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Ginsenosides That Show Antinociception in Writhing and Formalin Tests

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Abstract : We demonstrated in previous study that protopanaxadiol and protopanxatriol saponins show antinociceptive activity in acetic acid induced writhing test and in the second phase (11-40 min) of formalin test but not tail-flick test. To identify further which ginsenoside has antinociceptive activity among various ginseng saponins, we have investigated antinociceptive effects of several ginsenosides using writhing and formalin test. Ginsenoside Rc, Rd, Re, and Rf induced antinociception in writhing test. These four ginsenosides also induced antinociception in the second phase of formalin (11-40 min) test but these ginsenosides showed a slight antinociception in the first phase (010 min) of formalin test except ginsenoside Rf. The antinociceptive effects induced by the ginsenosides were dose dependent and were not blocked by an opioid receptor antagonist, naloxone. The order of antinociceptive potency was Rd > Rc > Re > Rf in the formalin test. However, these ginsenosides did not show any significant analgesic effects in a tail-flick test. These results suggest that ginsenosides such as Rc, Rd, Re, and Rf inhibit tonic pain rather than acute pain induced by noxious heat. These results also indicate that the antinociceptive activity. Induced by ginsenosides may be one of the actions for pharmacological effects of *Panax ginseng*.

Key words : Pain, Ginsenosides, Antinociception, Analgesic agent.

Introduction

Ginseng, the root of *Panax ginseng* C.A. Meyer (Araliaceae) has been used as a tonic for a long time. Recent studies showed that ginseng saponins, which are the mixtures of various types of ginsenosides, are the main biologically active components of ginseng root. For example, these ginsenosides show some physiological or pharmacological actions like adrenaline, opioid, muscarine, or histamine.[1]

Ginseng also has been used to alleviate some types of pain such as tooth ache, abdominal pain, chest pain, or neuralgia in traditional folk medicine. A line of evidence also supports those efficacies of ginseng relieving pain induced by chemicals or noxious heat in experimental animals. Nabata *et al.* (1973) reported that ginseng neutral saponins (mainly consisting of ginsenoside Rb_1, Rb_2, and Rc) have antinociception in writhing test and tail-pressure test in mouse.[2] Saito *et al.* (1973) also reported that ginseng saponin extract from ginseng leaves have antinociception in writhing test and tail-pressure test using mouse.[3] In tail-flick test, ginseng total saponins (GTS) show only a weak antinociception in rat.[4] In previous study, we reported that ginseng protopanaxadiol (PD) and protopanaxatriol (PT) saponins both have analgesic effects in writhing test and second phase of pain in formalin test but not tail-flick test in mice.[5]

The aim of the study was to investigate which

single components of ginseng saponins have analgesic activity in writhing, formalin, and tail-flick tests. We used six different ginsenosides, ginsenoside Rb_1, Rc, Rd, Re, Rf, and Rg_1. We found that ginsenoside Rc, Rd, Re, and Rf reduce pains induced by acetic acid and mainly second phase of pain induced by formalin but ginsenoside Rb_1 and Rg_1 had no effect on these two tests. These results suggest that ginsenosides such as Rc, Rd, Re, and Rf inhibit tonic pain rather than acute pain.

Materials and Methods

1. Materials

ICR (20~25 g) mice were used in all *in vivo* experiments. The number of mouse used for experiments was 8-10 per group. Equal number of mice from both sexes were used, since no sex differences were observed and data from both sexes were pooled for all reported analysis. Mice were purchased from Woo Jung Chemincal Co. (Seoul, Korea) or Sam Yuk animal breeding center (Suwon, Korea). Animals were maintained in a temperature-controlled environment (22±2℃), on a 12:12 hour light-dark cycle. Mice were given ad lib access to food and tap water. Six ginsenosides (ginsenoside Rb_1, Rc, Rd, Re, Rf, and Rg_1) were obtained from the Korean Ginseng and Tobacco Research Institute (Taejon, Korea). All other agents were purchased from Sigma. For the behavioral experiments, individual ginsenoside was suspended in 1% carboxymethylcellulose (CMC) following Kaku *et al.* (1975).[6] Ginsenoside and vehicle solution (1% CMC) were injected i.p. in a volume of 10 ml/kg. In one experiment mice were pretreated with naloxone (5 mg/kg) for 20 min or physiological saline prior to ginsenoside administration.

2. Methods

(1) Writhing test

The method that Koster *et al.* (1959) have described was used for abdominal constrictions with 0.9% glacial acetic acid.[7]

(2) Formalin test

A slightly modified version of the technique of Hunskaar and his collegues (1985) was used with mice.[8] 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. After twenty minutes i.p. injection of the test substance, 40 ml of 1% formalin was injected just under the skin of the plantar surface of the left hindpaw by use of a microsyringe with 30 gauge needle. Mice were returned to the cylinders and immediately observed for bitings and lickings of the injected hindpaw. The total time that spent for bitings and lickings over the next 40 min was measured 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 first phase was defined as 0 to 10 min post-injection of formalin and the second phase as 11 min to 40 min post-injection.[8]

(3) Tail-flick test

The tail-flick assay was performed according to the method of D'Amour and Smith (1941) using mice.[9]

(4) Data analysis

Antinociception was expressed as percent antinociception calculated as follows; A=mean no. of constriction/time spent bitings & lickings by control group. B=mean no. of constriction/time spent bitings & lickings by drug-treated group. % Antinociception=(A-B)/A×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 Litchfield and Wilcoxon (1949).[10] Data were analyzed by analysis of variance (ANOVA) and Dunnett's procedure for mutiple comparisons with a 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

We tested analgesic effects of six different ginsenosides such as ginsenoside Rb_1, Rc, Rd, Re, Rf, and Rg_1 using conventional algesiometric methods such as writhing, formalin, and tail-flick test. As shown in Fig. 1, ginsenoside Rc, Rd, Re, or Rf reduced 0.9% acetic acid induced abdominal pain with dose dependent manner. ED_{50} was 20.5 (7.3~57.4 mg/kg) for Rc, 17 (11~27.6 mg/kg) for Rd, 3.5 (1~12 mg/kg) for Re, 54 (36~81 mg/kg) for Rf, respectively. Interestingly, ginsenoside Rb_1 and Rg_1 at 50 mg/kg had no antinociceptive effects in the writhing test (data not shown). Therefore, ginsenoside Rb_1 and Rg_1 were excluded in following formalin and tail-flick tests.

We tested further the antinociceptive effect of its respective ginsenoside Rc, Rd, Re, or Rf using 1% formalin. As shown in Fig. 2~5, the administration of 1% formalin into the plantar surface of hindpaw induced typical two phases of pain behavior.[8] The first phase of pain or acute pain appears on 0~10 min after formalin injection and the second phase of pain or tonic

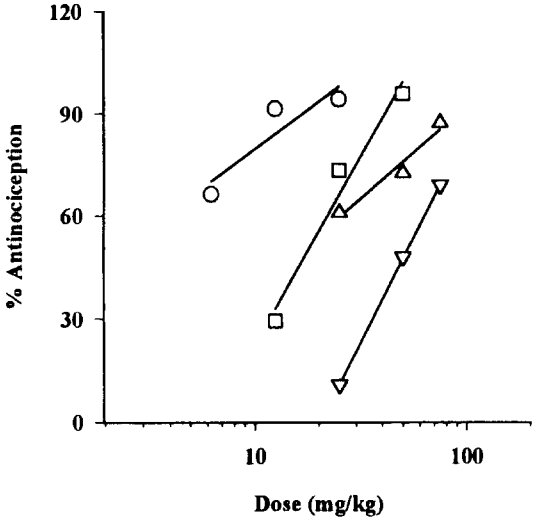

Fig. 1. Dose-dependent effects of ginsenoside Rc, Rd, Re, and Rf on 0.9% acetic acid-induced number of writhes. Ginsenoside Rc (△), Rd (□), Re (○), or Rf (▽) was administrated with indicated dose. After 20 min, writhings were induced by i.p. injection of 0.9% acetic acid and counted for 30 min. Error bars were omitted in the dose response curve for clarity.

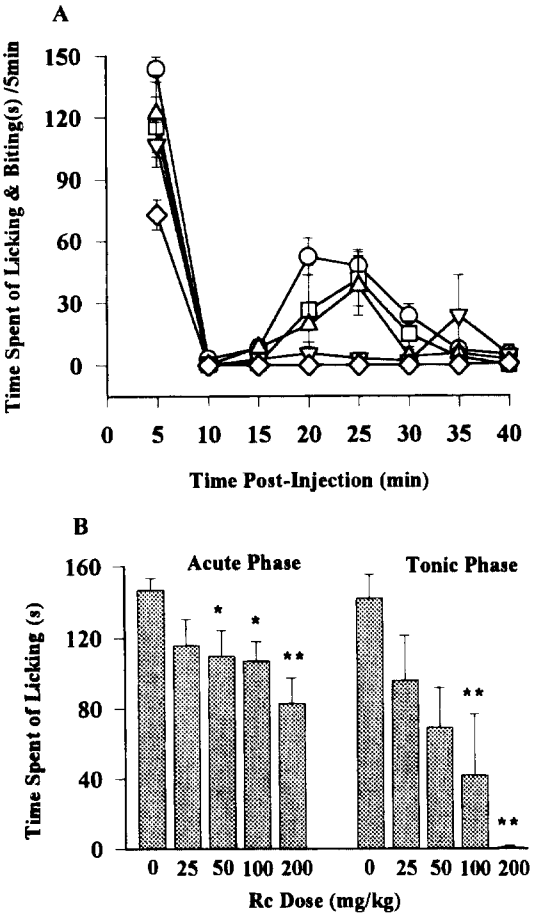

Fig. 2. The effect of ginsenoside Rc on pain induced by 1% formalin. A. Con (○), 25 (□), 50 (△), 100 (▽), or 200 (◇) mg/kg of ginsenoside Rc. Pain responses were measeured from immediately with 5 min block after intraplantar surface injection of 40 μl of 1% formalin. Pain responses are the time that spent licking and biting(s) of the injected hind paw or leg. Each value represents mean SEM. B. These histograms show the first (0-10 min) and second phase (11-40 min) of pain responses following the injection of formalin after pretreatment with different doses of ginsenoside Rc.
*$p<0.05$ or **$p<0.01$ compared to saline-injected controls (by ANOVA and Dunnett's procedure for multiple comparisons).

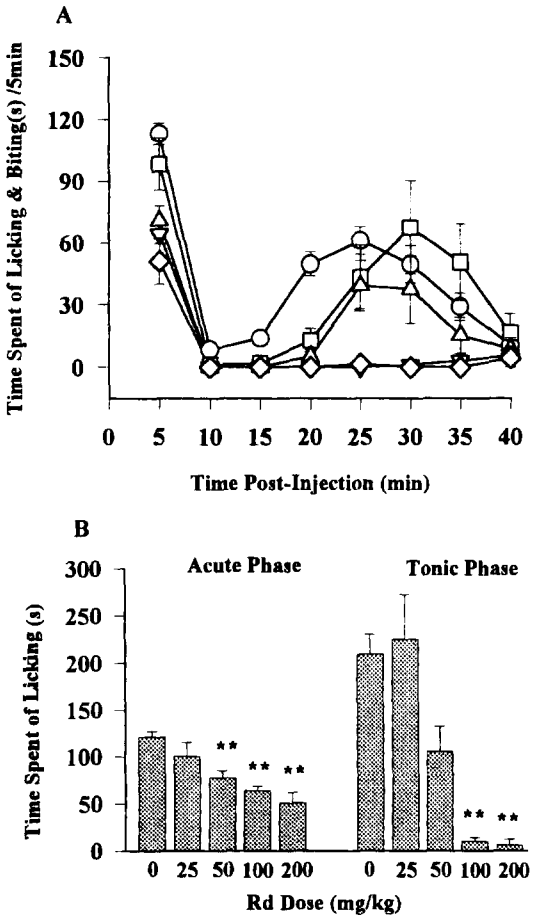

Fig. 3. The effect of ginsenoside Rd on pain induced by 1% formalin. A. Con (○), 25 (□), 50 (△), 100 (▽), or 200 (◇) mg/kg of ginsenoside Rd. Pain responses were measeured from immediately with 5 min block after intraplantar surface injection of 40 μl of 1% formalin. Pain responses are the time that spent licking and biting(s) of the injected hind paw or leg. Each value represents mean SEM. B. These histograms show the first (0-10 min) and second phase (11-40 min) of pain responses following the injection of formalin after pretreatment with different doses of ginsenoside Rd.
*p<0.05 or **p<0.01 compared to saline-injected controls (by ANOVA and Dunnett's procedure for multiple comparisons).

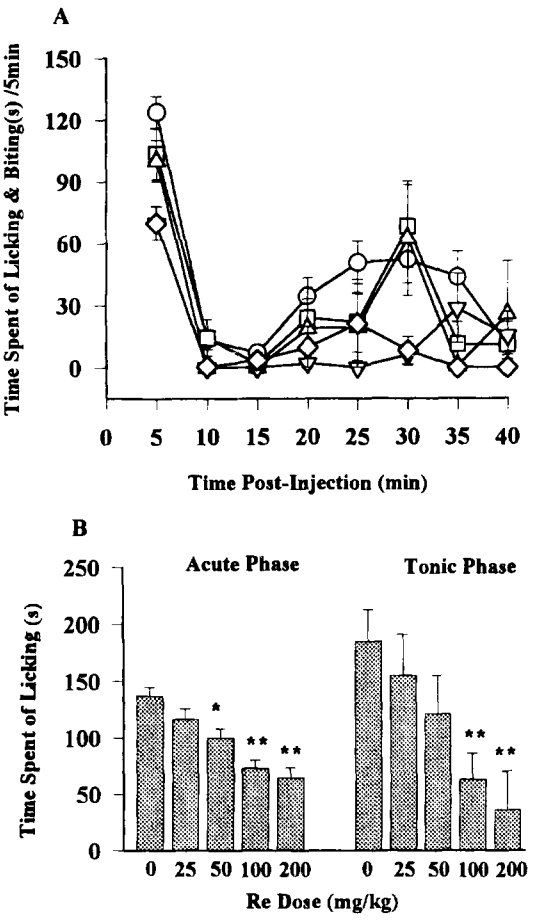

Fig. 4. The effect of ginsenoside Re on pain induced by 1% formalin. A. Con (○), 25 (□), 50 (△), 100 (▽), or 200 (◇ mg/kg of ginsenoside Re. Pain responses were measeured from immediately with 5 min block after intraplantar surface injection of 40 μl of 1% formalin. Pain responses are the time that spent licking and biting(s) of the injected hind paw or leg. Each value represents mean±SEM. B. These histograms show the first (0~10 min) and second phase (11~40 min) of pain responses following the injection of formalin after pretreatment with different doses of ginsenoside Re.
*p<0.05 or **p<0.01 compared to saline-injected controls (by ANOVA and Dunnett's procedure for multiple comparisons).

pain appears 11~40 min following short term period of interval. These two pains caused by formalin are different in their pain intensity and quality.[11] Pretreatment of each ginsenoside at dose of 25 mg/kg in the first phase of pain and 50 mg/kg in the second phase of pain did not attenuate pain induced by formalin. Ginsenoside Rc, Rd, and Re relieve slightly acute

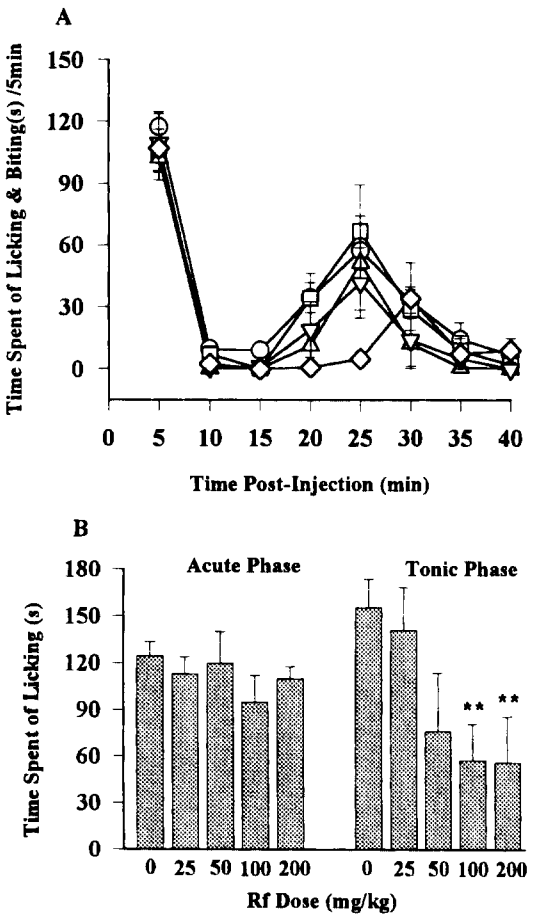

Fig. 5. The effect of ginsenoside Rf on pain induced by 1% formalin. A. Con (○), 25 (□), 50 (△), 100 (▽), or 200 (◇ mg/kg of ginsenoside Rf. Pain responses were measeured from immediately with 5 min block after intraplantar surface injection of 40 μl of 1% formalin. Pain responses are the time that spent licking and biting(s) of the injected hind paw or leg. Each value represents mean±SEM. B. These histograms show the first (0~10 min) and second phase (11~40 min) of pain responses following the injection of formalin after pretreatment with different doses of ginsenoside Rf.
*$p<0.05$ or **$p< 0.01$ compared to saline-injected controls (by ANOVA and Dunnett's procedure for multiple comparisons).

pain at dose over 50 mg/kg.

Interestingly, pretreatment of each ginsenoside at dose of 100 mg/kg inhibited strongly the second phase of pain, indicating that these ginsenosides exert their analgesic activity by inhibiting tonic pain rather than acute pain. Ginsenoside Rf did not show any analgesic effect at dose over even 200 mg/kg in first phase of pain (Fig. 5). Second phase of pain was relieved by pretreatment of ginsenoside Rf at dose over 100 mg/kg but % antinociception induced by ginsenoside Rf was less potent than that of other three ginsenosides tested. ED_{50} was 62 (42~90 mg/kg) for Rc, 45 (20.5~99 mg/kg) for Rd, 82 (48~139 mg/kg) for Re, and 92 (58~147 mg/kg) for Rf in second phase pain, respectively (Fig. 6). We tested whether dosages showing antinociception in formalin test relieve pain induced by noxious heat through tail-flick test. However, these ginsenosides did not show any antinociception (data not shown). We also tested whether analgesic efficacy induced by four gisenosdies is mediated via opioid receptor using opioid receptor antagonist naloxone but naloxone did not block the analgesic action of four ginsenosides (data not shown).

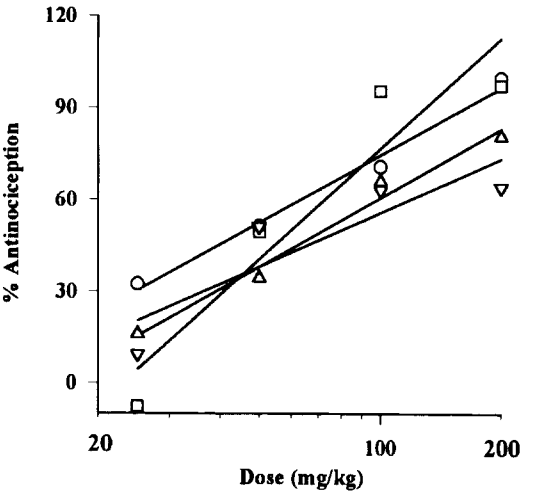

Fig. 6. Dose-dependent effects of ginsenoside Rc, Rd, Re, and Rf on 1% formalin-induced pain. Ginsenoside Rc (○), Rd (□), Re (△), or Rf (▽) was administrated with indicated dose. After 20 min, lickings and bitings were induced by intraplantar surface of hind paw injection of 1% formalin and counted for 40 min. Error bars were omitted in the dose response curve for sake of clarity.

Discussion

We demonstrated for the first time that ginsenoside such as ginsenoside Rc, Rd, Re, and Rf exerted antinociception in writhing and formalin test. We also used ginsenoside Rg_1 and Rb_1 but these two ginsenoside did not show analgesic activity at dose that other four ginsenosides induced antinociception in writhing test (data not shown). In further study using formalin we did not test Rb_1 or Rg_1 for its analgesic action, since these two ginsenosides having not analgesic activity in writhing test are probably not effective in formalin test, in which pain is much stronger than that of dilute acetic acid.[11]

We also observed that the values of ED_{50} from four ginsenosides obtained through writhing test was much lower than those obtained from formalin test, which means that less amounts of ginsenoside are needed to get analgesia in writhing test. This could be derived from the difference of pain intensity induced by acetic acid or formalin, since the administration of dilute formalin into under skin usually induce a persistent and stronger pain than that of acetic acid as mentioned above.[11]

Other four ginsenosides such as ginsenoside Rc, Rd, and Re except Rf relieved the first phase of pain but all these ginsenosides relieved the second phase of pain with dose-dependent manner as shown in figures 2-5. The order of analgesic potency was $Re > Rd \approx Rc > Rf$ in writhing test. The order of analgesic potency was $Rd > Rc > Re > Rf$ in formalin test. Interestingly, ginsenoside Re was most potent in writhing test but it appears that ginsenoside Rd was more potent than ginsenoside Re in formalin test. This discripancy is not yet clear but it could be derived from the site of pain induction and pain intensity. However, although four ginsenosides show analgesic activity in both algesiometric assays, these ginsenosides did not relieve pain induced by noxious heat, suggesting that ginseng saponins induce differential antinociception and these results are well consistent with previous report.[5] Interestingly, PD and PT saponins did not relieve the first phase of pain with even 200 mg/kg and only second phase of pain was attenuated.[5] In present study, ginsenoside Rc, Rd, and Re except Rf relieved first phase of pain. These results suggest that ginsenosides having not antinociception are probably contained in PD or PT saponins.[5] These results also suggest that the portion or ratio of ginsenoside(s) having antinociception could be an important factor for exerting analgesic action of ginseng saponins. For example, ginsenoside Rb_1 is one of PD saponins. The content of ginsenoside Rb_1 in PD saponins is usually higher than that of other minor PD saponins but Rb_1 had no analgesic effect in pain test. Ginsenoside Rg_1 is one of PT saponins. The content of ginsenoside Rg_1 in PT saponins is also higher than that of other PT saponins but Rg_1 also had no analgesic effect in pain test. Although we tested only six ginsenosides as mentioned above, we can not exclude the possibility that other ginsenoside(s) that we did not use in our experiments also play an important factor for relieving acute or tonic pain. The mechanism that ginsenoside(s) relieves chemogenic pains induced by chemicals such as acetic or formalin rather than thermal stimuli is not yet clear. However, recent studies show some evidences that ginseng saponins could exert their analgesic efficacy by acting on presynaptic site(s). For example, Nah and McCleskey (1994) reported at cellular level that ginseng saponins inhibit voltage-dependent Ca^{2+} channels on sensory neurons. The inhibition of voltage-dependent Ca^{2+} channels on sensory neurons by ginseng saponins provide one possible explanation of analgesic efficacy of ginseng saponins, since sensory neurons are involved in convey of sensory informations such as pain from peripheral nerve to central nervous system and voltage-dependent Ca^{2+} channels on sensory neuron also play an important role for the release of pain transmitters from afferent presynaptic nerve terminal into dorsal horn of spinal cord following peripheral stimulations such as formalin treatment.[12] Interestingly, these regulations of voltage-activated Ca^{2+} channels by ginseng saponins was

not mediated through opioid receptor.[12] Furthermore, Nah *et al.* (1995) also reported that ginsenoside Rf among several other ginsenosides such as Rb_1, Rc, Re, and Rg_1 exerts the inhibition of voltage-dependent Ca^{2+} channels on sensory neurons.[13]

In present study using experimental animals, both ginsenoside Rc and Re induce antinociception in writhing and formalin tests, although these two ginsenosides had no effects on voltage-dependent Ca^{2+} channels on sensory neurons.[13] These results show the possibilities that ginsenosides also act on postsynaptic site(s) as well as presynaptic site(s) to exert their analgesic effects. In fact, we recently found that ginseng saponins attenuate substance P(SP)-induced nociceptive behavior. Intrathecal(i.t.) administration of SP usually induce nociceptive behavior in rodents as noxious stimuli were applied to their body, since SP injected i.t. acts on postsynaptic site(s) of dorsal horn of spinal cord and transfers nociceptive information to sensory cortex to induce nociceptive behavior.[14,15] Interestingly, administration of ginseng saponins with SP through i.t. route blocked the effect of SP with dose-dependent manner.[16] Therefore, these results strongly support that ginseng saponins act on postsynaptic site(s) as well as presysnaptic site(s) in spinal cord level but not through opioid receptor. However, we can not exclude the possibility that the analgesic efficacy of ginseng saponins also could be achieved through other pathway(s).

In summary, we found that pretreatment of ginsenoside Rc, Rd, Re, or Rf through systemic administration relives mainly tonic pain rather than acute pain induced by formalin but spares noxious thermal pain.

요 약

앞 연구에서 본 연구실에서는 PD 사포닌과 PT 사포닌의 전 투여는 0.9% 초산과 1% 포르말린에 의하여 유도되는 통증을 억제하는 것으로 보고하였다. 그러나 뜨거운 열에 의하여 유도되는 통증은 억제되지 않은 것으로 나타났다. 본 연구에서는 여러 단일 진세노사이드 중 어느 진세노사이드가 앞에서 언급한 항통증 효능을 발휘하는가를 연구하였다. 본 연구를 위하여 진세노사이드 Rb_1, Rc, Rd, Re, Rf 및 Rg_1를 이용하였다. 이들 여섯가지 진세노사이드 중 진세노사이드 Rc, Rd, Re 및 Rf가 0.9% 초산과 1% 포르말린에 의하여 유도되는 통증을 억제하는 것으로 나타났다. 이들 네가지 진세노사이드에 의한 항통증 potency는 포르말린 테스트에서 Rd > Rc > Rf순이었다. 그러나 진세노사이드 Rb_1 과 Rg_1은 초산에 의하여 유도되는 통증을 억제하지 않은 것으로 나타났다. 흥미로운 것은, 포르말린 테스트에서 좋은 항통증 효능을 보여주는 진세노사이드들은 뜨거운 열에 의한 통증을 억제하지 않은 것으로 나타났다. 따라서 본 연구실에서는 인삼 사포닌이 특이적인 항통증 효능이 있다는 것을 보고하였고 또한 이러한 항통증작용에 기여하는 단일 진세노사이드가 진세노사이드 Rc, rd, Re 및 Rf라는 것을 발견하였다.

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