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Intracerebroventricular Gisenosides are Antinociceptive in Proinflammatory Cytokine-Induced Pain Behaviors of Mice

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Several ginsenoside (R_{b1}, R_{b2}, R_c, R_d, R_e, R_f, R_{g1} and R_{g3}) are neuroprotective and antinociceptive agents. In this study, we assessed the effects of these ginsenosides following intracerebroventricular (i.c.v.) administration on the nociceptive behaviors induced by intrathecal injection of pro-inflammatory cytokines (tumor necrosis factor-a (TNF-α), interleukin-1β (IL-1β), and interferon-γ (IFN-γ)). The ginsenosides, R_{b1}, R_{b2}, R_c, R_d, R_e, R_f and R_{g1}, significantly attenuated the nociceptive behavior induced by TNF-α, IL-1β, and IFN-γ injection, but ginsenoside-R_{g3} did not. These results suggest that several ginsenosides may regulate the nociceptive processing induced by pro-inflammatory cytokines.

Key words: Ginsenoside, Anti-nociception, Pain behaviors, Pro-inflammatory cytokines, TNF-α, IL-1β, IFN-γ

INTRODUCTION

Traditionally, *Panax ginseng* is used for a panacea or promoting longevity in far-east Asian traditional medicine. Most pharmacological action of ginseng is produced by ginsenosides, which belong to the steroidal saponin family (Tsang *et al.,* 1985; Attele *et al.,* 1999).

Ginsenosides administered systemically (i.p.), intracerebroventricularly (i.c.v.) or intrathecally (i.t.) regulate nociception in pain models (Shin *et al.,* 1999; Nah *et al.,* 2000; Yoon *et al.,* 1998; Suh *et al.,* 1997, 1999; Choi *et al.,* 2003b; Rhim *et al.,* 2002; Mogil *et al.,* 1998). Ginsenosides differ in the structure of their sugar moieties and their effects on the nervous system. For example, i.t. administered ginsenosides attenuated analgesia induced by morphine, a mu opioid ligand, and U-50,488H, a kappa opioid ligand, in mice (Kim *et al.,* 1992; Suh *et al.,* 1997, 2000), while ginsenoside R_f potentiated U-50,488H-mediated analgesia (Nemmani and Ramarao 2003). Ginsenosides can also regulate inflammatory pain processing. Ginsenosides R_c, R_d and R_e, produce analgesic effects on pain behaviors elicited by formalin injected subcutaneously (s.c.) or writhing responses induced by 1% acetic acid (i.p.) injection (Shin *et al.,* 1999). In addition, we also demonstrated that ginsenosides inhibit pain behavior induced by substance P (i.t.) injection (Choi *et al.,* 2003b), a typical neurotransmitter in inflammatory pain (Hunt and Mantyh 2001). Ginsenosides can regulate inflammatory pain processing (Shin *et al.,* 1999; Yoon *et al.,* 1998; Mogil *et al.,* 1998; Nah *et al.,* 2000; Choi *et al.,* 2003b), but little information is available on the effect of ginsenosides in pain behaviors related to pro-inflammatory cytokines (i.e. TNF-α, IL-1β, and IFN-γ).

Injection of mouse pro-inflammatory cytokines evoke nociceptive behaviors (Choi *et al.,* 2003c) and contribute to the pathophysiology of pathological pain states in hyperalgesia or allodynia (Watkins *et al.,* 1994; Tadano *et al.,* 1999; Reeve *et al.,* 2000). However, the supraspinal action of ginsenosides in regulating nociceptive behavior induced by pro-inflammatory cytokines by i.t. injection is not well-characterized. Thus, we examined the modulatory role of ginsenosides (R_{b1}, R_{b2}, R_c , R_d, R_e, R_f, R_{g1} and R_{g3}) injected supraspinally on the nociceptive behavior induced by pro-inflammatory cytokines (TNF-α, IL-1β, and IFN-γ) injected i.t.

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MATERIALS AND METHODS

These experiments were approved by the Hallym University Animal Care and Use Committee. All procedures were conducted in accordance with the 'Guide for Care and Use of Laboratory Animals' published by the National Institutes of Health and the ethical guidelines of the International Association for the Study of Pain.

Experimental animals

Male ICR mice (MJ Ltd., Seoul, Korea) weighing 23-25 g were used for all the experiments. Animals were housed 5 per cage in a room maintained at 22 ± 0.5°C with an alternating 12 h light-dark cycle for at least 5 days before the experiments were started and food and water were available ad libitum. The animals were allowed to adapt to the experimental condition in the laboratory for at least 2 h before pain testing. To reduce variation, all experiments were performed during the light phase of the cycle (10:00-17:00).

Intracerebroventricular (i.c.v.) and intrathecal (i.t.) injection of drugs

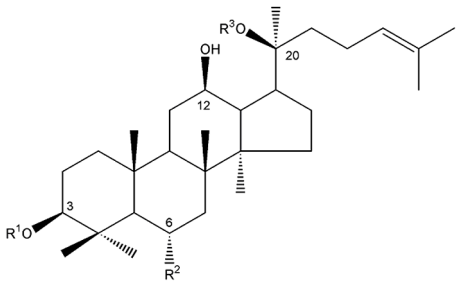
The i.t. injections were made according to the procedure of Hylden and Wilcox (Hylden and Wilcox 1981) using a 25 μL Hamilton syringe with a 30 gauge needle. The i.c.v. administration followed the method described by Haley and McCormick (Haley and McCormick 1957). The mouse was grasped firmly without anesthesia by the loose skin behind the head. The skin was pulled taut. A 30-gage needle attached to a 25 mL syringe was inserted perpendicularly through the skull into the brain and solution was injected. The injection site was 2 mm from either side of the midline on a line drawn through the anterior base of the ears. The i.c.v. and i.t. injection volumes were 5 μL and the injection sites were verified by injecting a similar volume of 1% methylene blue solution and determining the distribution of the injected dye in the ventricular space or in the spinal cord. The dye injected i.c.v. was distributed through the ventricular spaces and reached the ventral surface of the brain and the upper cervical portion of the spinal cord. The dye injected i.t. was distributed both rostrally and caudally but within a short distance (about 0.5 cm), and no dye was found visually in the brain. The success rate for the prior injections with this technique was over 95%.

Drug treatment and pro-inflammatory cytokine-induced nociceptive behavioral test

One group of mice was pretreated i.c.v. once with 50 μg of ginsenoside R_{b1}, R_{b2}, R_c, R_d, R_e, R_f, R_{g1} and R_{g3} 10 min before pro-inflammatory cytokines (TNF-α (100 pg/5 μL), IL-1β (100 pg/5 μL) or IFN-γ (100 pg/5 μL)) were i.t. injected. Immediately after cytokine injection, each mouse was placed in an observation chamber (20 cm high, 20 cm diameter) and responses such as licking, biting, and scratching directed toward the lumbar and caudal region of the spinal cord were recorded for 30 min. The cumulative response time(s) of scratching and biting episodes were measured with a stop-watch timer.

Drugs

Ginsenosides (R_{b1}, R_{b2}, R_c, R_d, R_e, R_f, R_{g1}, and R_{g3}) were obtained from Korea Ginseng and Tobacco Research Institute (Taejon, Korea). The purity of R_{b1}, R_{b2}, R_c, R_d, R_e, R_f, R_{g1}, and R_{g3} used in the present study was 98.63%, 98.13%, 95.97%, 97.87%, 99.23%, 99.33%, 98.10%, and 99.00%, respectively. TNF-α, IL-1β, and IFN-γ were purchased from R&D Systems Inc. (Minneapolis, MN., U.S.A.). Morphine hydrochloride was purchased from Sam-Sung Pharm. Co. (Seoul, Korea). All drugs for injection, except R_d, were dissolved in sterile saline (0.9% NaCl solution). Ginsenoside R_d was prepared in saline containing 20% dimethyl sulfoxide (DMSO) as vehicle. All drugs were prepared just before use. Drug doses were chosen based on our previous study (Choi *et al.,* 2003b).



Ginsenoside	R^1	R^2	R^3
R_{b1}	-Glc^2-Glc	-H	-Glc^6-Glc
R_{b2}	-Glc^2-Glc	-H	-Glc^6-Ara(p)
R_c	-Glc^2-Glc	-H	-Glc^6-Ara(f)
R_d	-Glc^2-Glc	-H	-Glc
R_e	-H	-O-Glc^2-Rha	-Glc
R_f	-H	-O-Glc^2-Glc	-H
R_{g1}	-H	-O-Glc	-Glc
R_{g3}	-Glc^2-Glc	-H	-H

Fig. 1. Structure of ginsenosides. Ginsenosides have a common steroid ring with three different side chains. Abbreviations for carbohydrates are as follows: Glc, glucopyranoside; Ara(p), arabinopyranoside; Ara(f), arabinofuranoside; Rha, rhamnopyranoside. Superscripts indicate the carbon in the glucose ring that links two carbohydrates.

Statistical analysis

Data are presented as the mean ± SEM. Statistical significance was assessed with ANOVA and Bonferroni's post-hoc test using GraphPad Prism version 4.0 for Windows XP (GraphPad Software, San Diego, CA, USA); $P < 0.05$ was considered significant.

RESULTS

The effects of ginsenosides on the nociceptive behavior elicited by TNF-α injection

Ginsenosides (100 μg/5 μL) decreased performance on the rotarod compared to the vehicle-injected mouse (data not shown), so we chose a maximal dose of 50 μg/5 μL. Groups of mice were pretreated i.c.v. with 50 μg/5 μL of saline or ginsenosides for 10 min, and the mice were injected with TNF-α (100 pg/5 μL, i.t.). TNF-α induced nociceptive behavior in mice (Fig. 2). Morphine (0.5 μg/5 μL; i.c.v.) pretreatment blocked the nociceptive behavior induced by TNF-α (24.57 ± 4.63 sec). All ginsenosides except R_{g3} also attenuated the nociceptive behaviors induced by TNF-α, with R_{b1}, R_{b2}, R_c and R_d showing remarkable efficacy (Fig. 2).

The effects of ginsenosides on the nociceptive behavior elicited by IL-1β injection

Groups of mice were pretreated i.c.v. with 50 μg/5 μL of saline or ginsenosides for 10 min, and the mice were injected with IL-1β (100 pg/5 μL, i.t.). IL-1β induced nocicep-

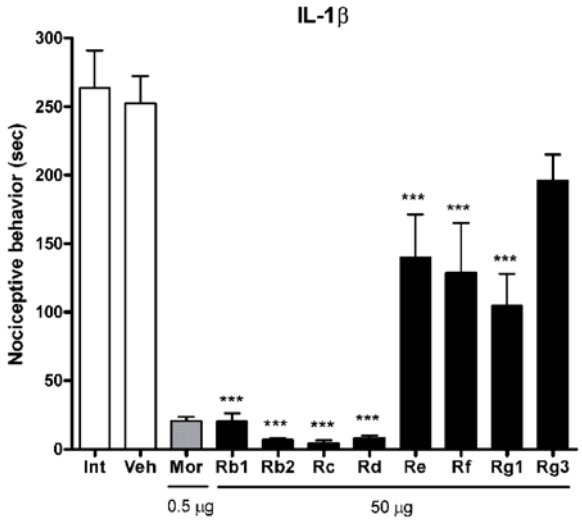


Fig. 3. The effect of ginsenosides on IL-1β-induced nociceptive behavior. Mice were treated i.c.v. with 50 μg/5 μL of saline or ginsenosides (R_{b1}, R_{b2}, R_c , R_d, R_e, R_f, R_{g1} and R_{g3}) for 10 min before IL-1β (100 pg/5 μL, i.t.). The cumulative response time of licking, biting and scratching directed toward the lumbar and caudal regions of the spinal cord was measured for 30 min. The vertical bars denote the standard error of the mean. The number of animal used for each group was 8 to 10. ***P<0.001 compared to the group of mice injected with vehicle (veh).

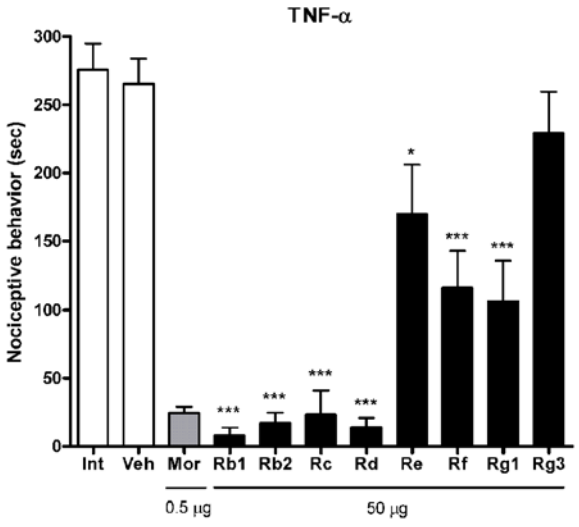


Fig. 2. The effect of ginsenosides on TNF-a-induced nociceptive behavior. Mice were treated i.c.v. with 50 μg/5 μL of saline or ginsenosides (R_{b1}, R_{b2}, R_c , R_d, R_e, R_f, R_{g1} and R_{g3}) for 10 min before TNF-a (100 pg/5 μL, i.t.). The cumulative response time of licking, biting and scratching directed toward the lumbar and caudal regions of the spinal cord was measured for 30 min. The vertical bars denote the standard error of the mean. The number of animal used for each group was 8 to 10. *P < 0.05, ***P<0.001 compared to the group of mice injected with vehicle (Veh).

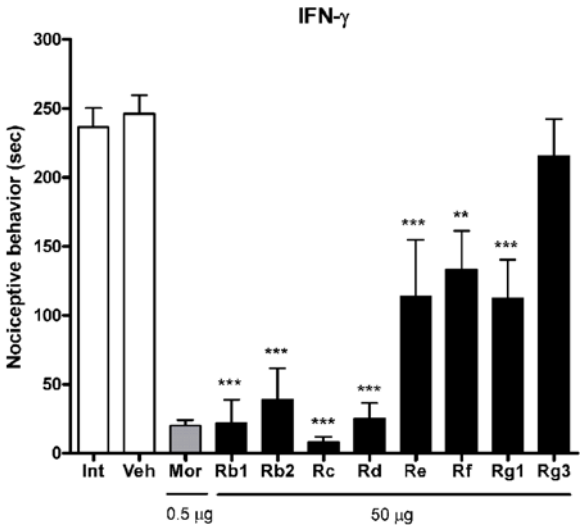


Fig. 4. The effect of ginsenosides on IFN-γ-induced nociceptive behavior. Mice were treated i.c.v. with 50 μg/5 mL of saline or ginsenosides (R_{b1}, R_{b2}, R_c , R_d, R_e, R_f, R_{g1} and R_{g3}) for 10 min before IFN-γ (100 pg/5 μL, i.t.). The cumulative response time of licking, biting and scratching directed toward the lumbar and caudal regions of the spinal cord was measured for 30 min. The vertical bars denote the standard error of the mean. The number of animal used for each group was 8 to 10. **P < 0.01, ***P<0.001 compared to the group of mice injected with vehicle (veh).

tive behavior in mice (Fig. 3). Morphine (0.5 μg/5 μL; i.c.v.) pretreatment blocked the nociceptive behavior induced by IL-1β (20.75 ± 3.04 sec). All ginsenosides except R_{g3} also attenuated the nociceptive behaviors induced by IL-1β, with R_{b1}, R_{b2}, R_c and R_d showing remarkable efficacy (Fig. 3).

The effects of ginsenosides on the nociceptive behavior elicited by IFN-γ injection

Groups of mice were pretreated i.c.v. with 50 μg/5 μL of saline or ginsenosides for 10 min, and the mice were injected with IFN-γ (100 pg/5 μL, i.t.). IFN-g induced nociceptive behavior in mice (Fig. 4). Morphine (0.5 μg/5 μL; i.c.v.) pretreatment blocked the nociceptive behavior induced by IFN-γ (20.00 ± 4.05 sec). All ginsenosides except R_{g3} also attenuated the nociceptive behaviors induced by IFN-γ, with R_{b1}, R_{b2}, R_c and R_d showing remarkable efficacy (Fig. 4).

DISCUSSION

Ginseng can energize or help restore homeostasis. Ginsenosides or ginseng total saponins, the major active ingredients in ginseng, have neuromodulatory and antinociceptive roles in the central nervous system. Here, we found that several ginsenosides (i.c.v.) blocked the nociceptive behavior elicited by pro-inflammatory cytokine (TNF-α, IL-1β, or IFN-γ) injection, particularly the panaxadiol group of R_{b1}, R_{b2}, R_c and R_d. Our results suggest that ginsenosides may regulate the nociceptive response in hyperalgesia or allodynia induced by pro-inflammatory cytokisne production.

Nociceptive stimuli with different modalities can be processed differently in the spinal and supraspinal regions (Chung *et al.,* 2000; Choi *et al.,* 2001; Hunt and Mantyh 2001). We also reported pro-inflammatory cytokines induce different pain behaviors than formalin injection or writhing responses (Seo *et al.,* 2006; Shim *et al.,* 2007). Spinal pro-inflammatory cytokines control central sensitization and neuropathic pain development (Watkins *et al.,* 1994; Tadano *et al.,* 1999; Reeve *et al.,* 2000; Robertson *et al.,* 1997; Martucci *et al.,* 2007; Kim *et al.,* 2007). Toll-like receptor 2 and its ligands (TNF-α, IL-6, and IL-1β) directly contribute to the nerve injury-induced spinal glia activation and central sensitization (Kim *et al.,* 2007). In addition, TNF-α and IL-1β are markers of the neuropathic pain state (Martucci *et al.,* 2007). Thus, nociceptive stimuli elicited by pro-inflammatory cytokines may relate to the spinal sensitization induced by peripheral nerve injury rather than peripheral inflammation.

Several ginsenosides are analgesic in pain models, including inflammatory or thermogenic pain stimuli (Shin *et al.,* 1999; Yoon *et al.,* 1998; Mogil *et al.,* 1998; Nah *et al.,* 2000; Choi *et al.,* 2003b). In this study, we report, for the first time, that ginsenosides block the nociceptive behaviors induced by pro-inflammatory cytokines. Although clinical predictions are complicated due to the different underlying mechanisms and pathologic states in nerve injury models and pro-inflammatory cytokines pain models, our results imply that ginsenosides R_{b1}, R_{b2}, R_c, R_d, R_e, R_f and R_{g1} may have clinical value in the treatment or prevention of spinal sensitization induced by neuropathic pain. However, the clinical benefits of ginsenosides on neuropathic pain induced by nerve injury should be elucidated in future studies.

Several mechanisms have been proposed to explain the antinociceptive effect of ginsenosides, including effects on neurotransmitters and ion channels. For example, ginseng total saponin shares signaling pathways with opioid receptors, particularly pertussis toxin-sensitive G-proteins and N- or P type Ca^{2+} channels (Rhim *et al.,* 2002; Suh *et al.,* 1999). Ginsenosides also increase biogenic amines in normal rat brain or interact with nicotinic or GABAergic receptors (Tachikawa *et al.,* 1999; Sala *et al.,* 2002; Choi *et al.,* 2003a; Radad *et al.,* 2006). We speculate that ginsenosides in our model work through supraspinal mechanisms.

Structurally, ginsenosides are classified into three groups: the panaxadiol group (e.g. R_{b1}, R_{b2}, R_{b3}, R_c, R_d, R_{g3}, R_{h2} and R_{h1}), the panaxatriol group (e.g. R_e, R_f, R_{g1}, R_{g2}, R_{h1}) and the oleanolic group (e.g. R_o) (Tachikawa *et al.,* 1999; Radad *et al.,* 2006). Although we did not find group-specific antinociceptive effects following substance P i.t. injection (Choi *et al.,* 2003b), here the panaxadiol group, except R_{g3}, was more potent than the panaxatriol group. Ginsenoside R_{g3} was the most active compound in ginseng total saponin-mediated anti-nociception, via modulating Ca^{2+} currents (Rhim *et al.,* 2002). In our result, however, R_{g3} did not show any antinociceptive effect at all. The explanation for this difference may be that the previous study used systemic administration. Therefore, this result implies that R_{g3} may work through spinal or peripheral sensory neurons, and not by supraspinal mechanisms.

In summary, we showed that several ginsenosides (R_{b1}, R_{b2}, R_c, R_d, R_e, R_f and R_{g1}) significantly attenuated the nociceptive behavior induced by pro-inflammatory cytokines (TNF-α, IL-1β, and IFN-γ). Our result suggests that ginsenosides or ginseng may reduce symptoms or severity of neuropathic pain. However, the exact mechanisms of ginsenosides in blocking pain behavior induced by pro-inflammatory cytokines need to be elucidated in a further study.

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