J. Ginseng Res.
Vol. 24, No. 3, 143-147(2000)

Ginsenosides Attenuate Formalin-Induced Pains Through Spinal and Supraspinal Sites

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(Received July 13, 2000)

Abstract : In previous studies we have demonstrated that several individual ginsenosides such as Rc, Rd, Re and Rf relieves formalin-induced pain following systemic treatment. But it is unknown where these single ginsenosides induce antinociception. We investigated the antinoiceptive effect of four individual ginsenosides on formalin-induced pain after intrathecal (i.t.), intracereventricular (i.c.v.), or subcutaneous (s.c.) administration using mice. We found that ginsenoside Rc, Rd, and Re except Rf attenuated both acute and tonic phase of pain. Ginsenoside Rf attenuated only tonic phase of pain after i.t. administration. The ED_{50} was 1.0 (0.55~1.75 mg/kg) for Rc, 1.15 (0.6~2.25 mg/kg) for Rd, and 8.9 (3.9~20.5 mg/kg) for Re in acute phase of pain. The ED_{50} was 0.3 (0.1~0.85 mg/kg) for Rc, 0.6 (0.35~1.1 mg/kg) for Rd, 2.45 (1.25~4.65 mg/kg) for Re, and 1.9 (1.5~4.25 mg/kg) for Rf in tonic phase of pain. We also found that ginsenoside Rc, Rd, Re, and Rf after i.c.v. administration attenuated both acute and tonic phase of pain. The ED_{50} for acute phase of pain was 0.9 (0.55~1.4 mg/kg) for Rc, 0.9 (0.45~1.7 mg/kg) for Rd, 0.93 (0.5~1.75 mg/kg) for Re, and 1.85 (0.95~3.5 mg/kg) for Rf. The ED_{50} for tonic phase of pain was 0.7 (0.45~1.05 mg/kg) for Rc, 1.25 (0.7~2.2 mg/kg) for Rd, 0.85 (0.45~1.6 mg/kg) for Re, and 0.8 (0.4~1.45 mg/kg) for Rf. Thus, the order of the analgesic potency was Rc≥Rd>Re>Rf in both i.t. and i.c.v. administration routes. However, s.c. pretreatment of four ginsenosides did not reduce formalin-induced pain. These results suggest that analgesic effect of ginsenosides is achieved through spinal or supraspinal site(s) in formalin test.

Key words : Ginsenoside Rc, Rd, Re, and Rf, pain, spinal and supraspinal sites, analgesia.

INTRODUCTION

Ginseng, the root of *Panax ginseng* C.A. Meyer, has claimed to have a variety of efficacies for a long time. For example, ginseng has been used to alleviate some types of pain such as toothache, abdominal pain, chest pain, or neuralgia in folk medicine. Recent studies showed that ginseng saponins, which consist of various ginsenosides, are the main biologically active ingredients of ginseng. Many evidences show that ginseng saponins are responsible for relieving pain induced by chemicals or noxious heat using experimental animals. Ginseng neutral saponins showed antinociception in writhing test and tail-pressure test in mice.[1] Saito *et al* (1973) also reported that ginseng saponins isolated from ginseng leaves have antinociception in writhing and tail-pressure test in mice.[2] However, in tail-flick test using rat ginseng total saponins showed only a weak antinociception.[3]

It was reported at the cellular level that ginseng root extract inhibits voltage-dependent Ca^{2+} channels in sensory neurons and ginsenoside Rf among several ginsenosides exerts the inhibitory effect of voltage-dependent Ca^{2+} channels in sensory neurons.[4,5] We reported that ginseng saponin produces analgesia in writhing and formalin tests but not in tail-flick test in mice.[6] We also found that the main responsible components for analgesia are ginsenoside Rc, Rd, Re, and Rf through systemic treatment in formalin test.[7,8]

However, it is still unknown whether these individual ginsenosides act on peripheral, spinal or supraspinal site(s) to induce antinociception. Therefore, we tested the analgesic effect of four individual ginsenosides to find out action site(s) after i.t., i.c.v., or s.c. pretreatment in formalin test.

MATERIALS AND METHODS

1. Materials

Four purified ginsenosides (ginsenoside Rc, Rd, Re, and

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Rf) were obtained from Korea Ginseng and Tobacco Research Institute (Taejon, Korea). All other agents were purchased from Sigma. For behavioral experiments, individual ginsenoside were dissolved in dimethyl sulfoxide (DMSO) as s stock solution (20 mg/0.1 m*l*) and was used after dilution with saline. The final DMSO concentration was less than 5% and 5% DMSO itself had no effect. Vehicle (5% DMSO) or individual ginsenoside solution were injected i.t. i.c.v. or s.c. in a volume of 5 μl/mouse.

2. Experimental animal and drug administration

ICR (20~25 g) mice were used in all experiments. The number of mouse used for experiments was 8~10 per animal group. Equal number of mice from both sexes were used, since no sex difference was observed and data from both sexes were pooled for all reported analysis. Mice were purchased from Dae-Han animal breeding center (Cheongju, Korea). Animals were maintained in a temperature-controlled environment (22±2°C), on a 12 : 12 hour light-dark cycle. Mice were given ad lib access to food and tap water. I.t. administration of ginsenosides was performed according to the method of Hylden and Wilcox (1980).[9] Injection procedure involves direct inserting a 30-gauge needle, matched to a 50 μl microsyringe, into an intervertebral space at approximately the level of the 5^{th} or 6^{th} lumber vertebrae. The success rate for the injection was consistently found to be over 95% using 1% methylene blue solution before the experiments were done. I.c.v. administration of ginsenosides was performed according to the method of Haley and McCormick (1957).[10]

3. Algesiometric assays

Formalin test was performed with a slightly modified version of the technique of Hunskaar and his colleagues with mice.[11] Briefly, 1% formalin was prepared from the aqueous solution of 37% w/w formaldehyde. In this assay, mice were introduced to the testing environment, i.e., 30 cm high, 20 cm diameter Plexiglas box for 60 min before any injection. A mirror was placed behind the cylinders for easy observation of whole body of testing animals. They were then weighed and returned to the cylinders. Following twenty minutes of i.t. or i.c.v. administration of test substances, 1% formalin was injected just under the skin of the plantar surface of the left hind paw by use of a microsyringe with 30 gauge needle. Mice were returned to the cylinders and immediately observed for biting and licking of the affected hind paw. The total time that spent for biting and licking over the next 40 min was measured with a stopwatch and recorded to the nearest second in 5 min blocks during both phases as an indicator of nociception. Based on pilot data and in keeping with the literature, the acute phase of pain was defined as 0 to 10 min post-injection of formalin and the tonic phase of pain as 11~40 min post-injection.

4. Rotorod test

In order to evaluate the possible nonspecific motor or sedative effects of ginsenosides, the mice were tested on the rotorod.[12] The apparatus consisted of a bar (2.5 cm in diameter) subdivided into six compartments by disks (25 cm in diameter) (Ugo Basile, Italy). Mice were injected i.t. or i.c.v. route with 2.5 or 1.25 mg/kg ginsenosides 20 min before testing, respectively. Vehicle group was injected with only saline. The time that they remained on the rotating bar (cut-off time: 60 s) was recorded. Rotorod test scores were calculated as the average of three trials.

5. Statistics

Antinociception was expressed as percent antinociception calculated as follows; % antinociception = (mean of time spent bitings & lickings by control group-mean of time spent bitings & lickings by drug-treated group/mean of time spent bitings & lickings by control group)×100. These values were then used to generate dose-response curves (DRCs). The DRCs were analyzed for slope and interpolated to ED_{50} by linear regression of probit-transformed percent analgesia scores by the method of Lichfield and Wilcoxon (1949).[13] Data were analyzed by analysis of variance (ANOVA) and Dunnetts procedure for multiple comparisons with single vehicle group was used to analyze the overall patterns of results. The level of significance was set to 5% ($p<0.05$). Results are given as mean±S.E.M.

RESULTS

1. Effects of i.t. administered ginsenosides on formalin-induced pain

We investigated the analgesic effect of i.t. administered ginsenoside Rc, Rd, Re, or Rf on formalin-induced pain. As shown in Table 1~4, the administration of 1% formalin into plantar surface of hind paw induced typical biphasic pain behavior such as licking and biting. Acute phase of pain or first phase of pain appears on 0~10 min after formalin injection and the second phase of pain or tonic pain appears 11~40 min following short term period of qui-

Table 1. The effect of ginsenoside Rc against the acute phase, 0 to 10 min, and the tonic phase, 11 to 40 min, in the formailin test on mice

	Dose (mg/kg)	Amount of licking and bitig (s)	
		0~10 min	11~40 min
Intrathecal	0	87.00±6.48	134.63±18.25
	0.5	76.25±4.35	138.13±9.42
	1.25	30.13±5.28*	12.25±6.57*
	2.5	6.63±4.50*	3.13±2.35*
	5	2.88±1.68*	0.50±0.50
Intracerebroventricular	0	70.63±6.88	81.13±17.31
	0.25	67.25±8.20	77.13±19.89
	0.5	56.00±4.54	53.75±7.46
	1.25	22.00±5.68*	8.80±4.10*
	2.5	3.88±1.41*	1.25±1.25*

Each value represent the mean±SEM. *p<0.01 compared to saline-treated control (by ANOVA of Dunnette's test).

Table 2. The effect of ginsenoside Rd against the acute phase, 0 to 10 min, and the tonic phase, 11 to 40 min, in thee formalin test on mice

	Dose (mg/kg)	Amount of licking and bitig (s)	
		0~10 min	11~40 min
Intrathecal	0	95.25±6.20	206.25±26.24
	0.5	71.38±13.34	108.88±32.55
	1.25	44.75±6.09*	37.26±10.24*
	2.5	20.63±4.91*	4.25±2.13*
	5	11.63±4.66*	8.50±5.06*
Intracerebroventricular	0	75.50±8.44	96.50±11.72
	0.25	58.88±3.83	88.88±5.92
	0.5	53.00±4.23*	86.13±15.12
	1.25	33.25±3.34	40.50±7.49*
	2.5	15.75±4.37	2 3.63±8.47*

Each value represent the mean±SEM. *p<0.01 compared to saline-treated control (by ANOVA of Dunnette's test).

Table 3. The effect of ginsenoside Rc against the acute phase, 0 to 10 min, and the tonic phase, 11 to 40 min, in the formailin test on mice

	Dose (mg/kg)	Amount of licking and bitig (s)	
		0~10 min	11~40 min
Intrathecal	0	9.500±6.07	141.75±13.79
	0.5	89.00±4.64	139.38±28.49
	1.25	82.00±8.20	89.13±14.37
	2.5	68.13±6.76*	66.25±18.80*
	5	67.13±5.82*	3.13±1.98*
Intracerebroventricular	0	62.88±6.13	83.75±15.12
	0.25	70.00±5.13	72.13±16.42
	0.5	41.63±3.78*	80.88±11.12
	1.25	22.38±5.04*	31.38±8.33*
	2.5	11.75±3.34*	8.00±3.29*

Each value represent the mean±SEM. *p<0.01 compared to saline-treated control (by ANOVA of Dunnette's test).

Table 4. The effect of ginsenoside Rc against the acute phase, 0 to 10 min, and the tonic phase, 11 to 40 min, in the formailin test on mice

	Dose (mg/kg)	Amount of licking and bitig (s)	
		0~10 min	11~40 min
Intrathecal	0	88.63±5.75	130.50±8.68
	0.5	96.63±1.19	101.88±11.85
	1.25	90.00±4.77	57.38±19.85*
	2.5	84.88±6.74	54.63±24.26*
	5	7.163±9.85	28.63±10.95*
Intracerebroventricular	0	73.13±7.42	108.38±15.47
	0.25	76.13±5.77	74.38±18.55
	0.5	76.88±6.97	95.13±15.14
	1.25	37.63±2.98*	37.50±11.40*
	2.5	28.50±2.98*	9.50±4.16*

Each value represent the mean±SEM. *p<0.01 compared to saline-treated control (by ANOVA of Dunnette's test).

escent interval. I.t. pretreatment of ginsenoside Rc attenuated pain induced by formalin at dose over 1.25 mg/kg in both acute phase of pain and tonic phase of pain. The ED_{50} for Rc was 1.0 (0.55~1.75 mg/kg) for acute phase of pain and 0.3 (0.1~0.85 mg/kg) for tonic phase of pain (Table 1). I.t. pretreatment of ginsenoside Rd also attenuated pain induced by formalin at dose over 1.25 mg/kg in acute phase of pain and at dose over 0.5 mg/kg in tonic phase of pain. The ED_{50} for Rd was 1.15 (0.6~2.25 mg/kg) for acute phase of pain and 0.6 (0.35~1.05 mg/kg) for tonic phase of pain (Table 2). I.t. pretreatment of ginsenoside Re attenuated pain induced by formalin at dose over 2.5 mg/kg both acute phase of pain and tonic phase of pain. The ED_{50} for Re was 8.9 (3.85~20.45 mg/kg) for acute phase of pain and 2.45 (1.25~4.65 mg/kg) for tonic phase of pain (Table 3). Thus, Re was required large amount to get antinociception in acute phase of pain compared to Rc or Rd. Interestingly, I.t. pretreatment of ginsenoside Rf had no effect on acute phase of pain induced by formalin over 5 mg/kg and at dose over 1.25 mg/kg Rf attenuated tonic phase of pain. The ED_{50} for Rf was 1.9 (1.5~4.25 mg/kg) for tonic phase of pain (Table 4).

2. Effects of i.c.v. administered ginsenosides on formalin-induced pain

We also investigated the analgesic effect of four ginsenosides after i.c.v. administration. As shown in Table 1, i.c.v. pretreatment of ginsenoside Rc attenuated pain

induced by formalin at dose over 1.25 mg/kg in both acute phase of pain and tonic phase of pain. The ED_{50} for Rc was 0.88 (0.55~1.4 mg/kg) for acute phase of pain and 0.7 (0.45~1.05 mg/kg) for tonic phase of pain. I.c.v. pretreatment of ginsenoside Rd attenuated pain induced by formalin at dose over 0.5 mg/kg in acute phase of pain and at dose over 1.25 mg/kg in tonic phase of pain. The ED_{50} for Rd was 0.9 (0.45~1.7 mg/kg) for acute phase of pain and 1.25 (0.7~2.2 mg/kg) for tonic phase of pain (Table 2). I.c.v. pretreatment of ginsenoside Re also attenuated pain induced by formalin at dose over 0.5 mg/kg in acute phase of pain and at dose over 1.25 mg/kg for tonic phase of pain. The ED_{50} for Re was 0.93 (0.5~1.75 mg/kg) for acute phase of pain and 0.85 (0.45~1.55 mg/kg) for tonic phase of pain (Table 3). I.c.v. pretreatment of ginsenoside Rf attenuate both acute phase and tonic phase of pain induced by formalin at dose over 1.25 mg/kg. The ED_{50} for Rf was 1.85 (0.95~3.5 mg/kg) for acute phase of pain and 0.8 (0.43~1.45 mg/kg) for tonic phase of pain (Fig. 8). However, s.c. pretreatment of four ginsenosides did not attenuate formalin-induced pain even at the dose of 5 mg/kg (data not shown). In rotorod test, i.t. or i.c.v. administered ginsenosides did not significantly affect the motor response of animal compared to vehicle (data not shown).

DISCUSSION

In the present study, we demonstrated that: (1) i.t. or i.c.v. administered ginsenosides such as ginsenoside Rc, Rd, Re, or Rf suppressed formalin-induced pain in a dose-dependent manner and (2) the effective dose of ginsenosides to suppress formalin-induced pain was much smaller for the central than previous intraperitoneal (i.p.) administration.[7,8] In addition, we showed that s.c. administered ginsenosides was ineffective in blocking formalin-induced pain. Taken together, these results strongly suggest that: (1) the sites of action of ginsenosides are in the central nervous system (CNS) and not in the periphery and (2) it is the sensory, not motor, system that is affected by ginsenosides, since ginsenosides have no effect in rotorod performance. These results are also well consistent with previous reports that systemic treatment of ginsenosides did not affect motor activity.[7,8]

In present study, ginsenoside Rc and Rd showed more potent antinociception than Re and Rf in both administration routes and the order of analgesic potency was Rc $\geq$Rd>Re>Rf. Interestingly, ginsenoside Rc and Rd belong to protopanaxadiol (PD) saponins and ginsenoside Re and Rf are protopanaxatriol (PT) saponins. These results suggest that PD saponins are more potent than PT saponins in their analgesic activity. These results are well consistent with previous report that PD saponins are more potent than PT saponins to relieve formalin-induced pain after systemic treatment.[6]

From the present results, however, it is unclear precisely where and how in the CNS ginsenosides act to produce the antinociceptive effects. One possible mechanism is that, at the spinal cord, ginsenosides may inhibit the glutamate/substance P release from primary sensory nerve terminals following formalin injection. Consistent with this hypothesis, it has been shown that ginseng root extract and ginsenoside Rf inhibit voltage-dependent Ca^{2+} channels in sensory neurons and in identified nociceptive neurons.[4,5,7]

Another possibility is that ginsenosides may work by attenuating the responsiveness to glutamate/substance P of postsynaptic neurons in the spinal dorsal horn. In support of this notion, we have shown that i.t. administered ginsenosides attenuates the pain-related behavior produced by i.t. injection of substance P or NMDA.[14,15] Thus, it is possible that ginsenosides act on both pre- and postsynaptic sites at the spinal cord level.

The effect of ginsenosides against formalin-induced pain seems unlikely to be mediated by opioid receptors. First, pretreatment of mice with naloxone did not block the effect of ginsenosides.[7] Second, ginsenosides had much less of an analgesic effect than morphine or other opioid receptor agonists.[3] Third and finally, the regulation of voltage-dependent Ca^{2+} channels in sensory neurons by ginseng root extract was shown not to be mediated by opioid receptors.[4]

In summary, this study showed that i.t. or i.c.v but not s.c. administered ginsenosides inhibited formalin-induced pain. Although the precise mechanism underlying the antinociceptive ginsenoside effect is unknown, our data suggest that the sites of ginsenosides action are located at both the spinal and supraspinal regions and these are not opioid receptors.

요 약

앞의 연구에서 우리는 진세노사이드 Rc, Rd, Re 및 Rf를 복강내 전 처리할 경우 포르말린으로 유도된 통증을 억제하다는 것을 보고하였다. 그러나 이러한 진세노사이드가 어느 위치에서 항통증 작용을 발휘하는가에 대하여서는 아직 알려지지 않고 있다. 본 연

구에서는 이들 진세노사이드를 뇌실내, 척수강내 혹은 피하내 전처리한 다음 포르말린에 의하여 유도되는 통증이 어느 위치에서 억제되는가를 연구하였다. 연구 결과 이들 진세노사이드는 척수강내 전처리할 경우 포르말린에 의하여 유도되는 통증을 억제하는 것으로 나타났다. 급성 통증 phase에서 ED_{50}는 Rc가 1.0 (0.55~1.75 mg/kg)이었고, Rd가 1.15 (0.6~2.25 mg/kg)이었고, Re가 8.9 (3.9~20.5 mg/kg)이었다. 지속성 통증 phase에서 ED_{50}는 Rc가 0.3 (0.1~0.85 mg/kg)이었고, Rd가 0.6 (0.35~1.1 mg/kg)이었고, Re가 2.45 (1.25~4.65 mg/kg)이었고, Rf가 1.9 (1.5~4.25 mg/kg)인 것으로 나타났다. 또한 뇌실내 전처리할 경우에도 이들 진세노사이드들은 포르말린에 의하여 유도되는 통증을 억제하였다. 급성 통증 phase에서 ED_{50}는 Rc가 0.9 (0.55~1.4 mg/kg)이었고, Rd가 0.9 (0.45~1.7 mg/kg)이었고, Re가 0.93 (0.5~1.75 mg/kg), Rf가 1.85 (0.95~3.5 mg/kg)인 것으로 나타났다. 지속성 통증 phase에서는 ED_{50}는 Rc가 0.7 (0.45~1.05 mg/kg)이었고, Rd가 1.25 (0.7~2.2 mg/kg)이었고, Re가 0.85 (0.45~1.6 mg/kg)이었고, Rf의 경우에는 0.8 (0.4~1.45 mg/kg) 이었다. 항통증 효능 potency는 두 가지 투여 경로에 있어서 Rc≥Rd>Re>Rf 인 것으로 나타났다. 그러나, 피하내 주사는 포르말린에 의하여 유도되는 통증을 억제하지 않은 것으로 나타났다. 이러한 연구 결과는 진세노사이드에 의한 항통증 작용은 척수 수준 및 척수위 수준에서 이루어진다는 것을 보여주고 있다.

ACKNOWLEDGEMENTS

This work was supported by grant of 97 Korea Ginseng and Tobacco Research Institute.

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