC12 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum in a humidified atmosphere of 5% CO2 at 37 °C. To produce oxidative stress, H2O2 was freshly prepared from 3% stock solution immediately before use. PC12 cells at a density of 1×10^5/cm² were treated with H2O2 400 μM in the presence of ginsenoside Rd (1—50 μM) or vehicle (saline containing 10% 1,3-propanediol) for 24 h at 37 °C. The control cells were treated with ginsenoside Rd or vehicle without H2O2. α-Tocopherol was used as the reference antioxidant compound.

**RESULTS**

**Ginsenoside Rd Ameliorated H2O2-Induced Cytotoxicity** First, it was demonstrated that ginsenoside Rd alone does not induce cytotoxicity in PC12 cells (Fig. 2A). Cells were treated with ginsenoside Rd at different concentrations (1—50 μM) or vehicle and cell viability was determined in the LDH assay.

Second, the effect of ginsenoside Rd on H2O2-induced PC12 cell cytotoxicity, as shown in the LDH assay, demonstrated that LDH leakage increased to 53.2% after treatment with H2O2 for 24 h compared with the control group. Ginsenoside Rd (1, 10 μM) significantly attenuated H2O2-induced cell death, reducing the LDH leakage from 54.1% (vehicle-treated group) to 35.5% and 27.6%, respectively, whereas ginsenoside Rd 50 μM did not exert any protective effect. α-Tocopherol, a potent antioxidant, at 50 μM also significantly attenuated this increase in LDH release. There was no significant difference between ginsenoside Rd and α-tocopherol (Fig. 2B).

We next evaluated cell injury through Hoechst 33342/PI staining. As illustrated in Fig. 3, when ginsenoside Rd (1,
as added to the cultures, the survival rate was elevated from 59.5% to 77.4% and 85.5%, respectively. The protective effects of ginsenoside Rd or \( \alpha \)-tocopherol were similar to those determined in the LDH assay.

### Ginsenoside Rd Ameliorated H\(_2\)O\(_2\)-Induced Oxidative Stress

When PC12 cells were incubated with H\(_2\)O\(_2\) 400 \( \mu \)M for 24 h, a 1.61-fold increase in intracellular ROS was found using the DCFH-DA assay. However, coincubation with ginsenoside Rd (1, 10 \( \mu \)M) significantly decreased ROS production in comparison with the vehicle-treated group (Fig. 4).

Figures 5 and 6 show that H\(_2\)O\(_2\) markedly increased the level of the lipid peroxidation product (MDA) and decreased antioxidant enzymatic activities (SOD, GPX) in the cultures. However, ginsenoside Rd (1, 10 \( \mu \)M) treatment resulted in a noticeable reduction of the MDA content and elevation in the activities of SOD and GPX in comparison with the vehicle-treated group. Additionally, the activity of SOD and GPX in the normal cultured PC12 cells treated with ginsenoside Rd for 24 h showed no significant difference compared with the control group.

### Ginsenoside Rd Attenuated H\(_2\)O\(_2\)-Induced Dissipation of the MMP

We further assessed the effect of ginsenoside Rd on the mitochondrial depolarization induced by H\(_2\)O\(_2\). As shown in Fig. 7, after 24-h exposure to H\(_2\)O\(_2\), the fluorescence intensity of Rh 123 in PC12 cells was rapidly reduced, representing a dissipation of the MMP. However, the MMP decrease recovered after incubation with ginsenoside Rd (1, 10 \( \mu \)M).
can exert neuroprotective effects against \( \text{H}_2\text{O}_2 \)-induced oxidative stress in PC12 cells. Concurrent treatment with ginsenoside Rd inhibits intracellular ROS formation, reduces the level of the lipid peroxidation product (MDA), and maintains cellular antioxidant activity (SOD and GPX). When cells were only exposed to exogenous \( \text{H}_2\text{O}_2 \), the DCF fluorescence significantly increased. Although a small part of \( \text{H}_2\text{O}_2 \) may be scavenged by cellular antioxidant enzymes, it can directly cause oxidation of various intracellular targets including the fluorescence probe DCFH-DA. The formation of hydroxyl radicals mediated by intracellular heavy metal ions could also contribute to the increased DCF fluorescence in response to \( \text{H}_2\text{O}_2 \). These results suggest that ginsenoside Rd exerts its antioxidant effects in the intracellular compartment. Ginsenosides are steroid-like molecules that have a gonane steroid nucleus with different sugar moieties attached. Many reports suggested that ginseng saponins are capable of accessing intracellular locations thanks to their steroid-like structures, justifying their ability to attenuate the oxidative stress caused by diverse stimuli.\(^{25,26}\) The chemical structure of ginsenoside Rd (sugar moiety attached to the 20-position of the triterpene dammarane) may possibly contribute to its direct antioxidant property.\(^{26}\) Scavenging of ROS may also occur via recruitment of the endogenous antioxidative system, such as induction of SOD and GPX activities by ginsenoside Rd. Alternatively, a possible direct scavenging of \( \text{H}_2\text{O}_2 \) by ginsenoside Rd during the incubation period cannot be ruled out. However, the antioxidant action was also found in other cellular models,\(^{14–16}\) and the concentrations of ginsenoside Rd required for neuroprotection are far lower than those of \( \text{H}_2\text{O}_2 \) used in the assay, suggesting that it may not be a simple stoichiometric reaction.

The antioxidant activity of ginsenoside Rd was observed in this study at doses of 1—10 \( \mu \text{M} \), whereas ginsenoside Rd 50 \( \mu \text{M} \) did not show protective effects. Lopez et al. previously evaluated individual ginsenosides in primary astrocyte cultures using an oxidative stress model with \( \text{H}_2\text{O}_2 \) and found that ginsenoside Rd decreased ROS formation at the dose of 5—100 \( \mu \text{M} \).\(^{27}\) The reason for this discrepancy may be the different cell cultures used. Different cell types have different functions that are determined by their genetic codes and enzyme content. Because of that, the responses to different stimuli depend on the function for which they are naturally prepared. The PC12 cells used in this research are clonal cells derived from rat pheochromocytoma. Treatment with nerve growth factor induces the differentiation of PC12 cells into a sympathetic neuron-like phenotype.\(^{27}\) It has been widely used as a model for neurobiologic, neuropharmacologic, and neurotoxicologic studies. The response of PC12 cells to ginsenoside Rd may not be exactly the same as that observed in other cells.

In addition to producing an increase in ROS and consequent lipid peroxidation, \( \text{H}_2\text{O}_2 \) exposure can cause an elevation of intracellular \( \text{Ca}^{2+} \) levels. The occurrence of large increases in intracellular \( \text{Ca}^{2+} \) represents a detrimental insult from oxidative stress imposed by ROS in the cells. Sustained elevated \( \text{Ca}^{2+} \) levels in cells may impair mitochondrial function and activate phospholipase, protease, and endonucleases leading to irreversible membrane, organelle, and chromatin damage and eventually to cell death. Therefore \( \text{Ca}^{2+} \) plays an important role in the development of oxidative injury.\(^{28}\) Re-
cently, it has been shown that ginsenoside Rd inhibits Ca\(^{2+}\) entry through receptor-operated and store-operated Ca\(^{2+}\) channels.\(^1\) This may possibly provide an explanation for the neuroprotection of ginsenoside Rd against H\(_2\)O\(_2\).

The protective effect of ginsenoside Rd was also supported by the higher MMP. Mitochondria are not only susceptible targets of free radical-mediated damage but also play a crucial role in cellular ROS production.\(^2\)\(^9\) The MMP reflects the performance of the electron transport chain and can indicate a pathologic disorder of this system. Dissipation of the MMP leads to apoptosis, high average MMP is related to higher viability, and vice versa.\(^10\) We used the cationic fluorescent dye Rh 123, which is sequestered in mitochondria based on the highly negative MMP and released upon mitochondrial depolarization.\(^11\)\(^12\) We found that H\(_2\)O\(_2\) led to mitochondrial membrane depolarization. Ginsenoside Rd prevented the loss of the MMP, suggesting that the electron transport chain was attenuated PC12 cell injury. Although more detailed mechanistic studies are necessary to clarify the neuroprotection of ginsenoside Rd, ginseng remains one of the top-selling natural species that have already had thousands of years of human exposure with little reported toxicity. Recent surveys have indicated that ginseng remains one of the top-selling natural product remedies in the U.S.A.\(^14\)\(^15\) Additionally, ginsenoside Rd is highly lipophilic and can easily diffuse across biological membranes and the blood-brain barrier.

In conclusion, ginsenoside Rd not only decreases oxidative stress-induced ROS overproduction and lipid peroxidation, but also maintains endogenous antioxidant enzymatic activities, stabilizes mitochondrial function, and subsequently attenuates PC12 cell injury. Although more detailed mechanistic studies are necessary to clarify the neuroprotection of ginsenoside Rd fully, these results should encourage further studies on neurologic diseases to explore the potential neuroprotective effects of ginsenoside Rd.

REFERENCES