Antinociceptive Profiles of Crude Extract from Roots of Angelica gigas NAKAI in Various Pain Models

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To characterize the antinociceptive profiles of Angelica gigas NAKAI (ANG; Korean angelica), methanol extract from the dried roots of ANG was made and mice were administered orally at the various doses (from 0.25 to 3 g/kg). ANG produced the increased latencies of the tail-flick and hot-plate paw-licking responses in a dose-dependent manner. In acetic acid-induced writhing test, ANG dose-dependently decreased writhing numbers. Moreover, the cumulative response time of nociceptive behaviors induced by intraplantar formalin injection was reduced during both the 1st and the 2nd phases in a dose-dependent manner in ANG-treated mice. Furthermore, oral administration of ANG did not cause licking, scratching and biting responses induced by TNF-α (100 pg), IFN-γ (100 pg) or IL-1β (100 pg) injected intrathecally (i.t.), especially at higher dose (3 g/kg). Additionally, in ANG treated mice, the cumulative nociceptive response time for i.t. administration of substance P or capsaicin was dose-dependently diminished. Finally, nociceptive responses elicited by i.t. injection of glutamate (20 μg), N-methyl-D-aspartic acid (60 ng), α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (13 ng) or kainic acid (12 ng) were decreased by oral administration of ANG. Our results suggest that ANG produces antinociception via acting on the central nervous system and shows antinociceptive profiles in various pain models, especially inflammatory pain.

Key words Angelica gigas; antinociception; formalin test; inflammatory pain; central nervous system

The dried roots Angelica gigas NAKAI (ANG; Umbelliferae), which is known as Korean angelica, are used in oriental traditional medicine as a treatment regimen for anemia or some circulatory disorders. Previously, it has been reported that ANG has some pharmacological effects, for example, depression of cardiac contraction, decreased effects on the carotid arterial pressure and decrease of the respiration.1) Several recent reports have shown that ANG have anticancer activity;2) immuno-stimulatory effects3) and acetylcholinesterase inhibitory effect.4) Additionally, some authors have revealed that another specy of angelica, Angelica acutiloba Kitakawa (Japanese angelica), has analgesic and anti-inflammatory effects.5,6) Since the chemical components of angelica species are quite different7) and there is little known antinociceptive effect of ANG, we attempted to characterize their antinociceptive profiles using various nociceptive pain models.

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 (25—30 g) from MJ LTD. (Seoul, Korea) were used for all experiments. The animals were housed 5 cage in a room maintained at 22±0.5°C with an alternating 12 h light–dark cycle. Food and water were available ad libitum. Each mouse was used only once.

Preparation of Crude Extract from Roots of ANG

The dried roots of ANG was broken into pieces. And then, crude extract was prepared as follow. The mixture of broken roots of ANG with 70% methanol at 100°C (5 ml/g of roots) was made and the temperature was maintained for 3 h. After cooling at the room temperature, the mixture was filtered thorough Whatman No. 1 filter paper. The filtered methanol extract was concentrated using rotary evaporator system (BÜCHI Labortechnik AG, Flawil, Swiss, Rotavapor R-124). The yield of methanol extract from roots of ANG corresponded to 25.93% of the original dry weight. The yellowish extract was dissolved in the 0.9% saline just before use.

Oral Administration and Intrathecal (i.t.) Injection

Oral administration was performed with gavage in a volume of 20 mL/kg body weight. The i.t. injection followed the method described by Hylden and Wilcox8) using 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 with 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 were done.

Tail-Flick and Hot-Plate Tests

At first, antinociception was determined by the tail-flick test9) and the hot-plate paw-licking test.10) 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. For the hot-plate test, mice were individually placed on the hot-plate (55°C) and the reaction time starting from the placement of the mouse on the hot-plate to the time of licking the front-paw was measured. The dimensions of the hot-plate apparatus were 30×30×30 cm (Itic Life Sci-
ence, Woodland Hills, CA, U.S.A., Model 39 Hot Plate). Control latencies for the hot-plate test were approximately 9 s. The tail-flick or hot-plate paw-licking latencies were measured before (T₀) and after (Tₜ) the treatment of crude extract from roots of ANG. Inhibition of the tail-flick and hot-plate paw-licking responses were expressed as “percent of maximal possible effect (% MPE)” which was calculated as \( [(T₀−Tₜ)/(T₀−T₀)] \times 100 \), where the cut-off time (T₀) was set at 10 and 30 s for the tail-flick and hot-plate tests, respectively.

**Acetic Acid-Induced Writhing and Intraplantar Formalin Tests** For the writhing test, mouse was administered intraperitoneally with 0.5 ml of 1% acetic acid dissolved in 0.9% saline. The number of writhes was counted during a 30 min period following the injection of acetic acid. A writh was defined as a contraction of the abdominal muscles accompanied by an extension of the forelimbs and elongation of the body. For the formalin test, 10 µl of 1.0% formalin solution, made up in 0.9% saline, was injected subcutaneously under the plantar surface of the left hindpaw. Two groups of mice (control and treated) were observed simultaneously from 0 to 40 min following formalin injection. The early phase of the nociceptive response normally peaked 0 to 5 min after formalin injection and the late phase 20 to 40 min after formalin injection, representing the direct effect on nociceptors and inflammatory nociceptive responses, respectively. Following intraplantar injection of formalin, the animals were immediately placed in an acrylic observation chamber (20 cm high, 20 cm diameter), and the time spent licking/flinching and biting the injected paw was measured with a stop-watch timer and considered as indicative of nociception.

**Pro-inflammatory Cytokine-Induced Nociceptive Test** Mice were acclimated in an observation chamber for at least 30 min before the injection of pro-inflammatory cytokines, TNF-α (100 pg), IFN-γ (100 pg) and IL-1β (100 pg). Immediately after the i.t. injection with pro-inflammatory cytokines, the mice were placed in an observation chamber and their nociceptive behavioral responses were recorded for 30 min. The cumulative response time(s) of licking, scratching and biting episodes directed toward the lumbar and caudal region of spinal cord were measured.

**Substance P- and Capsaicin-Induced Nociceptive Tests** Mice were acclimated in an observation chamber for at least 30 min before the injection of substance P (0.7 µg) or capsaicin (0.5 µg). Immediately after the i.t. injection with capsaicin or substance P, the mice were placed in an observation chamber and its behavioral response was recorded for 30 min. The cumulative response time(s) of licking, scratching and biting episodes directed toward the lumbar and caudal region of spinal cord were measured.

**Excitatory Amino Acids-Induced Nociceptive Test** Mice were acclimated in an observation chamber for at least 30 min before the injection of glutamate (20 µg), N-methyl-D-aspartic acid (NMDA; 60 ng), α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA; 13 ng) and kainic acid (KA; 12 ng). Immediately after the i.t. injection with excitatory amino acids, the mice were placed in an observation chamber and their behavioral response was recorded for 30 min. The cumulative response time(s) of licking, scratching and biting episodes directed toward the lumbar and caudal region of spinal cord were measured.

**Rotarod Test** To confirm the antinociceptive effects of ANG, which was not be associated with motor dysfunction or paralysis, the rotarod test was performed. The apparatus consisted of a bar, with a diameter of 2.5 cm, subdivided into six compartments by disks 25 cm in diameter (Ugo Basile, Italy). The bar rotated at a constant speed of 14 revolution/min. Mice were treated orally with saline or ANG at the maximal dose (3 g/kg) used in the present study. After 30 min ANG treatment, the rotarod test was conducted. The time that they remained on rotating bar (maximum of 60 s) was recorded.

**Statistical Analysis** The data were presented as the mean±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** Acetic acid, formalin, substance P, capsaicin, glutamate and kainic acid were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). NMDA and AMPA were purchased from Research Biomedicals Inc. (Natick, MA, U.S.A.). TNF-α, IFN-γ and IL-1β were purchased from R&D Systems Inc. (Minneapolis, MN, U.S.A.). All drugs were dissolved in sterile normal saline (0.9% w/v of NaCl). All drugs were prepared just before use.

**RESULTS**

**Effects of ANG Administered Orally on the Tail-Flick and Hot-Plate Paw-Licking Responses** Oral treatment of ANG at the dose of 3 g/kg produced the significant inhibition of the tail-flick response, which reached a peak at 30 min after the administration (12±3, 53±5 and 41±3 % MPE, for 10, 30 and 60 min, respectively). Similar pattern was observed in the hot-plate test (11±4, 71±6 and 49±4 % MPE, for 10, 30 and 60 min, respectively).

Mice were treated orally with crude extract from roots of ANG at the various doses (from 0.25 to 3 g/kg) and the tail-flick and hot-plate tests was performed 30 min after ANG administration. As revealed in Fig. 1, ANG produced the increased latencies (% MPE) of the tail-flick and hot-plate paw-licking responses in a dose-dependent manner. At the maximal dose (3 g/kg) used in the present study, no animal showed paralysis or motor function defect 30 min after oral administration of ANG (saline 57.9±2.0 vs. ANG 57.4±1.2 s in rotarod performance, n=8—10).

**Effects of ANG Administered Orally on the Acetic Acid-induced Writhing and Intraplantar Formalin Responses** After noxious visceral stimulation induced by 1% acetic acid intraperitoneally, mice showed the increased number of writhing response. In ANG (from 0.25 to 3 g/kg)-treated mice, writhing number reduced by acetic acid was diminished as compared with saline-treated control mice in a dose-dependent manner (Fig. 2a).

Subcutaneous injection of 1% formalin into plantar aspect of the hindpaw after saline treated control group caused an acute, immediate nociceptive formalin response, i.e., licking/flinching and biting the injected paw, which lasted for 5 min (1st phase response). The 2nd phase formalin response began about 20 min after formalin administration and lasted for about 20 min (20—40 min after formalin injection). In ANG
from 0.25 to 3 g/kg)-treated mice, the cumulative response time of nociceptive behaviors induced by intraplantar injection of formalin was decreased as compared with control mice during both the 1st and the 2nd phases in a dose-dependent manner (Fig. 2b, c). Notably, the effect of ANG was more prominent during the 2nd tonic inflammatory phase (Fig. 2b).

**Effects of ANG Administered Orally on the Nociceptive Behaviors Induced by i.t. Pro-inflammatory Cytokines**

In saline-treated control mice, i.t. injection of TNF-α (100 pg), IFN-γ (100 pg) and IL-1β (100 pg) caused an acute, immediate behavioral response, i.e., licking, scratching and biting, which lasted about 30 min. As shown in Fig. 3, in ANG (from 0.25 to 3 g/kg)-pretreated mice, the cumulative response time of nociceptive behaviors induced by i.t. cytokines was reduced. Pro-inflammatory cytokine-induced nociceptive response was completely blocked at higher dose (3 g/kg), although the antinociceptive effect of ANG showed dose-dependent pattern.

**Effects of ANG Administered Orally on the Nociceptive Behaviors Induced by i.t. Substance P and Capsaicin**

In saline-treated control mice, i.t. injection of substance P (0.7 μg) or capsaicin (0.5 μg) caused an acute, immediate behavioral response, i.e., licking, scratching and biting, which lasted about 30 min. As shown in Fig. 4, in ANG (from 0.25 to 3 g/kg) treated mice, the cumulative nociceptive response time for i.t. administration of substance P or capsaicin was significantly and dose-dependently diminished.

**Effects of ANG Administered Orally on the Nociceptive Behaviors Induced by i.t. Excitatory Amino Acids**

In saline-treated control mice, i.t. injection of glutamate (20 μg), NMDA (60 ng), AMPA (13 ng) and KA (12 ng) caused an acute, immediate behavioral response, i.e., licking, scratching and biting, which lasted about 30 min. As shown in Fig. 5, in ANG (from 0.25 to 3 g/kg) treated mice, the cumulative nociceptive response time for i.t. administration of excitatory amino acids was significantly and dose-dependently diminished.

**DISCUSSION**

In the present study, we found that ANG administered...
orally produced antinociceptive effect in various pain models and its antinociceptive effect was dose-dependent. In the tail-flick and hot-plate tests, ANG treated orally showed an antinociceptive effect on the acute noxious thermal stimulation. The tail-flick response has been believed to be a spinally mediated reflex and the paw-licking hot-plate response is a more complex supraspinally organized behavior.15) Moreover, Grumbach16) have shown that the effectiveness of analgesic agents in the tail-flick pain model is highly correlated with relief of human pain. Our results demonstrate that ANG has the ability to prolong response latencies indicating the increase of nociceptive threshold.

To investigate the effect of ANG in the tonic inflammatory pain models, we also performed the acetic acid-induced writhing and intraplantar formalin tests after oral administration of ANG. Intraperitoneal injection of acetic acid produced the peritoneal inflammation (acute peritonitis), which caused a response characterized by contraction of the abdominal muscles accompanying an extension of the forelimbs and elongation of the body. This writhing response has been considered a visceral inflammatory pain model.11,17) The ANG treatment diminished the number of writhing responses in a dose-dependent manner in acetic acid-induced visceral nociception. Furthermore, we observed that ANG-treated mice showed the decreased nociceptive behaviors induced by intraplantar formalin during both the 1st and the 2nd phases in a dose-dependent manner. The acute 1st phase of the nociceptive response in the mouse formalin test lasts for about 5 min after formalin injection and is followed by the tonic 2nd phase which is persistent from 20 to 40 min after formalin injection.12,18,19) It is widely agreed that the 1st and 2nd phases result from the direct effect on nociceptors activating primary afferent fiber and the tonic inflammatory nociceptive responses, respectively.13,20—22) Notably, the effect of ANG was more prominent during the 2nd tonic inflammatory phase as well as the 1st phase. Shibata et al.23) have reported that peripherally acting drugs such as aspirin and glucocorticoid only inhibited the 2nd phase in the formalin test. In contrast, aminopyrine and mefenamic acid, which acted on both central and peripheral sites, inhibited both phases. Therefore, our results about antinociceptive action of ANG implicate that ANG may be more effective on the tonic inflammatory pain and it can probably act on central nervous system when administered systemically. Recently, it has been known that

Fig. 3. Effects of Crude Extract from Roots of Angelica gigas NAKAI on the Nociceptive Response Induced by Proinflammatory Cytokines

Animals were treated orally with crude extract from roots of Angelica gigas NAKAI at various doses (from 0.25 to 3 g/kg) for 30 min prior to the TNF-α (a; 100 pg per 5 µl), IFN-γ (b; 100 pg per 5 µl) or IL-1β (c; 100 pg per 5 µl) injection intrathecally (i.t.). The cumulative response time of licking, scratching and biting episodes was measured for 30 min. The vertical bars denote the standard error of the mean. The number of animals used for each group was 8—10. *p<0.05 compared to the saline-treated control group of mice.

Fig. 4. Effects of Crude Extract from Roots of Angelica gigas NAKAI on the Substance P- and Capsaicin-Induced Nociceptive Response

Animals were pretreated orally with crude extract from roots of Angelica gigas NAKAI at various doses (from 0.25 to 3 g/kg) for 30 min prior to the substance P (a; 0.7 µg per 5 µl) or capsaicin (b; 0.5 µg per 5 µl) injection intrathecally (i.t.). The cumulative response time of licking, scratching and biting episodes was measured for 30 min. The vertical bars denote the standard error of the mean. The number of animals used for each group was 8—10. *p<0.05 compared to the saline-treated control group of mice.
Proinflammatory cytokines may play an important role in the development of inflammatory pain or hyperalgesia in the spinal cord. We found that the i.t. injection of TNF-α, IFN-γ and IL-1β did not cause nociceptive behavior in ANG-treated mice suggesting ANG also have analgesic potential in the inflammatory pain model.

It has been reported that i.t. injections of substance P and capsaicin in mice elicit behavioral responses similar to that caused by noxious stimulation, which nociceptive responses consist of biting, scratching and licking the caudal parts of the body. Furthermore, several lines of evidence have demonstrated that i.t. injection of excitatory amino acids cause the hyperalgesic response in the hot-plate test. In addition, iontophoretic application of excitatory amino acids show that the responses to noxious heat and pinch and innocuous tap stimuli are enhanced. We, in the present study, demonstrated that the cumulative nociceptive response time for i.t. administration of substance P or capsaicin was significantly and dose-dependently diminished in ANG-treated mice. In addition, ANG-treated mice were observed antinociceptive effects on the i.t. injection of excitatory amino acids, such as glutamate, NMDA, AMPA and KA. These results suggest that ANG administered systemically may exert their antinociceptive effect on the central sites, especially spinally mediated mechanisms. The present study indicates that unknown single compound or certain combinations of ANG have a possibility to be developed into new analgesic drugs.

Currently, antinociceptive effect of these single compounds have been examined in our laboratory.

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