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# Suppression of LPS-Induced Inflammatory and NF-κB Responses by Anomalin in RAW 264.7 Macrophages

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## ABSTRACT

The treatment of inflammatory diseases today is largely based on interrupting the synthesis or action of the mediators that drive the host's response to injury. It is on the basis of this concept that most of the anti-inflammatory drugs have been developed. In our continuous search for novel anti-inflammatory agents from traditional medicinal plants, *Saposhnikovia divaricata* has been a focus of our investigations. Anomalin, a pyranocoumarin constituent of *S. divaricata*, exhibits potent anti-inflammatory activity. To clarify the cellular signaling mechanisms underlying the anti-inflammatory action of anomalin, we investigated the effect of anomalin on the production of inflammatory molecules in LPS-stimulated murine macrophages. The anomalin dose-dependently inhibited inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) mRNA and protein expression in LPS-stimulated RAW 264.7 macrophage. Molecular analysis using quantitative real time polymerase chain reaction (qRT-PCR) revealed that several pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), were reduced by anomalin, and this reduction correlated with the down-regulation of the NF-κB signaling pathway. In addition, anomalin suppressed the LPS-induced phosphorylation and degradation of IκBα. To further study the mechanisms underlying its anti-inflammatory activity, an electrophoretic mobility shift assay (EMSA) using a $^{32}$P-labeled NF-κB probe was conducted. LPS-induced NF-κB DNA binding was drastically abolished by anomalin. The present data suggest that anomalin is a major anti-inflammatory agent and may be a potential therapeutic candidate for the treatment of inflammatory disorders. J. Cell. Biochem. 112: 2179–2188, 2011. © 2011 Wiley-Liss, Inc.

**KEY WORDS:** ANOMALIN; *Saposhnikovia divaricata*; ANTI-INFLAMMATION; NF-κB; TNF-α

Inflammation is an evolutionarily conserved host reaction that is initiated in response to trauma, tissue damage, and infection and leads to changes in tissue homeostasis and blood flow, immune cell activation and migration, and the secretion of cytokines and other mediators in a spatio-temporally coordinated manner [Matthias et al., 2009]. Inflammation involves diverse molecular pathways and is entangled with a wide array of physiological processes [Shyam et al., 2009]. Research on the mechanisms of the inflammatory response has identified various mediators, cytokines, and protein kinases that act as vital signaling components, which represent potential therapeutic targets [Matthias et al., 2009]. Acute inflammation is generally down-regulated after the removal of pathogens and cellular debris. However, a chronic inflammatory state leads to local and systemic deleterious effects on host cells and tissues. Atherosclerosis, Alzheimer's disease, rheumatoid arthritis, bowel disease, ischemic heart, brain diseases, and cancer are associated with chronic inflammation [Smolen and Steiner, 2003].

The transcription factor NF-κB has been of interest for inflammatory-mediated responses, primarily because several mediators and cytokines cause the activation of this transcription factor [Soren and Steven, 2004; Novotny et al., 2008; Shyam et al., 2009]. Upon infection, pathogenic microorganisms activate NF-κB via the triggering of Toll-like receptors (TLRs), which are expressed on the cells of the innate immune system, including macrophages, dendritic cells (DCs), and mucosal epithelial cells [Medzhitov, 2000; Van et al., 2000]. TLRs recognize invariant microbial molecules, including components of the bacterial cell wall, such as lipopolysaccharide (LPS) and microbial nucleic acids [Takeda et al., 2003]. The major subunits of activated NF-κB are p50 and p65 [Hart et al., 1998; Mark, 2005; Novotny et al., 2008]. These transcription factors are kept inactive in the cytoplasm of resting cells by IκB (inhibitors of NF-κB)

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proteins. The activation of NF-κB is regulated by IκB kinase (IKK) [Novotny et al., 2008]. Various stimuli, such as viruses, bacteria, pro-oxidants, pro-inflammatory cytokines, and IκB proteins, are first phosphorylated, ubiquitinated, and then rapidly degraded by the proteasome, allowing NF-κB nuclear translocation and the transcriptional initiation of NF-κB-dependent genes. NF-κB is activated when IκB is phosphorylated by IKK [Novotny et al., 2008]. The functions of NF-κB span diverse cellular processes, including adhesion, immune regulation, proliferation, apoptosis, differentiation, and angiogenesis [Sun and Zhang, 2007].

The functioning of the immune system is finely balanced by the activities of pro-inflammatory and anti-inflammatory enzymes and cytokines [Charles, 2010]. However, inflammatory inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) production need to be regulated by NF-κB because, these mediators contribute not only to the destruction of invading pathogens but also, if excessive, to harmful inflammatory responses such as those observed in chronic inflammatory diseases (e.g., rheumatoid arthritis and septic shock) [Soren et al., 2004; Sarada et al., 2008].

A variety of safe and effective anti-inflammatory agents are currently available, and many more drugs are under development. In particular, a new era of anti-inflammatory agents, the natural compounds, have received much attention because of their potential anti-inflammatory effects [Charles, 2010].

The dry root of *Saposhnikovia divaricata* (Turcz.) Schischk. (Umbelliferae) is a perennial herb that belongs to the carrot family and is also known as Fangfeng (Bangpung) in traditional herbal medicine. *S. divaricata* induces diaphoresis to dispel the pathogenic wind and is used to treat the headache associated with the common cold, to alleviate rheumatic conditions, to mitigate spasms and to treat tetanus. It also possesses analgesic, antipyretic, and antibiotic properties [Yan, 1977; Kuo et al., 2002].

Earlier phytochemical investigations showed that "*S. divaricata*" contains various types of compounds, including chromones, coumarins, alkanes, alkynes, and polysaccharides [Kang et al., 2008]. Naturally occurring coumarins show significant pharmacological properties [Duh et al., 1991]. The pyranocoumarins in plants have antagonistic effects on calcium [Asahara et al., 1984; Ikeshiro et al., 1992]. In addition, pyranocoumarins protect the liver from injury in mice [Yoshikawa et al., 2006]. However, limited studies regarding the influence of pyranocoumarin-type compounds on inflammatory activity have been performed.

The current investigation was undertaken to evaluate the anti-inflammatory effect and the anti-NF-κB activities of anomalin, a pyranocoumarin isolated from the ethyl acetate fraction of *S. divaricata*. The present study documented the promising activity of anomalin against the NF-κB signaling pathway in LPS-stimulated RAW 264.7 macrophages.

## MATERIALS AND METHODS

### ANIMALS

Male BALB/c mice (Samtaka, Osan, Korea), 3–4 weeks of age, weighing 25–30 g, were used in this study. Mice were acclimated for 1 week and fed a diet of animal chow and water ad lib. The animals were housed at 23 ± 0.5°C and 10% humidity in a 12 h light–dark cycle. All animal studies were performed in a pathogen-free barrier zone of the Seoul National University Animal Laboratory, according to the procedures outlined in the Guide for the Care and Use of Laboratory Animals (Seoul National University, Seoul, Korea).

### PLANT MATERIALS

The dried roots of *S. divaricata* (Turcz.) Schischk. (Umbelliferae) collected in China were obtained from the Omniherb Company (Daegu, South Korea) and identified by Professor Lee, Je-Hyun (College of Oriental Medicine, Dongguk University, South Korea). A voucher specimen has been deposited at the College of Pharmacy, Seoul National University, South Korea. The dried roots of *S. divaricata* (10 kg) were extracted with MeOH three times, using ultrasonic extraction three times and concentrated under a vacuum into a residue. The MeOH extract (1.9 kg) was suspended in $H_2O$ and subsequently partitioned with ethyl acetate (EtOAc). Anomalin (34 mg) was purified from the EtOAc-soluble fraction (565 g) through repeated silica gel column chromatographic separation, which has described in detail elsewhere [Kim et al., 2008]. Its structure was confirmed by the interpretation of 1D- and 2D-NMR spectroscopic data and comparison with previously published values [Kim et al., 2008]. The purity was assessed using HPLC analysis (Fig. 1) as previously described [Tosun et al., 2007].

### CELL CULTURE

RAW 264.7 murine macrophages were obtained from the American Type Culture Collection (Manassas, VA). These cells were maintained at sub-confluence in a 95% air and 5% $CO_2$ humidified atmosphere at 37°C. The medium used for the routine subculture was Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml). The RAW 264.7 cells harboring a pNF-κB secretory alkaline phosphatase (SEAP)–NPT reporter construct [Ahn et al.,

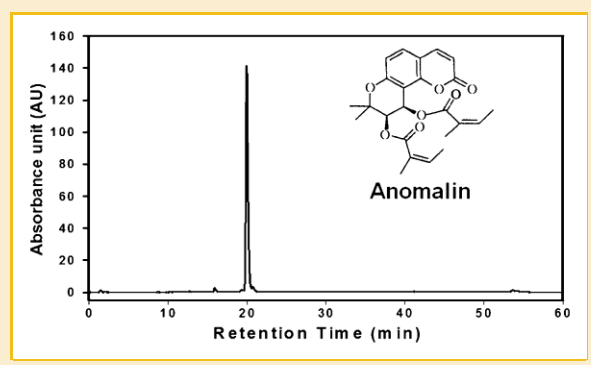


Fig. 1. Structure of anomalin and HPLC chromatogram of isolated anomalin. Extracts of *S. divaricata* were purified using an activity guided chromatographic fractionation on silica gel. On the subsequent HPLC, a single peak with a retention time of 20 min exhibited >98% of the absorbance eluted and was anomalin (~99% pure).

2005] were cultured under the same conditions, except that the media was supplemented with 500 μg/ml geneticin.

### THE MTT ASSAY FOR DETERMINATION OF CELL VIABILITY

The measurement of cell viability of anomalin was performed using the MTT (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. Briefly, RAW 264.7 cells were plated at a density of $1 \times 10^4$ per well in a 96-well plate and incubated at 37°C for 24 h. The cells were treated with various concentrations of anomalin (1, 10, and 50 μM) or vehicle alone. Anomalin was first dissolved in dimethyl sulfoxide (DMSO) to make 100 mM stock concentration and further diluted with DMSO for working concentration. Final DMSO concentrations on the cells were <0.25% and were shown not to interfere with the assay. After 20 h of incubation at 37°C, 10 μl of the MTT (2 mg/ml in saline) solution was added to each well and incubated under the same conditions for another 2 h. Mitochondrial succinate dehydrogenase in live cells converts MTT into visible formazan crystals during incubation. The formazan crystals were then solubilized in DMSO, and the absorbance was measured at 595 nm using an enzyme-linked immunosorbent assay (Emax, Molecular Devices, Sunnyvale, CA). Relative cell viability was calculated by comparing the absorbance of the treated group to the untreated control group. All experiments were performed in triplicate.

### NF-κB SEAP REPORTER GENE ASSAY

To determine the inhibitory activity of anomalin in LPS stimulated RAW 264.7 macrophages, NF-κB-dependent reporter gene transcription was analyzed using the SEAP assay as previously described, with some modifications [Shin et al., 2008]. In brief, $1 \times 10^5$ RAW 264.7 macrophages transfected with pNF-κB–SEAP–NPT encoding four copies of -κB sequences and the SEAP gene as a reporter were pre-incubated with different concentrations of anomalin for 2 h and challenged with LPS (1 μg/ml) for additional 18 h. Aliquots of the cell-free culture medium were heated at 65°C for 5 min and given an assay buffer (2 M diethanolamine, 1 mM $MgCl_2$, 500 μM 4-methylumbelliferyl phosphate (MUP)) in the dark at 37°C for 1 h. The fluorescence from the products of the SEAP/MUP was measured using a 96-well microplate fluorometer (Gemini XS, Molecular Devices) at an excitation of 360 nm and an emission at 449 nm. *N-p*-tosyl-L-phenylalanyl chloromethyl ketone (TPCK), 10 μM was used as a positive control in this experiment.

### DETERMINATION OF NITRIC OXIDE PRODUCTION IN RAW 264.7 MACROPHAGES

The inhibitory effect of anomalin on NO production in murine macrophage-like RAW 264.7 cells was evaluated in the medium using the Griess reaction method as described previously [Ahn et al., 2005]. In brief, $1 \times 10^5$ RAW 264.7 cells were plated in 24-well plates, incubated for 24 h, pre-treated with different concentrations of anomalin or vehicle for another 2 h and, challenged with LPS (1 μg/ml) for an additional 18 h. Equal volumes of cultured medium and Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphtylethylenediamine dihydrochloride in distilled water) were mixed, and the absorbance at 540 nm was determined with a microplate reader (Gemini XS, Molecular Devices). The absorption coefficient was calibrated using a sodium nitrite solution standard. For this experiment, 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine (AMT) was used as a positive control.

### ISOLATION OF PERITONEAL MACROPHAGES AND MEASUREMENT OF NITRITE PRODUCTION

For the primary cultures, peritoneal exudates cells were collected from the peritoneal cavities of male BALB/c mice by washing with ice-cold 3% FCS–PBS. The cells were suspended in supplemented DMEM and pre-cultured in 24-well microplates at 37°C in 5% $CO_2$ for 2 h. The non-adherent cells were removed and the adherent cells were cultured in the fresh medium. The cells were treated with different concentrations of anomalin for another 2 h then stimulated with LPS (10 μg/ml) for an additional 24 h. The NO production in each well was assessed as previously described.

### FLUORESCENCE ACTIVATING CELL SORTING (FACS)

The peritoneal macrophages were collected from the peritoneal cavity of the BALB/c mice elicited with cold 3% FCS–PBS. The peritoneal cells cultured at 37°C in 5% $CO_2$ for 2 h. The non-adherent cells were removed and the adhered cells were washed and collected for FACS analysis.

### WESTERN IMMUNOBLOT ANALYSIS

RAW 264.7 macrophages were pre-treated with the indicated concentrations of anomalin or vehicle for 2 h and stimulated with LPS (1 μg/ml) for 5, 10, 15, and 20 min (for the measurement of phospho-IκBα and IκBα), 18 h (for the measurement of COX-2, COX-1, and iNOS) and 3, 6, 12, and 14 h (for the measurement of phosphor-eIF2α). All of the primary and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), while the phosphor-eIF2α monoclonal antibody was purchased from Cell Signalling Technologies (Danvers, MA). Ten micrograms of total proteins for iNOS, IκBα, and phosphor-IκBα, phosphor-eIF2α and COX-1, while, 5 μg for COX-2, was separated on an SDS–PAGE, 8% (iNOS, COX-2, and COX-1) and 10% (phosphor-IκBα, IκBα, and β-actin), respectively. After electrophoresis, the proteins were electro-transferred to nitrocellulose membranes (Whatman GmbH, Dassel, Germany), blocked with 5% non-fat milk in TBS-T buffer and, blotted with each primary antibody (1:1,000) and its corresponding secondary antibody (1:5,000) according to the manufacturer's instructions. The antibodies were detected with the WEST-SAVE Up™ luminol-based ECL reagent (ABFrontier, Seoul, South Korea). The target bands were quantified using UN-SCAN-IT™ software version 6.1 (Silk Scientific Co., Orem, UT).

### RNA EXTRACTION AND REVERSE TRANSCRIPTASE (RT)-PCR

RT-PCR was performed with total RNA extracted using TRI REAGENT™, according to the manufacturer's recommendations (Sigma–Aldrich, St. Louis, MO). The purity of the RNA preparation was assessed by measuring the absorbance ratio at 260/280 nm. The primer used for the amplifications of iNOS, COX-2, and GAPDH transcripts and the condition for the amplifications were the same as previously described [Shin et al., 2008], with the modification of the use of 30–40 cycles for amplification. The sense and antisense primers for iNOS were 5′-CCCTTCCGAAGTTTCTGGCAGC-

3′ and 5′-GGCTGTCAGAGCCTCGTGGCTT-3′, respectively. The sense and antisense primers for COX-2 were 5′-GGAGAG-ACTATCAAGATAGTGATC-3′ and 5′-ATGGTCAGTAGACTTTTA-CAGCTC-3′, respectively. The sense and antisense primers for rat GAPDH mRNA expression (used as a control for total RNA content for each sample) were 5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′ and 5′-CATGTAGGCCATGAGGTCCACCAC-3′, respectively. RT-PCR was performed using the one-step-RT-PCR PreMix kit (Intron Biotechnology, Korea) according to the manufacturer's instructions. The amplified cDNA products were separated by 2% agarose gel electrophoresis and stained with ethidium bromide. The gels were viewed using the Doc-It LS Image Analysis software (UVP, Inc., Upland, CA) and quantified using the UN-SCAN-IT$^{TM}$ software version 6.1 (Silk Scientific Co). The PCR products were normalized to the amount of GAPDH for each band.

### QUANTITATIVE REAL TIME POLYMERASE CHAIN REACTION (qRT-PCR)

Total RNA was extracted from cells by the Tri-Reagent method (MRC, Cincinnati, OH), according to the manufacturer's instructions. Total RNA (1 μg) was reverse-transcribed into cDNA using the SuperScriptIII First-Strand Synthesis Supermix for qRT-PCR kit (Invitrogen, Carlsbad, CA), under the following conditions: 65°C for 5 min, 42°C for 50 min, and 70°C for 10 min. The expression of the genes was determined by qRT-PCR (LightCycler® 2.0 Real-Time PCR System, Roche Diagnostics, Mannheim, Germany) using the TaqMan Expression Assays (Roche Diagnostics). Two microliter of cDNA was used for qRT-PCR, and the reaction was performed at 95°C for 10 min, followed by 45 cycles of 94°C for 10 s and 55°C for 30 s. The expression level of the genes was normalized to GAPDH. The data was analyzed using the LightCycle software version 4.0 (Roche Diagnostics).

### MEASUREMENT OF TNF-α PRODUCTION IN THE MEDIUM

The TNF-α production in the culture medium was determined using commercially available TNF-α ELISA kit (eBioscience, Inc., CA).

### ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

EMSA was performed to investigate the inhibitory effect of anomalin on NF-κB DNA binding as previously described [Shin et al., 2008]. Briefly, nuclear extracts prepared from LPS-treated cells were incubated with $^{32}$P-end-labeled 22-mer double-stranded NF-κB consensus oligonucleotide (Promega, Madison, WI) with the sequence 5′-AGT TGA GGG GAC TTT CCC AGG C-3′ for 30 min at room temperature. To verify the specificity for NF-κB, a 50-fold excess of unlabeled NF-κB oligonucleotide was added to the reaction mixture as a competitor. For the super-shift assay, 5 μg of the p50, p65, and c-rel antibodies were added, followed by a 30-min incubation at room temperature. The samples were electrophoresed through a 6% native polyacrylamide gel. Finally, the gels were dried and exposed to X-ray film. The signals obtained from the dried gel were quantitated with an FLA-3000 apparatus (Fuji, Tokyo, Japan) using the BAS reader version 3.14 and Aida Version 3.22 software (Fuji-Raytest, Straubenhardt, Germany). The binding conditions have been previously optimized by Shin et al. [2008].

### STATISTICAL ANALYSIS

Unless otherwise stated, the results were expressed as means ± standard deviations (SD) from three different experiments. One-way analysis of variance (ANOVA) followed by Dunnett's *t*-test was applied to assess the statistical significance of the differences between the study groups (SPSS version 10.0, Chicago, IL). A value of $P < 0.05$ was chosen as the criterion for statistical significance.

## RESULTS

### EFFECT OF ANOMALIN ON CELL VIABILITY

To determine the effect of anomalin on cell viability, anomalin was tested in the MTT cell viability assay using RAW 264.7 murine macrophages. The cytotoxic effect was tested to establish the appropriate concentration ranges of anomalin for the analysis of ongoing experiments (Fig. 2A). The non-toxic concentrations (1, 10, and 50 μM) were used for the experiments in the entire experimental model.

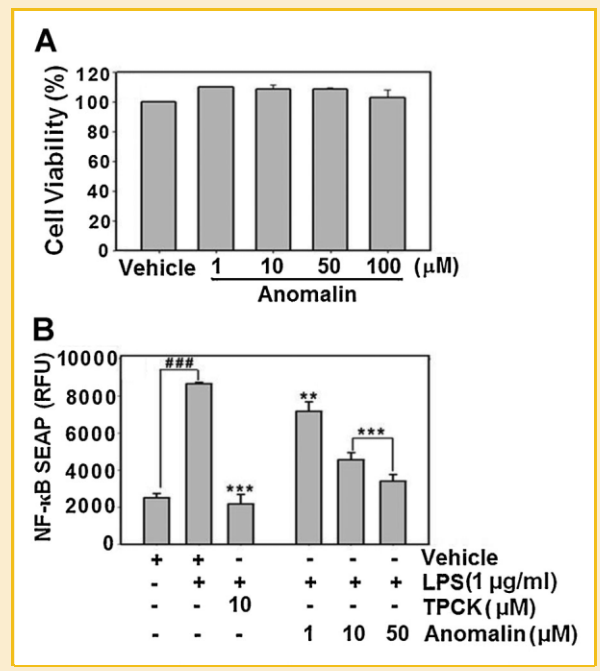


Fig. 2. A: Effect of anomalin of cell viability using RAW 264.7 macrophage cells. RAW 264.7 cells were cultured with the indicated concentrations of anomalin and incubated at 37°C in a 96-well plate for 24 h. Cell viability was evaluated as described in the "Materials and Methods Section," and is expressed as a percentage of the vehicle control. Values are expressed as the mean ± SD of three individual experiments. B: Dose-dependent suppression of LPS-induced and NF-κB-dependent alkaline phosphatase (SEAP) expression by anomalin in transfected RAW 264.7 macrophages. Data were derived from three independent experiments and are expressed as the mean ± SD. (**) $P < 0.01$ and (***) $P < 0.001$ indicate a significant difference from the LPS-challenged group. ### $P < 0.001$ indicates a significant difference from the unstimulated control group. Control (vehicle), LPS (LPS + vehicle)-treated cells alone, TPCK 10 μM, *N*-*p*-tosyl-L-phenylalanyl chloromethyl ketone were used as a positive control.

### INHIBITORY EFFECT OF ANOMALIN ON THE NF-κB SEAP REPORTER GENE IN LPS-STIMULATED RAW 264.7 MACROPHAGES

To evaluate the inhibitory activity of anomalin on NF-κB transcriptional activity, pNF-κB–SEAP–NPT construct-transfected RAW 264.7 macrophages were employed. After a challenge to LPS alone, SEAP expression increased 3.4-fold over basal expression, confirming the successful NF-κB dependent transcription in the cell system (Fig. 2B). Anomalin dramatically inhibited LPS-induced SEAP expression in a dose-dependent manner (Fig. 2B). TPCK was used as a control and inhibited LPS-induced SEAP expression more than 95% at 10 μM (Fig. 2B).

### INHIBITORY EFFECT OF ANOMALIN ON NITRITE PRODUCTION IN LPS-STIMULATED RAW 264.7 MACROPHAGES

We examined whether the inhibitory effects of anomalin on NF-κB-transcriptional activity translated into a reduction of the expression and activity of the pro-inflammatory enzyme iNOS. LPS-stimulated RAW 264.7 cells were used as a model system, and the quantified nitrite concentration was used as a measure of NO production catalyzed by the iNOS enzyme. After 18 h, LPS (1 μg/ml) produced considerable nitrite in the culture media. Anomalin drastically inhibited nitrite production in a dose-dependent manner with a maximum inhibitory effect at 50 μM (95%) (Fig. 3A).

Similar results were seen in the primary cultured peritoneal macrophages (Fig. 3B). The results clearly indicate that anomalin dose-dependently inhibited NO production in peritoneal macrophages (Fig. 3B). The positive iNOS inhibitor, AMT, significantly inhibited LPS-induced NO production (Fig. 3A,B). Moreover, the FACS analysis revealed that the peritoneal macrophages isolated from the mice elicited with 3% FCS–PBS were labeled 88.5% by the anti-CD14 FITC antibody, whereas, the RAW 264.7 cells were labeled 100% as shown in (Fig. 3C,D).

### INHIBITORY EFFECT OF ANOMALIN ON iNOS AND COX-2 mRNA AND PROTEIN EXPRESSION IN LPS-STIMULATED RAW 264.7 MACROPHAGES

The promising suppressive effect of anomalin, a reduction in de novo protein synthesis involved the suppression of iNOS activity. Therefore, the effect of anomalin on the expression level of iNOS protein was also evaluated. A Western immunoblot analysis was performed. Importantly, anomalin completely inhibited iNOS protein expression at 18 h (Fig. 4A).

Because iNOS expression is regulated at the level of transcription, one step-RT-PCR was performed to study the effect of anomalin on iNOS mRNA expression. The response of iNOS was observed 5 h after LPS stimulation. iNOS mRNA expression consistently decreased with increasing concentrations of anomalin (Fig. 4C). These results illustrated that the suppression of iNOS mRNA and protein expression was responsible for the inhibitory effect of anomalin on LPS-stimulated NO production.

COX-2 is an inflammatory factor associated with LPS stimulation. To investigate the anti-inflammatory activity of anomalin, the effect of anomalin on LPS-induced COX-2 protein up-regulation in RAW 264.7 cells was studied by Western blot analysis, and the results are

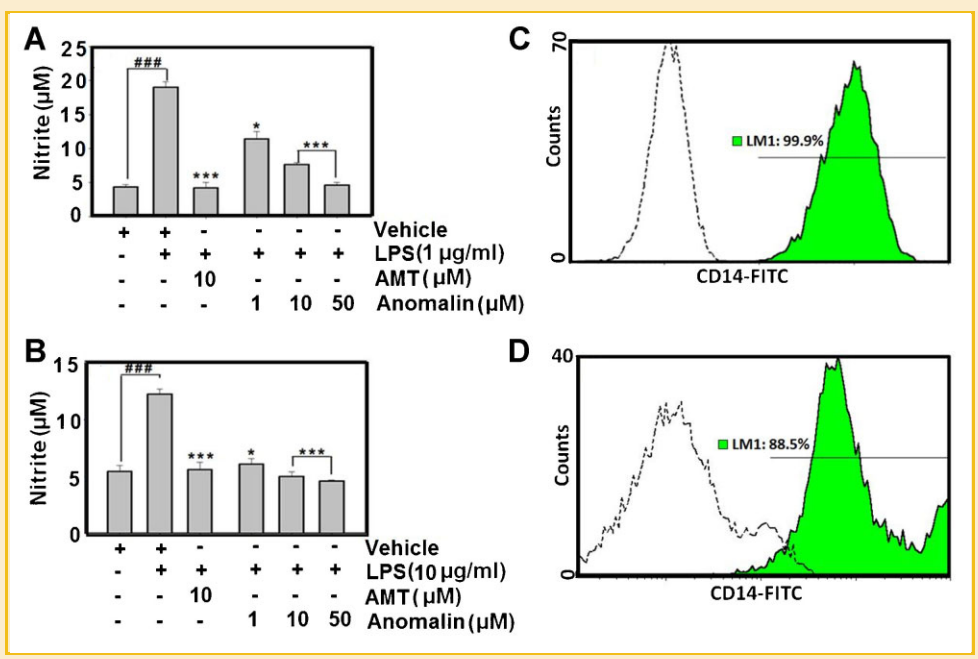


Fig. 3. A: Anomalin inhibited the NO production in a dose-dependent manner using LPS-stimulated RAW 264.7 macrophages. B: Inhibitory effect of anomalin using LPS-stimulated primary cultured peritoneal macrophages. The Griess reagent assay was performed to measure nitrite production as described in the "Materials and Methods Section". C: Fluorescence activating cell sorting (FACS) analysis of RAW 264.7 cells labeled with anti-CD FITC. D: Mice peritoneal macrophages labeled with anti-CD FITC. The data were obtained from three independent experiments and are expressed as the mean ± SD. (*) $P < 0.05$ and (***) $P < 0.001$ indicate a significant difference from the LPS-challenged group. ### $P < 0.001$ indicates a significant difference from the unstimulated control group. Control (vehicle), LPS (LPS + vehicle)-treated cells alone, AMT 10 μM, 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine were used as positive control.

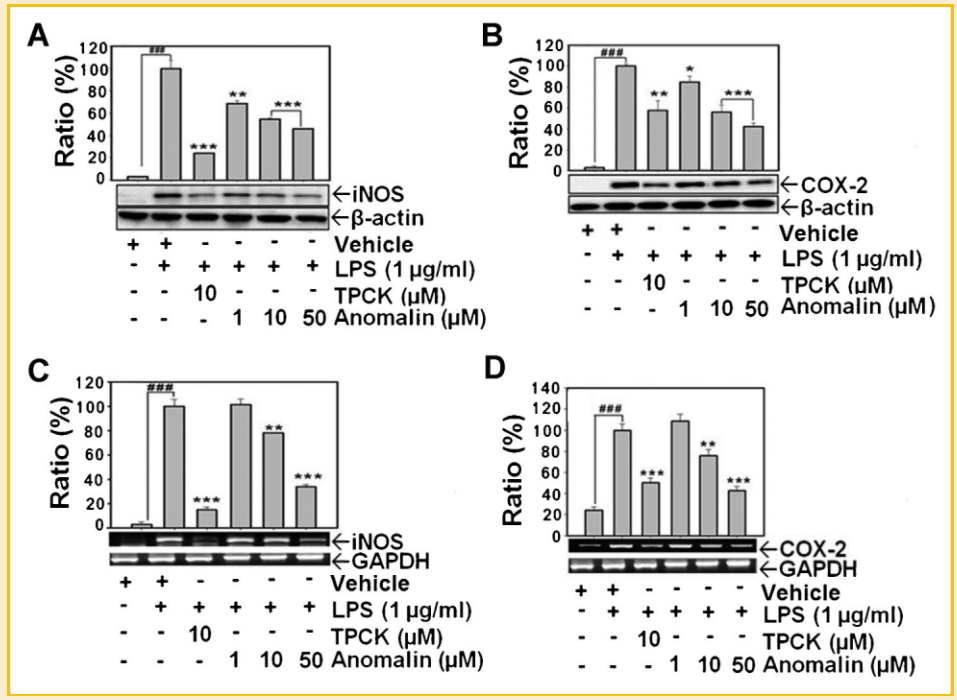


Fig. 4. Down-regulation of iNOS and COX-2 proteins and mRNA expression by anomalin in LPS-stimulated RAW 264.7 macrophages using Western blotting as described in "Material and Methods Section." A: iNOS protein expression. B: COX-2 protein expression. While the mRNA expression, using the One-Step-RT-PCR PreMix kit (Intron Biotechnology) as described in the "Materials and Methods Section." C: The effect of anomalin on iNOS mRNA expression and (D) COX-2 mRNA expression. Data are expressed as the mean ± SD from three separate experiments. For quantification, the mRNA expression data were normalized to the GAPDH signal. Control (vehicle), LPS (LPS + vehicle)-treated cells alone, and TPCK served as a positive control. (**) $P < 0.05$, (**) $P < 0.01$, and (***) $P < 0.001$ indicate significant differences from the LPS-treated group. ### $P < 0.001$ indicates a significant difference from the unstimulated control group.

summarized in (Fig. 4B). A considerable suppression of the COX-2 gene was observed in cells treated with anomalin at different concentrations (Fig. 4B). Additionally, COX-1 was also drastically inhibited by anomalin in the same concentrations (data not shown).

Anomalin displayed a remarkable inhibitory effect on COX-2 protein expression; therefore, LPS-induced mRNA expression was further investigated using One-Step-RT-PCR (Fig. 4D). Anomalin significantly inhibited LPS-induced COX-2 mRNA expression. In this regard, the suppressive effect of anomalin on COX-2 production may be primarily through transcriptional mechanisms.

### EFFECT OF ANOMALIN ON mRNA EXPRESSION OF THE PRO-INFLAMMATORY CYTOKINES, TNF-α AND IL-6, IN LPS-STIMULATED RAW 264.7 CELLS

As illustrated above, anomalin potently inhibited the LPS-induced production of pro-inflammatory iNOS and COX-2 mRNA and protein expression. To examine the effect of anomalin on the LPS-stimulated pro-inflammatory cytokines, TNF-α and IL-6, mRNA expression was further investigated using qRT-PCR. In response to LPS, TNF-α and IL-6 expression was significantly up-regulated (Fig. 5A,B). Treatment with anomalin considerably inhibited the LPS induction of TNF-α and IL-6 (Fig. 5A,B), respectively.

TNF-α production has been considered a pivotal cytokines in the pathogenesis of inflammatory diseases [Collins and Cybulsky, 2001]. Therefore, TNF-α was selected for the further study. The TNF-α production was determined in the medium after 6 h of LPS-stimulation in RAW 264.7 cells using TNF-α ELISA kit. The results demonstrated that anomalin inhibited the TNF-α production in the culture medium in concentration dependent manner as shown in (Fig. 6). The positive control, TPCK suppressed the TNF-α production at 20 μM (Fig. 6).

These results indicated that anomalin might have elicited its overall anti-inflammatory effects through the same transcription factor or pathway, including NF-κB, which regulates the transcription level of these pro-inflammatory enzymes and cytokines.

### EFFECT OF ANOMALIN ON THE PHOSPHORYLATION, DEGRADATION OF IκBα PROTEINS, AND MEASUREMENT OF THE PROTEIN SYNTHESIS CAPACITY USING WESTERN BLOTTING IN LPS-STIMULATED RAW 264.7 CELLS

To further investigate the molecular mechanism involved in the anomalin-mediated inhibitions of iNOS, COX-2, TNF-α, and IL-6, we focused on the NF-κB signaling pathway, which is known to be responsible for the transactivation of these genes. One of the major mechanisms involving the transcriptional activation of NF-κB is the phosphorylation of (IKK) α/β and the concomitant degradation of the IκBα protein, which allows the release of free NF-κB and its translocation to nucleus. The immunoblot analysis was performed

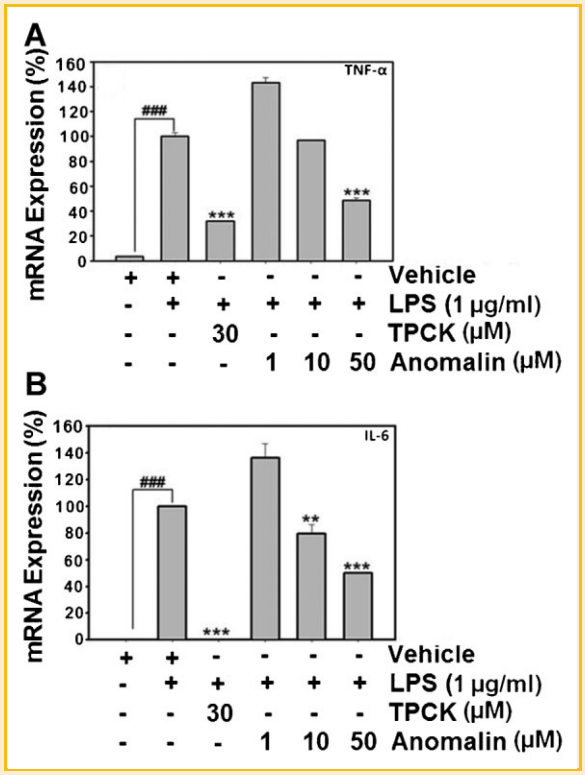


Fig. 5. Suppression effect of anomalin on the mRNA expression of the pro-inflammatory cytokines TNF-α (A) and IL-6 (B). The RAW 264.7 cells were stimulated with 1 μg/ml of LPS only or LPS plus different concentrations of anomalin (1, 10, and 50 μM) for 5 h. Total RNA was isolated and the expression of TNF-α and IL-6 was determined by qRT-PCR, as described in the "Material and Methods Section." GAPDH was used as a control. Control (vehicle), LPS (LPS + vehicle)-treated cells alone, and TPCK (30 μM) served as a positive control. (*) $P < 0.05$, (**) $P < 0.01$, and (***) $P < 0.001$ indicate significant differences from the LPS-treated group. ### $P < 0.001$ indicates a significant difference from the unstimulated control group.

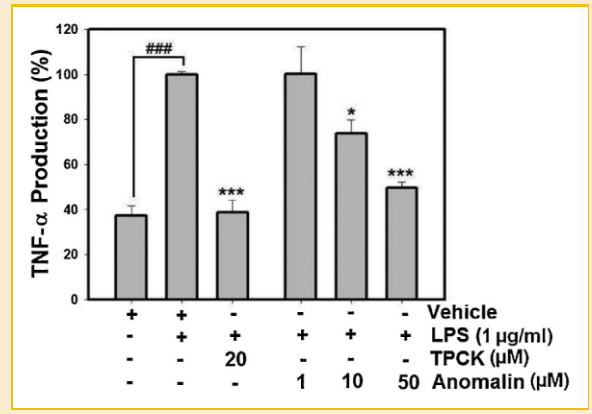


Fig. 6. Inhibitory effect of anomalin on TNF-α production in the culture medium was determined using commercially available TNF-α ELISA kit. The data were obtained from three independent experiments and are expressed as the mean ± SD. (*) $P < 0.05$ and (***) $P < 0.001$ indicate a significant difference from the LPS-challenged group. ### $P < 0.001$ indicates a significant difference from the unstimulated control group. Control (vehicle), LPS (LPS + vehicle)-treated cells alone, and TPCK 20 μM were used as positive control.

with cytoplasmic extracts of LPS-stimulated RAW 264.7 macrophages to understand whether anomalin could affect IκBα degradation and phosphorylation. After exposure to LPS alone, IκBα was degraded within 15 min (Fig. 7A). IκBα can be phosphorylated at Ser-32 and -36 residues by the IKK complex, which marks for ubiquitin-dependent IκBα degradation [Lee et al., 1998]. IκBα phosphorylation was also analyzed using Western immunoblotting. After exposure to LPS alone for 10 min, IκBα phosphorylation was markedly caused. After 10 min of LPS stimulation, IκBα phosphorylation was completely inhibited by anomalin (Fig. 7B).

The protein synthesis capacity was analyzed with phosphorylation eukaryotic initiation factor 2 α (phosphor-eIF2α), protein initiation factor using Western blotting (Fig. 7C). The phosphorylation of eIF2α was measured after 3, 6, 12, and 24 h of LPS stimulation using RAW 264.7 cells. The results revealed that anomalin showed no significant inhibitory effect on the stimulation of eIF2α phosphorylation. Thus, the protein synthesis was not interfered by the anomalin as shown in (Fig. 7C).

### EFFECT OF ANOMALIN ON NF-κB DNA BINDING ACTIVITY MEASURED BY ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA) IN LPS-STIMULATED RAW 264.7 CELLS

To investigate the effect of anomalin on the activation of NF-κB, the LPS-stimulated RAW 264.7 cells were incubated with various concentrations of anomalin for 1 h and EMSA was performed. The NF-κB DNA binding activity was elucidated through an analysis of EMSA with NF-κB $^{32}$P-labeled oligonucleotides. The RAW 264.7 cells showed a marked increase in NF-κB DNA binding activity after a 1 h exposure to LPS alone (Fig. 8A). Anomalin exhibited a promising inhibitory activity on LPS-induced NF-κB DNA binding, and the results are summarized in (Fig. 8A).

To determine the specificity and the identity of NF-κB in RAW 264.7 cells, EMSA was performed with excess amounts of unlabeled NF-κB oligonucleotides for the competition assay and with antibodies against the typical NF-κB subunit p50, p65, and c-rel for the supershift assay. As demonstrated in Figure 8B, the incubation of the LPS-stimulated nuclear extract with excess unlabeled NF-κB oligonucleotide before EMSA abolished the NF-κB DNA binding (Fig. 8B). The result indicates that the retarded band observed in the EMSA is indeed NF-κB. Moreover, the incubation of LPS-stimulated nuclear extracts with antibodies against p50, p65, and c-rel were dramatically supershifted (Fig. 8B). It has been shown that p50 protein have DNA-binding activity and p65 and c-rel proteins have transactivation domains in their C termini and thus are able to activate transcription of target genes [Lee et al., 2011]. This finding suggests that anomalin may inhibit the formation of either p50/c-rel or p50/p65 heterodimers based on the supershift studies (Fig. 8B).

## DISCUSSION

The transcription factor NF-κB plays a significant role in several signal transduction pathways involved in chronic inflammatory

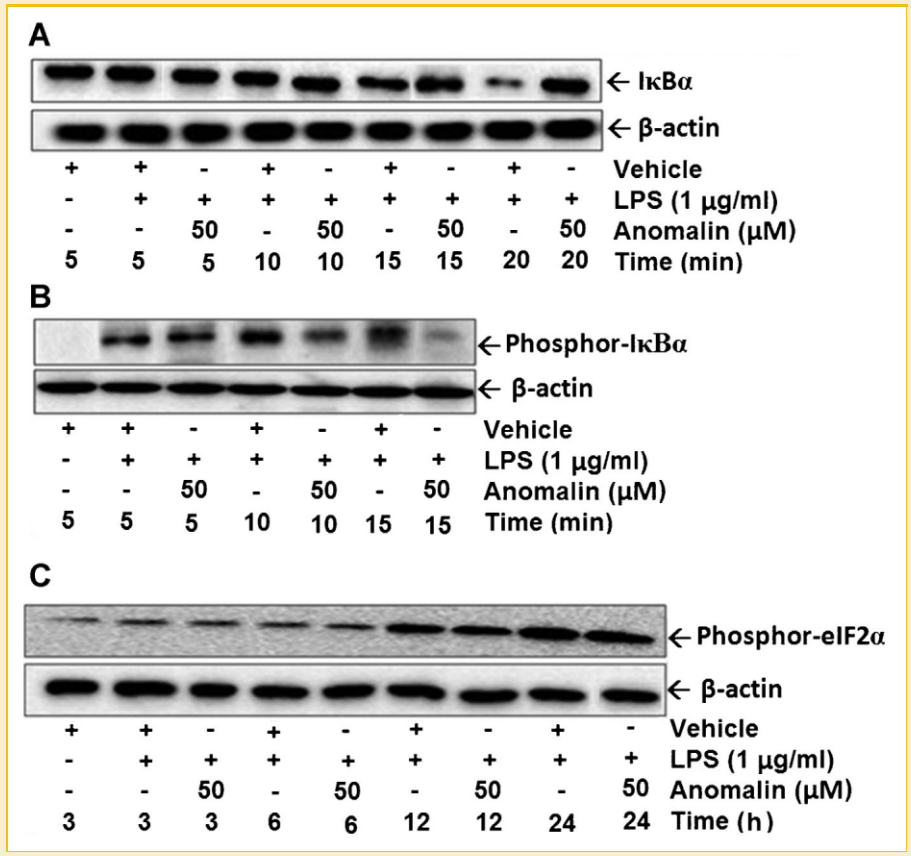


Fig. 7. (A) The expressions of IκBα, (B) phosphorylated IκBα, and (C) phosphor-eIF2α protein in the cytosolic extracts were determined by Western blot analysis, as described in the "Materials and Methods Section." The RAW 264.7 cells were pretreated with 50 μM of anomalin for 2 h and treated with LPS (1 μg/ml) for the specified time periods. A representative result from three separate experiments is shown.

diseases and various cancers [Surh et al., 2001]. The activation of NF-κB can protect cancer cells from apoptotic stimuli, apparently through the induction of survival genes. Therefore, agents that are inhibiting NF-κB transcriptional regulation and modulate the inflammatory response could have therapeutic use and a chemoprotective value. Recently, there has been a growing interest among researchers in the targeting of the NF-κB signaling pathway for the fight against carcinogenesis [Aggarwal and Shishodia, 2006; Han et al., 1998]. The current study was conducted to investigate the potential anti-inflammatory and anti-NF-κB responses of anomalin using RAW 264.7 macrophages. Because of the limited number of studies concerning anomalin on NF-κB transactivation, the effect of anomalin isolated from *S. divaricata* on the transcription activity of NF-κB was investigated. Because *S. divaricata* has been reported to have antipyretic and antiproliferative activity the effect of anomalin isolated from *S. divaricata* was assessed. To have a therapeutic purpose and a chemotherapeutic value, the suppression of NF-κB activity of anomalin needed to be translated into an anti-inflammatory action.

The regulation of enzymes (NO and $PGE_2$) and cytokines (TNF-α and IL-6) can be mediated by NF-κB [Naureckiene et al., 2007]. NF-κB is highly activated at sites of inflammation in a diverse set of diseases and can induce the transcription of pro-inflammatory mediators and cytokines. As a consequence of its critical role in several pathological conditions, NF-κB is major drug target in a variety of diseases [Yamamoto and Gaynor, 2001; Assche and Rutgeerts, 2005; Christina et al., 2006]. Naturally occurring compounds have played a significant role in the drug discovery of anti-inflammatory agents, especially for diseases that have existed since antiquity.

We found that the suppressive effect of anomalin on LPS-induced production of NO was mediated at the transcriptional level. Because of the strong anti-inflammatory properties of anomalin, we also assessed its ability to inhibit NO production using LPS-stimulated peritoneal macrophages isolated from mice. Our results clearly demonstrated that anomalin inhibited NO production in a concentration-dependent manner using peritoneal macrophages. Additionally, anomalin dose-dependently down-regulated LPS-induced iNOS and COX-2 mRNA and protein production. Furthermore, the mRNA expression of the pro-inflammatory cytokines, TNF-α and IL-6, stimulated by LPS treatment was attenuated by anomalin.

eIF2 is involved in the initiation of protein translation and plays a significant role in limiting rate of protein synthesis [Kimball, 1999]. Initiation of protein translation requires a ternary complex consisting of a GTP molecule bound to eIF2 and Met-tRNA. The Met-tRNA enables binding of 40S ribosomal subunit, and positioning of the AUG initiation codon to an mRNA [Kimball, 1999]. During translation initiation GTP is hydrolyzed to GDP plus inorganic phosphate. For the next cycle of translation initiation, the

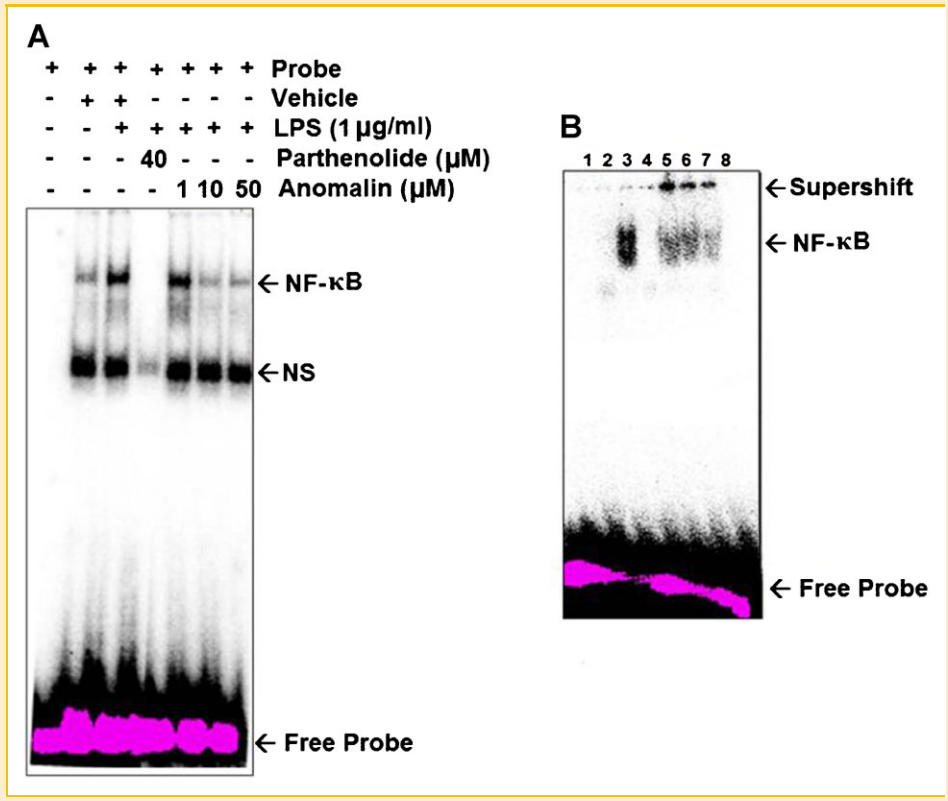


Fig. 8. A: Effects of anomalin on NF-κB DNA binding activity. Electrophoretic mobility shift assay (EMSA) was performed as described in the "Material and Methods Section." RAW 264.7 macrophages were pretreated with the indicated concentrations of anomalin for 2 h and stimulated with LPS (1 μg/ml) for 1 h. Five micrograms of nuclear extract was incubated with $^{32}$P-labeled oligonucleotide specific to NF-κB and electrophoresed on a 6% PAGE. An EMSA result is represented and NF-κB complexes, nonspecific signals (NS), and excessive probe are indicated by arrows. Parthenolide 40 μM was used as a positive control. B: Competition and supershift assays for NF-κB DNA binding using lane (1) probe alone, lane (2) the control or vehicle, lane (3) the LPS-stimulated nuclear extract, lane (4) anomalin 50 μM, lane (5) the p50 antibody, lane (6) p65 antibody, lane (7) c-rel antibody, and lane (8) the unlabeled NF-κB oligonucleotide.

GDP on eIF2 must be released and replaced by GTP; this is accomplished by eIF2B. However, if the eIF2 is phosphorylated on the α subunit by the eIF2 kinases, then the eIF2αPO4 binds to eIF2B irreversibly which prevent the GDP on eIF2 from being replaced by GTP. The number of eIF2B molecules is limited in the cell, and when free eIF2B is depleted, the initiation of protein synthesis is inhibited which leads to apoptosis. The results illustrated that anomalin showed no inhibitory effect on the initiation of protein synthesis (Fig. 7C).

LPS-induced gene expression is mediated by a series of signaling pathways, including NF-κB [Sweet and Hume, 1996]. Many compounds isolated from natural plants exhibit anti-inflammatory activity associated with their potency. The ability of NF-κB to regulate pro-inflammatory gene expression is controlled by chemical modifications and by interactions with other proteins, notably members of the IκB family [Hoffmann et al., 2002]. The phosphorylation of IκB leads to its degradation and the subsequent translocation of NF-κB to the nucleus, where it activates the transcription of target genes [Ghosh and Karin, 2002; Lee et al., 2003]. We examined these signaling pathways after an LPS challenge in RAW264.7 macrophages. To study a mechanism for anomalin inhibition, we evaluated the cytoplasmic degradation and phosphorylation of IκBα as shown in Figure 7A,B. Consistently, we found that anomalin inhibited LPS-mediated NF-κB activation via the prevention of IκB phosphorylation and degradation and the subsequent suppression of NF-κB DNA binding activity (Fig. 8A). It is clearly evident that anomalin inhibited the transactivation of NF-κB via the stabilization of IκBα.

The expression of inflammatory enzymes and cytokines is regulated via the MAPK pathway [Lee et al., 2011], but others have reported that anti-inflammatory agents may alter gene expression through a MAPK-dependent or a MAPK-independent pathway [Lee et al., 2011]. Because the anomalin protected the degradation and phosphorylation of IκBα, the upstream factors, such as members of the MAPK pathway, might be the next targets of anomalin for the inhibition of the NF-κB signaling pathway. In addition to NF-κB, another early transcription, $ATF_2$ (member of AP-1), regulates the expression of a number of pro-inflammatory genes either alone or by coupling with NF-κB. Further investigations are required to clarify the detailed mechanisms of anomalin's anti-inflammatory properties.

In summary, our data indicated that the naturally occurring pyranocoumarin, anomalin, was a potential target compound for the treatment of inflammatory diseases. Because anomalin suppressed the output of several pro-inflammatory mediators and cytokines, our results demonstrate that anomalin is a potential target compound for the treatment of inflammatory diseases.

## ACKNOWLEDGMENTS

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