Antinociceptive Effect of Amygdalin Isolated from *Prunus armeniaca* on Formalin-Induced Pain in Rats

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Amygdalin is a plant glucoside isolated from the stones of rosaceous fruits, such as apricots, peaches, almonds, cherries, and plums. To investigate the pain-relieving activity of amygdalin, we induced pain in rats through intraplantar injection of formalin, and evaluated the antinociceptive effect of amygdalin at doses of 0.1, 0.5, 1.0, and 10.0 mg/kg-body weight by observing nociceptive behavior such as licking, biting and shaking, the number of Fos-immunoreactive neurons in the spinal cord, and the mRNA expression of inflammatory cytokines in the plantar skin. The intramuscular injection of amygdalin significantly reduced the formalin-induced tonic pain in both early (the initial 10 min after formalin injection) and late phases (10—30 min following the initial formalin injection). During the late phase, amygdalin did reduce the formalin-induced pain in a dose-dependent manner in a dose range less than 1 mg/kg. Molecular analysis targeting c-Fos and inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β) also showed a significant effect of amygdalin, which matched the results of the behavioral pain analysis. These results suggest that amygdalin is effective at alleviating inflammatory pain and that it can be used as an analgesic with anti-nociceptive and anti-inflammatory activities.
reported the participation of TNF-α and IL-1β in nociceptive responses induced by various stimuli. This study evaluated the antinociceptive effect of amygdalin in the rat formalin test by analyzing formalin-inducing licking, biting, and shaking behaviors. The pain-relieving effect of amygdalin was confirmed by the decreases in c-Fos expression in the rat superficial dorsal horn, and the expression of TNF-α and IL-1β mRNAs in the rat paw skin.

MATERIALS AND METHODS

Animals Male Sprague–Dawley rats weighing 230—250 g were used for this experiment. The animals were purchased from Samtaco (Kyungki-do, Korea). They were kept in a controlled environment (20±2°C, 12 h/12 h light–dark) for at least 1 week before the study. Food and water were available ad libitum. In addition, all the rats were habituated to the formalin test chambers and handled with care to minimize stress. All methods used in the present study were approved by the Animal Care and Use Committee of Kyunghee University. All procedures were conducted in accordance with the “Guide for the Care and Use of Laboratory Animals,” published by the Korean National Institute of Health.

Preparation of Amygdalin from Apricot Seeds The amygdalin was generously supplied by Prof. Hong’s research group in the Department of Oriental Pharmaceutical Science, College of Pharmacy, Kyung Hee University, Seoul, Korea, as a powder. The purity of the amygdalin was confirmed to exceed 95.0% using high-pressure liquid chromatography. The powder form of amygdalin was manufactured using the following protocol. First, 500 g of armeniacae semen were macerated from the seed and 100 l of 4% citric acid solution were refluxed for 2 h. After filtering it when it was still hot, the filtrate was passed through a column packed with HP-20. The substance absorbed within the column was concentrated after it had been eluted by ethanol. Recrystallizing the extracts with ethanol gave 4.2 g of amygdalin (0.84% yield). The fixed amount of amygdalin powder was dissolved in saline solution for further experiments.

Experimental Groups The experimental groups used in this study were as follows: normal group without treatment (NOR, n=8), formalin-injected group without treatment (FOR, n=8), formalin+saline-injected group as a vehicle control (SAL, n=8), formalin+0.1 mg/kg of amygdalin-injected group (AMY-0.1, n=8), formalin+0.5 mg/kg of amygdalin-injected group (AMY-0.5, n=8), formalin+1 mg/kg of amygdalin-injected group (AMY-1, n=8), formalin+10 mg/kg of amygdalin-injected group (AMY-10, n=8), and 5 mg/kg of indomethacin-injected group (INDO, n=7). NOR group as a control was appended to the experiments of amygdalin-injected group (AMY -0.1, 0.5, 1.0, 10 mg/kg), or saline as a vehicle control. No restraint was applied to the rats during the behavioral observations.

Histological Examination One hour after formalin injection, all of the animals were anesthetized with sodium pentobarbital (80 mg/kg, i.p.) and perfused through the left ventricle with normal saline (0.9%), followed by 300 ml (per rat) of 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). The spinal cords were removed, post-fixed over-night, and cryoprotected with 30% sucrose in 0.1 M PBS at 4°C.

c-Fos Immunohistochemistry The specimens were sectioned on a cryostat in 40-μm coronal sections between L3 and L5. The sections were immunostained for Fos protein using the avidin–biotin–peroxidase method. The tissues were rinsed in PBST (PBS plus 0.05% Tween 20) three times before use. The primary rabbit anti-c-Fos polyclonal antibodies (1:5000) for c-Fos immunohistochemistry were purchased from Abcam (Cambridge, U.K.). The primary antibody was diluted with blocking solution (Vector Laboratories, Burlingame, CA, U.S.A.) and the tissues were incubated for 48 h at room temperature with constant agitation. After rinsing in PBS, the sections were incubated for 2 h at room temperature in the biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA, U.S.A.), diluted 1:200 in PBST containing 1% normal goat serum. The sections were placed in Vectastain™ Elite ABC reagent (Vector Laboratories, Burlingame, CA, U.S.A.) for 1 h at room temperature. After further rinsing in PBS, the tissues were developed using diaminobenzidine as the chromogen with nickel intensification. These slides were then air-dried and coverslipped for microscopic observation. All slides were examined under a light microscope (Carl Zeiss, Oberkochen, Germany). The superficial layers (superficial dorsal horn and lamina I and II) of the rat dorsal spinal cords were examined to assess the effect of the formalin injection on c-Fos expression in spinal neurons. The number of Fos-immunoreactive cells was counted at 100× magnification using a microscope with a grid measuring 100×100 μm. For counting spinal cord, the grid was placed on lamina I and II of superficial dorsal horn. Fos-immunoreactive cells were counted in three or four sections from each region in all spinal cords.

RT-PCR Analysis of TNF-α and IL-1β mRNAs One hour after formalin injection, the rats were killed and the paw skins were harvested by using a surgical scissor. The skin samples were immediately snap-frozen in liquid nitrogen, and stored at −80°C until use. Total RNA was extracted from the skin samples of rats using TRIzol™ (Invitrogen
RESULTS

was further confirmation. The criterion for statistical significance of variance (ANOVA) followed by the Tukey post hoc test for differences was chosen to show Fos immunohistochemistry. As shown in Fig. 2, many dark Fos-positive spots were observed in the superficial layers of the rat spinal cord (Rexed’s laminae I–II). Among the experimental groups in this study, three different groups, treated with 0.1, 1.0 and 10 mg/kg, was chosen to show Fos immunohistochemistry. As shown in Fig. 2, many dark Fos-positive spots were observed in the superficial layers of the dorsal horn. At a dose of 1 mg/kg, the intramuscular injection of amygdalin inhibited formalin-induced Fos expression in the spinal cord by 36%, while the pretreatment with other doses of amygdalin did not effectively suppress the formalin-induced Fos expression in the same area of the spinal cord. We found 219.5 ± 13.48, 222.5 ± 10.79, 194.7 ± 14.05, 142.3 ± 14.30, 243.83 ± 20.37, and 132.75 ± 6.9 Fos-immunoreactive neurons in the superficial layers of the dorsal horn. The AMY-0.1 group showed slight nonsignificant reductions in c-Fos expression compared to the FOR group. In AMY-0.1 group, the suppression of the formalin-induced Fos expression by amygdalin was comparable to that by indomethacin.

RT-PCR Analysis of TNF-α and IL-1β mRNAs in Paw Skin To determine the anti-inflammatory effect of amygdalin, we also examined the mRNA expression levels of TNF-α and IL-1β in paw skin injected with formalin in the presence and absence of amygdalin. The RT-PCR results were used to compare TNF-α and IL-1β mRNA expression at amygdalin dose of 1 mg/kg-body weight, recognized as the optimum from the previous results (Figs. 4, 5). Amygdalin
treatment significantly inhibited the expression of TNF-\( \alpha \) and IL-1\( \beta \) mRNAs in paw skin tissue, compared to the saline treatment as a vehicle control. And the inhibition levels were comparable to that by indomethacin as a positive control. Based on the RT-PCR analysis, the TNF-\( \alpha \) and IL-1\( \beta \) mRNA expression levels in skin tissue of rat paw in AMY group, treated with 1 mg/kg-body weight, was 53% and 64% less than that in SAL group without amygdalin treatment, respectively.

**DISCUSSION**

This study investigated the antinociceptive activity of amygdalin prepared from an aqueous extract of armeniacae semen in rats with formalin-induced pain. Amygdalin is a compound containing a cyanogenic glycoside found in the pits of many fruits and plants in the Rosaceae family. It consists of two molecules of glucose units, one is benzaldehyde and the other is hydrocyanic acid (HCN). As the metabolites of amygdalin, both molecules may possess anti-neoplastic properties which are believed to be an active anticancer ingredient. Amygdalin has been used widely to treat various

![Fig. 2. Representative Microphotographs of Coronal Sections Showing c-Fos Expression in the Spinal Cord](image)

Photomicrographs (200×) showing c-Fos immunoreactive neurons in the rat superficial dorsal horn (SDH) at levels L3—5. The arrows indicate c-Fos immunoreactive cells and dotted lines the boundaries of laminae I and II. Indomethacin was used as a positive control. (A) FOR, (B) SAL, (C) INDO, (D) AMY-0.1, (E) AMY-1 and (F) AMY-10 groups.

![Fig. 3. The Number of c-Fos Immunoreactive Cells in the Spinal Cord](image)

Indomethacin (INDO) was used as a positive control. The data were analyzed using one-way ANOVA and confirmed using the Tukey post hoc test (*\( p < 0.05 \) compared to SAL group).

![Fig. 4. Effect of Amygdalin on mRNA Expression of TNF-\( \alpha \) in the Rat Paw Skin](image)

NOR means normal rat without any treatments including formalin, saline or amygdalin injection. Indomethacin (INDO) was used as a positive control. Values are means with standard deviations from at least three independent experiments. The PCR products were normalized to GAPDH, a housekeeping gene (**\( p < 0.005 \) compared to SAL group).

![Fig. 5. Effect of Amygdalin on mRNA Expression of IL-1\( \beta \) in the Rat Paw Skin](image)

NOR means normal rat without any treatments including formalin, saline or amygdalin injection. Indomethacin (INDO) was used as a positive control. Values are means with standard deviations from at least three independent experiments. The PCR products were normalized to GAPDH, a housekeeping gene (**\( p < 0.005 \) compared to SAL group).
cancers. In particular, α-amylgladin selectively kills cancer cells. However, the anticancer activity of α-amylgladin is controversial, and α-amylgladin has not been approved by the FDA for medicinal use in the United States due to insufficient evidence of its efficacy and its potential toxicity. Even though there have been little reports to imply the mechanism of antinociceptive activity of α-amylgladin, we just noted that the chemical structure of α-amylgladin includes a benzaldehyde unit, which seems to play a key role in antinociceptive action of α-amylgladin. Recently, it was reported that α-amylgladin has anti-inflammatory and antinociceptive effects in mouse BV2 microglial cells.  

In the rat formalin test, the early (0–10 min) phase of formalin-induced pain behavior is produced by the direct activation of C-fiber primary afferent nociceptors, while the pain behaviors associated with the late (10–30 min) phase are related to the sensitization of dorsal horn neurons due to the initial barrage of primary afferent input during the early phase or the formalin-induced inflammatory reaction. Therefore, this behavioral test can be used to verify the antinociceptive effect of a test compound and to investigate its mechanism. Drugs affecting the central nervous system, such as opiates, inhibit the pain responses of both phases in the rat formalin test. In contrast, peripherally acting drugs, such as cyclooxygenase inhibitors (e.g., aspirin and indomethacin) and corticosteroids, inhibit the hypersensitivity to von Frey hair’s stimulus in the late phase. Indomethacin was used as a positive control in this study. As shown in Fig. 1, it seems to inhibit nociceptive behavior in the early phases even though it was not statistically significant. The antinociceptive activity of α-amylgladin was significantly observed in the early phase as well as in the late phase, even though the effect is more remarkable and dose-dependent in the late phase than in the early phase. In the previous thought describing the different mechanism of formalin-induced pain between the early and late phases in the formalin test, it would be explainable that α-amylgladin exerted to alleviate both nociceptive and inflammatory pain. However, in the previous reports on formalin-evoked responses in C-fibers in the rat formalin test, the increased C-fiber activity by formalin injection contributes to the pain response in the late phase, as well as in the early phase. It implies that formalin elicits the biphasic firing of C-fiber primary afferent nociceptors and the formalin-evoked behavior may be much more dependent on C-fiber primary afferent input than previous thought. We therefore postulated that the pain-relieving effect of α-amylgladin results from action on C-fiber primary peripheral nervous system which shows biphasic pattern in the rat formalin test. In this study, the α-amylgladin treatment at doses of 0.5 and 1.0 mg/kg significantly suppressed the formalin-induced paw licking behavior, especially in the late phase. In contrast, an α-amylgladin dose of 10 mg/kg did not reduce the formalin-induced paw-licking time during in the late or early phase (Fig. 1). With the lower doses to 1.0 mg/kg, the reduced antinociceptive activity of α-amylgladin was dose-dependent. With the higher dose of 10 mg/kg, the reason for the masking of pain-relieving activity of α-amylgladin in terms of nociceptive behavior has not been identified as yet. It might be in association with α-amylgladin poisoning at low concentration. The toxicity of α-amylgladin is to be dependent on the route of administration. Oral administration is associated with much more toxicity than intravenous, intraperitoneal, or intramuscular injection since most mammalian cells contain only trace amount of β-glucosidase which is responsible for breaking orally administered α-amylgladin and producing cyanide. The enzyme is present in gastrointestinal tract bacteria and in various food plants. General symptoms following cyanide poisoning include sudden, severe vomiting and epigastric pain followed by syncope, lethargy, coma, seizures, nausea and vomiting, headache, dizziness, mental obtundation, dermatitis, etc.) 

It has been reported that peripheral noxious stimulation increased Fos expression in the corresponding region of the spinal cord. As an immediate early (IE) gene, c-Fos is expressed extensively in the central nervous system, including the brain and spinal cord, on inducing noxious stimulation via nociceptors. Formalin injection into the plantar surface of the hind paw dramatically increases spinal Fos expression, while the intracerebroventricular injection of morphine dose-dependently reduces it. Analgesic drugs such as dexamethasone and aspirin also lower spinal Fos expression induced by nociceptive stimuli. In Fos expression in the spinal cord, the superficial dorsal horn, which is involved in nociceptive signaling, is the most important area in the formalin-induced pain behavior. In this study, we confirmed that formalin injection markedly stimulated Fos expression in the spinal cord, and the intramuscular injection of the optimal dose (1 mg/kg) of α-amylgladin significantly suppressed the Fos expression, as shown in Figs. 2 and 3. It was not confirmed if saline instead of formaldehyde could induce a substantial number of c-Fos expression in this study. In the rat formalin test, a dilute solution (2–10%) of formalin (37% formaldehyde solution) and saline is injected under the skin of a rats paw to induce tonic pain. It means that saline solution also contributes to induce a certain level of pain as well as formalin. Furthermore, commercially available formalin solution generally contains methanol as a preservative. Therefore, there is a possibility that methanol might act as an active agent in the test. In 1990, Wheeler-Aceto et al. performed a comparison between several possible irritants such as acetic, acid, carrageenan, formalin, kaolin, platelet-activating factor, mustard oil, serotonin, yeast, etc. for use as stimuli in a behavioral nociceptive test. Among them, it was concluded that subcutaneous injection of diluted formalin produced a reproducible persistent pain behavior, the response was biphasic and the duration was well suited for a nociceptive test. It is thus possible that intraplantar injection of 50 μL of saline instead of formalin can produce a certain level of c-Fos expression in the spinal cord. However, the important fact is that, irrespective of what component is the major factor to induce tonic pain, a diluted solution of formaldehyde, containing formaldehyde, saline and some methanol, can produce a persistent pain, and it was optimized for studying a clinical pain.

Pro-inflammatory cytokines such as TNF-α and IL-1β play an important role in the pathogenesis of autoimmune and inflammatory disorders, including arthritis. These signaling agents can induce self-expression or the expression of other inflammatory mediators once they are activated. Inflammatory stimuli such as lipopolysaccharide (LPS), carrageenan, and mitogen generally induce the expression of...
TNF-α and IL-1β.47 Consistent with the roles of TNF-α and IL-1β in inflammatory hyperalgesia, the expression of these cytokines in paw skin increased significantly after the injection of carrageenan or LPS.25,48,49 In this study, we verified that the amygdalin pretreatment significantly inhibited the hypersensitive pain response, induced by the subcutaneous injection of formalin into the hind paw skin. Moreover, amygdalin suppressed the expression of TNF-α and IL-1β mRNAs in the hind paw skin. The results suggest that the inhibitory effect of amygdalin can be attributed to transcriptional suppression of the mRNA expression of proinflammatory cytokines, which is closely related to the induction of inflammatory pain in the skin of the hind paw induced by formalin injection. Based on these results, amygdalin might be effective at alleviating inflammatory pain and could be used as an analgesic based on its anti-nociceptive and anti-inflammatory properties.

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