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Artemisolide from *Artemisia asiatica*: Nuclear Factor-κB (NF-κB) Inhibitor Suppressing Prostaglandin E_2 and Nitric Oxide Production in Macrophages

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Aerial parts of *Artemisia asiatica* (Compositae) have been traditionally used as an oriental medicine for the treatment of inflammatory and ulcerogenic diseases. In the present study, artemisolide was isolated as a nuclear factor (NF)-κB inhibitor from *A. asiatica* by activity-guided fractionation. Artemisolide inhibited NF-κB transcriptional activity in lipopolysaccharide (LPS)-stimulated macrophages RAW 264.7 with an IC_{50} value of 5.8 μM. The compound was also effective in blocking NF-κB transcriptional activities elicited by the expression vector encoding the NF-κB p65 or p50 subunits bypassing the inhibitory kB degradation signaling NF-κB activation. The macrophages markedly increased their PGE_2 and NO production upon exposure to LPS alone. Artemisolide inhibited LPS-induced PGE_2 and NO production with IC_{50} values of 8.7 μM and 6.4 μM, respectively, but also suppressed LPS-induced synthesis of cyclooxygenase (COX)-2 or inducible NO synthase (iNOS). Taken together, artemisolide is a NF-κB inhibitor that attenuates LPS-induced production of PGE_2 or NO *via* down-regulation of COX-2 or iNOS expression in macrophages RAW 264.7. Therefore, artemisolide could represent and provide the anti-inflammatory principle associated with the traditional medicine, *A. asiatica*.

Key words: Artemisolide, *Artemisia asiatica*, Nuclear factor-κB, Prostaglandin E_2, Nitric oxide, Macrophages

INTRODUCTION

Host defensive mechanisms against bacterial or viral infection require rapid and accurate transmission of signals from cell-surface receptors to the cell nucleus. These signaling pathways lead to the activation of specific transcription factors that induce the expression of appropriate target genes. Among the transcription factors, nuclear factor (NF)-κB is essential for innate immunity. NF-κB is functional as the hetero- or homo-dimeric form of Rel family proteins, including p65 and p50 subunits, and is sequestered as an inactive complex in the cytoplasm bound to inhibitory κB (IκB) proteins (Beg *et al.*, 1992; Baeuerle and Baltimore, 1996). Upon exposure to lipopolysaccharide (LPS), immune cells, such as macrophages, recognize LPS by their toll-like receptor 4, which triggers signaling pathways to activate IkB kinase (IKK) resulting in phosphorylation of IkB that can be further subjected to ubiquitin-dependent degradation (Israel, 2000; Magnani *et al.*, 2000; Akira, 2001). NF-κB moves into the nucleus and regulates the expression of immune and inflammatory genes, including cyclooxygenase (COX)-2 and inducible nitric oxide (NO) synthase (iNOS) (Guha and Mackman, 2001; Tian and Brasier, 2003).

Nonsteroidal anti-inflammatory drugs (NSAIDs) have been widely used for the relief of inflammation and pain. It is generally accepted that anti-inflammatory or analgesic efficacy of the NSAIDs is dependent on their inhibitory effects on the enzymic activity of COX, a key enzyme in the biosynthetic pathway of prostaglandins (PGs) starting from arachidonic acid (Vane and Botting, 1998). More recently, two distinct forms of COX were identified as constitutive COX-1 and inducible COX-2 (Vane *et al.*, 1998) wherein COX-1 provides PGs to maintain physiological

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functions e.g. cytoprotection of the stomach and regulation of renal blood flow (Vane *et al.*, 1998) and COX-2 is inducible in immune cells e.g. macrophages, in response to infection or injury for producing excess amounts of PGs that sensitize nociceptors and induce inflammatory states (Needleman and Isakson, 1997; Vane *et al.*, 1998). Even though COX inhibition could be important for the pharmacological activity of NSAIDs, some NSAIDs could modulate COX-independent cellular responses, including NF-κB activation, which is also involved in the anti-inflammatory activity of the drugs (Tegeder *et al.*, 2001).

NO is a free radical gas generated from L-arginine by the catalytic action of NO synthase (NOS). Endothelial NOS and neuronal NOS produce appropriate amounts of NO, which mediate physiological responses such as vasodilation and neurotransmission (Prast and Philippu, 2001; Ignarro, 2002). Meanwhile, NO is also produced in the immune system by iNOS, where NO facilitates the killing of invading bacteria or viruses (MacMicking *et al.*, 1997). High-output NO by iNOS, however, can provoke several inflammation-related diseases (Blantz and Munger, 2002).

In our earlier efforts to discover and develop anti-inflammatory agents from medicinal plants, we identified *trans*-cinnamaldehyde and methoxycinnamaldehyde as the NF-κB inhibitors (Reddy *et al.*, 2004). *Artemisia asiatica* is a plant of the Compositae family whose aerial parts have been traditionally used as an oriental medicine for treating inflammatory and ulcerogenic diseases. In the present study, artemisolide (Fig. 1) was isolated as a NF-κB inhibitor from *A. asiatica* by activity-guided fractionation and its inhibitory effects on PGE_2 and NO production were investigated by using LPS-stimulated macrophages RAW 264.7.

MATERIALS AND METHODS

Materials

Organic solvents were purchased from Ducksan (Taejon, Korea), and pre-coated silica gel plates from Merck (Darmstadt, Germany). Fetal bovine serum (FBS) and other culture materials were purchased from Invitrogen (Carlsbad, CA, U.S.A.), and antibodies against COX-2 or iNOS from Santa Cruz Biotech (Santa Cruz, CA, U.S.A.). The other chemicals including LPS (*Escherichia coli* 055:B5) and parthenolide, were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.).

Activity-guided extraction and isolation

Aerial parts of *A. asiatica* were collected at the hillsides of Geryong Mountain, Korea in October, 2002 and the plant materials were authenticated by Prof. K.S. Lee, Chungbuk National University, Korea. The plant materials (6 kg) were exclusively extracted with 80% methanol (MeOH) at room temperature for seven days and then evaporated at 40°C under reduced pressure to dryness. The MeOH extract (826 g) was resuspended in distilled water and then successively partitioned with methylene chloride (MC), ethyl acetate (EtOAc) and *n*-butanol (BuOH). As shown in Table I, extracts were further fractionated according to their inhibitory effects on NF-κB transcriptional activity. The MC extract (269 g) was loaded onto a silica gel column (15 × 100 cm, 5 kg) and eluted with 5 L each of EtOAc/*n*-hexane mixtures (0:1, 3:7, 1:1, 7:3, 1:0) to yield five fractions (F1 to F5), respectively. The F3 extract (12 g) was loaded onto a silica gel column (5 × 100 cm, 500 g), and eluted with 2 L each of EtOAc/*n*-hexane mixtures (1:9, 3:7, 1:1, 7:3, 9:1) to yield five fractions (F31 to F35), respectively. An NF-κB inhibitor (54 mg) was isolated from the F34 extract (2.9 g), and identified as artemisolide by comparison of spectra data (Kim *et al.*, 2002).

Artemisolide

Amorphous white powder; $C_{25}H_{32}O_4$ (M 396); ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 6.08 (1H, d, *J*=3.6 Hz), 6.03 (1H, d, *J*=5.6 Hz), 6.00 (1H, d, *J*=5.6 Hz), 5.33 (1H, d, *J*=3.6 Hz), 4.04 (1H, t, *J*=10.0 Hz), 3.32 (1H, m), 2.70 (1H, d, *J*=10.2 Hz), 2.49 (1H, dd, *J*=2.4, 18.4 Hz), 2.34 (1H, dd, *J*=2.4, 18.4 Hz), 2.27 (1H, d, *J*=8.0 Hz), 2.23 (1H, m), 1.85 (1H, m), 1.57 (1H, m), 1.37 (1H, m), 1.33 (1H, d, *J*=8.0 Hz), 1.26 (1H, dd, *J*=3.6, 8.0 Hz), 1.05 (1H, d, *J*=6.6 Hz), 0.87 (1H, d, *J*=6.9 Hz), 0.55 (1H, m), 0.12 (1H, m); ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 220.4, 170.6, 141.2, 138.1, 137.7, 118.3, 80.0, 76.7, 64.8, 64.2, 63.7, 60.0, 44.7, 43.1, 41.5, 34.7, 32.2, 29.8, 28.2, 27.3, 23.7, 20.1, 19.3, 15.2, 14.8.

Cell culture and sample treatment

Murine macrophages RAW 264.7 were cultured in DMEM (13.4 mg/mL Dulbecco's modified Eagle's medium, 24 mM $NaHCO_3$, 10 mM HEPES, 143 U/mL benzylpenicillin potassium, 100 μg/mL streptomycin sulfate, pH 7.1) containing 10% FBS and maintained at 37°C with 5% CO_2 atmosphere. The RAW 264.7 cells harboring pNF-κB-secretory alkaline phosphatase (SEAP)-neomycin phosphotransferase (NPT) reporter construct (Moon *et al.*, 2001) were cultured under the same conditions except supplement with geneticin (500 μg/mL). Artemisolide (purity >95%) was dissolved in 100% dimethylsulfoxide (DMSO) to make a 100 mM stock and freshly diluted to the indicated concentrations with culture media.

SEAP assay

Macrophages RAW 264.7 harboring the pNF-κB-SEAP-NPT construct ((Moon *et al.*, 2001) were pretreated with sample for 2 h and stimulated with LPS (1 μg/mL) for 16

h. Aliquots of the culture media were heated at 65°C for 5 min, reacted with 4-methylumbelliferyl phosphate (500 μM) in an assay buffer (2 M diethanolamine, 1 mM $MgCl_2$) in the dark for 1 h, and then relative fluorescence units (RFU) were measured by emission at 449 nm and excitation at 360 nm. In another experiment, macrophages RAW 264.7 harboring the pNF-κB-SEAP-NPT construct were transiently transfected with expression vector encoding the NF-κB p65 or p50 subunits (Garcia-Pineres *et al.*, 2004) and the cells were treated with samples for 16 h prior to the SEAP assay.

Enzyme-linked immunosorbent assay (ELISA)

Macrophages RAW 264.7 were pretreated with sample for 2 h and stimulated with LPS (1 μg/mL) for 24 h. The amounts of PGE_2 in the culture media were quantified using an ELISA kit following the supplier's protocol (Amersham-Pharmacia, San Francisco, CA, U.S.A.).

NO quantification

Macrophages RAW 264.7 were pretreated with samples for 2 h and stimulated with LPS (1 μg/mL) for 24 h. The amounts of NO in the culture media were measured using the Griess reaction (Archer, 1993) as follows. Briefly, cell-free culture media (100 μL) were reacted with an 1:1 mixture (100 μL) of 1% sulfanilamide in 5% H_3PO_4 and 0.1% *N*-(1-naphthyl)ethylenediamine in distilled water, and the absorbance values were measured at 540 nm.

Western blot analysis

Macrophages RAW 264.7 were pretreated with samples for 2 h and stimulated with LPS (1 μg/mL) for 18 h. Lysates of the cells were resolved on SDS-acrylamide gel by electrophoresis and transferred to polyvinylidine difluoride membranes. The membranes were incubated with anti-COX-2 antibody (1:500) or anti-iNOS antibody (1:300) overnight at room temperature. After incubation with horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:2500) for 5 h, the blots were treated with ECL reagents (Amersham-Pharmacia, San Francisco, CA, U.S.A.) and then exposed to X-ray film.

WST-1 assay

Macrophages RAW 264.7 were incubated with various concentrations of samples for 24 h. The cells were treated with WST-1 solution (Dojindo Lab, Kumamoto, Japan) and the absorbance values were measured at 450 nm.

Statistical analysis

The data are expressed as means ± standard error (SE) and analyzed by ANOVA and followed by the Dunnet test. Values of $p<0.01$ were considered significant.

RESULTS AND DISCUSSION

An ethanol extract (DA-9601) of *A. asiatica* was previously reported to reduce 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced ear edema in mice and to inhibit NF-κB activation resulting in down-regulation of iNOS and COX-2 expressions in TPA-stimulated mouse skin (Seo *et al.*, 2002). However, active constituents of DA-9601 showing anti-inflammatory activity remain to be elucidated. In the present study, *A. asiatica* was subjected to an activity-guided fractionation as shown in Table I and artemisolide (Fig. 1), a sesquiterpene-monoterpene lactone, was isolated as the inhibitor of NF-κB transcriptional activity in LPS-stimulated macrophages. NF-κB transcriptional activity was monitored using macrophages RAW 264.7 harboring the pNF-κB-SEAP-NPT construct encoding four copies of kB sequences fused to SEAP as a reporter

Table I. Inhibitory effects on LPS-induced NF-κB transcriptional activity by chromatographic fractions of *A. asiatica*

Sample	Dose	Inhibition
Fractions of MeOH extract		
MC layer	10 μg/mL	72.4 ± 2.4%*
EtOAc layer	10 μg/mL	33.5 ± 5.1%*
BuOH layer	10 μg/mL	4.1 ± 3.3%
Polar residue	10 μg/mL	0.7 ± 2.7%
Fractions of MC layer		
F1	7.5 μg/mL	1.7 ± 4.5%
F2	7.5 μg/mL	2.3 ± 3.9%
F3	7.5 μg/mL	89.7 ± 3.4%*
F4	7.5 μg/mL	63.0 ± 2.9%*
F5	7.5 μg/mL	56.3 ± 5.3%*
Fractions of F3		
F31	5 μg/mL	15.4 ± 3.7%
F32	5 μg/mL	23.5 ± 6.4%
F33	5 μg/mL	41.4 ± 5.0%*
F34	5 μg/mL	91.1 ± 4.6%*
F35	5 μg/mL	73.8 ± 3.3%*

* $p<0.01$ vs. LPS alone-treated group.

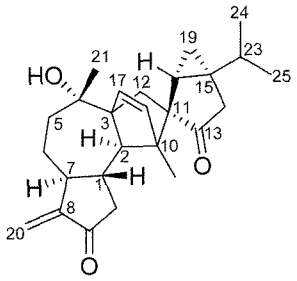

Fig. 1. Chemical structure of artemisolide

(Moon *et al.*, 2001). Upon exposure to LPS alone, the transfected cells increased SEAP expression by up to 3-fold over the basal level, indicating that cellular NF-κB is transcriptionally functional (Fig. 2A). No significant differences in the SEAP expression were found between resting RAW 264.7 cells and the cells treated with artemisolide (10 μM) (Fig. 2A). Artemisolide inhibited LPS-induced SEAP expression in a dose-dependent manner, corresponding to 35% inhibition at 3 μM, 59% at 6 μM and 86% at 10 μM with an IC_{50} value of 5.8 μM (Fig. 2A). As a positive control, parthenolide exhibited a dose-dependent inhibitory effect on the LPS-induced SEAP expression with an IC_{50} value of 3.2 μM (Fig. 2A). Neither artemisolide nor parthenolide, at the effective concentrations showed significant cytotoxicity to macrophages RAW 264.7 (data not shown).

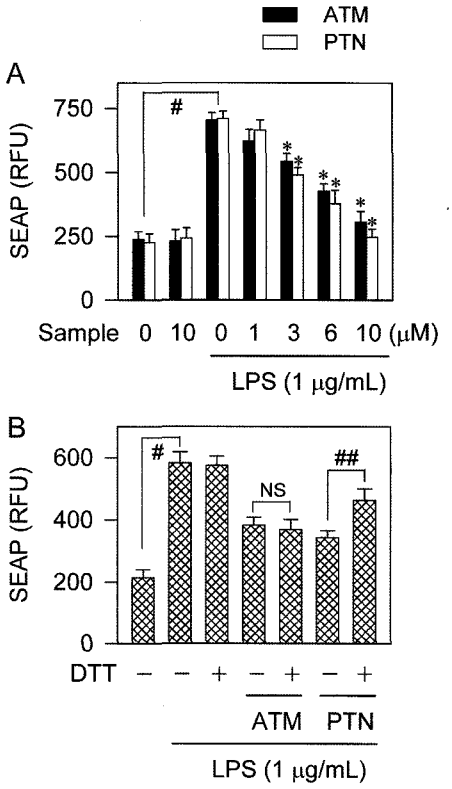

Fig. 2. Effect of artemisolide on LPS-induced NF-κB transcriptional activity. (A) Macrophages RAW 264.7 harboring the pNF-κB-SEAP-NPT construct were pretreated with artemisolide (ATM) or parthenolide (PTN) and then stimulated with LPS. (B) The RAW 264.7 cells harboring the pNF-κB-SEAP-NPT construct were pretreated with ATM (5 mM) or PTN (5 mM), in the presence or absence of dithiothreitol (DTT, 100 mM), and then stimulated with LPS. As a reporter for NF-κB transcriptional activity, SEAP expression was measured with the culture media and is represented as relative fluorescence units (RFU). Values are means ± SE from five independent experiments. $^{\#}p<0.01$ vs. media alone-treated group. $^{*}p<0.01$ vs. LPS alone-treated group. $^{\#\#}p<0.01$ between paired groups. NS, no significant difference.

Artemisolide and other guaianolide sesquiterpene lactones were isolated as NF-κB inhibitors from *A. sylvatica* (Jin *et al.*, 2004) while this study was in progress. However, an inhibitory mechanism of artemisolide on NF-κB activation has not been documented. Sesquiterpene lactones, such as parthenolide and helenalin, have been determined to block the NF-κB activating pathway targeting DNA binding activity of the NF-κB p65 subunit with a slight inhibition of IκB degradation (Hehner *et al.*, 1999; Garcia-Pineres *et al.*, 2001). The α,β- or α,β,γ-unsaturated carbonyl structures e.g. α-methylene-g-lactones or α,β-unsubstituted cyclopentenones, in the sesquiterpene lactones are known to react with nucleophiles. especially with cysteine sufhydryl groups. in the Michael-type addition (Schmidt, 1997). The inhibitory effect of parthenolide on LPS-induced NF-κB transcriptional activity was decreased in the presence of dithiothreitol, but not with artemisolide (Fig. 2B). Further, macrophages RAW 264.7 harboring the pNF-κB-SEAP-NPT construct were transiently transfected with expression vector encoding the NF-κB p65 or p50 subunits, in an attempt to understand whether artemisolide directly interacts with the cellular NF-κB complex, bypassing the IκB degradation signaling for LPS-induced NF-κB activation. Artemisolide was effective in inhibiting both NF-κB transcriptional activities elicited by overexpression of the NF-κB p65 or p50 subunits (Fig. 3). However, parthenolide inhibited the NF-κB transcriptional activity elicited by overexpression of the NF-κB p65 subunit but did not affect the NF-κB transcriptional activity by NF-κB p50 subunit (Fig. 3), which is consistent with previous reports (Garcia-Pineres *et al.*, 2001, 2004). Therefore, artemisolide seems to differ in its NF-κB-inhibitory mechanism compared to parthenolide, although the biochemical analyses remain to be clarified.

NF-κB is a transcription factor that has been shown to up-regulate the expression of the LPS-inducible COX-2 or iNOS genes (Guha and Mackman, 2001; Tian and Brasier, 2003). An ELISA was carried out to investigate whether artemisolide affects PGE_2 production in the LPS-stimulated macrophages RAW 264.7. While in a resting state, the macrophages produced 959 ± 372 pg/mL of PGE_2 during 24 h incubation, whereas the cells markedly increased PGE_2 production, up to 9870 ± 591 pg/mL, upon exposure to LPS (Fig. 4A). Artemisolide inhibited LPS-induced PGE_2 production in a dose-dependent manner, corresponding to 11% inhibition at 5 μM, 63% at 10 μM and 98% at 15 μM with an IC_{50} value of 8.7 μM (Fig. 4A). As a positive control, parthenolide (5 μM) also exhibited 73% inhibition on LPS-induced PGE_2 production (Fig. 4A). We next performed Western blot analysis to determine whether artemisolide can affect COX-2 expression in LPS-stimulated macrophages RAW 264.7. COX-2 protein was hardly undetectable in the resting macrophages, but

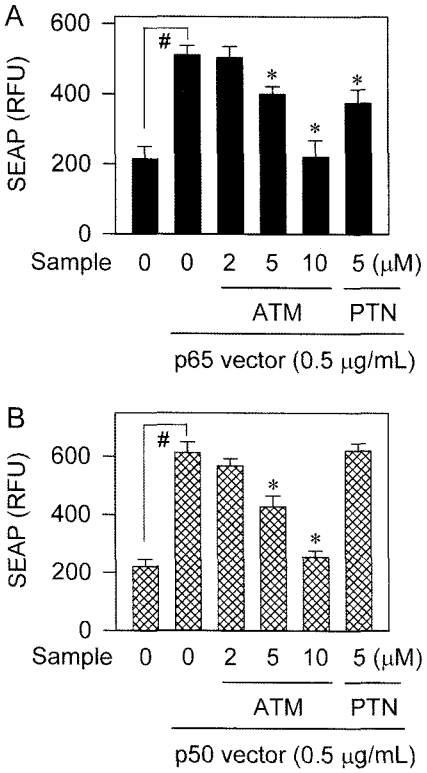

Fig. 3. Effect of artemisolide on NF-κB transcriptional activity elicited by the expression vector encoding the NF-κB p65 or p50 subunit. Macrophages RAW 264.7 harboring the pNF-κB-SEAP-NPT reporter construct were transiently transfected with expression vector encoding the NF-κB p65 (A) or p50 subunit (B). The transfected cells were treated with artemisolide (ATM) or parthenolide (PTN), and SEAP expression as the NF-κB transcriptional activity was then measured with the culture media and represented as relative fluorescence units (RFU). Values are means ± SE from three independent experiments. #$p<0.01$ vs. the reporter construct alone-transfected group. *$p<0.01$ vs. the reporter construct plus each expression vector alone-transfected group.

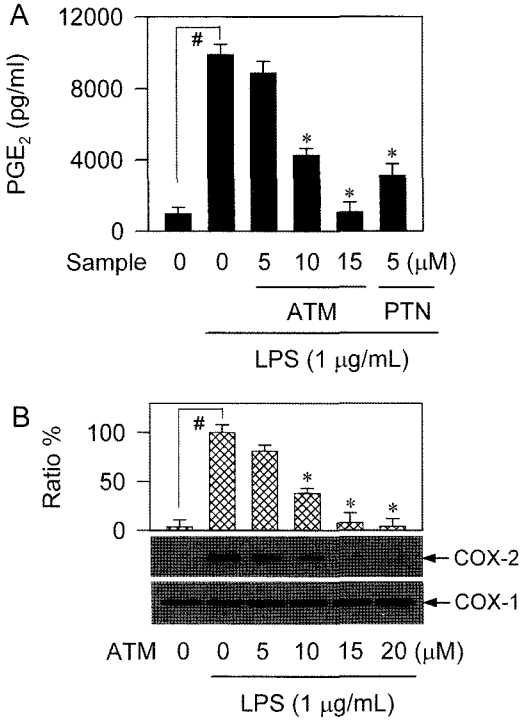

Fig. 4. Effect of artemisolide on LPS-induced PGE_2 production and COX-2 synthesis. Macrophages RAW 264.7 were pretreated with artemisolide (ATM) or parthenolide (PTN) and stimulated with LPS. (A) Amounts of PGE_2 in the culture media were quantified using an ELISA. (B) Lysates of the cells were subjected to Western blot analysis with anti-COX-2 antibody. A representative result is shown with the relative ratio % where the COX-2 signal was normalized to the COX-1 signal. Values are means ± SE from three independent experiments. #$p<0.01$ vs. media alone-treated group. *$p<0.01$ vs. LPS alone-treated group.

markedly increased upon exposure to LPS (Fig. 4B). Artemisolide inhibited LPS-induced synthesis of COX-2 protein in a dose-dependent manner, corresponding to 16% inhibition at 5 μM, 62% at 10 μM and 92% at 15 μM (Fig. 4B). However, the amounts of COX-1 protein were not changed by treatment of LPS or artemisolide (Fig. 4B).

NO is another chemical mediator implicated in LPS-induced inflammatory states (Blantz and Munger, 2002). Macrophages RAW 264.7 in the resting state produced 6.9 ± 2.1 μM of nitrite, a stable metabolite of NO, during incubation for 24 h, whereas the cells markedly increased nitrite production, up to 47.2 ± 3.5 μM, upon exposure to LPS (Fig. 5A). Artemisolide inhibited LPS-induced NO production in a dose-dependent manner, corresponding to 22% inhibition at 5 mM, 72% at 10 μM and 93% at 15 μM with an IC_{50} value of 6.4 μM (Fig. 5A). The positive control, parthenolide (5 μM) exhibited 64% inhibition on LPS-induced NO production (Fig. 5A). The expression of iNOS was also documented by Western blot analysis using LPS-stimulated macrophages RAW 264.7. The iNOS protein was undetectable in resting macrophages, but markedly increased upon exposure to LPS (Fig. 5B). Artemisolide inhibited LPS-induced synthesis of the iNOS protein in a dose-dependent manner, corresponding to 36% inhibition at 5 μM, 57% at 10 μM and 71% at 15 μM (Fig. 5B). The amounts of housekeeping glyceraldehyde 3-phosphate dehydrogenase (GAPDH), however, were not affected by treatment of LPS or artemisolide (Fig. 5B)

Aerial parts of *A. asiatica* have been traditionally used as an oriental medicine in treating inflammatory and ulcerogenic diseases. Eupatilin was reported as the antiulcer constituent from *A. asiatica* (Kim *et al.*, 1997) and other biological activities have also been characterized for the *Artemisia* species. Flavones of eupatilin and 4'-demethyleupatilin were identified as 5-lipoxygenase inhibitors from *A. rubripes*, and sesquiterpene lactones of

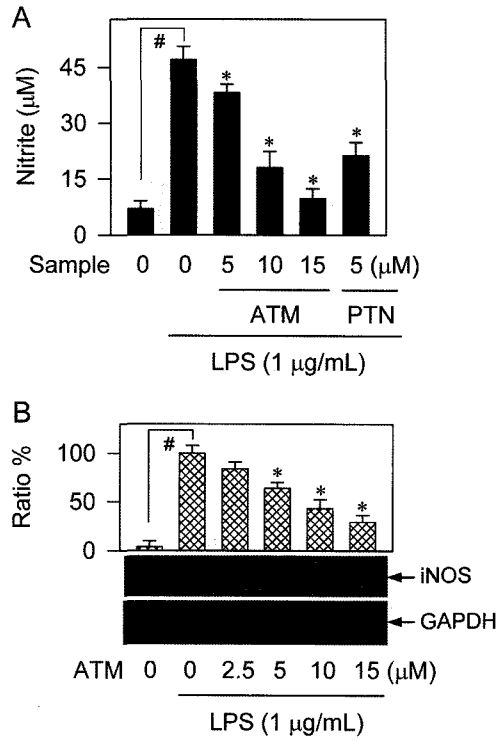

Fig. 5. Effect of artemisolide on LPS-induced NO production and iNOS synthesis. Macrophages RAW 264.7 were pretreated with artemisolide (ATM) or parthenolide (PTN) and stimulated with LPS. (A) Amounts of NO in the culture media were quantified using the Griess reaction. (B) Lysates of the cells were subjected to Western blot analysis with anti-iNOS antibody. A representative result is shown with the relative ratio % where the iNOS signal was normalized to the GAPDH signal. Values are means ± SE from three independent experiments. $^{\#}p<0.01$ vs. media alone-treated group. $^{*}p<0.01$ vs. LPS alone-treated group.

dihydroepideoxyarteannulin and deoxyartemisinin as the antiulcerogenic activity of *A. annua* (Koshihara *et al.*, 1983; Foglio *et al.*, 2002). Recently, extracts of *A. alba* were reported to inhibit IL-1-induced activation of NF-κB, but their active constituents remain to be determined (Stalinska *et al.*, 2005).

In conclusion, artemisolide is an NF-κB inhibitor isolated from *A. asiatica* by activity-guided fractionation, and was shown to inhibit LPS-induced production of PGE_2 or NO, via down-regulation of COX-2 or iNOS expression, in the macrophages RAW 264.7. Artemisolide likely represents an anti-inflammatory principle associated with the traditional medicine of *A. asiatica*.

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