Anisomycin protects against sepsis by attenuating IκB kinase-dependent NF-κB activation and inflammatory gene expression

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INTRODUCTION

Sepsis is an inflammatory response in whole body to infection and is related with high mortal rates and long-term morbidity (1). Recently, methods for the treatment of sepsis have developed rapidly. However, the incidence and mortality rates owing to septic shock are still increasing (1, 2). Inflammatory factor imbalance and the secretion of inflammatory cytokines as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-1β (IL-1β), are key in the pathogenesis of sepsis (3). These inflammatory mediators are also key regulators of the pathogenesis of several other human diseases as asthma and endotoxin-induced multiple organ injury (4, 5). Therefore, anti-inflammatory agents that target various inflammatory pathways can be used as effective therapies for inflammatory disorders.

Nuclear factor-κB (NF-κB) is one of transcription factors that are normally inactive in the cytoplasm owing to interactions with inhibitory molecules of the IκB family (6). Hyperactivation of NF-κB is frequently observed with the gene expression of various inflammatory molecules such as inducible nitric oxide synthase (iNOS), TNF-α, IL-6, and IL-1β (6). It is activated by post-translational modification as phosphorylation, and the IκB protein’s proteolytic degradation via enhanced IκB kinase (IKK) and NF-κB inducing kinase (NIK) (6). Activated NF-κB translocate to the nucleus and acts as a transcription factor that binds to the promoter region of inflammatory related genes, triggering their transcription (7). Hyperactivation of NF-κB increases inflammatory diseases and anti-inflammatory drugs inhibit the expression of inflammatory cytokine by attenuating the NF-κB pathway (7). Thus, NF-κB inhibitors have important clues for the drug development of inflammation responses and therapeutic paradigms to inhibit pathological inflammation.

Anisomycin is a pyrrolidine antibiotic from Streptomyces griseoviridis and inhibits eukaryotic protein and DNA synthesis by reducing the peptidyl transferase level or deactivating the 80S ribosomal system (8). Anisomycin also inhibits the biological activity of immune cells such as T cell and transplanted organ rejection, and its result is superior to that of cyclosporine A, which has toxic side effects (8). We have previously identified the underlying mechanism of anisomycin that impacts T cell activity and function, indicating its potential in treating autoimmune diseases and inhibiting transplantation rejection (8). Recently, anisomycin was observed to increase apoptosis...
of cancer cells in glucocorticoid-resistant acute lymphoblastic leukemia by facilitating the mitogen-activated protein kinase p38 phosphorylation and the JNK activation (9). Therefore, anisomycin may be a potential anti-inflammatory and chemotherapeutic drug. However, the mechanism of these circumstance about anti-inflammatory effects has not yet been explained.

In this study, we investigated the effect of anisomycin on the survival rate of mice performed with cecal ligation and puncture (CLP)-induced and lipopolysaccharide (LPS)-induced septic shock and on the proinflammatory mediators expression. We showed that anisomycin suppressed NF-κB activation, inflammatory gene expression, and cytokine secretion by reducing IKK activity during inflammation. These results indicate that anisomycin might attenuate the expression of proinflammatory genes by inhibiting NF-κB activation and might be effective in the inflammatory diseases treatment.

RESULTS
Anisomycin prevents septic shock-induced mortality and inflammatory responses in vivo

We determined the therapeutic effort of anisomycin on the survival rate of C57BL/6 mice subjected to CLP, which caused peritonitis-induced polymicrobial sepsis in mice (4). To this end, mice were intraperitoneally injected with anisomycin (20 mg/kg) at 24 h after CLP surgery, and the survival rates of the mice were measured for five days. We found that treatment with anisomycin significantly improved the survival rates of mice performed with CLP-stimulated sepsis compared with that of mice performed with CLP and injected with phosphate-buffered saline (PBS; Fig. 1A). We also investigated the effect of anisomycin treatment in mice with LPS-induced septic shock. Mice were either stimulated with LPS or LPS and anisomycin (20 mg/kg), and the mice mortality rate was measured for 12 days. Like CLP, the survival rate of anisomycin-treated mice was significantly more increased than that of mice treated with only LPS injection, suggesting that anisomycin affects mortality in mouse models of sepsis (Fig. 1B).

Anisomycin inhibits the inflammatory response in mice subjected to CLP-induced septic shock

Next, we demonstrated with the effort of anisomycin on the CLP-induced inflammatory mediators and cytokines production in mice. To this end, serum samples harvested from mice at 24 h after CLP surgery, with or without anisomycin treatment, were subjected to an enzyme-linked immunosorbent assay (ELISA). Consistent with the effect on survival rate, anisomycin treatment lowered the levels of inflammatory cytokines such as TNF-α, IL-6, and IL-1β in mice performed with CLP-induced septic shock compared with that of mice subjected to CLP-induced sepsis model with PBS treatment (Fig. 2A-C). In addition, CLP operation increased TNF-α, IL-6, and IL-1β protein levels in mouse organs as lung and liver tissue as compared to that of mice treated with PBS only, but this increase was inhibited by anisomycin treatment (Fig. 2D-I). Thus, anisomycin inhibited the expression of proinflammatory cytokine levels in mice subjected to CLP-induced sepsis mouse model.

Anisomycin attenuates LPS-stimulated expression of proinflammatory mediators in macrophages

Macrophages play an important responsibility in the pathogenesis of inflammatory diseases by producing nitric oxide (NO), prostaglandin mediators, and inflammatory cytokines during an inflammatory response (4, 6). To demonstrate the effort of
Anisomycin on the secretion of proinflammatory mediators by macrophages, we analyzed NO production in the culture media of RAW264.7 and J774A.1 macrophage stimulated with LPS with or without anisomycin treatment. Anisomycin repressed NO production levels which treated with a dose-dependent method (Fig. 3A) in J774A.1 cell. NO production in RAW264.7 cells was downregulated by anisomycin treatment in both a time- and concentration-dependent manner (Fig. 3B). Furthermore, RAW264.7 cells stimulated with LPS, with or without anisomycin treatment for varying durations, were subjected to western blot analysis. The translational expression of iNOS, COX-2, and inflammatory cytokines were elevated upon LPS stimulation compared to that of control cells, and it was also observed that anisomycin treatment attenuated this increase in protein expression (Fig. 3C). We also estimated the levels of inflammatory cytokines namely TNF-α, IL-6, and IL-1β in the media using ELISA. LPS stimulation of RAW264.7 cells drastically increased the levels of TNF-α, IL-6, and IL-1β compared to that of control cells. This increase in levels was inhibited by anisomycin in a time-dependent method (Fig. 3D-F). Consistent with our in vivo results, anisomycin downregulated the levels of inflammatory molecules and cytokines in in vitro. Thus, anisomycin repressed the production of inflammatory mediators and cytokines during sepsis.

Fig. 3. Anisomycin decreases the induction of inflammatory enzymes and cytokines in lipopolysaccharide (LPS)-stimulated macrophages. (A) Anisomycin inhibited production of nitric oxide (NO) in a time-dependent manner in J774A.1 cells. (B) RAW264.7 cells were activated with LPS (1 μg/ml) with or without different concentrations of anisomycin, and NO levels were measured by Griess reaction. Data are represented as mean ± standard deviation (each group per triplicates). (C) After stimulation for 16 hour, cells were isolated and iNOS, COX-2, TNF-α, and IL-1β levels were measured using western blotting. The blot was rehybridized with β-actin antibody to verify equal loading of protein. (D) TNF-α, (E) IL-1β, and (F) IL-6 production in RAW264.7 following treatment with 1 μg/ml of LPS for the indicated time with or without 100 μM of anisomycin. *P < 0.05, **P < 0.01.

Anisomycin inhibits NIK and IKKβ phosphorylation and attenuates the activation of NF-κB

A decrease in the gene expression of inflammatory mediators may be related with the downregulation of their protein levels. To examine the effect of anisomycin on macrophages, transcriptional activity of iNOS, COX-2, TNF-α, and IL-1β were performed by reverse transcription quantitative PCR (RT-qPCR) after stimulation with LPS (1 μg/ml) in the presence or absence with anisomycin treatment for varying durations. LPS stimulation increased the expression of iNOS, COX-2, and inflammation-induced cytokine genes which is TNF-α, and IL-1β in RAW264.7 cells compared to control cells (Fig. 4A, B). However, on the other hand, anisomycin treatment decreased the iNOS, COX-2, TNF-α, and IL-1β expression in LPS-activated macrophages compared to cells treated with LPS in a time-dependent method only (Fig. 4A-D). We next tried to determine if anisomycin can inhibit IkBα phosphorylation and proteolytic degradation and the NF-κB nuclear translocation, which are involved in mediating the NF-κB pathway. Anisomycin significantly inhibited the IkBα’s phosphorylation and degradation in macrophages stimulated with LPS for 30 min compared to control cells (Fig. 4E). In addition, western blot analysis showed that translocation of the cytosolic p65 which is NF-κB subunit to the nucleus was enhanced in LPS-induced macrophages compared to PBS-treat-

Fig. 4. Anisomycin inhibits the transcriptional activity of proinflammatory mediators through attenuating IKK activation in lipopolysaccharide (LPS)-stimulated macrophages. (A) iNOS, (B) COX-2, (C) TNF-α, and (D) IL-1β expression after stimulating RAW264.7 cells with 1 μg/ml of LPS for the indicated time durations (F) Western blot analysis to detect NIK (upper panel) and IKKβ (lower panel) phosphorylation after induction with 1 μg/ml of LPS for the indicated time durations, with or without anisomycin (100 nM) pretreatment for two h followed by induction with 1 μg/ml of LPS for 0.5 h. (H) Phosphorylated AKT and AKT (upper panel) or phosphorylated ERK and ERK (lower panel) levels by western blot analysis after preincubation with DMSO or anisomycin (100 nM) for two hours and subsequent stimulation with LPS (1 μg/ml) for the indicated time durations. *P < 0.05, **P < 0.01.
ment cells, and this was suppressed by anisomycin (Fig. 4F). The phosphorylation of NIK and IκBα leads to the activation and phosphorylation of IkBα (6), and interestingly, immunoblotting showed that anisomycin significantly suppressed the phosphorylation of NIK and IκBα in LPS-stimulated macrophages as compared to that of macrophages treated with LPS only (Fig. 4G). To confirm the putative role of anisomycin as an NF-κB inhibitor, we examined the activation of other downstream targets of NF-κB pathway, including Akt and ERK, in LPS-stimulated RAW264.7 cells treated with anisomycin for varying durations (Fig 4H). Anisomycin did not inhibit the phosphorylation of Akt and ERK in LPS-induced RAW264.7 cells compared to RAW264.7 cells with LPS treatment only. These results indicated that anisomycin suppressed LPS-stimulated inflammation by inhibiting NF-κB activation in macrophages.

**DISCUSSION**

Anisomycin is known to play roles in mediating the anti-inflammatory responses of macrophages (10); however, its anti-inflammatory response mechanism has not yet been demonstrated. In here, we showed that the anti-inflammatory effect of anisomycin was contributed to its ability to attenuate NF-κB activation and our results indicated that anisomycin could be a potential molecule for the treatment of various inflammatory diseases such as sepsis and multiple organ failure (4-6).

Anti-inflammatory drugs are known to prevent inflammatory diseases by attenuating the inflammatory cytokines’s expression and secretion as iNOS and COX-2 (4-6). The inhibition of inflammatory mediators as iNOS, TNF-α, IL-6, and IL-1β expression suppresses the progressive inflammatory diseases (11, 12) and protects against proinflammatory reactions during septic shock (13). Here, we reported that anisomycin decreased the iNOS and COX-2 expression, and NO, TNF-α, and IL-1β production in mouse macrophage cell lines following LPS stimulation. In addition, anisomycin treatment downregulated serum levels of iNOS, TNF-α, and IL-1β in animal models of sepsis. Thus, our data validated our expectation that anisomycin has a protective effect in mice performed with CLP- and LPS-induced sepsis.

Our results also revealed that anisomycin could be a potential candidate for septic shock therapy. Recent reports suggested that anisomycin might also function as an immunesuppressant, as it inhibits T cell behavior and transplant rejection in mice (6). Anti-inflammatory and immunesuppressive drugs suppress the NF-κB pathway, and attenuation of NF-κB activation is essential for the inhibition of inflammation (14). Our study provided evidence to suggest that anisomycin is an efficient anti-inflammatory agent that functions both in vivo and in vitro. Furthermore, we performed that inhibition of the inflammatory response in LPS-stimulated macrophages by anisomycin was time- and dose-dependent. Anisomycin was also found to decrease the NF-κB pathway by attenuating IkBα phosphorylation and proteolytic degradation, following a decrease in NIK and IκBα phosphorylation. Consequently, anisomycin blocked the NF-κB nuclear translocation and downregulated the NF-κB-dependent transcriptional and translational levels of target genes, including iNOS, COX-2, TNF-α, and IL-1β in LPS-induced macrophages.

Anisomycin was previously reported to have considerable inhibitory effects on a variety of tumors and is a promising chemotherapeutic drug candidate (15). Previous studies showed that anisomycin has natural killer cell-dependent immunomodulatory effects and is a novel therapeutic drug for hepatocellular carcinoma cells (15). Moreover, several immune-associated proteins such as major histocompatibility complex molecule class I and intercellular adhesion molecule 4 are also regulated by anisomycin, impacting immune synapse formation between immune and cancer cells. Collectively, our results provided important roles into the anti-inflammatory mechanisms of anisomycin in macrophages. Anisomycin is an activator of JNK, which is one of the mitogen-activated protein kinase (MAPK) superfamily (10, 16). The JNK and p38 MAPK signaling pathways were involved in the inflammatory reaction and the p38 modulators such as anisomycin, as anti-inflammatory reagents might require an analysis of the dose-dependent effects on the balance between the modulation of inflammatory processes and the protection of infection.

In conclusion, we are the first to report that anisomycin attenuated NF-κB activation by inhibiting IKK activation and subsequently downregulated the production of inflammatory enzymes and cytokines, resulting in the amelioration of septic shock. Therefore, our findings suggested that anisomycin could be a potential therapeutic anti-inflammatory molecule.

**MATERIALS AND METHODS**

**Animal studies**

Female C57BL/6 mice, 6-10 weeks of age, were purchased from Orient (Seongnam-si, Gyeonggi-do, Korea) and maintained in accordance with the guidelines and under the approval of the Institutional Review Committee for Animal Care and Use (Korea Research Institute of Bioscience and Biotechnology). For LPS induction of sepsis, LPS (10 mg/kg, in a 100 μl volume of sterile saline) was injected intraperitoneally in mice with or without anisomycin (20 mg/kg). For CLP induction of sepsis, CLP was surgically performed on mice according to the original protocol developed by Chaudry et al., with some modifications (6). Briefly, mice were anesthetized via an intraperitoneal injection of avertin (500 mg/kg). A midline incision was made, and the cecum was ligated 1 cm from the apex and punctured (one hole) with a 23-G needle. Next, a small amount of fecal mass from the punctured cecum was gently squeezed out to ensure patency of the punctures. The cecum was relocated, and 6-0 sutures were performed to close the peritoneum and skin. The sham group of mice underwent only incision and cecum exteriorization. Twenty-four hours after sham and CLP operations, mice were either injected with 1 ml
of anisomycin (20 mg/kg) prepared in PBS or with 1 ml of PBS only.

Reagents and antibodies
LPS was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Anisomycin was purchased from Calbiochem (San Diego, CA, USA). The following antibodies were used: iNOS and β-actin (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA); COX-2, IL-1β, TNF-α, IκBα, p-IκBα, p-NIK, NIK, IKKβ, p-IKKβ, p65, and PARP (Cell Signaling Technology Co., Danvers, MA, USA).

Cells and cell culture
Murine macrophage cell lines RAW264.7 and J774.A1 were cultured in Dulbecco’s Modified Eagle’s medium (DMEM; Gibco®, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco®) and 1× antibiotic-antimycotic (100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml fungizone®; Gibco®) at 37°C in a humidified incubator with 5% CO2. Cells were then treated with LPS (1 μg/ml) in the presence or absence of varying concentrations, namely, 10, 50, 100 nm of anisomycin.

Measurement of levels of NO metabolites, TNF-α, IL-1β, and IL-6
The level of nitrite, a stable oxidized product of NO, was measured using the culture media with Griess reagent. For this, triplicates of each sample were incubated with the same volume of sulfanilamide and N-(1-Naphthyl)ethylenediamine solution. After 5-10 min, the absorbance was measured at 550 nm using a microplate reader (Molecular Devices LLC., Sunnyvale, CA, USA). The levels of TNF-α, IL-1β, and IL-6 in culture medium and sera were detected using the Duoset ELISA system (R&D Systems, Minneapolis, MN, USA), according to the manufacturer’s instructions.

Western blot analysis
Cells were lysed on ice with radioimmunoprecipitation assay lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1× protease inhibitor cocktail [Sigma-Aldrich Chemical Co.]) for 30 min. Lysates were quantified using a Pierce® BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). Next, 10-50 μg of protein was separated using a 6%-15% gel with sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to a polyvinylidene fluoride membrane using a Trans-Blot® Turbo™ Transfer pack (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5% skim milk/tris-buffered saline-Tween for 1 h and incubated with primary antibodies overnight at 4°C. After three washes, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 40 min at room temperature. After washing for 1.5 h, protein bands were visualized using the chemiluminescent HRP substrate (Millipore, Billerica, MA, USA).

RT-qPCR
Total RNA was extracted from the macrophages using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Complementary DNA was synthesized from 5 μg of total RNA using a reverse transcription kit (Promega, Madison, WI, USA). Each cDNA sample was used for RT-qPCR, and triplicate reactions were performed using ABI Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK). Primer sequences were as follows: β-actin (forward: 5'- TTC TTA ACC TCT TCA AGT TCA-3', reverse: 5'-CTT GCT GGT TGC TAC TGA AG-3'), iNOS (forward: 5'-GCC ACC AAC ATG GAG GCG TGA GCT GTG AA-3', reverse: 5'-GAC GCC CAT GGA GTG GAC TTA AA-3'), COX-2 (forward: 5'-GGC CAT GGA GTG GAC GTA TTA AA-3', reverse: 5'-ACC TCT CCA CCA ATG ACC TG-3'), TNF-α (forward: 5'-AGC GCA TGG ATC TCA AAG AC-3', reverse: 5'-AGA TAG CAA ATC GGC TGA CG-3'), β-actin (forward: 5'-GAG CCC ACC TTC TCT GTC A-3', reverse: 5'-AGC TCA TAT GGG TCC GAC AG-3'), qPCR was performed using an ABI StepOnePlus instrument (Applied Biosystems). RNA expression for the gene of interest was normalized to β-actin expression, and gene expression was quantified using the 2^-ΔCT method.

Statistical analysis
Quantitative data are represented as mean ± standard deviation, and significance was determined by performing a two-tailed, unpaired Student’s t-test. Statistical significance was set at P < 0.05.

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CONFLICTS OF INTEREST
The authors have no conflicting interests.

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