Scrophularia Buergeriana inhibits the Production of NO through the Suppression of NF-κB activity in LPS-stimulated Mouse Peritoneal Macrophages

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Scrophularia buergeriana Miquel (Scrophulariaceae) has been used as an anti-inflammatory drug in the folk medicine recipe and been proved its anti-inflammatory effect in the oriental medicine. Since nitric oxide (NO) and superoxide anion (O_2^-) are ones of the major inflammatory parameters, we studied the effect of aqueous extracts of Scrophularia buergeriana (SB) on NO and O_2^- production in lipopolysaccharide (LPS)-stimulated mouse peritoneal macrophages. NO, O_2^- production and inducible NO synthase (iNOS) level were significantly reduced in LPS-activated macrophages by SB compared to those without. Electrophoretic mobility shift assay (EMSA) indicated that SB blocked the activation of NF-κB, which was considered to be a potential transcription factor for the iNOS expression. SB also blocked degradation of IκBα. Furthermore, IκB kinase alpha (IKKα), which phosphorylates serine residues of IκB directly, is inhibited by SB. These results suggest that SB could exert its anti-inflammatory actions by suppressing the activation of NF-κB through inhibition of IKK activity.

Key words : Scrophularia buergeriana Mique, Superoxide, Nitric oxide (NO), Inducible nitric oxide synthase (iNOS), Nuclear Factor kappa B (NF-κB), Inhibitor kappa B (IκB), IκB kinase (IKK)

Introduction

The root of Scrophularia buergeriana has been used to relieve fever from blood, to nourish yin, to quench fire, and to counteract toxicity traditionally in the oriental medicine recipe (Choi, 1994). Since these effects are regarded totally as anti-inflammatory functions, this drug may affect to the key parameters of inflammation such as production of reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI). Phagocytic cells including macrophages produce and release ROS (superoxide, hydrogen peroxide) and RNI (nitric oxide, peroxynitrite) in response to phagocytosis or stimulation with various agents. ROS and RNI are induced by endotoxin in vivo and their injurious effects may contribute to pathogenesis of several diseases (Milligan et al., 1988; Bautista et al., 1990; Zurovsky and Gispaan, 1995).

Nitric oxide (NO) is produced from conversion of L-arginine to citrulline in vivo by three distinct isoforms of NO synthase (NOS): neuronal NOS (nNOS or NOS I), inducible NOS (iNOS or NOS II), and endothelial NOS (eNOS or NOS III). While nNOS and eNOS are constitutively expressed and regulated at post-translational level by Ca^{2+}-calmodulin, the activity of iNOS is regulated at the transcriptional level by mediators such as Interlukin (IL)-2, Interferon (IFN)-g and inflammatory stimuli including bacterial cell wall molecule, lipopolysaccharide (LPS) (Nathan et al., 1994).

NO produced continuously by nNOS and eNOS has been known to be an important regulatory molecule in diverse physiological functions such as vasodilation, neural communication, and toxicity for bacteria and tumor cells. On the other hand, high level of NO catalyzed by iNOS has been described in variety of pathophysiological process including various forms of circulatory shock, inflammation and carcinogenesis. A portion of the 5'-flanking region of the murine iNOS gene has been cloned (Weisz et al., 1994). The promoter of the iNOS gene contains a TATA box and consensus sequences for the binding of transcription factors associated with stimuli that induce iNOS expression. It has been reported that the binding of NF-κB to the iNOS promoter plays important role in maximal expression of iNOS gene induced by LPS and IFN-γ (Kim et al., 1997). NF-κB is a dimeric transcription factor composed of p50 and p65 and controls a number of genes that are important for immunity and inflammation (May and Ghosh, 1998). In its unstimulated

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form, NF-κB is present in the cytosol bound to the inhibitory protein, collectively termed I kappa B (IκB) (Baldwin et al., 1996). Several IκB proteins have been identified, including IκBα, IκBβ, and the more recently cloned IκBε (Whiteside et al., 1997). In response to cell stimulation, IκB becomes phosphorylated and recognized by a specific E3 ubiquitin ligase complex and then degradated by the 26S proteasome. The free NF-κB from IκB, which are spared from degradation, translocates to the nucleus to activate gene transcription (Yaron et al., 1998; Spencer et al., 1999; Winston et al., 1999). Recent studies have identified an IκB kinase (IKK), which consists of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, IKKγ. IKK was characterized by its ability to catalyze the phosphorylation of the N-terminal regulatory serines on IκBα and IκBβ, as well as its rapid activation in response to cell stimulation by TNF-α, IL-1, LPS (DiDonato et al., 1997; Rothwarf et al, 1998). In this study, the effects of the aqueous extract of Scrophularia buergeriana (SB) on NO and O_2^- production and possible mechanisms of the effect of the medicine on NO production in LPS-stimulated mouse peritoneal macrophages were examined.

MATERIALS AND METHODS

1. Reagents

Thioglycollate (TG) broth (Brewer) was purchased from DIFCO (Detroit, MI). Anti-murine iNOS polyclonal antibody, anti-mouse IκBα polyclonal antibody, anti-IKKα monoclonal antibody, horseradish peroxidase-conjugated anti-rabbit antibody and GST-IκBα (1-317) fusion protein were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). LPS (phenol extracted Salmonella enteritidis), Tween-20, lucigenin, T4 polynucleotide kinase, protein A/G sepharose beads were purchased from Sigma Chemical Co. (St. Louis, MO). [^{32}P] ATP (3000 Ci/mmol at 10 μCi/ml) and poly dI/poly dC were purchased from Amersham Pharmacia. 24 well, 96 well tissue culture plates and 60, 100 mm culture dishes were purchased from Nunc, Inc. (North Aurora Road, IL). RPMI 1640 containing L-arginine (200 mg/L), FBS and other tissue culture reagents were purchased from GIBCO Life Technologies (Gaithersburg, MD).

2. Preparation of extract

The root of Scrophularia buergeriana were purchased from a local herb store, Kwang Myoung Dang (Pusan, Korea) in February 1999. The roots were identified and authenticated by Professor W. S. Ko, College of Oriental Medicine, Dongeui University (Pusan, Korea). A voucher specimen (number SB-99-2) has been deposited at the Department of Oriental Medicine, Dongeui University, Pusan, Korea. The dry roots (400 g) were extracted with distilled water at 100℃ for 3 h. The extract was filtered through 0.45 μm filter and the filtrate was freeze-dried (yield, 8 g) and kept at 4℃. The dried filtrate was dissolved in phosphate buffered saline (PBS) and filtered through 0.22 μm filter before use.

3. Macrophage culture

C57BL/6 mice purchased from Dae Han Laboratory Animal Center (Korea) were used between 8 to 12 weeks of age (25~30 g). TG-elicited macrophages were harvested 3 days after intraperitoneal injection of 2.5 ml TG into mice and isolated as reported previously (Kim et al., 2000). Peritoneal lavage was performed by using 8 ml HBSS. Cells were then suspended in RPMI 1640, which was supplemented with 10% FBS, and incubated at 37℃ in an atmosphere of 5% CO_2 for 5 h. Nonadherent cells were removed by suction, and then freshly prepared complete media were added.

4. Determination of NO concentration

NO level in cell cultures was measured by a microplate assay method, as described previously (Schmidt, et al., 1996). After cells were stimulated in 24 well for 24 h, 100 μl each cultured medium was mixed with the same volume of the Griess reagent (1% sulfanilamide/0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride/2.5% H_3PO_4). NO concentration was determined by measuring the absorbance at 540 nm with Vmax kinetic microplate reader.

5. Lucigenin chemiluminescence assay

O_2^- production was measured by chemiluminescence in the presence of the chemiluminogenic probe lucigenin. The medium was removed from 24 well plate and the cells washed once with PBS. The cells were scraped off into in scintillation vials containing new serum free media. Without delay after LPS treatment, 20 μM lucigenin were added. Luninescence were measured by using luminometer (model TD-20-20, Tuner designs). Science ROS generated from stimulated leukocytes plays some substantial roles in the initiation stages (Pisani et al., 1997), counts were obtained at 37℃ for 90 min.

6. Western blot analysis

The cells were washed with PBS three times and scraped off and lysed with lysis buffer [1% Triton X-100, 1% Deoxycholate, 0.1% NaN_3]. Protein concentration of lysates was determined according to Bradford (Bradford et al, 1976) and equal amounts of protein were separated electrophoretically

using SDS-PAGE, and then the gel was transferred to 0.45 μm polyvinylidene fluoride (PVDF: Millipore). The blot was incubated with anti-iNOS, IκBα, IKKα polyclonal antibody at room temperature and was detected by the enhanced chemiluminescence detection system according to the recommended procedure (ECL, Amersham).

7. Preparation of nuclear extract

Nuclear extracts were prepared as described previously with some modifications (Andrews and Faller, 1991). Briefly, cells were scraped off, washed with PBS three times, resuspended in ice-cold buffer A [10 mM HEPES-KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.2 mM phenylmethylsulfonyl fluoride (PMSF)] and allowed on ice for 15 min. Then cell extract was added Nonidet P-40 (NP-40), incubated on ice for 5 min and centrifuged at 12000 g for 30s at 4℃. After removal of the supernatant, nuclear proteins were extracted by addition of buffer B [20 mM HEPES, pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, protease inhibitor cocktail] for 30 min at 4℃ with occasional vortexing. After centrifugation at 13,000 g for 5 min at 4℃, supernatants were collected and stored at -70 ℃ for use as nuclear extract.

8. Eletrophoretic Mobility Shift Assay (EMSA)

Gel shift assay of nuclear extracts was performed according to the manufactures instructions (Promega, Madison, WI) with some modifications. In brief, the probe consisted of a double-stranded oligonucleotide containing the consensus binding sequence for NF-κB (5-AGTTGAGGGGACTTTCCC AGGC-3, Promega, Madison, WI) was end-labeled with [γ-^{32}P]-ATP (3000Ci/mmol at 10 μCi/ml) using T4 polynucleotide kinase. Nuclear extracts were incubated with gel shift binding buffer [10 mM HEPES, pH 7.9, 50 mM KCl, 0.2 mM EDTA, 2.5 mM DTT, 10% glycerol, 0.05% NP-40, 0.25 mg/ml poly dI/poly dC, protease inhibitor cocktail] for 10 min at room temperature and then the mixture was incubated with 32P-labeled probe for 20 min at room temperature. The incubation mixture was loaded onto nondenaturating gel and run in 0.25 Tris/borate/EDTA buffer. Gels were dried and exposed to X-ray film at -70℃.

9. IKK Assay

IKK was assayed as performed by Yomaoka et al. (1998), with some modification. Whole cell extracts were lysed with lysis buffer [10% glycerol, 1% Triton X-100, 1 mM EGTA, 5 mM EDTA, 1 mM Sodium pyrophosphate, 20 mM Tris-HCl (pH 7.9), 10 mM b-glycerophosphate, 137 mM NaCl, 1 mM PMSF, 10 mM NaF, 1 mM sodium orthivanadate, protease inhibitor cocktail] for 15 min at 4℃. The cell lysates were clarified by centrifugation at 12000 g for 10min at 4℃. Equal amounts of total cellular protein were immunoprecipitated with IKKα specific antibody in TNT buffer [20 mM NaCl, 20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 300 μM sodium orthovanadate, 2 mM PMSF, protease inhibitor cocktail]. The IKKα-antibody complex was precipitated with protein A/G sepharose beads, washed three times with TNT buffer, and finally washed with kinase buffer [20 mM HEPES, 10 mM $MgCl_2$, 50 mM NaCl, 20 μM β-glycerophosphate, 300 μM sodium orthovanadate, 1 mM NaF, 2 mM DTT, 500 μM PMSF, protease inhibitor cocktail]. To perform in vitro kinase assay, IKKα-antibody complexes from protein extracts of LPS stimulated cells were incubated with 5 mg/ml SB for 10 min at 37℃. The kinase assay was carried out in 10 μl of kinase buffer containing 5 μCi [γ-^{32}P] ATP and GST-IκBα fusion protein 500 ng as substrate and incubated for 30 min at 30℃. Each sample was mixed with Laemmli's loading buffer, heated for 10 min 100℃ and subjected to 10% SDS-PAGE. The gels were dried, visualized by autoradiography.

RESULTS

1. Suppressive effect of SB on O_2^- production from in LPS-stimulated cells in vitro.

To determine whether SB affects the production of in O_2^- in LPS stimulated mouse peritoneal macrophages, superoxide level was measured by Lucigenin chemiluminescence assay using luminometer. Even though LPS significantly stimulated macrophages to increase the production of O_2^-, pre-treatment of SB markedly inhibited LPS-induced increase of O_2^- level (Fig. 1).

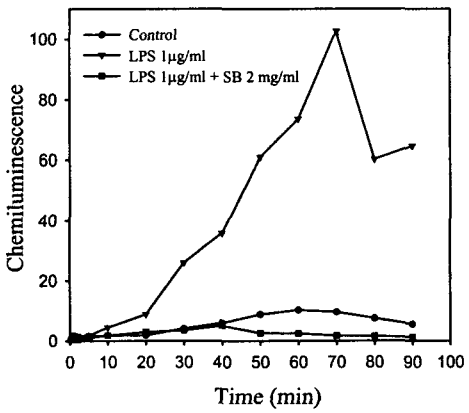

Fig. 1. Suppression of O_2^- level by SB in LPS-stimulated macrophages. The medium was removed from 24 well plate and the cells washed once with PBS. The cells were scraped off into in scintillation vials containing new serum free media. Without delay after LPS treatment, 20μ M lucigenin were added. Chemiluminescence counts were obtained at 37℃ for 90 min using TD-20-20 luminometer.

2. Suppression of NO production by SB by LPS-stimulated mouse peritoneal macrophages in vitro.

The effect of SB on NO generation in LPS-stimulated mouse peritoneal macrophages was also investigated. The accumulated NO, estimated by Griess method, in the culture medium was used as an index for NO synthesis from TG-elicited mouse peritoneal macrophages. Cells were pre-incubated in 24 well tissue culture plates ($2X10^5$ cells/well) with SB for 1 h and stimulated with 1 μg/ml LPS for 24 h. NO produced from unstimulated macrophages was considered as basal level. In LPS-treated cells, NO concentration released from macrophages was increased markedly about 8-fold in comparison with basal level. When macrophages were pre-incubated with various concentrations of SB and followed by stimulation with LPS for 24 h, significant inhibition of NO production was found (Fig. 2A). Furthermore, the inhibition of NO production seemed to be dose-dependent.

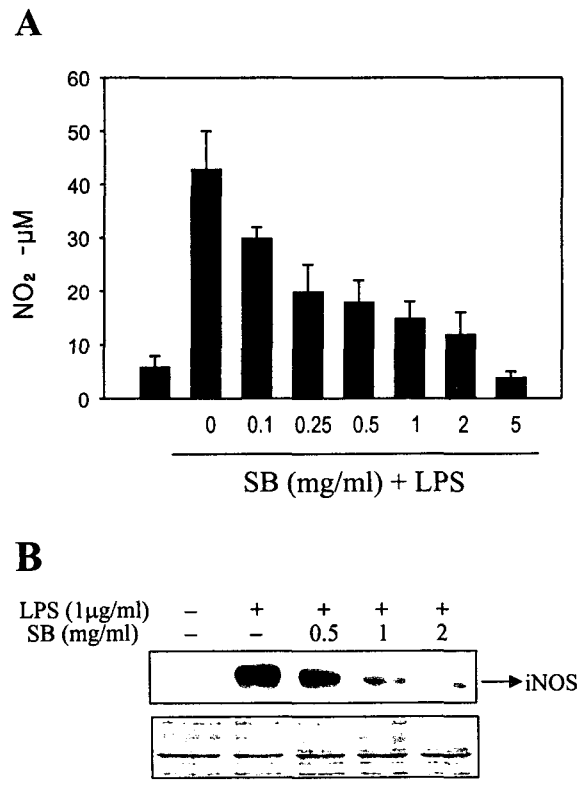

Fig. 2. Effect of SB on NO production and iNOS expression in LPS-stimulated macrophages. TG-elicited mouse peritoneal macrophages were incubated with various concentrations of SB for 1 h, and then stimulated with 1 μg/ml LPS for 24 h at 37°C. (A) At the end of incubation, the culture medium was collected for Griess reaction. Results were presented as the means S.E. of four individual experiments performed in duplicate. (B) Whole cell extracts were separated by SDS-PAGE and analyzed by western blotting (upper panel). Coomassie blue staining of PVDF was performed to confirm that an equal amount of total protein was loaded each lane (lower panel).

3. Inhibition of iNOS expression by SB in mouse peritoneal macrophages

To determine whether the decreased NO synthesis is correlated with iNOS expression, iNOS protein level was analyzed by Western blotting. TG-elicited mouse peritoneal macrophages were incubated with SB for 1 h and stimulated with 1 μg/ml LPS for 24 h. Mouse peritoneal macrophages did not express detectable iNOS protein when the cells were not stimulated by LPS, whereas 1 μg/ml of LPS induced a dramatic increase in the expression of the proteins (Fig. 2B). However, the amount of iNOS was gradually decreased by treatment of SB in a dose dependent manner. This result suggests that a significant decrease in NO release by SB is linked to the expression level of iNOS gene.

4. Suppression of NF-κB activation by SB

In mouse peritoneal macrophage stimulated by LPS, NF-κB has been known as a transcriptional factor for iNOS gene expression. To assess whether SB might suppress NF-κB activation, EMSA was performed. TG-elicited mouse peritoneal macrophages were incubated with various concentrations of SB for 1 h and stimulated with 1 μg/ml LPS for 30 min. EMSA result indicated a low level of binding activity of NF-κB in LPS-unstimulated macrophage, whereas LPS treatment induced a notable increase in NF-κB binding to its cognate DNA. As shown in Fig. 3, the binding activity of NF-κB to 32P-labeled oligonucleotide containing the consensus binding sequence for NF-κB was remarkably decreased by SB in a dose dependent manner.

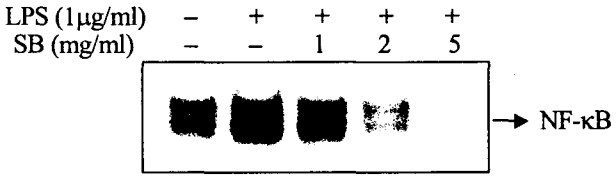

Fig. 3. Inhibition of LPS-stimulated NF-κB activity by SB. TG-elicited mouse peritoneal macrophages were incubated with various concentrations of SB for 1 h, and then stimulated with 1 μg/ml LPS for 30 min at 37°C. Nuclear proteins were extracted and assayed for NF-κB DNA binding affinity by EMSA.

5. Inhibition of degradation of IκBα by SB

The common pathway to NF-κB activation is triggered by degradation of IκB (Baldwin, 1996). To determine whether the action of SB is due to its inhibitory effect on IκB degradation, the level of IκBα protein in SB pre-treated cells was examined by Western blotting. IκBα was degradated transiently by LPS, but SB suppressed the degradation of IκBα in a dose dependent manner (Fig. 4).

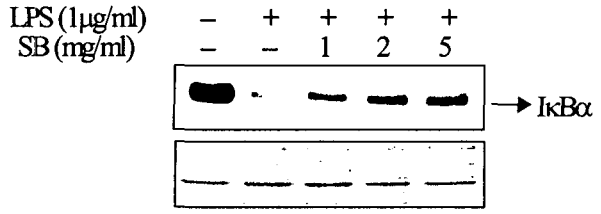

Fig. 4. Effect of SB on LPS-stimulated degradation of I κ B α in mouse peritoneal macrophages. Cells were incubated with various concentrations of SB for 1 h, and then stimulated with 1 μ g/ml LPS for 15 min at 37°C. Total cell extracts were prepared and analyzed by western blotting (upper panel). Coomassie blue staining of PVDF was performed to confirm that an equal amount of total protein was loaded each lane (lower panel).

6. Inhibition of IKK α activity by SB

In many mammal cells, I κ B phosphorylation is due to rapid activation of multisubunit protein kinase, IKK. To determine IKK activity in cell lysates, IKK α was immunoprecipitated by a monoclonal antibody and assayed by using recombinant GST-I κ B α (1-317) as a substrate. The result demonstrated that in mouse peritoneal macrophages IKK α activity is increased significantly by LPS treatment. In LPS treated cells, GST-I κ B α fusion protein was phosphorylated strongly, indicating stimulation of IKK α activity by LPS. This induction of IKK activity is suppressed by SB in a dose dependent manner (Fig. 5A) whereas Western blot analysis showed that the level of IKK α protein was not changed by the incubation with SB. The inhibitory effect of SB on LPS-induced IKK α activity was also confirmed in vitro kinase assay (Fig. 5B).

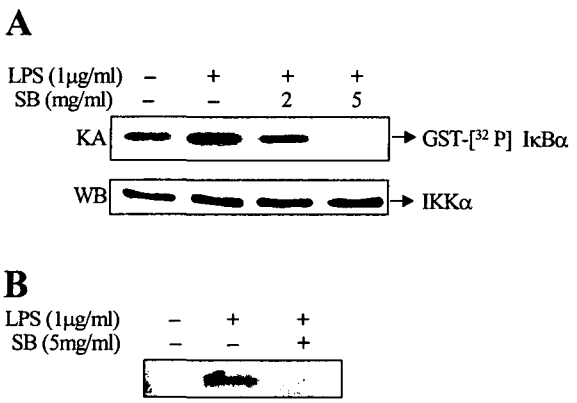

Fig. 5. Inhibition of LPS-stimulated IKK α activity by SB. (A) Mouse peritoneal macrophages were incubated with various concentrations of SB for 1 h, and then stimulated with 1 μ g/ml LPS for 10 min at 37°C. Whole cell extracts were immunoprecipitated with anti-IKKα antibody. The precipitates were incubated with GST-Iκ Bα (1-317) and [γ -32P] ATP, resolved by SDS-PAGE and analyzed by autoradiography (upper panel). Western blotting for IKK was performed as a loading control (lower panel). (B) In vitro kinase assay was performed. IKKα -antibody complexes from protein extracts of LPS stimulated cell were incubated with 5 mg/ml SB for 10 min at 37°C and then were incubated with GST-Iκ Bα (1-317) and [γ -32P] ATP, resolved by SDS-PAGE and analyzed by autoradiography.

DISCUSSION

Most species belonging to Scrophularia genus have been used as an anti-inflammatory drugs in the folk medicine and have been demonstrated to have bacteriostatic and anti-inflammatory properties (Bermejo et al., 2000). Especially, Scrophularia buergeriana have been used in treatment for febrile diseases, inflammation, constipation, scrofula, sore throat, rhinitis (Choi, 1994). However, the action mechanisms of Scrophularia buergeriana on anti-inflammatory effects have not been fully explained. Mammals are exposed in contact with Gram-negative bacteria and their LPS (Schletter et al., 1995). A low dose of LPS are thought to be beneficial for the host, e.g. in causing immunostimulation and enhancing resistance to infections and malignancies. However, large amounts of LPS can lead to inflammatory responses such as chemotaxis, differentiation and infiltration of inflammatory leukocytes, including neutrophils and macrophages. Those activated cells produce reactive oxygen species and reactive nitrogen intermediates including NO and O_2^- (Ducrocq et al., 1999), prostaglandins (PGs) and proinflammatory cytokines such as TNF and IL-1. A large amount of NO observed in a variety of cell types including macrophages, mesangial cells, vascular smooth muscle cells, keratinocytes, chondrocytes, osteoblasts, and hepatocytes (Moncada et al., 1991). NO is rapidly and non-enzymeticically reacted with O_2^- to form peroxynitrite anion, a highly toxic molecule causing a wide range of DNA and protein modifications.

The strong inflammatory response to foreign cells could also cause further damage for the neighboring cells and tissues of the host (MacMicking et al., 1997). Therefore, the reduction of the harmful effects caused by those inflammatory parameters is seemed to be important in inflammation therapy. In this study, LPS was employed to stimulate for the production of NO and O_2^- in mouse peritoneal macrophages. Pretreatment of SB, however, inhibited production of those molecules without appreciable cytotoxic effects (Fig.1, 2). These results suggest that SB could do potent anti-inflammatory action via inhibition of NO and O_2^-. Isozyme specific inhibitors of NOS are essential for therapeutic purposes and drugs that specifically inhibit iNOS could be useful in treating diseases mediated by NO production (Southan and Szabo, 1996). In fact administration of the selective inhibitors of iNOS has been reported to attenuate experimental autoimmune myocarditis (Shin et al., 1998), carrageenan-induced models of inflammation (Cuzzocrea et al., 1998), shock (Levy et al., 1999). Since anti-inflammatory effect of SB seems to be originated by suppressing iNOS gene expression, how SB affects on iNOS

gene expression was investigated. As the result, the LPS-induced high level of iNOS was gradually decreased with increasing concentration of SB.

The expression of murine macrophage iNOS is regulated at the transcriptional level and its essential transcription factor is NF-κB (Wang et al., 1997). Since dysregulation of NF-κB function is associated with inflammation, a development of drug that control NF-κB is one of promising candidates for the therapeutic strategy in the treatment of inflammatory disease (Karin et al., 2000). This study showed that NF-κB was positively regulated by LPS, and SB cotreatment significantly inhibited NF-κB activity in mouse peritoneal macrophages. In this result, SB inhibited NO production through suppression of NF-κB activity. SB also suppressed LPS-stimulated degradation of IκBα.

IKK was defined through its ability to catalyze the phosphorylation of the N-terminal regulatory serines on IκBα and IκBβ as well as its rapid activation in response to cell stimulation by LPS in several cell lines including THP-1 human monocytic cells and RAW264.7 cells (Karin et al., 2000; O'Connell et al., 1998; Jeon et al., 2000). In this study, we demonstrated that IKKα was activated by LPS stimulation and its activity was significantly suppressed by SB in mouse peritoneal macrophages. This result suggests that the reduction of IKKa activity by SB can be mediated by direct effect on the IKKα or on events upstream from IKKα in the signal transduction pathway. One may argue that SB could block by binding to LPS itself or hindering LPS-LPS receptor interaction. However, the result from in vitro kinase assay makes clear that the effect of SB is ascribed to action on intracellular signaling pathway especially to the inhibition of IKK.

In summary, these results suggest that SB could inhibit LPS-stimulated NO and O_2^- production. Moreover, biological effect of SB on NO prodution catalyzed by iNOS may involve the inhibition of NF-κB through negative regulation of IKK pathway. Going a step forward, the studies for isolation and characterization of the active chemical constitutes of SB are in progress now.

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