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

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Running title: Anisomycin is protective against septic shock

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Abstract

Anisomycin is known to inhibit eukaryotic protein synthesis and has been established as an antibiotic and anticancer drug. However, the molecular targets of anisomycin and its mechanism of action have not been elucidated in macrophages. Here, we investigated the anti-inflammatory effects of anisomycin both *in vivo* and *in vitro*. We found that anisomycin decreased the mortality rate of macrophages in models of cecal ligation and puncture (CLP)- and lipopolysaccharide (LPS)-induced acute sepsis. It also downregulated the gene expression of proinflammatory mediators, such as inducible nitric oxide synthase, cyclooxygenase-2, tumor necrosis factor-α, and interleukin-1β, as well as the production of nitric oxide and proinflammatory cytokines in macrophages subjected to LPS-induced acute sepsis. Furthermore, in LPS-stimulated macrophages, anisomycin attenuated nuclear factor (NF)-κB activation, which is correlated with the inhibition of phosphorylation of NF-κB-inducing kinase and IκB kinase, phosphorylation and proteolytic degradation of IκBα, and nuclear translocation of NF-κB p65 subunit. These results suggest that anisomycin prevents acute inflammation by inhibiting NF-κB-related inflammatory gene expression and is a potential therapeutic candidate for sepsis.

Keywords: Anisomycin, inflammation, septic shock, NF-κB, macrophages

Introduction

Sepsis is a common systemic inflammatory response to infection and is associated with high mortality and long-term morbidity (1). Recently, strategies for the treatment of sepsis have developed rapidly; however, the incidence of and mortality due to sepsis are still increasing (1, 2). Inflammatory factor imbalance and the release of proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6), are key to the pathogenesis of sepsis (3). These inflammatory mediators are also key regulators of the pathogeneses of several other human diseases, such as asthma and endotoxin-induced multiple organ injury (4, 5). Therefore, anti-inflammatory agents that target the various inflammatory pathways can be used as effective therapies for inflammatory disorders.

Nuclear factor-κB (NF-κB) is a family 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 mediators such as inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), TNF-α, IL-1β, and IL-6 (6). It is activated by phosphorylation, ubiquitination, and proteolytic degradation of the IκB protein via activated IκB kinase (IKK) and NF-κB inducing kinase (NIK) (6). Activated NF-κB translocates to the nucleus and acts as a transcription factor that binds to κB motifs located in the promoter regions of target genes, triggering their transcription (7). Hyperactivation of NF-κB is associated with various inflammatory diseases, and most anti-inflammatory drugs attenuate inflammatory cytokine expression by inhibiting the NF-κB pathway (7). Thus, NF-κB inhibitors have important implications for the development of anti-inflammatory drugs and strategies to limit pathological inflammation.

Anisomycin is a pyrrolidine antibiotic isolated from *Streptomyces griseolus* 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 T cells and transplantation rejection, and its effect is superior to that of cyclosporine A, which has relatively toxic side effects (8). We have previously identified the underlying mechanism of anisomycin that impacts T cell behavior and function, indicating its potential in treating some autoimmune diseases and inhibiting transplantation rejection (8). Recently, anisomycin was shown to induce apoptosis of cancer cells in glucocorticoid-resistant acute lymphoblastic leukemia by facilitating the phosphorylation of the mitogen-activated protein kinase p38 and the activation of JNK (9). Therefore, anisomycin may be a potential anti-inflammatory and chemotherapeutic drug. However, the mechanism underlying its anti-inflammatory effects has not yet been elucidated.

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

Results

Anisomycin prevents septic shock-induced mortality and inflammatory responses *in vivo*. We determined the effect of anisomycin on the survival rate of C57BL/6J mice subjected to CLP, which causes 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 monitored for 5 days. We found that treatment with anisomycin significantly improved the survival rates of mice subjected to CLP-induced sepsis compared with that of mice subjected to CLP and treated 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 injected with LPS or LPS and anisomycin (20 mg/kg), and the survival rate was monitored for 12 days. Similar to CLP, the survival rate of anisomycin-treated mice was significantly higher than that of mice treated with only LPS injection, suggesting that anisomycin affect mortality in mouse models of sepsis (Fig. 1B).

Anisomycin inhibits the inflammatory response in mice subjected to CLP-induced septic shock. Next, we investigated the effect of anisomycin on the CLP-induced production of inflammatory mediators and cytokines 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 proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 in mice subjected to CLP-induced septic shock compared with that of mice subjected to CLP-induced septic shock with PBS treatment (Fig. 2A to 2C). In addition, CLP operation increased the protein levels of TNF-α, IL-1β, and IL-6 in lung and liver tissue as compared to

that of mice treated with PBS only, but this increase was inhibited by anisomycin treatment (Fig. 2D to 2I). Thus, anisomycin inhibits the expression of proinflammatory cytokine levels in mice subjected to CLP-induced septic shock.

Anisomycin attenuates LPS-induced expression of proinflammatory mediators in macrophages. Macrophages play an important role in the pathogenesis of inflammatory diseases by producing nitric oxide (NO), prostaglandin mediators, and inflammatory cytokines during the inflammatory response (4, 6). To investigate the effect of anisomycin treatment on the secretion of proinflammatory mediators by macrophages, we analyzed NO production in the culture media of RAW264.7 and J774A.1 macrophage cells stimulated with LPS with or without anisomycin treatment. Anisomycin repressed NO production in a dose-dependent manner (Fig. 3A) in J774A.1 cells. NO production in RAW264.7 cells was inhibited 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. Protein levels of iNOS, COX2, TNF-α, and IL-1β were elevated upon LPS stimulation as compared to that of control cells, and anisomycin treatment attenuated this increase in protein expression (Fig. 3C). We also estimated the levels of inflammatory cytokines, namely, TNF-α, IL-1β, and IL-6, in the culture media using ELISA. LPS stimulation of RAW264.7 cells dramatically increased levels of TNF-α, IL-1β, and IL-6 as compared to that of control cells, and these increases were inhibited by anisomycin in a time-dependent manner (Fig. 3D–3F). Consistent with our *in vivo* results, anisomycin downregulated the levels of inflammatory mediators and cytokines *in vitro*. Thus, anisomycin represses the production of inflammatory mediators and cytokines during sepsis.

Anisomycin inhibits NIK and IKKβ phosphorylation and attenuates NF-κB activation. A decrease in the gene expression of inflammatory mediators may lead to the downregulation of their protein levels. To examine the effect of anisomycin on macrophages, mRNA levels of *iNOS*, *COX2, TNF-α*, and *IL-1β* were measured by reverse transcription quantitative PCR (RT-qPCR) after stimulation with LPS (1 μg/mL), with or without anisomycin treatment for varying durations. LPS stimulation increased the expression of *iNOS, COX2, TNF-α*, and *IL-1β*, which are inflammation-induced genes, in RAW264.7 cells compared with that of control cells (Fig. 4A and 4B). In contrast, anisomycin treatment decreased the expression of *iNOS*, *COX2, TNF-α*, and *IL-1β* in LPS-stimulated macrophages as compared to that of cells treated with LPS only in a time-dependent fashion (Fig. 4A–4D). We next sought to determine if anisomycin inhibits the phosphorylation and proteolytic degradation of IκBα and the nuclear translocation of the p65 subunit of NF-κB, which are involved in mediating the NF-κB pathway. Anisomycin significantly inhibited the phosphorylation and degradation of IκBα in macrophages treated with LPS for 30 min as compared to that in control cells (Fig. 4E). In addition, western blot analysis showed that translocation of the cytosolic NF-κB subunit p65 to the nucleus was enhanced in LPS-stimulated macrophages as compared to that in control cells, and this was suppressed by anisomycin (Fig. 4F). The phosphorylation of NIK and IKKβ leads to the activation and phosphorylation of IκBα (6), and interestingly, immunoblotting showed that anisomycin significantly suppressed the phosphorylation of NIK and IKKβ 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-stimulated RAW264.7 cells as compared to that of RAW264.7 cells treated with LPS only. These results indicate that anisomycin suppresses LPS-induced inflammation by inhibiting NF-κB activation in macrophages.

Discussion

Anisomycin is known to play a role in mediating the anti-inflammatory responses of macrophages (10); however, its mechanism of action has not yet been demonstrated. In this study, we showed that the anti-inflammatory effect of anisomycin is attributed to its ability to attenuate NF-κB activation, and our results indicate that anisomycin is a potential candidate for the treatment of various inflammatory diseases such as sepsis and multiple organ failure (4-6). Anti-inflammatory drugs are known to prevent human inflammatory diseases by suppressing the expression and secretion of proinflammatory cytokines and proteins such as iNOS and COX-2 (4-6). The inhibition of iNOS, TNF-α, IL-1β, and IL-6 expression suppresses the progression of inflammatory diseases (11, 12) and protects against proinflammatory reactions during septic shock (13). Here, we report that anisomycin decreased the expression of iNOS and COX-2, resulting in reduced production of NO, TNF-α, and IL-1β in mouse macrophage cell lines following LPS stimulation. In addition, anisomycin treatment downregulated plasma levels of NO, TNF-α, and IL-1β, in animal models of sepsis. Thus, our results validate our hypothesis that anisomycin has a therapeutic effect on mice subjected to CLP- and LPS-induced sepsis.

Our results also revealed that anisomycin is a potential candidate for septic shock therapy. Recent reports have suggested that anisomycin might also function as an immunosuppressant, as it inhibits T cell behavior and transplant rejection in mice (8). Anti-inflammatory and immunosuppressive drugs suppress the NF-κB pathway, and attenuation of NF-κB activation is essential for the inhibition of inflammation (14). Our study provides evidence to suggest that anisomycin is an efficient anti-inflammatory agent that functions both *in vivo* and *in vitro*. Further, we show that inhibition of the inflammatory response in LPS-stimulated macrophages

by anisomycin is time- and dose-dependent. Anisomycin was also found to decrease activation of the NF-κB pathway by inhibiting IκBα phosphorylation and proteolytic degradation, following a decrease in NIK and IKKβ phosphorylation. Consequently, anisomycin blocked the nuclear translocation of the NF-κB p65 subunit and inhibited the NF-κB-dependent transcriptional and translational levels of target genes, including *iNOS*, *COX2*, *TNF-α*, and *IL-1β* in LPS-stimulated macrophages.

Anisomycin has been previously reported to have significant inhibitory effects on a variety of solid tumors and is a promising chemotherapeutic drug candidate (15). Previous studies have shown 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 provide important insights into the anti-inflammatory mechanisms of anisomycin in macrophages. Anisomycin is an activator of JNK, which is a member of the mitogen-activated protein kinase (MAPK) superfamily (10, 16). The JNK and p38 MAPK signaling pathways are involved in the inflammatory response, and thus, the use of p38 modulators, such as anisomycin, as anti-inflammatory agents might require an in-depth analysis of the dose-dependent effects on the balance between the regulation of inflammatory processes and the prevention of infection.

In conclusion, we are the first to report that anisomycin attenuates NF-κB activation by inhibiting IKK activation and subsequently downregulates the production of inflammatory enzymes and cytokines, resulting in the amelioration of septic shock. Therefore, our findings suggest that anisomycin is a potentially 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); COX2, 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% CO_2. 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 in the culture media using 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% NP40, 0.25% sodium deoxycholate, 1 mM phenylmethylsulfonylfluoride, 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′- CTC TTC CAG CCT TCC TTC CT-3′, reverse: 5′-AGC ACT GTG TTG GCG TAC AG-3′), *iNOS* (forward: 5′-GCC ACC AAC AAT GGC AAC A-3′, reverse: 5′-CGT ACC GGA TGA GCT GTG AAT T-3′); *COX-2* (forward: 5′- GGC CAT GGA GTG GAC TTA AA-3′, reverse: 5′-ACC TCT CCA CCA ATG ACC TG-3′); *TNF-α* (forward: 5′-ACG GCA TGG ATC TCA AAG AC-3′, reverse: 5′-AGA TAG CAA ATC GGC TGA CG-3′), *IL-1β* (forward: 5′-GAG CCC ATC CTC TGT GAC TC-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 $2^{-\Delta Ct}$ 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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Conflict of Interest

The authors declare that they have no conflict of interest.

Author contributions statement

G.L.P. and S.J.L. performed research, analyzed data, and wrote the manuscript. G.L.P. performed cell culture experiments; M.K.P., J.K.M., and Y.J.P. performed some experiments and analyzed the data. S.W.C. and S.J.L. reviewed and discussed the manuscript. S.J.L designed the project, supervised the research, and reviewed the manuscript.

Figure legends

Figure 1. Anisomycin decreases the mortality of mice subjected to lipopolysaccharide (LPS)- and cecal ligation and puncture (CLP)-induced sepsis. (A) Mice were subjected to CLP or sham operation, then intraperitoneally injected with or without anisomycin (20 mg/kg). Mortality of each group, namely, CLP with phosphate-buffered saline (PBS; sham operation, white) and CLP with anisomycin (black) was monitored daily for 5 days after surgery. (B) C57BL/6 mice were intraperitoneally injected with LPS (10 mg/kg) simultaneously with or without anisomycin (20 mg/kg). Mortality of each group, namely, LPS only (black box) and LPS + anisomycin (white box), was monitored daily for 12 days after injection. n =10 per group

Figure 2. Anisomycin downregulates TNF-α, IL-1β, and IL-6 secretion in tissues of mice subjected to cecal ligation and puncture (CLP)-induced sepsis. Mice subjected to CLP were injected with anisomycin or phosphate-buffered saline. (A, D, G) TNF-α, (B, E, H) IL-1β, and (C, F, I) IL-6 levels in mouse serum, lung, and liver tissues at 24 h after the injection. The levels of TNF-α, IL-1β, and IL-6 secretion were measured using enzyme-linked immunosorbent assay. Data are represented as mean ± standard deviation (n = 10). $^{*}P < 0.05$, $^{\#}P < 0.01$.

Figure 3. Anisomycin decreases the induction of inflammatory enzymes and cytokines in lipopolysaccharide (LPS)-stimulated macrophages. (A) Anisomycin inhibits production of nitic oxide (NO) in a time-dependent manner in J774A.1 cells. (B) RAW264.7 cells were stimulated with LPS (1 μg/mL) in the presence or absence of different concentrations of anisomycin, and nitrite levels were measured by Griess reaction. Data are represented as mean ± standard deviation (n ≥ 3). (C) After 16 h of stimulation, cells were harvested, and levels of iNOS, COX-2, TNF-α, and IL-1β were measured by 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 stimulation with 1 μg/mL of LPS for 4, 8, 16, and 24 h with or without 100 μM of anisomycin. $^{*}P < 0.05$, $^{\#}P < 0.01$.

Figure 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 with or without 100 nM of anisomycin. (E) Western blot analysis of p-IκBα and IκBα levels after preincubation with DMSO or anisomycin (100 nM) for 2 h and subsequent stimulation 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 μM) pretreatment for 2 h. (G) Western blot analysis of the nuclear translocation of NF-κB subunit p65 after pretreatment with or without 20 μM of erastin for 2 h followed by induction with medium containing 1 μg/mL of LPS for 0.5 h. (H) Western blot analysis of p-AKT and AKT (upper panel) or p-ERK and ERK (lower panel) levels after preincubation with DMSO or anisomycin (100 nM) for 2 h and subsequent stimulation with 1 μg/mL of LPS for the indicated time durations. $^{*}P < 0.05$, $^{\#}P < 0.01$.

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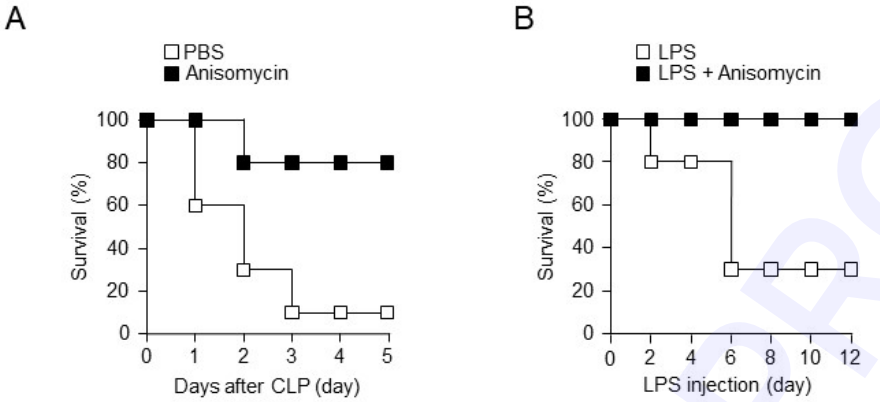

Figure 1

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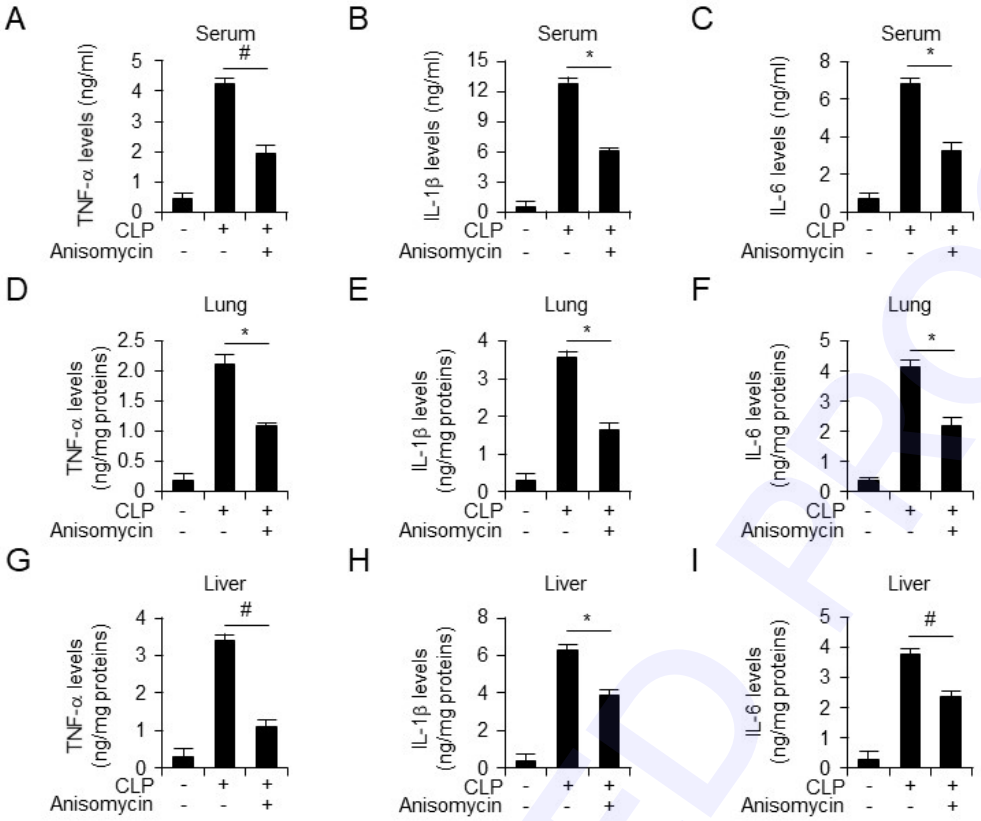

Figure 2

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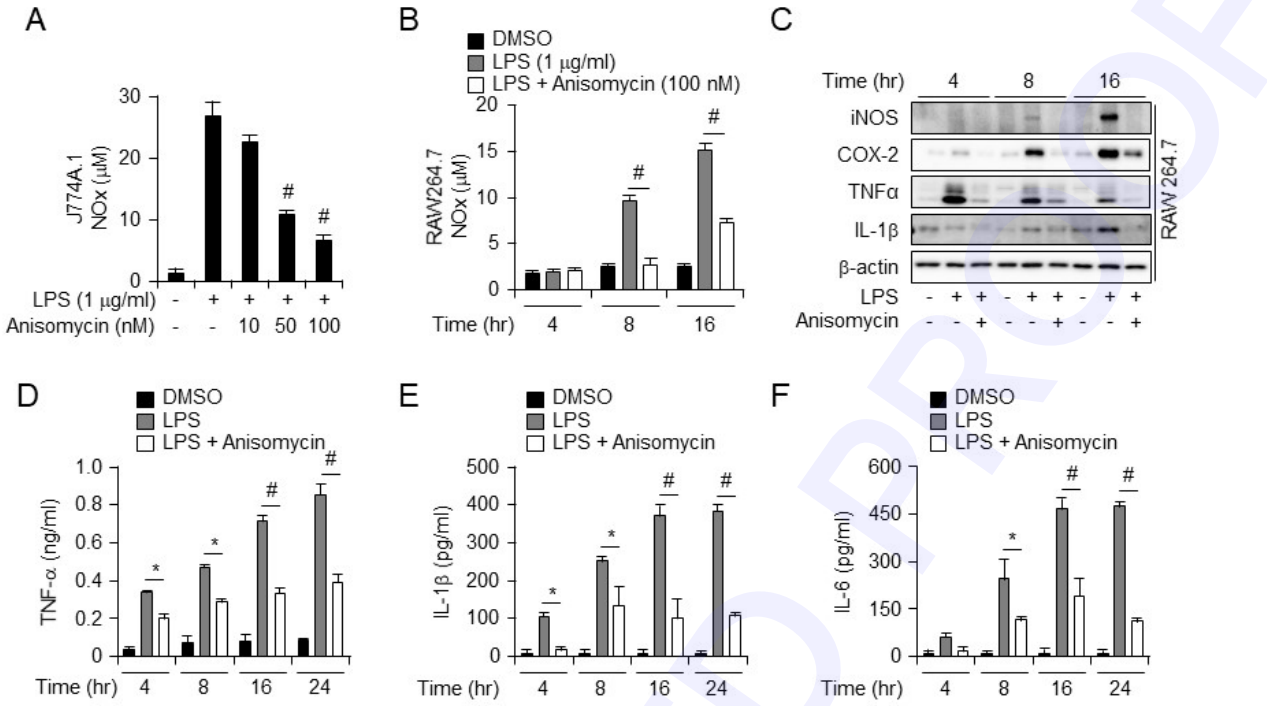

Figure 3

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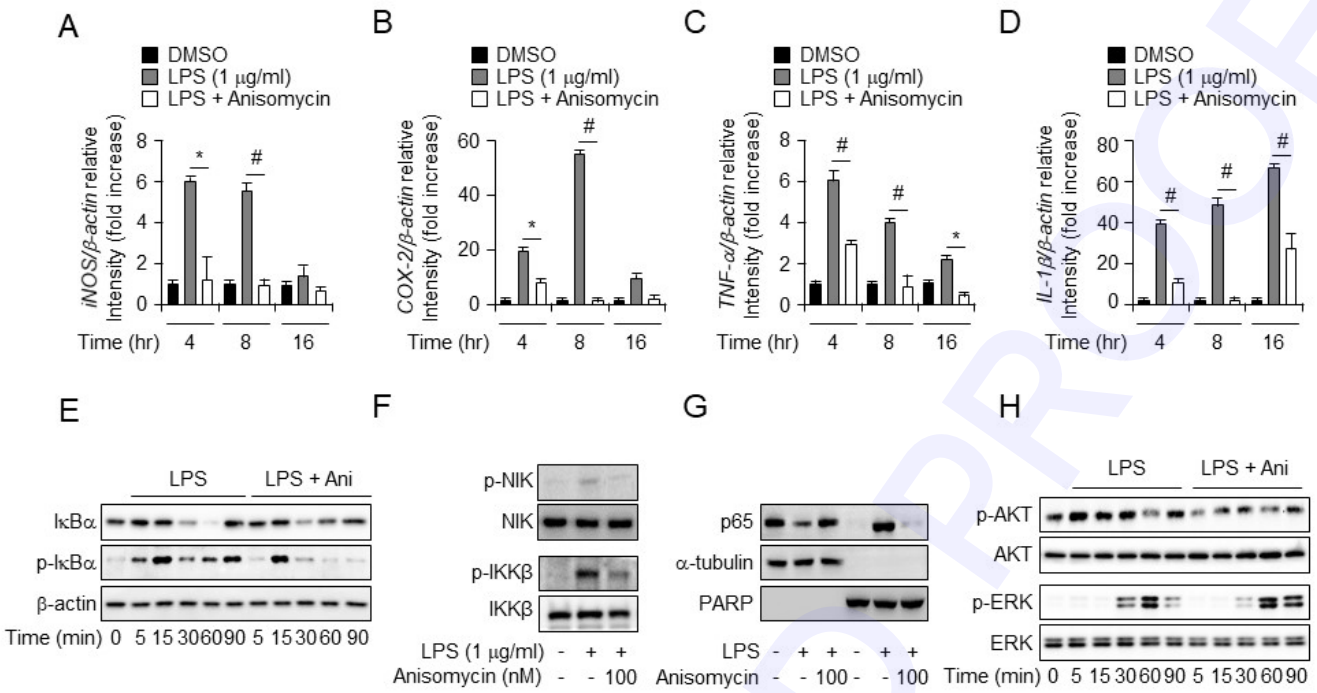

Figure 4

Fig. 4.