Polyacetylenes from *Angelica gigas* and Their Inhibitory Activity on Nitric Oxide Synthesis in Activated Macrophages

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In activated macrophages the inducible form of nitric oxide synthase (i-NOS) generates high amounts of the toxic mediator, nitric oxide (NO) which contributes to the circulatory failure associated with septic shock. Two polyacetylenes were isolated from the medicinal plant *Angelica gigas* and their structures were elucidated as octadeca-1,9-dien-4,6-diyne-3,8,18-triol (1) and 18-acetox-octadeca-1,9-dien-4,6-diyne-3,8-diol (2) by spectroscopic method. These polyacetylenes and their peracetate, 3, 8, 18-triacetoxy-octadeca-1, 9-dien-4, 6-diyne (3) inhibited the production of NO in LPS-activated RAW 264.7 cells by suppressing the i-NOS enzyme expression. These new inhibitors of i-NOS expression may have potential in the treatment of endotoxemia and inflammation accompanied by the overproduction of NO.

**Key words** *Angelica gigas*; polyacetylene; nitric oxide synthase; induction; LPS; inhibitor

L-Arginine-derived nitric oxide (NO) is an intracellular mediator produced in mammalian cells by two types of nitric oxide synthase (NOS). Constitutive NOS (c-NOS) is Ca²⁺-dependent and releases small amounts of NO which is required for physiological functions; the other form of inducible NOS (i-NOS) is Ca²⁺-independent and induced by LPS or proinflammatory cytokines TNF-α, IL-1 and IFN-γ. 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. NO is also known to be responsible for the vasodilation and hypotension observed in septic shock. The inhibitor of i-NOS may thus be available as a therapeutic agent of septic shock and inflammation. Recently, we reported several i-NOS inhibitors from the medicinal plants *Artemisia princeps*, *Saussurea lappa* and *Tussilago farfara*. In this paper, we report the isolation of two polyacetylenes from *Angelica gigas* and their inhibitory activity of NO production in LPS-activated macrophages.

**MATERIALS AND METHODS**

**Materials** DMEM was purchased from Gibco Laboratories (Detroit, MI) and LPS (Escherichia coli, 0127:B8) and Nω-monomethyl-l-arginine (l-NMMA) were purchased from Calbiochem Co. (San Diego, CA). Bovine serum albumin, sodium nitrite, naphthylthelylene diamine, sulfanilamide, aminoguanidine, l-arginine and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Anti-mouse i-NOS polyclonal antibody was purchased from Transduction Laboratories (Lexington, KY) and alkaline phosphatase-labeled goat anti-rabbit antibody was purchased from Gibco-BRL (Grand Island, NY).

**Instruments** NMR spectra were obtained on a Bruker AMX-400 spectrometer. El mass spectra were taken with a direct inlet and recorded with a Jeol JMS-DX 303 mass spectrometer. The optical rotations were determined on an Autopol III automatic polarimeter and optical density was measured with a Dynatech MR 5000 microplate reader.

**Extraction and Isolation** *A. gigas* was purchased from the Kyungdong oriental drug market in Seoul, Korea in April 1997. The methanolic extract (1.1 kg) of dried plant materials (5 kg) was suspended in H₂O and extracted with EtOAc to give EtOAc soluble layer (312 g). Ten grams of this EtOAc soluble layer was chromatographed on a silica gel column eluted with hexane-aceton (10:1, 5:1, 3:1 and 1:1) gradients to yield eight sub-fractions. The most potent inhibitory activity of NO production in LPS-activated macrophages was observed in fr. 2 (780 mg) and fr. 6 (160 mg). Further chromatography on silicagel with hexane-EtOAc as eluent gave active compounds 1 (21 mg) and 2 (29 mg) from fr. 6 and fr. 2, respectively.

**Acetylation of Compounds 1 and 2** Compounds 1 and 2 (5 mg each) were dissolved in 200 μl of pyridine, added with 200 μl of acetic anhydride and stirred for 12 h at room temperature. The products were extracted with ether and identified as the same acetates (3) on silica gel TLC.

**Cell Culture** The murine macrophage cell line (RAW 264.7) was obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). 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% CO₂ in fully humidified air, and were split twice a week. RAW 264.7 cells were seeded at 8×10⁵ 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 dimethyl sulfoxide (DMSO). The supernatants were collected as the source of secreted NO. The final concentration of DMSO in culture media was 0.1% and this concentration did not show any effect on the assay systems.

**Nitrite Assay** NO released from macrophages was assessed by the determination of NO concentration in the culture supernatant. Samples (100 μl) of culture media were incubated with 150 μl of Griess reagent (1% sulfanilamide, 0.1% naphthylthelylene diamine in 2.5% phosphoric acid solution) at room temperature for 10 min in 96-well microplates. Absorbance at 540 nm was read using an ELISA plate reader. Standard calibration curves were prepared using sodium nitrite.

**Cell Viability** Cell viability was assessed using a 3-[NII-Electronic Library Service]
(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based colorimetric assay. Cells in 96-well plates (5 × 10^4 cells/well) were exposed to various concentrations of sample at 37 °C, 5% CO₂ in air for 24 h. The 10 μl MTT solution (5 mg/ml in phosphate buffered saline) was added and further incubation followed for 4 h at 37 °C. After aspirating the supernatant from the wells, 100 μl of extraction buffer was added to dissolve formazan crystals. The absorbance of each well was then read at 570 nm using an ELISA plate reader.

**Western Blot Analysis of i-NOS** The cells were rinsed with phosphate buffered saline and lysed by boiling with lysis buffer (1% SDS, 10 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 polyvinylidene difluoride (PVDF) membrane by the standard method. The membrane was blocked with a solution containing 3% BSA for 1 h. It was then incubated with anti-mouse i-NOS polyclonal antibody as primary antibody for 2 h and was washed 3 times with phosphate buffered saline. After incubation with alkaline phosphatase-labeled goat anti-rabbit antibody for 1 h, the bands were visualized using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate as substrate for phosphatase (Bio Rad Laboratories, Hercules, CA, U.S.A.).

**RESULTS AND DISCUSSION**

To find new i-NOS inhibitors from medicinal plants, we have screened the inhibitory activity of NO production in LPS-stimulated RAW 264.7 cells. Among the tested plants that have been used for the treatment of inflammation in oriental medicine, the methanolic extract of *A. gigas* exhibited strong activity of 55.9% inhibition of NO production at the concentration of 50 μg/ml in culture media. The activity-guided fractionation and repetitive chromatographic procedures with the EtOAc soluble fraction of *A. gigas* resulted in the isolation of two active polyacetylenes. Their structures of compounds 1 and 2 were elucidated by the analysis of spectral data including two dimensional NMR spectra, and comparison with those reported in the literature. The acetates from compounds 1 and 2 resulted in the same compound 3.

In LPS (1 μg/ml) stimulated RAW 264.7 cell culture system, the production of NO was increased by the enzymatic reaction of induced i-NOS. When polyacetylenes from *A. gigas* were added to the culture media at the time cell stimulation, they inhibited the production of NO in a dose-dependent manner. The concentrations required to inhibit the production of NO by 50% (IC₅₀ value) were calculated on the basis of calculations of nitrite released into the culture media as shown in Fig. 1. The peracetate of polyacetylene (3) showed the most potent inhibition of NO production. Cell viability was assessed to be above 90% by the MTT method at the concentrations of nitrite assay. The accumulation of NO was also inhibited by treatment with 0.1 mM N²-monomethyl-l-arginine (l-NMMA), an inhibitor of NOS through substrate competition, and the production of NO was restored by addition of 1 mM arginine from 10.0 ± 0.2 to 17.3 ± 0.2 μM. Inhibition of NO production by compound 1, however, was not restored by the addition of arginine as the substrate of NOS (Fig. 2). These data implied that the inhibition of NO production by compound 1 was not due to substrate competition for NOS like l-NMMA was. When compound 1 (10 μM) was treated after induction of i-NOS by 18 h activation of RAW 264.7 cells, the inhibition of NO production was just 10.6% compared with LPS-control, while the cotreatment of compound 1 with LPS resulted in 71.9% inhibition (Fig. 3). The treatment of l-NMMA (0.1 mM) and aminoguanidine (0.1 mM) strongly inhibited NO production even though treatment to cells was made after 18 h activation of RAW 264.7 cells. Aminoguanidine is a specific inhibitor of i-NOS, and can inhibit the enzymatic production of NO by i-NOS. Thus, compound 1 may not be an inhibitor of i-NOS, but rather an inhibitor of i-NOS induction by LPS in RAW 264.7 cells. This postulation was further confirmed in another experiment which showed time-dependent inhibition of NO synthesis by compound 1 during LPS-activation of RAW 264.7 cells (Fig. 4). Maximum inhibition was observed when compound 1 was added to the culture media 2 h before the onset of LPS activation. However, when compound 1 was added 6 or 18 h after LPS addition into the media, or added 2 h before the activation followed by washing out, the inhibitions were weak. The incubation time with compound 1 during LPS-activation was closely related to the decrease in NO production by RAW 264.7 cells. Thus, compound 1 is an inhibitor of i-NOS expression rather than an enzyme inhibitor of i-NOS such as aminoguanidine. Compounds 2 and 3...
Fig. 3. The Effects of Compound 1 (10 μM), l-NMMA (0.1 mM) and Aminoguanidine (AG, 0.1 mM) on the NO Production in LPS-Activated RAW 264.7 Cells

All the media were replaced by fresh ones after 18 h LPS-activation. a) Incubation with compound 1 during 18 h LPS-activation and wash out. b) Incubation with effectors for another 18 h after LPS-activation. Nitrite assay was performed 36 h after LPS treatment. Results are expressed as mean±S.D. of 3 experiments. Significant difference from LPS control, * p<0.001.

Fig. 4. Time Course for the Inhibition of NO Production by Compound 1 (10 μM) in LPS-Activated RAW 264.7 Cells

Compound 1 was treated to RAW 264.7 culture media at different times before/after activation with LPS. LPS: LPS alone, (-)2 h: compound 1 was treated at 2 h before LPS-activation, (-)2 h/6 h: compound 1 was treated at 2 h before LPS-activation and the media were replaced by fresh ones, 0 h: co-treated with LPS, 3 h (6 h, 18 h): treated at 3 h (6 h, 18 h) after addition of LPS into culture media. After 20 h LPS-activation, nitrite assay was performed. Results are expressed as mean±S.D. of 3 experiments. Significant difference from LPS control, * p<0.001.

showed the same pattern of activity as those of compound 1. Western blot analysis (Fig. 5) showed that macrophages acti-

Fig. 5. Western Blot Analysis of i-NOS in Lysates of RAW 264.7 Cells (30 μg Protein/Lane)

Cell lysates were prepared as described in the Materials and Methods section after 18 h post-LPS treatment. lane 1: LPS-activated cells, lane 2, 3 and 4: LPS-activated cells in the presence of 10 μM of compounds 1, 2 and 3, respectively.

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REFERENCES