Yomogin, an Inhibitor of Nitric Oxide Production in LPS-Activated Macrophages

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In activated macrophages the inducible form of nitric oxide synthase (i-NOS) generates high amounts of toxic mediator, nitric oxide (NO) which contributes to the circulatory failure associated with septic shock. A sesquiterpene lactone compound (yomogin) isolated from medicinal plant *Artemisia princeps* Pampan inhibited the production of NO in LPS-activated RAW 264.7 cells by suppressing i-NOS enzyme expression. Thus, yomogin may be a useful candidate for the development of new drugs to treat endotoxemia and inflammation accompanied by the overproduction of NO.

Key words : Yomogin, *Artemisia princeps,* Nitric oxide, Nitric oxide synthase, Induction, LPS, Inhibitor

INTRODUCTION

L-Arginine-derived nitric oxide (NO) is a intracellular mediator produced in mammalian cells by two types of nitric oxide synthase (NOS) (Forstermann *et al.*, 1991). A constitutive NOS (c-NOS) is Ca^{2+}-dependent and releases small amounts of NO which is required for physiological functions (Bredt *et al.*, 1990). And the other form of inducible NOS (i-NOS) is Ca^{2+}-independent and induced by LPS or some proinflammatory cytokines such as TNF-α, IL-1β and IFN-γ (Stuehr *et al.*, 1991; Idia *et al.*, 1992; Kilbourn and Belloni, 1990). The NO produced in large amounts by the i-NOS 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). And NO is also known to be responsible for the vasodilation and hypotension observed in septic shock (Kilbourn *et al.*, 1990; Thiemermann and Vane, 1990). Therefore, the inhibitor of i-NOS may be effective as a therapeutic agent for septic shock and inflammation. Recently, several i-NOS inhibitors were reported from plants such as benzoquinones (Niwa *et al.*, 1997), costunolide (Park *et al.*, 1996), curcumin (Brouet and Oshima, 1995) and bisbenzylisoquinoline alkaloids (Kondo *et al.*, 1993). The extracts of the whole herb of *Artemisia princeps* Pampan (Asteraceae) have been used for the treatment of inflammation, diarrhoea and many circulatory disorders in Chinese traditional medicine. From the methanol extract of *Artemisia princeps* Pampan, we purified and identified an active principle having inhibitory activity of NO production in lipopolysaccharide (LPS) activated macrophage cell line, RAW 264.7 cells.

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MATERIALS AND METHODS

General

NMR spectra were obtained on a Brucker AM-300. EI mass spectra were taken with a direct inlet and recorded with a JMS-DX 303 mass spectrometer (JEOL). The optical rotations were determined on Autopol III automatic polarimeter. DMEM was purchased from Gibco Laboratories (Detroit, MI) and LPS (*Escherichia coli*, 0127:B8), sodium nitrite, N-(1-naphthyl) ethylenediamine, N^G-monomethyl-L-arginine (L-NMMA) and L-arginine were obtained from Sigma Chemical Co. (St. Louis, MO).

Extraction and isolation

The herb of *Artemisia princeps* Pampan was collected in Chungnam Province, Korea in September 1992, and a voucher specimen (SNU-9254) is deposited at the Herbarium of Natural Products Research Institute of Seoul National University, Korea. The dried herb (1.2 kg) was extracted with methanol at room temperature for 7 days. Concentration gave the syrupy pro-

duct (160 g), which was suspended in H_2O and partitioned successively with CH_2Cl_2 (48 g) and EtOAc (17 g). Six subfractions (Fr.1 -Fr.6) were obtained by silicagel column chromatography of the CH_2Cl_2 soluble fraction (48 g) using gradient elution with MeOH in CH_2Cl_2. Each subfraction was evaluated individually by current bioassay system and only the Fr.3 (7g) was revealed to be active. Thus, the Fr.3 was further purified by repeated silicagel column chromatography using hexane and EtOAc (7:3) as an eluent, which finally gave 140 mg of a colorless needle in EtOAc. This active component was identified as an eudesmane-sesquiterpene constituent, yomogin (**1**) by the direct comparison of physical and spectral properties with those in literature (Jakupovic *et al.*, 1988).

yomogin (1): colorless needle (EtOAc), $C_{15}H_{16}O_3$; mp. 201°C; $[\alpha]_D$=-88° (c 0.1, $CHCl_3$); UV λ_{max} nm (MeOH): 36, 265 (shoulder); EI-Mass m/z (rel. int.): 244 (M^+, 6.8), 183 (20), 130 (17), 120 (35), 105 (100), 91 (38).

^{1}H-NMR δ (300 MHz, $CDCl_3$): 1.31 (3H, s, CH_3-14), 1.67 (1H, dd, *J*=4.8, 15.3 Hz, H-9a), 1.95 (3H, s, CH_3-15), 2.28 (1H, dd, *J*=12.9, 14.0 Hz, H-6a), 2.43 (1H, dd, *J*=2.8, 15.3 Hz, H-9b), 2.97 (1H, dd, *J*=7.0, 14.0 Hz, H-6b), 3.08 (1H, m, H-7), 4.48 (1H, m, H-8), 5.72 and 6.25 (each 1H, d, *J*=1.1 Hz, H-13), 6.22 (1H, d, *J* =9.6 Hz, H-2), 6.78 (1H, d, *J*=9.6 Hz, H-1).

^{13}C-NMR δ (75 MHz, $CDCl_3$): 10.78 (C-15), 25.66 (C-14), 29.94 (C-6), 38.55 (C-10), 38.94 (C-9), 41.88 (C-7), 75.34 (C-8), 121.96 (C-13), 126.38 (C-2), 131.17 (C-4), 140.36 (C-11), 154.44 (C-5), 155.23 (C-1), 169.65 (C-12), 185.58 (C-3).

Cell culture

The murine macrophage cell line (RAW 264.7) was obtained 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 8×10^5 cells/ml in 24 well plates and were activated by incubation in medium containing LPS (1 μg/ml) and various concentrations of test compounds. The supernatant was collected as a source of secreted NO.

Nitrite assay

NO released from macrophages was assessed by determining of NO_2^- concentration in the 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.

RESULTS AND DISCUSSION

Among the solvent fractions made from the methanol extract of *A. princeps* Pampan, the CH_2Cl_2 soluble fraction showed the most potent inhibitory activity of NO production in LPS-activated macrophages. The CH_2Cl_2 soluble fraction was investigated extensively through serial fractionations by column chromatography followed by the nitrite assay, which finally led to the isolation of an active compound (**1**). All the spectral data of **1** were matched well with those of yomogin previously published by Jakupovic *et al.* (1988) (Fig. 1).

Fig. 2 shows the effect of yomogin on the production of NO_2^- in the LPS-activated macrophages. Murine macrophage cell line, RAW 264.7 cells were stimulated by 1 μg/ml of LPS for 18hr, and the production of NO_2^- was increased by the induced i-NOS up to 46 μM in the cell culture media. When yomogin was added to the culture media at the onset time of LPS stimulation, it inhibited the production of NO_2^- with dose-dependent manner. The concentration required to inhibit the production of NO_2^- by 50% (IC_{50} value) was calculated on the basis of NO_2^- con-

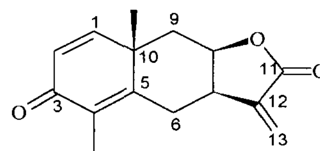

Fig. 1. Structure of yomogin (**1**) from *Artemisia princeps.*

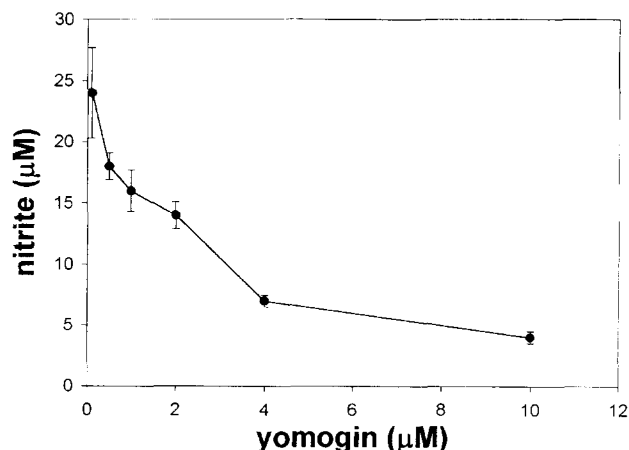

Fig. 2. Yomogin inhibits the production of NO released into the media of LPS-activated RAW 264.7 cells. Conditioned media were collected after 18 hr activation of RAW 264.7 cells with yomogin. The NO_2^- levels were determined as described in the materials and methods section. Results represent the mean±S.D. of three experiments.

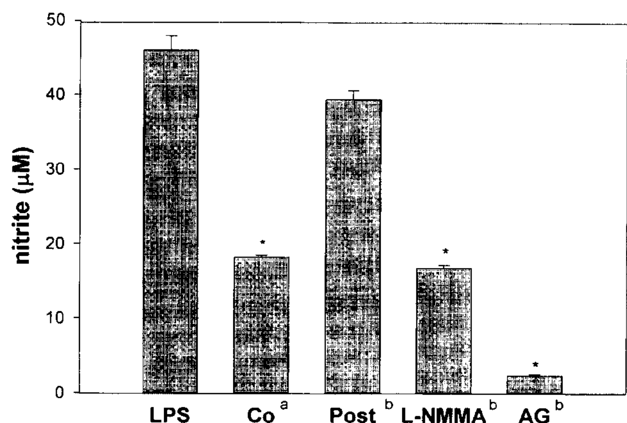

Fig. 3. Effects of yomogin (Co and Post, 5 μM), L-NMMA (0. 1 mM) and aminoguanidine (AG, 1 mM) on the NO production in LPS-activated RAW 264.7 cells. All the media was exchanged by fresh one after 18 hr LPS-activation. The NO_2^- levels were determined after another 18 hr incubation with fresh media and/or effectors.
[a]Incubation with yomogin during 18 hr LPS-activation.
[b]Incubation with effectors for 18 hr after LPS-activation.
Results represent the mean±S.D. of three experiments. Significantly different from LPS control, *p<0.001.

centrations released into the culture media. The IC_{50} value of yomogin was 2.4 μM. This inhibition of NO_2^- production by yomogin may come from either the inhibition of NOS enzyme activity or the inhibition of NOS induction by LPS.

As shown in Fig. 3, when yomogin (5 μM) was treated after the 18 hr activation of RAW 264.7 cells, the concentration of NO in media was 49.4 ±1.2 μM, while the co-treatment of yomogin with LPS resulted in the much lower concentration of NO, 18.2±0.3 μM. And the treatment of L-NMMA (0.1 mM) and aminoguanidine (1 mM) after the 18 hr activation of RAW 264.7 cells, resulted in the strong inhibition of NO production. These results showed that NOS inhibitors, L-NMMA as arginine analog and aminoguanidine as i-NOS specific inhibitor, inhibited the enzymatic production of NO by induced i-NOS. But yomogin could not inhibit the NO production when treated after the completion of i-NOS induction by LPS. Thus, yomogin may be not an inhibitor of NOS but rather than inhibitor of i-NOS induction by LPS in RAW 264.7 cells.

In conclusion, yomogin isolated from *Artemisia princeps* Pampan reduced the levels of NO_2^- production in LPS-activated macrophages by inhibiting of i-NOS expression. Owing to these biological property of yomogin, it may thus have potential in the treatment of endotoxemia and inflammation accompanied by the overproduction of NO.

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