Protective Effects of Ginsenoside Rg3 against Cholesterol Oxide-Induced Neurotoxicity in the Rat

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Abstract : Ginsenosides are among the most well-known traditional herbal medicines frequently used for the treatment of various symptoms in South Korea. The neuroprotective effects of ginsenoside Rg3 (G-Rg3) on cholesterol-oxide-(CO)-induced neurotoxicity were investigated through the analyses of rat brains. The recently accumulated reports show that ginseng saponins (GTS), the major active ingredients of Panax ginseng, have protective effects against neurotoxin insults. In the present study, the neuroprotective effects of G-Rg3 on CO-induced hippocampal excitotoxicity were examined in vivo. The in-vitro studies using rat cultured hippocampal neurons revealed that G-Rg3 treatment significantly inhibited CO-induced hippocampal cell death. G-Rg3 treatment not only significantly reduced CO-induced DNA damage but also attenuated CO-induced apoptosis. The in-vivo studies that were conducted revealed that the intracerebroventricular (i.c.v.) pre-administration of G-Rg3 significantly reduced i.c.v. CO-induced hippocampal damage in rats. To examine the mechanisms underlying the in-vitro and in-vivo neuroprotective effects of G-Rg3 against CO-induced hippocampal excitotoxicity, the effect of G-Rg3 on the CO-induced elevations of the apoptotic cells in cultured hippocampal cells was examined, and it was found that G-Rg3 treatment inhibited CO-induced apoptosis. The histopathological evaluation demonstrated that G-Rg3 significantly diminished the apoptosis in the hippocampus and also spared the hippocampal CA1, CA3, and dentate gyrus neurons. G-Rg3 also significantly improved the CO-caused behavioral impairment. G-Rg3 itself had no effect, however, on the CO-induced inhibition of succinate dehydrogenase activity (data not shown). These results collectively indicate the G-Rg3-induced neuroprotection against CO in rat hippocampus. With regard to the wide use of G-Rg3, this agent is potentially beneficial in treating CO-induced brain injury.

Key words : ginsenoside-Rg3, cholesterol-oxide, neurotoxicity, behavioral test, neuroprotection

INTRODUCTION

Cholesterol has been associated with the genesis of atherosclerosis, and it was shown that cholesterol oxides, the oxygenated derivatives of cholesterol, might be the real causative agent of this pathological condition.1) Cholesterol oxides (CO) are products of cholesterol catabolism in animals2,3) and are found in food, such as powdered milk, cheese and egg products.4) These compounds are potent inhibitors of 3-hydroxy-3-methylglutaryl (HMG) CoA reductase, a key enzyme in the endogenous biosynthesis of cholesterol.5) The possible involvement of cholesterol oxides in the initiation and formation of atherosclerosis has been the subject of intensive study. For example, an excess amount of cholesterol oxides was shown to damage endothelial cells,6,7) smooth muscle cells,8,1) and fibroblasts,9,10) all of which are major components of the arterial wall. Cholesterol oxides are also toxic to cells derived from the immune system, such as thymocytes, lymphoma cells and leukemic T-cells.11,12,13) The direct toxicity of cholesterol oxides on cells of neural origin, however, has been largely neglected.

The root of Panax ginseng, which has been used as a tonic in traditional medicine, contains more than 30 types of its main active ingredients, the ginsenosides.14) Recently, ginsenosides have been shown to have protective effects against glutamate toxicity-induced neuronal and glial cell death. In rat cortical cultures, ginsenosides Rb1 and Rg3 (G-Rg3) were shown to attenuate glutamate- and N-methyl-D-aspartic acid (NMDA) induced neurotoxicity by inhibiting the overproduction of nitric oxide and the formation of malondialdehyde.15) Liao et al. (2002) also reported that ginsenosides Rb1 and Rg3 protected spinal
neurons from glutamate- or kainite-induced excitotoxicity.\textsuperscript{16} In addition to neurons, Seong \textit{et al.} (1995) showed that ginsenosides attenuated glutamate-induced swelling of cultured rat astrocytes.\textsuperscript{17} In vivo, intracerebroventricular (i.c.v.) administration of ginsenoside Rb\textsubscript{1} was shown to significantly inhibit the magnitude of long term potentiation induced by strong tetanus in the dentate gyrus.\textsuperscript{18} Ginsenoside pretreatment via the intrathecal route attenuated NMDA- or substance P-induced nociceptive behaviors in mice,\textsuperscript{19} and ginsenoside pretreatment via the intraperitoneal route attenuated kainite-induced cell death of hippocampal neurons in rats.\textsuperscript{20} These results collectively indicate that ginsenosides could regulate NMDA receptors as well as other excitatory neurotransmitter receptors. Kim \textit{et al.} (2002) suggested that the underlying mechanism of ginsenoside induced protection against glutamate- or NMDA-induced excitotoxicity might be due to attenuation of the intracellular Ca\textsuperscript{2+} elevations induced by these excitatory amino acids in hippocampal neurons.\textsuperscript{21} Kim \textit{et al.} (2002) showed that among various ginsenosides, Rg\textsubscript{3} was the most potent inhibitor of NMDA-induced intracellular Ca\textsuperscript{2+} elevation in hippocampal neurons.\textsuperscript{21} Furthermore, Bae \textit{et al.} (2004) and Tian \textit{et al.} (2005) showed that oral or intravenous administration of Rg\textsubscript{3} exhibited significant neuroprotective effects against focal cerebral ischemic injury in rats.\textsuperscript{22,23} However, it is not yet known whether G-Rg\textsubscript{3} exhibits a neuroprotective effect on CO-induced excitotoxicity in vitro and/or in vivo. In the present study, we examined the effects of Rg\textsubscript{3} on CO-induced in vivo excitotoxicity in the rat hippocampus since the hippocampus is selectively damaged in brain disorders such as stroke, epileptic seizures and Alzheimer’s disease.\textsuperscript{24,25,26} Our results revealed that G-Rg\textsubscript{3} treatment greatly attenuated CO- in vivo hippocampal excitotoxicity by inhibiting neurodegeneration of hippocampus.

**MATERIALS AND METHODS**

**Materials**

The 20(S) G-Rg\textsubscript{3} (Fig. 1) was kindly provided by the Korean Ginseng Cooperation (Daejon, Korea). G-Rg\textsubscript{3} and CO were dissolved in DMSO as a concentrated stock and further diluted to its final concentration in saline. We found the final concentration of DMSO (<0.01\%) had no effect on the neuronal cell (data not shown). All other chemical agents were purchased from Sigma (St. Louis, MO, USA) unless otherwise specified.

**Animals**

Male Sprague-Dawley rats weighing 150 to 200 g were supplied by Charles River (KFT). The rats were fed a standard rodent pellet chow and housed with free access to commercial food pellets (LSM, Bacutil, Poland), as well as tap water ad libitum under strictly controlled conditions (room temperature: 23±1°C, relative humidity: 50±10\%). All the animals was kept in light (L)-dark (D) conditions L:D=12:12. And, the rats were acclimatized to their environment for 2 weeks before commencement of the experiments. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the the “Guideline for Institutional Animal Care and Use Committees (IACUC)” of Chonbuk National University (Jeonju, Korea).

**Research design**

The animals were divided into four groups (n=7-8, respectively): normal control, G-Rg\textsubscript{3} control, CO alone control, and G-Rg\textsubscript{3}+CO-treated group. By our previous study,\textsuperscript{27} G-Rg\textsubscript{3} (1 nmol) was determined to be the appropriate dosage of the G-Rg\textsubscript{3} to optimize the neuroprotective effects on rat brain. Animals of normal control group did not receive any treatment. Cholesterol oxide-group and CO+G-Rg\textsubscript{3} group treated intracerebroventricularly with 0.5 nmol CO.\textsuperscript{28} Cholesterol oxide was prepared as a solution in sterile normal saline. The G-Rg\textsubscript{3} and G-Rg\textsubscript{3}+CO group received an intracerebroventricular injection of G-Rg\textsubscript{3} (1 nmol) 1 h before CO injection intracerebroventricularly. Namely, G-Rg\textsubscript{3} was injected 1 h before CO was injected in the rat brain.
**Primary cultures of hippocampal neurons**

Rat hippocampal neuronal cultures were prepared using a technique modified from Kim et al. (2002). Briefly, hippocampi were isolated from 16- to 18-day-old fetal Sprague–Dawley rats and incubated with 0.25% trypsin in Leibovitz L-15 medium at 37°C for 20 min. Cells were then mechanically dissociated by trituration with fire-polished Pasteur pipettes and were plated on poly-L-lysine coated covers lips in 35-mm culture dishes or on 24-well plates. The cells were maintained in MEM culture medium containing 10% NU-serum, 2% B-27 supplement, 20 mM D-glucose, 26.2 mM sodium bicarbonate, 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. After 1 day in vitro, the culture medium was exchanged, and the cultures were treated with cytosine β-arabinofuranoside (1 µM). Experiments were carried out on neurons after 10-15 days in vitro.

**Intracerebroventricular injection and histological evaluation**

In each group, male Sprague–Dawley rat were removed from their dams 1 h prior to experiments and placed in plastic observation chambers on electric pads heated to 34°C (i.e., the temperature of the nest). For i.c.v. infusion of drugs, the rats were anesthetized by intraperitoneal (i.p.) injection of 1.0 µl/g body weight of a 3.5:1 mixture of ketamine and xylazine hydrochloride. The anesthetized animals were fixed in a stereotaxic apparatus that had been modified for rat pups, and operations were performed as previously described. Briefly, bilateral stainless steel guide cannulae (26 gauge) were stereotactically implanted 1 mm above each lateral ventricle (AP: 0.2-0.3 mm caudal from the bregma; L: 1.6 mm; V: 3.3 mm from the skull surface). The cannulae were secured with fast curing dental acrylic and the rats were allowed to recover from surgery for a minimum of 90 min. For experiments, animals in the normal control group received normal saline. All brains were perfused by 10% formalin under ether anesthesia. Blocks were cut into 6 µm sections and 20 μm slides. The slides were mounted with glass coverslips. In each treatment group, a total of 600 cells from 10 different fields (including TUNEL-negative and -positive neurons) were counted using an Olympus BX-51 light microscope (Olympus, Tokyo, Japan) at 40× magnification.

**Histopathological evaluation**

All histopathological analyses described below were performed by an investigator blind to rats treatment. Twenty-four hours after the first dose of CO, all animals were perfused by 10% formalin under ether anesthesia. Brain tissues were removed and fixed in 10% formalin in phosphate buffer for 24 h. To study the protective effects of G-Rg₃ against CO-induced apoptotic neurodegeneration, the time point 24 h after initiation of CO was chosen because 24 h is considered an important time point in the execution phase of apoptotic neuronal degeneration. Brain tissues were processed and embedded in paraffin blocks. The blocks were cut into 6 µm sections at multiple levels and stained with cresyl violet. The hemispheric areas were determined at each cross sectional level by one morphometrist with no prior knowledge of the experimental data, using a computer assisted image analyzer system.
consisting of a microscope (Olympus BH-2) equipped with a high-resolution video camera (JVC TK 890E, Japan). The images were processed by an IBM-compatible personal computer, high-resolution video monitor, and image analysis software. Briefly, the images were grabbed with the video camera at 3.3× magnification; the hemispheric areas were viewed on the monitor and outlined by drawing. The localization of cannulae was checked, the sections were photographed, and neuronal loss was assessed in the pyramidal layers (CA1 and CA3) and the dorsal blade of the dentate gyrus, using total live cell counts from the same optical field of each region in 10 animals. Data were acquired in blinded manner using a 0–6 rating scale: 0, no observable damage; +1, <10%; +2, 11–25%; +3, 26–50%; +4, 51–75%; +5, 76–90%; +6, >90% observable cell loss and statistical significances among rating scales were estimated.

Estimation of hippocampus neuron density
Each sample was subjected to the estimation of hippocampus (CA1, CA2, CA3, and dentate gyrus) density. The boundary of hippocampus was defined in accordance with The Mouse Brain Library/C57BL/6J Mouse Brain Atlas. CA1, CA2, CA3, and dentate gyrus neuron densities of hippocampus were estimated. The images were analyzed by using a computer assisted image analyzer system consisting of a microscope (Olympus BH-2 Tokyo, Japan) equipped with high-resolution video camera (JVC TK 890E, Japan). The numbers of CA1, CA2, CA3, and dentate gyrus were counted by the help of a 6000-Am2 counting frame viewed through a 20x Nikon lens at the monitor. The counting frame was placed randomly ten times on the image analyzer system monitor and the neuron numbers of CA1, CA2, CA3, and dentate gyrus regions of hippocampus were counted (UTHSCA Image Tool for windows version 3.0 software) and the average was taken. All counting and measurement procedures were performed blindly. The neuron numbers of CA1, CA2, CA3, and gyrus dentatus regions of the hippocampus were calculated separately for the right and left hemispheres.

Behavioral test
Animals were randomly divided into normal control, G-Rg3 alone, CO alone and G-Rg3 (1.0 nmol, i.c.v)+CO (0.5 nmol, i.c.v) group (n=7-8, each group). Other procedures for drug administrations were same as described above. Animals in each group were examined for sensorimotor ability 12 and 24 h after administration of saline or CO at once every 12 h for 24 h. Before dividing into each experimental group, animals were trained on apparatus for a maximum of 180 s 3 consecutive times for 3 days and animals that did not master this task were excluded from further experiments. The apparatus consisted of a bar, with a diameter of 6.0 cm, subdivided into four compartments by disks 50 cm in diameter (Ugo Basile, Italy). The bar rotated at an accelerated speed from 5 to 25 revolutions/min. For each trial, the duration that the animal was able to spend on the apparatus prior to falling was measured with a trial maximum latency of 180 s. The three separate results were averaged and recorded.

RESULTS AND DISCUSSION
Effects of G-Rg3 in CO-induced apoptosis
TUNEL-positive cells were counted in each layer (CA1, CA2, CA3, and dentate gyrus) of the hippocampus using a Zeiss Axiphot microscope at ×200 magnification connected to a 14-in. monitor. TUNEL-positive cells showed the typical morphological features of apoptosis such as the chromatino condensation, cytoplasmic budding, and apoptotic bodies. Twenty-four hours after CO injection intracerebroventricularly only a few cells with fragmented DNA labeled by TUNEL assay were found in the hippocampus (CA1 and CA3), especially dentate gyrus. When compared with the control group, the number of TUNEL+ neurons in the CA1 and CA3 regions of the hippocampus increased on the CO-treated mice (p<0.001, respectively). G-Rg3 significantly reduced the number of TUNEL+ neurons in the CA1, CA3 and dentate gyrus regions of the hippocampus as compared with the ethanol + saline-treated group (Table 1 and Fig. 2) (p<0.001, respectively). For quantitative measurement of the number of cells that underwent apoptosis, we counted 100 cells randomly in these different areas and calculated the percentage of the apoptotic cells (Table 2).

Effects of G-Rg3 in C.O-induced neuronal density
Twenty-four hours after administration of CO (0.5 nmol, i.v.c) with G-Rg3 (1 nmol) significantly preserved the number of neurons of CA1, CA3 and MB regions of dentate gyrus of the hippocampus when compared with the CO-treated group ( **p<0.01, respectively). There were no significant difference in neuron density of the CA2 regions of the hippocampus between G-Rg3+CO and CO -treated groups (p>0.05) (Table 2 and Fig. 3). The number of neurons of CA1, CA3, and MB regions of dentate gyrus regions were significantly less in the CO treated
group in comparison with the normal control group ($p<0.01$, respectively) (Table 2 and Fig. 3).

**Effects of G-Rg$_3$ on CO-induced hippocampal damage in vivo**

We next examined the neuroprotective effects of G-Rg$_3$ against *in vivo* CO-induced hippocampal lesions in the rats. Control experiments revealed that i.c.v. administration of saline or G-Rg$_3$ alone had no significant effect on hippocampal brain regions, as examined by cresyl violet staining (Fig. 3, 4). Next, we administered CO (0.5 nmol, i.c.v., n=7-8, each group) into both sides of the hippocam-
pus and examined hippocampal lesions after 24 hours. Our results revealed that i.c.v. administration of 0.5 nmol CO alone resulted in selective neuronal death, more prominently in the medial blade than the lateral region, with maximum cell loss seen at the crest of the dentate gyrus region of the hippocampus (Fig. 4). A bilateral loss of CA3 pyramidal cells was also observed in all animals, and neuronal cell death was seen in several other areas of the brain, including the neocortex, septum, striatum and thalamus (data not shown). In contrast, pre-administration of 1.0 nmol of G-Rg₃ 1 h before 0.5 nmol CO treatment significantly suppressed CO-induced CA1, CA3 loss and hippocampal cell death in the crest of the dentate gyrus (Figs. 4). Interestingly, 0.25 and 0.5 nmol G-Rg₃ was slightly less neuroprotective against CO (0.5 nmol) than G-Rg₃ (1 nmol), although this difference was not statistically significant (data not shown). To quantitatively examine the levels of neuronal cell death, we used a previously described six-level rating scale²⁵,³⁶ in which +1 indicated cell death of less than 10% and +6 indicated cell death of more than 90% in the pyramidal cell layer and estimated statistical significance among rating scales. In rats treated with CO alone (0.5 nmol), the average cell death grade was +3.51±0.43 in the CA1 region, +3.67±0.52 in the CA3 region and +5.16±0.41 in the medial blade of the dentate gyrus. Rats receiving G-Rg₃ alone showed no significant differences compared to normal control (B).

**Table 2.** The effect of systemic ginsenoside Rg₃ treatment on neuronal density of the hippocampus of rats with cholesterol oxides-induced brain injury

<table>
<thead>
<tr>
<th>Group</th>
<th>CA1</th>
<th>CA2</th>
<th>CA3</th>
<th>MB</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Normal control</td>
<td>95.70±8.65</td>
<td>84.85±10.88</td>
<td>78±12.65</td>
<td>133.78±32.58</td>
</tr>
<tr>
<td>2. G-Rg₃ (alone)</td>
<td>105.23±12.38</td>
<td>95.53±13.76</td>
<td>85.47±9.62</td>
<td>123.85±28.42</td>
</tr>
<tr>
<td>3. CO</td>
<td>53.25±7.56</td>
<td>65.87±13.54</td>
<td>48.71±8.78</td>
<td>34.53±12.67</td>
</tr>
<tr>
<td>4. G-Rg₃+CO</td>
<td>69.87±9.76*</td>
<td>77.83±15.46</td>
<td>78.15±13.57**</td>
<td>97.38±27.35**</td>
</tr>
</tbody>
</table>

(***p<0.01; n=7-8, respectively)

**Fig. 3.** Effects of G-Rg₃ in cholesterol oxide-induced hippocampal cell death in rats (A–D). Cresyl violet staining of hippocampal brain sections prepared 24 h after i.c.v. administration of normal control (A), G-Rg₃ alone (B), (0.5 nmol/side) CO alone (C), and G-Rg₃+CO co-treated group (D). Note selective neuronal loss in the dorsal blade of dentate gyrus and the CA3 regions of hippocampus. Pre-administration of G-Rg₃ (1 nmol, i.c.v.) 1 h prior to administration of CO reduced CO-induced neuronal loss in the dentate gyrus and some CA3 regions of the hippocampus (C). But, neuronal loss in the dorsal blade of dentate gyrus and the CA3 regions of hippocampus was inhibited in G-Rg₃ treatment group (D). Rats receiving G-Rg₃ alone showed no significant differences compared to normal control (B).
Effect of G-Rg3 on C.O-induced behavioral deficits

Since hippocampus of rats was related with normal behavior, we investigated whether G-Rg3-induced neuroprotection against CO-induced hippocampus lesions also reduces the degree of motor impairment derived from 0.5 nmol CO intoxication. For this, we performed rota-rod sensorimotor test in normal control, CO alone- and G-Rg3+CO group. In these experiments we excluded animals showing abnormal movements by dystonia, limb paralysis, or other abnormal behaviors after CO administration in CO alone and G-Rg3+CO group. Cholesterol oxide alone-treated animals began to show impaired rota-rod performances 24 h after administration of CO. However, the animals of CO+G-Rg3 group showed much improved rota-rod performance (Fig. 6). Thus, co-treatment of G-Rg3 with CO reduced behavioral deficit compared with CO alone-treated animals (CO : G-Rg3+CO = 163.4±13.2 : 204.7±16.3 secs 24 h after i.c.v. injection; **p<0.01, n=7-8 each group). On the other hand, in G-Rg3 alone treatment group we could observe a slight decrease of body weight (<5%) at the beginning of 0.5 nmol G-Rg3 administration without any effects on behavioral activity but this phenomenon was disappeared soon (data not shown).

It is becoming clear that lipid metabolism is involved in some pathological conditions associated with cells of the central nervous system. For example, altered lipid metabolism is found in retinas of Swedish Briard dogs with a slowly progressive retinal dystrophy. In addition, lipid oxidation is involved in retinal ganglion cell death induced by chemical hypoxia and hypoglycemia. While

Fig. 4. Effect of G-Rg3 treatment on the neuron density in the hippocampus. Representative pictures obtained by cresyl violet staining. When compared with the control group (A), the number of neurons of CA1, CA3, and dentate gyrus region of the hippocampus decreased on CO-treated group (C). G-Rg3 (1 nmol) significantly preserved the number of neurons of CA1, CA3 and dentate gyrus region of the hippocampus as compared with the C.O-treated group (D). Scale bar, 200 µm.

Fig. 5. Summary histograms of neuroprotection by G-Rg3 (1 nmol, i.c.v.) against CO-induced losses in the CA1, CA3 and dentate gyrus. Severity of neuronal loss in the dentate gyrus and some CA3 regions of the ipsilateral hippocampus was semi-quantitatively evaluated using a rating scale of 0–6, as described in the Experimental procedures section. Values are means±SEM. **p<0.01 versus CO-injected controls.
the possible involvement of cholesterol oxides in the initiation and formation of atherosclerosis has been a subject of intense study, the findings that cholesterol oxides are toxic to cerebellar granule cells suggest that neurons of the central nervous system might be a target of the damaging effects of these compounds. While there is currently no information in the literature concerning the level of different cholesterol oxides in the brain, a recent study indicated that brain synaptosomes and mitochondria under oxidative stress can generate cholesterol oxides, with 7-keto- and 7-OH- cholesterol being the major species formed. It was reported that cholesterol oxides can inhibit DNA synthesis in replicating cells. This should not be the basis for the toxicity observed in cerebellar granule cells, because they are post-mitotic. On the other hand, there is evidence that cholesterol oxides can induce excess lipid accumulation in cells, which can potentially disturb normal cellular metabolism and homeostasis. It should also be noted that exogenously added cholesterol oxides can inhibit DNA synthesis in replicating cells. This should not be the basis for the toxicity observed in cerebellar granule cells, because they are post-mitotic. On the other hand, there is evidence that cholesterol oxides can induce excess lipid accumulation in cells, which can potentially disturb normal cellular metabolism and homeostasis.

Ginsenosides, which are unique saponins found only in *Panax ginseng*, are physiologically and pharmacologically active ingredients that regulate various voltage-dependent channels (e.g. Ca\(^{2+}\), K\(^+\) and Na\(^+\) channels) and receptors (e.g. 5-HT\(_{1A}\), nicotinic acetylcholine and NMDA receptors) in the central and peripheral nervous systems. Recent studies have shown that ginsenoside-induced regulations of ion channels and ligand-gated ion channels could be coupled to neuroprotection against excitatory neurotransmitters in vitro and in vivo. The present study further extends this by showing that G-Rg3, one of the more active ginsenosides, exhibits protective effects against CO-induced hippocampal cell death in vivo. What is the mechanism underlying the protective effect of G-Rg3 against CO-caused rat hippocampus neurotoxicity? One possibility is that G-Rg3-induced protection against CO-induced neurotoxicity might be derived from the inhibition on CO-induced Ca\(^{2+}\) influx via L- and other types of Ca\(^{2+}\) channel. Previous report showed that intracellular Ca\(^{2+}\) elevation was mediated via a L-type and other types of Ca\(^{2+}\) channels. Also, it was reported that ginsenosides inhibit L-, N-, and P/Q-types of Ca\(^{2+}\) channels. Thus, G-Rg3-induced inhibitions on various types of Ca\(^{2+}\) channels could be the basis of attenuation of CO-induced intracellular Ca\(^{2+}\) elevation. The second possibility might be derived from G-Rg3-induced attenuation of extracellular Ca\(^{2+}\) influx caused by NMDA receptor activation. In previous studies, other group showed that ginsenosides not only inhibit NMDA receptor-mediated current and Ca\(^{2+}\) influx but also attenuate kainate-induced hippocampal neuron death. Thus, these G-Rg3-induced limiting actions on extracellular CO-induced Ca\(^{2+}\) influx via Ca\(^{2+}\) channels and subsequent Ca\(^{2+}\) influx via secondarily NMDA receptor activation might help not to aggravate CO-induced intracellular Ca\(^{2+}\) unbalance. Moreover, these contributions of G-Rg3 might help to diminish ATP consumption needed for maintaining intracellular ionic balances in hippocampus cells under CO treatment and finally ameliorate CO-induced neurotoxicity. Similarly, MK801, a NMDA receptor antagonist, or riluzole, which inhibits neuronal voltage-dependent Ca\(^{2+}\) and Na\(^+\) channel activity, not only attenuated intracellular Ca\(^{2+}\) elevation but also exhibited neuroprotective effects. The last possibility is that G-Rg3-induced neuroprotection against CO neurotoxicity might be derived from the attenuation of oxidative stress, since ginsenosides inhibit glutamate-mediated overproduction of NO (nitric oxide) and malonyldiadehyde and prevented a decrease of superoxide dismutase activity in glutamate-
treated cortical neurons. However, it is unlikely that the protective effects of G-Rg$_3$ are due to direct interaction with CO, activation of succinate dehydrogenase (SDH), or blocking action of SDH inhibitor, since ginseng saponin itself had no effect on SDH activity and ginseng saponin administered 3 h before neurotoxin treatment also did not affect inhibition of SDH activity. Ginseng saponin could not reverse malonate-induced inhibition of SDH activity. It is also possible to say that G-Rg$_3$ treatment may accelerate the elimination of CO, rendering the neurotoxicity less active. Here, this possibility can be clearly ruled out. Taken together, the main contributing factors on G-Rg$_3$-induced protection against CO neurotoxicity could be due to the inhibitory effects on intracellular Ca$^{2+}$ elevations that might be due to Ca$^{2+}$ channel and NMDA receptor activations.

In summary, using a rodent model system that shows a hippocampal lesion by CO treatment, we obtained results suggesting that G-Rg$_3$ has neuroprotective effects against CO-induced neurotoxicity. Moreover, since the pathological symptoms induced by long-term treatment of CO share many common characteristic features observed in human patients, the present findings further suggest that treatment of G-Rg$_3$ might be a novel preventive strategy against neurodegeneration.

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