Anti-inflammatory Activity of Ailanthus altissima in Ovalbumin-Induced Lung Inflammation

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As part of an ongoing investigation to find bioactive medicinal herbs exerting anti-inflammation activity, the effect of an ethanol extract from the parts of Ailanthus altissima (Simaroubaceae) was evaluated in both in vitro and in vivo system. The ethanol extract of A. altissima (EAA) inhibited generation of the cyclooxygenase-2 (COX-2) dependent phases of prostaglandin D2 in bone marrow-derived mast cells (BMMC) in a concentration-dependent manner with an IC50 value of 214.6 μg/ml. However, this compound did not inhibit COX-2 protein expression up to a concentration of 400 μg/ml in the BMMC, indicating that EAA directly inhibits COX-2 activity. In addition, EAA inhibited leukotriene C4 production with an IC50 value of 25.7 μg/ml. Furthermore, this compound inhibited degranulation reaction in a dose dependent manner, with an IC50 value of 27.3 μg/ml. Ovalbumin (OVA)-sensitized mice were orally pretreated with EAA before aerosol challenges. EAA reduced the eosinophil infiltration into the airway and the eosinophil, IL-4, and IL-13 mRNA expression levels. These results suggest that the anti-inflammation activity of A. altissima in OVA-induced lung inflammation may occur in part via the down regulation of TNF-α cytokines and etoxacin transcripts as well as the inhibition of inflammatory mediators.

Key words Ailanthus altissima; cyclooxygenase-2; 5-lipoxygenase; asthma; eosinophil; TNF-α cytokine

Ailanthus altissima, commonly known as the ‘tree of heaven,’ is used in traditional medicine in many parts of Asia including China to treat cold and gastric diseases. Extracts of A. altissima, have demonstrated anti-proliferative1) and central nervous depressant activities.2) Among the bioactive compounds isolated from A. altissima,3)–6) quassinoids have various biological activities such as anti-tumor, anti-viral, and anti-inflammatory effects.7)

Asthma is one of the most common diseases characterized by airway hyper-responsiveness (AHR) associated with elevated serum IgE and bronchial inflammation. In bronchial asthma, various factors result in infiltration of mast cells, eosinophils and Tn2 lymphocytes into the lesions with a downstream mediator.8) Measurements of eicosanoids and their metabolites in bronchoalveolar lavage fluid (BALF) after inhaled allergen challenge of asthmatics suggest that resident mast cells, infiltrating eosinophils, basophils and macrophages are responsible for leukotrienes (LTs) and prostaglandins (PGs) in the early and late bronchoconstrictor responses.9,10)

Eicosanoids are inflammatory mediators that are biosynthesized in many cell types by cyclooxygenases (COX) and lipoxygenases (LOX). They are strongly associated with inflammatory disorders, acute as well as chronic inflammation. The inhibition of eicosanoid production is one of the important therapeutic strategies in various inflammatory diseases. Among the eicosanoid generating enzymes, COX-2 was found to be essential for the production of PGs in inflammatory sites.11) LTs produced by 5-LOX also have biological properties that would be expected for molecules that participate in the pathogenesis of bronchial asthma.12,13) Therefore, the dual inhibition of COX-2/5-LOX is believed to be the ideal treatment for allergic diseases and asthma.14)

Although many biological activities of A. altissima were reported, the anti-asthmatic activity of A. altissima has not been reported. In the present study, we describe a new biological function of A. altissima for the arachidonic cascade metabolism enzymes such as COX-2 and 5-LOX along with the potential for use in the treatment of asthma.

MATERIALS AND METHODS

Plant Material The leaf and branch of A. altissima were collected in July 2004 in Daejeon, Korea. This plant material was authenticated by one of the authors (KH Bae) and a voucher specimen has been deposited in the Herbarium of College of Pharmacy, Chungnam National University under the registration number CNU 923. The dried sample (1 kg) was extracted in ethanol (5 l) by refluxing for 24 h twice to give 212 g of an extract and used throughout this study.

Preparation of Bone Marrow-Derived Mast Cells (BMMC) Bone marrow cells from male BALB/c mice (Hyochang Science, Daegu, Korea) were cultured for up to 10 weeks in 50% enriched medium (RPMI 1640 containing 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mm L-glutamine, 0.1 mm nonessential amino acids, and 10% fetal calf serum) and 50% WEHI-3 cell conditioned medium as a source of IL-3. After 3 weeks >98% of the cells were found to be BMMC when checked by the previously described procedure.14) For the activation of BMMC, c-kit ligand (KL), LPS (Difco, Detroit, MI, U.S.A.) and IL-10 were obtained by their expression in baculovirus, as described previously.15,16)

PGD2 Production and COX-2 Activity BMCC at a density of 5×10⁶ cells/ml in enriched medium were preincubated with aspirin (1 μg/ml) for 2 h to irreversibly inactivate preexisting COX-1. After washing, cells were acti-
vated with KL (100 ng/ml), IL-10 (100 U/ml) and LPS (200 ng/ml) at 37°C for 8 h in the presence or absence of ethanol extract of *A. altissima* (EAA) previously dissolved in DMSO. All reactions were stopped by centrifugation at 120×g at 4°C for 5 min. The supernatant and cell pellets were immediately frozen in liquid N2 and stored at −80°C for further analysis. Under the conditions employed, COX-2-dependent phases of PGD2 generation reached approximately 4.7 ng/10⁶ cells without EAA. For the effect of EAA on COX-2 activity, COX-2 enzyme activity was measured using the colorimetric COX (ovine) inhibitor screening assay kit (Cayman Chemical, Ann Arbor, MI, U.S.A.) according to manufacturer’s instruction.

**Western Blot Analysis** After activation with KL, IL-10 and LPS, BMMC were washed once with 10 mM PBS (pH 7.4) containing 150 mM NaCl and lysed in PBS containing 0.1% SDS and 10 mM β-mercaptoethanol at 1×10⁶ cells/ml. The lysate (2×10⁵ cells equivalent) was applied to 10% SDS-polyacrylamide gels. After running the gel, the proteins were transferred in 20% methanol, 25 mM Tris, and 192 mM glycine to a nitrocellulose membrane. The membrane was blocked with 5% non-fat dry milk in TTBS (25 mM Tris–HCl, 150 mM NaCl, and 0.2% Tween-20) and probed with the COX-2 antibody (Cayman Chemical). After incubation for 1 h followed by washing three times, the membranes were incubated for 1 h with a secondary HRP-conjugated antibody. The protein bands were visualized using an ECL system (Amersham Biosciences, Piscataway, NJ, U.S.A.).

**Effect of EAA on 5-LOX Activity** BMMC suspended in enriched medium at cell density of 1×10⁶ cells/ml were pretreated with EAA for 30 min at 37°C. After 15 min of stimulation with KL (100 ng/ml), the supernatants were isolated for further analysis by EIA. LTC₄ was determined using laminar flow microplate reader (Perkin Elmer Life Sciences, Norwalk, CT, U.S.A.). One microgram of total RNA from each sample was used as template for RT reaction using 2.5 μmol oligo (dT)₁₅ primers and 5 U of Avian Myeloblastosis Virus (AMV) reverse transcriptase in 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 5 mM MgCl₂, 1 μg/ml RNase inhibitor and 1 mM dNTP mixture. Two micro-liter of RT product was used for PCR. PCR was carried out in a final volume of 20 μl containing 1 mM MgCl₂, 1X PCR buffer, 1 μM dNTP mixture, 1 μl Taq DNA polymerase, 0.2 μM each primer. Amplifications were carried out using a thermal cycler with the following profile: 5 min at 94°C before the first cycle, 30 s for denaturation at 94°C, 30 s for primer annealing, 30 s for extension at 72°C, and 5 min at 72°C after the last cycle. Primer sequences and PCR product sizes were as follows: eotaxin (5'-CCA AGG ACT TGG CTT CAT GTA G-3') and 5'-ATT CTG GCT TGG CAT GGT AGC-3', 495 bp, 25 cycles; IL-4, 5'-ACG GGA CCA CAG AGT TAT TGA TG-3' and 5'-ATG GTG GTG CAG TAC TAC GA-3', 454 bp, 35 cycles; IL-13, 5'-ACA GCA CCG TGG TTC TCT CA-3' and 5'-CCA GAA ACG TCT GAT GTG AG-3', 500 bp, 35 cycles. Actin-specific primers (5'-CAC CGG CCA CCA GTT CCC CA-3' and 5'-GAG TTC CCG GCC AGC CAG GT-3', 574 bp, 25 cycles) were used as the positive comparative controls. After amplification, 10 μl of each reaction mixture was analyzed by 1.2% agarose gel electrophoresis, and the bands were visualized with ethidium bromide staining.

**Allergen Sensitization, Challenge and EAA Treatment** Six weeks old female BALB/c were sensitized by intraperitoneal (i.p.) administration on days 0, 7, and 14 with 100 μg of OVA and equal volumes of alum (Pierce Biotechnology, Rockford, IL, U.S.A.) as an adjuvant in a total volume of 200 μl. On days 22 and 24, mice were exposed to aerosolized OVA (1% OVA in PBS) or PBS for 1 h. EAA or dexamethasone (DEX, Sigma) was treated for 7 times at 12 h intervals from days 21 to 23. At day 24, 18 h after the last aerosol, BALF and lung were obtained.

**Preparation of cDNA and RT-PCR** Total RNA from mouse lung was extracted with TRI ZOL reagent (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer’s instructions. RT-PCR was carried out using RNA PCR kit (Takara, Shiga, Japan) by thermal cycler (GeneAmp PCR system 2400, Perkin-Elmer, Norwalk, CT, U.S.A.). One microgram of total RNA from each sample was used as template for RT reaction using 2.5 μmol oligo (dT)₁₅ primers and 5 U of Avian Myeloblastosis Virus (AMV) reverse transcriptase in 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 5 mM MgCl₂, 1 μg/ml RNase inhibitor and 1 mM dNTP mixture. Two micro-liter of RT product was used for PCR. PCR was carried out in a final volume of 20 μl containing 1 mM MgCl₂, 1X PCR buffer, 1 μM dNTP mixture, 1 μl Taq DNA polymerase, 0.2 μM each primer. Amplifications were carried out using a thermal cycler with the following profile: 5 min at 94°C before the first cycle, 30 s for denaturation at 94°C, 30 s for primer annealing, 30 s for extension at 72°C, and 5 min at 72°C after the last cycle. Primer sequences and PCR product sizes were as follows: eotaxin (5'-CCA AGG ACT TGG CTT CAT GTA G-3') and 5'-ATT CTG GCT TGG CAT GGT AGC-3', 495 bp, 25 cycles; IL-4, 5'-ACG GGA CCA CAG AGT TAT TGA TG-3' and 5'-ATG GTG GTG CAG TAC TAC GA-3', 454 bp, 35 cycles; IL-13, 5'-ACA GCA CCG TGG TTC TCT CA-3' and 5'-CCA GAA ACG TCT GAT GTG AG-3', 500 bp, 35 cycles. Actin-specific primers (5'-CAC CGG CCA CCA GTT CCC CA-3' and 5'-GAG TTC CCG GCC AGC CAG GT-3', 574 bp, 25 cycles) were used as the positive comparative controls. After amplification, 10 μl of each reaction mixture was analyzed by 1.2% agarose gel electrophoresis, and the bands were visualized with ethidium bromide staining.

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dependent delayed responses. The immediate PGD2 generation occurs within 2 h and is associated with the coupling of COX-1 with the delayed PGD2 generation, which is observed after several hours of culturing (during 2—10 h). This is associated with the de novo induction and function of COX-2 after stimulation with particular combinations of cytokines and LPS.16) As shown in Fig. 2A, COX-2-dependent PGD2 generation was inhibited in a dose-dependent manner in the presence of EAa when the BMMC were stimulated with KL, IL-10, and LPS with an IC50 value of approximately 214.6 µg/ml. However, EAa did not affect the expression of COX-2 protein (Fig. 2B). In order to determine if EAa inhibited the delayed phase PGD2 generation via direct inhibition of COX-2 activity, the enzyme activity was measured. EAa inhibited the COX-2 dependent PGD2 generation in a concentration-dependent manner with an IC50 value of 47.4 µg/ml (Fig. 2C). These results clearly show that the reduced PGD2 production by EAa in the BMMC was due to the inhibition of COX-2 enzyme activity. For COX-1 dependent PGD2 generation, EAa inhibited PGD2 production in a dose-dependent manner with IC50 value of 131.66 µg/ml (data not shown).

RESULTS AND DISCUSSION

BMMC exhibit biphasic PGD2 synthetic responses over time, as well as COX-1-dependent immediate and COX-2-dependent delayed responses. The immediate PGD2 generation occurring within 2 h is associated with the coupling of COX-1 with the delayed PGD2 generation, which is observed after several hours of culturing (during 2—10 h). This is associated with the de novo induction and function of COX-2 after stimulation with particular combinations of cytokines and LPS.16) As shown in Fig. 2A, COX-2-dependent PGD2 generation was inhibited in a dose-dependent manner in the presence of EAa when the BMMC were stimulated with KL, IL-10, and LPS with an IC50 value of approximately 214.6 µg/ml. However, EAa did not affect the expression of COX-2 protein (Fig. 2B). In order to determine if EAa inhibited the delayed phase PGD2 generation via direct inhibition of COX-2 activity, the enzyme activity was measured. EAa inhibited the COX-2 dependent PGD2 generation in a concentration-dependent manner with an IC50 value of 47.4 µg/ml (Fig. 2C). These results clearly show that the reduced PGD2 production by EAa in the BMMC was due to the inhibition of COX-2 enzyme activity. For COX-1 dependent PGD2 generation, EAa inhibited PGD2 production in a dose-dependent manner with IC50 value of 131.66 µg/ml (data not shown).

BMMC have been used in our laboratory for screening 5-LOX inhibitors because the immediate LTC4 generation elicited by the IgE-dependent or cytokines occurs in BMMC via 5-LOX.17) Therefore, the inhibitory activity of EAa on the generation of LTC4 in the BMMC was examined. Figure 3 shows that the BMMC stimulated with KL for 15 min produced approximately 2.0 ng/ml of LTC4, and preincubation of the BMMC with EAa resulted in the dose-dependent suppression of LTC4 generation with an IC50 value of 25.7 µg/ml.

Mast cells are involved in allergic disorders by virtue of their ability to be activated through FcεRI or cytokine receptors. Activated mast cells release a number of biologically active molecules, including histamine, serotonin, proteoglycans and neutral proteases. Among these molecules, histamine is one of the most important chemical mediators in the pathologic allergic reaction.18) The release of histamine bears a close parallel to that of β-HEX, which is one of degranulation marker. Therefore, the inhibitory activity of EAa on the degranulation reaction in the BMMC was examined. As shown in Fig. 4, EAa caused the dose-dependent inhibition of β-HEX release with an IC50 value of 27.3 µg/ml. Under the conditions employed, β-HEX was released about 33—35% without the pretreatment of EAa.

On allergen challenge of airways, the mast cells release preformed and synthesized mediators. Among those mediators, histamine, LTs, and PGs whose increased production has been demonstrated in asthma have significant effects on the eosinophil activation and chemotaxis.19—23) Because EAa inhibited PGD2 and LTC4 production as well as β-HEX release in BMMC, these results have led us to determine the biological activity of EAa in in vivo animal model. The number of total leukocytes in the BALF obtained from the PBS-
challenged group was 0.88\(\pm\)0.21\(\times\)10^5 cells, but relatively few eosinophils was detected in this group. On the other hand, the total number of leukocytes (6.2\(\pm\)1.5\(\times\)10^6) and eosinophils (3.84\(\pm\)0.04\(\times\)10^5) in the BALF of the OVA-challenged lung tissues was significantly higher compared with the PBS-challenged group (data not shown). When 100—400 mg/kg of EAa was administered orally to the mice 7 times, the number of eosinophils was weakly reduced in a dose-dependent manner (Fig. 5). Under the same conditions, DEX (5 mg/kg) significantly inhibited the recruitment of eosinophils. EAa was orally administered to mice up to 800 g/kg for 14 d to determine the acute toxicity. No apparent dose-effect was observed (data not shown), indicating that this plant material by oral administration may be used safely in human at moderate doses.

Clinical and experimental investigations have reported a strong correlation between the presence of CD4^+ T_{H2} cells and disease severity, suggesting an integral role for these cells in the pathophysiology of asthma.24—25 Among the T_{H2} cytokines, high levels of IL-4 and IL-13 were produced at an asthmatic lung and are believed to be the key regulators of many of hallmark features of disease.26—28 Accumulated studies have also suggested an important role of eotaxin in allergic inflammatory disease, especially in the early of eosinophil recruitment after allergen challenge.29—32 Therefore, the effect of EAa on the mRNA expression level of the eotaxin and T_{H2} cytokines was examined because EAa reduced the number of infiltrated eosinophils in the BALF. As shown in Fig. 6, the expression of eotaxin and T_{H2} cytokine mRNA was very weak and almost undetectable, respectively in the normal lung tissue whereas those mRNA were induced in the OVA-challenged mice compared with the PBS-challenged mice. However, pretreatment of OVA-challenged mice with EAa reduced the transcription levels of eotaxin, IL-4, and IL-13. DEX also inhibited mRNA expression of those genes from the OVA-challenged lung. Since IL-5 is an important factor in recruiting eosinophils in allergenic tissues, the effect of EAa on the mRNA expression level of IL-5 was examined. Pretreating the OVA-challenged mice with EAa did not effectivly inhibit IL-5 mRNA expression (Fig. 6), which indicating IL-5 in conjunction with eotaxin may cause a marked increase in eosinophils. Fujitani et al. reported that PGD_{2} overproduction increased IL-4, IL-5 and eotaxin in BALF of transgenic mice overexpressing human PGD synthase.33 In addition, deletion of PGD_{2} receptor in mice markedly reduced the pulmonary eosinophilia and AHR, which was accompanied by reduced levels of the T_{H2} cytokines.34 Furthermore, it was shown that LTC_{4} could stimulate eotaxin production by IL-13-treated fibroblasts, thereby indirectly inducing eosinophilic inflammation.35 Overproduction of eicosanoids in the airway asthma may cause an increase in the level of T_{H2} cytokines and an eotaxin accompanied by the enhanced accumulation of eosinophils in the lung. Therefore, the reduction of infiltrated eosinophils in the lung by EAa may occur in part via the inhibition of eicosanoid generation, which reduced directly or indirectly the level of eotaxin and T_{H2} cytokines.

In conclusion, the present paper shows that A. altissima has anti-inflammatory activities in in vitro as well as in vivo OVA-induced lung. This study suggests that some herbal medicines such as A. altissima may be useful for treating inflammation related disorders such as asthma.

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