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Protective Effects of Ginsenosides on Cyanide-induced Neurotoxicity in Cultured Rat Cerebellar Granule Cells

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Abstract : Effects of ginsenosides on NaCN-induced neuronal cell death were studied in cultured rat cerebellar granule cells. NaCN produced a concentration-dependent (1~10 mM) reduction of cell viability (measured by trypan blue exclusion test), that was blocked by N-methyl-D-aspartate receptor antagonist (MK-801) and L-type Ca^{2+} channel blocker (verapamil). Pretreatment with ginsenosides (Rb_1, Rc, Re, Rf and Rg_1) significantly decreased the neuronal cell death in a concentration range of 0.5~5 μg/m*l*. Ginsenosides Rb_1 and Rc (5 μg/m*l*) inhibited glutamate release into medium induced by NaCN (5 mM). NaCN (1 mM)-induced increase of $[Ca^{2+}]_i$ was significantly inhibited by the pretreatment of Rb_1 and Rc (5 μg/m*l*). Other ginsenosides caused relatively little inhibition on the elevation of glutamate release and of $[Ca^{2+}]_i$. These results suggest that the NaCN-induced neurotoxicity was related to a series of cell responses consisting of glutamate release and $[Ca^{2+}]_i$ elevation via glutamate (NMDA and kainate) receptors and resultant cell death, and that ginsenosides, especially Rb_1 and Rc, prevented the neuronal cell death by the blockade of the NaCN-induced Ca^{2+} influx.

Key words : Ginsenosides, Rb_1, Rc, cerebellar granule neuron, NaCN, Ca^{2+} influx

INTRODUCTION

Cyanide is one of the most rapid-action poisons available to mammals. Central nervous system is a primary target organ in cyanide neurotoxicity which includes respiratory distress, seizures, convulsions[1-3] and in some individuals a Parkinson-like condition may develop as a post-toxicity sequela.[4-6] Cyanide exerts its toxic action by interfering with cellular respiration through the inhibition of cytochrome oxidase. Therefore, cyanide poisoning has been frequently used as a model of chemical hypoxia. Excitatory amino acids such as glutamate and aspartate, which are important and abundant neurotransmitters in CNS, plays an important part in cyanide neurotoxicity. Cyanide induces the release of glutamate from neuronal stores and alters the brain levels of glutamate.[7] The increased extracellular levels of glutamate may result in overstimulation of glutamate receptors, leading to excitotoxic responses.[8] NMDA receptor-mediated Ca^{2+} influx appears to be a key in the neurotoxic process initiated by cyanide.[9] Therefore, in neuronal cells, specific-NMDA receptor antagonists such as APV and MK-801 block cyanide-induced intracellular Ca^{2+} ($[Ca^{2+}]_i$) elevation and prevent neuronal cytotoxicity.[10,11] Oxidative stress also play a role in cyanide neurotoxicity. Johnson *et al.* proposed that increased intracellular Ca^{2+} after KCN treatment generates reactive oxygen species (O^{2-}, OH) and nitric oxide (NO) leading to lipid peroxidation and neuronal damage.[12] Primary cultures of cerebellar granule cells have been used extensively to study mechanisms of neuronal death. This is in part due to the fact that these are predominantly glutamatergic neurons, and glutamate-receptor-mediated excitotoxicity is believed to play a role in the pathophysiology of neurodegenerative diseases.[13]

Much attention has been paid to ginseng saponins, main effective component of ginseng, because of their multiple pharmacological actions. They have central actions such as suppression of exploratory and spontaneous movements, prolongation of hexobarbital sleeping time and inhibition of reverse-tolerance development to dependence-liable drugs.[14-16] Liu *et al.* (1992) have reported that ginsenosides could significantly increase the survival rate of mice exposed to acute hypoxia.[17] There are several reports of *in vitro* actions of some active components extracted from *Panax ginseng* on cultured cell line. Ginsenoside Rb_1 and Rd, saponins isolated from *Panax ginseng*, were reported

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to potentiate the nerve growth factor-mediated neurite extension in organ cultures of chicken embryonic dorsal root ganglia and lumbar sympathetic ganglia.[18] A protective effect of the lipophilic components of *Panax ginseng* on the differentiation of an established culture cell of rat pheochromocytoma, PC12 cells, was also reported.[19] In previous studies, we reported that ginseng total saponins prevented hypoxia-induced cellular dysfunction and glutamate-induced swelling of cultured rat cortical astrocytes, and that ginsenosides reduced NMDA-induced elevation of glutamate release in cultured rat cerebellar granule cells.[20-22] Furthermore, we confirmed that Rb_2 and Rc inhibited glutamate-induced astrocytic swelling via blockade of Ca^{2+} influx.[23] Thus, in the present study, we examined the protective effects of ginsenosides on cyanide-induced neurotoxicity using cultured rat cerebellar granule neurons. The effects of ginsenosides on NaCN-induced neuronal cell death, glutamate release and $[Ca^{2+}]_i$ elevation were studied.

MATERIALS AND METHODS

1. Materials

Ginsenosides were supplied from Korea Ginseng and Tobacco Research Institute. MK-801, NAME and 6,7-dinitroquinoxaline-2,3-dione (DNQX) were purchased from Research Biochemicals International (Natick, MA, USA). Fetal bovine serum was from Hyclone (Logan, Utah, USA). NaCN, verapamil, fura-2/acetoxymethyl ester (Fura-2 AM) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used were of the highest grade available.

2. Cerebellar granule cell culture

Cerebellar granule cells were cultured as described in previous report.[23] Briefly, 7 to 8-day old rat pups (Sprague-Dawley) were decapitated, and the heads were partially sterilized by dipping in 95% ethanol. The cerebellum was dissected from the tissue and placed in medium which lacks serum and bicarbonate, and contains trypsin (0.25 mg/m*l*). Dissociated cells by trypsinization and slight trituration were collected by centrifugation and resuspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with sodium pyruvate (0.9 mM), glutamine (3.64 mM), sodium bicarbonate (40 mM), glucose (22.73 mM) and 10% fetal bovine serum. Cells were seeded at a density of about 2 $\times 10^6$ cells/ml into poly-L-lysine coated 12 well- or 24 well-plates or cover glass for $[Ca^{2+}]_i$ measurement. After 2 days incubation, growth medium was aspirated from the cultures and new growth medium containing 25 mM KCl and 20 uM cytosine arabinoside to prevent proliferation of nonneuronal cells was added. Cultures were kept at 37°C in a 7% CO_2 atmosphere. Cells were used after 8 to 10 days in culture.

3. Measurement of neuronal cell death induced by NaCN

The neurotoxic effects of NaCN were quantitatively assessed by the trypan blue exclusion method as described.[24] For the experiment, the growth medium was replaced with an incubation buffer consisting of (in mM) : NaCl 154, KCl 5.6, $CaCl_2$ 2.3, $MgCl_2$ 1 and HEPES 8.6 adjusted to pH 7.4 with NaOH. Cells were incubated at 37°C in the incubation buffer for 30 min prior to the addition of NaCN. Ginsenosides and other inhibitors were added during this preincubation period for the last 15 min. Then, NaCN was added to the cells and cells were further incubated for 1 hr. At the end of the incubation, cells were stained with 0.4% trypan blue solution at room temperature for 10 min. Only dead cells with a damaged cell membrane are permeable to trypan blue. The number of trypan blue-permeable blue cells and viable white cells was counted in six randomly chosen fields/culture dish. Experiments were performed in triplicate with at least 3 different batches.

4. Measurement of Glutamate

Growth medium was removed and cells were washed with the incubation buffer and equilibrated in the buffer for 30 min prior to the addition of NaCN. Gingsenosides and other inhibitors were added during this preincubation period for the last 15 min. After an additional 1 hr incubation with NaCN, a sample was taken from the buffer for the determination of glutamate secreted into the buffer. Glutamate was quantified by a high performance liquid chromatography with an electrochemical detector.[25] Briefly, after a small aliquots was collected from the culture plates, glutamate was separated on an analytical column (ODS2; particle size, 5 μm; 4.6×100 mm) after prederivatization with *o*-phthaldialdehyde/2-mercaptoethanol. Derivatives were detected by electrochemistry at 0.1 μA/V, and the reference electrode was set at 0.7 V. The column was eluted with mobile phase (pH 5.20) containing 0.1 M sodium phosphate buffer with 37% (v/v) HPLC-grade methanol at a flow rate of 0.5 m*l*/min.

5. Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was determined by ratio fluorometry as described by others.[26,27] Cells grown on glass cover slides were loaded with 5 μM Fura-2 AM for 40 min in serum-free DMEM at 37°C in the CO_2 incubator, and washed with the incubation buffer. Cell culture slides were mounted into spectrophotometer cuvette containing 3 ml incubation buffer. Fluorescence was measured with a 3FLUORO-LOG-2-spectrophotometer (PTI Ind. Inc., USA) by exciting cells at 340 and 380 nm and measuring light emission at 510 nm. Baseline of $[Ca^{2+}]_i$ was measured for 180 sec prior to the addition of NaCN. In order to test the effects of ginsenosides, cells were pretreated with ginsenosides for 15 min. Ionomycin and EGTA (final concentration of 10 μM and 20 mM, respectively) were added at the end of experiments to determine the emission of dye saturated with Ca^{2+} and free of Ca^{2+}, respectively. The change of $[Ca^{2+}]_i$ was expressed as the ratio of fluorescent intensity measured at 340 nm and 380 nm excitation wavelength (F 340/F 380).

6. Statistical analysis

Data were expressed as Mean±SEM and statistical significance was assessed by one-way analysis of variance (ANOVA) followed by the Student's *t*-test in all experiments.

RESULTS

1. Inhibitory effects of ginsenosides on neuronal cell death

One hour exposure of the cells to NaCN (≥1 mM) increased the number of cells stained by trypan blue (Fig. 1). As shown in Fig. 2, NaCN (10 mM) produced significant neuronal cell death from 79.0% of control cells to 45.5%. MK-801 (10 μM), verapamil (10 μM), a Ca^{2+} channel blocker, and L-NAME (1 mM) inhibited the NaCN-induced cell death, showing 76.2%, 71.1% and 70.8% of cell viability, respectively. All of ginsenosides used in the present experiments produced significant inhibitory effects on NaCN (10 mM)-induced neuronal cell death at the concentrations of 0.5 and 5 μg/ml (Fig. 2).

2. Inhibitory effects of ginsenosides on NaCN-induced elevation of glutamate release

Glutamate release, as an indicator of cyanide-induced neurotoxicity, was measured after the incubation of cells with NaCN for 1 hr. NaCN (5 mM) caused a significant elevation of glutamate release (control; 1.6 μM, 5 mM;

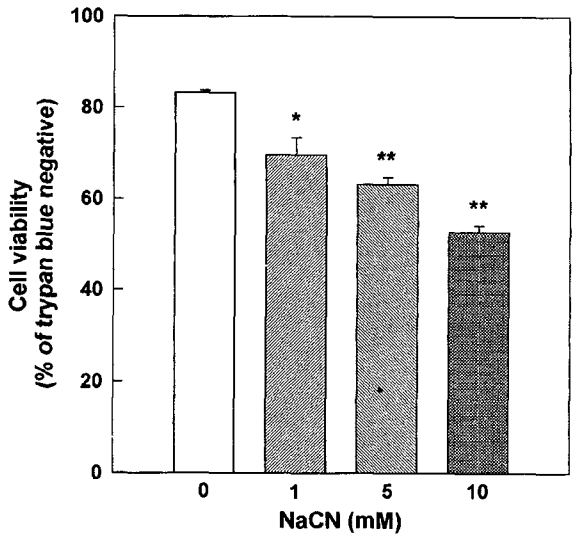

Fig. 1. Concentration-response of NaCN on cell viability of cultured cerebellar granule neurons. After washing and equilibration of 30 min with incubation buffer, cells were incubated with various concentrations of NaCN (1~10 mM) for 1 hr at 37°C . At the end of the incubation, trypan blue exclusion test was performed. Values represent mean±SEM. *p<0.05, **p<0.01 compared to control.

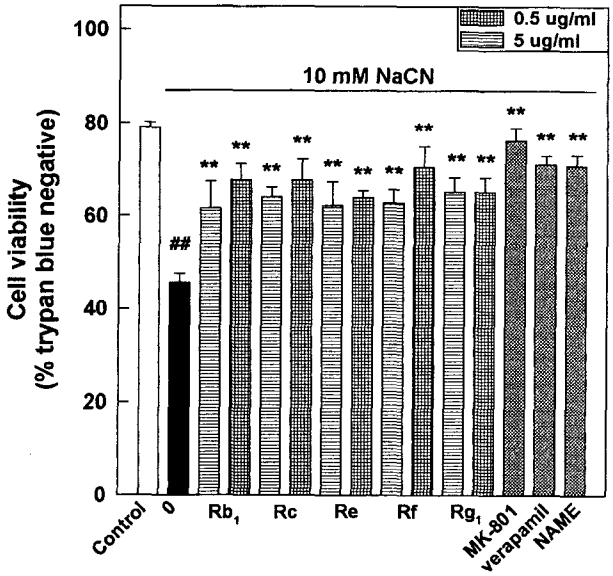

Fig. 2. Effects of ginsenosides and various compounds on NaCN-induced neuronal cell death. After washing and equilibration of 30 min at 37°C with incubation buffer, cells were incubated with NaCN for 1 hr in the presence or absence of compounds. Ginsenosides, MK-801 (10 μM), verapamil (10 μM) and NAME (1 mM) were applied 15 min prior to NaCN treatment. At the end of the incubation, trypan blue exclusion test was performed. Values represent mean±SEM. ##p<0.01 compared to control. *p<0.05, **p<0.01 compared to 10 mM NaCN.

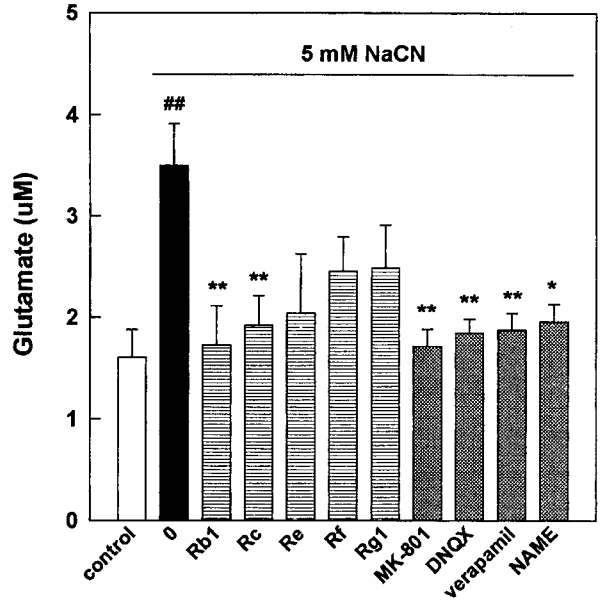

Fig. 3. Effects of ginsenosides and various compounds on NaCN-induced glutamate release into medium. After washing and equilibration of 30 min at 37°C with incubation buffer, cells were incubated with NaCN for 1 hr in the presence or absence of compounds. Ginsenosides (5 μg/m*l*), MK-801 (10 μM), DNQX (20 μM), verapamil (10 μM) and NAME (1 mM) were applied 15 min prior to NaCN treatment. At the end of the incubation, released glutamate was measured by HPLC with ECD. Values represent mean ± SEM. $^{\#\#}p<0.01$ compared to control. *p<0.05, **p<0.01 compared to 5 mM NaCN.

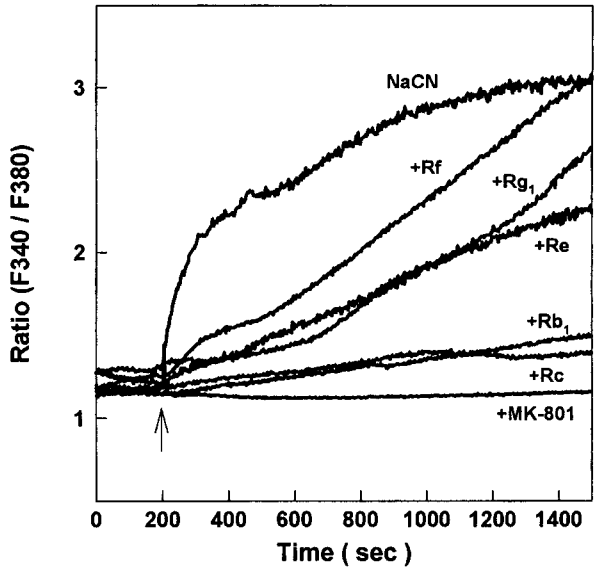

Fig. 4. Effects of ginsenosides and MK-801 on NaCN-induced elevation of $[Ca^{2+}]_i$ shown by fluorescence ratio (F340/F380) in cultured cerebellar granule neuron. After equilibrium of 1 hr with serum-free DMEM, cells were labelled with 5 μM Fura-2 AM for 40 min. Fura-2 AM loaded cells were stimulated with NaCN (1 mM) as indicated by arrow in the presence or absence of ginsenosides. Ginsenosides were treated 15 min before NaCN treatment.

3.5 μM). MK-801 (10 μM), DNQX (10 μM), verapamil (10 μM) and NAME (1 mM) markedly inhibited NaCN-induced elevation of glutamate release (Fig. 3). Ginsenoside Rb_1 and Rc at 5 μg/m*l* strongly blocked the NaCN-induced elevation of glutamate release. However, ginsenosides Re, Rf and Rg_1 showed slight, but not significant inhibition on NaCN (5 mM)-induced elevation of glutamate release (Fig. 3).

3. Inhibitory effects of ginsenosides on NaCN-induced elevation of $[Ca^{2+}]_i$

There are many reports that cyanide enhances Ca^{2+} influx following glutamate release. Thus, elevation of $[Ca^{2+}]_i$ by NaCN was measured by ratio fluorometry after loading fluorescent dye, Fura-2 AM to the cells. When 1 mM NaCN was applied to cerebellar granule cells, fluorescent intensity representing $[Ca^{2+}]_i$ was increased slowly and gradually, and reached plateau level about 15 min after the treatment with NaCN. NaCN-induced elevation of $[Ca^{2+}]_i$ was almost completely inhibited by ginsenosides Rb_1 and Rc (5 μg/m*l*). MK-801 also completely inhibited the $[Ca^{2+}]_i$ elevation. Meanwhile, ginsenosides Re, Rf and Rg_1 blocked the initial phase, but failed to block the later plateau phase of the $[Ca^{2+}]_i$ elevation induced by 1 mM NaCN (Fig. 4).

DISCUSSION

Cyanide-induced neurotoxicity is associated with glutamate release from intracelluar stores. Cyanide initiates an excitotoxic-like reaction in cerebellar granule cells which is primarily mediated by activation of the NMDA receptor. Activation of the NMDA receptor promotes the influx of cations, including Na^+ and Ca^{2+}, which can lead to membrane depolarization. In turn, depolarization can activate membrane voltage-sensitive Ca^{2+} channels leading to additional Ca^{2+} influx. Influx of Ca^{2+} stimulates concurrent generation of nitric oxide and reactive oxygen species which then results in lipid peroxidation and cellular injury.[28] Therefore, the resultant neuronal damage causes irreversible physiological disorder.

The present study confirmed previous observations in cerebellar granule cells that cyanide stimulated glutamate

release and $[Ca^{2+}]_i$ elevation which in turn led to neuronal cell death.[7,10,11] The present study also demonstrated that ginsenosides such as Rb_1, Rc, Re, Rf and Rg_1 protected neurons against the neuronal damage induced by NaCN. protective effects of ginsenosides on NaCN-induced neuronal cell death were significant from 0.5 to 50 μg/m*l*. Similarly, MK-801, verapamil, and NAME showed the protective effect on the NaCN-induced cell death, implying that the NaCN-induced neurotoxicity would be related to NMDA-receptor, L-type Ca^{2+} channel and nitric oxide.

In order to investigate the mechanism of the neuroprotective effect of ginsenosides, the effects of ginsenosides on the NaCN-induced glutamate release and $[Ca^{2+}]_i$ elevation were studied. When cerebellar granule neurons were exposed to NaCN (5 mM) for 1 hr, extracellular glutamate level was significantly increased. Glutamate release induced by NaCN was significantly prevented by MK-801 and DNQX, implying that neurotoxicity of NaCN was induced via activation of not only NMDA receptor but also kinate receptor. And, NAME, nitric oxide synthase inhibitor, also inhibited the NaCN-induced glutamate release. Ginsenosides Rb_1 and Rc, but not Re, Rf and Rg_1, at 5 μg/m*l* concentration, produced significant inhibition on NaCN (5 mM)-induced elevation of glutamate release. It was reported that activation of NMDA receptor-linked cation channels leads to an increase in $[Ca^{2+}]_i$, which may cause further glutamate release.[29] Thus, increase in $[Ca^{2+}]_i$ were thought to regulate glutamate release elevated by NaCN. Ginsenosides Rb_1 and Rc (5 μg/m*l*) almost completely inhibited NaCN (1 mM)-induced elevation of $[Ca^{2+}]_i$. Several reports have shown that ginsenosides have a property of Ca^{2+} channel antagonist. Nah *et al* (1995) reported that ginsenosides, especially Rf, inhibited N-type Ca^{2+} channels in rat sensory neurons.[30] Tachikawa *et al.* (1995) reported that ginseng saponins blocked nicotinic acetylcholine receptor-coupled Na^+ channel and consequently reduced Ca^{2+} influx.[31] Therefore, it is assumed that this inhibitory effect of Rb_1 and Rc on $[Ca^{2+}]_i$ elevation resulted in the inhibition on glutamate release and, moreover, neuronal cell death. However, ginsenosides Re, Rf and Rg_1 only showed blockade of initial phase of $[Ca^{2+}]_i$ increase induced by NaCN. This property of these ginsenosides may explain the failure in the significant inhibition of glutamate release. In support of the present results, we confirmed that ginsenosides Rb_2 and Rc inhibited glutamate-induced swelling of cultured astrocytes via blockade of Ca^{2+} influx in the previous study.[23] However, further experiments would be helpful to clear the precise mechanism.

In conclusion, together with the previous reports, the present results demonstrated that ginsenosides, active components of *Panax ginseng*, have beneficial effects on hypoxic brain cell damage *in vitro*.

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요 약

흰쥐 소뇌로부터 과립신경세포를 배양하여 NaCN으로 유도되는 신경세포손상에 대한 ginsenosides의 보호효과를 검토하였다. NaCN (1~10 mM)을 배양된 세포에 1시간 동안 처리하면 농도 의존적으로 신경세포사를 일으켰다. Ginsenosides(Rb_1, Rc, Re, Rf, Rg_1) (0.5, 5 μg/m*l*)를 세포에 전처치하면 10 mM NaCN으로 유도되는 세포사가 현저히 감소되었다. Rb1과 Rc(5 μg/m*l*)는 5 mM NaCN에 의하여 배양액 중으로 유리되는 glutamate의 증가를 현저히 억제하였으며, 1 mM NaCN에 의하여 유발되는 세포내 Ca^{2+}농도의 증가를 억제하였다. NaCN으로 유발되는 세포독성은 또한 MK-801, verapamil, NAME에 의하여도 억제되었다. 따라서, NaCN으로 유도되는 신경세포사는 glutamate release를 통한 NMDA수용체의 활성화와 그에 따른 Ca^{2+}의 세포내유입에 의한 것임을 알 수 있고, ginsenosides, 특히 Rb_1과 Rc는 Ca^{2+}의 유입을 억제하므로서 NaCN에 의한 신경세포사를 억제하는 것으로 생각된다.

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