Inhibitory Effect of Ginsenosides on NMDA Receptor-mediated Signals in Rat Hippocampal Neurons

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Abstract

Ginseng is the best known and most popular herbal medicine used worldwide. Ameliorating effects of ginseng were observed on the models of scopolamine-induced, aged or hippocampal lesioned learning and memory deficits. Further beneficial effects of ginseng were observed on neuronal cell death associated with ischemia or glutamate toxicity. In spite of these beneficial effects of ginseng on the CNS, little scientific evidence shows at the cellular level. In the present study, we have employed cultures of rat hippocampal neurons and examined the direct modulation of ginseng on NMDA receptor-induced changes in $[Ca^{2+}]_i$ and -gated currents using fura-2-based digital imaging and perforated whole-cell patch-clamp techniques, respectively. We found that ginseng total saponins inhibited NMDA-induced but less effectively glutamate-induced increase in $[Ca^{2+}]_i$. Ginseng total saponins also modulated Ca^{2+} transients evoked by depolarization with 50 mM KCl along with its own effects on $[Ca^{2+}]_i$. Among ginsenosides tested, ginsenoside Rg_3 was found to be the most potent component for ginseng actions on NMDA receptors. Furthermore, we examined the inhibitory effects of biotransformants of ginsenosides on NMDA receptor using purified stereoisomers of ginsenosides. 20(*S*)-ginsenoside Rg_3 and its metabolite, 20(*S*)-ginsenoside Rh_2, produced the strongest inhibition while 20(*S*)-ginsenoside Rh_1 and Compound K produced the moderate inhibition on NMDA-induced increase in $[Ca^{2+}]_i$. The data obtained suggest that the inhibition of NMDA receptors by ginseng, in particular by 20(*S*)-ginsenoside Rg_3 and its metabolite, 20(*S*)-ginsenoside Rh_2, could be one of mechanisms for ginseng-mediated neuroprotective actions.

Introduction

Ginseng, the root of *Panax ginseng* C.A. Meyer (Araliaceae), is well known traditional herbal medicine. Ginsenosides, which are triterpene derivatives that contain sugar moieties, are the main active ingredients of ginseng and more than thirty ginsenosides have been isolated from roots of *Panax ginseng* (1-2). Among the efficacies of ginseng, which produces an array of pharmacological responses, recent studies demonstrated beneficial effects of ginseng on the CNS. For example, it was reported that ginseng ameliorated learning and memory deficits in different disease models (3-5). Ameliorating effects of ginseng were observed on the models of scopolamine-induced (3), aged (4-5) or hippocampal lesioned learning and memory deficits (5). Further beneficial effects of ginseng were observed on neuronal cell death associated with ischemia or glutamate toxicity. Administration of ginseng extracts prevented neuronal death in forebrain (6) or myocardial ischemia animal (7). As a study using purified compounds of ginsenosides, it has been reported that ginsenoside Rb_1 protected hippocampal neurons against ischemia (8). Recently it has been also shown ginsenosides Rb_1 and Rg_3 protected cultured cortical neurons from glutamate-induced neurotoxicity (9). These results raise the possibility that therapeutic use of this herbal medicine as the blocking agent in primary cause of neuronal death or memory impairment linked to neurodegeneration diseases. However, the cellular and molecular mechanisms that underlie the actions of ginseng extracts or ginsenosides are not fully understood.

The accumulation of glutamate in the extracellular space in the CNS can induce neuronal death associated with ischemia, hypoglycemia, and trauma. This glutamate toxicity has been clearly attributed to a massive influx of Ca^{2+} through non-NMDA and primarily NMDA receptor channels. A sustained increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) initiates the excitotoxic processes culminating in delayed neuronal death (10-12). Glutamate-evoked Na^+ influx has been also proposed to contribute to acute form of neurotoxicity (13-14).

In an effort to elucidate the mechanism of ginseng actions at the cellular level and the identity of the active substance, we investigated the modulation of ginseng on the activation of NMDA receptors due to the significant role of NMDA receptors in both acute and delayed forms of neuronal death. We have employed cultures of rat hippocampal neurons and examined the direct modulation of ginseng on NMDA receptor-induced changes in $[Ca^{2+}]_i$ and -gated currents using fura-2-based digital imaging and perforated whole-cell patch-clamp techniques, respectively. In the current study, we first report the rapid modulation of ginseng total saponins on NMDA recep-

tor-mediated signals and ginsenoside Rg_3 to be one of the major active components for the effects of ginseng total saponins. Furthermore, 20(S)-ginsenoside Rg_3 and its metabolite, 20(*S*)-ginsenoside Rh_2, produced the strongest inhibition of NMDA-induced $[Ca^{2+}]_i$ increase in hippocampal neurons.

Materials and Methods

Cell preparation

Cell preparation and whole-cell recordings using perforated patch-clamp methods were done as previously described (15). Cells were maintained in Neurobasal/B27 medium containing 0.5 mM L-glutamine, 25 μM glutamate, 25 μM 2-mercaptoethanol, 100 U/ml penicillin and 100 μg/ml streptomycin under a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Cultures were fed twice a week with the same medium without glutamate. Experiments were carried out on neurons after 7-15 days in culture.

Intracellular Ca^{2+} imaging

Fura-2/AM was used as the fluorescent Ca^{2+} indicator. Cells were illuminated using a xenon arc lamp and excitation wavelengths (340 and 380 nm) were selected by a computer-controlled filter wheel. Data were acquired every 2 s and a shutter in the light path between exposures was interposed to protect cells from photo-toxicity. Emitter fluorescence light was reflected through a 515 nm long-pass filter to a frame transfer cooled CCD camera and ratios of emitted fluorescence were calculated using a digital fluorescence analyzer and converted to intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$). All imaging data were collected and analyzed using Universal Imaging software. All the data are represented as means ± S.E.M.

Results and Discussion

Effects of ginseng total saponins on NMDA receptor-mediated signals

In order to examine the direct modulation of ginseng total saponins on NMDA-mediated signals, we initially examined the effect of multiple applications of NMDA on the increases in $[Ca^{2+}]_i$ using fura-2-based digital imaging techniques (Fig. 1A). In most cultured hippocampal cells, the acute application of NMDA (100 μM, a 10 s-duration) produced a rapid increase of

$[Ca^{2+}]_i$ in Mg^{2+}-free and 1 μM glycine-containing recording solution. This NMDA-induced increase in $[Ca^{2+}]_i$ was reproducible by repeated applications of NMDA with 4-5 min interval up to 1 h. The increase in $[Ca^{2+}]_i$ was calculated from the difference between peak and basal values of $[Ca^{2+}]_i$ and not diminished significantly by multiple applications of NMDA (362.6 nM for first and 356.8 nM for 12^{th} application of NMDA), suggesting a valuable system for the examination of NMDA receptor-mediated signals.

Under these conditions, we examined the effect of ginseng total saponins on NMDA-induced $[Ca^{2+}]_i$ increase in cultured hippocampal neurons. After pretreatment of ginseng total saponins (100 μg/ml) for 1 min, co-application of ginseng total saponins with NMDA produced 70.2% inhibition of NMDA-induced $[Ca^{2+}]_i$ increase (Fig. 1B). The mean percentage inhibition by 100 μg/ml ginseng total saponins was 62.0±2.1% from 93 cells. Fig. 1C shows the dose-response relationship for the ginseng total saponins-mediated inhibition on NMDA-induced $[Ca^{2+}]_i$ increase with an IC_{50} of 26.6 μg/ml. In some cells, NMDA-induced $[Ca^{2+}]_i$ increase was not fully recovered when used higher than 100 μg/ml concentration of ginseng total saponins. Thus, only cells showing more than 85% recovery were included in data analysis. This ginseng-mediated effect was also time-dependent. Although the co-application of ginseng total saponins and NMDA produced around 20% inhibition, longer than a 45 s-duration of pretreatment was required for maximum effect (Fig. 1C, *inset*). We, therefore, pretreated cells for 1 min with ginseng total saponins for further experiments. When the NMDA receptor antagonist, D(-)-2-amino-5 phosphonopentanoic acid (D-APV), was applied in cells under the same condition as ginseng total saponins, 250 μM D-APV completely inhibited NMDA-induced $[Ca^{2+}]_i$ increase (Fig. 1B). The IC_{50}, estimated from the graph of the concentration-response relationship, was 8.9 μM which is close to the IC_{50} for D-AVP to NMDA receptors in other studies (16-18).

In order to confirm the direct modulation of NMDA by ginseng total saponins, we measured NMDA receptor-gated whole-cell currents using the nystatin perforated patch-clamp method. At the holding membrane potential of −60 mV, application of NMDA (100 μM, 5 s) induced an inward current which decreased to a plateau level after an initial fast rise. Fig. 1D illustrated representative currents evoked by NMDA alone and co-applied with ginseng total saponins with 1 min pretreatment. Application of ginseng total saponins (100 μg/ml) inhibited NMDA-mediated currents at both peak and the mean values of sustained currents in this cell. This ginseng-mediated inhibition was also occurred in a dose-dependent manner: 39.8±6.8% and 68.2±4.6% inhibition of peak current by 30 and 100 μg/ml and 29.7±6.4% and 60.8±4.6% inhibition of the means

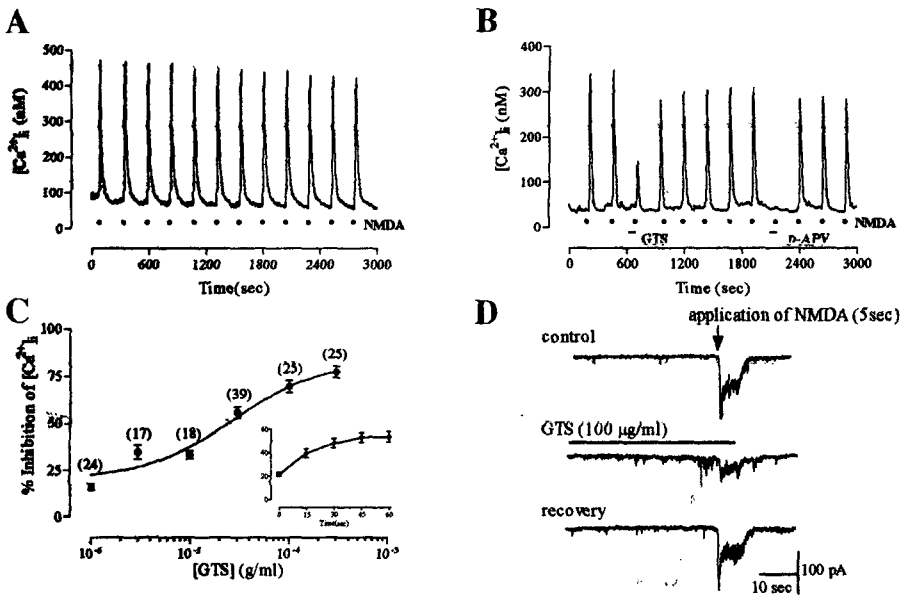

Fig. 1. Effects of ginseng total saponins on NMDA receptor-mediated signals in cultured rat hippocampal neurons. (a) Multiple applications of NMDA (100 μM) on the increases in $[Ca^{2+}]_i$ using fura-2-based digital imaging techniques. (B) Co-application of ginseng total saponins (GTS, 100 μg/ml) with NMDA caused 70.2% inhibition of NMDA-induced $[Ca^{2+}]_i$ increase in this cell. Ginseng total saponins were pretreated for 1 min in the cell before the application of NMDA. Under the same condition, D-APV (250 μM), the NMDA receptor antagonist, produced a complete inhibition of NMDA-mediated response in this cell. (C) Dose-response relationship for inhibition of NMDA-induced $[Ca^{2+}]_i$ increase by ginseng total saponins. The IC_{50} of ginseng total saponins is 26.6 μg/ml. *Inset*, Time-dependent relationship for inhibition of NMDA-induced $[Ca^{2+}]_i$ increase by 30 μg/ml ginseng total saponins. (D) The effect of ginseng total saponins on current responses induced by application of NMDA (5 s) using perforated whole-cell voltage-clamp recordings. Ginseng total saponins rapidly and reversibly inhibited NMDA-mediated currents with 1 min pretreatment.

values of sustained currents by 30 (n=5) and 100 μg/ml (n=6), respectively. This rapid inhibition of ginseng on NMDA receptor-gated ionic currents could provide a feasible explanation of ginseng's action on NMDA-induced $[Ca^{2+}]_i$ increase and all these data together strongly provide a possible mechanism of ginseng's beneficial effects on neuronal cell death, especially associated with glutamate toxicity.

Effects on glutamate- and high K^+-induced changes in $[Ca^{2+}]_i$ and direct modulation of $[Ca^{2+}]_i$ by ginseng total saponins

Since glutamate is an endogenous ligand to non-NMDA and NMDA receptors, we investigated

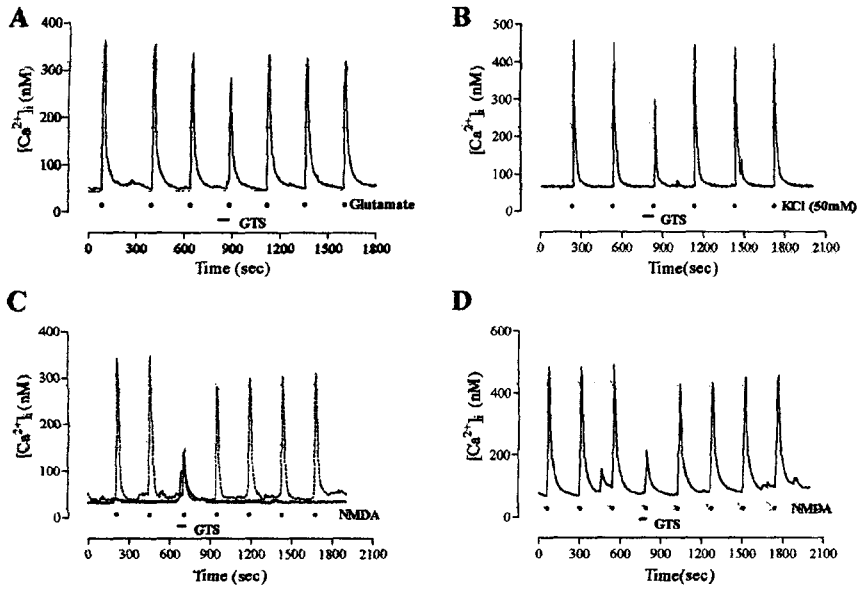

Fig. 2. Effects on glutamate- and high K^+-induced changes in $[Ca^{2+}]_i$ and direct effect on $[Ca^{2+}]_i$. (A) Time course of ginseng total saponins-mediated inhibition on glutamate-induced $[Ca^{2+}]_i$ increase in normal HEPES-buffered solution. (B) Inhibition of 50 mM KCl-induced $[Ca^{2+}]_i$ increase by ginseng total saponins (39.8% in this cell). (C) In addition to the effect on NMDA receptors (dotted line), ginseng total saponins itself directly increased $[Ca^{2+}]_i$ of the cells, which was mostly observed in small sized cells having no neurite processes and NMDA-insensitive cells (solid line). (D) Inhibition of ginseng on NMDA receptors recorded in acutely dissociated hippocampal neurons (66.0%). Hippocampal neurons were isolated from 9-15 day old rats.

the effect of ginseng total saponins on glutamate-induced $[Ca^{2+}]_i$ increase in normal HEPES-buffered solution.

Fig. 2A shows the effect of ginseng total saponins on $[Ca^{2+}]_i$ increase by repeated applications of glutamate (100 μM) instead of NMDA. Application of ginseng total saponins (100 μg/ml) also inhibited 23.8% of glutamate-induced $[Ca^{2+}]_i$ increase. However, the mean percentage inhibition of ginseng total saponins on glutamate from 40 cells tested (19.7±1.7%) was much smaller than the one on NMDA (62.0±2.1%). This ginseng total saponins-mediated effect on glutamate was further examined using the selective agonists of non-NMDA receptors, AMPA and KA (100 μM), in normal HEPES-buffered solution. Increases in $[Ca^{2+}]_i$ evoked by AMAP or KA were also reduced by ginseng total saponins but the degrees of inhibition were smaller than the one on NMDA; 31.6±2.4% (n=76) on AMPA- and 29.1±3.7% (n=26) on KA-mediated signals. These results suggest that ginseng could produce its inhibitory effect through both NMDA and non-NMDA receptors, but mainly through NMDA receptors, which provides a significant therapeutic

role of ginseng by decreasing an excessive activation of NMDA and non-NMDA receptors resulting from abnormal releases of glutamate in injury to the CNS.

Our previous study showed the modulation of ginseng total saponins on high-threshold Ca^{2+} channels in sensory neurons (19). We, therefore, examined whether ginseng could modulate Ca^{2+} currents in the CNS as did in DRG neurons. The effect of ginseng total saponins on Ca^{2+} channels was indirectly examined on the increase in $[Ca^{2+}]_i$ evoked by depolarization using 50 mM KCl. Fig. 2B shows that ginseng total saponins (100 μg/ml) suppressed the 50 mM KCl-induced $[Ca^{2+}]_i$ increase by 35.3±1.7% (n=44). Treatment with the L-type Ca^{2+} channel blocker, nifedipine (10 μM), decreased the inhibition from 38.4±2.4 to 14.7±2.9 (62% occlusion of the effect, n=16). On the other hand, co-treatment of the N-type blocker, ω-conotoxin-GVIA (1 μM), with nifedipine did not further change the inhibition (14.3±2.7, n=14), suggesting ginseng could modulate L- and other uncharacterized-types, but not N-type of Ca^{2+} channel in cultured hippocampal neurons. Neurotransmitter secretion seems to be preferentially coupled to one or more of high-threshold Ca^{2+} channel types under different circumstances. Since excessive release of neurotransmitters is reported to contribute to the neuronal cell death (10-12), neuroprotective effect of Ca^{2+} channel blockers, which effect probably results from its inhibition of the excessive release of neurotransmitters, including excitatory amino acids, were reported (20-23). Especially, the neuroprotective effects of L-type blockers were reported in several types of neurotoxicity such as growth factor deprived, KA-induced, or Zn^{2+}-induced neurotoxicity (20-22). Chronic treatment of hippocampal neurons with the L-type Ca^{2+} channel antagonist, nimodipine, significantly enhanced survival in long-term culture (23). Therefore, the ability of ginseng to decrease Ca^{2+} influx mainly through L-type Ca^{2+} channels in hippocampal neurons may therefore contribute to the neuroprotective effects. This could provide a more powerful therapeutic role of ginseng by decreasing Ca^{2+} influx both through Ca^{2+} channels and NMDA receptors in neurodegenerative diseases which are characterized by a disturbance of cellular Ca^{2+} homeostasis.

In addition to the effects of ginseng on the Ca^{2+} influx through Ca^{2+} channels and NMDA receptors, ginseng total saponins itself directly modulated $[Ca^{2+}]_i$ of the cells (Fig. 2C). Application of ginseng total saponins (100 μg/ml) increased $[Ca^{2+}]_i$ from a resting level (58.9±2.2 nM) to a broad peak level (148.4±8.6 nM, n=54) within a 1min, which was diminished by repeated application. Most of these responses were observed in small sized cells having no neurite processes and NMDA-insensitive but rarely in NMDA-sensitive cells, suggesting that ginseng total saponins modulate Ca^{2+} signals in non-neuronal cells. However, we can not rule out the possibil-

ity that ginseng total saponins modulate $[Ca^{2+}]_i$ in neuronal cells. Although ginseng has been already shown to increase $[Ca^{2+}]_i$ of cells in non-neuronal system (24-26), it is first observed in the neuronal system. Results of previous studies suggested that this process involves PLC activation, the release of Ca^{2+} from the IP_3-sensitive intracellular store and pertussis toxin-insensitive G protein activation in Xenopus oocytes (25, 26). Further experiments for the identification of cell types and source of Ca^{2+} increase as well as the significance in neurotoxicity will be interesting.

Although methods of primary culturing neurons provide useful ways to study the CNS neurons, they might be different from ones dissociated acutely depending on culturing conditions. Therefore, we examined whether ginseng can also modulate NMDA receptors in acutely dissociated hippocampal neurons from 9-15 days old rats. The reduction of ginseng total saponins (100 μg/ml) on NMDA-induced $[Ca^{2+}]_i$ increase was observed in acutely dissociated hippocampal neurons (Fig. 2D) as shown in cultured ones (Fig. 1). The mean percentage inhibition of ginseng total saponins was 76.3±3.2% in acutely isolated cells (n=6). The recovery time of $[Ca^{2+}]_i$ from peak to basal level after NMDA application was seemed to be a little longer in acutely isolated cells. In addition to the modulation of NMDA receptors, direct effect of ginseng on $[Ca^{2+}]_i$ was also observed in these isolated cells. Therefore, the effects of ginseng which we observed in the present study are the general characteristics observed in hippocampal neurons regardless of cell culture conditions.

Effects of individual ginsenosides on NMDA-induced $[Ca^{2+}]_i$ increase

For the application of natural compounds as a potential pharmacological agent, it is necessary to identify the main component(s) that is responsible for its effect. Ginsenosides or ginseng saponins are the main molecular components responsible for the actions of ginseng and more than 30 types of ginsenosides have been identified (1, 2). Therefore, we examined the effects of purified individual ginsenosides on NMDA-mediated $[Ca^{2+}]_i$ increase for the identity of the active compound(s) in the action of ginseng (Fig. 3). The list of 10 individual ginsenosides tested -Rb_1, Rb_2, Rc, Rd, Re, Rf, Rg_2, Rg_3, Rh_1, and Rh_2- was chosen from ginsenosides comprised most of the main components of ginseng and their chemical structures were reported previously (19). At the concentration of 10 μM, ginsenosides Rb_2, Rd, Re, Rf, and Rg_2 had a little or no significant effect (<10% inhibition) while ginsenosides Rb_1, Rc, Rh_1, and Rh_2 produced the moderate inhibition on NMDA-induced $[Ca^{2+}]_i$ increase (about 20-27% inhibition). However, we found ginsenoside Rg_3 produced the biggest inhibitory effect (70.0±4.5% at 10 μM, n=29, Fig. 3A &

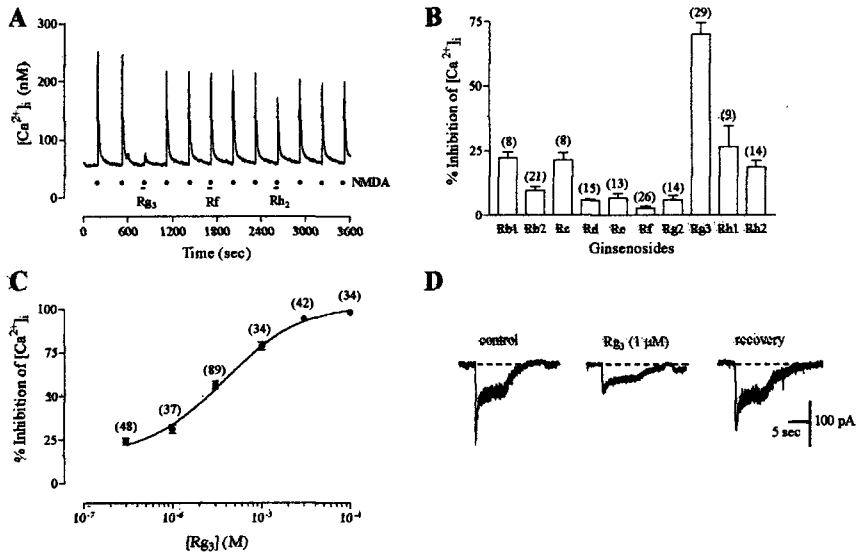

Fig. 3. Identification of active components responsible for ginseng-mediated inhibition on NMDA receptors. (A) Effects of different ginsenosides Rg_3, Rf and Rh_2 on NMDA-induced $[Ca^{2+}]_i$ increase in a one cell. At 10 μM concentration, ginsenoside Rg_3 (Rg_3) and ginsenoside Rh_2 (Rh_2) inhibited NMDA-induced $[Ca^{2+}]_i$ increase by 89.3% and 28.8%, respectively, while ginsenoside Rf(Rf) produced no significant effect on NMDA response in this cell. (B) The pooled results illustrating the mean percentage inhibition of NMDA-induced $[Ca^{2+}]_i$ increase by individual ginsenosides. Among 10 ginsenosides tested, ginsenoside Rg_3 produced the biggest effect (70.0±4.5%) on NMDA-induced $[Ca^{2+}]_i$ increase at 10 μM. (C) Dose-response relationship for inhibition of NMDA-induced $[Ca^{2+}]_i$ increase by ginsenoside Rg_3. The IC_{50} value of ginsenoside Rg_3 is 3.8 μM. (D) Ginsenoside Rg_3, the major active component of ginseng on the inhibition of NMDA-induced $[Ca^{2+}]_i$ increase, also inhibited NMDA receptor-gated currents.

3B) on NMDA-induced $[Ca^{2+}]_i$ increase. This ginsenoside Rg_3-mediated inhibition also occurred in a dose-dependent manner with an IC_{50} of 3.8 μM (Fig. 3C).

Since we found that ginsenoside Rg_3 is a major component for the inhibition of NMDA-induced $[Ca^{2+}]_i$, we next examined the effect of ginsenoside Rg_3 on NMDA receptor-gated currents as did with ginseng total saponins (Fig. 1D). Ginsenoside Rg_3 (1 μM) inhibited NMDA-mediated currents at both peak and the mean values of sustained currents (49.4±10.2% and 32.5±9.5% inhibition, respectively, n=5), which was recovered after washout of the drug as shown in Fig. 3D.

Effects of biotransformants of ginsenosides on NMDA-induced $[Ca^{2+}]_i$ increase

Some ginsenosides being taken orally are known to be metabolited into its biotransformate by

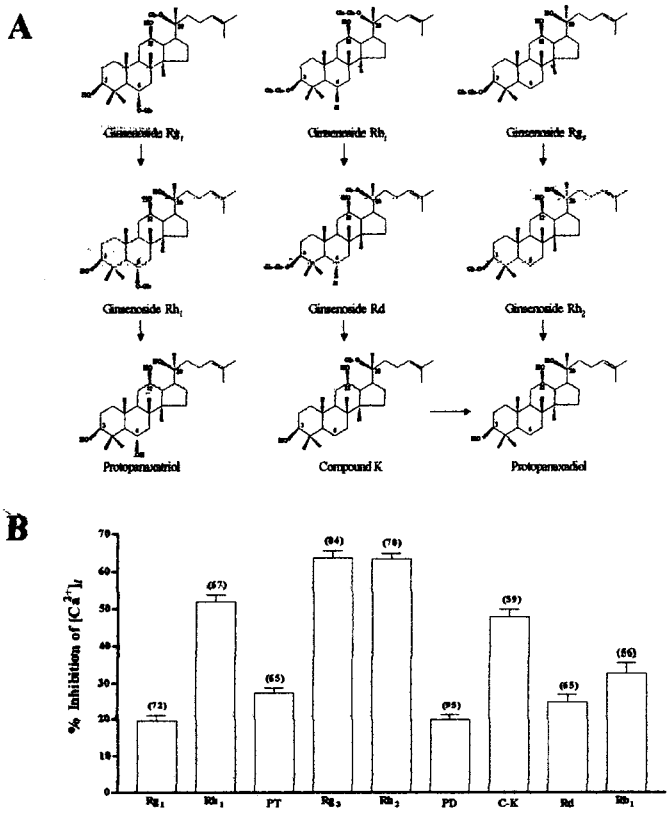

Fig. 4. Inhibitory effects of biotransformants of ginsenosides on NMDA receptors. (A) Structures of main 20(S)-ginsenosides of *Panax ginseng*, and the proposed metabolic pathways of ginsenosides by human intestinal bacteria. (B) The pooled results illustrating the mean percentage inhibition of NMDA-induced $[Ca^{2+}]_i$ increase by biotransformants of ginsenosides. Among 9 ginsenosides tested, ginsenoside Rg_3 and its metabolite, 20(*S*)-ginsenoside Rh_2, produced the biggest inhibitory effect (63.55±1.96% and 63.26±1.64%) on NMDA-induced $[Ca^{2+}]_i$ increase at 10 μM.

as shown in Fig. 4A. For example, 20(S)-ginsenoside Rg_3 are transformed to 20(S)-ginsenosides Rh_2 and finally to 20(S)-protopanaxadiol.

Therefore, we examined the effects of purified 20(S)-ginsenosides and its metabolites of ginsenosides on NMDA-mediated $[Ca^{2+}]_i$ increase. At the concentration of 10 μM, 20(S)-ginsenosides Rg_1, Rd, protopanaxatriol and protopanaxadiol had a little or no significant effect (about 20% inhibition) while ginsenosides Rh_1, Rb_1 and Compound K produced the moderate inhibition on NMDA-induced $[Ca^{2+}]_i$ increase (about 30-50% inhibition). However, among 20(S)-forms of ginsenosides tested, 20(*S*)-ginsenoside Rg_3 and its metabolite, 20(*S*)-ginsenoside Rh_2, produced

the strongest inhibitory effect (63.55±1.96% and 63.26±1.64% inhibition, n=82 and 73, respective) (Fig. 4B).

Our finding of ginsenoside Rg_3, as a main component, is interesting because it is consistent with results of the previous studies showing ginsenoside Rg_3 is one of the identified main component(s) that are responsible for the actions of ginseng (9, 21, 29-30). Kim et al. (9) reported that ginsenosides Rb_1 and Rg_3 significantly attenuated glutamate-induced neurotoxicity in cultured cortical neurons. Ginsenoside Rg_3 is the most potent compound of ginseng attenuated NMDA-mediated signals and ginsenosides Rb_1 also produced the moderate inhibition on the signals in cultured hippocampal neurons from the present study. In addition, we previously found that ginseng total saponins inhibit high-threshold Ca^{2+} channels and identified ginsenoside Rg_3 as an active component among the major fractions of ginseng saponins for the inhibition of Ca^{2+} channels in sensory neurons (19). The results from the present study provide possible mechanisms of ginseng in protecting neuronal cell death, especially glutamate-mediated death. First, ginseng's action on the modulation of NMDA receptors will diminish excessive activation of glutamate receptors, primarily NMDA subtypes, which were excessively released from injury to the CNS. Secondly, ginseng's antagonistic effects of Ca^{2+} channels will potentiate its neuroprotective action by reducing Ca^{2+}-dependent signal cascades in neurotoxicity or inhibiting excessive release of neurotransmitters, including glutamate. Thirdly, for long-term effects, the previous study showed that ginsenosides Rb_1 and Rg_3 inhibited the overproduction of nitric oxide, which routinely follows glutamate neurotoxicity, and preserved the level of superoxide dismutase in glutamate-treated cells (9). Although we initially set the experiment focused on the acute modulation of NMDA receptors for fast screening system of ginseng's activities, it is necessary to investigate the effects of ginseng and ginsenoside Rg_3 on long-term treatment of NMDA. We are currently investigating the effects of ginseng and ginsenosides on long-term NMDA-induced change of $[Ca^{2+}]i$ level and NMDA-mediated toxicity, which are more relevant conditions of the delayed form of excitotoxic neuronal injury.

From the present study, it is not clearly elucidated yet whether ginseng mediates its action by affecting NMDA receptors directly or indirectly. The co-application of ginseng total saponins with NMDA produced a considerable inhibition (~20%, Fig. 1C), which suggests a possibility of NMDA receptors as a direct target of ginseng. Recent studies (29, 30) showed that ginsenoside Rg_2 directly blocks nicotinic acetylcholine receptors expressed in *Xenopus* oocytes. They showed ginsenoside Rg_2 acts as a noncompetitive antagonist on acetylcholine receptors which belong to

ligand-gated channels as do NMDA receptors. These studies provide a possibility of ginsenoside Rg_3 as a direct modulator on NMDA receptors in hippocampal neurons. However, the requirement of pretreatment may reflect certain signal cascade for the full action of ginseng although the possibility of direct pre-occupation of ginseng on NMDA receptors during pretreatment is still remained. There are several studies showing indirect modulation of ginsenosides on ion channels. Ginseng saponins reversibly block high-threshold Ca^{2+} currents in chromaffin cells (31) and sensory neurons (19, 32), and it has been suggested that this effect is mediated via activation of pertussis toxin-sensitive G protein(s). As pertussis toxin-insensitive G protein pathway, Choi et al. (25, 26) reported that ginsenosides increased Ca^{2+}-activated Cl^- current by activating $G\alpha_{q/11}$ protein coupled to PLC, and the release of Ca^{2+} from the IP_3-sensitive intracellular store in *Xenopus* oocytes. This is more feasible pathway for direct modulation of ginseng on $[Ca^{2+}]_i$, which mostly observed in NMDA-insensitive cells from the present study. Recently, we also reported that ginsenoside Rf activates G protein-coupled inwardly rectifying K^+ (GIRK) channels after co-injection of subfractions of rat brain mRNA with GIRK1/4 channel cRNAs in *Xenopus* oocytes (33). Furthermore, we provided evidence that ginsenoside Rf produces its signal through pertussis toxin-insensitive G protein and might interact with unidentified protein derived from rat brain. Taken together, these studies suggest that ginsenosides could modulate ion channels indirectly using various signal pathways.

Although further experiments will be needed for the detailed mechanisms of ginseng's action on NMDA receptors, our data clearly demonstrate that ginseng total saponins, in particular ginsenoside Rg_3 and its metabolite, 20(S)-ginsenoside Rh_2, attenuate the influx of Ca^{2+} via NMDA-receptors on cultured and acutely isolated hippocampal cells. Therefore, for the first time, our findings provide a cellular evidence that the rapid inhibition of NMDA receptors by ginsenoside Rg_3 and metabolite of 20(S)-ginsenoside Rg_3, 20(S)-ginsenosides Rh_2, in hippocampal neurons and offer a useful therapeutic choice in the treatment of neurodegenerative disorders.

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References

1. Liu, C.X., Xiao, P.G. (1992) *J. Ethnopharmacol.* **36**, 27-38.
2. Baek, N.I., Kim, D.S., Lee, Y.H., Park, J.D., Lee, C.B., Kim, S.I. (1996) *Planta Med.* **62**, 86-87.
3. Hsieh, M.T., Peng, W.H., Wu, C.R., Wang, W.H. (2000) *Phytother. Res.* **14**, 375-377.
4. Wesnes, K.A., Ward, T., McGinty, A., Petrini, O. (2000) *Psychopharmacology (Berl)* **152**, 353-361.
5. Zhong, Y.M., Nishijo, H., Uwano, T., Tamura, R., Kawanishi, K., Ono, T. (2000) *Physiol. Behav.* **69**, 511-525.
6. Wen, T.C., Yoshimura, H., Matsuda, S., Lim, J.H., Sakanaka, M. (1996) *Acta. Neuropathol. (Berl)* **91**, 15-22.
7. Maffei Facino, R., Carini, M., Aldini, G., Berti, F., Rossoni, G. (1999) *Planta Med.* **65**, 614-619.
8. Lim, J.H., Wen, T.C., Matsuda, S., Tanaka, J., Maeda, N., Peng, H., Aburaya, J., Ishihara, K., Sakanaka, M. (1997) *Neurosci Res.* **28**, 191-200.
9. Kim, Y.C., Kim, S.R., Markelonis, G.J., Oh, T.H. (1998) *J. Neurosci. Res.* **53**, 426-432.
10. Coyle, J.T., Puttfarcken, P. (1993) *Science* **262**, 689-695.
11. Choi, D.W., Rothman, S.M. (1990) *Annu. Rev. Neurosci.* **13**, 171-182.
12. Sattler, R., Tymianski, M.J. (2000) *Mol. Med.* **78**, 3-13.
13. Rothman, S.M. (1985) *J. Neurosci.* **5**, 1483-1489.
14. Olney, J.W., Price, M.T., Samson, L., Labruyere, J. (1986) *Neurosci. Lett.* **65**, 65-71.
15. Kim S, Ahn K, Oh T, Nah S, Rhim H. (2002) *Biochem Biophys Res Commun.* **296**, 247-254.
16. Gean, P.W., and Shinnick-Gallagher, P. (1988) *Neuropharmacology* **27**, 557-562.
17. Lodge, D., Davies, S.N., Jones, M.G., Millar, J., Manallack, D.T., Ornstein, P.L., Verberne, A.J., Young, N., Beart, P.M. (1988) *Br. J. Pharmacol.* **95**, 957-965.
18. Debono, M.W., Le Guern, J., Canton, T., Doble, A., Pradier, L. (1993) *Eur. J. Pharmacol.* **235**, 283-289.
19. Rhim, H., Kim, H., Lee, D.Y., Oh, T.H., Nah, S.Y. (2002) *Eur. J. Pharmacol.* **436**, 151-158.
20. Xia, S., Lampe, P.A., Deshmukh, M., Yang, A., Brown, B.S., Rothman, S.M., Johnson, E.M. Jr, Yu, S.P. (2002) *J. Neurosci.* **22**, 114-122.
21. Leski, M.L., Valentine, S.L., Coyle, J.T. (1999) *Brain Res.* **828**, 27-40.

22. Kim, A.H., Sheline, C.T., Tian, M., Higashi, T., McMahon, R.J., Cousins, R.J., Choi, D.W. (2000) *Brain Res.* **886**, 99-107.
23. Porter, N.M., Thibault, O., Thibault, V., Chen, K.C., Landfield, P.W. (1997) *J. Neurosci.* **17**, 5629-5639.
24.. Li, Z., Niwa, Y., Sakamoto, S., Shono, M., Chen, X., Nakaya, Y. (2000) *Life Sci.* **67**, 2983-2989.
25. Choi, S., Rho, S.H., Jung, S.Y., Kim, S.C., Park, C.S., Nah, S.Y. (2001) *Br. J. Pharmacol.* **132**, 641-648.
26. Choi, S., Kim, H.J., Ko, Y.S., Jeong, S.W., Kim, Y.I., Simonds, W.F., Oh, J.W., Nah, S.Y. (2001) *J. Biol. Chem.* **276**, 48797-48802.
27. Kim, N.D., Kang, S.Y., Kim, M.J., Park, J.H., Schini-Kerth, V.B. (1999) *Eur. J. Pharmacol.* **367**, 51-57.
28. Liu, W.K., Xu, S.X., Che, C.T. (2000) *Life Sci.* **67**, 1297-1306.
29. Choi, S., Jung, S.Y., Lee, J.H., Sala, F., Criado, M., Mulet, J., Valor, L.M., Sala, S., Engel, A.G., Nah, S.Y. (2002) *Eur. J. Pharmacol.* **442**, 37-45.
30. Sala, F., Mulet, J., Choi, S., Jung, S.Y., Nah, S.Y., Rhim, H., Valor, L.M., Criado, M., Sala, S. (2002) *J. Pharmacol. Exp. Ther.* **301**, 1052-1059.
31. Kim, H.S., Lee, J.H., Goo, Y.S., Nah, S.Y. (1998) *Brain Res. Bull.* **46**, 245-251.
32. Nah, S.Y., Park, H.J., McCleskey, E.W. (1995) *Proc. Natl. Acad. Sci. USA,* **92**, 8739-8743.
33. Choi, S., Jung, S.Y., Ko, Y.S., Koh, S.R., Rhim, H., Nah, S.Y. (2002) *Mol Pharmacol.* **61**, 928-93.