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Inhibitory Effects of Ginsenosides on Glutamate-Induced Swelling of Cultured Astrocytes

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Abstract : Effects of ginsenosides (Rb_1, Rb_2, Rc, Re, Rg_1, Rf) on L-glutamate (glutamate)-induced swelling of cultured astrocytes from rat brain cerebral cortex were studied. Following the exposure to 0.5 mM glutamate for 1 hr, the intracellular water space (as measured by [^{3}H]O-methyl-D-glucose uptake) of astrocytes increased by about two-fold. Simultaneous addition of ginsenosides Rb_2 and Rc with glutamate reduced the astrocytic swelling in a dose-dependent manner. These ginsenosides at 0.5 mg/ml did not affect the viability of astrocytes for up to 24 hr which was determined by a colorimetric assay (MTT assay) for cellular growth and survival. These ginsenosides at 0.3 mg/ml inhibited the increase of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) induced by glutamate. These data suggest ginsenosides Rb_2 and Rc prevent the cell swelling of astrocytes induced by glutamate, maybe via inhibition of Ca^{2+} influx.

Key words : Ginsenosides, glutamate, astrocytic swelling, Ca^{2+} influx.

INTRODUCTION

Astrocytes are involved in the regulation of electrolyte concentration and water volume in the extracellular space in central nervous system (CNS). Astrocytes are known to swell in some pathological states, such as hypoxia or ischemia.[1] L-Glutamate (glutamate), the major excitatory neurotransmitter amino acid, is known to be involved in the pathophysiology of neuronal cell death in hypoxic-ischemic brain injury,[2,3] epileptic brain damage[4] and other neurodegenerative disorders.[5-7] Glutamate, at concentrations similar to those required to induce neuronal cell death, causes swelling of brain slices and astrocytes in primary culture.[8-11] Ketamine and MK-801, noncompetitive NMDA receptor antagonists that bind to the ion channel complexes, are effective in combatting the swelling of cultured astrocytes induced by glutamate.[12,13]

Much attention has been paid to ginseng saponins, the main effective components of ginseng, because of their multiple pharmacological actions. Their actions on CNS include the suppression of exploratory and spontaneous movements,[14] prolongation of hexobarbital sleeping time[15] and inhibition of reverse-tolerance development to dependence-liable drugs.[16] There have been several reports on the *in vitro* actions of some active components extracted from *Panax ginseng* on cultured cell lines. Ginsenosides Rb_1 and Rd, saponins isolated from *Panax ginseng*, were reported to potentiate the nerve growth factor-mediated neurite extension in organ cultures of chicken embryonic dorsal root ganglia and lumbar sympathetic ganglia.[17] 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.[18] In previous report, we confirmed that ginseng total saponins (GTS), as an active component fraction of ginseng extract, inhibited glutamate-induced swelling of cultured astrocytes.[19] And GTS has protective effect on hypoxic astrocytic damage.[20]

Since a lot of experiments were performed using GTS, a mixture of several components, it was not easy to define the mechanism of actions. In this study, the effects of several ginsenosides, Rb_1, Rb_2, Rc, Re, Rf and Rg_1, isolated and purified from ginseng saponin fraction on glutamate-induced swelling of cultured rat cortical astrocytes were investigated.

MATERIALS AND METHODS

1. Materials

[^{3}H]O-methyl-D-glucose ([^{3}H]OMG) was purchased from

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American Radiolabeled Chemicals (St. Louis, MO, U.S.A.). Eagle's minimum essential medium (MEM) and fetal bovine serum were from Gibco (U.S.A.). Dibutyryl cyclic AMP (dBcAMP), phroletin, FURA-2 AM and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (ST. Louis, MO, U.S.A). Ginsenosides were supplied from Korea Ginseng and Tobacco Research Institute. All other chemicals used were of the highest grade available.

2. Cell cultures

Cultured rat astrocytes were prepared from cerebral cortices of 1 to 2 day old Sprague-Dawley rats by the method of Frangakis and Kimelberg.[21] Briefly, the dissociated cells were suspended in the culture medium (MEM with 10% fetal bovine serum) and plated at 1×10^4 cells/cm^2 in plastic tissue culture plates (12 wells, Corning) or poly-L-lysine coated glass cover slides for the measurement of intracelluar Ca^{2+} concentration ($[Ca^{2+}]_i$). The cells were grown in a 5% CO_2/95% air humidified atmosphere at 37°C with exchange of growth medium twice a week. The cultures were grown to confluence in 2 weeks and then further differentiated in the culture medium containing 0.5 mM dBcAMP. The astrocyte cultures were used 3~5 days after dBcAMP treatment. At this stage, more than 90% of the cells were glial fibrillary acidic protein positive by immunoreactivity as described previously.[19]

3. Exposure to glutamate and ginsenosides, and determination of [^{3}H]OMG space

After aspiration of the growth medium, cultured astrocytes were rinsed three times with HEPES-buffered Krebs-Ringer solution (HBKR (in mM): NaCl 156, KCl 5.6, $NaHCO_3$ 11, D-glucose 10, $MgSO_4$ 1, $CaCl_2$ 1, NaH_2PO_4 1, HEPES-Na 20, pH 7.4) and incubated at 37°C for 30 min in HBKR. The cells were exposed to glutamate and/or ginsenosides in the fresh HBKR at 37°C for 60 min. Astrocytic swelling observed at the end of the incubations was quantitatively studied by measuring the intracellular water space by [^{3}H]OMG equilibrium uptake in intact cell cultures, as described elsewhere.[20] The uptake of [^{3}H]OMG (0.5 μCi/well, 1 mM) was carried out for the last 20 min of incubation. The uptake was terminated by aspiration of the medium and subsequent rinsing 3 times with ice-cold HBKR containing 0.1 mM phloretin. The cells were then digested by 0.1 N NaOH and the subsequent aliquots was taken for protein determination and scintillation counting. Experiments were performed in duplicate with at least 3 different batches. Protein was determined by the method of Lowry *et al.*[22]

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

$[Ca^{2+}]_i$ was determined by ratio fluorometry as described.[23,24] Cells grown on glass cover slides were loaded with 5 μM FURA-2 AM for 1 hr in serum-free MEM at 37°C in the CO_2 incubator, and washed with HBKR. Cell culture slides were mounted into spectrophotometer cuvettes containing 3 m*l* HBKR. Fluorescence was measured with a fluorocytometry (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 glutamate. In order to test the effects of ginsenosides, cells were pretreated with ginsenosides for 15 min. Ionomycin and EGTA (final concentration 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. Calcium concentrations were calculated according to the method of Grynkiewicz *et al.*[25]

5. MTT colorimetric assay

This method is based on the reduction of the tetrazolium salt MTT into a blue formazan product mainly by the mitochondrial enzyme succinate-dehydrogenase. Therefore, the amount of formazan produced is proportional to the number of living cells.[26] Briefly, after incubation of cells with ginsenosides, the culture medium was replaced by a solution of MTT (0.5 mg/m*l*) in serum-free MEM. After 4 hr incubation at 37°C, this solution was removed and the produced blue formazan was solubilized in acid-isopropanol (0.04 N HCl in isopropanol). The optical density of the formed blue formazan was estimated on a microelisa reader, using a test wavelength of 570 nm and a reference wavelength of 630 nm.

RESULTS

1. Effects of ginsenosides on glutamate-induced swelling

Treatment with 0.5 mM glutamate caused an increase in cellular volume of astrocytes. As described in previous report,[19] the swelling of astrocytes was characterized by swollen nuclei and the disappearance of obvious cell bodies and processes. Fig. 1 shows the inhibitory effects of ginsenosides on glutamate-induced increase in astrocytic cell volume. The basal cellular volume of 5.45 μ*l*/mg protein increased to 12.09 μ*l*/mg protein after 60 min expo-

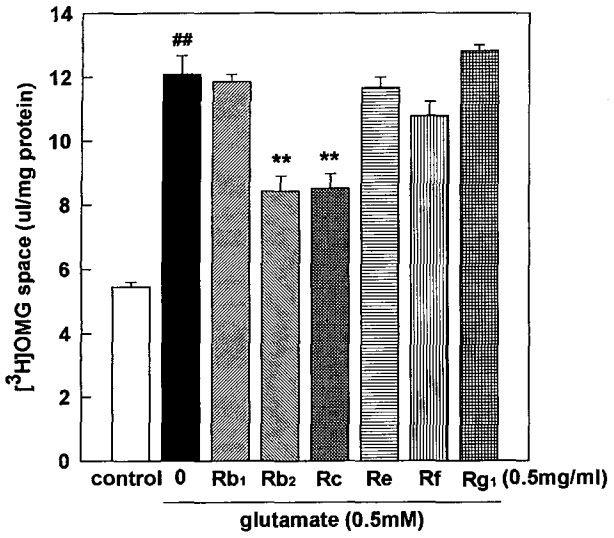

Fig. 1. Effects of ginsenosides on glutamate-induced increase in intracellular water space (measured as [^{3}H]OMG space) in cultured astrocytes. Cultured astrocytes were incubated with 0.5 mM glutamate for 1 hr in the presence or absence of ginsenosides. Each ginsenosides (0.5 mg/m*l*) was added simultaneously with glutamate to the cells. The uptake of [^{3}H]OMG was carried out for the last 20 min of incubation. [^{3}H]OMG space was measured in 3~5 different cultures by duplicate determination. Values are means ± SEM. $^{\#\#}p<0.01$, compared to control. $^{**}p<0.01$, compared to glutamate.

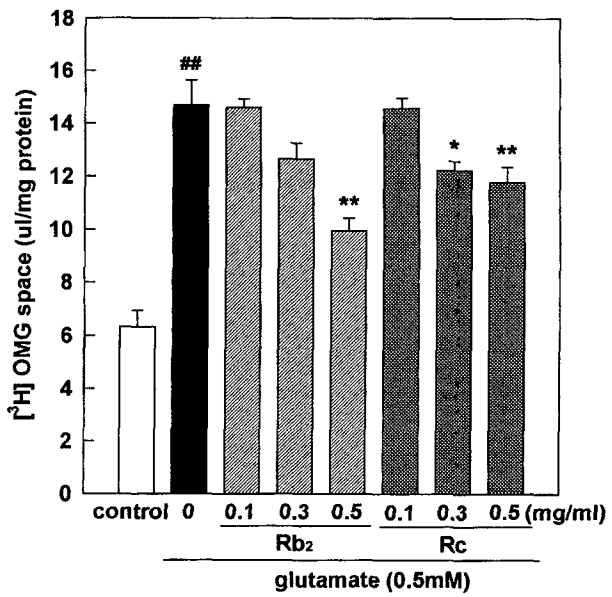

Fig. 2. Inhibitory effects of ginsenosides Rb_2 and Rc on glutamate-induced increase in intracellular water space in cultured astrocytes. Experimental conditions were similar to those described in Fig. 1 legend. Values are means ± SEM. $^{\#\#}p<0.01$; compared to control. $^{*}p<0.05$, $^{**}p<0.01$; compared to glutamate.

sure to 0.5 mM glutamate. Ginsenosides Rb_2 and Rc at 0.5 mg/m*l*, when added to the cultures simultaneously with glutamate, caused significant inhibition of glutamate-induced astrocytic swelling. However, ginsenosides Rb_1, Re, Rf and Rg_1 did not affect the glutamate-induced astrocytic swelling. The inhibition of glutamate-induced swelling by Rb_2 and Rc responded to an increase in their concentrations (Fig. 2).

2. Effects of Rb_2 and Rc on cell viability

Since the MTT assay is a sensitive, quantitative and reliable colorimetric assay for cell viability, the assay was performed for the astrocytes incubated with 0.5 mg/m*l* ginsenosides at various time intervals. Cell numbers of the astrocytes did not decrease even up to 24 hr following the incubation with ginsenosides Rb_2 and Rc (Table 1). This result indicates that these ginsenosides do not injure the intact integrity of cellular membrane and cell viability.

3. Effects of Rb_2 and Rc on glutamate-induced elevation of $[Ca^{2+}]_i$

To determine the mechanism of the inhibitory effects of ginsenosides on glutamate-induced astrocytic swelling, $[Ca^{2+}]_i$ was measured by ratio fluorometry with fluorescence dye, FURA-2 AM. Glutamate (0.5 mM) produced sharp and transient increase in $[Ca^{2+}]_i$ followed by gradual decrease (Fig. 3). The glutamate-induced elevation of $[Ca^{2+}]_i$ was completely and partially but significantly blocked by

Table 1. Changes in cell numbers of cultured astrocytes by treatment with ginsenosides Rb_2 and Rc

	Cell number (10^5/cm^2)				
Treatment time (h)	1	2	4	8	24
control	-	-	-	-	1.03 ± 0.69
Rb_2	1.11 ± 0.38	1.14 ± 0.51	1.11 ± 0.24	1.13 ± 0.37	1.10 ± 0.37
Rc	1.14 ± 0.60	1.13 ± 0.56	1.13 ± 0.61	1.16 ± 0.32	1.12 ± 0.45

Cultured astrocytes were further grown for various times in the culture medium containing 0.5 mg/m*l* ginsenosides. Cell numbers were measured by MTT assay. Results are mean ± SEM of four wells.

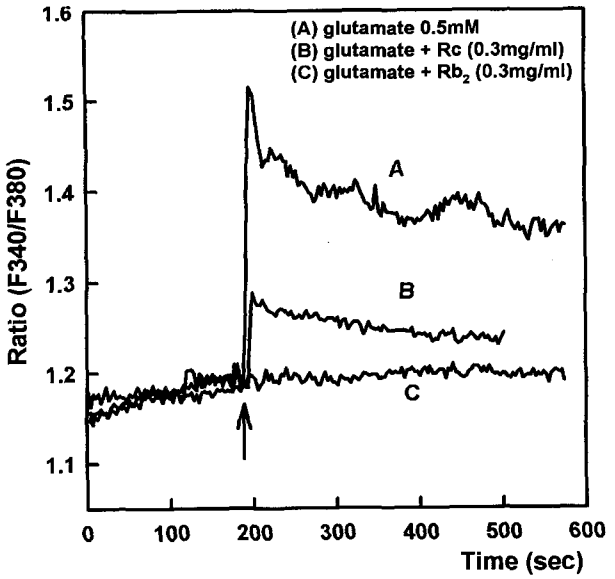

Fig. 3. Inhibitory effects of ginsenosides Rb_2 and Rc on glutamate-induced increase in intracellular Ca^{2+} concentration shown by fluorescence ratio (F340/F380) in cultured astrocytes. FURA-2 AM loaded cells were stimulated with glutamate as indicated by arrow in the presence or absence of ginsenosides. Ginsenosides were treated 15 min before glutamate treatment.

the pretreatment with Rb_2 and Rc (0.3 mg/m*l*), respectively.

DISCUSSION

Glutamate (0.5 mM) caused a swelling of astrocytes in primary culture, as evidenced by the increase in intracellular water space in the present experiments. This glutamate-induced astrocytic swelling was reduced by ginsenosides Rb_2 and Rc in a dose-dependent manner. The mechanisms underlying the beneficial effects of the ginsenosides are not clear at present. However, it was concluded that the inhibitory effects of Rb_2 and Rc on the glutamate-induced increase in [^{3}H]OMG space were not due to a nonspecific detergent-like effect of saponins, because the level of cell numbers was not decreased after the co-incubation with the ginsenosides, even up to 24 hr. LDH release into medium during the incubation was not affected by the treatment with Rb_2 and Rc (0.3 mg/m*l*) (data not shown). Cellular swelling is usually triggered by increased Na^+ influx followed by the influx of water. Glutamate opens Na^+ channels and increases Na^+ uptake in astrocytes.[27,28] Thus, it is likely that the ginsenosides reduced the astrocytic swelling by the inhibition of glutamate-induced Na^+ influx. Since it is not known whether ginsenosides Rb_2 and Rc specifically interact with Na^+ channel, the action of these ginsenosides on astrocytic swelling may be due to a membrane stabilizing effect. Moreover, it has been shown that the increase in intracellular Ca^{2+} and Cl^- following the increase of Na^+ influx with various insults is important for cell swelling.[1,7] Koyama *et al.*[10] indicated that swelling of astrocytes induced by glutamate was characterized by an accompanied influx of Ca^{2+}. Some reports demonstrated that ginseng root extract and ginsenosides inhibited various type of Ca^{2+} channels on sensory neuron or rat adrenal chromaffin cells.[29,30] Ginsenosides Rb_2 and Rc (0.3 mg/m*l*) significantly blocked glutamate-induced elevation of $[Ca^{2+}]_i$ in the present study. Therefore, it is likely that the reduction of astrocytic swelling was mainly attributable to the blockade of glutamate-induced Ca^{2+} influx by these ginsenosides.

In previous reports, we indicated the central actions of ginsenosides administered systemically (100~200 mg/kg) in mice. GTS injected intraperitoneally showed an antagonism of opioid agonists- induced antinociception.[31] And, GTS and ginsenosides Rb_1 and Rg_1 produced the blockade of development of cocaine- and methamphetamine-induced reverse tolerance and dopamine receptor supersensitivity in mice.[32-34] These findings suggest that active components of ginseng saponins can pass through the blood-brain barrier and thus interact with brain cells. We have no evidence regarding whether the concentration of ginsenosides *in vitro* in the present study is within the range pharmacologically available *in vivo*. In view of the concentration used here, it does not seem to be high, because Namba *et al.*[35] suggested that GTS has a protective activity against hemolytic action of a saponin, producing 50% effect at 2.6 mg/m*l* concentration. Furthermore, in support of the present results, Liu *et al.*[36] have reported that ginsenosides could significantly increase the survival in mice exposed to acute hypoxia.

In conclusion, the present results indicate that ginsenosides Rb_2 and Rc have protective effects on glutamate-induced astrocytic swelling *in vitro* indicating these ginsenosides may be the major active components of ginseng saponin. To assess the precise mechanism of these ginsenosides, further studies are necessary.

요 약

흰쥐 대뇌겉질로부터 별아교세포를 배양하여 흥분성아미노산인 L-glutamate에 의하여 유발되는 세포종창(astrocytic swelling)에

대한 ginsenosides의 억제효과를 검토하였다. Glutamate(0.5 mM)를 세포에 가하고 1시간동안 배양하면 swelling을 일으켜, 세포내의 물의 용적([^{3}H]OMG의 uptake량으로 측정)은 대조세포에 비하여 약 2배의 증가를 나타냈다. Glutamate와 함께 ginsenosides Rb_2와 Rc를 가하고 배양하면 glutamate에 의한 astrocytic swelling이 용량의존적으로 감소하였다. 세포는 Rb_2와 Rc(0.5 mg/ml)에 24시간까지 노출시켜도 MTT reduction이 감소하지 않는 것으로 보아 이 ginsenosides에 의한 swelling의 억제효과는 세포막의 손상에 의한 것이 아님을 알 수 있었다. Rb_2와 Rc는 glutamate에 의한 세포내 Ca^{2+}농도의 상승을 억제하였다. 따라서 Rb_2와 Rc는 Ca^{2+}의 유입을 억제하므로서 glutamate에 의한 astrocytic swelling을 억제하는 것으로 생각된다.

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