Inhibitory Effect of Amygdalin on Lipopolysaccharide-Inducible TNF-α and IL-1β mRNA Expression and Carrageenan-Induced Rat Arthritis

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Amygdalin is a cyanogenic glycoside plant compound found in the seeds of rosaceous stone fruits. We evaluated the anti-inflammatory and analgesic activities of amygdalin, using an in vitro lipopolysaccharide (LPS)-induced cell line and a rat model with carrageenan-induced ankle arthritis. One mM amygdalin significantly inhibited the expression of TNF-α and IL-1β mRNAs in LPS-treated RAW 264.7 cells. Amygdalin (0.005, 0.05, and 0.1 mg/kg) was intramuscularly injected immediately after the induction of carrageenan-induced arthritic pain in rats, and the anti-arthritic effect of amygdalin was assessed by measuring the weight distribution ratio of the bearing forces of both feet and the ankle circumference, and by analyzing the expression levels of three molecular markers of pain and inflammation (c-Fos, TNF-α, and IL-1β) in the spinal cord. The hyperalgesia of the arthritic ankle was alleviated most significantly by the injection of 0.005 mg/kg amygdalin. At this dosage, the expressions of c-Fos, TNF-α, and IL-1β in the spinal cord were significantly inhibited. However, at dosage greater than 0.005 mg/kg, the pain-relieving effect of amygdalin was not observed. Thus, amygdalin treatment effectively alleviated responses to LPS-treatment in RAW 264.7 cells and carrageenan-induced arthritis in rats, and may serve as an analgesic for relieving inflammatory pain.

Keywords: Amygdalin, arthritis, inflammation, pain, c-Fos, TNF-α, IL-1β

Amygdalin (α-mandelonitrile-β-gentiobioside) is a major component of the seeds of prunasin family plants, such as apricots, almonds, peaches, apples, and other rosaceous plants [8]. It has been used as a traditional drug because of its wide range of medicinal benefits, including curing or preventing cancer, relieving fever, suppressing cough, and quenching thirst. In the late 1970s and early 1980s, amygdalin was reported to selectively kill cancer cells at the tumor site without systemic toxicity and to effectively relieve pain in cancer patients [7]. However, the Food and Drug Administration (FDA) has not approved amygdalin as a cancer treatment owing to insufficient clinical evidence of its efficacy and potential toxicity [14]. Despite the failure of clinical tests to demonstrate the anticancer effects of amygdalin in the U.S. and in Europe, amygdalin continues to be manufactured and administered as an anticancer therapy in northern Europe and Mexico, and few studies have investigated its other pharmacological effects. Recently, Chang et al. [4] reported the effect of Armeriaceae semen extract on the lipopolysaccharide (LPS)-stimulated expression of cyclooxygenase-I and -II and inducible nitric oxide synthase in mouse BV2 microglial cells, based on the results of a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, reverse transcription-polymerase chain reaction (RT-PCR) analysis, Western blot analysis, prostaglandin E₂ immunoassay, and nitric oxide detection.

In the present study, carrageenan-induced arthritis was used as a model of arthritis pain for assessing the pain-relieving effect of amygdalin. Arthritis, a term originated from the Greek word for joint, is a multifactorial disease that is induced when the immune system is attacked and the body’s joints start to be degraded. Although arthritic diseases are not a primary cause of death, a disorder of senorimotor function resulting from arthritis can seriously affect the quality of a patient’s life. Arthritic pain can interfere with the social life of those afflicted and can produce feelings of hopelessness and depression. Therefore, the development of new therapies for alleviating arthritic pain is important in the treatment of arthritic diseases.

Carrageenan, a sulfated mucopolysaccharide, is extracted from the seaweeds Chondrus spp. and Gigartina spp., which are commonly known as Irish moss or carrageen moss. It has been used to induce various rat models of inflammation,
including the footpad inflammation and paw edema model [24] and the air pouch model [19]. Moreover, carrageenan has been used to induce acute arthritis [13] and deteriorating pathological symptoms of inflammatory arthritis in other models [20]. The responses of ascending tract cells in the cat spinal cord were enhanced by carrageenan-induced inflammation of the knee joint [17]. Intra-articular injection of lambda carrageenan into the knee joint resulted in localized inflammation, which caused decreased weight bearing, guarding of the affected limb, and hyperalgesia [23].

In the present study, the anti-inflammatory and analgesic effects of amygdalin were investigated in LPS-induced inflammation of an in vitro cell line and in carrageenan-induced arthritis in rat by measuring the weight distribution ratio (WDR) and ankle circumference. The analgesic effect of amygdalin was also shown to be associated with significant decreases in the expression levels of c-fos and the inflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β, in the superficial dorsal horn of rat.

**Materials and Methods**

**In Vitro Anti-Inflammatory Effect of Amygdalin**

Amygdalin (over 99% purity) and LPS (from _E. coli_ serotype 055:B5) were purchased from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.). RAW 264.7 cells (1.5×10⁵) were adapted to serum-free DMEM for 3 h. The cells were treated with 1, 10, or 100 µM amygdalin for 2 h; LPS (1 µg/ml) was added, and the cells were incubated for another 6 h. Total RNA was prepared from the LPS-treated RAW 264.7 cells using TRIzol reagent (Gibco BRL, Gaithersburg, MD, U.S.A.), according to the manufacturer’s protocol. Semiquantitative RT reactions were conducted using Mol.V reverse transcriptase. The PCR reaction mixture contained 2 µl of cDNA, 4 µM primers for TNF-α (forward, 5′-CGTTG-TGACCCAGCTGCAAGC-3′; reverse, 5′-TTGACCATGCGCCTGAA-3′) (96 bp, AY 427675) and 11-β (forward, 5′-GGCTACAAGGCTCATTG-3′; reverse, 5′-CGATCCACCGAGCTGAG-3′) (414 bp, NM_031512), 10× buffer [10 mM Tris–HCl (pH 8.3), 50 mM KCl, 0.1% Triton X-100], 250 µM dNTPs, 25 mM MgCl₂, and 1 unit of Taq polymerase (TaKaRa Co., Shiga, Japan). The primers were designed using a primer selection software, offered through a Web site, Primer 3 (http://www.genome.wi.mil.edu; The Whitehead Institute for Biomedical Research, MA, U.S.A.). The reaction conditions were 30 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 60°C, and extension for 30 s at 72°C, with a final extension of 10 min at 72°C.

**Animals**

Sprague-Dawley male rats weighing 230–250 g were used for these experiments. All experimental animals were obtained from Samtaco Animal Co. (Kyungki-do, Korea). They were kept under controlled environmental conditions (20±2°C and a light-dark cycle of 12/12 h) for at least 1 week prior to the start of the experiment. Food and water were available ad libitum. All subjects were habituated to the behavioral test chambers and handled with special care to minimize stress. All methods were approved by the Animal Care and Use Committee of Kyung Hee 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.

**Experimental Groups**

Rats were randomly divided into five treatment groups: normal group (NOR, n=6), carrageenan-induced and saline-treated arthritic group (CR, n=4), carrageenan-induced and 0.1 mg/kg amygdalin-treated group (CR plus 0.1-AMY, n=7), carrageenan-induced and 0.05 mg/kg amygdalin-treated group (CR plus 0.05-AMY, n=6), and carrageenan-induced and 0.005 mg/kg amygdalin-treated group (CR plus 0.005-AMY, n=6). Amygdalin dissolved in saline solution with the concentrations of 0.005, 0.05, and 0.1 mg/kg was intramuscularly injected immediately after the induction of carrageenan-induced arthritic pain in rats.

**Carrageenan-induced Ankle Arthritis and Behavioral Analysis**

Arthritic inflammation was induced by injection of 0.7% lambda carrageenan (Sigma-Aldrich, St. Louis, MO, U.S.A.) in 50 µl of pyrogen-free saline into the right tibiotaral joint. To estimate the level of pain in the arthritic ankle, the weight-bearing force of the feet and the ankle circumference difference were measured before injection and then at 2, 4, 6, and 8 h after injection. The animals were placed in a test box with a slanted plank. The weight distribution ratio (WDR) was measured by an incapacitation meter using a mechanotransducer (Cass Co., Seoul, Korea) placed below the hind paw, allowing the bearing force of each foot to be weighed separately. The bearing force of each hind paw was estimated as a 5-s average, and the mean bearing force was calculated from four separate estimations. The WDR percentage was calculated as %WDR=100×(weight borne by ipsilateral paw/total weight borne by both paws). The circumference of the right ankle was measured around the lower edge of the lateral and medial malleolus, using a scaled soft ruler without elasticity. The difference in the ankle circumference between the initial value and that at each time point after injection was calculated using the following equation: % difference in ankle circumference=100×[(circumference post-circumference pret)/circumference pret].

**Histological Examination of the Spinal Cord**

Eight hours after the carrageenan 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 30 ml of 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). The spinal cord was removed, post-fixed overnight, and cryoprotected with 30% sucrose in 0.1 M PBS at 4°C. Using a cryostat, coronal sections of 40-µm thickness were cut between L3 and L5.

**c-Fos Immunohistochemistry**

The sections were rinsed three times in PBS containing Triton X-100 (PBST) and immunostained for Fos using the avidin-biotin-peroxidase method. For the primary antibody, rabbit polyclonal antibody against c-Fos (Abcam Ltd., Cambridge, U.K.) was diluted (1:5000) with blocking solution (Vector Laboratories, Burlingame, CA, U.S.A.) and incubated with the sections for 48 h at room temperature, with constant agitation. After rinsing in PBST, the sections were incubated with biotinylated rabbit anti-rabbit (Vector Laboratories) diluted 1:200 in PBST containing 1% normal goat serum, for 2 h at room temperature. The sections were then placed in Vectorstain Elite ABC reagent (Vector Laboratories) for 1 h at room temperature. After further rinsing in PBST, the reaction was developed using diaminobenzidine (DAB) as a chromagen with nickel intensification. The slides were air-dried and
coverslipped for microscopic observation under a light microscope (Carl Zeiss, Oberkochen, Germany). The superficial layers (SDH, laminae I and II) of the rat dorsal horn of the spinal cord were examined to assess the effect of amygdalin treatment on c-Fos expression in spinal neurons.

**Immunohistochemistry of TNF-α and IL-1β**

The sections were immunostained for TNF-α and IL-1β. After antigen retrieval, endogenous peroxidase was blocked with 0.3% hydrogen peroxidase for 30 min. The sections were washed in PBS and then incubated in 1.5% normal goat serum for 30 min at room temperature to block nonspecific background staining. After three 5-min washes with PBS, the sections were incubated overnight at room temperature with primary rabbit anti-rat TNF-α antibody (diluted 1:500 in PBS) and primary rabbit anti-rat IL-1β antibody (diluted 1:100 in PBS). After rinsing with PBS, the reactivity was detected using peroxidase-conjugated secondary antibodies (Dako, Glostrup, Denmark). Peroxidase activity was visualized with DAB (Dako, Glostrup, Denmark). The reaction was stopped by rinsing with PBS, and the sections were counterstained with hematoxylin, dehydrated in a series of alcohol and xylene, and permanently mounted with permount (Fisher Scientific International, Pittsburg, PA, U.S.A.).

**Statistical Analysis**

The data are presented as means ± SEM. Weight distribution ratio (WDR) and ankle circumference were analyzed by repeated ANOVA, followed by the Tukey post hoc test for further confirmation. TNF-α mRNA expression and the number of Fos-reactive neurons were analyzed by one-way ANOVA, followed by the Tukey post hoc test for further confirmation. The criterion for statistical significance was p < 0.05.

**RESULTS**

**Inhibition of LPS-inducible Expression of TNF-α and IL-1β in RAW 264.7 Cells**

To determine the anti-inflammatory effect of amygdalin, we examined the mRNA expression levels of TNF-α and IL-1β in RAW 264.7 cells stimulated with LPS (1 μg/ml) for 6 h, in the presence and absence of amygdalin. RT-PCR was performed, and TNF-α and IL-1β mRNA expressions at three amygdalin concentrations (1, 10, and 100 mM) were compared. At 1 mM concentration, amygdalin significantly inhibited the expression of TNF-α and IL-1β mRNAs in LPS-treated cells, whereas 100 mM amygdalin did not inhibit TNF-α and IL-1β expression (Fig. 1). RT-PCR analysis also revealed that the TNF-α and IL-1β mRNA expression levels in LPS-induced cells, when treated with 1 mM amygdalin, was 32% and 44% less than those in LPS-induced cells without amygdalin treatment, respectively.

**Effect of Amygdalin on the Weight Distribution Ratio**

Fig. 2 shows the effect of amygdalin treatment on the weight distribution between the paws of a carrageenan-induced monoarthritis rat. The WDR of the unilateral paws in the NOR group was normal at 50%. As the pain and swelling of the knee or ankle progressed from induction of monoarthritis, the balance of the weight distribution was disrupted, resulting in a decrease of WDR in the arthritic leg. To investigate the analgesic effect of amygdalin, three different doses of amygdalin, 0.1, 0.05, and 0.005 mg/kg-weight, were used. Thus, carrageenan-induced monoarthritis was developed in the rat ankle as an acute pain model, and the WDRs from the bearing forces of both feet were analyzed to assess pain behavior. As shown in Fig. 2, WDR values (%) for each group at 0, 2, 4, and 8 h, respectively, after the carrageenan injection were as follows: NOR (50.35 ± 2.08, 46.28 ± 1.65, 45.36 ± 1.96, 48.22 ± 1.22, 46.86 ± 2.26); CR (49.90 ± 1.23, 43.41 ± 1.67, 34.67 ± 1.48, 16.10 ± 2.47, 12.85 ± 2.17); CR plus 0.1-AMY (48.42 ± 1.38, 47.78 ± 1.65, 9.90 ± 4.77, 11.84 ± 4.63, 4.80 ± 2.04); CR plus 0.05-AMY (30.54 ± 0.79, 46.99 ± 0.88, 30.50 ± 3.87, 17.53 ± 4.20, 11.61 ± 2.52); and CR plus 0.005-AMY.

![Fig. 1. Effect of amygdalin on mRNA expression of TNF-α and IL-1β in RAW 264.7 cells.](image)

PCR product was normalized to GAPDH, a housekeeping gene. Values are means with standard deviations from at least three independent experiments. (**p < 0.001 vs. LPS non-treated control, and *p < 0.05 and **p < 0.01 vs. LPS-treated control)**
Before the carrageenan injection (time 0), the mean WDR did not differ significantly among the experimental groups. However, significant changes in the ratio were seen at 2 h after the carrageenan injection, and the change of WDR in the CR group reached 36% ($p<0.01$ vs. NOR group) at 8 h. A significant recovery of WDR was observed only in the CR plus 0.005-AMY group. The higher doses of amygndalin (0.1 and 0.05 mg/kg) did not produce a significant difference in WDR compared with that in the CR group. This indicates that intramuscular injection of amygndalin at doses greater than 0.005 mg/kg might produce a detrimental effect on the arthritic symptoms of carrageenan-induced rat arthritis.

(48.95±1.08, 47.34±1.90, 36.97±4.60, 40.24±4.07, 38.55±6.24)

**Effect of Amygdalin on Ankle Circumference**

To evaluate the anti-inflammatory effect of amygndalin, the carrageenan-induced edema in rats was analyzed by measuring ankle circumference. As shown in Fig. 3, the ankle circumference at time 0 did not differ significantly among the experimental groups. However, the ankle circumference began to increase after arthritis was induced, reaching a maximum value at 8 h. In the CR group, the percentage difference in ankle circumference gradually increased from 0 at time 0 to 11.34±1.66, 36.63±1.47, 44.82±1.79, and 49.43±2.32 at 2, 4, 6, and 8 h, respectively. The percentage differences in ankle circumference at 2, 4, 6, and 8 h for the CR plus 0.1-AMY, CR plus 0.05-AMY, and CR plus 0.005-AMY groups were as follows: CR plus 0.1-AMY:

![Fig. 2. Effect of amygndalin on the weight distribution between the paws of the carrageenan-induced ankle arthritic rat. Data were analyzed by repeated ANOVA, followed by Tukey's test. ($^* p<0.001$, $^{***} p<0.001$ vs. NOR group and $^{**} p<0.001$ vs. CR group)](image)

![Fig. 3. Effect of amygndalin on ankle circumference in carrageenan-induced ankle arthritic rats. Data were analyzed by repeated ANOVA, followed by Tukey's test. ($^* p<0.05$, $^{**} p<0.001$ vs. CR group)](image)

![Fig. 4. Effect of amygndalin on c-Fos expression in the spinal cord of the carrageenan-induced ankle arthritic rat. Left: Photomicrographs (200×) illustrate c-Fos immunoreactive neurons in the rat superficial dorsal horn (SD1) at L3-5 levels. Normal (A), carrageenan-induced ankle arthritis (B), and 0.005 mg/kg body weight amygndalin treatment (C). Amygdalin was intramuscularly injected immediately after carrageenan injection and treated for eight hours. The arrows indicate c-Fos immunopositive neurons and the scale bar in A indicates 15 µm. This figure is representative of at least three experiments performed on different experimental days. Right: Separate measures of one-way ANOVA for number of c-Fos immunoreactive cells among the groups were followed by Tukey's test. Values are means with standard deviations from at least three independent experiments. ($^* p<0.01$ vs. NOR group; $^{**} p<0.01$ vs. CR group)](image)
The present study showed that amygdalin treatment significantly inhibited algesia and edema induced by carrageenan, thus establishing amygdalin as a new candidate analgesic and anti-inflammatory agent originated from natural herbs. For more than 40 years, amygdalin has been one of the most popular “alternative cancer cures” in many European and South American countries. In particular, D-amygdalin was shown to selectively kill cancer cells [3, 7, 8, 15]. However, it has not yet received FDA approval for use in the United States owing to insufficient clinical verification of its therapeutic efficacy, and the anticancer effect of amygdalin remains controversial. We also performed the toxicity test of amygdalin in vitro and in vivo using RAW 264.7 cells. MTT assay indicated no significant growth inhibition at 100 mM.
concentration, and there was no distinct toxic effect observed with 2 mg/kg dosage by a single and short-term exposure of mice (data not shown).

Amygdalin is composed of two molecules of glucose, one benzaldehyde, which is a known analgesic, and one hydrocyanic acid, which is proposed as an antineoplastic compound. From these chemical and pharmacological points of view, we considered that the benzaldehyde component of amygdalin might be the key unit to exert an analgesic action. In a recent report, Chang et al. [4] showed that amygdalin exerted anti-inflammatory and analgesic effects in an in vitro culture of mouse BV2 microglial cells.

In the present study, the anti-inflammatory and analgesic effects of amygdalin were investigated in an in vitro cell line and an in vivo arthritic rat model. In LPS-treated RAW 264.7 cells, the transcriptions of TNF-α and IL-1β, cytokines representative of the inflammation response, were differentially suppressed by 1 mM amygdalin. The maximum inhibition occurred at 1 mM amygdalin, implying that amygdalin did not inhibit TNF-α and IL-1β mRNA expressions in a dose-dependent manner. Thus, a part of the glycoside or other degradation products of amygdalin might exert an inhibitory effect on the inflammation response to LPS, although amygdalin's overall cytotoxic effect was not observed in the RAW 264.7 cell line within the range of amygdalin concentrations tested here (data not shown).

Carrageenan has been used to induce various types of inflammation, paw edema, and acute monoarthritis in many animal studies. It induces hyperalgesia and inflammation in rodents, which have widely been accepted as an appropriate model for examining the efficacy of anti-inflammatory and/or analgesic drug candidates. Previously, the injection of carrageenan into the ankle joint of one leg in rats produced edema and pain-related behaviors, and the WDR was primarily used to assess the severity of pain [18]. Decrease of the WDR is one of the most commonly observed functional disabilities in monoarticular animals [23]. In this study, 0.005 mg/kg amygdalin significantly inhibited the decrease of the WDR that resulted from carrageenan-induced pain and swelling in the arthritic rats, suggesting that amygdalin may be a strong candidate as an analgesic drug to control both pain and inflammation. Arthritic edema usually results from local action of multiple inflammatory mediators, including prostaglandins [27], bradykinin [26], nitric oxide [9], 5-hydroxytryptamine [10], and histamine [21]. The ability of amygdalin to inhibit carrageenan-induced ankle edema in animals further supports its action as an anti-inflammatory drug.

We tried additional concentrations and doses of amygdalin in addition to those described above; for example, concentrations (0.01 mM, 0.05 mM, 0.1 mM, and 0.5 mM) below 1 mM in vitro, and doses (0.0005 and 0.001 mg/kg) below 0.005 mg/kg in vivo. Even though we were able to obtain clear results showing the analgesic and anti-inflammation effects of amygdalin, it exhibited little dependency on these concentrations or doses. The inhibition of amygdalin at the concentrations or doses below 1 mM or 0.005 mg/kg, respectively, exhibited almost similar patterns and very close to each other. Therefore, there appears to be an experimental limitation to show a significant dose-dependency in the case of analgesic activity of amygdalin. It was thought that this might be due to interference of other metabolites of amygdalin, even though it has not yet been defined. Nevertheless, the present preliminary experiments indicated that the optimum concentration or dosage, which produced the maximum analgesic activity, was determined to be 1 mM in vitro and 0.005 mg/kg in vivo.

In the present study, amygdalin treatment suppressed Fos expression in the spinal cord evoked by carrageenan injection. It has been shown that carrageenan-induced hyperalgesia parallels not only to the development of peripheral inflammation, but also the expression of Fos protein in the spinal cord [2, 11]. Numerous studies have used spinal cord Fos expression to evaluate the effectiveness of anti-inflammatory drugs and analgesics in carrageenan-induced hyperalgesia/inflammation models [11, 16]. Those studies showed a clear relationship between the inhibitory effect of the drugs on spinal Fos expression and their ability to alleviate preclinical signs of carrageenan-induced inflammation. Similarly, amygdalin treatment was shown to decrease ankle edema in our study and suppress the number of Fos-positive neurons in the spinal cord of arthritic rats.

The expressions of IL-1β and TNF-α mRNA in the arthritic rat spinal cord was significantly increased. Astrocytes and microglial cells, two major glial cell types of the CNS, typically produce several cytokines. Pro-inflammatory cytokines, such as IL-1β and TNF-α, are normally expressed at low concentrations in the CNS. However, their expressions markedly increase after injury to the brain, spinal cord [1], peripheral neurons [6], and with autoimmune and inflammatory disorders, including rheumatoid arthritis [5, 22]. The enhanced expression of pro-inflammatory cytokines in such pathological states suggests that they play a crucial role in the initiation or maintenance of these types of diseases. It has been shown that circulating peripheral cytokines stimulate afferent nerves to either directly enhance pain responses or increase cytokine levels in the CNS [23]. Peripheral pain stimuli could also evoke cytokine release in the CNS [12]. The upregulation of these cytokines, that is, an inflammatory response in the CNS of the arthritic rat, might be necessary in hyperalgesic mechanisms relevant to individual tissues and organs.

This study verified that amygdalin treatment significantly inhibited the inflammation and hypernociception induced in an in vitro cell line by LPS and by carrageenan injection into the rat ankle, which served as an inflammatory monoarthritic model. Amygdalin treatment also suppressed c-Fos, TNF-α, and IL-1β expressions in the spinal cord. Therefore, the inhibitory effect of amygdalin was most likely due to suppression of pro-inflammatory cytokine release, which is closely related to inducing the inflammatory response in...
the CNS. These results indicate that amygdalin is a plausible candidate for the development of a new drug to treat inflammatory pain.

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References


