Effect of Ginsenosides Administered Intrathecally on the Antinociception Induced by Cold Water Swimming Stress in the Mouse

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We examined the effects of ginseng total saponin (GTS) and several ginsenosides injected intrathecally (i.t.) or intracerebroventricularly (i.c.v.) on the antinociception induced by cold water swimming. The tail-flick response was used as an antinociceptive parameter. We found that i.t. injection of GTS time- and dose-dependently attenuated inhibition of the tail-flick response induced by cold water swimming, although GTS given i.c.v. had no significant effect on the latency of the tail-flick response induced by cold water swimming. To identify those responsible for antagonism of GTS against cold water swimming-induced antinociception, the effects of various kinds of ginsenosides (R_{b1} , R_{b2} , R_c , R_d , R_e , and R_{g1}) on inhibition of the tail-flick response induced by cold water swimming were examined. R_{b1} , R_d , R_e , and R_{g1} effectively attenuated the inhibition of the tail-flick response induced by cold water swimming stress. Our results suggest that GTS injected spinally, but not supraspinally, reduces the antinociception induced by this stress, and that the responsible ginsenosides against antinociception induced by cold water swimming may be R_{b1} , R_d , R_e , and R_{g1} . Moreover, the possible involvement of the opioid system in the regulation of cold water swimming stress-induced antinociception by these four ginsenosides is discussed.

Key words ginseng total saponin; ginsenoside; cold water swimming stress; antinociception; spinal cord; mouse

Recent studies have shown that cold water swimming stress induces antinociception. Although the exact mechanisms involved have not been identified, several reports have stated that supraspinal or spinal opioid receptors appear to be related to cold water swimming-induced antinociception.^{1–3)} In addition, the opioidergic system appears to play an important role.^{2,4,5)} We also previously demonstrated that pertussis toxin-sensitive G-protein, cAMP phosphodiesterase and supraspinal glutamate and GABA receptors may be involved in the antinociception produced by cold water swimming.^{6,7)}

Neither the action of ginseng total fraction, active component of the root of *Panax ginseng* used in Far-east Asian traditional medicine, against the production of antinociception induced by cold water swimming stress nor the responsible ginsenosides of the ginseng total saponin (GTS) fraction have yet been characterized. Our group as well as others previously observed that various doses of several ginsenosides injected intrathecally (i.t.) dose-dependently attenuated antinociception induced by U50,488H (a kappa opioid receptor agonist) administered intracerebroventricularly (i.c.v.), suggesting that ginsenosides administered spinally appear to be responsible for blocking the antinociception induced by this agonist when administered supraspinally.^{8,9)} We also reported that GTS or ginsenosides dose-dependently attenuated antinociception induced by morphine administered i.c.v. or systemically.^{9,10)} Thus, the present studies were designed to determine the possible role of GTS and various kinds of ginsenosides (R_{b1} , R_{b2} , R_c , R_d , R_e , and R_{g1}) injected supraspinally or spinally on antinociception induced by cold water swimming stress.

MATERIALS AND METHODS

These experiments were approved by the Animal Care and Use Committee of Hallym University. 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 (20–30 g) from Daehan Laboratories (Seoul, Korea) were used for all experiments. The animals were housed 5 per cage in a room maintained at $22 \pm 0.5^\circ\text{C}$ with an alternating 12 h light–dark cycle. Food and water were available ad libitum. Each mouse was used only once.

Cold Water Swimming Stress The mice were forced to swim in cold (4°C) water for 3 min. They swam in a container 15 cm in diameter and 20 cm tall with water filled to a depth of 11 cm. After the swimming, the mice were gently dried by patting their bodies with paper towels.

Assessment of Antinociception Antinociception was determined by the tail-flick test.¹¹⁾ To measure the latency of the tail-flick response, mice were gently held with one hand with the tail positioned in an apparatus (EMDIE Instrument Co., Maidens, VA, U.S.A., Model TF6) for radiant heat stimulation. The tail-flick response was elicited by applying radiant heat to the dorsal surface of the tail. The intensity of heat stimulus in the tail-flick test was adjusted so that the animal flicked its tail within 3 to 5 s. The tail-flick latencies were measured before (T_0) and after (T_1) the swimming stress. Inhibition of the tail-flick response was expressed as "percent of maximal possible effect (% MPE)" which was calculated as $[(T_1 - T_0)/(T_2 - T_0)] \times 100$, where the cut-off time (T_2) was set at 10 s for the tail-flick test.

Intrathecal Injection Intrathecal injection (i.t.) followed the method described Hylden and Wilcox¹²⁾ using a 25 μl Hamilton syringe with 30 gauge needle. The i.t. injection volume was 5 μl , and the injection site was verified by injecting a similar volume of 1% methylene blue solution and determining the distribution of injected dye in the spinal cord. The dye injected i.t. was distributed both rostrally and caudally but for only a short distance (about 1 cm) and no dye was found in the brain. The success rate for the injections was consistently found to be 95% before the experiments

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were done.

Experimental Protocol Mice were pretreated i.t. with either saline or various doses (1, 10 μ g) of GTS, R_{b1} , R_{b2} , R_c , R_d , R_e , and R_{g1} 10 min prior to cold water swimming. This period was chosen based on preliminary time course studies in which inhibition of the tail-flick response reached maximum after the swimming; the tail-flick response was tested 10, 20 and 30 min after the swimming. All treatment doses were determined from our previous study that showed effects reached a maximum after injection.⁸⁾

Statistical Analysis The data were presented as the mean $\pm$ S.E.M. Statistical analysis was carried out by one-way analysis of variance (ANOVA) with post-hoc test. *p* values less than 0.05 were considered to indicate statistical significance.

Drugs GTS and its fractions (R_{b1} , R_{b2} , R_c , R_d , R_e , and R_{g1}) were obtained from Korea Ginseng and Tobacco Research Institute (Taejon, Korea). All drugs used for injection were dissolved in sterile saline (0.9% NaCl solution).

RESULTS

Effects of GTS Injected i.c.v. and i.t. on Inhibition of the Tail-Flick Responses Induced by Cold Water Swimming Stress Groups of mice were pretreated i.c.v. or i.t. with 5 μ l of saline or various doses of ginseng total saponin (1, 10 μ g) for 10 min, and the mice were forced to swim in cold (4 $^{\circ}$ C) water for 3 min. The tail-flick response was mea-

sured 10, 20 and 30 min after swimming. As shown in Fig. 1, cold water swimming alone profoundly deterred the tail-flick response. GTS at 1 and 10 μ g given i.c.v. had no significant effect on the latencies of the tail-flick response induced by cold water swimming (Fig. 1a), while inhibition of the tail-flick response induced by cold water swimming stress was attenuated time- and dose-dependently by GTS (1, 10 μ g) injected i.t. (Fig. 1b).

Effects of Ginsenosides Injected i.t. on the Inhibition of Tail-Flick Responses Induced by Cold Water Swimming Stress Groups of mice were pretreated i.t. with 5 μ l of saline or various doses of ginsenosides (1, 10 μ g) for 10 min and were forced to swim in cold (4 $^{\circ}$ C) water for 3 min. The tail-flick response was measured 10, 20 and 30 min after swimming. Inhibition of the tail-flick responses induced by cold water swimming was attenuated time- and dose-dependently by ginsenosides R_d and R_e (1, 10 μ g) injected i.t. (Figs. 2b, 3a). In contrast, R_{b1} and R_{g1} injected i.t. at a dose of only 10 μ g reduced the cold water swimming stress-induced inhibition of tail-flick response in a time-dependent manner (Figs. 3b, 4a). Ginsenosides R_{b2} and R_c injected i.t., however, had no significant effect on the latencies of the tail-flick response induced by cold water swimming (Figs. 2a, 4b).

DISCUSSION AND CONCLUSIONS

To determine its action of GTS against cold water swim-

Fig. 1. Time-Course and Dose-Dependent Experiments Examining the Effect of Ginseng Total Saponin (GTS) Injected Intracerebroventricularly (i.c.v.) and Intrathecally (i.t.) on Inhibition of the Tail-Flick Response Induced by Cold Water Swimming

Mice were pretreated i.c.v. (a) or i.t. (b) with either saline (5 μ l) or GTS (1, 10 μ g) 10 min prior to being forced to swim in 4 $^{\circ}$ C water for 3 min. The tail-flick response was measured 10, 20 and 30 min after the swimming. The vertical bars denote the standard error of the mean. The number of animals used for each group was 8 to 10. **p* < 0.05 compared to the control group of mice injected i.c.v. or i.t. with saline.

Fig. 2. Time-Course and Dose-Dependent Experiments Examining the Effect of R_c and R_d Injected Intrathecally (i.t.) on Inhibition of the Tail-Flick Response Induced by Cold Water Swimming

Mice were pretreated i.t. with either saline (5 μ l), R_c (1, 10 μ g; a) or R_d (1, 10 μ g; b) 10 min prior to being forced to swim in 4 $^{\circ}$ C water for 3 min. The tail-flick response was measured 10, 20 and 30 min after the swimming. The vertical bars denote the standard error of the mean. The number of animals used for each group was 8 to 10. **p* < 0.05 compared to the control group of mice injected i.t. with saline.

Fig. 3. Time-Course and Dose-Dependent Experiments Examining the Effect of R_e and R_{g1} Injected Intrathecally (i.t.) on Inhibition of the Tail-Flick Response Induced by Cold Water Swimming

Mice were pretreated i.t. with either saline (5 µl), R_e (1, 10 µg; a) or R_{g1} (1, 10 µg; b) 10 min prior being forced to swim in 4 °C water for 3 min. The tail-flick response was measured 10, 20 and 30 min after the swimming. The vertical bars denote the standard error of the mean. The number of animals used for each group was 8 to 10. * $p < 0.05$ compared to the control group of mice injected i.t. with saline.

ming stress-induced antinociception, the effect of GTS injected i.c.v. or i.t. on inhibition of the tail-flick response induced by cold water swimming stress was examined in the present study. We found that i.t. injection of GTS time- and dose-dependently attenuated inhibition of the tail-flick response induced by cold water swimming stress, although when given i.c.v. it had no significant effect on the latencies of the tail-flick response induced by cold water swimming. Our results suggest that GTS injected spinally, but not supraspinally, reduces the antinociception induced by cold water swimming stress.

To identify those responsible for antagonism of GTS against cold water swimming stress-induced antinociception, the effects of the ginsenosides R_{b1} , R_{b2} , R_c , R_d , R_e , and R_{g1} on inhibition of tail-flick response induced by cold water swimming stress were examined; R_{b1} , R_d , R_e , and R_{g1} effectively attenuated the inhibition of the tail-flick response induced by cold water swimming. Although the reason for this is not currently clear, some ginsenosides in GTS may have opposing effects against the antinociception induced by cold water swimming stress. In addition, the mechanism of time-dependent suppression of GTS and ginsenoside R_{b1} , R_d , R_e , and R_{g1} in the cold water swimming stress-induced antinociception may be due to their structures. Ginsenosides possess a four trans-ring rigid steroid skeleton with a modified side chain at C-20, and share structural features with steroid hormones.¹³ It can therefore be speculated that the time-dependent or relatively delayed response (30 min) of ginsenosides

Fig. 4. Time-Course and Dose-Dependent Experiments Examining the Effect of R_{b1} and R_{b2} Injected Intrathecally (i.t.) on Inhibition of the Tail-Flick Response Induced by Cold Water Swimming

Mice were pretreated i.t. with either saline (5 µl), R_{b1} (1, 10 µg; a) or R_{b2} (1, 10 µg; b) 10 min prior to being forced to swim in 4 °C water for 3 min. The tail-flick response was measured 10, 20 and 30 min after the swimming. The vertical bars denote the standard error of the mean. The number of animals used for each group was 8 to 10. * $p < 0.05$ compared to the control group of mice injected with saline.

may be attributable to their lipid-solubility.

It is already well known that the opioid systems mediate cold water swimming stress-induced antinociception. Previously, Briggs *et al.*³ reported that spiradoline injected systemically acts as a kappa opioid receptor-mediated antinociceptive agonist in the cold-water tail-flick assay. An additional experiment demonstrated that both beta-funaltrexamine (a selective mu opioid receptor antagonist) and mu-antisense oligodeoxynucleotide administered i.c.v. reduced the maximal analgesic effect of morphine and produced rightward shifts in its dose-effect curves using the cold water tail-flick test.¹⁴ However, Mizoguchi *et al.* previously demonstrated the involvement of spinal delta opioid receptor in the cold-water swimming stress-induced antinociception.^{15,16} Recently our laboratory reported that various doses (from 0.1 to 1 µg) of ginsenosides R_{b1} , R_{b2} , R_c , R_d , and R_{g1} , but not R_e , injected i.t. dose-dependently attenuated antinociception induced by U50,488H (a kappa opioid receptor agonist) administered i.c.v.; this suggested that R_{b1} , R_{b2} , R_c , R_d , and R_{g1} administered spinally appear to be responsible for blocking the antinociception induced by U50,488H administered supraspinally.⁸ We also reported that ginsenosides R_{b2} , R_c , R_d and R_{g1} , but not R_{b1} and R_e , injected i.t. dose-dependently attenuated antinociception induced by morphine administered i.c.v.¹⁰ Furthermore, it was reported that the antagonism of U50,488H-induced antinociception in mice pretreated with GTS was abolished by pretreatment with a serotonin precursor, 5-hydroxytryptophan, in the tail-flick test.⁹

Although not exactly understood at the present time, the results of the present study raise the possibility that ginsenosides R_{b1} may mediate the kappa opioid system. In addition, R_d and R_{g1} may mediate the kappa and mu opioid systems, thus modulating cold water swimming-induced antinociception by several actions, although R_e employs a different underlying mechanism. First, it can be speculated that ginsenosides R_{b1} , R_d , and R_{g1} may modulate, presynaptically, the release of a certain neurotransmitter or neuropeptide such as dynorphin or enkephalin from the spinal cord. Second, descending serotonergic system may be involved in the attenuation of the cold water swimming-induced inhibition of the tail-flick response. To examine this hypothesis, the effects of coadministration of supraspinal serotonin receptor antagonist plus spinal GTS or ginsenosides on antinociception induced by cold water swimming stress should be further assessed.

The present study has clearly demonstrated that GTS or its fractions R_{b1} , R_d , R_e , and R_{g1} injected spinally, but not supraspinally, have a significant inhibitory effect on cold water swimming-induced antinociception.

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