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Neuroprotective Effects of Ginsenoside Rg_3 against 24-OH-cholesterol-induced Cytotoxicity in Cortical Neurons

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Ginsenoside Rg_3 (Rg_3), one of the active ingredients in *Panax ginseng*, attenuates NMDA receptor-mediated currents *in vitro* and antagonizes NMDA receptors through a glycine modulatory site in rat cultured hippocampal neurons. In the present study, we examined the neuroprotective effects of Rg_3 on 24-hydroxycholesterol (24-OH-chol)-induced cytotoxicity *in vitro*. The results showed that Rg_3 treatment significantly and dose-dependently inhibited 24-OH-chol-induced cell death in rat cultured cortical neurons, with an IC_{50} value of 28.7±7.5 μm. Furthermore, the Rg_3 treatment not only significantly reduced DNA damage, but also dose-dependently attenuated 24-OH-chol-induced caspase-3 activity. To study the mechanisms underlying the *in vitro* neuroprotective effects of Rg_3 against 25-OH-chol-induced cytotoxicity, we also examined the effect of Rg_3 on intracellular Ca^{2+} elevations in cultured neurons and found that Rg_3 treatment dose-dependently inhibited increases in intracellular Ca^{2+}, with an IC_{50} value of 40.37±12.88 μm. Additionally, Rg_3 treatment dose-dependently inhibited apoptosis with an IC_{50} of 47.3±14.2 μm. Finally, after confirming the protective effect of Rg_3 using a terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling assay, we found that Rg_3 is an active component in ginseng-mediated neuroprotection. These results collectively indicate that Rg_3-induced neuroprotection against 24-OH-chol in rat cortical neurons might be achieved via inhibition of a 24-OH-chol-mediated Ca^{2+} channel. This is the first report to employ cortical neurons to study the neuroprotective effects of Rg_3 against 24-OH-chol. In conclusion, Rg_3 was effective for protecting cells against 24-OH-chol-induced cytotoxicity in rat cortical neurons. This protective ability makes Rg_3 a promising agent in pathologies implicating neurodegeneration such as apoptosis or neuronal cell death.

Keywords: Ginsenoside Rg_3, Cortical neurons, 24-OH-cholesterol oxides, Excitotoxicity, Neuroprotection

INTRODUCTION

Cholesterol and oxidized lipoproteins have been associated with the genesis of diseases [1], and cholesterol oxides (also termed oxysterols), the oxygenated derivatives of cholesterol, might be causative agents [2-7]. An excessive amount of cholesterol oxides damages endothelial cells [8,9], smooth muscle cells [2,10], and fibroblasts [11,12], and accumulating evidence suggests that cholesterol oxides are toxic to neural cells in nerve growth factor-differentiated neuronal PC12 cells as a model for sympathetic neurons [13,14], cultured cerebellar granule cells [1], and microglial cells [15]. Although the molecular mechanisms by which cholesterol

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oxides impair neuronal cells and promote neuropathy are still not clearly understood, it is believed that a high concentration of cholesterol oxides is the main cause of the neurodegenerative injury by inducing increased oxidative stress and decreasing cell viability [1,15]. The conversion of cholesterol into the polar metabolite 24-hydroxycholesterol (24-OH-chol) appears to be one of the most important mechanisms for eliminating cholesterol from the brain, where concentrations of up to 30 μm free 24-OH-chol are present and act as a neurotoxin [16].

Panax ginseng, which is a tonic in traditional medicine, contains more than 30 types of active ingredients, the ginsenosides [17]. Recently, ginsenosides have been shown to have protective effects against glutamate toxicity-induced neuronal and glial cell death. In rat cortical cultures, the ginsenosides Rb1 and Rg_3 attenuate glutamate- and NMDA-induced neurotoxicity by inhibiting nitric oxide overproduction and malondialdehyde formation [18,19]. Ginsenoside Rb1 and Rg1 protect spinal neurons from glutamate- or kainite-induced excitotoxicity. In addition to neurons, Seong *et al.* [20] showed that ginsenosides attenuate glutamate-induced swelling in cultured rat astrocytes. Intracerebroventricular administration of ginsenoside Rb1 significantly inhibits the magnitude of long-term potentiation induced by strong tetanus in the dentate gyrus [21]. Furthermore, intrathecal ginsenoside pretreatment attenuates NMDA- or substance P-induced nociceptive behaviors in mice [22], and intraperitoneal ginsenoside pretreatment attenuates kainite-induced cell death in hippocampal neurons of rats [23]. Kim *et al*. [24] suggested that the neuroprotection of hippocampal neurons by ginsenoside against glutamate- or NMDA-induced excitotoxicity might be due to attenuation of the intracellular Ca^{2+} elevation and that among various ginsenosides, ginsenoside Rg_3 (Rg_3) is the most potent inhibitor of NMDA-induced intracellular Ca^{2+} elevation in hippocampal neurons. Furthermore, oral or intravenous administration of Rg_3 exhibits significant neuroprotective effects against focal cerebral ischemic injury in rats. However, it is not yet known whether Rg_3 exhibits a neuroprotective effect on 24-OH-chol-induced neurotoxicity *in vitro* [25,26]. Therefore, in the present study, we investigated whether Rg_3 treatment could attenuate 24-OH-chol-induced neurotoxicity such as a decrease in cell viability and changes in intracellular Ca^{2+}, oxidative stress, and apoptosis in rat cortical neurons.

Our data showed that Rg_3 greatly contributed to the neuroprotective effects on 24-OH-chol-induced neurotoxicity, partly through its ability to inhibit changes in intracellular Ca^{2+}, resulting in increased cell viability and reduced apoptosis.

MATERIALS AND METHODS

Materials

Fig. 1 shows the chemical structure of 20(*S*)-Rg_3, which was kindly provided by the Korea Ginseng Cooperation (Daejon, Korea). All chemicals for rat cortical cell culture were purchased from Life Technologies (Grand Island, NY, USA). 24-OH-chol and all other chemical agents were purchased from Sigma (St. Louis, MO, USA), unless otherwise specified.

Preparation of cortical neurons

Mixed cortical cell cultures containing both neuronal and glial elements were prepared as previously described [27] from fetal mice at 14 to 17 day gestation. Dissociated cortical cells were plated in Primaria (Falcon) 15-mM multiwell vessels (approximately 0.4×10^6 cells/well) in Eagle's minimal essential medium (Earle's salts) supplemented with 10% heat-inactivated horse serum, 10% fetal bovine serum, 2 mM glutamine, and l0 to 20 mM glucose. Cultures were maintained at 37°C in a humidified CO-containing incubator (initial pH 7.4). After 5 to 10 days, non-neuronal cell division was halted by exposure to 10^{-5} M cytosine arabinoside for 1 day, and the cells were transferred to a maintenance medium identical to the plating media but lacking fetal serum. Subsequent media replacement was performed twice per week. Only mature (14 to 24 days *in vitro*) cortical cultures were selected for study; key comparisons were made on sister cultures derived from a single plating.

Measurement of cell death

Metabolic inhibition- or mitochondrial dysfunction-

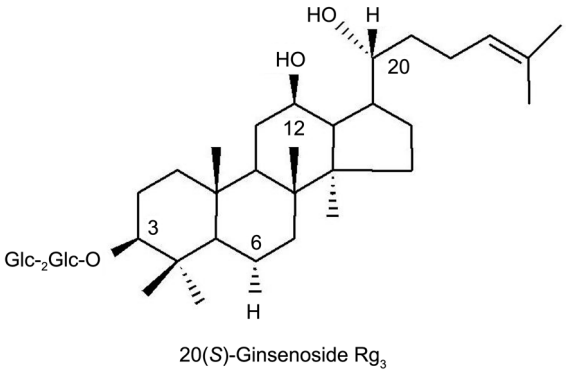

Fig. 1. Chemical structure of ginsenoside Rg_3. Various ginsenosides exist. They differ in the three side chains attached to a common steroid ring. Side chains for 20(*S*)-ginsenoside Rg_3 are R_1, -Glc_2-Glc, R_2, -H, and R_3, -OH.

induced cell death was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [28]. Cortical cells were washed with serum-free medium and then incubated with 50 μm 24-OH-chol alone or in the presence of various concentrations of Rg_3 for 24 hours at 37°C. Cortical cells were pretreated with Rg_3 for 1 hour before exposure to 24-OH-chol. After a 24-hour incubation in serum-free culture medium, the cultures were assessed for viability. Cell viability was measured by detecting dehydrogenase activity retained in living cells using the MTT assay. An aliquot (50 μL) of MTT solution (1 mg/mL) in PBS was added directly to the cells, and the cells were incubated for 4 hours to allow the MTT to metabolize to formazan. The supernatant was then aspirated, and 100 μL of DMSO was added to dissolve the formazan. The optical density was measured with an automated spectrophotometric plate reader at a wavelength of 560 nm. Relative survival in comparison with untreated controls was then determined. In all cases, the Rg_3 solutions were freshly prepared, and the final DMSO concentrations were <0.1%. DMSO did not have any detectable effect on cell survival in the vehicle-only group [29].

Measurements of intracellular Ca^{2+} concentration

The acetoxymethyl-ester form of fura-2 (fura-2/AM; Molecular Probes, Eugene, OR, USA) was used as the fluorescent Ca^{2+} indicator. Cortical cells were incubated for 40 to 60 minutes at room temperature with 5 μm fura-2/AM and 0.001% pluronic F-127 in a HEPES-buffered solution composed of (in mM): 150 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, and 10 glucose, with pH adjusted to 7.4 with NaOH. The cells were then washed with HEPES-buffered solution and placed on an inverted microscope (Olympus, Tokyo, Japan). Cells were illuminated using a xenon arc lamp, and excitation wavelengths (340 and 380 nm) were selected by a computer-controlled filter wheel (Sutter Instruments, Novato, CA, USA). Data were acquired every 2 to 5 seconds, and a shutter was interposed in the light path between exposures to protect the cells from phototoxicity. Emitter fluorescence was reflected through a 515 nm long-pass filter to a frame transfer-cooled CCD camera, and the ratios of emitted fluorescence were calculated using a digital fluorescence analyzer and converted to intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$). Data were collected and analyzed using Universal Imaging software (West Chester, PA, USA) [24].

Intracellular malondialdehyde measurement

Malondialdehyde (MDA), a cell lipid peroxidation product, was assayed using the thiobarbituric acid fluorometric method [30] with 515 nm excitation/552 nm emission wavelengths and 1,1,3,3-tetramethoxypropane as the standard. Briefly, cortical cells were harvested and homogenized with 0.5% Triton X-100. The reaction mixture containing 300 mL of TCA buffer (0.25 N HCl/0.375% thiobarbituric acid/15% trichloroacetic acid/0.01% butyl hydroxytoluene) was mixed with cell lysate. After centrifugation, the supernatant was incubated in a shaking water bath at 85°C for 30 minutes, an equal amount of *n*-butanol was added, and the fluorometric measurement was performed. The protein concentration was determined by Lowry's method.

TUNEL staining to detect DNA fragmentation

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) was conducted using the In Situ Cell Death Detection kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions, with minor modifications [29]. Briefly, cells were cultured on coverslips and divided into groups receiving control vehicle, 100 μm Rg_3 alone, 50 μm 24-OH-chol alone, and 100 μm Rg_3 + 50 μm 24-OH-chol. An Rg_3 dose of 100 μm was determined to be the optimum for cell viability. One hour later, the 24-OH-chol alone group and the Rg_3+24-OH-chol group were treated with 50 μm 24-OH-chol. After a 24-hour incubation, the cells were washed with PBS, fixed with 4% paraformaldehyde in PBS for 30 minutes, and permeabilized with 0.25% Triton X-100 for 10 minutes. The cells were rinsed with PBS and covered with a labeling reaction mixture containing terminal deoxynucleotidyl transferase for 60 minutes at 37°C. After three rinses with PBS, peroxidase was added and the cells were incubated for 30 minutes at 37°C, followed by three more rinses with PBS and a 10-minutes incubation with diaminobenzidine. Finally, the reactions were terminated by washing the cells with PBS, and the slides were mounted with glass coverslips. In each treatment group, 400 cells from 7 to 8 different fields (including TUNEL-negative and -positive neurons) were counted using an Olympus BX-51 light microscope (Olympus) at 40× magnification.

Caspase-3 activity assay

To confirm the results of the TUNEL assay, a second apoptosis marker (caspase-3 activity) was examined by immunocytochemistry. Briefly, cortical neurons were prepared in 24 well plates as described previously [31]. After 7 days, the cortical cells were washed with PBS (pH 7.4), blocked with 4% normal goat serum (Jackson

Immuno Research, West Grove, PA, USA) for 90 minutes, and incubated overnight at 4°C with a primary polyclonal antibody against active caspase-3 (1:250; R&D Systems, Minneapolis, MN, USA). The negative control group was treated identically, but was incubated with buffer only or with normal rabbit serum. Cultured cells from each group were rinsed and incubated for 1 hour with FITC-conjugated AffinPure goat anti-rabbit IgG (1:100; Dako, Glostrup, Denmark) and examined by epifluorescence using a Zeiss Axioplan 2 microscope (Carl Zeiss, Inc., Hamburg, Germany) equipped with a Spot camera and Spot software ver. 2.2 (Diagnostic Instruments, Sterling Heights, MI, USA). An analysis of variance was used to determine the significant differences in fluorescence between the groups.

Data analysis

All numerical values are represented as means±SEMs. Tests for statistical significance were performed on the data using the unpaired Student's *t*-test. A *p*-value of <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Effects of ginsenoside Rg_3 on cortical neuron viability

Because 50 μm 24-OH-chol induces neurotoxicity [16], we first examined the preventive effect of Rg_3 on 24-OH-chol-induced cytotoxicity using the MTT assay. Exposure of primary cultured cortical cells to 50 μm 24-OH-chol for 24 hours resulted in metabolic suppression, as shown by decreased mitochondrial reducing capacity on MTT, and cell viability decreased significantly by 45.88±4.27% (**$p<0.01$, compared with control) (Fig. 2). Pretreatment of cortical cells with Rg_3 1 hour before 24-OH-chol treatment dose-dependently increased cell viability to 33.78±6.57%, 42.76±5.78%, 50.38±7.03%, 71.82±12.54%, 78.95±7.73%, and 75.83 ±8.76% in cultures receiving 1, 3, 10, 30, 100, and 300 μm Rg_3, respectively (*$p<0.05$, **$p<0.01$) (Fig. 2). However, no significant differences were observed between groups treated with Rg_3 doses of 1, 3, and 10 μm (Fig. 2). These results indicate that Rg_3 inhibits 24-OH-chol-induced cytotoxicity in rat cortical cells.

Inhibition of intracellular Ca^{2+} levels

We next investigated the mechanism of Rg_3 protection against 24-OH-chol-induced neurotoxicity. We examined whether Rg_3 could inhibit the 24-OH-chol-induced intracellular Ca^{2+} ($[Ca^{2+}]_i$) elevation in cultured cortical

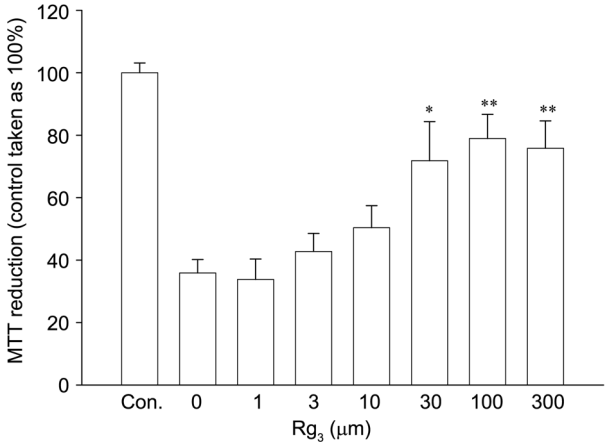

Fig. 2. Effects of ginsenoside Rg_3 (Rg_3) on 24-hydroxycholesterol (24-OH-chol)-induced mitochondrial activity. Cortical cells were pretreated with various concentrations of Rg_3 for 24 hours and then exposed to 24-OH-chol (50 μm) for 60 minutes, and mitochondrial activities were determined by the MTT assay. Values are expressed as percentages of the untreated control. Results are expressed as the means±SEMs from seven separate triplicate experiments. *$p<0.05$, **$p<0.01$, compared with 24-OH-chol treatment alone.

neurons, as acute treatment of 24-OH-chol-induces a slow $[Ca^{2+}]_i$ increase in cultured neurons [16]. We measured the $[Ca^{2+}]_i$ level using a Ca^{2+} imaging technique with fura-2. As shown in Fig. 3, 50 μm 24-OH-chol treatment led to a significant elevation in $[Ca^{2+}]_i$ (data not shown). The percent inhibition by Rg_3 on $[Ca^{2+}]_i$ elevation in cortical neurons was 18.7±4.22%, 17.58±5.35%, 24.67±4.25%, 43.77±12.30%, 68.35±8.63%, and 70.45 ±10.51% at Rg_3 doses of 1, 3, 10, 30, 100, and 300 μm, respectively, with levels in normal controls taken as 100% (Fig. 3). Therefore, treatment with 1, 3, or 10 μM Rg_3 did not attenuate the 24-OH-chol-induced $[Ca^{2+}]_i$ elevation, but 30, 100, and 300 μm Rg_3 significantly and dose-dependently inhibited the $[Ca^{2+}]_i$ elevation (IC_{50}=40.37±12.88 μm; *$p<0.05$, **$p<0.01$, n=30.37) (Fig. 3). Interestingly, 300 μm Rg_3 was slightly less neuroprotective against 24-OH-chol than 100 μm Rg_3, although this difference was not statistically significant (Fig. 3). These results indicate that 50 μm 24-OH-chol elevated $[Ca^{2+}]_i$ in cultured cortical neurons, and this effect was attenuated by Rg_3 concentrations of 30, 100, and 300 μm.

Effects of ginsenoside Rg_3 on intracellular malondialdehyde

Because oxysterols induce oxidative damage in cultured murine neurons [32], we also examined the preventive effect of Rg_3 on 50 μm 24-OH-chol-induced neurotoxicity by determining the MDA content, a compound produced by lipid peroxidation during cellular oxidative stress. The MDA determination was performed

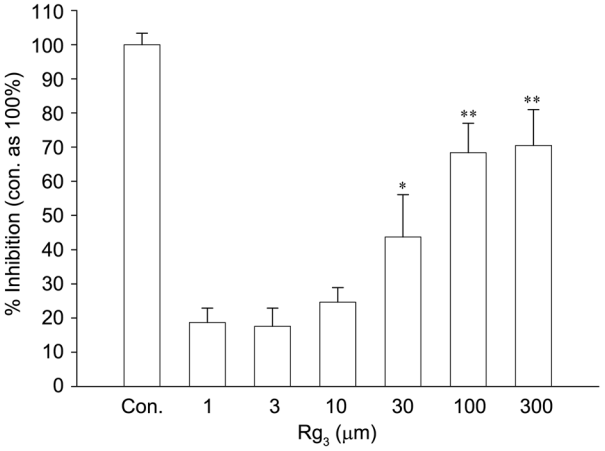

Fig. 3. Effects of ginsenoside Rg_3 (Rg_3) on 50 μm 24-hydroxycholesterol (24-OH-chol)-induced $[Ca^{2+}]_i$ increase in cultured rat cortical neurons. Inhibitory effect of Rg_3 on 24-OH-chol-induced $[Ca^{2+}]_i$ increase using Ca^{2+} imaging techniques. Responses evoked by 24-OH-chol were quantified based on the Rg_3 dose (1–300 mM) to detect the maximal anti-calcium effect. Data are expressed as percentage inhibition compared with a 100% control response. Each bar represents the mean±SEM from seven or eight cells. $*p < 0.05$, $**p < 0.01$, compared with 24-OH-chol treatment alone.

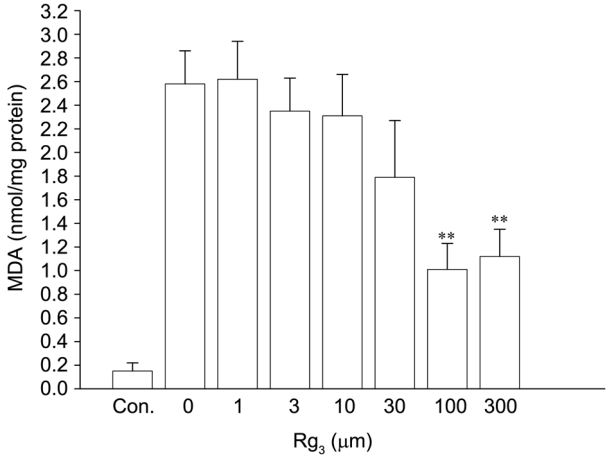

Fig. 4. Effects of ginsenoside Rg_3 (Rg_3) on malondialdehyde (MDA) levels in rat cortical cells. MDA levels were determined in cortical cells after a 24 hour incubation with 50 μm 24-hydroxycholesterol with or without Rg_3. Results are presented as nmol MDA/mg protein. Data are the mean±SEM ($**p < 0.01$, $n = 7–8$).

to estimate the degree of free radical production in the neuron culture. Low levels of MDA were found in the control (0.15±0.07 nmol/mg protein), and these values were considered physiological. In contrast, a significant increase in MDA concentration (2.58±0.28 nmol/mg protein) occurred in the 24 hour 50 μm 24-OH-chol treatment. The MDA concentrations were 2.62±0.32, 2.35±0.28, 2.31±0.35, and 1.79±0.48 nmol/mg protein at Rg_3 doses of 1, 3, 10, and 30 μm (Fig. 4). These Rg_3 concentrations did not have a significant impact on the 24-OH-chol-induced MDA level. Rg_3 exerted a more profound concentration-dependent inhibition of MDA production at Rg_3 doses of 100 and 300 μm (1.01±0.22, and 1.12±0.23 nmol/mg protein, respectively; $**p<0.01$) (Fig. 4). Thus, Rg_3 concentrations <30 μm did not inhibit the change in MDA production. These results indicate that 50 μm 24-OH-chol elevated MDA production and that pretreatment with 100 to 300 μm Rg_3 significantly decreased MDA production (Fig. 4).

Effects of ginsenoside Rg_3 on TUNEL staining

As it is well known that cholesterol oxides exert DNA damage on neuronal cells [15], we next examined the protective effects of Rg_3 against *in vitro* 24-OH-chol-induced DNA fragmentation in cortical neurons. Control experiments revealed that saline or 100 μm Rg_3 alone had no significant affect on cortical neurons. This result suggests that Rg_3 itself does not influence neuronal cell viability. Next, we administered 50 μm 24-OH-chol into cell culture plates ($n=8$) and examined cytotoxicity after 24 hour. The maximum effect on neuronal cell viability after the 24-OH-chol treatment was obtained with 100 μm Rg_3 (Fig. 2). Thus, 100 μm was determined to be the appropriate Rg_3 dose to optimize the neuroprotective effect on 24-OH-chol-induced neuronal cytotoxicity. To quantitatively examine the level of neuronal cell death, we counted the number of TUNEL-positive cells. Treating cells with control vehicle or Rg_3 alone yielded only a few TUNEL-positive neurons (Fig. 5). However, a

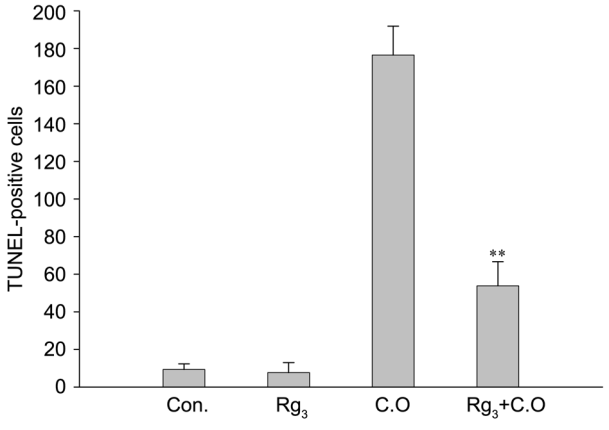

Fig. 5. Effects of ginsenoside Rg_3 (Rg_3) on 24-hydroxycholesterol (24-OH-chol)-induced DNA strand breakage as assessed by Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining. Cells were divided into control vehicle (Con), Rg_3 alone, 24-OH-chol (C.O) alone, and Rg_3+C.O groups. In the Rg_3+C.O co-treatment group, the cells were pretreated with 100 μm Rg_3 1 hour before adding 50 μm C.O. The density of TUNEL-positive neurons was quantified, and the results are expressed as the means±SEMs from seven separate triplicate experiments ($*p < 0.01$, compared with cells treated with C.O alone).

24 hour treatment with 24-OH-chol induced dramatic increases in TUNEL positivity compared with control or Rg_3 alone, whereas Rg_3 pretreatment markedly reduced 24-OH-chol-induced TUNEL positivity (**$p < 0.01$, compared with cells treated with 24-OH-chol + Rg_3) (Fig. 5). These results indicate that Rg_3 pretreatment attenuated 24-OH-chol-induced mitochondrial dysfunction and DNA damage.

Effects of ginsenoside Rg_3 on apoptosis

Because 50 μm 24-OH-chol increases apoptosis in neurons [15], we next examined whether Rg_3 would affect 50 μm 24-OH-chol-induced apoptosis. Treatment with 50 μm 24-OH-chol alone for 24 hours significantly increased caspase-3 activity levels compared with controls (Fig. 6). Although the effect of Rg_3 on 24-OH-chol-induced caspase-3 activity was not significant in the range of 1 to 30 μm, a 1 hour pretreatment with 100 and 300 μm Rg_3 significantly and dose-dependently decreased caspase-3 protease activity (Fig. 6). Treatment with Rg_3 alone had no significant effect on basal caspase-3 activity (data not shown). The percentages of caspase-3 activity (with levels in controls taken as 100%) were 163.78±7.89%, 161.85±8.68%, 160.58±9.65%, 151.75±7.87%, 143.78±10.77%, 119.18±8.93%, and 110.80±9.65% in cultures pretreated with 0, 1, 3, 10, 30, 100, and 300 μm Rg_3, respectively (**$p < 0.01$) (Fig. 6). These results indicate that Rg_3 inhibits 24-OH-chol-induced apoptosis in rat cortical neurons.

Ginsenosides are unique saponins that only exist in *Panax ginseng* and that have pharmacological effects in the central and peripheral nervous systems [33]. In a previous study, ginsenosides exerted *in vitro* and *in vivo* protective actions against acute excessive stimulation of excitatory neurotransmitters [18,34,35]. Furthermore, ginsenoside-induced regulation of ion channels and ligand-gated ion channels may be coupled with neuroprotection against excitatory neurotransmitters *in vitro* and *in vivo* [18-20,23,35]. The present study further extended the finding that Rg_3, one of the active ingredients in *Panax ginseng*, can protect the central nervous system from repeated neurotoxic insults. Thus, we demonstrated that *in vitro* administration of Rg_3 exhibited protective effects against 24-OH-chol-induced cytotoxicity in rat cortical neurons. One of the main indicators of neural excitotoxicity or excitotoxin-induced cell death is derived from a disturbance in intracellular Ca^{2+} homeostasis. Previous reports have shown that 24-OH-chol-induced neurotoxicity is coupled with increased intracellular Ca^{2+} [16]. In the present study, we showed that 24-OH-chol alone induced a rapid elevation in intracellular Ca^{2+} in cortical neurons, as shown in previous reports [16], and pretreatment with Rg_3 prior to 24-OH-chol treatment greatly attenuated the 24-OH-chol-induced intracellular Ca^{2+} elevation (Fig. 3). We also showed that pretreating cultured cortical neurons with Rg_3 rescued 24-OH-chol-induced decreases in mitochondrial ability, diminished caspase-3 activity, and subsequent apoptosis (Fig. 6) as well as abrogated increases in TUNEL positivity (Fig. 5). The inhibition of lipid peroxidation by Rg_3 and ginseng saponins accounts for the marked anti-oxidative stress effect [26]. We showed that Rg_3 reduced reactive oxygen species production indirectly (Fig. 4). Collectively, these findings provide a strong case for Rg_3-induced neuroprotection against 24-OH-chol-induced cytotoxicity. One possible mechanism underlying the protective effect of Rg_3 against 24-OH-chol-induced rat neurotoxicity might be derived from the inhibition on 24-OH-chol-induced Ca^{2+} influx via L- and other types of Ca^{2+} channels [36]. Moreover, ginsenosides inhibit L-, N-, and P/Q-types of Ca^{2+} channels [37-40]. A second possibility might be derived from ginsenoside-induced attenuation of extracellular Ca^{2+} entry caused by NMDA receptor activation. Previous studies have shown that ginsenosides not only inhibit NMDA receptor-mediated current and Ca^{2+} influx but also attenuate kainate-induced hippocampal neuron death [23,24]. Thus, these Rg_3-induced limiting actions on extracellular Ca^{2+} influx via Ca^{2+} channels and subsequent Ca^{2+} influx via secondary NMDA receptor activation might prevent aggravation of 24-OH-chol-induced intracellular Ca^{2+} elevation. Moreover, these Rg_3 contributions might help to diminish the ATP consumption needed for maintaining intracellular ionic

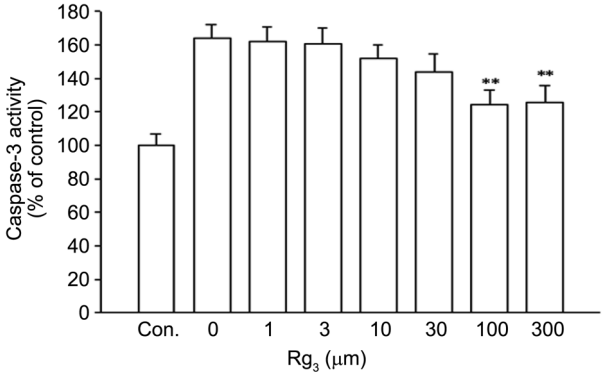

Fig. 6. Effect of ginsenoside Rg_3 (Rg_3) on caspase-3 activation in 24-hydroxycholesterol (24-OH-chol)-induced cortical cell death. Cultured rat cortical cells were pretreated with the indicated concentrations of Rg_3 1 hour prior to adding 50 μm 24-OH-chol, and caspase-3 activity was measured. Values are means±SEM from 7 to 8 separate experiments run in triplicate (**$p < 0.01$, compared with values from 24-OH-chol alone).

balance in neurons under 24-OH-chol-induced toxicity. The last possibility is that Rg_3-induced neuroprotection might be derived from an attenuation of oxidative stress, as ginsenosides inhibit glutamate-mediated overproduction of nitric oxid and malondialdehyde and prevent a decrease in superoxide dismutase activity in glutamate-treated cortical neurons [18,34].

Taken together with previous reports, the present findings suggest that Rg_3-induced neuroprotection against 24-OH-chol could be due at least in part to inhibition of NMDA receptor activation and the subsequent enhancement of intracellular Ca^{2+}. In summary, for various neural injuries associated with increased cholesterol oxidation products [41], our findings further suggest that Rg_3 could be useful as a novel preventive agent against 24-OH-chol-induced neurocytotoxicity.

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