Protection of ischemic hippocampal neurons by ginsenoside Rb$_1$, a main ingredient of ginseng root

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Abstract

Our previous study showed that the oral administration of red ginseng powder before but not after transient forebrain ischemia prevented delayed neuronal death in gerbils, and that a neuroprotective molecule within red ginseng powder was ginsenoside Rb$_1$. However, it remains to be clarified whether or not ginsenoside Rb$_1$ acts directly on the ischemic brain, and the mechanism by which ginsenoside Rb$_1$ protects the ischemic CA1 neurons is not determined. Without elucidation of the pharmacological property of ginsenoside Rb$_1$, the drug would not be accepted as a neuroprotective agent. The present study demonstrated that the intracerebroventricular infusion of ginsenoside Rb$_1$ after 3.5 min or 3 min forebrain ischemia, precluded significantly the ischemia-induced shortening of response latency in a step-down passive avoidance task and rescued a significant number of hippocampal CA1 neurons from lethal ischemic damage. The intracerebroventricular infusion of ginsenoside Rb$_1$ did not affect hippocampal blood flow or hippocampal temperature except that it caused a slight increase in hippocampal blood flow at 5 min after transient forebrain ischemia. Furthermore, ginsenoside Rb$_1$ at concentrations of 0.1–100 fg/ml (0.09–90 fM) rescued hippocampal neurons from lethal damage caused by the hydroxyl radical-promoting agent FeSO$_4$ in vitro, and the Fenton reaction system containing p-nitrosodimethylaniline confirmed the hydroxyl radical-scavenging activity of ginsenoside Rb$_1$. These findings suggest that the central infusion of ginsenoside Rb$_1$ after forebrain ischemia protects hippocampal CA1 neurons against lethal ischemic damage possibly by scavenging free radicals which are overproduced in situ after brain ischemia and reperfusion. The present study may validate the empirical usage of ginseng root over thousands of years for the prevention of cerebrovascular diseases. © 1997 Elsevier Science Ireland Ltd.

Keywords: Ginsenoside Rb$_1$; Passive avoidance test; Brain ischemia; Hippocampus; Hydroxyl radical

1. Introduction

Ginseng root (Panax ginseng C.A. Meyer) has served as an important component of Chinese prescriptions (Kampou) for thousands of years. Since the introduction of ginseng root into oriental medicine, this crude drug has been thought to prevent neuronal degeneration associated with brain ischemia, but experimental proof in support of this speculation is limited. Ginseng root consists of two major ingredients: crude ginseng saponin and crude ginseng non-saponin fractions. To date, more than 20 saponins have been isolated from ginseng root and identified chemically. They can be classified into three major groups according to their chemical structures: protopanaxadiol, protopanaxatriol and oleanolic saponins. Ginsenoside Rb$_1$, ginsenoside Rg$_1$, and ginsenoside R$_0$ are representative substances, respectively (Shibata et al., 1985).
Using a passive avoidance task whose results correlated well with hippocampal CA1 neuron number (Araki et al., 1986, 1987; Sano et al., 1994; Wen et al., 1995a,b; K otani et al., 1996a; Matsu da et al., 1996), we recently showed that the oral administration of red ginseng powder before transient forebrain ischemia prevented the occurrence of ischemia-induced learning disability and hippocampal neuron loss in gerbils, and that a neuroprotective molecule within red ginseng powder was ginsenoside Rb1 (Wen et al., 1996). However, in this study the intraperitoneal injections of ginsenoside Rb1 starting immediately after ischemic insult did not rescue the ischemic CA1 neurons (Wen et al., 1996). A possible explanation for this is that peripherally administered ginsenoside Rb1, even though having neuroprotective action, could not reach the ischemic brain by the time when neuronal death or survival was determined. If this speculation is the case, the central administration of ginsenoside Rb1 after ischemic insult should rescue hippocampal CA1 neurons.

In our previous study (Wen et al., 1996) the mechanism(s) by which ginsenoside Rb1 protected the hippocampus against ischemic damage was not determined, although the possible free radical-scavenging action of ginsenoside Rb1 was discussed on the basis of the chemical structure of ginsenoside Rb1 similar to that of 21-aminosteroids (Braughler et al., 1987; Hall et al., 1988; Young et al., 1988; Lesiuk et al., 1991). Since the administration of red ginseng powder in humans is empirically known to cause vasodilatation and slight elevation of body temperature, the possibility is also left open that ginsenoside Rb1 protects ischemic CA1 neurons by modulating hippocampal temperature and/or blood flow before and after ischemic insult.

To determine the central action(s) of ginsenoside Rb1 in cases of brain ischemia, the first set of the present experiments was designed to see the effect of the postischemic intracerebroventricular infusion of ginsenoside Rb1 on the hippocampal CA1 region of normothermic gerbils with 3.5 min forebrain ischemia, which is known to yield neuronal damage similar to that of hypothermic gerbils with 5 min forebrain ischemia (Kirino, 1982; Mitani et al., 1991; Andou et al., 1992). We also investigated the neuroprotective action of centrally administered ginsenoside Rb1 in 3 min ischemic gerbils, since most of the previous studies dealing with the neurotrophism of peptide growth factors used 3 min ischemic gerbils (Sano et al., 1994; Wen et al., 1995a,b; K otani et al., 1996a; Matsu da et al., 1996). The second set of experiments focused on changes in hippocampal blood flow and brain temperature in ischemic animals with or without ginsenoside Rb1 infusion. In the third set of experiments, we investigated whether or not ginsenoside Rb1, like several peptide growth factors (Zhang et al., 1993; Cheng and Matson, 1995), rescued hippocampal neurons from lethal damage caused by the hydroxyl radical-promoting agent FeSO4 in vitro (Braughler et al., 1986; Floyd, 1990; Stadtman and Oliver, 1991). Subsequently, the Fenton reaction system containing p-nitrosodimethylaniline was used to see the hydroxyl radical-scavenging activity of ginsenoside Rb1 (Bors et al., 1979). The results of these experiments would shed light on the central mechanism underlying the neurotrophism of ginsenoside Rb1.

2. Materials and methods

2.1. Animals

Male Mongolian gerbils weighing 70–80 g (approximately 12 weeks of age) were housed communally at a constant temperature (22 ± 1°C) with a 12:12 h light–dark cycle, and given food and water ad libitum. They were handled once a week for cage cleaning. The following experiments were conducted in accordance with the Guide for Animal Experimentation at Ehime University School of Medicine.

2.2. Experiment 1

The gerbils were subjected to 3.5 min or 3 min forebrain ischemia (Andou et al., 1992; Wen et al., 1995a,b, 1996), while the body and brain temperatures were maintained at 37.0 ± 0.2°C during forebrain ischemia (Mitani et al., 1991). Immediately after transient forebrain ischemia, 2 μl of isotonic saline containing 2.5 ng or 25 ng of ginsenoside Rb1 (kindly supplied by Korea Tobacco and Ginseng Institute) or the same volume of saline alone was injected into the left lateral ventricle through a Hamilton syringe. The purity of ginsenoside Rb1 used in this study was more than 98% as determined by thin-layered chromatography and nuclear magnetic resonance (Fig. 1A) (Kawashima and Sumukawa, 1986). An osmotic minipump (Alza Palo Alto, CA) was implanted subcutaneously into the back of each animal, and a needle from the minipump was placed in the left lateral ventricle (Sano et al., 1994; Wen et al., 1995a,b; Matsuda et al., 1996). Ginsenoside Rb1 was dissolved in isotonic saline and infused at a dose of 60 or 600 ng/day for 7 days into the lateral ventricle of 3.5 min or 3 min ischemic gerbils which had been injected with 2.5 ng or 25 ng of ginsenoside Rb1 equivalent to one-twenty fourth of the daily infusion dose (n = 8 in each group). Control animals, one group with 3.5 min or 3 min ischemia and one group of sham-operated animals, received saline infusion (n = 8 in each group). The dose of a single injection of ginsenoside Rb1 was determined on the basis of our preliminary experiments which showed that 60 or 600 ng/day ginsenoside Rb1 infusion starting 1 h before ischemic insult and lasting 1 week...
Fig. 1. The chemical structure of ginsenoside Rb1 (A) and effects of intracerebroventricular ginsenoside Rb1 infusion on response latency in a passive avoidance task and on number of hippocampal CA1 neurons in 3.5 min (B, C) ischemic gerbils (closed columns). When ginsenoside Rb1 infusion into the lateral ventricle started just after 3.5 min forebrain ischemia and continued for 7 days, it caused a significant dose-dependent increase in response latency (B) and in the number of viable neurons within the hippocampal CA1 region (C), in comparison with the latency and CA1 neuronal density of 3.5 min ischemic gerbils infused with saline (shaded columns). The open columns indicate the mean response latency and neuronal density of sham-operated (sham-op) animals. Each value represents mean $\pm$ S.D. ($n=8$). * $P<0.05$, ** $P<0.01$, significantly different from the corresponding saline-infused ischemic group.

2.3. Experiment 2

The blood flow and temperature of the hippocampus prior to and after 3 or 3.5 min ischemia were investigated in gerbils with the continuous infusion of ginsenoside Rb1 at a dose of 600 ng/day or with saline infusion into the lateral ventricle ($n=8$ in each group). The hippocampal blood flow was determined by the hydrogen clearance method with the use of a digital blood flow meter (MHG-D1; Unique Medical, Tokyo, Japan) under inhalation anesthesia. The hippocampal temperature was monitored with the combination of a temperature-sensitive probe (Mini Mitter, Sunriver, USA) inserted into the hippocampus and the Telemetry System receiving signals from the probe (Datascience, Minneapolis, USA), while the animals gained free access to food and water except for the periods of minipump implantation and forebrain ischemia.

The effects of ginsenoside Rb1 were evaluated by the two-tailed Mann-Whitney U-test. All data were represented as $\text{mean } \pm \text{ S.D.}$ All experiments were done blind with respect to the experimental group.

2.4. Experiment 3

The hippocampi of 17 day old rat embryos were aseptically dissected out. Hippocampal neurons were dissociated from the tissues as described elsewhere (Kira et al., 1995). The dissociated cells were seeded on 24 well plastic plates (Corning, NY) coated with EHS-laminin (Koken, Japan) at a density of $3.5 \times 10^4$ cells/cm². The cells were cultured at 37°C in Dulbecco's...
modified Eagle's medium (DMEM, 0.1% glucose; Nipro, Japan) supplemented with 10% fetal calf serum (FCS; Equitech-Bio, TX) under an atmosphere of 5% CO₂ and nearly 100% humidity. On the second day of culture, one half of the culture medium was replaced with serum-free DMEM containing 20 mM HEPES, 0.45% glucose, 5 μg/ml pig insulin (Boehringer-Mannheim), 5 μg/ml human transferrin (Boehringer-Mannheim), 5 ng/ml sodium selenite (Boehringer-Mannheim), 25 nM progesterone (Sigma), 0.1% bovine serum albumin (BSA; Sigma) and ginsenoside Rb₁ (0–10 ng/ml). To introduce oxidative damage to the cultured neurons, freshly prepared 90 μM FeSO₄ solution was added to the medium on the third day of culture and the neurons were maintained for 24 h at 37°C (Braughler et al., 1986; Floyd, 1990; Stadtman and Oliver, 1991; Zhang et al., 1993; Cheng and Mattson, 1995). Then, the cells were fixed with PHEM buffer (60 mM PIPES, 25 mM HEPES, 2 mM MgCl₂ and 1 mM EGTA, pH 6.9) containing 4% paraformaldehyde and 0.2% glutaraldehyde for morphological examination as previously described (Tanaka and Maeda, 1996).

For subsequent immunoblotting studies, the cells were solubilized with Laemmli's sample solution (Laemmli, 1970). The survival rate of neuronal cells after FeSO₄ treatment was evaluated with immunoblotting against microtubule-associated protein 2 (MAP2) which is known to be a specific marker for neuronal cells. The cells were solubilized in the sample solution containing SDS and electrophoresed with the use of 6% polyacrylamide gel in the Laemmli's buffer system. The electrophoretic bands were transferred to nitrocellulose sheets and immunoblotted with an anti-MAP2 monoclonal antibody (Sternberger Monoclonals, Baltimore). Anti-mouse IgG antibody coupled with alkaline phosphatase (Promega, Madison, WI) was used for the second immunoreaction. The immunoreactive bands were visualized as described elsewhere (Kira et al., 1995). Prestained molecular weight markers were purchased from Bio Rad (Richmond, CA). For quantitative evaluation, the immunoreactive bands of MAP2 were subjected to densitometric analysis (Tanaka and Sobue, 1994). Statistical analyses were conducted by the analysis of variance followed by post hoc test (Fisher's PLSD).

A cell for the measurement of light absorbance was filled with 1 ml of saline, 940 μl of different concentrations of ginsenoside Rb₁, 20 μl of 5 mM p-nitrosodimethylaniline (p-NDA) and 23 μl of 88 mM H₂O₂. After measuring light absorbance at 440 nm, 20 μl of 10 mM FeSO₄ was added to the solution which had been stirred and kept at 30°C. Then, light absorbance at 440 nm was measured again and the difference (ΔA) in light absorbance before and after FeSO₄ addition was calculated. When the reaction system did not contain ginsenoside Rb₁, the difference in light absorbance before and after FeSO₄ treatment was defined as ΔA₀ (Bors et al., 1979).

3. Results

3.1. Experiment 1

The mean response latency and CA1 neuronal density of sham-operated animals with intraventricular saline infusion were 223.0 ± 60.1 s and 251.0 ± 15.5 cells/mm, respectively, while those of 3.5 min ischemic gerbils infused with saline alone were 55.1 ± 46.4 s and 51.3 ± 21.5 cells/mm, respectively. There were significant differences in response latency (U = 0, P < 0.01) and CA1 neuronal density (U = 0, P < 0.01) between the two groups (Fig. 1B and 1C). In histological sections the CA1 region of 3.5 min ischemic exhibited a marked decline in neuron number and an apparent increase in astrocytes, when compared with
the CA1 field of sham-operated animals (Fig. 2A and 2B).

Ginsenoside Rb1 infusion into the lateral ventricle, starting just after 3.5 min forebrain ischemia and continuing for 7 days, caused a significant dose-dependent prolongation in response latency in the step-down passive avoidance task (60 ng/day ginsenoside Rb1 versus saline in ischemic gerbils: U = 10.0, P < 0.05; 600 ng/day ginsenoside Rb1 versus saline in ischemic gerbils: U = 1, P < 0.01) (Fig. 1B). Subsequent histological examinations revealed that ginsenoside Rb1 treatment rescued dose-dependently many ischemic neurons that were destined to degenerate without the treatment (60 ng/day ginsenoside Rb1 versus saline in ischemic gerbils: U = 11.5, P < 0.05; 600 ng/day ginsenoside Rb1 versus saline in ischemic gerbils: U = 5.0, P < 0.01) (Fig. 1C and Fig. 2B and 2C). The infusion of ginsenoside Rb1 at a dose of 6 μg/day was ineffective in preventing ischemia-induced neuronal damage in gerbils and ischemic animals with 60 μg/day ginsenoside Rb1 infusion exhibited a higher mortality than those with saline infusion (data not shown). The intracerebroventricular infusion of ginsenoside Rb1 after 3 min forebrain ischemia also resulted in significant increases in response latency time (60 ng/day ginsenoside Rb1 versus saline in ischemic gerbils: U = 9.5, P < 0.05; 600 ng/day ginsenoside Rb1 versus saline in ischemic gerbils: U = 7.0, P < 0.01) and in CA1 neuronal density (60 ng/day ginsenoside Rb1 versus saline in ischemic gerbils: U = 10.0, P < 0.05; 600 ng/day ginsenoside Rb1 versus saline in ischemic gerbils: U = 7.0, P < 0.01) (Fig. 3).

When ginsenoside Rb1 at a dose of 10 or 20 mg/kg per day was intraperitoneally injected for 7 days before 3.5 min forebrain ischemia, the response latency and the CA1 neuronal density were significantly increased in a dose-dependent manner, in comparison with those of saline-injected ischemic gerbils (ginsenoside Rb1 10 mg/kg per day versus saline in ischemic gerbils: response latency, U = 7, P < 0.01; neuronal density, U = 4, P < 0.01; ginsenoside Rb1 20 mg/kg per day versus saline in ischemic gerbils: response latency, U = 3, P < 0.01; neuronal density, U = 2, P < 0.01) (Fig. 4). The neuroprotective effect of ginsenoside Rb1 administered intraperitoneally prior to 3.5 min forebrain ischemia was comparable with that of ginsenoside Rb1 infused intraventricularly after the ischemic insult (Fig. 1B, 1C and Fig. 4). However, the intraperitoneal injection of ginsenoside Rb1 after 3.5 min forebrain ischemia was ineffective in preventing delayed neuronal death (data not shown) (see Section 4).

3.2. Experiment 2

There was no significant difference in hippocampal blood flow or hippocampal temperature between ginsenoside Rb1-infused (600 ng/day) and saline-treated gerbils 2 h before and 5–120 min after 3 min or 3.5 min forebrain ischemia, except that the ginsenoside Rb1-treated gerbils exhibited a slight increase in hippocampal blood flow at 5 min after 3 min forebrain ischemia in comparison with the hippocampal blood flow of saline-infused ischemic animals (U = 13, P < 0.05) (Fig. 5).

3.3. Experiment 3

Hippocampal neurons on 24 well plastic plates gave rise to numerous processes by 4 days after cell culture (Fig. 6A). As the hydroxyl radical-promoting agent FeSO4 was added to the culture medium, the long processes of hippocampal neurons became fragmentary.
within 16 h (Fig. 6B) and they were no longer visible within 24 h. When hippocampal neurons were cultured in the presence of 1 pg/ml or higher concentrations of ginsenoside Rb1, neurite outgrowth was apparently impeded before adding FeSO4 to the culture medium. However, lower concentrations (around 1 fg/ml) of ginsenoside Rb1 significantly facilitated neurite extension and protected hippocampal neurons from lethal damage caused by FeSO4 (Fig. 6C).

To quantify the neuroprotective action of ginsenoside Rb1 against FeSO4 treatment, the cultured hippocampal neurons were solubilized after FeSO4 treatment, electrophoresed and immunoblotted with a monoclonal antibody specific to microtubule-associated protein 2 (MAP2) in neurons (Fig. 7A). MAP2 was apparently detected in the control cultured neurons (Fig. 7A, lane Fe2+), but was not detected in FeSO4-treated neurons (Fig. 7A, lane Fe2+). When ginsenoside Rb1 was added to the culture medium beforehand, FeSO4-induced neuron death was prevented at a rather narrow range of doses between 0.1 and 100 fg/ml (Fig. 7A). The neuroprotective effect of 0.1–100 fg/ml ginsenoside Rb1 against lethal oxidative injury by FeSO4 was quantitatively evaluated by densitometric analysis of the immunoreactive bands (Fig. 7B).

When the Fenton reaction system with p-NDA did not contain ginsenoside Rb1, p-NDA was bleached rapidly by hydroxyl radicals (·OH) which were generated by the following Fenton reaction: Fe2+ + H2O2 → Fe3+ + ·OH + OH-. In this case, the difference (ΔA0) in light absorbance before and after FeSO4 addition was maximal. With ginsenoside Rb1 at concentrations less than 20 μg/ml, bleaching of p-NDA by hydroxyl radicals generated by the Fenton reaction was attenuated; in other words, ΔA/ΔA0 declined gradually (Fig. 7C).

4. Discussion

During forebrain ischemia, brain temperature has been shown to fall differently in individual animals, thereby affecting the number of viable CA1 neurons after ischemia (Mitani et al., 1991). To avoid the effect of unstable brain temperature on ischemic neuronal loss, we kept the brain temperature at 37.0 ± 0.2°C while clamping the common carotid arteries. This enabled us to apply the same intensity of ischemic insult to all animals and to evaluate with accuracy the neuroprotective effect of ginsenoside Rb1 on 3.5-min ischemic animals which exhibit a neuronal damage similar to that of hypothermic gerbils with 5-min ischemia (Kirino, 1982; Mitani et al., 1991; Andou et al., 1992) and on 3-min hippocampal CA1 region unless neuroprotective agents are administered.

The present study demonstrated that the intracerebroventricular infusion of ginsenoside Rb1 after transient forebrain ischemia prevents disability in the passive avoidance task and rescued hippocampal CA1 pyramidal cells from delayed neuronal death. The intraperitoneal injections of ginsenoside Rb1 before forebrain ischemia exhibited a similar neurotrotective action, but those after the ischemic insult failed to preclude delayed neuronal death. These findings suggest that ginsenoside Rb1 is a centrally acting neuroprotective agent, and that ginsenoside Rb1 administered peripherally after transient forebrain ischemia, even though having a neuroprotective action, could not reach the hippocampus by the time when neuronal death or survival was determined after brain ischemia. However, there is no available information on the amount of centrally transported Rb1 after systemic administration of the drug. Based on the
growth factors. Since (1) ginsenoside Rb1, unlike peptide growth factors, can be administered peripherally to prevent ischemic neuronal death and (2) ginseng root containing ginsenoside Rb1 has been prescribed for thousands of years without exhibiting any apparent ill-effects on humans, ginsenoside Rb1 appears to be a promising agent for the prevention of ischemic neuronal damage.

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