Anti-Nociceptive and Anti-Inflammatory Effects of Angelicae Dahuricae Radix Through Inhibition of the Expression of Inducible Nitric Oxide Synthase and NO Production

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Abstract: The extract of Angelicae Dahuricae Radix has traditionally been used as an anti-nociceptive remedy in China. In this study, the methanol extract of Angelicae Dahuricae Radix (MEAD) was evaluated to determine if it has anti-nociceptive and anti-inflammatory action. The anti-nociceptive activities of MEAD were evaluated by determining the writhing response and sleeping time, as well as by a formalin test. In addition, the anti-inflammatory activities of MEAD were evaluated by a vascular permeability test as well as by measuring the carrageenan-induced paw edema and conducting a myeloperoxidase (MPO) assay. MEAD (600 and 1200 mg/kg) exhibited anti-inflammatory effects on acetic acid-induced vascular permeability, carrageenan-induced paw edema, and MPO activity. Moreover, the results of the formalin test, the acetic acid-induced writhing response and the pentobarbital-induced sleeping time indicated that MEAD had anti-nociceptive effects that occurred in a concentration-dependent manner. To determine the mechanism by which MEAD exerted its effects on the expression of inducible nitric oxide synthase (iNOS) and the production of nitric oxide (NO) by treated murine macrophage RAW 264.7 cells was evaluated. Similar to the in vivo activities, both the iNOS expression and NO production were significantly suppressed by MEAD in a dose-dependent manner. Furthermore, MEAD inhibited the activating phosphorylation

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of ERK1/2. These results provide a scientific basis that explains the mechanism by which Angelicae Dahuricae Radix relieves inflammatory pain.

Keywords: Angelicae Dahuricae Radix; Anti-Nociceptive; Anti-Inflammatory; Nitric Oxide; Inducible Nitric Oxide Synthase.

Introduction

Nitric oxide (NO), which is an important regulatory molecule for diverse physiological functions such as host defense, is produced by inducible NO synthase (iNOS) in activated macrophages (Moncada et al., 1991; MacMicking et al., 1997). NO is produced from L-arginine through the action of the NO synthase (NOS) enzyme. NO has many positive functions in biological systems, such as vasodilation, eurotransmission, and inflammatory response (Pieretti et al., 1991). However, excess the production of NO can cause the formation of mutagenic peroxynitrites (ONOO-), which leads to septic shock, autoimmune disorders, DNA modifications and other forms of cellular injury (Deraedt et al., 1980). In addition, NO itself can induce harmful responses, such as apoptosis, septic shock, and tissue injury (Wager, 1999), which have been attributed to the iNOS mediated production of NO and the associated generation of potent reactive radicals such as peroxynitrite (Shah and Billiar, 1998). The high amount of NO produced by iNOS in response to stimulation by pro-inflammatory cytokines, free radicals and lipopolysaccharide (LPS) may be a critical mediator in the pathogenesis of inflammatory diseases (Vane et al., 1994; Szabo and Thiemermann, 1995), and attenuation of the iNOS/NO pathway has been found to be beneficial for the treatment of sepsis and other inflammation-related diseases (Blantz and Munger, 2002; MacMicking et al., 1997). Accordingly, agents that inhibit the overproduction of NO derived from iNOS in macrophages may have anti-inflammatory activity.

Angelicae Dahuricae Radix (Umbelliferae) is a perennial plant that grows naturally in the mountains of Korea. Umbelliferae has a strong scent and its leaves are used to make incense. In addition, the roots of Umbelliferae (also known as Bai Zhi) are used in traditional Chinese medicine to counter harmful external influences on the skin, such as cold, heat, dampness and dryness (Chevallier, 2001). Additionally, it has been claimed that umbelliferae is useful for treatment of acne, erythema, headache, toothache, sinusitis, colds and flu (Wagner, 1999). Furthermore, Angelicae Dahuricae Radix has been shown to protect against sepsis and have anti-staphylococcal activity (Song et al., 2005; Lechner et al., 2004). Pharmacological studies on the Chinese drug radix Angelicae dahuricae, have investigated its anti-inflammatory, analgesic and antipyretic actions and acute toxicity as a guideline for clinic application (Li et al., 1991). Essential oil of Angelicae Dahuricae Radix has analgesic effect in pain model rats, and has been reported that it was associated with endogenous opiate-like substance such as beta-endorphin (Nie and Shen, 2002). Moreover, we recently reported that ethyl acetate extract of Angelica Dahuricae Radix inhibits LPS-induced production of inflammatory mediators through mitogen-activated protein kinases (MAPKs) and nuclear factor-kB (NF-kB) (Kang et al., 2007). However, the anti-nociceptive and anti-inflammatory
mechanism by which the methanol extract of Angelicae Dahuricae Radix (MEAD) functions is still unknown.

In this study, we evaluated the anti-nociceptive and anti-inflammatory effects of MEAD. The in vivo anti-nociceptive activity was investigated using a formalin test, as well as evaluating pentobarbital induced sleep and acetic acid-induced writhing in mice. The in vivo anti-inflammatory activity was investigated using acetic acid and carrageenan-induced edema animal models. Additionally, the effects of MEAD expression on iNOS and NO production were investigated using LPS-stimulated RAW 264.7 macrophages.

Material and Methods

Reagents

Evans Blue, acetic acid, carrageenan, aspirin, indomethacin, formalin, hexa-decyltrimethylammonium bromide (HTAB), bovine serum albumin, LPS and o-dianisidine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Tween 80 was obtained from Duksan (Korea) and NaHCO₃ and Na₂CO₃ were purchased from Daejung (Korea). RPMI 1640, penicillin, and streptomycin were obtained from Hyclone (Logan, UT, USA). iNOS, p38, phosphorylated p38, ERK, phosphorylated ERK, JNK, phosphorylated JNK, β-actin, and peroxidase-conjugated secondary antibody were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). In addition, an RNA extraction kit was purchased from iNtRON Biotech (Daejeong, Republic of Korea). Finally, iNOS and β-actin oligonucleotide primers were purchased from Bioneer Corp. (Daejeong, Korea).

Plant Material and Extraction

The root of Angelica dahurica was purchased from an oriental drug company, Daehak Pharmacy (Iksan, Jeonbuk, Korea), and authenticated by Professor D.Y. Kwon, Wonkwang University, Iksan, Jeonbuk, Korea. A voucher specimen was deposited at the Herbarium of the College of Pharmacy, Wonkwang University. The dried roots of Angelica dahurica (1 kg) were then extracted under reflux with hot MeOH (2 l) 3 times for 2 hours each time. The resultant extract was then filtered and condensed to 1 liter in a rotary vacuum evaporator (N-1000S, EYELA, Japan) to obtain a dried powder extract (298 g). All samples were stored at 4°C until use. The samples and reference drugs were suspended in 5% Tween 80.

Animals

Male Sprague-Dawley rats (100 ~ 120 g, 180 ~ 200 g) and male ICR mice (30 ± 3 g) that were used for this study were purchased from SAMTAKO BIOKOREA (Korea). All animals were kept in a temperature-controlled room with a 12 hours light-12 hours dark cycle. The animals had free access to commercial solid food (SCF Co., Ltd. Korea) and were provided with water ad libitum. All animals were acclimatized for at least 1 week prior to beginning the experiments.
Formalin-Induced Licking Time

The formalin test comprises 2 distinctive phases, possibly reflecting different types of pain. For this study, a slightly modified version of the method described Hunskaar and Hole was used (Hunskaar and Hole, 1987). Briefly, each mouse was administered MEAD (100, 200, 400 mg/kg, p.o.), aspirin (300 mg/kg) or 5% Tween 80 (p.o.) 60 min prior to having 20 µl of 2.5% formalin (1% formaldehyde, Sigma) in distilled water injected into the subplantar of its right hind paw. Each mouse was then individually placed in a transparent plexiglass cage (25 cm × 25 cm × 20 cm) observation chamber and the amount of time spent licking and biting the injected paw was recorded 0 ∼ 5 min (early phase) and 15 ∼ 30 min (late phase) after injection.

Pentobarbital-Induced Sleeping Time

The effect of MEAD on pentobarbital-induced sleeping time was studied in mice as described previously (Pieretti et al., 1991). Briefly, mice were treated with either 5% Tween 80 or MEAD and then treated with pentobarbital sodium (40 ml/kg, i.p.) 30 min. later. The time between the loss and subsequent recovery of the righting reflex was taken to be the sleeping time and recorded.

Acetic Acid-Induced Writhing Response

Male Swiss albino mice weighing 30 ± 3 g were used to evaluate the acetic acid-induced writhing response following a previously described method (Nakamura et al., 1986). Briefly, test drugs and control vehicles were orally administered 60 min prior to injection of an aqueous solution of 0.75% acetic acid (0.1 ml/10 g body weight) into the peritoneal cavity. The animals were then placed in a transparent plexiglass chamber and the number of writhes, which was described as a response consisting of contraction of the abdominal wall and pelvic rotation followed by hind limb extension, was counted during continuous observation for 15 min beginning 5 min following the acetic acid injection.

Acetic Acid-Induced Vascular Permeability

The acetic acid-induced vascular permeability test was performed as described previously (Whittle, 1964). Briefly, male mice weighing 27 ∼ 33 g were fasted for 10 hours prior to the experiments and then orally administered either the test drugs or a vehicle. Each animal then received an intravenous injection of 1% Evans blue (0.1 ml/10 g) 30 min after the oral treatment. The vascular permeability inducer, 0.1 ml/10 g of 0.6% acetic acid in saline, was then injected intraperitoneally at 30 min after the Evans blue injection. After 20 min, the mice were sacrificed by dislocation of the neck and 10 ml of normal saline was injected intraperitoneally, after which the wash solution was collected in tubes and centrifuged at 2,000 rpm for 10 min. The absorbance of the supernatant was then measured at 610 nm using a spectrophotometer (UV-2401PC, Shimadzu, Japan). The vascular permeability was then expressed in terms of the amount of total dye (µg/mouse) that leaked into the intraperitoneal cavity.
Carrageenan-Induced Paw Edema

The anti-inflammatory activity of MEAD was evaluated using a previously described method (Winter et al., 1962). Briefly, 1 hour after sample administration, edema was induced by injecting 0.02 ml of 1% carrageenan in sterile saline into the plantar side of the right hind paw. The paw thickness of hind paw was measured using a dial thickness gauge (Mitutoyo, Japan) before and at 1 hour after carrageenan injection, and the differences in the thickness were then calculated. The size of edema was assessed as an increase of paw thickness (mm).

Assay for Myeloperoxidase Activity

Mice from different groups that were administered samples 1 hour earlier were sacrificed by local dislocation 4 hours after carrageenan injection. The paws were then weighed and the myeloperoxidase activity was evaluated using a modified myeloperoxidase (MPO) assay as described previously (Bradley et al., 1982). Briefly, tissue was minced and homogenized in KH₂PO₄/K₂HPO₄ buffer (pH 6.0) containing 0.5% hexa-decyltrimethyl-ammonium bromide (HTAB) (Sigma) at 0°C for 45 sec in a motor driven homogenizer and then centrifuged at 3,000 rpm for 20 min at 4°C. The myeloperoxidase activity was then assayed by adding the following reagents to wells in a 96-well microtiter plate: 50 µl of supernatant, 50 µl of phosphate buffer containing 0.5% HTAB (pH 6.0) and 50 µl of o-dianisidine (0.68 mg/ml in distilled water) (Sigma). The reaction was then started by adding 50 µl of freshly prepared 0.003% hydrogen peroxide to each of the wells. The change in absorbance at 450 nm was then measured spectrophotometrically.

Cell Culture

The murine macrophage cell line, RAW 264.7, was obtained from the Korea Research Institute of Bioscience and Biotechnology (Seoul, Republic of Korea) and grown in RPMI 1640 medium containing 10% fetal bovine serum and 100 U/ml of penicillin/streptomycin sulfate in a humidified 5% CO₂ atmosphere at 37°C. The cells were then stimulated by replacing the medium with fresh RPMI 1640. LPS was then added in the presence or absence of MEAD for the indicated periods.

Measurement of NO Production

NO production was assayed by measuring the level of nitrite in the supernatants of cultured RAW 264.7 cells. Briefly, the cells were seeded at a density of 5 x 10⁵/ml in 96-well culture plates and then preincubated for 18 hours. Next, the cells were pretreated with MEAD and stimulated with LPS (200 ng/ml) for 24 hours. The supernatant was then mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylthelylenediamine dihydrochloride, and 2.5% phosphoric acid) and incubated at room temperature for 5 min. The concentrations of nitrite were then determined by measuring the absorbance at 570 nm and comparing the values to a standard curve generated using known concentrations of sodium nitrite (NaNO₂).
Western Blot Analysis

Protein expression was assessed by Western blot analysis according to a standard procedure. Briefly, RAW 264.7 cells were cultured in 60 mm culture dishes (2 × 10^6/ml) and then pretreated with various concentrations of MEAD (200 µg/ml and 500 µg/ml). After 30 min, LPS (200 ng/ml) were added to the culture medium and the cells were then incubated at 37°C. Following incubation, the cells were washed twice in ice-cold PBS (pH 7.4). Cell pellets were resuspended in lysis buffer on ice for 15 min, after which the cell debris was removed by centrifugation. The protein concentration was then determined using the BIO-RAPID protein assay reagent according to the manufacturer’s instructions. Briefly, equal amounts of protein (20 µg) were subjected to SDS-PAGE and then transferred onto a PVDF membrane (Millipore). The membrane was then blocked with 5% non-fat milk in TBS/T buffer (150 mM NaCl, 20 mM Tris-HCl, 0.05% Tween 20, pH 7.4), after which it was incubated with primary antibodies for 18 hours. Next, the membrane was washed with TBS/T and then incubated with anti-mouse or anti-rabbit IgG horseradish peroxidase-conjugated secondary antibodies. The immuno-reactivity was then detected using enhanced chemiluminescence (ECL: Amersham, Milan).

RNA Extraction and Reverse-Transcription PCR (RT-PCR)

Total cellular RNA was isolated using the easy-BLUE™ RNA extraction kit according to the manufacturer’s instructions. Total RNA (2 µg) was then converted to cDNA by treatment with 200 units of reverse transcriptase and 500 ng of oligo-dT primer in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, and 1 mM dNTPs at 42°C for 1 hour. The reaction was then stopped by heating at 70°C for 15 min, after which 3 µl of the cDNA mixture was used for enzymatic amplification. PCR was conducted by subjecting a reaction mixture comprised of 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 0.2 mM dNTPs, 2.5 units of Taq DNA polymerase, and 0.1 µM each of primers for iNOS and β-actin to the following conditions: initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 56°C for 45 sec, and extension at 72°C for 90 sec, with a final extension at 72°C for 7 min. The PCR products were then electrophoresed on a 2% agarose gel and stained with ethidium bromide. The primers used were 5'-AGC CCA ACA ATA CAA ATG ACC CTA-3’ (sense) and 5'- TTC CTG TTG TTT CTA TTT CCT TTG T-3’ (antisense) for iNOS, and 5'-ATG AAG ATC CTG ACC GAG CGT-3’ (sense) and 5'-AAC GCA GCT CAG TAA CAG TCC G -3’ (antisense) for β-actin.

Statistical Analysis

Statistical analyses were conducted using one-way analysis of variance (ANOVA) followed by a Dunnett’s t-test for multiple comparisons, and the Student’s test for single comparisons. The data from the experiments are presented as the mean ± S.E. The numbers of independent experiments assessed are provided in the figure legends.
ANTI-NOCICEPTIVE AND ANTI-INFLAMMATORY EFFECTS

Table 1. The Effect of MEAD on Formalin-Induced Licking Time in Mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg, p.o)</th>
<th>Licking Time(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Early Phase 0–5 min</td>
</tr>
<tr>
<td>Control</td>
<td>—</td>
<td>125.3 ± 8.9</td>
</tr>
<tr>
<td>ASA</td>
<td>300</td>
<td>94.3 ± 6.3</td>
</tr>
<tr>
<td>MEAD</td>
<td>100</td>
<td>117.2 ± 7.3</td>
</tr>
<tr>
<td>MEAD</td>
<td>200</td>
<td>98.0 ± 5.8</td>
</tr>
<tr>
<td>MEAD</td>
<td>400</td>
<td>67.0 ± 3.9**</td>
</tr>
</tbody>
</table>

Results are expressed as the mean ± SEM (n = 6). *p < 0.01, **p < 0.001, significant as compared to the control group by Dunnett’s t-test. ASA: aspirin.

Results

Formalin-Induced Licking Time

The effects of MEAD in the early and late phases of the formalin test are shown in Table 1. MEAD was active in both the early and late phases of the formalin test; however, ASA inhibition was only observed in late phase. The results of this test indicate that the extract induced a moderate inhibitory effect on the licking response at a dose of 400 mg/kg.

Pentobarbital-Induced Sleeping Time

The sleeping times are presented in Fig. 1. MEAD significantly extended the pentobarbital-induced sleeping time in a concentration-dependent manner.

![Figure 1](image-url)

Figure 1. The effect of MEAD on pentobarbital-induced sleeping time in mice. Values are expressed as the mean ± S.E.M. (n = 6). *p < 0.001 compared to the control by Dunnett’s t-test. Indo: indomethacin.
Acetic Acid-Induced Writhing Response

The effects of MEAD on writhing response in mice are shown in Fig. 2. MEAD was found to inhibit the writhing response in a dose-dependent manner. In addition, indomethacin (10 mg/kg) significantly inhibited the writhing response.

Acetic Acid-Induced Vascular Permeability

The oral administration of MEAD at doses of 600 and 1200 mg/kg inhibited the leakage of dye into the peritoneal cavity in mice when compared with indomethacin (Table 2). In addition, when the acetic acid-induced vascular permeability was evaluated, MEAD was found to exert significant inhibition at doses of 300 mg/kg (10.5% inhibition), 600 mg/kg (26.4% inhibition) and 1200 mg/kg (33.7% inhibition).

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg, p.o)</th>
<th>Amount of Dye Leakage (µg/mouse)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>—</td>
<td>86.1 ± 3.2</td>
<td>—</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>10</td>
<td>67.9 ± 2.6*</td>
<td>21.1</td>
</tr>
<tr>
<td>MEAD</td>
<td>300</td>
<td>82.1 ± 4.2</td>
<td>4.6</td>
</tr>
<tr>
<td>MEAD</td>
<td>600</td>
<td>63.4 ± 3.0**</td>
<td>26.4</td>
</tr>
<tr>
<td>MEAD</td>
<td>1200</td>
<td>57.1 ± 2.9**</td>
<td>33.7</td>
</tr>
</tbody>
</table>

Results are expressed as the mean ± SEM (n = 6). *p < 0.01, **p < 0.001 significant compared to the control group by Dunnett’s t-test.
Figure 3. The effect of MEAD on carrageenan-induced paw edema in mice. Indo: indomethacin. Values are expressed as the mean ± SEM (n = 6). *p < 0.001 significant compared to the control by Dunnett’s t-test.

**Carrageenan-Induced Paw Edema**

The results of MEAD on carrageenan-induced hind paw edema in mice are shown in Fig. 3. Treatment with 600 and 1200 mg/kg of MEAD produced a significant reduction in paw edema that occurred in a dose dependent manner.

**Myeloperoxidase Activity**

Subplantar injection of carrageenan into the right hind paw of mice led to an extensive cellular infiltration 4 hours after injection with an agonist. Treatment with 600 and 1200 mg/kg of MEAD extract significantly inhibited the MPO activity of the paw tissue by 73.4% and 78.4%, respectively (Fig. 4).

**Effects of MEAD on LPS-Induced NO Production**

RAW 264.7 macrophages were stimulated with LPS for 24 hours in order to induce NO synthesis. The production of NO was then estimated based on the accumulation of nitrite, which is a stable product of NO metabolism, in medium using Griess reagent. The LPS-stimulated RAW 264.7 cells produced 13.3 ± 0.9 M of nitrite over a 24 hour period (Fig. 5); however, incubation of the cells with MEAD significantly inhibited the production of nitrite in a dose-dependent manner (Fig. 5). Although nitrite production was decreased by MEAD, the viability of the cells was not (data not shown).

**Effects of MEAD on LPS-Induced iNOS Protein and iNOS mRNA Expression**

The effect of MEAD on the LPS-induced expression of iNOS by RAW 264.7 macrophages was evaluated to identify the anti-inflammatory mechanism. Western blot analysis revealed
Figure 4. The effect of MEAD on carrageenan-induced MPO activity in mice. Values are expressed as the mean ± SEM (n = 6). *p < 0.001 compared to the control by Dunnett’s t-test. Indo: indomethacin.

Figure 5. The effect of MEAD on LPS-induced NO production in RAW 264.7 macrophages. RAW 264.7 cells were pretreated with the indicated concentrations of MEAD for 30 min prior to being incubated with LPS (200 ng/ml) for 24 hours. The culture supernatants were subsequently isolated and analyzed for nitrite levels. Values are the mean ± SEM of duplicate determinations from 3 separate experiments. *p < 0.01 compared to the LPS alone by Dunnett’s t-test. LPS, lipopolysaccharides.

that induction of the iNOS protein in the cells occurred 24 hours after the LPS treatment, but that the iNOS protein and mRNA was not produced by unstimulated RAW 264.7 cells. Furthermore, iNOS was strongly expressed in cells that were treated by LPS, and this increase was significantly inhibited by treatment with MEAD in a dose-dependent manner (Fig. 6a). Additionally, RT-PCR analysis showed that iNOS mRNA expression was related to the protein levels (Fig. 6b).
Figure 6. The effect of MEAD on LPS-induced iNOS protein and mRNA expression in RAW 264.7 macrophages. (a) RAW 264.7 cells were pretreated with the indicated concentrations of MEAD for 30 min before being incubated with LPS (200 ng/ml) for 24 hours. Equal amounts of protein (20 µg) were separated by SDS-polyacrylamide gel electrophoresis and immunoblotted with iNOS antibodies. Equal loading of protein was verified by β-actin. (b) iNOS mRNA were assessed by RT-PCR in RAW 264.7 cells. Cells were pretreated with the indicated concentrations of MEAD for 30 min prior to being incubated with LPS (200 ng/ml) for 24 hours. Analysis of β-actin mRNA was conducted in parallel to confirm equivalency of the cDNA preparation. The experiment was repeated 3 times and similar results were obtained. The values were expressed as a percentage of the maximal band intensity in the culture treated with LPS alone. Data are the means ± SEM of iNOS/β-actin based on at least 3 separate experiments. *p < 0.01 compared to the LPS alone by Dunnett’s t-test. LPS, lipopolysaccharides.
Effects of MEAD on the Activating Phosphorylation of MAPKs

MAPKs are essential for LPS-induced iNOS expression to occur in RAW 264.7 macrophages (Chen et al., 1999); therefore, the effect of MEAD on the activation of ERK 1/2 and p38 MAPK in LPS-stimulated RAW 264.7 cells was evaluated. As shown in Fig. 7, MEAD markedly inhibited the activating phosphorylation of ERK 1/2, whereas phosphorylation of JNK 1/2 and p38 MAPK phosphorylation were unaffected by treatment with MEAD (data not show). Taken together, these results indicate that MAPK phosphorylation was inhibited by MEAD pretreatment.

Discussion

Recently, many studies have evaluated the inhibitory effects of plant-derived anti-nociceptive and anti-inflammatory agents in vivo and in vitro. Bai Zhi, which is the dried root of Angelicae Dahuricae (Umbelliferae), has been used in Korea and China as a traditional treatment for acne, erythema, headache, colds, and flu. Angelica Dahuricae Radix contains various compounds including isoimperatorin, imperatorin, oxypeucedanin, byakangelicol, and byakangelicin. Of these agents, imperatorin, which is found in various plants used in traditional medicine, has recently been shown to have an inhibitory effect on mice concanavalin A-induced hepatitis (Okamoto et al., 1987). However, to the best of our knowledge, no studies
conducted to date have reported the mechanism by which the anti-nociceptive and anti-inflammatory action of methanol extract of Angelicae Dahuricae Radix (MEAD) occurs.

In this study, we evaluated the pharmacological basis for traditional use of MEAD for the treatment of various inflammatory diseases. The results of this study established the anti-nociceptive activity of MEAD using several experimental animal models. First, the effects of MEAD were examined using a topical nociceptive model, pentobarbital-induced sleeping time. The results showed that MEAD prolonged sleeping time in a dose-dependent manner (Fig. 1). Next, the anti-nociceptive activities were estimated based on the formalin-induced licking time and acetic acid-induced writhing response. The formalin test possesses two distinctive phases, possibly reflecting different types of pain. The early phase of the test, which occurs immediately after the formalin is injected and continues for 5 min, can be used to evaluate the direct effect of formalin on a nociceptive agent. The late phase of the test begins 15–30 min after the injection of formalin and continues for 60 min. This phase is marked by a return to high levels of nociception and believed to reflect inflammatory pain (Olajide et al., 2000). It is well known that substance P and bradykinin participate in the early phase of the formalin test, whereas histamine, serotonin, prostaglandins and bradykinin are involved in the late phase (Shibata et al., 1989). NSAIDs, such as indomethacin, reduce nociceptive behavior during the late phase, whereas the early phase appears to be unaffected by these agents (Hunskaar and Hole 1987; Rosland et al., 1990). The results of this study indicated that MEAD exerted a significant analgesic effect in both phases at a dose of 400 mg/kg (Table 1). The acetic acid-induced writhing response showed that the oral administration of MEAD inhibited the writhing response in a dose-dependent manner (Fig. 2). The writhing response of the mouse to an intraperitoneal injection of a noxious chemical is used to screen for both peripherally and centrally acting anti-nociceptive activity. Acetic acid causes pain by liberating many substances, including endogenous substances, and exciting pain nerve endings (Collier et al., 1968).

NSAIDs can inhibit COX in peripheral tissues, thereby interfering with the mechanism of transduction in primary afferent nociceptors. Therefore, the analgesic action of MEAD may be due to a blockade of the effect or the release of endogenous substances that excite pain nerve endings, similar to the mechanism of indomethacin, which is mediated via a peripheral mechanism.

Additionally, the anti-inflammatory activities were evaluated by acetic acid-induced vascular permeability, carrageenan-induced paw edema, and MPO activity in vivo. The acetic acid-induced vascular permeability showed that MEAD inhibited the vascular permeability in a dose-dependent manner (Table 2). Carrageenan-induced paw edema is a good animal model, in which peak edema is characterized by the presence of prostaglandins (Yang et al., 1996). In this study, MEAD was found to significantly reduce carrageenan-induced paw edema and MPO activity (Fig. 3 and Fig. 4). In addition, the results of all the animal models used in this study clearly demonstrated that MEAD had anti-inflammatory activity, as well as anti-nociceptive effects.

The effects of MEAD on macrophage functions related to inflammation were investigated to verify possible mechanisms underlying its beneficial effects. NO is an important mediator in the inflammatory process and is produced at inflamed sites by iNOS. High levels
of NO have been reported in a variety of pathological processes including various forms of inflammation, circulatory shock, and carcinogenesis (Ohshima and Bartsch, 1994; Szabo, 1995; MacMicking et al., 1997). Therefore, an inhibitor of NOS might be effective as a therapeutic agent for inflammatory diseases (Koo et al., 2001). The results of this study showed that MEAD inhibited LPS-induced NO production in RAW 264.7 macrophages. To further investigate the mechanism underlying these inhibitions by MEAD, the expression of iNOS protein and iNOS mRNA levels were examined by Western blot and RT-PCR, respectively, which revealed that MEAD reduced iNOS protein and iNOS mRNA expression (Fig. 6).

Taken together, these results indicate that MEAD has a potent anti-inflammatory effect that occurs through inhibition of the expression of iNOS and NO production. Additional experiments were conducted to determine if MEAD inhibited the cyclooxygenase pathway; however, only a minimal effect on the expression of COX-2 and PGE2 production in LPS-stimulated RAW 264.7 macrophages was observed (data not shown). Overall, these results suggest that another mechanism is involved in the inhibition of carrageenan-induced edema.

The MAPKs play a critical role in the regulation of cell growth and differentiation, particularly in response to cytokines and stress (Johnson and Lapadat, 2002). Several studies have demonstrated that MAPKs are involved in LPS-induced iNOS expression (Kang et al., 2007; Chen et al., 1999; Kim et al., 2004). Therefore, the effects of MEAD on the LPS-induced phosphorylation of extracellular signal regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK were evaluated in this study. Interestingly, pretreatment of macrophages with MEAD inhibited ERK1/2 phosphorylation, but not JNK1/2 and p38 MAPK phosphorylation (Fig. 7), which indicates that ERK1/2 is likely responsible for the suppressive effect of MEAD on iNOS induction.

In conclusion, the results of this study demonstrate the anti-inflammatory and anti-nociceptive effects of MEAD. In addition, MEAD was found to potently inhibit LPS-induced iNOS expression and NO production. Furthermore, these inhibitions were found to be caused by blockage of MAPK activation in RAW 264.7 macrophages. Taken together, these findings indicate that MEAD may represent a potential new source of drugs for the treatment of inflammatory and pain diseases.

Acknowledgments

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References

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