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Anti-inflammatory effect of a human prothrombin fragment-2-derived peptide, NSA9, in EOC2 microglia

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Abstract

Pro-inflammatory mediators, such as nitric oxide (NO), prostaglandin E_2 (PGE_2), and several cytokines (tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6) are responsible for central nervous system (CNS) injuries that include ischemia, Alzheimer's disease, and neural death. Inhibition of these pro-inflammatory mediators would be an effective therapy to reduce the progression of neurodegenerative diseases. In this study, we examined the anti-inflammatory effects of a human prothrombin fragment-2-derived peptide, NSA9 (NSAVQLVEN), on the production of pro-inflammatory mediators in lipopolysaccharide (LPS)-activated brain microglia. NSA9 significantly inhibited the release of NO, PGE_2, and pro-inflammatory cytokines in a dose-dependent manner. Furthermore, NSA9 reduced the expression of inducible NO synthase (iNOS) and cyclooxygenase (COX)-2 mRNA and protein, which control the production of NO and PGE_2, respectively. Moreover, NSA9 suppressed the LPS-induced nuclear translocation and activation of nuclear factor-κB (NF-κB). These results suggest that NSA9 strongly inhibits the pro-inflammatory responses of microglia through the modulation of NF-κB activity.

Keywords: Microglia; Human prothrombin fragment-2-derived peptide; Lipopolysaccharide; Pro-inflammatory mediators; Nuclear factor-κB

Microglia cells are the resident macrophages of the brain. They play a pivotal role in the innate immune response of the central nervous system (CNS) and are the first line of defense against microorganism invasion and injury [1]. When microglia cells are activated in the CNS, they become inflammatory cells, which have been implicated in pathogenesis and neuronal degeneration through production of pro-inflammatory mediators and neurotoxic compounds. These include pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, COX-2, NO, ROS, and PGE_2 [2–5]. Induction of these pro-inflammatory cytokines is associated with the cytotoxic phenomenon of inflammation in neuropathological diseases such as Alzheimer's disease, cerebral ischemia, and multiple sclerosis [6,7]. However, the mechanisms underlying microglia activation in the injured brain have not been clearly established.

Prothrombin, a zymogen of thrombin, is converted to thrombin, fragment-1 and -2 by factor Xa, which in turn induces blood coagulation by catalyzing the cleavage of fibrinogen into fibrin [8]. We previously reported that recombinant human prothrombin kringle-1, -2, and -1-2 all have potent anti-angiogenic activities [9,10]. The recombinant kringle proteins inhibit chorioallantoic membrane (CAM) angiogenesis in chick embryos and reduce Lewis lung carcinoma (LLC) tumor growth and metastases in C57BL6/J mice. In addition, we constructed an overlapping synthetic peptide library representing the entire sequence of human prothrombin kringle-2, and demonstrated that the nona-peptide, NSA9 (NSAVQLVEN), strongly inhibits the proliferation of bovine capillary endothelial (BCE) cells and CAM angiogenesis through endocytosis and energy-dependent pathways [11,12].

According to our recent studies, prothrombin, thrombin, and prothrombin fragment-2 (kringle-2) all could activate brain microglia [13]. This microglial cell activation

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induces the release of NO and the production of iNOS, IL-1β, and TNF-α. In this study, we investigated the effects of a human prothrombin kringle-2-derived peptide, NSA9, on the production of NO, iNOS, PGE_2, COX-2, and pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) in LPS-activated microglia. Our results indicate that NSA9 significantly inhibited the production of inflammatory mediators in LPS-stimulated microglia through the inhibition of NF-κB pathways, suggesting that NSA9 may function as an internal antagonist of the prothrombin fragment-2 domain and thus may act to prevent the activation of microglia.

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 conditioned medium containing colony stimulating factor-1 (CSF-1) derived from LADMAC cells, a bone marrow stromal cell line purchased from ATCC (CRL-2420). The LADMAC cells were maintained in minimum essential medium (MEM) containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. All cells were maintained at 37 °C under a humidified atmosphere of 10% CO_2.

Nitrite assay. EOC2 cells were plated at 3×10^4 cells/well in DMEM containing 10% FBS and 20% LADMAC supernatant and stimulated for 24 h with 100 ng/ml LPS in the presence or absence of NSA9. The production of NO was determined by measuring the accumulation of nitrite, the stable metabolite of NO, in culture medium. The 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. The absorbance at 540 nm was measured with an ELISA reader using a calibration curve with a range of 0–100 μM concentrations of $NaNO_2$. All experiments were performed in triplicate.

Measurement of prostaglandin E_2. PGE_2 levels in LPS-stimulated microglia were determined using a PGE_2 enzyme immunoassay kit (Cayman Chemical Co., Ann Arbor, MI) according to the manufacturer's instructions. In brief, EOC2 cells (1×10^5/well) were seeded into each well of 24-well plates and allowed to adhere during an overnight incubation at 37 °C and 10% CO_2. After incubation, cells were activated with 100 ng/ml LPS in the presence or absence of the indicated concentration of NSA9 (range, 10–100 μM) for 24 h. The released PGE_2 into culture medium was determined by measuring the absorbance at 405 nm using an ELISA reader.

RNA extraction and RT-PCR. For RNA extraction, EOC2 cells were plated onto 6-well dishes at 1×10^6 cells/well. After a 24-h incubation, cells were treated with 100 ng/ml LPS and between 10 and 100 μM of NSA9. Total RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA) and then reverse transcribed into cDNA using reverse transcriptase (Promega, Madison, WI) according to the manufacturer's instructions. Reverse transcription-polymerase chain reaction (RT-PCR) was carried out with 1 μg of cDNA, 5 pmol of each primer (Table 1), and the *HiPi* PCR PreMix (Elpis biotech, Daejeon, Korea). The PCR products were assessed by separation on a 1.0% agarose gel and visualized by ethidium bromide staining.

Western blot analysis. To assess iNOS and COX-2 protein expression, LPS- and NSA9-treated cells were harvested, washed with cold PBS, and resuspended in 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_3VO_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 inhibitor (aprotinin, pepstatin, leupeptin). The lysates were centrifuged for 5 min at 12,000*g* at 4 °C, and the supernatants were collected. Nuclear extracts were collected using the Nuclear Extraction Kit (Chemicon, USA) according to the manufacturer's procedures. Fifty micrograms of total protein or 30 μg of nuclear extract were loaded on a 4–12% gradient sodium dodecyl sulfate–polyacrylamide gel, separated by SDS–PAGE, transferred to a nitrocellulose membrane and incubated with a primary antibody. After overnight incubation at 4 °C, washed membranes were incubated with HRP-conjugated secondary antibodies, and then visualized using an ECL system (Amersham, Piscataway, NJ).

Enzyme-linked immunosorbent assay (ELISA). The amounts of pro-inflammatory cytokines, TNF-α, IL-1β, and IL-6, were measured by ELISA kits according to the manufacturer's procedures (R&D Systems Inc., Minneapolis, MN). The absorbance at 450 nm was determined using an ELISA reader.

NF-κB luciferase assay. To determine NF-κB luciferase activity, 1×10^6 cells were transfected with 2 μg of NF-κB luciferase reporter plasmids (BD Sciences, San Jose, CA) using Lipofectamine according to the manufacturer's procedures (Gibco/BRL, Gaithersburg, MD). After a 24-h incubation, the EOC2 cells were stimulated with 100 ng/ml LPS in the presence or absence of NSA9 and, after 24 h, the cells were washed, lysed, and harvested. The luciferase activity in 100 μl of cell lysates was detected using the Luciferase Assay System kit (Promega, Madison, WI) according to the manufacturer's instructions.

Statistical analysis. Data were presented as means ± SEM of at least three separate experiments. Statistically significant differences were assessed by the Student's *t*-test. In addition, $p < 0.01$ (#) or $p < 0.001$ (##) was compared to the control group, while $p < 0.05$ (*), $p < 0.01$(**), or $p < 0.001$ (***) was compared to the LPS-treated groups.

Table 1
Gene-specific primers for RT-PCR

Gene	Sequence
iNOS	Forward primer 5′-ATGGCTTGCCCCTGGAAGTTTCTC-3′ Reverse primer 5′-TCAGAGCCTCGTGGCTTTGGGCTC-3′
COX-2	Forward primer 5′-ATGCTCTTCCGAGCTGTGCTGCTC-3′ Reverse primer 5′-TTACAGCTCAGTTGAACGCCTTTT-3′
TNF-α	Forward primer 5′-GACCCTCACACTCAGATCAT-3′ Reverse primer 5′-TTGAAGAGAACCTGGGAGTA-3′
IL-1β	Forward primer 5′-AGCAATGGTCGGGACA-3′ Reverse primer 5′-TGTGC-CACGGTTTTCTT-3′
IL-6	Forward primer 5′-ATGAAGTTCCTCTCTGCAAGAGAC-3′ Reverse primer 5′-CTAGGTTTGCCGAGTAGATCTCAA-3′
GAPDH	Forward primer 5′-CCAAGGAGTAAGAAACC-3′ Reverse primer 5′-GCAGCGAACTTTATTGA-3′

Abbreviations: iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; IL-6, interleukin-6; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Results

NSA9 decreases NO and PGE_2 production in LPS-activated microglia in a dose-dependent manner

It is well known that LPS-activated microglia release many cytotoxic compounds such as NO and PGE_2 [14,15]. To test whether NSA9 has the potential to reduce the release of NO or PGE_2 in LPS-activated microglia, EOC2 cells were treated with 100 ng/ml LPS and 10-100 μM NSA9 for 24 h. We treated LPS-activated