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Ginsenoside Rg3 from Red Ginseng Prevents Damage of Neuronal Cells through the Phosphorylation of the Cell Survival Protein Akt

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Abstract Neuronal cell death significantly contributes to neuronal loss in neurological injury and disease. Typically, neuronal loss or destruction upon exposure to neurotoxins, oxidative stress, or DNA damage causes neurodegenerative diseases such as Alzheimer's disease. In this study, we attempted to determine whether ginsenoside Rg3 from red ginseng has a neuroprotective effect via an anti-apoptotic role induced by S-nitroso-N-acetylpenicillamine (SNAP) at the molecular level. We also investigated the antioxidant effect of Rg3 using a metal-catalyzed reaction with Cu^{2+}/H_2O_2. Our results showed that Rg3 (40-100 μg/mL) protected SK-N-MC neuroblastoma cells under cytotoxic conditions and effectively protected DNA from fragmentation. In the signal pathway, caspase-3, and poly (ADP-ribose) polymerase (PARP) were kept at an inactivated status when pretreated with Rg3 in all ranges. In particular, the important upstream p-Akt signal pathway was increased in a dose-dependent manner, which indicates that Rg3 may contribute to cell survival. We also found that oxidative stress can be mitigated by Rg3. Therefore, we have concluded that Rg3 plays a certain role in neurodegenerative pathogenesis via an anti-apoptotic, antioxidative effect.

Keywords: ginsenoside Rg3, apoptosis, neurodegenerative disease, NO, Akt

Introduction

Apoptosis, or programmed cell death, is a normal component of the development and health of multicellular organisms. Cells die in response to a variety of stimuli; during apoptosis they do so in a controlled, regulated fashion. This makes apoptosis distinct from another form of cell death, called necrosis, in which uncontrolled cell death leads to cell lysis, inflammatory responses, and other potentially serious health problems. In contrast, apoptosis is a process in which cells play an active role in their own deaths. Diseases in which apoptosis have been involved can be divided into two groups: inhibitive apoptosis and hyperactive apoptosis (1-3).

Neurodegenerative diseases, such as Alzheimer's disease (AD) and Parkinson's disease, are associated with selective neuronal apoptosis. This neuronal death appears to be associated with increased susceptibility to apoptosis and oxidative stress. Mitochondrial defects and neurotoxic agents have been postulated as the inductors of neuronal death, which involves activation of caspase signal pathways (4). In particular, AD, the most common cause of dementia in people over 65 years of age, is associated with senile plaque and neurofibrillary tangles. However, the relationship between the two neuropathogenic lesions has not yet been elucidated (5).

Although several approaches to neuronal death have been identified to be associated with diseases such as AD, an examination of the enzymes involved in the mediation of programmed cell death can provide deeper molecular evidence in regards to apoptotic AD. Upstream or downstream regulation of caspase signal pathways can also be a promising target for AD treatment, while beta amyloid peptides (Aβ) initiate the activation of an apoptotic mechanism through the extrinsic or intrinsic pathway (6-8). With respect to the enzymes that are involved in the protection of cells against programmed cell death, the PI3-K/Akt pathway is known as a key player in the prevention of apoptosis. It is also known that its downstream effectors are necessary for the survival of a number of cell types, including neurons (9-10). The cause of neurodegeneration in AD remains controversial, but it is believed that Aβ might induce apoptosis by interacting with neuronal receptors (11). These receptor interactions might activate several different cell-death signaling pathways, including the caspase activation pathway (12).

In the arena of research of promising neuroprotective agents from natural sources, a family of ginsenosides has been broadly investigated (13-16). It has suggested that ginsenosides may be potential therapeutic agents for neurological diseases, including AD (17-18). In previous studies, we reported that Rg3 has a potential effect in enhancing the phagocytosis of Aβ peptides through microglia. Additionally, panaxadiols and panaxatriols can provide a synergic outcome in cell protection as both of these ginsenosides were focused against AD (19-20). In the present study, we examined the anti-apoptotic properties at the molecular level in neuroblastoma cells, mainly focusing on the anti-apoptotic protein, Akt. The purpose of our study was to evaluate whether ginsenoside Rg3 can contribute to the cell death signaling pathways of neurons, so that neurodegenerative diseases such as AD can be pre-regulated prior to aggravation.

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Materials and Methods

Cell culture The SK-N-MC neuroblastoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM,

GibcoBRL, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (GibcoBRL) at 37°C in 5% CO_2, 95% air in a humidified cell incubator.

Treatment with SNAP and Rg3 S-nitroso-N-acetylpenicillamine (SNAP) was synthesized as described previously (21). Cells were treated with SNAP for 24 hr, and Rg3 was added 10 hr prior to treatment with SNAP.

Cell viability assay The 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was performed as follows. SK-N-MC cells were cultured in each well of a 96-well plate (Corning Inc., Corning, NY, USA) at a density of 5×10^3 cells per well. The cells were treated with varying concentrations of SNAP, an nitric oxide (NO) donor, and with or without Rg3 pretreatment. After 24 hr, the cells were washed and treated with MTT. The plate was incubated in the dark for 4 hr, and absorbencies were measured at 570 nm using a microtiter plate reader (Bio-Tek, Winooski, VT, USA). In order to determine cell viability, percent viability was calculated as (absorbance of drug-treated sample/control absorbance)×100.

DNA fragmentation analysis Cell pellets were resuspended in 750 μL of lysis buffer (20 mM Tris-HCl, 10 mM EDTA, and 0.5% Triton X-100, pH 8.0) and left on ice for 45 min with occasional shaking. DNA was extracted with phenol/chloroform and precipitated with alcohol. The precipitate was dried and resuspended in 100 μL of 20 mM Tris-HCl, pH 8.0. After degradation of RNA with RNase (0.1 mg/mL) at 37°C for 1 hr, samples (15 μL) were electrophoresed on a 1.2% agarose gel in 450 mM Tris borate-EDTA buffer (TBE, pH 8.0) and photographed under UV light.

Western blot analysis Cells were harvested, washed two times with ice-cold phosphate buffered saline (PBS), and homogenized on ice in 2.5 mL TKM buffer (50 mM Tris-HCl, pH 7.5, 25 mM KCl, 0.32 M sucrose, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 1 mM phenyl methyl sulfonyl fluoride (PMSF), 1 mM Na_3VO_4, and 2 mM NaF). The homogenate was centrifuged at 600×g for 10 min. The supernatant was centrifuged again at 7000×g for 10 min. Then, cytosolic proteins were prepared for Western blot analysis of caspase-3, poly (ADP-ribose) polymerase (PARP), Akt, p-Akt, Bcl-2, and cytochrome c. Protein concentration was determined using the bovine serum albumin (BCA) assay (Sigma, St. Louis, MO, USA). Proteins (40 μg) were separated on an 8% SDS-PAGE gel for analysis of poly (ADP-ribose)polymerase (PARP) or a 12% SDS-PAGE gel for analysis of caspase-3, Bcl-2, Bax, cytochrome c, p-Akt, and Akt. The proteins were then transferred to a nitrocellulose membrane. The membrane was hybridized with PARP antibody (PharMingen, San Diego, CA, USA) (1:1000), caspase-3 antibody (Transduction Laboratories, Lexington, KY, USA) (1:500), Bcl-2 and Bax antibodies (Transduction Laboratories, (1:1000), cytochrome c antibody (PharMingen) (1:1000), p-Akt and Akt antibodies (Cell Signaling Technology, Beverly, MA, USA) and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as a internal control. Protein bands were visualized by exposure to X-ray film.

Metal-catalyzed oxidation of bovine serum albumin (BSA) Hydroxyl radical-mediated oxidation experiments were performed using a metal-catalyzed reaction. BSA (Sigma) was dissolved in 150 mM phosphate buffer (pH 7.3) at a final concentration of 0.5 mg/mL. The liquid BSA was incubated with or without 100 μM copper (Cu^{2+}) and 2.5 mM hydroperoxide (H_2O_2) in the presence or absence of Rg3 for the amount of time indicated. 50 mg of ascorbate was directly dissolved in 1X PBS and used as a control antioxidant. Reactions were carried out in open tubes placed in a shaking water bath maintained at 37°C. 1 mg/mL of Rg3 was prepared by dissolving in 10% dimethyl sulfoxide (DMSO), and was then diluted to the final reaction concentration in order to minimize false readings.

Statistical analysis Data are presented as mean ± SEM. of at least three separate experiments. A statistical comparison between the values was analyzed using one-way ANOVA with Dunnet's test. A p value <0.05 was considered statistically significant.

Results and Discussion

Cytoprotective effect of Rg3 in SNAP-induced cell death First, we studied the effects of Rg3 on mitigating the potential neurotoxic effects of NO. SK-N-MC cells were treated with SNAP in order to expose cells to NO. In a preliminary study, we found that cell death was induced in SK-N-MC cells when treated with 1 mM of SNAP for 24 hr. As shown in Fig. 1A, cell death induced by SNAP was inhibited at all ranges of Rg3 concentration (40, 60, 80, 100 μg/mL) in a dose-dependent manner. Moreover, DNA fragmentation by SNAP also was not detected in the presence of Rg3 (Fig. 1B). These results suggest that Rg3 can inhibit cell death induced by NO treatment.

Anti-apoptotic effect of Rg3 As apoptotic cell death in various cell lines can be induced by NO, SK-N-MC cells were treated with SNAP in order to induce apoptotic cell death. Fig. 2A shows that caspase-3, a major enzyme for apoptotic cell death, can be activated by 1 mM SNAP. However, this cell death was inhibited when treated with Rg3 at all concentrations. A line-up study was performed with PARP, a DNA repair enzyme found in the nucleus, and the result showed that Rg3 plays a role in inhibiting the cleavage of PARP through caspase-3 (Fig. 2B). Intracellular cytochrome c accelerates cell death when released from mitochondria. However, Rg3 inhibited the release of cytochrome c (Fig. 3A). The level of Bcl-2 in the mitochondrial membrane, which is related to cell survival, increased upon treatment with Rg3, while Bcl-2 decreased upon treatment with SNAP (Fig. 3B). In order to determine whether Rg3 contributes in the upstream signal pathway, we examined Akt activation (Akt phosphorylation). The results indicated that Rg3 increased the p-Akt level in a dose-dependent manner (Fig. 4).

Antioxidant effect of Rg3 In order to determine if Rg3 possesses antioxidant activity, we performed experiments

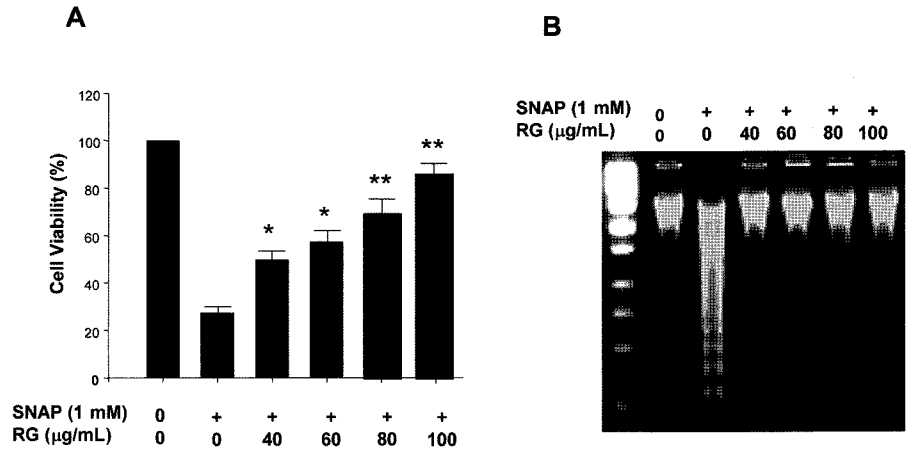

Fig. 1. Protective effect of Rg3 against SNAP-induced death of SK-N-MC cells. SK-N-MC cells were incubated in DMEM containing 10% fetal bovine serum without or with Rg3 for 10 hr before treatment with SNAP for 24 hr. (A) MTT assay was performed. (B) DNA fragmentation determined by gel electrophoresis. Significant statistical differences are shown for comparison with control (*$p< 0.05$, **$p <0.01$).

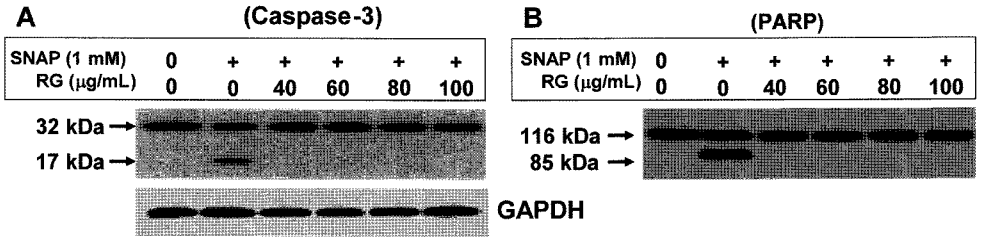

Fig. 2. Inhibition of caspase-3 activity by Rg3. (A) Western blot analysis of caspase-3 fragmentation, (B) Western blot analysis of PARP fragmentation.

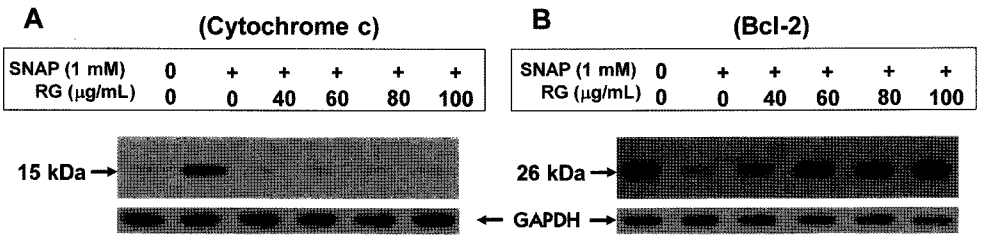

Fig. 3. Western blot analyses of cytochrome c (A) and Bcl-2 (B) expression. Induction of Bcl-2 expression and the resultant suppression of the release of cytochrome c by Rg3 treatment were observed in cytosol.

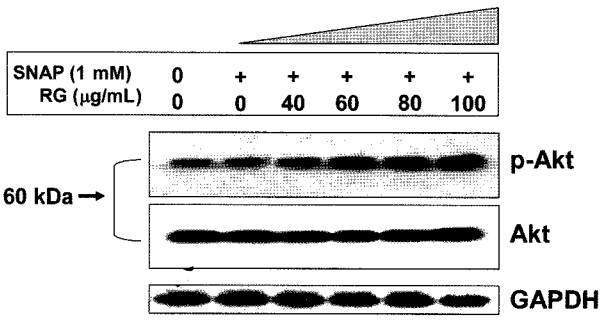

Fig. 4. Western blot analyses of p-Akt and Akt. Induction of p-Akt and the resultant expression of Bcl-2 by Rg3 treatment were observed.

to determine the amount of protein damage induced by the metal-catalyzed reaction with Cu^{2+}/H_2O_2 in the presence of varying concentrations of Rg3. Figure 5 shows that rapid degradation occurs when BSA is incubated in the presence of Cu^{2+}/H_2O_2, and half of the protein is structurally damaged after approximately 1 hr. Our results indicate that all ranges of Rg3 from 10 to 100 mg/mL prevent Cu^{2+}/H_2O_2-induced BSA degradation and have antioxidant effects. This result also suggests that Rg3 may be a hydroxyl radical scavenger that protects DNA and lipids from free radical attack.

Apoptosis as a mechanism for neuronal death in AD, and has been a subject of interest in recent years with the growing recognition that apoptotic mechanisms may play a role in disease pathogenesis in the absence of overt apoptosis (22). In view of the apoptotic signal pathways, a ginseng-derived Rg3 may affect the pathogenesis of neurodegenerative diseases such as AD, which may possibly be caused by the escalation of reactive oxygen species, pro-inflammatory factors, and neurotoxic substances (23).

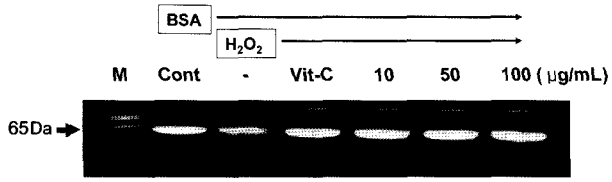

Fig. 5. Gel electrophoresis of BSA protein after treatment with copper (Cu^{2+}) and hydroperoxide (H_2O_2) in the presence of Rg3 from 10 to 100 μg/mL and control antioxidant ascorbate (50 μg). After 1 hr incubation at 37°C, the reaction mixture was separated on 12% SDS-PAGE gel and stained using coomassie blue.

In other words, our study suggests that Rg3 treatment in neuronal cells may result in cell survival under a stimulative environment, such as NO treatment in cells. This was evidenced by the attenuation of caspase-3 (Fig. 2A), PARP (Fig. 2B), and cytochrome c (Fig. 3A). In addition, the cell survival promoting protein, Bcl-2, was increased in the presence of Rg3 (Fig. 3B). Interestingly, Akt/PI-3 kinase, a component in the upstream anti-apoptotic signal pathway, was regulated by Rg3 treatment (elevation of p-Akt). These results suggest that an increase of Akt phosphorylation may be followed by the inhibition of caspase-3 activation through Rg3. Moreover, NO, which has known to be associated with AD pathogenesis (23), was attenuated according to its stimulative property. This may extend the therapeutic concept into neuroprotection by the anti-inflammatory effect of Rg3. In conclusion, although these results (signal pathway and hydroxyl radical scavenging) indicate that Rg3 prevents the apoptosis of neuronal cells, a process which causes neurodegenerative disease, more advanced and *in vivo* studies are necessary for a future clinical use.

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