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Effect of Various Herbal Extracts on Nitric Oxide Production in Lipopolysaccharide-induced Murine Peritoneal Macrophages

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Abstract–Nitric oxide (NO) can mediate numerous physiological processes, including vasodilation, neurotransmission, cytotoxicity, secretion and inflammatory response. The regulation of NO production by inducible NO synthase (iNOS) is considered to be the possible target of the development of anti-inflammatory agent, based on the observation that NO can activate cyclooxygenase, which results in the synthesis of prostaglandins. In an effort to screen new inhibitor of NO production from about 352 species of herbal extracts, we found 9 species with 50% or more inhibitory effect on NO production. Especially, the dose-dependent inhibition of NO production in lipopolysaccharide-treated macrophages by two of the herbal extracts (*Artemisiae asiaticae Herba* and *Saussureae Radix*) was due to the decrease in the expression of iNOS.

Key words ☐ nitric oxide, nitric oxide synthase, *Artemisiae asiaticae Herba*, *Saussureae Radix*

Nitric oxide (NO) is biosynthesized in mammalian systems via the enzymatic oxidation of the terminal guanidino nitrogen on the amino acid L-arginine through an N-hydroxy-L-arginine intermediate yielding L-citrulline as a coproduct (Fukuto and Chaudhuri, 1995). This reaction is catalyzed by NO synthase [NOS, EC 1.14.13.39] (Morris and Billiar, 1994).

The distinctive class of NOS isoforms generally falls into two categories, constitutive and inducible forms. A constitutive, Ca^{2+}/calmodulin-dependent type may be active under basal conditions and can be further stimulated by increase in intracellular calcium that occur in response to receptor-mediated agonists or calcium ionophore. In contrast to the constitutive form of the enzyme, an inducible, Ca^{2+}-independent form can occur in response to appropriate stimuli in many cell types including macrophages. Inducible NOS (iNOS) exhibits negligible activity under basal condition, but in response to factors such as lipopolysaccharide (LPS) and certain cytokines, produces much greater amounts of NO than the constitutive isoform does (Marletta, 1993).

Constitutive NOS (cNOS) isoforms are regulated by intracellular Ca^{2+} level via the Ca^{2+}-binding protein calmodulin (Bredt and Snyder, 1994) and phosphorylation by Ca^{2+}- and calmodulin-dependent protein kinase II and protein kinase C (Nakane *et al.*, 1991). Recently, activation of endothelial NOS was shown to be regulated by protein kinase B via phosphorylation (Dimmeler *et al.*, 1999 ; Fulton *et al.*, 1999). Also, iNOS appears to be regulated primarily via phosphorylation as well as induction of *de novo* protein synthesis (Salh *et al.*, 1998). Furthermore, the availability of substrate, cofactors, and inhibitors may play a role in iNOS regulation in macrophages and smooth muscle as well as cNOS (Nathan and Xie, 1994).

Because these studies of NO synthesis and regulation in mammalian systems have been greatly facilitated by various inhibitors of NOS, we have investigated the inhibitory effect of various herbal extracts on NO production in LPS-stimulated murine peritoneal macrophages *in vitro*. This first screening of NOS inhibition by herbal extracts may provide basic information for elucidating the mechanism of NOS regulation by pure compound purified from the herbal extracts showing inhibitory action and further developing the anti-inflammatory agent against NO-based diseases.

MATERIALS AND METHODS

Materials

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Fetal bovine serum, penicillin/streptomycin, and RPMI 1640 medium were purchased from Gibco BRL (U.S.A.). Lipopolysaccharide (*E. coli* serotype O111:B4), N^G-monomethyl-$_L$-arginine, and other reagents were obtained from Sigma Chemical Co. (U.S.A.) and were of the highest purity available. Herbal extracts were obtained from Pacific Corporation/ Research & Development Center (Korea).

Animals

Male ICR mice (8-10 weeks) were obtained from Ansung Cheil Inc. (Ansung, Korea) and housed under conditions of controlled temperature (22-24°C) and illumination (12 h light cycle starting at 8 A.M.) and received food and water *ad libitum* for 1 weeks in GLP room in College of Pharmacy, Sungkyunkwan university.

Preparation of herb extracts

Various herbs from Korean, Japan, and China were extracted with methanol and pellet was removed. The extracts were collected and evaporated. The residues were suspended in the concentration of one milligram per one milliliter of 20% ethanol. Herbal extracts (1 mg/ml) were diluted with RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum (FBS), penicillin (200 IU/ml), and streptomycin (200 μg/ml), in use into final concentration of 10 microgram per milliliter of the medium.

Isolation of murine peritoneal macrophages

Macrophages were isolated from peritoneal exudate cells following previous methods (Kirikae *et al.*, 1996). Thioglycollate (Difco Laboratories, U.S.A.)-elicited peritoneal exudate cells were obtained from 8-10 weeks old ICR male mice by intraperitoneal injection of 1 ml Brewer's thioglycollate broth (4.05% w/v) and lavage of the peritoneal cavity with 5-6 ml of RPMI 1640 medium 3 days later. The cells were washed twice and suspended in RPMI-1640 containing 10% heat-inactivated FBS, penicillin (200 IU/ml), and streptomycin (200 μg/ml). Peritoneal exudate cells were seeded at densities of 1×10^5 cells/well in 96-well tissue culture plates or 1×10^7 cells in 6-cm tissue culture dishes, and the macrophages were allowed to adhere for 2 hours at 37°C in 5% CO_2 incubator. The nonadherent cells were removed by pouring off the medium and rinsing the wells twice with pre-warm medium. The adherent cells were incubated for 24 hours at the same condition.

Co-treatment of lipopolysaccharide and herbal extracts in murine peritoneal macrophage cells

The isolated macrophages in 96-well tissue culture plates (1×10^5 cells/ml) or 6-cm tissue culture dishes (1×10^7 cells/dish) with LPS (10 μg/ml) in the absence or presence of herbal extracts were incubated to screen the herbal extracts for inhibition of NO production under the same condition. After incubation, the supernatants were collected to measure nitrite contents and the cells in 6-cm culture dishes were scrapped and disrupted for 10 sec with Vibra Cell (VCX 400, Sonics & Materials Inc., U.S.A.). The supernatants were obtained by centrifugation at 50,000 g for 30 min and stored at 4°C until use.

Nitrite determination

NO production was measured by estimating the stable NO metabolite, nitrite, in conditioned medium using Griess reaction. One hundred microliter aliquots of cell-free supernatants were mixed with 100 μl Griess reagent (1% sulfanilamide in 5% phosphoric acid : 0.1% naphthylethylenediamine dihydrochloride=1 : 1) and incubated for 15 minutes at room temperature. After incubation, the absorbances of the wells were determined using a microplate reader (Multiscan MCC/ 340 P version 2.3, USA) equipped with a 540 nm filter. Concentrations of nitrite were determined from a linear regression analysis of standards (sodium nitrite) generated for each plate.

SDS-polyacrylamide gel electrophoresis and Western blot analysis

Samples (1 μg protein) of the cell sonicates were subjected to electrophoresis on 1.5 mm thick 15% polyacrylamide gels. The separated proteins were transferred to PVDF membrane using semi-dry transfer cell (Trans-Blot, BIO-RAD, U.S.A.). The membrane was blocked for 30 minutes at room temperature in the blot buffer (50 mM Tris-HCl, 5% skim milk, 200 mM NaCl, and 0.05% Tween 20, pH 7.5). And the membrane was incubated with rabbit anti-mouse iNOS antibody (1 : 2000 dilution) in the same blot buffer overnight at 4°C. After the membrane was washed twice with the same blot buffer, the membrane was incubated with alkaline phosphatase-conjugated goat anti-rabbit antibody (1 : 1000 dilution) in blot buffer for 2 hours at room temperature and then washed three times in blot buffer for 3 minutes and once in Tris-buffered saline (pH 7.4) for 20 minutes. The membrane was then incubated with alkaline phosphatase substrate for 1-10 minutes. When the immunoreactive bands regarded as iNOS protein (Mr 130 kDa) appeared, the reactions were terminated by removing the alkaline phosphatase substrate.

Cell viability

After incubation, the media were removed by pouring off and macrophages were incubated with MTT to a final concentration of 0.5 mg/ml for 4 hours at 37°C. The media were

removed and 0.1 ml of DMSO were added to solubilize formazan crystals. Optical densities were measured at 540 nm in a microplate reader (Multiscan MCC/340 P version 2.3, U.S.A.).

Protein determination

Protein contents were measured by the Bradford method (Bradford, 1976), using bovine serum albumin as a standard.

Statistical analysis

The results were expressed as mean plus or minus the standard error. Statistical analysis was performed by using a two-tailed Student's t test. A difference with a p value of <0.05 was considered statistically significant.

RESULTS

Screening 352 species of herbal extracts against NOS inhibition

To search for herbal extracts with inhibitory effect on NO production, 352 herbal species were screened. Macrophages were stimulated with LPS (10 μg/ml) in the absence or presence of herbal extracts (10 μg/ml) for 24 hours at 37°C. In first screening, 15 species among the 352 herbal extract had more than 40% inhibitory effect (Table I). Only *Gentiana squarrosa* among 15 herbal extracts showed cytotoxic effect.

The 9 extracts showing more than 50% inhibition of NO production were represented (Table I and Fig. 1). However, their inhibitory effects on NO production in macrophage are not clear whether it results from their direct inhibition of iNOS enzyme activity or suppression of iNOS protein expression in response to LPS. *Artemisiae asiaticae Herba* and *Saussureae Radix* of the herbal extracts showed the strongest inhibitory effect on NO production (Fig. 1A). The decrease in NO production by the two herbal extracts was accompanied by their reduction of iNOS protein expression as measured by Western blot using anti-iNOS antibody (Fig. 1B).

Inhibitory effect of *Artemisiae asiaticae Herba* and *Saussureae Radix* on NO production

Herbal extracts, *Artemisiae asiaticae Herba* and *Saussureae Radix*, with the strongest inhibitory effect on NO production were further characterized. To examine the dose-response on NOS inhibition, macrophages were treated with increasing concentrations of both herbal extracts. Both herbal extracts at high concentration (1 μg/ml) caused the strong inhibition of NO production, while low concentration

Table I. Effect of herbal extracts on nitrite release from LPS-treated macrophages

Herbal extracts	% release of stimulated macrophage		
	1 (μg/ml)	5 (μg/ml)	10 (μg/ml)
Acronychia pedunculata	78.40±7.00	53.56±2.17*	57.26±1.14**
Alpiniae Fructus	90.68±2.65	76.68±4.25	62.96±4.60*
Alpiniae Katsumadaii Semen	91.26±10.18	72.82±9.16	60.39±1.41**
Artemisiae asiaticae Herba	67.03±3.80*	22.93±4.51***	13.08±5.45***
Caesalpiniae Lignum	72.27±3.29*	25.80±3.50***	11.07±3.45***
Carpesii Fructus	81.79±19.75	85.10±18.20	31.28±22.56*
Curcumae Tuber	58.51±15.26*	51.37±17.78*	35.02±12.03**
Gentiana squarrosa	3.38±0.78***	1.51±1.65***	2.64±2.67***
Inulae Flos	37.85±5.96**	14.75±3.77***	10.27±5.15***
Meliae Cortex	22.10±8.26***	17.91±8.39***	18.48±8.26***
Meliae Fructus	58.37±14.03*	44.45±12.54**	41.99±15.00*
Psoraleae Semen	94.62±0.82	65.62±2.17*	56.51±3.63**
Saussureae Radix	15.53±3.45***	11.42±5.11***	3.90±1.71***
Taraxaci Herba	84.93±11.99	29.87±19.47*	19.28±10.61***
Zingiberis Rhizoma	65.64±4.19*	65.00±9.49*	52.47±7.22**

Macrophages (1×10^5 cells/well) were incubated with LPS (10 μg/ml) at 96-well tissue culture plate in the absence or presence of herbal extract for 24 hours. After incubation, nitrite contents were 0.44 and 52.41 μM, compared control with LPS-treated in murine peritoneal macrophages, respectively. In the presence of N^G-monomethyl-L-arginine (10, 100, and 500 μM) as a competitive inihbitor of NOS, amounts of nitrite produced by LPS were reduced to 35.27, 10.35, and 1.2 μM, respectively. Results are expressed as the percentage inhibition of the LPS-induced nitrite release (without inhibitor) and represent the means±S.E. of at 4-6 separate experiments. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ indicate significant inhibition of NO production by herbal extracts in the presence of LPS.

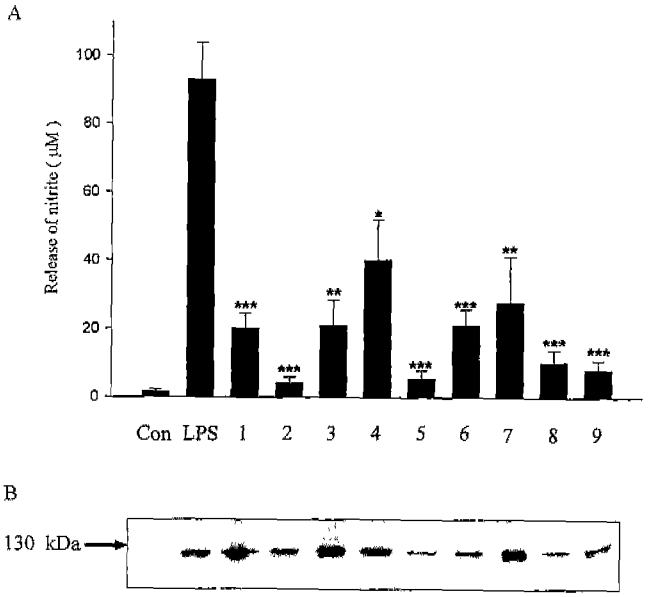

Fig. 1. Effect of various herbal extracts on iNOS expression and nitrite release in LPS-stimulated macrophages. Macrophages (1×10^7 cells/dish) were incubated with LPS (10 μg/ml) in the absence or presence of various herbal extracts (each concentration of 10 μg/ml) as the described Methods. Cells were disrupted and equal quantities of protein (each 1 μg) were separated on a 15% SDS-PAGE. After transfer to a PVDF membrane the presence of iNOS protein was detected using a polyclonal antibody against murine iNOS at a dilution of 1 : 2000. The iNOS protein was shown as a protein band at about 130 kDa. The changes of expression is shown in a number of various herbal extracts including (1) *Caesalpiniae Lignum*, (2) *Artemisiae asiaticae Herba*, (3) *Meliae Cortex*, (4) *Meliae Fructus*, (5) *Saussureae Radix*, (6) *Curcumae Rhizoma*, (7) *Carpesii Fructus*, (8) *Taraxaci Herba*, (9) *Inulae Flos*. Nitrite contents were determined using Griess reaction (panel A). Con, control ; LPS, LPS-stimulated (panel B). Results are expressed as the percentage inhibition of the LPS-induced nitrite release (without inhibitor) and represent the means±S.E. of at 4 separate experiments. $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ indicate significant inhibition of NO production by herbal extracts in the presence of LPS.

(0.1 μg/ml) was insufficient (Fig. 2A). The inhibitory effects of *Artemisiae asiaticae Herba* and *Saussureae Radix* on NO production were paralleled with a decrease in iNOS expression as measured by Western blot (Fig. 2B) indicating that the inhibitory effect of the two herbal extracts on NO production results from in part the reduction of iNOS expression.

Cell viability

Cell viability was determined using MTT assay. There was no cytotoxicity at the concentration of 10 μg/ml herbal extracts except *Gentiana squarrosa* (below 5%).

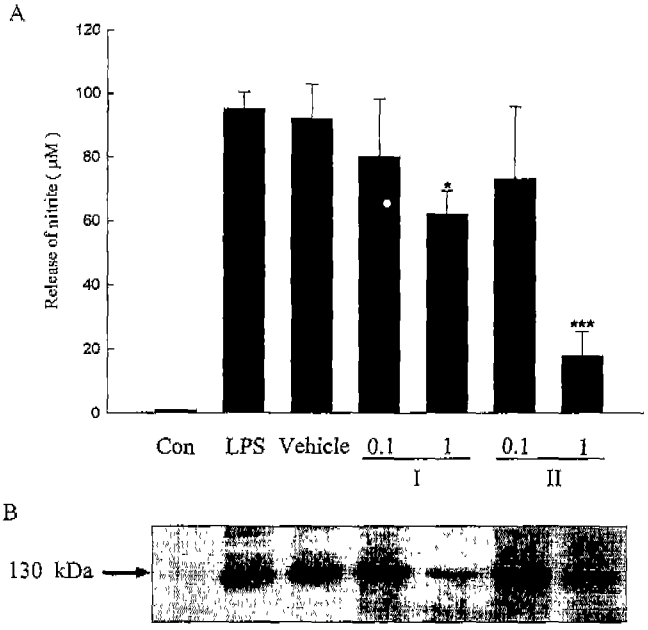

Fig. 2. Decrease of iNOS expression by treatment of *Artemisiae asiaticae Herba* and *Saussureae Radix*. Macrophages (1×10^7 cells/dish) were incubated with LPS (10 μg/ml) in the absence or presence of herbal extracts (0.1 and 1 μg/ml), *Artemisiae asiaticae Herba* or *Saussureae Radix* under described Methods. The effects of both herbal extracts on nitrite contents (panel A) and iNOS expression (panel B) were measured as the described Methods. Nitrite content in medium from non-stimulated macrophage was 1.03 μM. I, *Artemisiae asiaticae Herba*; II, *Saussureae Radix*; Con, control ; LPS, LPS-stimulated; Vehicle, LPS plus 0.2% ethanol. Data shown as the percentage inhibition of the LPS-induced nitrite release represent the means±S.E. of at 4 separate experiments. $^*P<0.05$ and $^{***}P<0.001$ indicate significant inhibition of NO production by herbal extracts in the presence of LPS.

DISCUSSION

Activated macrophages secrete many cytotoxic agents, including lysosomal enzymes, proteolytic enzymes, oxygen-derived free radicals, and reactive nitrogen intermediates such as NO. Recent studies have shown that microbicidal and tumoricidal activity of activated macrophages are associated with the production of NO (Nathan, 1992). Also, NO may play a role in autoimmune diseases such as type I diabetes (Kroncke *et al.*, 1993). The mechanisms by which NO exerts its effects may include ADP ribosylation (Zhang and Snyder, 1992), inhibition of mitochondrial respiration (Stuehr and Nathan, 1989) and electron transport, and inhibition of ribonucleotide reductase (Kwon *et al.*, 1991).

Agents that stimulate macrophages to produce NO include cytokines such as IFN-γ (Karupiah *et al.*, 1993), and endot-

oxins such as LPS (Nathan, 1992). Furthermore, TNF-α, IL-2, IL-1, and LPS can synergistically enhance the production of NO by IFN-γ treated macrophages (Cox *et al.*, 1992). However, cytokines such as IL-10 (Cunha *et al.*, 1992), TGF-1, TGF-2, and TGF-3 (Ding *et al.*, 1990) have shown to inhibit IFN-γ stimulated NO production.

Recently, NO has been implicated in a number of pathological conditions, including graft-versus-host disease (Garside *et al.*, 1992) and experimentally induced neurological diseases (Koprowski *et al.*, 1993). Especially, the role of NO has been demonstrated in inflammation. In rats, NO is involved in the acute inflammatory responses following footpad injection of carrageenan (Ialenti *et al.*, 1992). Serum from patients with rheumatoid arthritis or osteoarthritis contains significantly higher levels of nitrite compared to those of matched controls (Farrell *et al.*, 1992). However, the mechanisms involved and the regulation of NO synthesis in these models are obscure.

Therefore, in this study we have screened the various herbal extracts to develop the NOS inhibitor and determine its regulation. As a result, the 9 species have shown more than 50% inhibitory effect on NO production in LPS-stimulated macrophages (Table I, Fig. 1), and both *Artemisiae asiaticae Herba* and *Saussureae Radix* represented strongly inhibitory effect (Fig. 2A). And it is thought that this effect might be based on inhibition of NOS at the translation level (Fig. 2B). To investigate the inhibitory mechanism of the herbal extracts on NOS it is necessary to examine whether both herbal extracts can inhibit the NOS activity at the transcription level and further purify active component of both herbal extracts. Also, study of other herbal extracts showing strong effect for NO inhibition might be important to elucidate the mechanism of between NO and inflammatory action.

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