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Inhibitory Activity of Chinese Medicinal Plants on Nitric Oxide Synthesis in Lipopolysaccharide-Activated Macrophages

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Abstract – Nitric oxide (NO) produced in large amounts by the inducible nitric oxide synthase (iNOS) is known to be responsible for the vasodilation and hypotension observed in septic shock and inflammation. The inhibitors of iNOS, thus, may be useful candidate for the treatment of inflammatory diseases accompanied by the overproduction of NO. We prepared alcoholic extracts of Chinese medicinal plants and screened their inhibitory activity against NO production in lipopolysaccharide (LPS)-activated macrophages. Among the 80 kinds of extracts of herbal drugs, 15 extracts showed potent inhibitory activity of NO production above 80% at the concentration of 50 μg/ml. These potent extracts showed dose dependent inhibition of NO production of LPS-activated macrophages at the concentration of 50, 30, 10 μg/ml. Especially, *Rhus chinensis, Senecio scandens* and *Wikstroemia indica* showed most potent inhibition above 50% at the concentration of 10 μg/ml. These plants are promising candidates for the study of the activity-guided purification of active compounds and would be useful for the treatment of inflammatory diseases and endotoxemia accompanying the overproduction of NO.

Key words ☐ Nitric oxide, inhibitor, nitric oxide synthase, screening, Chinese plant, macrophage

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). And 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). NO produced in large amounts by iNOS and its derivatives, such as peroxynitrite and nitrogen dioxide, play 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 the inhibitor of iNOS may be available as therapeutic agent of septic shock and inflammaion. Recently, several iNOS inhibitors were reported from plants such as bisbenzylisoquinoline alkaloids (Kondo *et al.*, 1993), benzoquinones (Niwa *et al.*, 1997), sesquiterpene lactones (Park *et al.*, 1996; Lee *et al.*, 1999), curcuminoids (Brouet and Oshima, 1995), lignans (Son *et al.*, 2000) and polyacetylenes (Choi *et al.*, 2000). Most of these compounds showed their inhibitory activity of NO production through the inhibition of iNOS expression.

In order to find new iNOS inhibitors from Chinese medicinal plants, we have screened the inhibitory activity of NO production by measuring the NO production in LPS-stimulated RAW 264.7 cells.

MATERIALS AND METHODS

Plant materials

Plants were collected in the garden of Guangxi Institute of Botany, China in 1997 through 1998. The dried plant materials were extracted with 70% MeOH with reflux and the extracts were dried *in vacuo*.

Reagents and materials

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Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco Laboratories (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).

Cell culture

Murine macrophage cell line (RAW 264.7) was obtained from 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 8×10^5 cells/ml in 24 well plates and were activated by incubation in medium containing LPS (1 μg/ml) and various concentrations of test compounds dissolved in water or DMSO. The supernatant was collected as a source 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 540 nm was read using an ELISA plate reader. Standard calibration curves were prepared using sodium nitrite as standard.

RESULTS AND DISCUSSION

In order to find new iNOS inhibitors from medicinal plants, we have screened their inhibitory activity against NO production in LPS-stimulated 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 quantitation of NO, standard calibration curve was prepared by using sodium nitrite as standard. All the plant samples were dissolved in dimethyl sulfoxide (DMSO) and diluted with sterile water 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 the assay systems. The production of NO was dependent on the concentration of LPS in culture media and

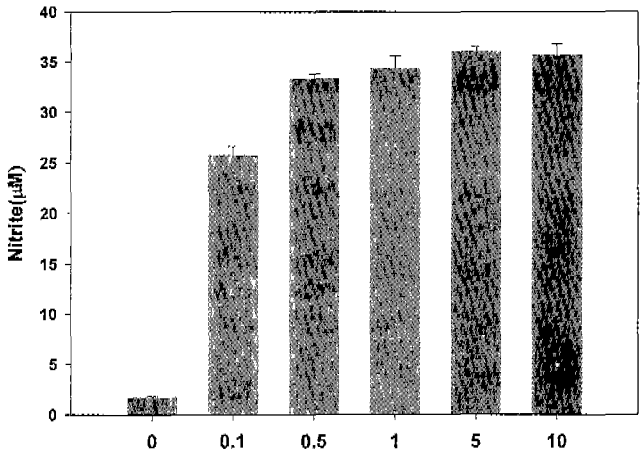

Fig. 1. Dose dependent production of NO with various concentrations (μM) of LPS in culture media of RAW 264.7 cells. The amounts of NO were measured as described in materials and methods section after incubation with LPS for 20 hr.

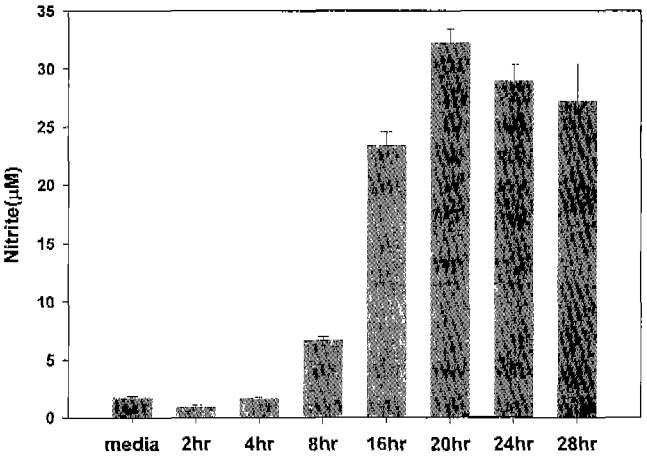

Fig. 2. Time dependent concentrations of NO produced by LPS-activated RAW 264.7 cells. After incubation with LPS (1 μg/ml) for indicated times, the amounts of NO released into culture media were measured as described in materials and methods section. The NO of media control group was measured after 20 hr incubation without LPS treatment.

incubation time with LPS (Fig. 1 and Fig. 2). The optimum concentration of LPS was adopted as 1 μg/ml that is enough for the induction of iNOS without cell toxicity. As shown in Fig. 2, the accumulated amounts of NO was reached maximum after incubation with LPS for 20 hr. In LPS (1 μg/ml) stimulated 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 that of media treated group was less than 3 μM. 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 calcu-

Table I. Inhibitory activities of the plant extracts on the LPS-activated NO production in RAW 264.7 cells

Botanical names of plants	Parts*	Inhibition (%)**
Acer palmatum Thunb.	B, L	33
Agave sisalana Perrine ex Engelm. (*A. rigida* Mill.)	L	40
Aglaonema modestum Schott ex Engl.	W	74
Agrimonia pilosa Ledeb.	W	42
Alpinia zerumbet (Pers.) Burtt. et Smith	W	32
Arenga pinnata (Kuntze) Merr.	B, L	72
Aspidistra elatior Bl.	W	68
Baphicacanthus cusia (Nees) Bremek.	W	11
Bougainvillea glabra Choisy	B, L	45
Camellia japonica L.(*Thea japonica* Baill.)	B, L	77
Campsis grandiflora (Thunb.) Loisel.	B, L	73
Canna indica L. (*C. chinensis* Willd.)	W	51
Chirita eburnea Hance. (*C. faurei* Fr.)	W	59
Cinnamomum camphora (L.) Presl	B, L	18
Cinnamomum burmanni (Nees) Bl.	B, L	48
Clausena lansium (Lour.) Skeels	B, L	51
Cratoxylon ligustrinum (Spach) Bl. (*C. polyanthum* Korth.)	B, L	64
Cycas revoluta Thunb.	L	36
Daucus carota L. var. *sativa* DC.	R	37
Drynaria fortunei (Kze.) J. Sm.	W	31
Erigeron canadensis L.	W	79
Euphorbia hirta L.	W	45
Excoecaria cochinchinensis Lour.	B, L	52
Gleditsia sinensis Lam. (*G. horrida* Willd.)	P	24
Gossampinus malabarica (DC.) Merr.	B, L	56
Gynura crepidioides Benth.	W	58
Homalocladium platycladum (F. Muell.) Bail.	W	52
Humata tyermanni Moore	W	37
Ilex cornuta Lindl.	B, L	48
Illicium verum Hk. f.	B, L	75
Iris tectorum Maxim.	W	62
Kalimeris indica (L.) Sch-Bip.	W	70
Lophatherum gracile Brongn.	W	39
Lycoris aurea Herb.	W	40
Melastoma candidum D. Don (*M. septemnervium* Lour.)	W	34
Michelia alba DC.	B, L	79
Michelia champaca L.	B, L	78
Mimosa pudica L.	W	51
Mirabilis jalapa L.	W	41
Murraya paniculata (L.) Jack.	B, L	54
Nandina domestica Thunb.	B, L	58
Nerium indicum Mill.	B, L	37
Ormosia henryi Prain	B, L	44
Oxalis corymbosa DC. (*O. matiima* Zucc)	W	31
Paliurus ramosissimus (Lour.) Poir.	B, L	63
Paulownia fortunei (Seem.) Hemsl.	B, L	71
Pharbitis nil (L.) Choisy	W	50
Pinus massoniana Lamb.	B, L	71
Pinus yunnanensis Fr. var. *tenuifolia* Cheng et Y.W. Law	B, L	62
Podocarpus macrophyllus Thunb. D. Don	B, L	34
Polygonum multiflorum Thunb.	R	71
Pseudolarix amabilis (Nelson) Rehd.	B, L	43
Pterideum aquilinum (L.) Kuhn var. *latiusculum* (Desv.) Underwex Hell	W	28
Rauvolfia verticillata (Lour.) Baill.	B, L	61
Reineckea carnea (Andr.) Kunth	W	58
Rhododendron simsii pl.	B, L	38
Rosa chinensis Jacq.	B, L	50
Rubus alceaefolius Poir	W	35
Saxifraga stolonifera Meerb.	W	67
Selaginella uncinata (Desv.) Spring	W	39
Tecomaria capensis Spach	B, L	62
Uncaria rhynchophylla (Miq.) Jacks.	B, L	40
Verbena officinalis L.	W	39
Wedelia chinensis (Osb.) Merr.	W	51

*W : whole plant, B : branches, L : leaves, R : rhizome, P : pod
**at concentration of 50 μg/ml

lated by using the followed equation;

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

The values of OD were measured at 540 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) which is an inhibitor of NOS through substrate competition (data not shown). Table I showed the inhibitory activity of NO production by the extracts of Chinese medicinal plants in LPS-activated macrophages. From 80 kinds of extracts, fifteen species showed higher than 80% inhibition of NO production at the concentration of 50 μg/ml of samples in culture media. As shown in Table II, these fifteen species showed dose dependent inhibition of NO production, especially, the extracts of *Rhus chinensis, Senecio scandens* and *Wikstroemia indica* showed higher than 50% inhibition at the concentration of 10 μg/ml in culture media. The anti-inflammatory and immunomodulatory effects of the methanolic extracts of *Bidens pilosa* were reported (Pereira *et al.*, 1999) and an acetylene compound was purified as an active component. This

Table II. Inhibitory activities of the plant extracts on the LPS-activated NO production in RAW 264.7 cells

Botanical names of plants	Parts*	Inhibition (%)		
		50 μg/ml	30 μg/ml	10 μg/ml
Bidens pilosa L.	W	95	57	26
Canarium album (Lour.) Raensch.	B, L	81	31	21
Crinum asiaticum L. var. *sinicum* Baker	W	82	33	30
Euonymus japonica Thunb.	B,L	92	42	14
Ficus pumila L.	W	95	41	34
Jaminum amplexicaule Buch.-Ham.	B, L	80	29	<10
Ophiopogon japonicus (Thunb.) Ker-Gawl.	W	85	14	12
Osmanthus fragrans Lour.	B, L	87	30	<10
Parthenocissus heterophylla (Bl.) Merr.	W	82	20	<10
Podocarpus nagi (Thunb.) Zoll. Et Moritz. Ex Zoll.	B, L	81	31	19
Radermachera sinica (Hance) Hemsl.	B, L	87	61	36
Rhus chinensis Mill. (*R. semialata* Merr.)	B, L	87	70	52
Senecio scandens Buch.-Ham.	W	117	82	52
Wikstroemia indica (L.) C.A. Mey.	W	92	69	60
Wisteria sinensis Sweet (*W. chinensis* DC.)	B, L	91	40	28

*W : whole plant, B : branches, L : leaves, R : rhizome

activity may be explained by the inhibitory activity of NO production of this plant. And ethnomedicinal application of *Bidens pilosa*, *Radermachera sinica*, *Osmanthus fragrans* and *Rhus chinensis* as promotion of blood circulation may also be related with the inhibition of NO production. The viabilities of RAW 264.7 cells were assessed to be above 85% by MTT method (Mosmann, 1983) at the sample concentrations for the nitrite assay. The inhibitory activity of NO production by medicinal plants may come from the inhibition of iNOS enzyme activity and/or expression of nitric oxide synthase. Many compounds from medicinal plants have been reported as inhibitors of expression of iNOS in LPS-activated macrophages. Their structures can be categorized as sesquiterpene (Park *et al.*, 1996; Lee *et al.*, 1999), flavonoid (Kim *et al.*, 1999; Kobuchi *et al.*, 1997), polyacetylenes (Choi *et al.*, 2000) and lignans (Son *et al.*, 2000). The plants showing inhibitory activity of NO production can be promising candidates for the activity-guided isolation of active components which may have potential for the treatment of endotoxemia and inflammation accompanying overproduction of NO. The identification of active components and action mechanism of these screened plants are under investigation.

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