Induction of Nitric Oxide Synthase by *Oldenlandia diffusa* in Mouse Peritoneal Macrophages

Hwan-Suck CHUNG,*a,b Hyun-Ja JEONG,a,b Seung-Heon HONG,a,b Mi-Sun KIM,a,b So-Jin KIM,a,b Bong-Keun SONG,c In-Seok JEONG,c Eon-Jeong LEE,c Jong-Woong AHN,d Seung-Hwa BAEK,e and Hyung-Min KIM* a,b

*c Department of Oriental Pharmacy, College of Pharmacy, Wonkwang University; b Ki Co. Ltd.; Iksan, Chonbuk, 570–749, South Korea; c College of Oriental Medicine, Wonkwang University; Iksan, Chonbuk, 570–749, South Korea; d Natural Products Research Team, Korea Research Institute of Chemical Technology; P.O. Box 107, Yusong, Taejon 305–600, South Korea; and e Department of Herbal Resources, Professional Graduate School of Oriental Medicine, Wonkwang University; Iksan, Chonbuk, 570–749, South Korea. Received January 21, 2002; accepted May 7, 2002

*To whom correspondence should be addressed. e-mail: hmkim@wonkwang.ac.kr © 2002 Pharmaceutical Society of Japan

*Oldenlandia diffusa* (OD) has been used to treat malignant tumors. In this study using mouse peritoneal macrophages, we have examined the mechanism by which OD regulates nitric oxide (NO) production. When OD (1 mg/ml) was used in combination with 10 U/ml of recombinant interferon-γ (rIFN-γ), there was a marked cooperative induction of NO production (36.13±7.12 μM) by the Griess method (nitrite). Treatment of macrophages with rIFN-γ plus OD (1 mg/ml) caused a significant increase in tumor necrosis factor-α (TNF-α) production (4.49±1.0 μM) by enzyme-linked immunosorbent assay. The increased production of NO and TNF-α from rIFN-γ plus OD-stimulated cells was almost completely inhibited by pretreatment with 100 μM of pyrrolidine dithiocarbamate (PDTC), an inhibitor of nuclear factor kappa B (NF-κB). PDTC also inhibited phosphorylation of 1κB in rIFN-γ plus OD-stimulated cells. These findings demonstrate that OD increases the production of NO and TNF-α by rIFN-γ primed macrophages and suggest that NF-κB plays a critical role in mediating these effects of OD.

**Key words** *Oldenlandia diffusa*; nitric oxide; peritoneal macrophages; tumor necrosis factor-α

Oldenlandia diffusa (OD, Rubiaceae), an herbal medicine, has been used in traditional Oriental medicine for treating liver, lung, and rectal tumors. Previous investigations showed that Oldenlandiae diffusae Herba and its major compound, ursolic acid, has antitumor effects and that Oldenlandiae diffusae Herba inhibited mutagenesis, tumor growth in vivo, DNA binding, and metabolism of aflatoxin B1 and benzo(a)pyrene bioactivated by Aroclor 1254 (polychlorinated biphenyl)-induced rat liver 9000×g supernatant (S9).

Nitric oxide (NO) is a highly reactive molecule produced from a guanidino nitrogen of arginine by NO synthase (NOS) enzymes. Over the past decade, NO as a potent macrophage-derived effector molecule against a variety of bacteria, parasites, and tumors has received increasing attention. Original evidence of tumor cell cytostasis and cytotoxicity was found in macrophage-tumor cell cocultures in which the presence of interferon-γ (IFN-γ) enhances the expression of wild-type p53 cells. The expression of NO has been linked with DNA damage. These signals stimulate immune functions (iNOS).

The proinflammatory cytokine tumor necrosis factor-α (TNF-α) regulates systemic responses to microbial infection or tissue injury. These signals stimulate immune functions and induce expression of acute-phase reactants in the liver, among other effects. Production of TNF-α protein is enhanced by the presence of interferon-γ (IFN-γ). TNF-α then acts as an autocrine signal to amplify IFN-γ-induced production of NO in macrophages. Macrophages are a major source of cytokines such as TNF-α, and induction of cytokine gene expression by LPS occurs primarily at the level of transcription and involves the action of several transcription factors, including members of the nuclear factor-κB (NF-κB)/rel, C/EBP, Ets, and AP-1 protein families.

In particular, NF-κB bound to specific consensus DNA elements present on the promoters of target genes initiates the transcription of TNF-α, iNOS, cyclooxygenase-2, and interleukin-6 (IL-6).

Oldenlandia diffusa (OD) has been used to treat malignant tumors and it stimulates the reticuloendothelial system. We hypothesized that the antiinfectious and antitumor effects of OD are due to its ability to enhance the nonspecific immunologic function of NO.

In the present study, we show that OD synergistically induces NO and TNF-α production by peritoneal macrophages when the cells are treated with recombinant IFN-γ (rIFN-γ). To investigate the mechanism of OD-induced NO and TNF-α production, we examined the ability of NF-κB inhibitors such as pyrrolidine dithiocarbamate (PDTC) to block the OD-induced effect. PDTC decreased NO and TNF-α production induced by rIFN-γ plus OD. These findings may explain how OD influences NO and TNF-α production via the NF-κB signaling pathway.

**MATERIALS AND METHODS**

**Reagents** Murine rIFN-γ (1×10⁶ U/ml) was purchased from Genzyme (München, Germany). Dulbecco’s Modified Eagle’s Medium (DMEM), N-(1-naphyl)-ethylenediamine dihydrochloride, LPS, sodium nitrite, PDTC, and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, U.S.A.). Rabbit polyclonal antisera to iNOS were obtained from Transduction Laboratories (Lexington, KY, U.S.A.). Phosphated-1κBα (p-1κBα) antibody was purchased from New England Bio-
labs (Beverly, MA, U.S.A.). Recombinant TNF-α (rTNF-α), biotinylated TNF-α, and anti-murine TNF-α were purchased from R & D System Inc., U.S.A. Nω-monomethyl-L-arginine (NMMA) was purchased from Calbiochem (San Diego, CA, U.S.A.). Thioglycollate (TG) was purchased from Difco Laboratories (Detroit, MI, U.S.A.). Syringe filters (0.2 μm), 4-well and 96-well tissue culture plates, and 100-mm diameter dishes were purchased from Nunc (Naperville, IL, U.S.A.). DMEM containing L-arginine (84 mg/l), Hank’s balanced salt solution (HBSS), fetal bovine serum (FBS), and other tissue culture reagents were purchased from Life Technologies (Grand Island, NY, U.S.A.). Male C57BL/6 mice were purchased from Dae Han Experimental Animal Center (Eum- sung, Republic of Korea).

**Peritoneal Macrophage Cultures** TG-elicited macrophages were harvested 3–4 d after injection of TG 2.5 ml i.p. to the mice and isolated as reported previously. Using 8 phages were harvested 3–4 d after injection of TG 2.5 ml saline (PBS) containing 0.05% Tween-20. All reagents used were purchased from Dae Han Experimental Animal Center (Eum-sung, Republic of Korea).

**Peritoneal Macrophage Cultures** TG-elicited macrophages were harvested 3–4 d after injection of TG 2.5 ml i.p. to the mice and isolated as reported previously. Using 8 phages were harvested 3–4 d after injection of TG 2.5 ml saline (PBS) containing 0.05% Tween-20. All reagents used were purchased from Dae Han Experimental Animal Center (Eum-sung, Republic of Korea).

**Preparation of OD** The plant sample was obtained from the Oriental Drug Store, Duckhyun Dang (Iksan, Republic of Korea). An extract of OD was prepared by decocting the dried prescription of herbs with boiling distilled water. The duration of decoction was about 3 h. The decoction was filtered, lyophilized, and stored at 4 °C. The yield of dried extract from starting materials was about 8%. Dilutions were made in saline and then filtered through a 0.2-μm syringe filter.

**Measurement of Nitrite Concentration** Peritoneal macrophages (2.5×10^5 cells/well) were cultured with rIFN-γ (10 U/ml) for 6 h. The cells were then stimulated with various concentrations of OD. NO synthesis in cell cultures was measured using a microplate assay method, as previously described. To measure nitrite, 100–μl aliquots were removed from conditioned medium and incubated with an equal volume of Griess reagent (1% sulfanilamide/0.1% N(1-naphthyl)-ethylenediamine dihydrochloride/2.5% H₃PO₄) at room temperature for 10 min. The absorbance at 540 nm was determined in a Titertek Multiskan (Flow Laboratories, North Ryde, Australia). NO₂ was determined by using sodium nitrite as a standard. The cell-free medium alone contained 5 to 8 μM of NO₂. This value was determined in each experiment and subtracted from the value obtained from the medium with peritoneal macrophages.

**Assay of TNF-α Release** Peritoneal macrophages (2.5×10^5 cells/well) were incubated with rIFN-γ (10 U/ml), OD, rIFN-γ plus LPS (10 μg/ml), and rIFN-γ plus various concentrations of OD for 24 h. Then the amount of TNF-α secreted by the cells was measured using a modified enzyme-linked immunosorbent assay (ELISA), as described previously. The ELISA was devised by coating 96-well plates containing murine monoclonal antibody with specificity for TNF-α. Before use and between subsequent steps in the assay, coated plates were washed with phosphate-buffered saline (PBS) containing 0.05% Tween-20. All reagents used in this assay were incubated for 2 h at 37 °C. The rTNF-α was diluted and used as a standard. Serial dilutions starting from 1 pg/ml were used to establish the standard curve. Assay plates were exposed sequentially to alkaline-phosphatase-conjugated goat anti-rabbit IgG. Optical density readings were made within 10 min of the substrate on a Titertek Multiskan with a 405-nm filter. Appropriate specificity controls were included.

**Western Blot Analysis** Peritoneal macrophages (5×10^6 cells/well) were incubated for 6 h with rIFN-γ (10 U/ml). The cells were then stimulated with OD (1 mg/ml) or LPS (10 μg/ml) for 12 h. Whole-cell lysates were made by boiling peritoneal macrophages in sample buffer (62.5 mm Tris–HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 20% glycerol, and 10% 2-mercaptoethanol). Proteins in the cell lysates were then separated by 7% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose paper. The membrane was then blocked with 5% skim milk in PBS-Tween-20 for 1 h at room temperature and then incubated with anti-iNOS and p-1αBz antibodies. After washing in PBS-Tween-20 three times, the blot was incubated with secondary antibody for 30 min and the antibody-specific proteins were visualized using the enhanced chemiluminescence detection system according to the recommended procedure (Amersham Corp., Newark, NJ, U.S.A.).

**Determination of Endotoxin** OD extracts used in this experiment were found to be less than 10 pg/ml of endotoxin as determined within the limits of assay E-TOXATE kit (Sigma), performed according to manufacturer’s protocol.

**Assay of MTT** To investigate the viability of cancer cells, MTT assay was performed. Briefly, 500 μl of HepG2 human hepatoma cells (ATCC-HB-8065, Rockville, MD, U.S.A.) or HT-29 cells human colon cancer cell, (ATCC-HTB-38, Rockville, MD, U.S.A.) in suspension (2.5×10^5 cells/well) were cultured in 4-well plates for 24 h after treatment with each concentration of OD. Twenty microliters of MTT solution (5 mg/ml) was added and the cells were cultured at 37 °C for an additional 4 h. The crystallized MTT was dissolved by the addition of dimethyl sulfoxide 500 μl to the well. The amount of dark blue formazan was determined by measuring the absorbance at 570 nm.

**Statistical Analysis** Results were expressed as the mean±S.E.M. of independent experiments, and statistical analysis was performed using the Student’s t-test to express the difference between two groups.

**RESULTS**

**Effects of OD on NO Production in Activated Peritoneal Macrophages** To determine the effect of OD on the production of NO by mouse peritoneal macrophages, we treated nonprimed (resting) and rIFN-γ-primed cells with OD. The resultant NO production was determined by detecting nitrite concentrations in the cell supernatants after 48 h treatment. As shown in Fig. 1, only OD had little effect on NO production in resting mouse peritoneal macrophages. However, when mouse peritoneal macrophages were primed for 6 h with murine rIFN-γ and then treated with various concentrations of OD (0.01—1 mg/ml), NO production was increased compared to nonprimed conditions.

**Effects of OD on rIFN-γ-Primed iNOS Expression** Data in Fig. 2A show the effects of rIFN-γ plus OD treat-
Peritoneal macrophages (2.5×10^6 cells/well) were cultured with rIFN-γ (10 U/ml). The peritoneal macrophages were then stimulated with various concentrations of OD for 6 h after incubation. After 48 h of culture, NO release was measured by the Griess method (nitrite). NO (nitrite) released into the medium is presented as the mean±S.E.M. of three independent experiments duplicated in each run. *p<0.05 compared with rIFN-γ alone.

**Table 1. Effects of PDTC on rIFN-γ Plus OD-Induced NO Production in Peritoneal Macrophages**

<table>
<thead>
<tr>
<th>Addition</th>
<th>PDTC (μM)</th>
<th>Nitrite concentration (μM)_c</th>
</tr>
</thead>
<tbody>
<tr>
<td>rIFN-γ</td>
<td>OD (1 mg/ml)_a</td>
<td></td>
</tr>
<tr>
<td>−</td>
<td>−</td>
<td>10.1±0.6</td>
</tr>
<tr>
<td>+</td>
<td>−</td>
<td>19.8±5.5</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>36.1±7.1</td>
</tr>
<tr>
<td>+</td>
<td>1</td>
<td>33.2±2.13</td>
</tr>
<tr>
<td>+</td>
<td>10</td>
<td>13.7±0.58_c</td>
</tr>
<tr>
<td>+</td>
<td>50</td>
<td>10.28±0.09_c</td>
</tr>
<tr>
<td>+</td>
<td>100</td>
<td>9.58±0.18_c</td>
</tr>
</tbody>
</table>

a) Peritoneal macrophages (2.5×10^6 cells/well) were stimulated with rIFN-γ plus OD or rIFN-γ plus OD plus various concentrations of PDTC. b) The amount of nitrite released by peritoneal macrophages was measured after 48 h using the Griess method. Values are the mean±S.E.M. of three independent experiments duplicated in each run. c) p<0.01 compared with rIFN-γ plus OD.

**Inhibition of OD-Induced NO Production by PDTC**

It is known that PDTC, an antioxidant compound, inhibits NF-κB activation.21 As an approach to define the signaling mechanism of OD in NO production, we examined the influence of PDTC in rIFN-γ- plus OD-treated mouse peritoneal macrophages. Adding PDTC (1—100 μM) to the rIFN-γ- plus OD-treated mouse peritoneal macrophages decreased NO production in a dose-dependent manner (Table 1).

**Effects of OD on rIFN-γ-Induced TNF-α Production**

The synergistic cooperative effect of OD on rIFN-γ-induced TNF-α production was examined. Mouse peritoneal macrophages secreted very low levels of TNF-α after 24-h incubation with medium alone or rIFN-γ alone. However, OD in combination with rIFN-γ markedly increased TNF-α production in a dose-dependent manner (Fig. 4). We also investigated the effect of OD on the signal transduction pathway of TNF-α production. Many actions of TNF-α can be ascribed to its ability to activate the transcription factor NF-κB.22 Disruption of NF-κB-activating pathways can be envisioned as a means to influence various immune response conditions. As shown in Fig. 4, adding the NF-κB inhibitor...
experiments duplicated in each run.

ELISA method after 24-h incubation. Values are the mean

between 0.80 and 2.80 ppm. 1H-NMR analysis of the

4.65 (d) and 5.25 (d) ppm, indicative of a glucoside group

obvious were chemical shifts between 3.20 and 3.95 ppm and

visible in the 1H-NMR spectrum of the extract. Especially

main biological compound of which the signals were clearly

main biological compound of which the signals were clearly

to the rIFN-γ plus OD (1 mg/ml). PDTC to the rIFN-γ plus OD-treated mouse peritoneal

macrophages decreased the synergistic effects of OD on

TNF-α production significantly.

Inhibition of OD-Induced Phosphorylation of IκBα by

PDTC The translocation of NF-κB to the nucleus is pre-

ceded by the proteolytic degradation of IκBα.23) Degradation

of IκBα, the inhibitory protein bound to NF-κB, is a key

step required for the activation of NF-κB. This process is ini-
tiated through signal-induced phosphorylation of two serines

(Ser 32, Ser 36) on the IκBα molecule.24,25) To determine the

inhibitory effect of PDTC on NF-κB activity correlated with

IκB degradation, the cytoplasmic p-IκBα level of OD-
treated peritoneal macrophages was examined by Western

blotting using a specific antibody to p-IκBα. The results indi-
cated that pretreatment with PDTC prevented IκBα phos-

phorylation after 60-min incubation with rIFN-γ plus OD (Fig. 5).

Direct Effect of OD on Cancer Cells To test whether

OD could affect the growth of cancer cells, we evaluated cell

viability by MTT assay in HepG2 cells and HT-29 cells. OD

(1 mg/ml) showed a direct effect in the HepG2 cells but no

effect in the HT-29 cells (Fig. 6).

Characterization of the Principal Components of OD

Finally, we standardized OD by nuclear magnetic resonance

(NMR) spectrometry.26—28) The water extract of OD was ana-

lyzed by 1H-NMR spectroscopy of D2O solubles. It gave the

main biological compound of which the signals were clearly

visible in the 1H-NMR spectrum of the extract. Especially

obvious were chemical shifts between 3.20 and 3.95 ppm and

4.65 (d) and 5.25 (d) ppm, indicative of a glucoside group

and between 0.80 and 2.80 ppm. 1H-NMR analysis of the

water extract showed that it contained glucoside as the main

component with a steroid (Fig. 7).

DISCUSSION

In this study, we demonstrated that NO production in

mouse peritoneal macrophages by OD could be highly stimu-

lated in combination with rIFN-γ. OD had a maximal effect

on NO production at a concentration of 1 mg/ml in rIFN-γ-
treated mouse peritoneal macrophages. The results of this

study suggest that OD may provide a second signal for syner-

gistic induction of NO production in mouse peritoneal

macrophages. NMMMA, an analogue of l-arginine, inhibited

rIFN-γ- plus OD-induced NO production in peritoneal

macrophages. The effective concentration of NMMMA for NO

inhibition without any toxicity on cells is up to 10 mM, which

is the concentration commonly used. The strong inhibition of

nitrite production by NMMMA indicates that it is likely to de-

pend upon NOS.

At present, the precise physiological significance of NO

production by OD is unknown. However, an important role

for the synthesis of NO in host defense against pathogens

and tumor cells has been recognized.7) NO generation by
iNOS also influences the cytotoxicity of macrophages and tumor-induced immunosuppression. NO produced by OD indicates that it may provide various activities such as antimicrobial, antitumoral, and antiviral activity under specific conditions in vivo. In addition, since NO has emerged as an important intracellular and intercellular regulatory molecule with functions as diverse as vasodilation, neural communication, cell growth regulation, and host defense, it is tempting to hypothesize that this molecule is involved in the local control of various fundamental processes.

NO synthesis was greatly diminished by neutralizing antibody for TNF-α. Our results also suggest that the synergism between IFN-γ and OD in increasing NO synthesis by stimulated macrophages could be due to TNF-γ secretion triggered by OD in IFN-γ-primed macrophages. It has previously been reported that LPS stimulation of rIFN-γ-primed macrophages induces NF-κB activation. NF-κB is now known to be ubiquitously expressed and to play a major role in controlling the expression of proteins involved in immune, inflammatory, and acute-phase responses. Expression of iNOS and TNF-α genes is dependent on the activation of NF-κB. We found that the addition of the NF-κB inhibitor PDTC inhibits the synergistic effect of OD with rIFN-γ on NO and TNF-α production. It also inhibits the phosphorylation of IκB in OD-plus rIFN-γ-primed macrophages. These results suggest that OD increases NO and TNF-α production through NF-κB activation. The NF-κB system may provide a future target of anticancer therapy.

In conclusion, our results demonstrate that OD acts as an accelerator of peritoneal macrophage activation by rIFN-γ via a process involving l-arginine-dependent NO production and that it increases the production of TNF-α significantly via NF-κB activation. Although the precise mechanism by which OD promotes NO and TNF-α production induced by rIFN-γ remains to be elucidated, NO and TNF-α production by OD might explain its beneficial effect in the treatment of tumors.

The amounts of OD used in this study were high concentrations, raising the possibility that the active agent or agents in the OD represent a small component of the total mass. Therefore further investigation is necessary to clarify unknown constituents that may be more active than OD itself. Studies on the isolation and characterization of the active chemical constituents are in progress.

Acknowledgments This work was supported by the Medicinal Resources Research Center of Wonkwang University and partially by Wonkwang University in 2001.

REFERENCES