대한한의학회지 제26권 제1호(2005년 3월)
J Korean Oriental Med 2005;26(1):26-36

원 저

Inhibition of Nitric Oxide Synthesis by Methanol and Butanol Extracts of *Euonymus Alatus* (Thunb.) Sieb in Murine Macrophages

Hyo-Hyun Lee, Young-Soo Park, Ra-Young Kim, Dong-Il Kim, Tae-Kyun Lee[1)]

Department of Gynecology, College of Oriental Medicine, Dongguk University.
Dr. Lee's Traditional Korean Medical Clinic[1)]

Objective : Many traditional herbal remedies exhibit several beneficial effects including anti-inflammation. *Euonymus alatus* (Thunb.) Sieb (EA), known as *Gui jun woo* in Korea, has long been used in folk medicine to regulate Qi (bodily energy) and blood circulation, relieve pain, eliminate stagnant blood, and treat dysmenorrhea in oriental countries. The exact mechanism of the anti-inflammatory action of *Euonymus alatus* (Thunb.) Sieb (EA), however, has not been determined.

Methods : Since there is increasing evidence that nitric oxide (NO) plays a crucial role in the pathogenesis of inflammatory diseases, this study was undertaken to address whether the methanol (MeOH) extract and its fractions of the bark of EA could modulate the expression of inducible NO synthase (iNOS) in thioglycollate-elicited murine peritoneal macrophages and murine macrophage cell line, RAW264.7 cells.

Results : Stimulation of the peritoneal macrophages and RAW264.7 cells with interferon-γ (IFN-γ) and lipopolysaccharide (LPS) resulted in increased production of NO in the medium. However, the butanol (BuOH) fraction of the MeOH extract of EA barks showed marked inhibition of NO synthesis in a dose-dependent manner. The inhibition of NO synthesis was reflected in the decreased amount of iNOS protein, as determined by Western blotting. The BuOH fraction did not affect the viability of RAW264.7 cells, as assessed by methylthiazol-2-yl-2, 5-diphenyl tetrazolium bromide (MTT) assay; rather, it reduced endogenous NO-induced apoptotic cell death via inhibition of NO synthesis in RAW264.7 cells. On the other hand, the MeOH and BuOH fraction showed no inhibitory effect on the synthesis of NO by RAW264.7 cells, when iNOS was already expressed by the stimulation with IFN-γ and LPS.

Conclusion : Collectively, these results demonstrate that the MeOH and BuOH fraction inhibits NO synthesis by inhibition of the induction of iNOS in murine macrophages.

Key Words: *Euonymus alatus* (Thunb.) Sieb (EA), nitric oxide (NO), inducible NO synthase (iNOS), apoptotic cell death, murine macrophages

Introduction

Nitric oxide (NO) plays a dual role in the inflammatory process. There is substantial evidence that NO has an anti-inflammatory role, usually shown under short-term experimental conditions[1)]. NO has also been shown to be an important regulatory molecule in diverse physiological functions such as vasodilation, neural communication, and host defense[2,3)]. These beneficial effects primarily involve smooth muscle relaxation and decreased leukocyte-blood vessel wall interactions. Both effects are mediated by increasing concentrations of intracellular 5' -cyclic guanosine monophosphate. The contrasting, pro-inflammatory

· 접수 : 2004년 8월 28일 · 논문심사 : 2004년 11월 6일
· 채택 : 2004년 12월 9일
· Correspondence to : Dong-Il Kim, Gangnam Oriental Medical Hospital, Dongguk University 37-21 Nonhyun-Dong, Gangnam-Gu, Seoul 135-010, Korea; Tel: 82-2-3416-9737, Fax: 82-2-3416-9770, E-mail: obgykdi@netian.com

effects of NO seem to be mediated by free radical mechanisms and associated with the expression of the inducible form of NO synthase (iNOS)[4,5]. Molecular cloning and sequencing analysis have revealed the existence of at least three main types of NOS isoforms[6-8]. NOS present in the vascular endothelium (eNOS) and that in central and peripheral neurons (nNOS) are constitutive (cNOS), and their activation is Ca^{2+}-dependent. Continuous release of NO by cNOS plays a role in keeping the vasculature in an active state of vasodilation. Various agonists such as bradykinin and acetylcholine have been shown to trigger cNOS-mediated NO production through increasing intracellular Ca^{2+}. NOS in macrophages and hepatocytes, on the other hand, is inducible (iNOS), and its activation is Ca^{2+}-independent[9,10]. After exposure to endotoxin and/or cytokines, iNOS can be induced in various cells such as macrophages, Kupffer cells, smooth muscle cells, and hepatocytes. The induced iNOS catalyzes the formation and release of a large amount of NO, which plays a key role in the pathophysiology of a variety of diseases including septic shock[11-14]. NO production catalyzed by iNOS, therefore, may reflect the degree of inflammation and provides a measure by which effects of drugs on the inflammatory process can be assessed. Expression of COX-2 in various tissue preparations following LPS treatment also has been reported[15-17]. This enzyme is considered to play a major role in the inflammatory process by catalyzing the production of prostaglandins. Although many studies have demonstrated that cytokine-induced NO production can lead to destruction or damage of parasites or tumor cells, NO has also been linked to damage and death of normal cells. Increased NO production has been associated with a range of inflammatory diseases including rheumatoid arthritis, ulcerative colitis, and in experimental models of gut injury[18-22].

A number of traditional plant-derived medicines have been found to be rich in polyphenolic compounds[23]. These include flavonoids, tannins, and anthraquinones and have been shown to exhibit anti-inflammatory activities[24-26]. The exact mechanism of anti-inflammatory action of these compounds is not established. The possibility that these compounds exhibit their biological effects by blocking iNOS and COX-2 expression, therefore, was examined in the present study. *Euonymus alatus* (Thunb.) Sieb (EA) is a deciduous tree which is widely distributed in Korea. The barks of the stem and the root of this plant have been used in oriental traditional medicine for the treatment of oedema, mastitis, gastric cancer, and inflammation[27]. EA, known as 'gui-jun woo' in Korea, was used in folk medicine to regulate *qi*(bodily energy) and blood circulation, relieve pain, eliminate stagnant blood, and treat dysmenorrhea in oriental countries. It can increase tolerance to oxygen deprivation, and has a significant, albeit temporary, hypotensive effect. It acts as a depressant on the CNS and can lengthen barbiturate-induced sleeping times. Its effects on metabolism include a reduction of blood sugar levels via stimulation of the beta cells of pancreatic islets. Additionally, quercetin has been found to be a good expectorant[28]. The antimetastatic and cytotoxic activity of the crude extract or the isolated compounds, however, have not yet been demonstrated. The stems of EA, commonly known as winged euonymus, have been used in traditional medicine for cancer treatment. Previous phytochemical and biological studies on winged euonymus have resulted in the isolation of cardenolides[29]. Substances isolated from EA have been documented toexhibit antioxidant capabilities, and recent studies also indicated that EA has anti-tumor potential[30]. It was reported that the crude extract of EA markedly prolonged the survival period of cervical carcinoma-bearing mice[30]. Methanol and buthanol extracts were also found to have anti-tumor activity in

mice[31]. It was recently found that the methanolic extract of EA exhibited a significant anti-proliferation effect against cultured human cancer cell lines[32]. Our recent findings also suggest that EA is a potent antioxidant in protecting primary hepatocytes from oxidative damage induced by aflatoxin B1, a well recognized hepatocarcinogen [unpublished results]. As a part of our search for new biologically active substances from traditional medicines, we evaluated whether extracts of EA stem barks could modulate the induction of NO synthesis in thioglycollate (TG)-elicited murine peritoneal macrophages or murine macrophage cell line, RAW264.7 cells.

Materials and Methods

1. Cells

RAW264.7, a mouse macrophage cell line, was obtained from the American Type Culture Collection. Cells were cultured in RPMI-1640 medium supplemented with 2 mM glutamine, antibiotics (100 U/mL of penicillin A and 100 U/mL of streptomycin) and 10% heat-inactivated fetal bovine serum (Gibco/BRL) and maintained at 37℃ in a humidified incubator containing 5% CO_2.

2. Processing of the plant material

The stems of EA (Thunb.) Sieb were collected in Kyungju city, the Republic of Korea, and the sample and voucher specimen (number 4-99-221) are kept in the herbarium of the College of Oriental Medicine, Dongguk University. Air-dried barks were milled to small chips (1.0 kg) and extracted twice with hot methanol (MeOH). The MeOH solution was filtered and evaporated under vacuum to give the MeOH extract (40.2 g). The MeOH extract (40.2 g) was dissolved in distilled water (500 ml) and partitioned with n-BuOH (300 ml × 3). These solutions were evaporated to afford the n-BuOH-soluble fraction (25.3 g, 2.36%) and water-soluble fraction (10.2 g, 0.97%). Samples were dissolved in dimethyl sulfoxide (DMSO) and further diluted in incubation buffer.

3. Mice

The original stock of C57BL/6 mice was purchased from the Jackson Laboratory (Bar Harbor, ME) and the mice were maintained in the Department of Microbiology and Immunology, WonkwangUniversity School of Medicine, Iksan, South Korea. To obtain peritoneal macrophages, mice were used at 8~12 weeks of age.

4. Reagents

Murine IFN-γ (1×10^5 U/mg) was purchased from Genzyme (Munchen, Germany). Rabbit anti-murine iNOS monoclonal antibody and peroxidase-labeled anti-rabbit IgG (H+L) was purchased from Affinity Bioreagents (Golden, CO). LPS from Escherichia coli, N-(1-naphthyl)-ethylenediamine dihydrochloride, sodium nitrite, and sulfanilamide were purchased from Sigma (St. Louis, MO). A molecular size marker of DNA was purchased from Bethesda Research Laboratories (Bethesda, MD). Genomic DNA purification kit was obtained from Promega (Madison, WI). A total of 12-well tissue culture plates and 100 mm diameter plastic Petri dishes were purchased from Nunc (Naperville, IL). Dulbecco's modified Eagle's minimal essential medium (DMEM) containing L-arginine (84 mg/l), Hanks' balanced salt solution (HBSS), fetal bovine serum (FBS) and other tissue culture reagents were purchased from Life Technologies.

5. Cell cultures

TG-elicited macrophages were harvested 3 days after intraperitoneal (i.p.) injection of 2.5 ml TG to 8-12-week-old mice and isolated, as reported previously (Jun et al., 1994). Peritoneal lavage was performed using 8

ml of HBSS, which contained 5 U/ml heparin. Then, cells were distributed in DMEM, which was supplemented with 10% (v/v) FBS, in either 24-well tissue culture plates (1×10^6 cells/well) or 100 mm diameter plastic Petri dishes (1×10^6 cells/dish), incubated for 3 h at 37℃ in an atmosphere of 5% CO_2, washed three times with HBSS to remove nonadherent cells, and equilibrated with DMEM that contained 10% FBS before treatment, as indicated in the text.

Murine macrophage RAW264.7 cell line was maintained at 1×10^6 cells/ml in DMEM supplemented with 10% heat-inactivated FBS, penicillin G (100 IU/ml), streptomycin (100㎍/ml), and glutamine (2 mM) and incubated at 37℃ in a humidified atmosphere containing 5% CO_2 and 95% air. On the following day, exponentially growing cells were exposed to various agents indicated below.

6. Measurement of nitrite concentration

NO synthesis in cell cultures was measured by a microplate assay method as described[33,34]. To measure nitrite, 100 ㎕ aliquots were removed from conditioned medium and incubated with an equal volume of the Griess reagent (1% sulfanilamide/0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride/2.5% H3PO4) at room temperature for 10 min. The absorbance at 540 nm was determined in a Titertek Multiskan, Flow Laboratories (North Ryde, Australia). NO-2 was determined by using sodium nitrite as standard. Cell-free medium alone contained 5-8 M of NO-2; this value was determined in each experiment and subtracted from the value obtained with cells.

7. Preparation of cell extracts

1×10^7 TG-elicited macrophages or RAW264.7 cells were collected by centrifugation and washed once with phosphate-buffered saline. Cells were resuspended in 0.4 ml of a hypotonic buffer that contained 10 mM HEPES, 10 mM KCl, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.2 mM EDTA (pH 7.4). After 15 min of swelling on ice, cells were disrupted on ice by 60 strokes in a Dounce homogenizer, and a 0.2 vol. of a buffer containing 50 mM HEPES and 250 mM KCl (pH 7.4) was added. Nuclei and cell debris were removed by centrifugation at 500 x g for 5 min at 4℃, and the lysate was returned to ice. Typically cell lysates prepared by this procedure contained 4-7 mg/ml protein.

8. Western blotting analysis

Protein samples (50-100 ㎍) were mixed with an equal volume of 2×sample buffer, boiled for 5 min, and then separated through 8 or 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. After electrophoresis, proteins were transferred to nylon membranes by semi-dry electrophoretic transfer. The membranes were blocked in 5% dry milk (1 h), rinsed, and incubated with primary antibodies (1:2000 anti-iNOS) in Tris-buffered saline (TBS) overnight at 4℃. Primary antibody was removed, membranes were washed four times in TBS, and 0.1 ㎍ /ml peroxidase-labeled goat secondary antibody was added for 1 h. Following four washes in TBS, bands were visualized by ECL and exposure to X-ray film.

9. DNA extraction and electrophoresis

The pattern of DNA cleavage was analyzed by agarose gel electrophoresis as described[35]. Briefly, genomic DNA was purified by Wizard TM Genomic DNA purification Kit. After ethanol precipitation, samples of 10 ㎍ in each lane were subjected to electrophoresis on a 1.4% agarose at 50 V for 3 h. DNA was stained with ethidium bromide.

10. Cell viability and morphological assessment

For determination of cell viability, 50 ㎍/ml of

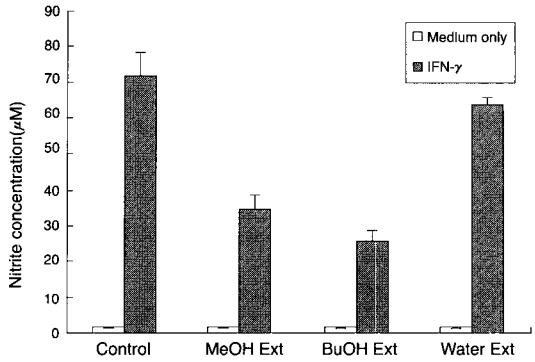

Fig. 1. Effects of MeOH extract of EA and its fractions on NO synthesis in RAW264.7 cells stimulated with IFN-γ and LPS.
RAW264.7 cells were cultured for 48 h with MeOH extract (20 μg/ml), BuOH fraction (20 μg/ml), or water fraction (20 μg/ml) in the presence or absence of IFN-γ (2 U/ml) and LPS (50 ng/ml) and NO release was measured using the method of Griess (nitrite). Results are expressed as mean±S.E. of three independent experiments.

methylthiazol-2-yl-2,5-diphenyl tetrazolium bromide (MTT), Sigma, was added to 1 ml of cell suspension (1×10^6 cells/ml in 24-well plates) for 4 h, and the formazan formed was dissolved in acidic 2-propanol; optical density was measured using an enzyme-linked immunosorbent assay reader at 590 nm. The optical density (OD) of formazan formed by control (untreated) cells was taken as 100%. To determine morphological changes, the cells were photographed at x 400 using bright-field optics after 48 h of incubation with agents, as indicated in the text.

11. Data analysis

50 values were calculated from non-linear regression analysis of the data. The statistical differences ($p < 0.05$) of values was determined with ANOVA.

Results

1. Effect of the MeOH extract and its fractions of EA on NO synthesis and iNOS protein expression

As shown in Fig. 1, among three extracts of EA, the BuOH fraction significantly reduced NO synthesis in cultured RAW264.7 cells. Therefore, we used the BuOH fraction to further evaluate the exact role of that material on NO synthesis in murine macrophages.

The dose-dependent effects of the BuOH and MeOH fractions in the presence of IFN-γ (2 U/ml) and LPS (50 ng/ml) are shown in Fig. 2A. The inhibition effect was always maximal at 20 μg/ml of BuOH fraction of the MeOH extract. The viability of RAW264.7 cells was not altered in the presence or absence of the fraction, as determined by MTT assay (data not shown). To further evaluate whether the reduced NO synthesis is correlated with iNOS protein levels, we examined iNOS protein content. On the basis of Western blotting data, expressed iNOS enzyme levels were correlated with NO synthesis (Fig. 2B). In unstimulated RAW264.7 cells, iNOS was not detectable; however, IFN-γ in combination with LPS markedly increased iNOS. The treatment of RAW264.7 cells with the BuOH fraction significantly reduced iNOS in a dose-dependent manner. The MeOH and BuOH fractions also inhibited NO synthesis induced by TG-elicited murine peritoneal macrophages stimulated with IFN-γ and LPS (Table 1).

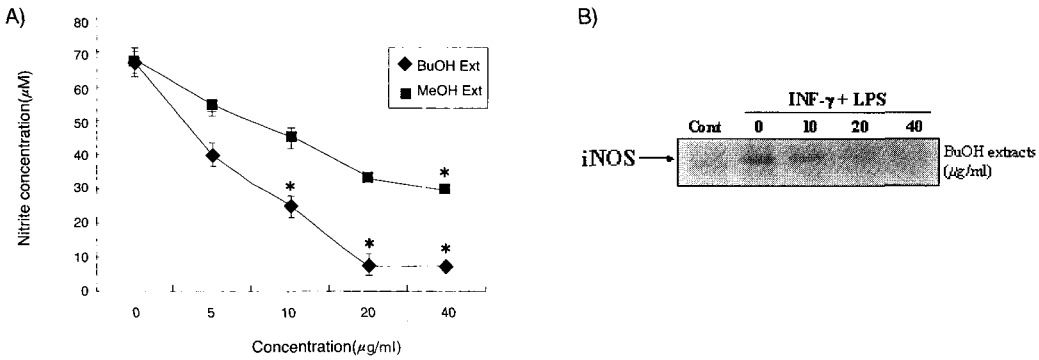

Fig. 2. Effect of the MeOH and BuOH fraction of EA on NO synthesis and iNOS induction in RAW264.7 cells stimulated with IFN-γ and LPS.

(A) Dose-dependent effects of the MeOH and BuOH extractsfor NO release. RAW264.7 cells were cultured for 48 h with various concentrations of BuOH fraction (5-40 μg/ml) in the presence of IFN-γ (2 U/ml) and LPS (50 ng/ml) and NO release was measured using the method of Griess (nitrite). A higher concentrations of the MeOH and BuOH fractions (40 μg/ml) slightly decreased cell viability (5-15% of control) as assessed by MTT assays *. Results are expressed as mean±S.E. of three independent experiments.

(B) Dose-dependent effects of the BuOH fraction for iNOS induction. Cells were treated as in (A). After 24 h, iNOS induction was tested by Western blot with the anti-iNOS antibody.

Table 1. Effect of the MeOH and BuOH Fraction on NO Synthesis in Murine Peritoneal Macrophages Stimulated with IFN-γ and LPS.

Treatment	Fractions added		Nutrite concentration (μM)	
			12 h	24 h
Medium only	-		<3	<3
IFN-γ+ LPS	-		NDb	68.8±6.3
	+ BuOH extract	(0 h)	16.4±2.3	31.3±2.7
		(12 h)	45.3±7.2	60.7±8.4
	+ MeOH extract	(0 h)	18.5±1.8	36.5±4.3
		(12 h)	49.3±4.6	64.3±5.7

TG-elicited macrophages were cultured with IFN-γ (2 U/ml)+ LPS (50 ng/ml). Then, the cells were treated with the MeOH and BuOH extract (20 μg/ml) simultaneously (0 h) or after 24 h of incubation (12 h). The amounts of NO release was measured after 12 or 24 h of incubation. Results are expressed as means±S.E. of six independent experiments.

To determine whether the MeOH and BuOH fractions also inhibited NO synthesis when iNOS was already expressed by the treatment with IFN-γ and LPS, TG-elicited murine peritoneal macrophages were stimulated for 12 h with IFN-γ and LPS. Then, the cells were cultured for an additional 12 h with the MeOH or BuOH fraction (20 μg/ml). However, it showed no inhibitory effect on the synthesis of NO (Table 1), implying that the MeOH or BuOH fractions may inhibit signal pathways corresponding to iNOS induction in murine macrophages.

2. Effects of the MeOH and BuOH fraction of EA on the Apoptosis of RAW264.7 cells induced by IFN-γ and LPS

As Fig. 3 shows, agarose gel electrophoresis revealed the typical 'laddered' profile of oligonucleosomal fragments in IFN-γ plus LPS-treated cells. However, treatment of the cells with the MeOH and BuOH fraction (5-40 μg/ml) clearly protected IFN-γ plus LPS-

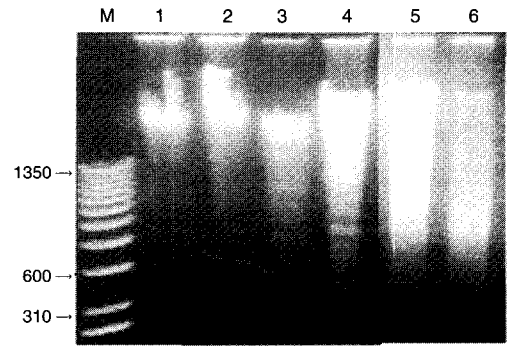

Fig. 3. Effects of the MeOH and BuOH fraction on IFN-γ plus LPS-induced apoptosis in RAW264.7 cells.

RAW264.7 cells (1 x 10^7 cells/dish) were cultured for 48 h with various concentrations of MeOH and BuOH fraction (5- 40 μg/ml) in the presence of IFN-γ (2 U/ml) and LPS (50 ng/ml). Then, genomic DNA was purified and subjected to electrophoresis. Lane M, molecular size marker; 1, untreated control cells; 2-5, cells treated with IFN-γ (2 U/ml) plus LPS (50 ng/ml) (lane 2) with various concentrations of the MeOH and BuOH fraction (lane 3, 10 μg/ml; lane 4, 20 μg/ml; lane 5, 40 μg/ml lane 6, 100 μg/ml).

induced DNA ladder in a dose-dependent manner, indicating that the extract could protect cell damage via inhibition of NO synthesis in activated RAW264.7 cells.

Discussion

EA has been used in Oriental traditional medicine as a remedy for treating inflammation, suppurative dermatitis, allergic diseases, hyperlipidemia, and arteriosclerosis[32,37]. The active principle in EA that exhibits these beneficial effects, however, has not been determined fully. Results of the present study indicated that EA inhibited the LPS induction of iNOS and COX-2 gene expression in macrophages without appreciable cytotoxic effects. These findings were consistent with a decrease caused by EA in LPS-induced NO and PGE_2 production. These results suggest that EA may play an important role in inhibiting inflammatory processes. Although the barks of EA stem and root have been used in oriental traditional medicine for inflammatory diseases, the action mechanisms of this species are not nearly understood. It may be important to understand how this plant extract performs anti-inflammatory action in vivo. To evaluate the role of EA on inflammatory diseases, we studied the effect of the MeOH extract of EA and its fractions on the production of NO in murine macrophage cell line, RAW264.7 cells and TG-elicited murine peritoneal macrophages in vitro.

Macrophage activation is known to occur through a series of steps ranging from a level equivalent to resident tissue macrophages and culminating in an activated state[33,34,38]. During this activation process, NO contributes to the cytotoxic and cytostatic actions of macrophages against microorganisms and tumors[39]. However, this molecule is also thought to be responsible for the deleterious aspects of immune responses, such as tissue destruction[40]. Thus, the suppression of iNOS expression is probably as important to its regulation as its induction. It has been established that RAW264.7 macrophages are highly susceptible to endogenously generated or exogenously supplied NO[41]. Treatment of RAW264.7 macrophages with LPS and IFN-γ has been shown to result in NOS induction and apoptosis. Both nitrite accumulation and apoptosis were blocked by the NOS inhibitor NG-

monomethyl-arginine. Memer et al.[42] reported that Bcl-2-overexpressing RAW264.7 macrophages appeared highly resistant to LPS/IFN-γ-induced apoptosis, although inducible NO synthase levels increased with concomitant nitrite production similar to parental cells[42]. The expression of iNOS in murine macrophages has been shown to be dependent on NF-kB activation[43]. The possibility that oroxylin A may inhibit the activity of NF-kB was examined. The results indicated that oroxylin A inhibition of expression of iNOS mRNA and proteins was most likely due to oroxylin A suppression of NF-kB activation. This is consistent with the reports that NF-kB response elements are present on the promoters for iNOS gene[43-48]. NF-kB is composed mainly of two proteins, p50 and p65[43]. Under unstimulated conditions, NF-kB is present in the cytosol and is bound to the inhibitory protein IB. After induction by a variety of agents such as LPS, IB is phosphorylated to trigger proteolytic degradation of IB. NF-kB is then released from IB and is translocated into the nucleus. There are several transcription factor binding sites located on the promoter of the murine iNOS gene such as IFN-γ response elements (IRF), GAS, OCT-1, and NF-kB sites. In the present study, inhibition of LPS-induced iNOS gene expression by EA was found to correlate well with the inhibition of NF-kB binding and trans-activating activities as seen by EMSA and NF-B-Luc transient transfection assays. The binding activities of AP-1, IRF-1, and OCT-1, however, did not change significantly in EA-treated macrophages (our unpublished data). These results again suggest that inhibition of LPS-induced iNOS expression by EA most likely occurs through blocking NF-kB. The failure of emodin to block LPS-induced NF-kB activation suggests that emodin inhibits LPS-activated iNOS gene expression by blocking binding activities of different transcription factors[49,50].

Our results showed that the MeOH and BuOH fraction of EA clearly reduced iNOS expression and NO production in macrophages. In addition, it protected IFN-γ and LPS-induced apoptotic cell death of RAW264.7 cells in a dose-dependent manner. The protection of cell death might correspond with the inhibition of NO synthesis, because it has been known that NO production causes apoptosis in either murine peritoneal macrophages or RAW 264.6 cells[36]. Furthermore, the treatment of RAW264.7 cells with NG-monomethyl-arginine, a specific inhibitor for NOS, blocked IFN-γ and LPS-induced apoptotic cell death (data not shown).

The MeOH and BuOH fraction showed no inhibitory effect on the synthesis of NO by RAW264.7 cells, when iNOS was already expressed by the stimulation of the cells with IFN-γ and LPS. This result indicates that the MeOH and BuOH fraction may inhibit not only signal pathways corresponding to iNOS induction but also the post-transcriptional modification of the enzyme. Although we did not measure iNOS enzyme activity directly, this result further suggests that it might have no significant effect on iNOS activity by itself.

Overall, our results suggest that the effect of EA in the inhibition of inflammatory diseases may be partially associated with the inhibition of NO synthesis and iNOS induction. Therefore, the extract may cause, depending on the extent and duration of NO synthesis, an inhibition of NO synthesis, thereby protecting the tissue microenvironment from the harmful effects of activated macrophages.

References

1. Caplan, MS., Hedland, E., Hill, N. and MacKendrick, W. The role of endogenous nitric oxide and platelet-activating factor in hypoxia-induced intestinal injury in rats. Gastroenterology. 1994;106:346-352.
2. Moncada S, R.M.J. Palmer and D.A. Higgs. Nitric

oxide: Physiology, pathophysiology, and pharmacology. Pharmacol Rev. 1992;43:109-142.

3. Marletta MA, P.S. Yoon, R. Iyengar, C.D. Leaf and J.S. Wishnok, Macrophage oxidation of L-arginine to nitrite and nitrate: Nitric oxide is an intermediate. Biochemistry. 1988;27:8706-8711.
4. Miller, M.J.S. and Clark, D.A.. Nitric oxide synthase inhibition can initiate or prevent inflammation: role of enzyme source. Agents Actions. 1994;41:C231-C232.
5. Thompson, J.H., Sadowska-Krowicka, H., Rossi, J., Clark, D.A. and Miller, M.J.S.. Inducible nitric oxide synthase gave expression in guinea pig ileitis: a model of IBD prevented by aminoguanidine (abstract). Gastroenterology. 1994;106:A782.
6. Nathan C. Nitric oxide as a secretory product of mammalian cells. FASEB J. 1992;6:3051-3064.
7. Marletta MA. Mammalian synthesis of nitrite, nitrate, nitric oxide and N-nitrosating agents. Chem Res Toxicol. 1988;1:249-257.
8. Marletta MA. Nitric oxide synthase structure and mechanism. J Biol Chem. 1993;268:12231-12234.
9. Duval DL, D.R. Miller, J. Collier and R.E. Billings. Characterization of hepatic nitric oxide synthase: Identification as the cytokine-inducible form primarily regulated by oxidants. Mol Pharmacol. 1996;50:277-284.
10. Yuan T, H.T. Vogel, C. Sutherland and M.P. Walsh. Characterization of the Ca^{2+}-dependent and -independent interactions between calmodulin and its binding domain of inducible nitric oxide synthase. FEBS Lett. 1998;431:210-214.
11. Liu RH, and J.H. Hotchkiss. Potential genotoxicity of chronically elevated nitric oxide: A review. Mutat Res. 1995;339:73-89.
12. Pedoto A, A.K. Tassiopoulos, A. Oler, D.J. McGraw, S.P. Hoffmann, E.M. Camporesi and T.S. Hakim. Treatment of septic shock in rats with nitric oxide synthase inhibitors and inhaled nitric oxide. Crit Care Med. 1998;26:2021-2028.
13. Tunctan B, O. Uludag, S. Altug and N. Abacioglu. Effects of nitric oxide synthase inhibition in lipopolysaccharide-induced sepsis in mice. Pharmacol Res. 1998;38:405-411.
14. Ueno M. and T.J.-F. Lee. Endotoxin decreases the contractile responses of the porcine basilar artery to vasoactive substances. J Cereb Blood Flow Metab. 1993;13:712-719.
15. Cao C, K. Matsumura, K. Yamagata and Y. Watanabe. Induction by lipopolysaccharide of cyclooxygenase-2 mRNA in rat brain; its possible role in the febrile response. Brain Res. 1995;697:187-196.
16. Quan N, M. Whiteside and M. Herkenham. Cyclooxygenase 2 mRNA expression in rat brain after peripheral injection of lipopolysaccharide. Brain Res. 1998;802:189-197.
17. Lee SH, E. Soyoola, P. Chanmugam, S. Hart, W. Sun, H. Zhong, S. Liou, D. Simmons and D. Hwang. Selective expression of mitogen-inducible cyclooxygenase in macrophages stimulated with lipopolysaccharide. J Biol Chem. 1992;267:25934-25938.
18. Roediger, W.E.W., Lawson, M.J., Nance, S.H. and Radcliffe, B.C. Detectable colonic nitrite levels in inflammatory bowel disease mucosal or bacterial malfunction Diegestion. 1986;35:199-204.
19. Lalenti, A., Moncada, S. and Di Rosa, M. Modulation of adjuvant arthritis by endogenous nitric oxide. British Journal of Pharmacology. 1993;110:701-706.
20. McCartney-Francis, N., Allenm, J.B., Mizel, D.E., Albina, J.E., Xie, Q.W., Nathan, C.F. and Wahl, S.M Suppression of arthritis by an inhibitor of nitric oxide synthase. Journal of Experimental Medicine. 1993;178: 749-754.
21. Middleton, S.J., Shorthous, M. and Hunter, J.D. Increased nitric oxide synthesis in ulcerative colitis. Lancet. 1993;341:456-466.
22. Miller, M.J.S., Sadowska-Krowicka, H., Chotinaruemol,

S., Kakkis, J.L. and Clark, D.A. Amelioration of chronic ileitis by nitric oxide synthase inhibition. Journal of Pharmacological Experimental Therapy. 1993;264:11-16.

23. Haslam E, T.H. Lelly, C. Ya,R. Martin and D. Magnolato. Traditional herbal medicine. The role of plant polyphenols. Planta Med. 1989;55:1-8.
24. Aoki K, H. Maruta, F. Uchiumi, T. Hatano, T. Yoshida and S. Tanuma. A macrocircular ellagitannin, oenothein B, suppresses mouse mammary tumor gene expression via inhibition of poly(ADP-ribose) glycohydrolase. Biochem Biophys Res Commun. 1995;210:329-337.
25. Yasukawa K, M. Takido, M. Takeuchi, Y. Sato, K. Nitta and S. Nakagawa. Inhibitory effects of flavonol glycosides on 12-O-tetradecanoylphorbol-13-acetate - induced tumor promotion. Chem Biol Bull. 1990;38: 774-776.
26. Miyamoto K, M. Nomura, M. Sasakura, E. Matsui, R. Koshiura, T. Murayama, T. Furukawa, T. Hatano, T. Yoshida and T. Okuda. Anti-tumor activity of oenothein B, a unique macrocyclic ellagitannin. Jpn J Cancer Res. 1993;84:99-103.
27. Huang K.C. The Pharmacology of Chinese herbs. CRC Press, Boca Raton. In. 1993:9394.
28. Kitanaka, S., Takido, M., Mizoue, K., Nakaike, S. Cytotoxic cardenolides from woods of Euonymus alata. Chemi. Pharm. Bulletin. Tokyo. 1996;44:615-617.
29. Change H.M. and P.P. But, Danshen. In: H.-M. Chang and P. But Editors, Pharmacology and Applications of Chinese Materia Medica World Scientific, Singapore. 1986:255-268.
30. Kim CH. Inhibitory effect of *Euonymus alatus* on cervical cancer growth. Kor. J. Orin. Gynecol. 2003;8: 28-36.
31. Cha BY, Park CJ, Lee DG, Lee YC, Kim DW, Kim JD, Seo WG, Moon SK and CH Kim. Inhibitory effect of methanol extract from *Euonymus alatus* on matrix metalloproteinase-9. J. Ethnopharmacol. 2003;85:163-167.
32. Lee, S.J. Korean Folk Medicine, Monographs Series No. 3. Publishing Center of Seoul National University, Seoul, South Korea. 1996:39.
33. Jun, C.D., Choi, B.M., Ryu, H., Um, J.Y., Kwak, H.J., Lee, B.S., Paik, S.G., Kim, H.M. and Chung, H.T. Synergistic cooperation between phorbol ester and IFN-γ for induction of nitric oxide synthesis in murine peritoneal macrophages. Journal of Immunology. 1994;153:3684-3690.
34. Jun, C.D., Pae, HO., Kim YC, Jeong SJ, Yoo JC, Lee EJ, Choi BM, Chae SW, Park RK, and Chung HT. Inhibition of nitric oxide synthesis by buthanol fraction of the methanol extract of Ulmus davidiana in murine macrophages. J. Ethnopharm. 1998;62:129-135.
35. Jun, C.D., Park, S.J., Choi, B.M., Kwak, H.J., Park, Y.C., Kim, M.S., Park, R.K. and Chung, .T. Potentiation of the activity of nitric oxide by the protein kinase C activator phorbol ester in human myeloid leukemic HL-60 cells: association with enhanced fragmentation of mature genomic DNA. Cellular Immunology. 1997;176: 41-49.
36. Albina, J.E., Cui, S., Mateo, R.B. and Reichner, J.S., Nitric oxide-mediated apoptosis in murine peritoneal macrophages. Journal of Immunology. 1993;150: 5080-5085.
37. Huang K.C. The Pharmacology of Chinese herbs. CRC Press, Boca Raton. In. 1993:9394.
38. Adams, D.O. and Hamilton, T.A. The cell biology of macrophage activation. Annual Review of Immunology. 1984;2:283-318.
39. Moncada, S., Palmer, R.M.J. and Higgs, E.A. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacological Review. 1991;43:109-142.
40. Vodovotz, Y., Bogdan, C., Paik, J., Xie, Q.W. and Nathan, C.F. Mechanisms of suppression of macrophage

nitric oxide release by transforming growth factor. Journal of Experimental Medicine. 1993;178:605-613.

41. Memer UK, E.G. Lapetina and B. Brune, Nitric oxide-induced apoptosis in RAW 264.7 macrophages is antagonized by protein kinase C- and protein kinase A-activating compounds. Mol Pharmacol. 1995;47:757-765.
42. Memer UK, J.C. Reed and B. Brune, Bcl-2 protects macrophages from nitric oxide-induced apoptosis. J Biol Chem. 1996;271:20192-20197.
43. Barnes PJ. and Karin M. Nuclear factor-kB a pivotal transcription factor in chronic inflammatory diseases. N Engl J Med. 1997;336:1066-1071.
44. Xie W, J.R. Merrill, W.S. Bradshaw and D.L. Simmons, Structural determination and promoter analysis of the chicken mitogen-inducible prostaglandin G/H synthase gene and genetic mapping of the murine homolog. Arch. Biochem. Biophys. 1993;300:247-252.
45. Xie QW, Y. Kashiwabara and C. Nathan. Role of transcription factor NF-B/Rel in induction of nitric oxide synthase. J Biol Chem. 1994;269:4705-4708.
46. Roshak AK, J.R. Jackson, K. McGough, M. Chabot-Fletcher, E. Mochan and L.A. Marshall. Manipulation of distinct NFB proteins alters interleukin-1-induced human rheumatoid synovial fibroblast prostaglandin E2 formation. J Biol Chem. 1996;271:31496-31501.
47. Chen F, D.C. Kuhn, S.-C. Sun, L.J. Gaydos and L.M. Demers, Dependence and reversal of nitric oxide production on NF-kB in silica and lipopolysaccharide-induced macrophages. Biochem Biophys Res Commun. 1995;214:839-846.
48. Schmedtje JF, Jr, Y.-S. Ji, W.-L. Liu, R.N. DuBois and M.S. Runge. Hypoxia induces cyclooxygenase-2 via the NF-B p65 transcription factor in human vascular endothelial cells. J Biol Chem. 1997;272:601-608.
49. Martin E, C. Nathan and Q.W. Xie. Role of interferon regulatory factor 1 in induction of nitric oxide synthase. J Exp Med. 1994;180:977-984.
50. Chu SC, J. Marks-Konczalik, H.P. Wu, T.C. Banks and J. Moss. Analysis of the cytokine-stimulated human inducible nitric oxide synthase (iNOS) gene: Characterization of differences between human and mouse iNOS promoters. Biochem. Biophys. Res. Commun. 1998;248:871-878.