Ginsenosides Rb_1 and Rg_3 Attenuate Glutamate-induced Neurotoxicity in Primary Cultures of Rat Cortical Cells

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ABSTRACT

In the present study, we assayed a number of compounds isolated from *Panax ginseng* C. A. Meyer (Araliaceae) for an ability to protect rat cortical cell cultures from the deleterious effects of the neurotoxicant, glutamate. We found that ginsenosides Rb_1 and Rg_3 significantly attenuated glutamate-induced neurotoxicity. Brief exposure of cultures to excess glutamate caused extensive neuronal death. Glutamate-induced neuronal cell damage was significantly reduced by pretreatment with Rb_1 and Rg_3. 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. Furthermore, in cultures treated with glutamate, these ginsenosides inhibited the formation of malondialdehyde, a compound produced during lipid peroxidation, and diminished the influx of calcium. These results show that ginsenosides Rb_1 and Rg_3 exerted significant neuroprotective effects on cultured cortical cells. As such, these compounds may be efficacious in protecting neurons from oxidative damage produced by exposure to excess glutamate.

Key words: *Panax ginseng;* neuroprotective activity; oxidative stress; nitric oxide; malondialdehyde; calcium influx; superoxide dismutase

Introduction

The root of *Panax ginseng* C. A. Meyer (Araliaceae) has been used as an herbal medicine for several thousand years in China, Korea and Japan (Cho *et al.*, 1995). In general, ginseng is known to increase arousal, stamina and resistance to stress (Takagi *et al.*, 1972a; Saito *et al.*, 1974; Cho *et al.*, 1995). Ginseng' s stimulant effects on the central nervous system (CNS), its action against fatigue and enhancement of non-specific resistance have been attributed to the saponin fraction of its constituents (Takagi *et al.*, 1972b; Nabata *et al.*, 1973). More than 30 ginsenosides, triterpene-derivatives containing sugars, have been isolated from the ginseng saponin fraction. The chemical structures of the individual ginsenosides have been identified (Baek *et al.*, 1996; Kaku and Kawashima, 1980; Kitagawa *et al.*, 1983; Nagai et al, 1971; Shao, 1984; Shibata *et al.*, 1963; Shibata *et al.*,

1965). It was reported that ginsenosides Rb and Rc exhibited a sedative effect while ginsenoside Rg showed stimulatory actions on the CNS (Takagi *et al.*, 1972b; Saito *et al.*, 1974). Ginsenoside Rb_1 enhanced the stimulatory effect of nerve growth factor on neurite outgrowth (Saito *et al.*, 1977), increased CNS cholinergic metabolism (Benishin, 1992), and protected hippocampal neurons against either ischemia (Lim *et al.*, 1997) or glutamate neurotoxicity (Liu and Zhang, 1995). Ginsenoside Rc strongly increased glutamate release and Ca^{2+} concentration in cultured rat cerebellar neurons (Oh *et al.*, 1995). Ginsenoside Rg_1 inhibits rat brain cAMP phosphodiesterase activity (Stancheva and Alova, 1993). Chu and Chen (1989, 1990) reported that a mixture of ginsenosides significantly improved superoxide dismutase (SOD) activity and reduced lipid peroxidation in cerebral ischemia/reperfusion in rats.

Glutamate is found in millimolar levels in the brain and plays a dominant role in central excitatory neurotransmission. Glutamate is also known to cause neuronal cell loss in the CNS (Choi, 1988). This excitatory amino acid produces its effect by acting on N-methyl-D-aspartate (NMDA) receptors and on non-NMDA receptors including those activated by kainic acid and amino-3-hydroxy-5-methylisoxazole-4-propionic acid (Monaghan *et al.*, 1989). Several studies have indicated that excitatory amino acids including glutamate are involved in neuronal survival, synaptogenesis, neuronal plasticity, learning and memory processes (Kruk and Pycock, 1991; Monaghan *et al.*, 1989). Abnormalities in glutamate neurotransmitter systems may be involved in neurological disorders such as seizures (Zaczek and Coyle, 1982), Alzheimer' s disease (Greenamyre and Young, 1989), ischemia and spinal cord trauma (Alber *et al.*, 1989). The neurotoxicity produced by glutamate can be separated into two distinct forms, acute and delayed (Choi, 1985; Choi *et al.*, 1989). Acute neurotoxicity appears to be mediated by the entry of Na^+ and K^+ into neurons resulting in cell swelling which leads to cell death. Delayed neurotoxicity is Ca^{2+}-dependent. The Ca^{2+}-mediated effects of glutamate receptor activation lead to neuronal degeneration via oxidative stress. Activation of both NMDA and non-NMDA receptors stimulated phospholipase A_2 with the subsequent liberation of arachidonic acid leading to the generation of such oxygen radicals as superoxide anion and hydroxyl radicals (Coyle and Puttfarcken, 1993).

In the present study, primary cultures of rat cortical cells were used in an *in vitro* assay system to evaluate the neuroprotective effects of ginsenosides. Among the 10 ginsenosides tested--Rb_1, Rb_2, Rc, Re, Rg_1, Rg_2, Rg_3, Rg_5, Rh_1 and Rh_2--only ginsenosides Rb_1 (Liu and Zhang, 1995) and Rg_3 protected against neuronal damage induced by excess glutamate.

Materials and Methods

Cell Culture

Primary cultures of mixed cortical cells containing both neuronal and glial cells were prepared

from 17-19 day-old fetal Sprague-Dawley rats as described previously (Park *et al.*, 1995). Cultures were allowed to mature for 2 weeks before being used for experiments.

Neurotoxicity

All ginsenosides were dissolved in DMSO (final culture concentration, 0.1%). Cortical cell cultures were washed with DMEM and incubated with ginsenosides for one hr. The cultures were then exposed to 50μM glutamate for 15 min and washed. After 24 hr incubation in DMEM in the presence of ginsenosides, the cultures were assessed for the extent of neuronal damage *(treatment throughout; see below).*

Assessment of Neurotoxicity

Neuronal viability was quantified by measuring dehydrogenase activity retained in living cells using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay (Mosmann, 1993); neuronal integrity was assessed by spectrophotometric measurement of the efflux of lactate dehydrogenase (LDH) into the culture medium (Choi and Koh, 1987).

Assays for Antioxidative Enzymes

Cells from four culture plates were pooled in 2 ml of 0.1 M phosphate buffer (pH 7.4) and homogenized. The homogenate was centrifuged for 30 min at 3,000 x g at 4℃ and the supernatant was used in enzyme assays. Cellular activities of SOD (McCord and Frivich, 1969), catalase (Beers and Sizer, 1952) and glutathione peroxidase (GSH-px) (Lawrence and Burk, 1976) were measured according to the methods described with minor modifications.

Nitrite Content

The level of nitric oxide formed was determined by measuring the content of nitrite released into the culture medium by the method of Dawson *et al.* (1994).

Lipid Peroxides

The content of malondialdehyde, a compound produced during lipid peroxidation, was determined using the thiobarbituric acid method (Yagi *et al. 1988).*

Measurement of Intracellular Calcium $[Ca^{2+}]i$

Intracellular calcium, $[Ca^{2+}]_i$, was determined by ratio fluorometry according to the method of Grynkiewicz *et al.* (1985).

Protein Assay

The content of protein in cells was measured by the method of Lowry *et al.* (1951).

Data Analysis

Data were evaluated for statistical significance by an "ANOVA" test using a computerized statistical package. The data were considered to be statistically significant if the probability value was $\leq$ 0.05.

Results

All the ginsenosides studied were initially screened in pilot experiments to assess their neuroprotective capabilites against glutamate toxicity. Only ginsenosides Rb_1 and Rg_3 had beneficial effects in our initial screening assays so they were evaluated more fully.

Neuroprotective activity of ginsenosides Rb_1 and Rg_3 was initially evaluated by assessing the integrity and viability of cortical cells after treatment with glutamate. Ginsenosides Rb_1 and Rg_3 significantly preserved cellular integrity as measured by a reduction in the release of LDH from glutamate-treated rat cortical cells, and preserved the viability of glutamate-treated rat cortical cells as assessed by the MTT assay (Fig. 1). Under phase-contrast microscopy, over seventy percent of neurons died in cortical cultures exposed to 50μM glutamate for 15 min when assessed after a lag interval of 24 hr; the viability of non-neuronal cells was not affected (data not shown). Ginsenoside Rb_1 protected neurons from glutamate-induced damage to a significant degree at concentrations ranging from 100 nM to 10μM. Ginsenoside Rg_3 also significantly attenuated glutamate-induced neuronal death at concentrations ranging from 10μM to 1μM. These results showed that ginsenoside Rg_3 has neuroprotective activity equivalent to that of ginsenoside Rb_1 but was active at one-tenth the concentration. The neuroprotective effects of ginsenosides Rb_1 and Rg_3 began to decrease, however, at 10μM (Fig. 1). In some experiments, cultures were pretreated with ginsenosides for one hr before a brief exposure (15 min) to glutamate, washed and maintained in DMEM for 24 hr in the absence of ginsenosides *(pre-treatment)*. Also some cultures were exposed to glutamate for 15 min, washed and maintained in DMEM for 24 hr in the presence of ginsenosides *(post-treatment)*. Both pre- and post-treatment with ginsenosides Rb_1 and Rg_3 significantly attenuated glutamate-induced neurotoxicity. However, post-treatment with ginsenosides appeared to be less effective against glutamate-induced neurotoxicity than when ginsenosides were present throughout the experiment or as a pre-treatment.

Since oxidative stress is a well-known mechanism responsible for glutamate-induced neuronal degeneration (Coyle and Puttfarcken, 1993), we determined the activities of the antioxidative enzymes, SOD, catalase and GSH-px in glutamate-treated cells. Glutamate treatment markedly reduced the activity of SOD in cortical cultures (Fig. 2). Pretreatment with ginsenosides Rb_1 and Rg_3

at 100 nM significantly attenuated the decrease in SOD activity in glutamate-treated cortical cultures (Fig. 2). SOD activities in cultures treated with glutamate plus ginsenosides fell slightly, while cultures treated with glutamate alone experienced a precipitous drop in SOD activities. Ginsenosides Rb_1 and Rg_3 had no significant effects on the activities of either catalase or GSH-px in glutamate-treated cultures (Fig. 2).

The formation of nitric oxide (NO) is involved in glutamate neurotoxicity by generating oxygen radicals. Since free radicals cause lipid peroxidation (Coyle and Puttfarcken, 1993), we determined both the level of NO formed, as measured after conversion to nitrite, and the content of cellular malondialdehyde, a compound produced by lipid peroxidation, in glutamate-treated cortical cultures. Ginsenosides Rb_1 and Rg_3 at 100 nM significantly reduced the nitrite released into the medium, and the content of cellular malondialdehyde in glutamate-treated cultures (Fig. 3).

Changes mediated by Ca^{2+}-influx through glutamate receptor activation lead to neuronal degeneration *via* oxidative stress (Coyle and Puttfarcken, 1993). We examined therefore the effects of ginsenosides on Ca^{2+} influx induced by high concentrations of glutamate. As shown in Fig. 4, a brief exposure (15 min) to 50 μM glutamate caused a significant increase in intracellular Ca^{2+} levels in cortical cell cultures. Ginsenosides Rb_1 and Rg_3 at concentrations ranging from 0.1 to 10 μM significantly reduced calcium influx caused by glutamate (see Liu and Zhang, 1995).

Discussion

The results of the present study demonstrate that ginsenosides Rb_1 and Rg_3 have significant neuroprotective effects against glutamate-induced cortical neuron damage *in vitro*. These compounds protected neuronal viability against glutamate-induced injury and reduced the release of LDH. They also prevented a decrease in SOD activity, and inhibited overproduction of NO, the formation of malondialdehyde and the influx of calcium induced by the neurotoxicant. These findings are in accord with two recent studies that demonstrated a neuroprotective effect by Rb_1 on hippocampal neurons in ischemia and glutamate neurotoxicity, respectively (Lim *et al.*, 1997; Liu and Zhang, 1995). For these reasons, ginsenosides Rb_1 and Rg_3 could be useful neuroprotective agents that mitigate the oxidative stress caused by excess glutamate.

The effects of ginsenosides Rb_1 and Rg_3 on cell viability demonstrate a dose-response "plateau" using concentrations ranging from 10nM to 1.0μM (see Fig. 1). The neuroprotective effects of these ginsenosides then began to decrease at 10 μM. Thus, at higher concentrations, the compounds appear to be cytotoxic to neuronal cells. This phenomenon is also seen with saponin at concentrations above 10μM (Hostettmann and Marston, 1995). The experimental protocols using *treatment throughout* (Fig. 1) and *pre-treatment (vide supra)* with ginsenosides at concentrations ranging from 0.1 to 1.0μ M produced comparable neuroprotective effects on glutamate-treated cells. However, while *post-*

treating with the ginsenosides *(vide supra)* showed some significant effects, this protocol was less effective in preserving viability than was the *pre-treatment* and *treatment throughout* protocols. This suggests that these compounds are more efficacious in protecting neurons against glutamate-induced damage when given prior to glutamate exposure.

Chu and Chen (1989; 1990) have shown that a mixture of ginsenosides significantly improved SOD activity and reduced the extent of lipid peroxidation in cerebral ischemia/reperfusion of rats. In the present study, we demonstrated that among the ten ginsenosides tested in cortical cell cultures, ginsenosides Rb_1 (Lim *et al.,* 1997; Liu and Zhang, 1995) and Rg_3 exerted significant neuroprotective effects on cortical cell cultures. This effect was demonstrated by an inhibition in the production of NO, an inhibition of lipid peroxidation, the prevention of a decrease in SOD activity and a decrease in the influx of calcium. Thus, in a mixture of ginsenosides, it seems likely that ginsenosides Rb_1 and Rg_3 may be responsible for the neuroprotective effects observed in acute cerebral ischemia-reperfusion injuries in rats (Chu and Chen, 1989; 1990, Lim *et al.,* 1997; Liu and Zhang, 1995).

Superoxide anion ($O_2^{\cdot-}$) has a higher affinity for NO than for SOD under certain conditions (Kohno *et al.,* 1995). The decrease in $O_2^{\cdot-}$ inactivation *via* a reduction in SOD activity promoted the overproduction of peroxynitrite radicals ($ONOO^-$), a product of the reaction of $O_2^{\cdot-}$ and NO (Kohno *et al.,* 1995). Our results showed that ginsenosides Rb_1 and Rg_3 at 100 nM inhibited the overproduction of NO which routinely follows glutamate neurotoxicity and rapidly reacts with $O_2^{\cdot-}$. to form $ONOO^-$ (Fig. 3). These results suggest that the retention of SOD activity by treatment with ginsenosides Rb_1 and Rg_3 promotes $O_2^{\cdot-}$ inactivation and in turn inhibits overproduction of NO and $ONOO^-$. Furthermore, ginsenosides Rb_1 and Rg_3 inhibited the formation of malondialdehyde in cortical cell cultures exposed to glutamate (Fig. 3), suggesting that lipid peroxidation is reduced as the result of a decrease in $ONOO^-$ production by treatment with ginsenosides Rb_1 and Rg_3.

It is well known that Ca^{2+} mediates a delayed neuronal degeneration caused by the activation of NMDA and non-NMDA receptors (Coyle and Puttfarcken, 1993). The Ca^{2+} influx by glutamate receptor activation causes oxidative stress that leads to neuronal degeneration. The ability of ginsenosides Rb_1 and Rg_3 to attenuate neuronal damage induced by glutamate suggests that these compounds may exert neuroprotective effects, in part, by reducing Ca^{2+} entry (see Lim *et al.,* 1997). The present experiments provide evidence that ginsenosides Rb_1 and Rg_3 significantly reduce the increase in intracellular Ca^{2+} caused by excessive glutamate (see Fig. 4). However, the mode of action of the ginsenosides on Ca^{2+} entry still is not known.

At present, the cellular and molecular mechanisms that underlie the action of ginsenosides Rb_1 and Rg_3 are not fully understood. However, our data clearly agree with the data from other laboratories (Lim et al, 1997; Liu and Zhang, 1995) and demonstrate unequivocally that these compounds exert significant neuroprotective effects on cultured cortical cells. The decrease in glutamate-induced

Ca^{2+} entry by the ginsenosides might explain the neuroprotective effects observed in the present *in vitro* study. Natural products that attenuate the influx of Ca^{2+} induced by excessive glutamate might offer a useful therapeutic choice in the treatment of neurodegenerative disorders caused by oxidative stress.

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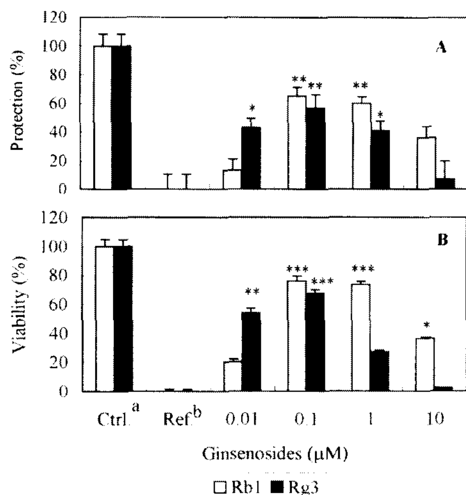

Figure 1.
Effects of ginsenosides Rb1 and Rg3 on cell viability and level of LDH released from glutamate-treated cortical cell culturesa.
Cortical cells cultures were washed with DMEM and incubated with ginsenosides for one hr. The cultures was then exposed to 50 M glutamate for 15 min and washed. After 24 hr incubation in DMEM in the presence of ginsenosides, the cultures were assessed for the extent of neuronal damage (treatment throughout). The values shown are the mean SEM of three experiments.
aLDH and Optical densities (OD) of control were 120.9 8.3 mU/ml and 1.03 0.08, respectively.
bLDH and Optical densities (OD) of reference were 197.6 10.6 mU/ml and 0.58 0.02, respectively. Reference value differs significantly from the untreated, control at a level of $p < 0.001$.
cProtection or cell viability was calculated as 100 x (LDH or OD of reference + ginsenoside-treated LDH or OD of reference) / (LDH or OD of control LDH or OD of reference).
Results differ significantly from the reference at a level of : $*p < 0.05$, $** p < 0.01$, $*** p$ 0.001.

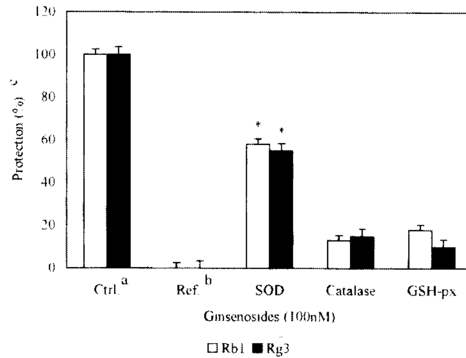

Figure 2.
Effects of ginsenosides Rb1 and Rg3 on the activities of superoxide dismutase (SOD), catalase and glutathione peroxidase (GSH-px) in glutamate-treated cortical cell cultures.
Cortical cultures were treated with ginsenosides Rb1 and Rg3 at a concentration of 100 nM for one hr before glutamate-induced neurotoxicity. The cultures was then exposed to 50 M glutamate for 15 min and washed. After 24 hr incubation in DMEM in the presence of ginsenosides, the cultures were assessed for the extent of neuronal damage (treatment throughout). The values shown are the mean SEM of three experiments.a SOD, catalase and GSH-px of control were 53.5 4.4 mU/ml, 38.1 5.2 ?mol H2O2 consumed/min/mg protein and 17.8 2.7 ?mol NADPH consumed/min/mg protein, respectively. b SOD, catalase and GSH-px of reference were 27.5 2.7 mU/ml, 21.1 3.2 ?mol H2O2 consumed/min/mg protein and 7.2 0.9 ?mol NADPH consumed/min/mg protein, respectively. Reference value differs significantly from the untreated, control at a level of $p < 0.001$.cProtection was calculated as 100 x (SOD, catalase or GSH-px of reference + ginsenoside-treated SOD, catalase or GSH-px of reference) / (SOD, catalase or GSH-px of control SOD, catalase or GSH-px of reference).
**Results differ significantly from the reference at a level of $p < 0.01$.

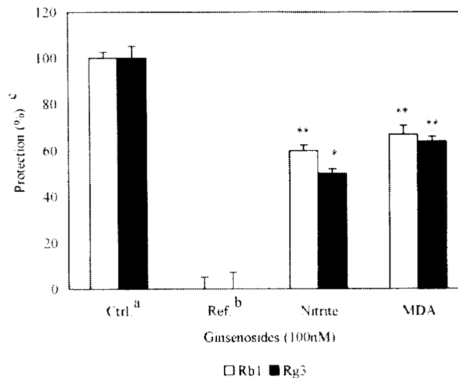

Figure 3. Effects of ginsenosides Rb1 and Rg3 on the contents of nitrite released and malondialdehyde in glutamate-treated cortical cell cultures Cortical cultures were treated with ginsenosides Rb1 and Rg3 at a concentration of 100 nM for one hr before glutamate-induced neurotoxicity (treatment throughout). The values shown are the mean unit SEM of three experiments.
a Nitrite and malondialdehyde of control were 75.5 2.7 nM and 80.9 5.2 pmol/mg protein, respectively.
b Nitrite and malondialdehyde of control were 145.7 5.2 nM and 216.3 19.2 pmol/mg protein, respectively. Reference value differs significantly from the untreated, control at a level of $p < 0.001$.
cProtection was calculated as 100 x (nitrite or malondialdehyde of reference + ginsenoside-treated nitrite or malondialdehyde of reference) / (nitrite or malondialdehyde of control nitrite or malondialdehyde of reference).
Results differ significantly from the reference at a level of $p < 0.01$ **, $p < 0.001$ ***.

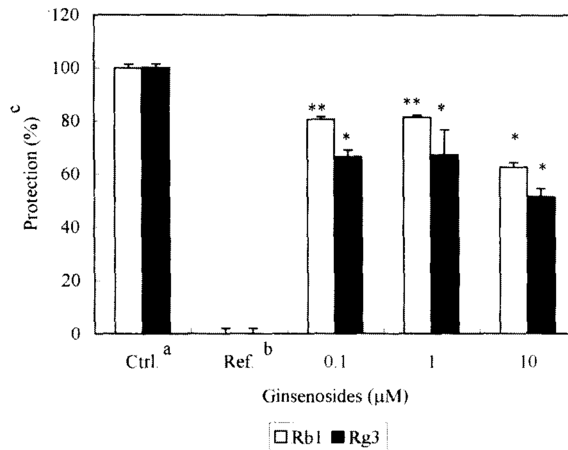

Figure 4. Effects of ginsenosides Rb1 and Rg3 on intracellular [Ca2+]i in primary cultures of rat cortical cells.

The values shown are the mean unit SEM of three experiments. a Intracellular [Ca2+]i of control was 75.0 15.0 nM.

bIntracellular [Ca2+]i of reference was 423.0 20.0 nM. Reference value differs significantly from the untreated, control at a level of $p < 0.001$.cProtection was calculated as 100 x (intracellular [Ca2+]i of reference + ginsenoside-treated intracellular [Ca2+]i of reference) / (intracellular [Ca2+]i of control - intracellular [Ca2+]i of reference).

Results differ significantly from the reference at a level of $p < 0.01$ **, $p < 0.001$ ***.