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Inhibitory Activity of Medicinal Herbs on Nitric Oxide Synthesis in Activated Macrophages

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Abstract – Nitric Oxide (NO), derived from L-arginine, is produced by two types (constitutive and inducible) of nitric oxide synthase (NOS: cNOS and iNOS). The NO produced in large amounts by the iNOS is known to be responsible for the vasodilation and hypotension observed in septic shock, cancer metastasis and inflammation. The inhibitors of iNOS, thus, may be useful candidates for the treatment of inflammatory diseases accompanied by the overproduction of NO. We prepared alcoholic extracts of herbal drugs which have been used for the treatment of inflammation in oriental medicine. We have screened the inhibitory activity of NO production in lipopolysaccharide (LPS)-activated macrophages after the treatment of these extracts. Among 82 kinds of extracts of herbal drugs, 35 extracts showed the potent inhibitory activity of NO production above 50% at the concentration of 50 μg/mL. The inhibitory activities of NO production were also evaluated for several solvent fractions at two different concentrations. Especially, hexane and EtOAc fractions of *Alpinia officinarum*, *Angelica gigas*, *Ostericum koreanum*, *Saussurea lappa*, *Torilis japonica*, and hexane fractions of *Agrimonia pilosa*, *Machilus thunbergii*, *Hydrangea serrata*, *Magnolia obovata*, *Prunella vulgaris*, *Tussilago farfara*, and EtOAC fractions of *Perilla frutescence* showed a significant activity at 10 and/or 25 μg/mL. In Western blot analysis, the hexane fractions (5 μg/mL) of *Magnolia obovata* and *Saussurea lappa*, and EtOAc fractions (20 μg/mL) of *Hydrangea Serrata*, *Perilla frutescence* and *Torilis japonica* inhibited the expression of iNOS protein in LPS-activated macrophages. These plants may be promising candidates for the study of the activity-guided purification of active compounds and might be useful for the treatment of inflammatory diseases and endotoxemia accompanying overproduction of NO.

Keywords – nitric oxide, inhibitor, nitric oxide synthase, screening, herbal drug, macrophage

Introduction

L-Arginine-derived nitric oxide (NO) is an intracellular mediator produced in mammalian cells by two types of nitric oxide synthase (NOS) (Forstermann *et al.*, 1991). A constitutive NOS (cNOS) is Ca^{2+}-dependent and releases small amounts of NO which is required for physiological functions (Bredt and Snyder, 1990). The other form of inducible NOS (iNOS) is Ca^{2+}-independent and induced by lipopolysaccharide (LPS) or some proinflammatory cytokines such as TNF-α, IL-1β, and IFN-γ (Stuehr *et al.*, 1991; Billiar *et al.*, 1990; Iida *et al.*, 1992; Kilbourn and Belloni, 1990). The NO produced in large amounts by iNOS and its derivatives, such as peroxynitrite and nitrogen dioxide, plays a role in inflammation and also possibly in the multistage process of carcinogenesis (Oshima and Bartsch, 1994). NO is also known to be responsible for the vasodilation and hypotension observed in septic shock (Kilbourn *et al.*, 1990; Thiemermann and Vane, 1990). So inhibitors of iNOS may be available as a therapeutic agent of septic shock and inflammation.

There are many plant-derived constituents that can affect NO signaling pathway (Achike and Kwan, 2003), and most of these compounds showed their inhibitory activity of NO production through the inhibition of iNOS expression (Ryu *et al.*, 2002; Hong *et al.*, 2002; Kim *et al.*, 2001; Chi *et al.*, 2001). In order to find new iNOS inhibitors from medicinal plants, we have screened the inhibitory activity of NO production by measuring the NO production in LPS-activated RAW 264.7 cells (Ryu *et al.*, 2003).

Experimental

Reagents and materials – Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco Laboratories

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(Detroit, MI), and LPS (*Escherichia coli*, 0127:B8), bovine serum albumin, sodium nitrite, N-(1-naphthyl) ethylenediamine and N^G-monomethyl-L-arginine (L-NMMA) were obtained from Sigma Chemical Co. (St. Louis, MO).

Extraction and solvent fractionation – The medicinal herbs were purchased from Kyungdong oriental drug market in Seoul. The dried plant materials were extracted three times with MeOH under reflux and the solvent was evaporated under reduced pressure. The selected extracts of plant materials were dispersed in water and extracted with n-hexane to give hexane soluble fraction. The remained water layer was extracted again with EtOAc and *n*-BuOH, sequentially to yield EtOAc and BuOH soluble fractions, respectively.

Cell culture – The murine macrophage cell line (RAW 264.7) was purchased from the American Type Culture Collection (Rockville, MD, USA). Cells were cultured in DMEM containing 10% fetal bovine serum, 2 mM glutamine, 1 mM pyruvate, penicillin (100 U/mL) and streptomycin (10 μg/mL). Cells were grown at 37°C, 5% CO_2 in fully humidified air, and were split twice a week. RAW 264.7 cells were seeded at 8×10^5cells/mL in 24 well plates and were activated by incubation in medium containing LPS (1 μg/mL) and various concentrations of test compounds. The supernatants were collected as sources of secreted NO.

Nitrite assay – NO released from macrophages was assessed by determination of NO_2^- concentration in culture supernatant. Samples (100 μL) of culture media were incubated with an 150 μL of Griess reagent (1% sulfanilamide, 0.1% naphthylethylene diamine in 2.5% phosphoric acid solution) at room temperature for 10 min in 96-well microplate (Green *et al.*, 1982). Absorbance at 570 nm was read using an ELISA plate reader. Standard calibration curves were prepared using sodium nitrite as a standard.

Western blot analysis of iNOS – The cells were rinsed with phosphate buffered saline and lysed by boiling with lysis buffer (1% SDS, 1.0 mM sod. vanadate, 10 mM Tris, pH 7.4) for 5 min. Thirty μg protein of cell lysates was applied on 8% SDS-polyacrylamide gels and transferred to PVDF membrane by the standard method. The membrane was probed with antibodies for anti-mouse iNOS (Transduction Laboratories, Lexington, KY) and anti-β-actin (Sigma). The Western blot was visualized using an enhanced chemiluminescence (ECL) detection kit (Amersham Bioscience, Piscataway, NJ) according to the manufacturer's instruction.

Densitometric analysis – The images of immunoblotting were digitalized and imported using VersaDoc3000 (Bio-Rad, Herclues, CA, USA). The area density was quantified using Quantity One software (Bio-Rad, Herclues, CA, USA). The unit given is an arbitrary unit that stands for the total units per band in the analyzed samples.

Statistical analysis – The results were expressed as mean±S.D. of three experiments, and statistical analysis was performed by the Student's *t*-test, and a P value of <0.01 was considered to indicate a significant difference.

Results and Discussion

In order to find new iNOS inhibitors from medicinal herbs, we have screened the inhibitory activity of NO production by measuring the NO production in LPS-activated RAW 264.7 cells. The amounts of NO were measured as the form of NO_2^- after incubation of culture media with Griess reagent. For the quantitation of NO, a standard calibration curve was prepared by using sodium nitrite as a standard. Plant samples were dissolved in dimethyl sulfoxide (DMSO) and diluted with culture media for adjusting the concentrations of test samples. The final concentration of DMSO in culture media was 0.1% and this concentration of DMSO did not show any effect on assay systems. For optimal condition for screening of inhibitory activity of NO production by plant materials, macrophages were activated with 1 μg/mL of LPS for 20 hr. In LPS-activated RAW 264.7 cell culture system, the production of NO was increased by the enzymatic reaction of induced iNOS. The concentration of NO_2^- of LPS-treated group was 30 - 40 μM, while those of vehicle treated group was less than 8 μM (data not shown). The assay samples were added into the culture media of RAW 264.7 cells during LPS-activation for 20 hr, and the inhibitory activity of NO production by samples was calculated by using a following equation;

$$\text{Inhibition (\%)} = 100\times[OD_{lps} - OD_{sample}]/[OD_{lps} - OD_{vehicle}]$$

The values of OD were measured at 570 nm as described in materials and methods section for each treated groups. The inhibition of NO accumulation in culture media was 65% by treatment with 0.1 mM N^G-monomethyl-L-arginine (L-NMMA) as a positive control which is an inhibitor of NOS through substrate competition (data not shown).

Table 1 shows the inhibitory activity of NO production by extracts of medicinal herbs in LPS-activated macrophages. From 82 kinds of extracts, 35 species showed higher than 50% inhibition of NO production at the concentration of 50 μg/mL of samples in culture media. Especially, the methanolic extracts of *Angelica gigas, Artemisia asiatica, Magnolia denudata, Ostericum koreanum, Machillus thunbergii, Rhus verniciflua* and *Saussurea lappa* showed

inhibition of NO production higher than 30% at 10 μg/mL. From sixteen herbs including species that showed strong inhibition of NO production, methanol extracts were prepared in a large scale, and solvent fractions of them were prepared with increasing the polarity of solvents. The inhibitory activities of NO production were concentrated to certain solvent fractions; to hexane and EtOAc fractions for *Alpinia officinarum, Angelica gigas, Curcuma zedoaria, Forsythia koreana, Hydrangea serrata, Saussurea lappa* and *Torilis japonica*, to hexane fractions for *Agrimonia pilosa, Machilus thunbergii, Magnolia obovata, Poncirus trifoliata, Prunella vulgaris, Schizandra chinensis* and *Tussilago farfara*, to EtOAC fractions for *Prunella vulgaris*. The BuOH fraction of *Magnolia obovata* and *Ostericum koreanum* also showed the strong inhibition of NO production in LPS-activated macrophages (Table 2). The viabilities of RAW 264.7 cells treated with these samples were assessed to be above 85% by MTT method (Mosmann, 1983) at the sample concentrations used for the nitrite assay. The hexane soluble fraction of *Machilus thunbergii* and *Ostericum koreanum* was toxic against RAW 264.7 cells at 50 μg/mL in cell culture media. The inhibitory activity of NO production by medicinal herbs may come from the inhibition of iNOS enzyme activity and/or expression of nitric oxide synthase.

Table 1. Inhibitory activities of the methanol extracts of herbal drugs against the LPS-activated NO production in macrophages

Medicinal herbs	Family	Inhibition (%)	
		50 μg/ml	10 μg/ml
Aconitum koreanum	Ranunculaceae	24	-
Agrimmonia pilosa	Polypodiaceae	67	16
Alpinia officinarum	Zingiberaceae	91	-
Amomum xanthioides	Zingiberaceae	34	-
Anemarrhena asphodeloides	Liliaceae	7	-
Angelica dahurica	Umbelliferae	35	-
Angelica gigas	Umbelliferae	84	30
Angelica tenuissima	Umbelliferae	76	-
Anthriscus sylvestris	Umbelliferae	58	-
Aralia continentalis	Araliaceae	40	-
Areca catechu	Palmae	59	-
Aristolochia contorta	Aristolochiaceae	14	-
Artemisia asiatica	Compositae	98	86
Asparagus cochinchinensis	Liliaceae	4	-
Aster tataricus	Compositae	32	-
Atractylodes japonica	Compositae	61	-
Bupleurum falcatum	Umbelliferae	55	-
Carthamus tinctoris	Compositae	31	-
Chelidonium major	Papaveraceae	43	-
Chrisanthemum sinense	Compositae	26	-
Chrysanthemum sibiricum	Compositae	51	5
Cimicifuga heracleifolia	Ranunculaceae	38	-
Cnidium officinale	Umbelliferae	31	-
Curcuma zedoaria	Zingiberaceae	85	-
Dioscorea quinqueloba	Dioscoreaceae	17	-
Embelia parviflora	Myrsinaceae	97	27
Ephedra sinica	Ephedraceae	6	-
Epimedium koreanum	Berberidaceae	41	-
Eriobotrya japonica	Rosaceae	29	-
Eucheresta japonica	Leguminosae	18	-

Table 1. continued

Medicinal herbs	Family	Inhibition (%)	
		50 μg/ml	10 μg/ml
Eucommia ulmoides	Eucommiaceae	63	12
Evodia officinalis	Rutaceae	52	-
Ferula scoradosma	Umbelliferae	41	-
Foeniculum vulgare	Umbelliferae	75	18
Forsythia koreana	Oleaceae	64	24
Fritillaria verticillata	Liliaceae	14	-
Gastrodia elata	Orchidaceae	35	-
Gleditschia japonica	Leguminosae	58	-
Glycyrrhiza uralensis	Leguminosae	48	-
Hydrangea serrata	Saxifragaceae	50	4
Juncus decipiens	Juncaceae	61	-
Kalopanax pictum	Araliaceae	38	-
Lindesera obtusiloba	Lauraceae	61	-
Liriope platyphylla	Liliaceae	6	-
Machillus thunbergii	Lauraceae	81	34
Magnolia denudata	Magnoliaceae	78	31
Magnolia obovata	Magnoliaceae	82	16
Morus alba	Moraceae	47	18
Ostericum koreanum	Umbelliferae	83	38
Paeonia albiflora	Paeoniaceae	30	-
Paeonia moutan	Ranunculaceae	11	-
Patrinia scabiosaefolia	Valerianaceae	18	-
Perilla frutescence	Labiatae	85	28
Pogostemon heyneanus	Labiatae	57	-
Polygonum aviculare	Polygonaceae	58	-
Poncirus trifoliata	Rutaceae	62	12
Poria cocos	Polyporaceae	44	-
Prunella vulgaris	Labiatae	48	3
Prunus persica	Rosaceae	39	-

Table 1. continued

Medicinal herbs	Family	Inhibition (%)	
		50 μg/ml	10 μg/ml
Pteridium aquilinum	Pteridaceae	44	-
Pueraria thunbergiana	Leguminosae	36	-
Pulsatilla koreana	Ranunculaceae	70	-
Rhaphanus sativa	Cruciferae	28	-
Rhus verniciflua	Anacardiaceae	84	44
Rubus coreana	Rosacea	18	-
Santalum album	Santalaceae	87	22
Saussurea lappa	Compositae	99	96
Schizandra chinensis	Magnoliaceae	43	12
Schizonepeta tenuifolia	Labiatae	34	-
Simomenium acutum	Menispermaceae	6	-
Smilax china	Liliaceae	15	-
Sophora japonica	Leguminosae	37	-
Stemona japonica	Stemonaceae	7	-
Taraxacum platycarpum	Compositae	32	-
Torilis japonica	Umbelliferae	93	28
Tussilago farfara	Compositae	74	27
Ulmus davidiana	Ulmaceae	28	-
Viscum album	Loranthaceae	47	-
Xanthium stramonium	Compositae	28	-
Zingiber officinale	Zingiberaceae	5	-
Ziziphus japonica	Rhamnaceae	4	-

They did not show any significant inhibitory activities of NO production when they were added into cell culture media after induction of iNOS by LPS (data not shown). As shown in Western blot analysis of cell lysates (Fig. 1), several solvent fractions of herbs inhibited the expression of iNOS protein compared with the LPS control group.

Diverse groups of compounds from medicinal plants have been known as inhibitors of expression of iNOS in LPS-activated macrophages. Their structures can be categorized as sesquiterpenes (Park *et al.*, 1996; Lee *et al.*, 1999), flavonoids (Kim *et al.*, 1999; Kobuchi *et al.*, 1997), polyacetylenes (Choi *et al.*, 2000), lignans (Son *et al.*, 2000), curcuminoids (Brouet and Oshima, 1995; Jang *et al.*, 2004), triterpenes and saponins (Oh *et al.*, 2004; Tapondjou *et al.*, 2003; Choi *et al.*, 2003; Jeong and Choi, 2002). The plants showing inhibitory activity of NO production can be promising candidates for the activity-guided isolation of active components having iNOS inhibitory activity, which may have potential for the treatment of endotoxemia and inflammation accompanying overproduction of NO. Further investigation is underway to characterize active constituents present in the extract of plants.

Table 2. Inhibitory activities of the solvent fractions of herbal drugs against the LPS-activated NO production in macrophages

Medicinal Plants		Inhibition(%)		Medicinal Plants		Inhibition(%)	
		50 μg/ml	25 μg/ml			50 μg/ml	10 μg/ml
	Hexane	95	63		Hexane	100	52
Agrimonia pilosa	EtOAc	36	5	*Angelica officinarum*	EtOAc	101	49
	BuOH	12	6		BuOH	50	26
	Hexane	80	ND		Hexane	toxic	59
Curcuma zedoaria	EtOAc	78	ND	*Angelica gigas*	EtOAc	toxic	64
	BuOH	53	ND		BuOH	ND	37
	Hexane	102	21		Hexane	toxic	70
Forsythia koreana	EtOAc	105	29	*Machilus thunbergii*	EtOAc	35	21
	BuOH	48	3		BuOH	ND	3
	Hexane	101	61		Hexane	ND	70
Hydrangea serrata	EtOAc	98	44	*Magnolia obovata*	EtOAc	ND	6
	BuOH	13	2		BuOH	ND	77
	Hexane	ND	ND		Hexane	toxic	95
Perilla frutescence	EtOAc	105	89	*Ostericum koreanum*	EtOAc	32	80
	BuOH	7	6		BuOH	62	30
	Hexane	69	38		Hexane	ND	100
Poncirus trifoliata	EtOAc	24	ND	*Saussurea lappa*	EtOAc	ND	95
	BuOH	5	ND		BuOH	ND	ND
	Hexane	82	53		Hexane	100	50
Prunella vulgaris	EtOAc	43	4	*Torilis japonica*	EtOAc	98	51
	BuOH	16			BuOH	91	47
	Hexane	76	41		Hexane	ND	98
Schizandra chinensis	EtOAc	6	ND	*Tussilago farfara*	EtOAc	ND	32
	BuOH	8	ND		BuOH	ND	13

*ND: not determined.
toxic: samples are toxic against RAW 264.7 cells at concentrations treated.

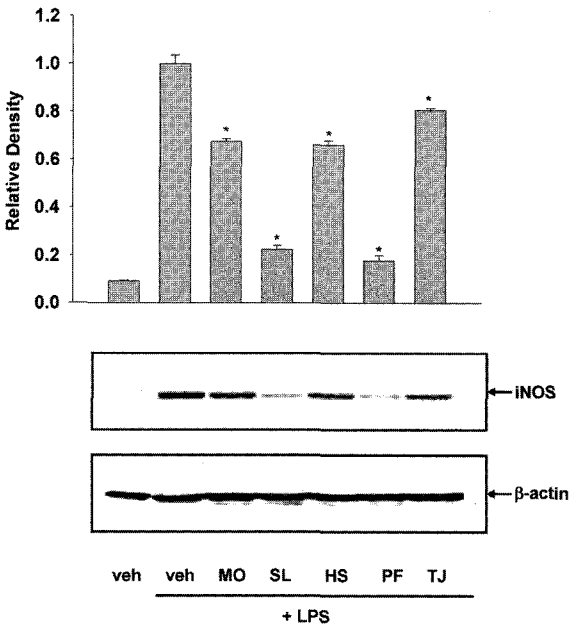

Fig. 1. Suppression of LPS-induced iNOS expression by solvent fractions of medicinal herbs in LPS-activated macrophages. Cell lysates were prepared as described in materials and methods section after 20 hr LPS (1 μg/mL) treatment with samples. Lane 1: vehicle control, lane 2: LPS control, lane 3, 4: hexane fractions (5 μg/mL) of *Magnolia obovata* (MO), *Saussurea lappa* (SL), lane 5~7: EtOAc fractions (20 μg/mL) of *Hydrangea serrata* (HS), *Perills frutescence* (PF), *Torilis japonica* (TJ), respectively. The result of iNOS Western blot was subjected to densitometric analysis (upper panel). Densitometric units were normalized to β-actin and represented as mean±SD of three separate measurements. Values with asterisk represent significant ($p<0.01$) differences of iNOS expression compared with LPS/vehicle control.

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