Leonurus sibiricus induces nitric oxide and tumor necrosis factor-α in mouse peritoneal macrophages

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Using mouse peritoneal macrophages, we have examined the mechanism by whichLeonurus sibiricus (LS) regulates nitric oxide (NO) production. When LSwas used in combination with recombinant interferon-γ (rIFN-γ), there was a marked cooperative induction of NO production. However, LS had no effect on NO production by itself. The increased production of NO from rIFN-γ plus LS-stimulated cells was almost completely inhibited by pre-treatment with pyrrolidine dithiocarbamate (PDTC), an inhibitor of nuclear factor kappa B. Furthermore, treatment of peritoneal macrophages with rIFN-γ plus LS caused a significant increase in tumor necrosis factor-α (TNF-α) production. PDTC also decreased the effect of LS on TNF-α production significantly. As NO and TNF-α play an important role in immune function and host defense, LS treatment could modulate several aspects of host defense mechanisms due to stimulation of the inducible nitric oxide synthase.

Keywords: *Leonurus sibiricus;* Peritoneal macrophages; Nitric oxide; Tumor necrosis factor-α

1. Introduction

Leonurus sibiricus (LS, Labiatae) is a respiratory stimulant, has a curare like effect on motor endings, its roots and leaves are used as febrifuge, and leaves cause contraction of uterus. In the local traditional medicine practice, leaves are used in chronic rheumatism; their juice is antibacterial and extensively

applied in psoriasis, scabies and chronic skin eruptions, and also used to relieve menstrual pain and excessive bleeding [1]. A number of research works have been performed to evaluate the biological activities, specially its effect on mammary glands [2], uterus [3], myocardial cells [4] and on blood viscosity [5]. Lee reported that LS has an anti-cancer effect against the human lung carcinoma, colorectal adenocarcinoma, endometrium malignant mixed mullerian tumor [6]. LS can induce the apoptosis of cancer cell directly, but to date studies to understand the effects of LS on immune cells like macrophages has not been determined.

Nitric oxide (NO) is a highly reactive molecule produced from a guanidino nitrogen of arginine by NO synthase (NOS) [7]. 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 co-cultures in which cytokine and/or lipopolysaccharides (LPS) - stimulated macrophages inhibited metabolic functioning of co-cultured tumor cells [8].

Three isoforms of NOS have been identified and are classified into two major categories, namely, constitutive and inducible NOS (iNOS). Neuronal and endothelial NOSs, which are constitutively expressed, are activated by calcium and calmodulin [9]. Of the three NO synthases, iNOS, the high-output isoform, is the most widely expressed in various cell types after its transcriptional activation [10]. Most importantly, iNOS is highly expressed in LPS-activated macrophages, and this contributes to the pathogenesis of septic shock [11]. NO may also be induced in target cells themselves yielding apoptotic cell death induced by autoexpression of iNOS [12]. The activity of NOS can be inhibited by N^{G}-monomethyl-L-arginine (N^{G}MMA, substrate analogs) or N^{a}-Tosyl-Phe Chloromethyl Ketone (TPCK, an inhibitor of iNOS) [13].

The proinflammatory cytokine, tumor necrosis factor-α (TNF-α) regulates systemic responses to microbial infection or tissue injury [14]. These signals stimulate immune functions and induce expression of acute phase reactants in the liver, among other effects. Production of TNF-α protein was enhanced by the presence of interferon-γ (IFN-γ). TNF-α then acted as an autocrine

signal to amplify IFN-ɣ-induced production of NO in macrophages [15]. Macrophages are a major source of cytokine 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 [16].

The transcription factor NF-κB is dimeric transcription factor formed by the homo- or hetero-dimerization of Rel family proteins, including p50 and p65 [16]. In unstimulated cells, NF-κB exists in an inactivated state, in the cytoplasm, complexed with an inhibitory protein, called IB. NF-κB is activated by a variety of stimuli and regulates diverse gene expressions and biological responses. Upon stimulation, IκB undergoes phosphorylation and degradation, and NF-κB is translocated into the nucleus, where it binds to DNA and activates transcription of variety of genes [17]. The activated NF-κB induces transcription of inflammatory cytokines such as TNF-α and interleukin (IL)-1 as well as iNOS [18].

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

2. Materials and Methods

2.1. Reagents

The LS were purchased from Omniherb (Youngchun, Republic of Korea). Murine rIFN-ɣ (1×10^7 U/㎖), recombinant TNF-α (rTNF-α), biotinylated TNF-α, and anti-murine TNF-α was purchased from R&D Systems (Minneapolis, Minnesota, USA). Dulbecco's Modified Eagle's Medium (DMEM), N- (1-naphtyl) -ethylenediamine dihydrochloride, LPS, sodium nitrite, PDTC, and TPCK, 2, 2'- azino - bis (3-ethylbenzthiazoline-6-sulfonic acid (ABTS) were purchased from Sigma (St. Louis, Missouri, USA). Rabbit polyclonal antisera to iNOS were obtained from Santa

Cruz Biotechnology, Inc. (Santa Cruz, California, USA). N^GMMA was purchased from Calbiochem (San Diego, California, USA). Thioglycollate (TG) was purchased from Difco Laboratories (Detroit, Michigan, USA). DMEM containing L-arginine (84 ㎎/ℓ), Hank's balanced salt solution (HBSS), fetal bovine serum (FBS), and other tissue culture reagents were purchased from Gibco BRL (Grand Island, New York, USA). Male C57BL/6J mice were purchased from Daehan Biolink (Dae-Jeon, South Korea).

2.2 Peritoneal macrophage cultures

TG-elicited macrophages were harvested 3~4 days after i.p. injection of 2.5 ㎖ of TG to the mice and isolated, as reported previously [19]. Using 8 ㎖ of HBSS containing 10 U/㎖ heparin, peritoneal lavage was performed. Then, the cells were distributed in DMEM, which was supplemented with 10% heat-inactivated FBS, in 24-well tissue culture plates (2.5×10^5 cells/well) incubated for 3 h at 37℃ in an atmosphere of 5% CO_2, washed three times with HBSS to remove non-adherent cells, and equilibrated with DMEM that contained 10% FBS before treatment

2.3. Preparation of LS

The LS was prepared by decocting the dried herb (100 g) with boiling distilled water (1 L). The duration of decoction was about 3 h and filtered through a nylon mesh. After centrifugation at 5,000 **g** for 20 min at 4℃, the supernatant of the LS was lyophilized and kept at 4℃. The yield of dried extract from starting materials was about 10%. Dilutions were made in normal saline then filtered through a 0.45 ㎛ syringe filter. The amounts of LS in dried plant for adult Korean (average body weight 60 ㎏) are about 5 - 7.5 g. The yield of powdered extraction is about 10% (w/w). Upon this basis, the dose of LS in powdered extraction for an adult person can be about 0.01 g/㎏. The dose range of 0.01 - 1 ㎎/㎖ was chosen to see the dose-dependency.

2.4. Measurement of nitrite concentration

Peritoneal macrophages (2.5×10^5 cells/well) were cultured with rIFN-γ (10 U/㎖) for 6 h. The cells were then stimulated with various concentrations of LS. NO synthesis in cell cultures

was measured by a microplate assay method, as previously described [20]. The NO concentrations were measured at 6, 12, 24, 48, 72h after LS treatment. To measure nitrite, 100l aliquots were removed from conditioned medium and incubated with an equal volume of Griess reagent (1% sulfanilamide/ 0.1% N-(1-naphtyl)-ethylenediamine dihydrochloride/ 2.5% H_3PO_4) at room temperature for 10 min. The absorbance at 540 nm was determined by an automatic microplate reader. $NO2^-$ 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.

2.5. MTT assay

Peritoneal macrophages (2.5 × 10^5 cells/well) were cultured with various inhibitor (N^GMMA, PDTC, TPCK) for 30 min and then IFN-ɣ and LS (0.01 - 1 mg/ml). Cell aliquots (2.5 × 10^5 cells/well) were seeded in microplate wells and incubated with 50 ℓ of a MTT solution (5 mg/ml) for 4 h at 37℃ under 5% CO_2 and 95% air. Consecutively, 250 ℓ of DMSO was added to extract the MTT formazan and the absorbance of each well at 540 nm was read by an automatic microplate reader.

2.6. Assay of TNF-α release

Peritoneal macrophages (2.5 × 10^5 cells/well) were incubated with rIFN-ɣ (10^8 U/㎖), LS, rIFN-ɣ plus LPS (10 g/㎖), and rIFN-ɣ plus various concentrations of LS for 6, 12, 24, 48, 72 h. Then the amount of TNF-α secreted by the cells was measured by a modified enzyme-linked immunosorbent assay (ELISA), as described previously [21]. The ELISA was devised by coating 96-well plates of 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℃. The rTNF-α was diluted and used as a standard. Serial dilutions starting from 10 ng/㎖ were used to establish the standard curve. After 2 h incubation at 37℃, the wells were washed and then each of 0.2 g/㎖ of biotinylated

anti-mouse TNF-α was added and the plates were incubated at 37℃ for 2 h. After washing the wells, avidin-peroxidase was added and the plates were incubated for 20 min at 37℃. Wells were again washed and ABTS substrate was added. Color development was measured at 405 nm using an automated microplate ELISA reader.

2.7. 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 LS (1 mg/mℓ) or LPS (10 g/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 antibodies. After washing in PBS-Tween-20 three times, the membrane was incubated with secondary antibody for 30 min and the antibody-specific proteins were visualized by the enhanced chemiluminescence detection system according to the recommended procedure (Amersham Corp., Newark, New Jersey, USA).

2.8. RNA isolation and RT-PCR

Total RNA was isolated from macrophages according to the manufacturers specifications using an easy-BLUE RNA extraction kit (iNtRON Biotech, Seoul, South Korea). The concentration of total RNA in the final elutes was determined by spectrophotometry. Total RNA (2.0 μg) was heated at 65℃ for 10 min and then chilled on ice. Each sample was reverse-transcribed to cDNA for 90 min at 37C using a cDNA synthesis kit (iNtRON Biotech, Seoul, South Korea). RT-PCR was carried out with 1 μℓ of a cDNA mixture, in 20 μℓ final volume with 2.5 mM $MgCl_2$, 200 mM dNTPs, 25 pM of cytokine primers, and 2.5 U of Taq DNA polymerase in the reaction buffer (50 mM KCl, 10 mM Tris-HCl, pH 9, and 0.1% Triton X-100). PCR was performed with the following primers for mouse (m) iNOS (5′ TCA CTG GGA CAG CAC AGA AT3′ 5′ TGT GTC TGC AGA TGT GCT GA-3′), TNF- (5′ ATG AGA ACA GAA

AGC ATG ATC-3′ 5′ TAC AGG CTT GTC ACT CGA ATT 3′), and GAPDH (5′ GGC ATG GAC TGT GGT CAT GA 3′ 5′ TTC ACC ACC ATG GAG AAG GC 3′) to verify if equal amounts of RNA were used for reverse transcription and PCR amplification from different experimental conditions. The annealing temperature was 58℃ for iNOS, 60℃ for TNF-α, and 6 2℃ for GAPDH, respectively. Products were electrophoresed on a 1.5% agarose gel and visualized by staining with ethidium bromide.

2.9. Preparation of nuclear extracts and electrophoretic mobility shift assays

To prepare nuclear extracts, cells were first resuspended in buffer containing 10 mM HEPES (pH 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, and 0.2 mM phenylmethylsulphonyl fluoride, followed by vigorous mixing for 15 min before standing at 4℃ for 10 samples were then centrifuged at 15,000 g for 20 min. The pelleted nuclei were resuspended in 30 ㎕ buffer containing 20 mM HEPES (pH 7.9), 2.5% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 MEDTA, 0.5 mM dithiothreitol, and 0.2 mM phenyl -methylsulphonyl fluoride and incubated for 20 min on ice, and nuclear lysates were then centrifuged at 15,000 g for 20 min Supernatants containing the solubilized nuclear proteins were stored at -70℃ for subsequent electrophoretic mobility shift assays (EMSAs). The binding reaction for NF-κB was performed in 15 ㎕ reaction mixtures containing nuclear extracts, 0.25 ㎍ of poly (dI-dC) and 20,000 d.p.m. of ^{32}P-labelled DNA probe in binding buffer (10 mM Tris, pH7.5 1mM EDTA, 4% Ficoll, 1mM dithiothreitol, 75 mM KCl). After incubation for 30 min at room temperature, the reaction mixtures were fractionated on 5% native polyacrylamide gels and then visualized by autoradiography [22]. For supershift experiments, nuclear extracts were incubated with anti-p65 antibody for 15 min before the addition of binding reaction mixture.

2.10. Determination of endotoxin

LS extract used in this experiment was found to be less than 10 pg/㎖ of endotoxin as determined within the limits of assay E-TOXATE kit (Sigma, St. Louis, Missouri, USA), performed according to manufacturer's protocol.

2.11. Statistical analysis

Results were expressed as the mean ± S.E.M. of independent experiments, and statistical analyses were performed by one-way analysis of variance (ANOVA) with Tukey, and Duncan post hoc test to express the difference between groups. All statistical analyses were performed using SPSS v11.0 statistical analysis software. A value of $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Effects of LS on NO production in peritoneal macrophages

To determine the effects of LS on the production of NO by mouse peritoneal macrophages, we treated non-primed (resting) and rIFN-ɣ-primed cells with LS. IFN-ɣ (10 U/㎖) alone does not cause the induction of NO production in accordance with previous reports [23-24]. However, when IFN-ɣ was used in conjunction with LPS, there was marked augmentation of the production of NO, and in previous study, the investigators usually treated 10 U/㎖ rIFN-ɣ to prime the macrophage [20].

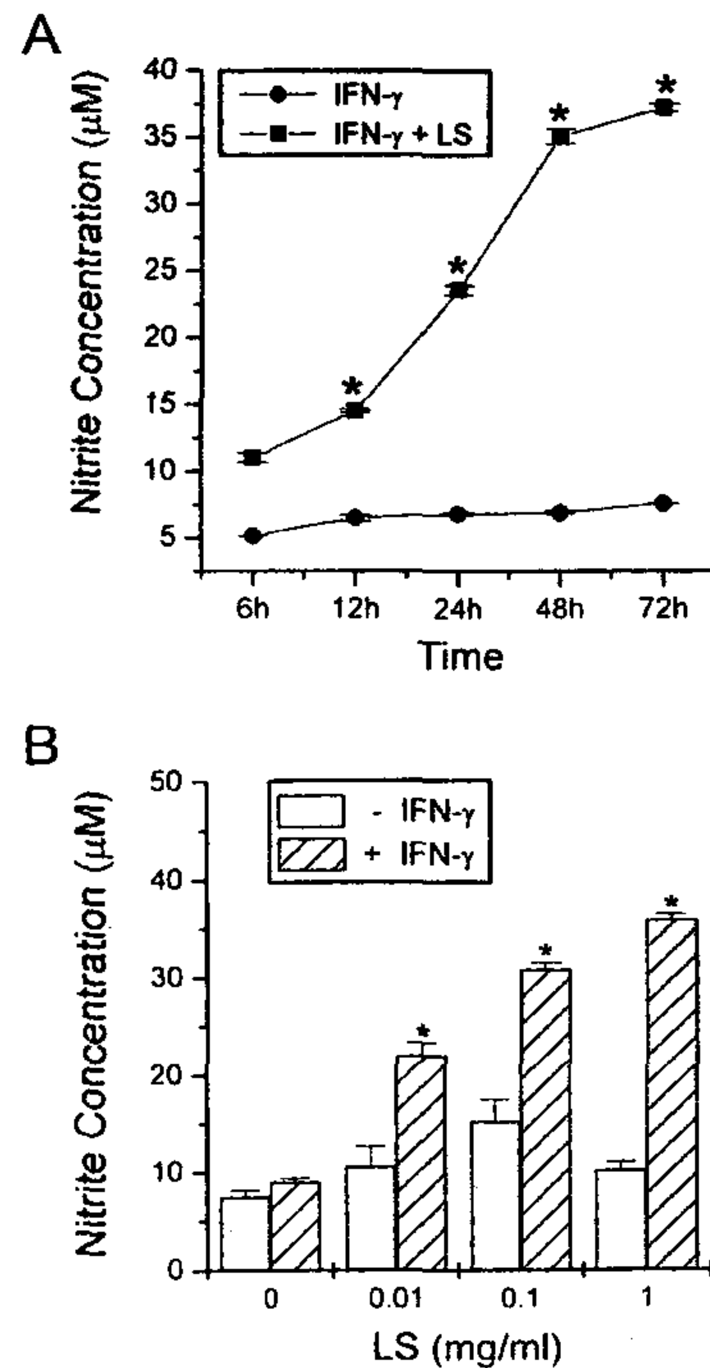

Fig. 1. (A) Time-dependent effects of LS on NO synthesis in rIFN-ɣ-treated peritoneal macrophages.
The cells (2.5 × 10^5 cells/well) were cultured with rIFN-ɣ (10 U/㎖). The cells were then stimulated with 1 ㎎/㎖ LS for 6, 12, 24, 48, and 72 h after incubation. NO release was measured by the Griess method. NO released into the medium is presented as the mean S.E.M. of three independent experiments duplicate in each run. * $P < 0.05$ compared to rIFN-ɣ alone.
(B) Dose-dependent effects of LS on NO synthesis in rIFN-ɣ-treated peritoneal macrophages. The cells (2.5 × 10^5 cells/well) were cultured with rIFN-ɣ (10 U/㎖). The cells were then stimulated with various concentrations of LS for 6 h after incubation. After 48 h of culture, NO release was measured by the Griess method. NO released into the medium is presented as the mean S.E.M. of three independent experiments duplicate in each run. * $P < 0.05$ compared to rIFN-ɣ alone.

The resultant NO production was determined by detecting nitrite concentrations in the cell supernatants after 6, 12, 24, 48, 72 h treatment. As shown in Fig. 1B, LS had no 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 LS, NO production was increased compared to non-primed conditions. The NO production was increased significantly after 12 h treatment. As shown in Fig. 1A, NO productions were increased time-dependent. Because there is little difference between NO concentrations of 48 h and 72 h, NO concentrations were assayed by various concentrations of LS (0.01, 0.1, and 1 ㎎/㎖) after 48 h treatment.

3.2. Effects of LS on rIFN-γ -primed iNOS expression

Data in Fig. 2 show the effects of rIFN-γ plus LS treatments on the expression of iNOS protein in mouse peritoneal macrophages. rIFN-γ plus LS increased synergistically the expression of iNOS protein in mouse peritoneal macrophages.

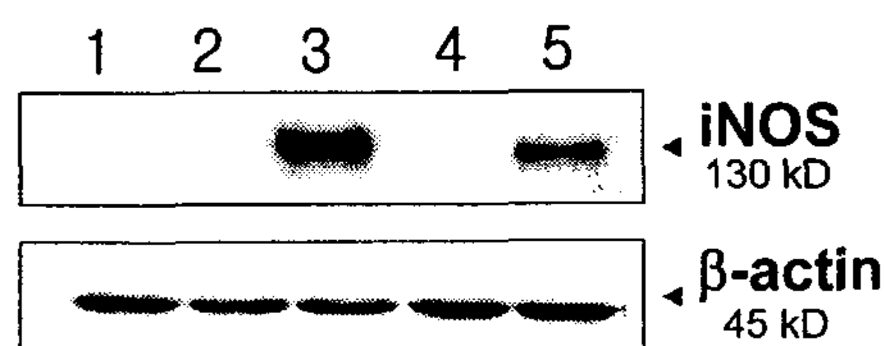

Fig. 2. Effects of LS on the expression of iNOS by rIFN-γ plus LS-induced peritoneal macrophages.
The cells (5×10^6 cells/well) were incubated for 6 h with rIFN-γ (10 U/㎖). The cells were then stimulated with LS (1 ㎎/㎖) or LPS (10 g/㎖) for 12 h. The protein extracts were prepared, and then the samples were analyzed for iNOS expression by Western blotting as described in the method. (1) Control (cell only); (2) rIFN-γ; (3) rIFN-γ + LPS; (4) LS (1㎎/㎖); and (5) rIFN-γ + LS (1 ㎎/㎖)

3.3. Inhibition of LS-induced NO production by N^GMMA

To determine if the signaling mechanism in LS-induced NO production participates in the L-arginine-dependent pathway in mouse peritoneal macrophages, the cells were incubated for 6 h in the presence of rIFN-γ plus N^GMMA. To determine the effects of N^GMMA on viability of mouse peritoneal macrophages, we carried out MTT assay. When we treated the cells with NGMMA (0.01 - 1 M), the viability was 99.58 ± 2.3% compared with non-treated group as 100%. The production of nitrite by rIFN-γ plus LS in mouse peritoneal macrophages

was progressively inhibited with increasing amount of N^{G}MMA. The LS-induced accumulation of nitrite was significantly blocked by N^{G}MMA (0.01 - 1 M) (Fig. 3).

3.4. Inhibition of LS-induced NO production by PDTC or TPCK

It is known that PDTC, an anti-oxidant compound, inhibits NF-B activation [22]. As an approach to define the signaling mechanism of LS on NO production, we examined the influence of PDTC or TPCK in rIFN- plus LS-treated mouse peritoneal macrophages. The method and inhibitors' concentrations were according to the previous study [25]. Adding PDTC (100 μM) or TPCK (40 μM) to the rIFN-γ plus LS-treated mouse peritoneal macrophages decreased the synergistic effects of LS on NO production (Fig. 3).

3.5. Effects of LS on rIFN-γ -induced TNF-α production

We next examined the synergistic cooperative effects of LS on rIFN-γ-induced TNF-α production. As shown in Fig. 4A, TNF-α productions were increased time-dependent. Because there is little difference

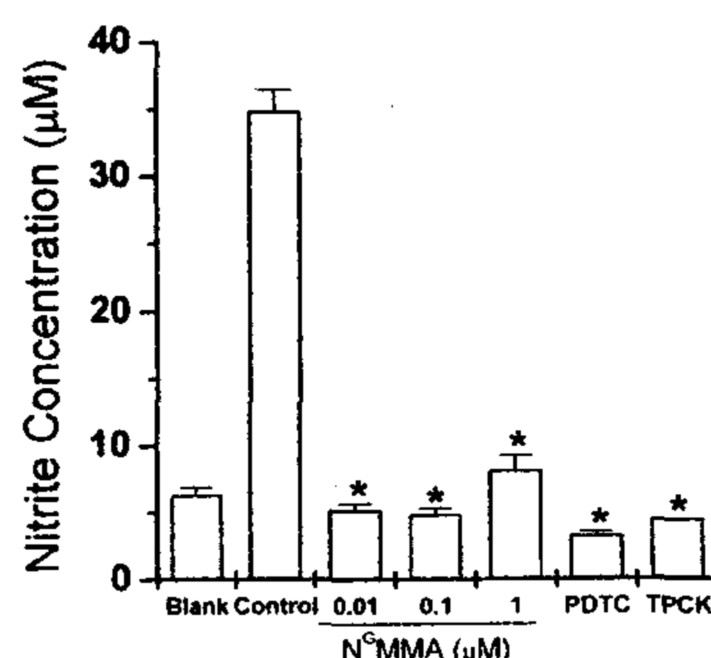

Fig. 3. Effects of NGMMA, PDTC, or TPCK on LS-induced nitrite accumulation in the cultured medium of peritoneal macrophages.
The cells (2.5 × 10^{5} cells/well) were incubated for 6 h with rIFN-γ (10 U/mℓ) plus various concentrations of N^{G}MMA, PDTC, or TPCK. The cells were then treated with LS (1 mg/mℓ) and cultured for 48 h. NO release was measured by the Griess method. NO released into the medium is presented as the mean S.E.M. of three independent experiments duplicate in each run. * $P < 0.05$ compared to control (absence of N^{G}MMA).

between TNF-α concentrations of 24 h, 48 h and 72 h, TNF-α concentrations were assayed by various concentrations of LS (0.01, 0.1, and 1 mg/mℓ) after 24 h treatment. Mouse peritoneal macrophages secreted very low levels of TNF-α after 24 h incubation with medium alone or rIFN-γ alone. However, LS in combination with rIFN-γ markedly increased TNF-α production in a dose-dependent manner (Fig. 4B). We also investigated the effects of LS on

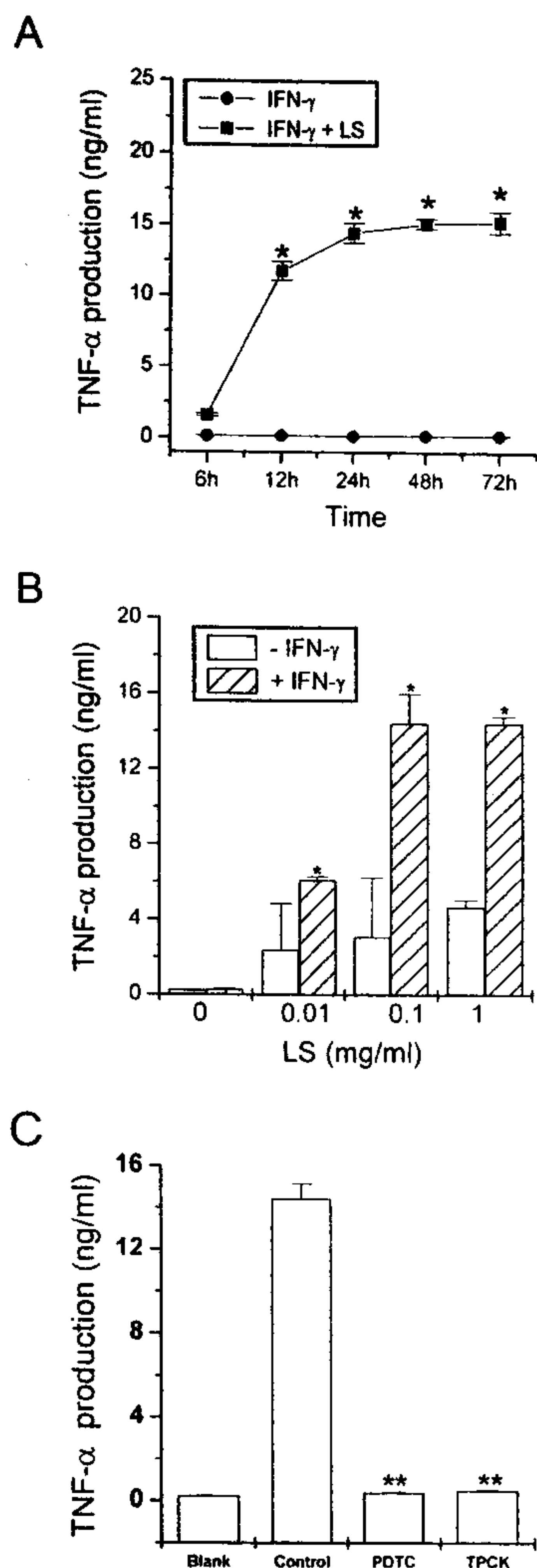

Fig. 4. (A) Time-dependent effects of LS on TNF-α in rIFN-γ-treated peritoneal macrophages. The cells (2.5 × 10^5 cells/well) were cultured with rIFN-γ (10 U/ml). The cells were then stimulated with 1 mg/ml LS for 6, 12, 24, 48, and 72 h after incubation. The amount of TNF-α secreted by peritoneal macrophages was measured by ELISA method. Values are the mean S.E.M. of three independent experiments duplicate in each run. * $P < 0.05$ compared to rIFN-γ alone.

(B) Dose-dependent effects of LS on the production of TNF-α by plus various concentrations of LS or rIFN-γ plus LS in peritoneal macrophages.
The cells (2.5 × 10^5 cells/well) were stimulated with various concentrations of LS or rIFN-γ (10 U/㎖) plus LS. The amount of TNF-α secreted by peritoneal macrophages was measured by ELISA method after 24 h of incubation. Values are the mean S.E.M. of three independent experiments duplicate in each run. * $P < 0.05$ compared to rIFN-γ alone.

(C) Effects of PDTC or TPCK on rIFN-γ plus LS-induced TNF-α secretion in peritoneal macrophages.
Peritoneal macrophages (2.5 × 10^5 cells/well) were stimulated with rIFN-γ plus LS or rIFN-γ plus PDTC or TPCK plus various concentrations of LS. The amount of TNF-α secreted by peritoneal macrophages was measured by ELISA method after 24 h of incubation. Values are the mean S.E.M. of three independent experiments duplicate in each run. ** $P < 0.05$ compared to rIFN-γ plus LS.

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 [26]. Disruption of NF-κB activating pathways can be envisioned as a means to influence various immune response conditions. As shown in Fig. 4C, adding the NF-κB inhibitor, PDTC (100 μM) or TPCK (40 μM), to the rIFN-γ plus LS (1 ㎎/㎖)-treated mouse peritoneal macrophages decreased the synergistic effects of LS on TNF-α production significantly.

3.6. Effects of LS on iNOS and

TNF-α mRNA expression

The iNOS and TNF-α expression level in macrophages was investigated by RT-PCR under the stimulation of LS (10 μg/mℓ of LPS as positive control). As shown in Fig. 5, the transcription of iNOS and TNF-α mRNA was induced by LS in macrophages. This was further evidenced that LS exhibited a potential inductive effect on iNOS and TNF-α gene expression in macrophages, resulting in the enhancement of NO release.

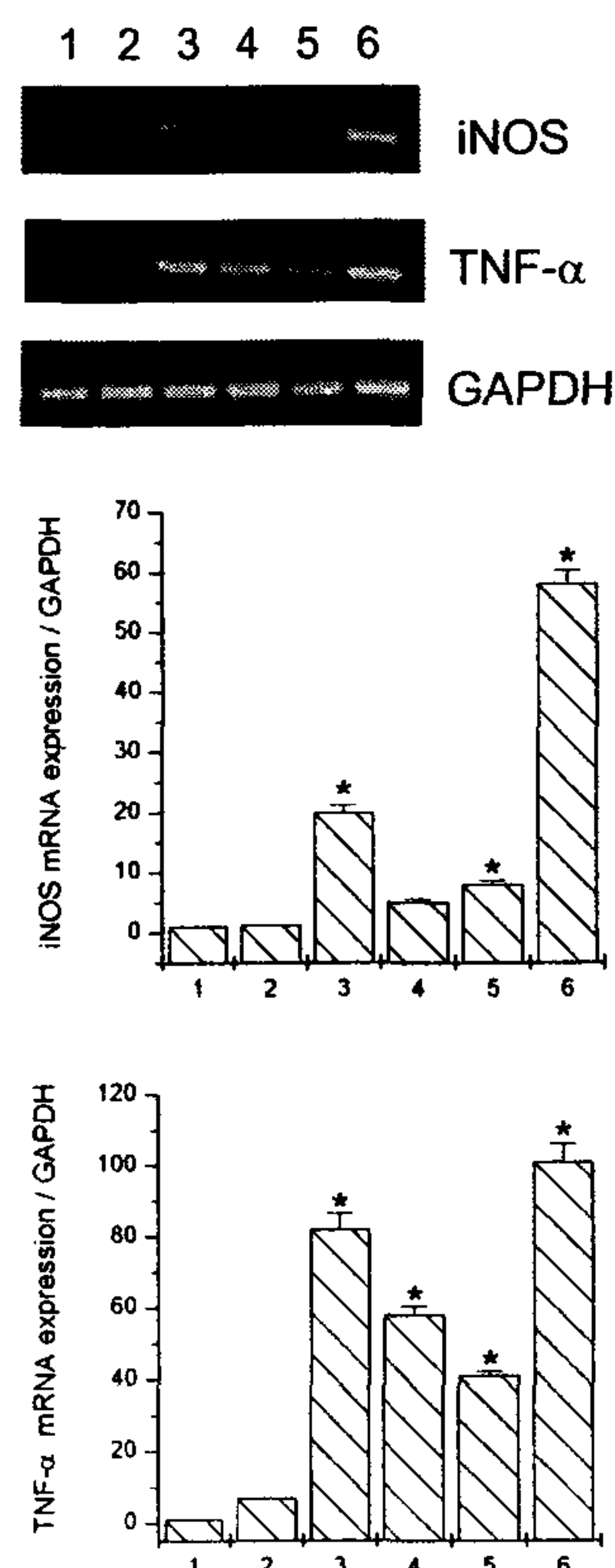

Fig. 5. Effects of LS on iNOS and TNF-α mRNA expression.
Cells (3 × 10^6) were stimulated with various concentrations of LS or rIFN-γ (10 U/mℓ) plus LS, and then mRNA level was measured using RT-PCR. Products were electrophoresed on a 1.5% agarose gel and visualized by staining with ethidium bromide. 1, unstimulated cells; 2, rIFN-γ ; 3, rIFN-γ plus LPS; 4, rIFN-γ plus 0.01 mg/mℓ LS; 5, rIFN-γ plus 0.1 mg/mℓ LS; 6, rIFN-γ plus 1 mg/mℓ LS. All data represent the mean ± S.E.M. of three independent experiments.

3.7. Effects of LS on NF-κB expression

Since NF-κB activation requires nuclear translocation of Rel/p65 subunit of NF-κB, we examined the effect of LS on the nucleus levels of Rel/p65 was examined after IFN-γ-stimulation by western blot analysis. In IFN-γ plus LPS-stimulated cells, the expression level of Rel/p65 was increased as positive control. Treatment of LS increased the expression level of Rel/p65 (Figure 6A). In order to further investigate the putative mechanism, the effect of LS on the activation of NF-κB was monitored using an EMSA. As shown in Fig. 6B, incubating the cells with LS for 1 h markedly increased the level of NF-κB binding at its conserved site, which was visualized as a distinct band (indicated by an arrow).

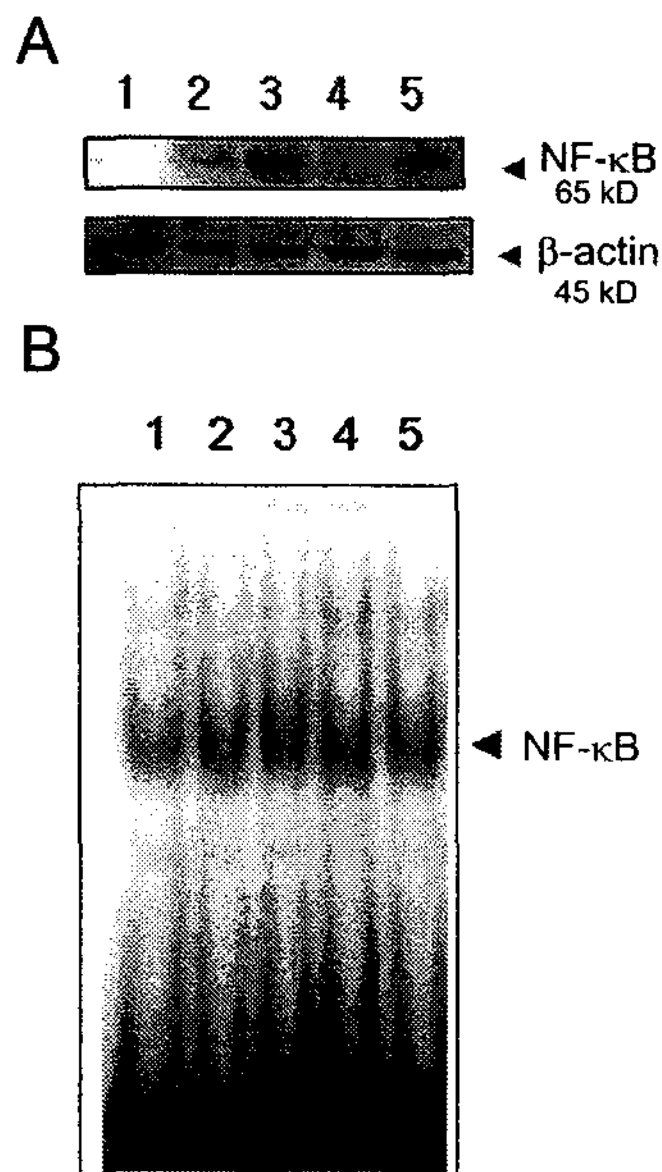

Fig. 6. (A) Effects of LS on nuclear translocation of the Rel/p65.
Cells (5 × 10^6) were stimulated with various concentrations of LS or rIFN-γ (10 U/mℓ) plus LS. Nuclear extracts were prepared as described in Materials and Methods and determined for RelA/p65 by Western blot anlaysis.
(B) Effects of LS on NF- B-binding.
Cells (5 × 10^6) were stimulated with various concentrations of LS or rIFN-γ (10 U/mℓ) plus LS. Nuclear extracts were isolated and used in an electrophoretic mobility shift assay with ^{32}P-labeled NF-κB oligonucleotide as a probe, as described under Materials and Methods The arrowhead indicates the NF-κB binding complex. 1, unstimulated cells; 2, rIFN-γ; 3, rIFN-γ plus LPS; 4, 1 mg/mℓ LS; 5, rIFN-γ plus 1 mg/mℓ LS.

4. Discussion

In this study, we demonstrated that NO production in mouse peritoneal macrophages by LS could be highly stimulated in combination with rIFN-γ. LS had a maximal effect on NO production at a concentration of 1 mg/mℓ in rIFN-γ-treated mouse peritoneal macrophages. Actually, the effect of LS is dose-dependent on NO production in mouse peritoneal macrophages. When the macrophages were treated with less than 1 g/mℓ LS, the NO production was not affected. The results of this study suggest that LS may provide a second signal for synergistic induction of NO production in mouse peritoneal macrophages. N^GMMA, an analog of L-arginine, inhibited rIFN-γ plus LS-induced NO production in peritoneal macrophages. The effective concentration of N^GMMA for NO inhibition without any toxicity on cells is up to 10 mM is the concentration commonly used [25]. The strong inhibition of nitrite production by N^GMMA indicates that it is likely to depend upon NOS.

At present, the precise physiological significance of NO production by LS is unknown. However, over the past decade, NO as a potent macrophage-derived effector molecule against a variety of bacteria, parasites, and tumors has received increasing attention [9]. NO has been reported to

play an important role for the mitogenic effect of angiogenic factor, e.g., in the angiogenic process. Eroglu *et al.* [27] showed that large tumor burden was associated with significantly increased levels of vascular endothelial growth factor and NO. In addition, Klotz *et al.* [28] reported that bladder carcinoma tissue had high iNOS content while benign tissue did not. NO generation by iNOS also influences the cytotoxicity of macrophages and tumor-induced immunosuppression. NO production by LS indicates that it may provide various activities such as antimicrobial, anti-tumoral, and anti-viral under specific conditions *in vivo*. In addition, since NO has emerged as an important intracellular and intercellular regulatory molecule having functions as diverse as vasodilation, neural communication, cell growth regulation, and host defense [29]. It is tempting to hypothesize that this molecule is involved in the local control of the various fundamental processes.

NF-κB appears to play a primary role in the transcriptional regulation of various genes such as iNOS and the proinflammatory cytokines [16]. In resting cells, the NF-κB dimer is held in the cytosol via an interaction with the inhibitor κB (IκB) inhibitory proteins [30]. After exposing the cells to proinflammatory stimuli, IκB becomes phosphorylated by IκB kinase α and β, ubiquitinated, and then degraded. Therefore, the liberated NF-κB dimers are translocated to the nucleus, where the transcription of the target gene is induced [6]. Expression of iNOS and TNF-α genes is dependent on the activation of NF-κB [31]. We found that the addition of NF-κB modulator, PDTC or TPCK, inhibits the synergistic effect of LS with rIFN-γ on NO, TNF-α production. These results suggest that LS increases NO and TNF-α production through NF-κB activation. In next step, we confirm that LS increases the level of NF-κB activation in macrophages using western blotting and EMSA (Fig.6). In previous study, LS can induce the apoptosis of cancer cell directly, but to date studies to understand the effects of LS on immune cells like macrophages has not been determined [6]. Therefore, this study is the first finding to indicate that LS can induce NO and TNF-α production via NF-κB activation and this NF-κB system may provide a cancer therapy.

In conclusion, our results

demonstrated that LS acted as an accelerator of peritoneal macrophages activation by rIFN-γ via a process involving L-arginine-dependent NO production and that it increased the production of TNF-α significantly via NF-κB activation. Although the precise mechanism of LS to promote NO and TNF-α production induced by rIFN-γ remains to be further elucidated, NO and TNF-α production by LS might explain its beneficial effect in the treatment of tumors.

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

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