NSA9, a human prothrombin kringle-2-derived peptide, acts as an inhibitor of kringle-2-induced activation in EOC2 microglia

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In neurodegenerative diseases, such as Alzheimer's and Parkinson's, microglial cell activation is thought to contribute to CNS injury by producing neurotoxic compounds. Prothrombin and kringle-2 increase levels of NO and the mRNA expression of iNOS, IL-1β, and TNF-α in microglial cells. In contrast, the human prothrombin kringle-2 derived peptide NSA9 inhibits NO release and the production of pro-inflammatory cytokines such as IL-1β, TNF-α, and IL-6 in LPS-activated EOC2 microglia. In this study, we investigated the anti-inflammatory effects of NSA9 in human prothrombin- and kringle-2-stimulated EOC2 microglia. Treatment with 20-100 μM of NSA9 attenuated both prothrombin- and kringle-2-induced microglial activation. NO production induced by MAPKs and NF-κB was similarly reduced by inhibitors of ERK (PD98059), p38 (SB203580), NF-κB (*N*-acetylcysteine), and NSA9. These results suggest that NSA9 acts independently as an inhibitor of microglial activation and that its effects in EOC2 microglia are not influenced by the presence of kringle-2. [BMB reports 2009; 42(6): 380-386]

INTRODUCTION

Microglia play a critical role in the innate immune response of the central nervous system (CNS) against microorganisms or injury as the first line of defense (1), but excessive microglial response to CNS injury induce microglial activation (2). In the CNS, activated microglia secrete reactive oxygen species (ROS), reactive nitrogen species, and pro-inflammatory cytokines, including nitric oxide (NO), tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6, in response to immunological stimuli, resulting in neurotoxicity (3, 4). This phenomenon is associated with neurodegenerative disorders such as Alzheimer's disease, cerebral ischemia, and multiple sclerosis (5, 6). Liberatore et al. reported that inducible nitric oxide syn-

thase (iNOS) induces dopaminergic neurodegeneration in the MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) mouse model of Parkinson's disease (7). However, the relationship between the neurotoxic phenomenon and neurodegenerative disease has not yet been clearly defined.

We previously reported that thrombin, prothrombin, and prothrombin kringle-2 all activate rat brain microglia, inducing nitric oxide (NO) release and mRNA expression of iNOS, IL-1β, and TNF-α (8). Those results suggested that the kringle-2 domain of prothrombin can activate microglia and that kringle-2 mimics the effect of prothrombin in inducing mRNA expression of iNOS, IL-1β, and TNF-α, along with mitogen-activated protein kinases (MAPKs) and NF-κB activation. Intracellular signaling mechanisms such as MAPKs and NF-κB, which are induced by lipopolysaccharide (LPS), interferon-γ (IFN-γ), β-amyloid and thrombin, may affect microglial activation. Overproduction of pro-inflammatory factors stimulated by prothrombin and kringle-2 may contribute to creating neurotoxic circumstances in the brain, resulting in various neurological diseases.

NSA9 (NSAVQLVEN), a human prothrombin kringle-2-derived peptide (residues 209-217), is known to inhibit blood vessel cell proliferation (9, 10). In addition, this nonapeptide exerts strong anti-inflammatory effects in LPS-activated murine microglia through suppression of NF-κB-mediated signal transduction (11). Because prothrombin and kringle-2 induce microglial cell activation and the release of cytotoxic compounds such as NO, iNOS, TNF-α, and IL-1β, which influence neuronal cell damage, understanding the effect of NSA9 on prothrombin or kringle-2-induced microglial activation will help clarify the correlation between the kringle-2 domain of prothrombin and NSA9. Therefore, we tested the effects of NSA9 in human prothrombin or kringle-2-activated EOC2 microglia to determine whether NSA9 could independently act as an internal antagonist of the human prothrombin kringle-2 domain. In this study, we provide evidence that NSA9 can independently inhibit microglial activation induced by human prothrombin or kringle-2 in EOC2 microglia.

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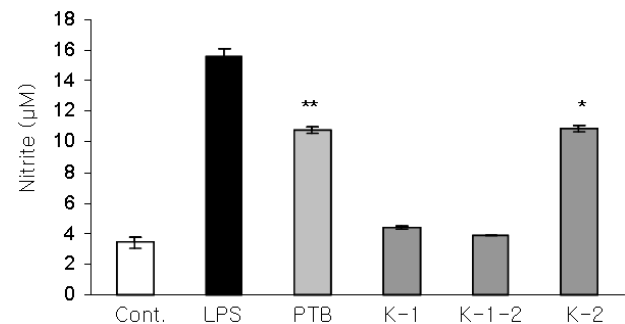
RESULTS

Human prothrombin and kringle-2 induce NO release in EOC2 microglia

Human prothrombin and kringle-2 can induce cultured rat microglial activation (8). To test whether human prothrombin and the internal domains of prothrombin such as kringle-1, kringle-1-2, and kringle-2 could stimulate the murine microglial cell line EOC2, cells were treated with 100 ng/ml LPS, 1 U/ml human prothrombin, or 10 µg/ml human prothrombin kringle-1, kringle-1-2, or kringle-2 for 24 h. The concentration of nitrite in the media increased in cells treated with LPS, prothrombin, and kringle-2 by about 4.57-, 3.15-, and 3.19-fold compared to control group, respectively, but kringle-1 and kringle-1-2 did not affect nitrite production (Fig. 1). These results demonstrate that human prothrombin and kringle-2 can stimulate NO production in both the EOC2 microglial cell line and cultured rat microglial primary cells.

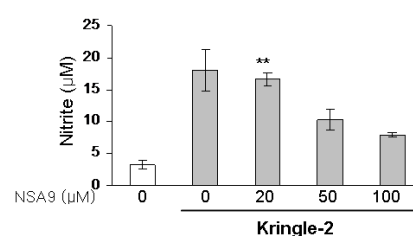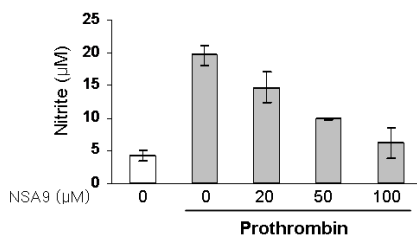### NSA9 inhibits microglial activation induced by human prothrombin and kringle-2

To verify whether NSA9 could affect microglial activation induced by human prothrombin or kringle-2, cells were treated

with 20-100 µM NSA9 for 24 h. As shown in Fig. 2A, the concentration of nitrite decreased in a dose-dependent manner with both human prothrombin and kringle-2. In addition, the protein expression of iNOS, which induces NO production,


Fig. 1. Human prothrombin and kringle-2 induce NO release in EOC2 microglia. EOC2 cells were treated with LPS (100 ng/ml), human prothrombin (1 U/ml), kringle-1 (10 µg/ml), kringle-1-2 (10 µg/ml), or kringle-2 (10 µg/ml) for 24 h. Nitrite in the culture medium was measured using the Griess assay. The data are presented as mean ± S.E.M. of three independent experiments performed in triplicate.

A

B

C

Fig. 2. Human prothrombin kringle-2-derived peptide, NSA9, inhibits microglial activation. (A) EOC2 cells were treated with human prothrombin (1 U/ml) or kringle-2 (10 µg/ml) and NSA9 (20-100 µM) for 24 h. Nitrite in the culture medium was measured using the Griess assay. The data represent mean ± S.E.M. of three independent experiments performed in triplicate. (B) Cell lysates were prepared from 1.5 × 10^6 EOC2 microglia that were treated with prothrombin (1 U/ml) or kringle-2 (10 µg/ml) and NSA9 (20-100 µM) for 24 h. Protein expression of iNOS was determined by western blot analysis using antibodies for iNOS. β-actin was used as a loading control. (C) mRNA expression of TNF-α and IL-1β was assessed by RT-PCR. Cells were treated with human prothrombin (1 U/ml) or kringle-2 (10 µg/ml) and NSA9 (20-100 µM) for 24 h. RT-PCR products were separated on 1.0% agarose gels and visualized by ethidium bromide staining. GAPDH levels were measured as a control. Data shown here are representative of three independent experiments.
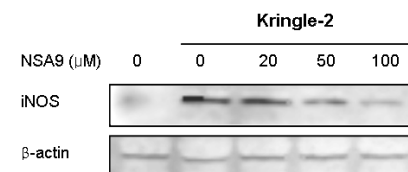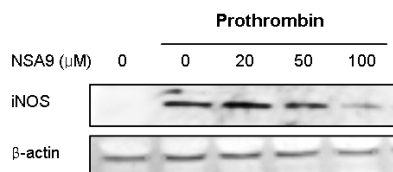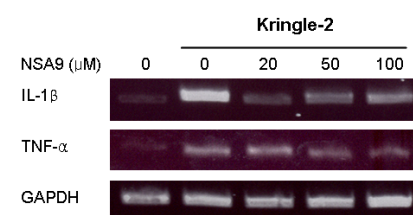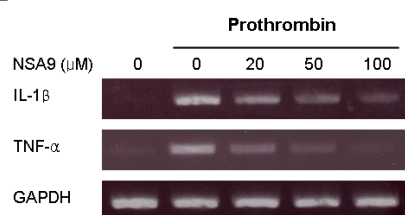
was determined using western blot analysis. iNOS protein level, stimulated by prothrombin- and kringle-2-treatment, declined in a dose-dependent manner after treatment with NSA9 (Fig. 2B).

Previously, we reported that prothrombin and kringle-2 induce mRNA expression of pro-inflammatory cytokines such as TNF-α and IL-1β (9), and that NSA9 has anti-inflammatory effects in LPS-stimulated EOC2 microglia (11). We next examined whether NSA9 would inhibit mRNA expression of TNF-α and IL-1β induced by prothrombin and kringle-2 in EOC2 microglia. As shown in Fig. 2C, mRNA levels of TNF-α and IL-1β, relative to those of GAPDH, decreased following treatment with 20-100 μM of NSA9. These results suggest that NSA9 decreases the expression of pro-inflammatory mediators such as iNOS, TNF-α, and IL-1β that are stimulated by human prothrombin or kringle-2 treatment, and that the anti-inflammatory effects of NSA9 in EOC2 microglia occur independent of human prothrombin and kringle-2 activation.

Effect of NSA9 on activation of MAPKs induced by human prothrombin and kringle-2

MAPKs are important signaling molecules that modulate the release of pro-inflammatory mediators from activated microglia (12). We examined whether NSA9 would inhibit the phosphorylation of prothrombin or kringle-2-induced MAPKs using western blot analysis. The prothrombin-induced phosphorylation of ERK, JNK, and p38 declined within 120 min of treatment with 50 μM NSA9 (Fig. 3A; left panel). Similarly, the kringle-2-induced phosphorylation of ERK, JNK, and p38 was inhibited within 60 min of treatment with 50 μM NSA9 (Fig.

3A; right panel). To determine whether inhibition of MAPKs by NSA9 treatment was related to the observed reduction of nitrite production, we assessed the production of nitrite in the presence of PD98059 and SB203580, inhibitors of the ERK pathway and p38, respectively. In the presence of 5 and 10 μM of PD98059, nitrite production dropped to 70.4 ± 0.5% and 47.4 ± 3.2%, respectively. In addition, 5 and 10 μM of SB203580 decreased nitrite production to 69.5 ± 6.4% and 50.8 ± 3.5%, respectively. Similarly, 20 and 50 μM of NSA9 reduced nitrite production to 68.8 ± 7.7% and 50.7 ± 5.4% compared to PD98059 and SB203580 treated groups, respectively (Fig. 3B; left panel). Kringle-2-induced nitrite production was also decreased by PD98059, SB203580, and NSA9. In the presence of 5 and 10 μM of PD98059, nitrite production was reduced to 77.0 ± 1.9% and 58.1 ± 2.4%, respectively, while 5 and 10 μM of SB203580 decreased nitrite production to 71.7 ± 1.4% and 47.7 ± 2.7%, respectively. Similarly, 20 and 50 μM of NSA9 decreased nitrite production to 73.2 ± 2.0% and 54.3 ± 1.99%, respectively (Fig. 2B; right panel). In summary, these data demonstrate that NO release induced by MAPKs activation was inhibited by NSA9 treatment.

NSA9 inhibits NF-κB activation induced by human prothrombin and kringle-2

We previously demonstrated that NSA9 inhibits LPS-induced NF-κB activation in EOC2 cells (11). In this study, we attempted to confirm the effect of NSA9 on NF-κB activity in prothrombin or kringle-2-induced EOC2 cells using the luciferase assay and Western blot analysis. Cells were treated with 1

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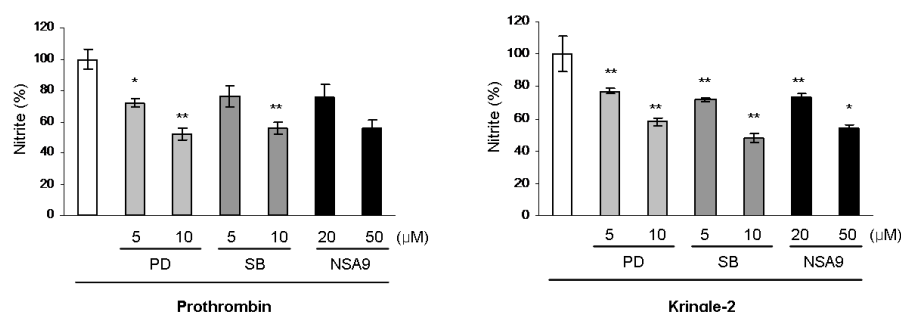
Fig. 3. Effects of PD98059, SB203580, and NSA9 on activation of MAPKs and NO release in human prothrombin and kringle-2-stimulated EOC2 cells. (A) Cells were treated with human prothrombin (1 U/ml) or kringle-2 (10 μg/ml) and NSA9 (50 μM) for the indicated time periods. Phosphorylated ERK (p-ERK), p38 (p-p38), and JNK (p-JNK) were detected by western blot analysis. (B) Cells were activated with human prothrombin (1 U/ml) or kringle-2 (10 μg/ml) for 24 h in the presence or absence of the indicated concentrations of PD98059, SB203580, and NSA9. The percent nitrite was determined by comparison with cells treated with only prothrombin or kringle-2, and the data are presented as mean ± S.E.M. of three independent experiments performed in triplicate.

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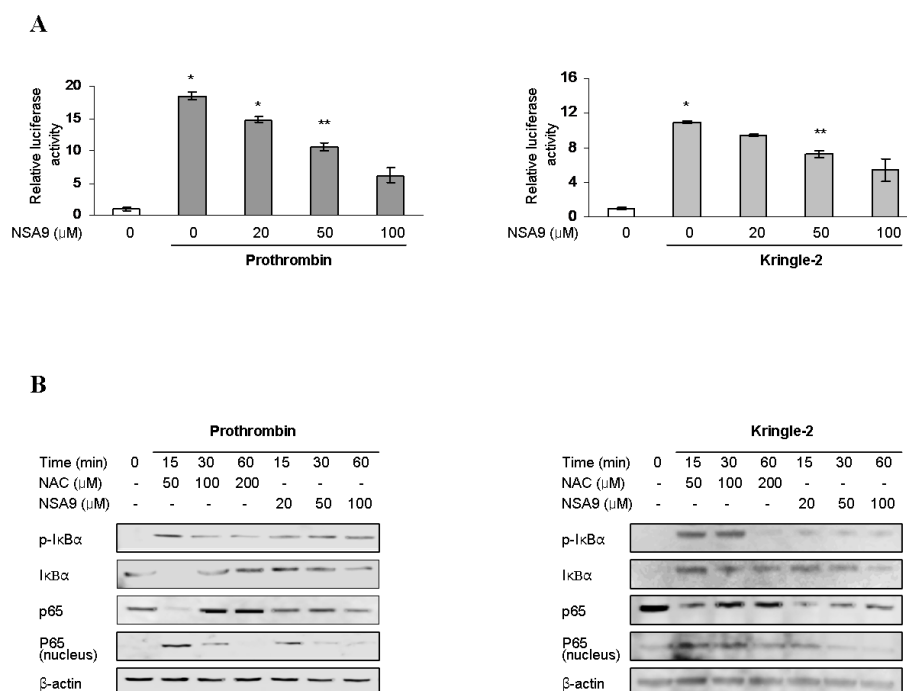
Fig. 4. Effects of NSA9 on activation of NF-κB in human prothrombin and kringle-2-induced microglia. (A) Transfected cells were treated with human prothrombin (1 U/ml) or kringle-2 (10 μg/ml) and NSA9 (20-100 μM) for 24 h. NF-κB activity was expressed as relative luciferase activity. (B) Cells were treated with human prothrombin (1 U/ml) or kringle-2 (10 μg/ml), NAC (50-200 μM), and NSA9 (20-100 μM) for the indicated time periods. Cytosolic or nuclear extracts were subjected to 4-12% SDS-PAGE followed by western blot analysis using antibodies for NF-κB p65, IκBα, and phospho-IκBα. β-actin was used as a cytosolic control. Data shown here are representative of three independent experiments.

U/ml prothrombin or 10 μg/ml kringle-2 and 20-100 μM of NSA9 for 24 h. The expression of an NF-κB reporter construct was assessed by relative luciferase activity. As shown in Fig. 4A, prothrombin and kringle-2 increased luciferase activity by about 18-fold and 10-fold, respectively, but luciferase activity was suppressed by 20-50 μM NSA9 in a dose-dependent manner. In addition, the expression of phospho-IκB, IκB degradation, and nuclear translocation of the NF-κB p65 subunit were investigated. NAC is known to inhibit NF-κB activation. In the presence of 50-200 μM of NAC and 20-100 μM of NSA9, the phosphorylation of IκBα, IκBα degradation, and p65 nuclear translocation stimulated by prothrombin or kringle-2 were inhibited (Fig. 4B).

DISCUSSION

As the resident macrophages of the brain, microglial cells have malleable roles when activated by pathogens. Indeed, activated cultured microglia can induce either neuroprotection or neurotoxicity (13). In the CNS, production of pro-inflammatory mediators such as NO, ROS, TNF-α, and IL-1β lead to neuronal cell death from endotoxin shock in animal models (14, 15). Moreover, recent studies have demonstrated that neuronal inflammation mediated by microglia induces neurodegenerative diseases such as Parkinson's, ischemia, and Alzheimer's (3, 16, 17).

NO is a well-known pro-inflammatory mediator that is induced by the expression of iNOS. Mammalian cells require specific stimuli such as LPS or IFN-γ to trigger iNOS expression

(18). Because modulation of iNOS expression affects subsequent NO production, agents with the ability to inhibit iNOS expression could potentially be used to help treat conditions associated with the overproduction of NO (19).

Human prothrombin and the kringle-2 domain of prothrombin can both activate rat microglia (8). Human prothrombin and kringle-2 mimic the effect of LPS in inducing NO production, leading to mRNA expression of pro-inflammatory mediators such as iNOS, TNF-α, and IL-1β, along with MAPKs and NF-κB activation. The kringle-2 domain of human prothrombin independently functions as a microglial activator. However, we recently found that NSA9 exerts inhibitory effects on NO production, iNOS, and pro-inflammatory mediators in LPS-stimulated EOC2 microglia through the suppression of NF-κB activation (11). In the present study, NSA9 attenuated NO release induced by iNOS expression, as well as mRNA expression of pro-inflammatory cytokines such as TNF-α and IL-1β (Fig. 2). In addition, NSA9 inhibited activation of MAPKs (Fig. 3A) and NF-κB (Fig. 4A, 4B).

Numerous protein fragments have been identified as microglial activators, including LPS, β-amyloid, gangliosides, and thrombin. These microglial activators are involved in the pathogenesis of the inflammatory response (20-23) and several studies have explored the intracellular signaling mechanisms that modulate microglial activation. However, the control of neurotoxic agents under pathological conditions is still unknown. Recently, it was reported that LPS-induced endogenously expressed IL-10 contributes to neuronal survival by inhibiting brain inflammation (24). This study provides evidence

that IL-10 regulates both the production of pro-inflammatory mediators and the deleterious effects of inflammation under neuropathological conditions. In the same manner, our study suggests that human prothrombin kringle-2 and NSA9 stimulate or inhibit microglial activation through the MAPKs and NF-κB signaling pathways.

There are some challenges in the therapeutic use of anti-inflammatory agents, such as metabolic instability, immunoreactivity, limited tissue distribution, and appropriate routes of administration due to their large molecular size. In contrast, protein-derived peptides are much smaller in size and have less immunogenicity than protein-based drugs (25). Uncontrolled activation of innate and adaptive immunity and sustained production of inflammatory mediators are deleterious for the host. Acute inflammation can lead to chronic inflammation, scarring, tissue destruction, and eventually to organ failure (26). Recently, in the neuronal system, the potent anti-inflammatory activity of neuropeptides/hormones, such as vasoactive intestinal peptide (VIP), endomorphin 1 and 2, and alpha-melanocyte stimulating hormone (α-MSH), has been demonstrated (27-31). These neuropeptides may serve as a platform to develop novel therapeutic agents able to not only block factors that initiate and drive inflammation, but also restore immune tolerance. As small molecules, neuropeptides have more efficient permeability at the site of inflammation than proteins. *In vitro* synthesis of neuropeptides is also straightforward and permits easy modification if necessary. In spite of the potential side effects that may arise from several neuropeptides in blood vessels, reproductive organs, and CNS function, some neuropeptides have been already tested in humans for the treatment of sepsis and other disorders (32).

In summary, we found that the kringle-2 domain of human prothrombin can contribute to microglial activation and that the synthetic peptide of human prothrombin kringle-2, NSA9, can independently inhibit microglial activation through suppression of MAPKs and NF-κB activation. Before these findings can be applied in a clinical setting, an effective drug delivery system must be developed to adequately infiltrate neuronal cells for the treatment of inflammatory disorders and neurodegenerative diseases.

MATERIALS AND METHODS

Cell culture

The microglia cell line EOC2 was purchased from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 4 mM glutamine, 10% heat inactivated fetal bovine serum (FBS), 20% LADMAC supernatant, 100 U/ml penicillin, and 100 μg/ml streptomycin. LADMAC supernatant is a conditioned medium that contains colony stimulating factor-1 (CSF-1) derived from the LADMAC cell line, a bone marrow stromal cell line purchased from ATCC and maintained in minimal essential medium (MEM) containing 10% FBS, 100 U/ml

penicillin and 100 μg/ml streptomycin. Cells were maintained at 37°C under a humidified atmosphere of 10% CO_2.

Materials

FBS, DMEM, MEM, phosphate-buffered saline (PBS), and antibiotic-antimycotics were purchased from Gibco/BRL (Gaithersburg, MD). *Salmonella enterica* lipopolysaccharide (LPS), *N*-acetylcysteine (NAC), and dimethylsulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, MO). Human prothrombin, and fragments (kringle)-1, kringle-1-2, and kringle-2 were purchased from Haematologic Technologies, Inc., (Essex Junction, VT). Inhibitors of MAPKs were purchased from Calbiochem (La Jolla, CA). Anti-iNOS antibody was purchased from Upstate Biotechnology (Lake Placid, NY) and anti-phospho-ERK, anti-phospho-p38, anti-phospho-JNK, and anti-β-actin antibodies were purchased from Cell Signaling Technology (Berverley, MA). Gene-specific primers were synthesized by Cosmogenetech (Seoul, Korea) and human prothrombin kringle-2 derived peptide (NSA9) was synthesized by Peptron, Inc., (Daejeon, Korea).

Measurement of nitric oxide (NO)

EOC2 cells were plated at 96-well plates (3×10^4 cells/well) in 100 μl of culture medium and stimulated with 100 ng/ml LPS, human prothrombin, or kringle-1, kringle-1-2, or kringle-2 for 24 h. NO production was determined by measuring the accumulation of nitrite, the stable metabolite of NO, in culture medium. Isolated supernatants were mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2% phosphoric acid) and incubated at room temperature for 10 min. Absorbance at 540 nm was measured with an ELISA reader.

RT-PCR analysis

Total RNA was isolated using the illustra RNAspin Mini RNA isolation kit (GE Healthcare, UK) and reverse transcribed into cDNA using the Reverse Transcription System (Promega, Madison, WI) according to the manufacturer's instructions. The following PCR primers were used: TNF-α, 5'-GACCCTCACACTCAGATCAT-3' (forward primer) and 5'-TTGAAGAGAACCTGGGAGTA-3' (reverse primer); IL-1β, 5'-AGCAATGGTCGGGACA-3' (forward primer) and 5'-TGTGCCACGGTTTTCTT-3' (reverse primer); and GAPDH, 5'-CCAAGGAGTAAGAAACC-3' (forward primer) and 5'-GCAGCGAACTTTATTGA-3' (reverse primer). RT-PCR was carried out using 1 μg cDNA, 5 pmol of each primer, and *HiPi* PCR PreMix (Elpis Biotech, Daejeon, Korea). PCR products were separated by gel electrophoresis on 1.0% agarose gels and visualized by ethidium bromide staining.

Western blot analysis

For Western blot analysis, harvested cells were lysed using cell lysis buffer containing 50 mM Tris (pH 7.5), 1 mM ethylene diamine tetraacetic acid (EDTA), 1 mM ethylene glycol bis

(2-aminoethyl ether)-*N,N,N'N'*-tetraacetic acid (EGTA), 0.5 mM Na$_3$VO$_4$, 0.1% β-mercaptoethanol, 1% Triton X-100, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 10 mM sodium β-glycerophosphate, 0.1 mM phenyl methyl sulfonyl fluoride (PMSF), and 1 μg/ml protease inhibitors (aprotinin, pepstatin, leupeptin). Fifty micrograms of total protein were separated by gel electrophoresis on a 4-12% gradient sodium dodecyl sulfate-polyacrylamide gel, transferred to a nitro-cellulose membrane, and incubated with a primary antibody. After overnight incubation at 4°C, washed membranes were incubated with HRP-conjugated secondary antibodies, and visualized using ECL (Amersham, Piscataway, NJ).

NF-κB luciferase assay

To assess NF-κB luciferase activity, 1.5×10^6 cells were transfected with 2 μg of NF-κB luciferase reporter plasmid (BD Sciences, San Jose, CA) using Lipofectamine (Gibco/BRL, Gaithersburg, MD) according to the manufacturer's instructions. After 24 h incubation, cells were treated with 1 U/ml prothrombin, 10 μg/ml kringle-2, and 50 μM NSA9 for 24 h. Luciferase activity in 100 μl of cell lysate was detected using the Lucifease Assay System kit (Promega, Madison, WI) according to the manufacturer's instructions.

Statistical analysis

Data are presented as the mean ± S.E.M. of at least three separate experiments. Statistical significance was assessed by the Student's *t*-test, and was defined as $P < 0.001$ (*) or $P < 0.005$ (**) compared to the control group.

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