Abstract. – BACKGROUND AND OBJECTIVES: Sinomenine is an alkaloid compound and a prominent anti-inflammatory agent found in the root of the climbing plant Sinomenium acutum. However, its effects on the mechanism of human mast cell line (HMC)-1 mediated inflammation remained unknown.

MATERIALS AND METHODS: To provide insight into the biological effects of sinomenine, we examined its influence on the pro-inflammatory cytokine production in HMC-1 cells stimulated by phorbol 12-myristate-13-acetate (PMA) plus A23187 by evaluating the stimulated cells in the presence or absence of sinomenine. In the present study, the pro-inflammatory cytokine production was measured using ELISA, Reverse Transcription-polymerase chain reaction (RT-PCR) and nuclear factor (NF)-κB, mitogen-activated protein kinases (MAPKs) pathway activation, as determined by Western blot analysis. Also, cyclooxygenase (COX)-2 expression was measured through Western blot and RT-PCR analysis.

RESULTS: Sinomenine inhibited the pro-inflammatory cytokine production induced by PMA plus A23187 in a dose-dependent manner. Furthermore, sinomenine inhibited the phosphorylations of extracellular signal-regulated kinase (ERK) and p38 MAPKs as well as the translocation of NF-kappaB p65 through reduced IkappaBalpha degradation. In addition, sinomenine suppressed COX-2 protein and mRNA expression dose-dependently.

CONCLUSIONS: Taken together, the results of this study indicate that the anti-inflammatory effects of sinomenine may occur via the inhibition of pro-inflammatory cytokine and COX-2 production through the inhibition of MAPKs and NF-kappaB pathway activation by PMA plus A23187 stimulation in HMC-1 cells.

Key Words: Sinomenine, Inflammatory cytokine, Cyclooxygenase-2, Mitogen-activated protein kinases, Nuclear factor-kappaB.
such as TNF-α, IL-6 and IL-8 are released in a coordinate network and play an important role in chronic inflammation. The pattern of cytokine expression largely determines the nature and persistence of the inflammatory response.\(^\text{14}\)

Stimulation of mast cells with PMA plus A23187 leads to the phosphorylation of the inhibitor of NF-κB (IκBα). NF-κB activation is involved in the expression of cytokine genes, including TNF-α, IL-6 and IL-8.\(^\text{15-16}\) In the human mast cell line HMC-1, stimulation with PMA plus A23187 is required for the NF-κB-dependent expression of inflammatory cytokines.

We aimed to determine whether sinomenine suppresses the pro-inflammatory cytokine production and COX-2 expression in HMC-1 cells induced by PMA plus A23187, and whether it modulates cytokine production and COX-2 expression in HMC-1 cells stimulated by PMA plus A23187 by interfering with extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinases (MAPKs) phosphorylation, and through interference with nuclear factor κB (NF-κB) activity via IκB phosphorylation.

Materials and Methods

Materials

Sinomenine was purchased from Wako (Osaka, Japan). Iscove’s modified Dulbecco’s medium (IMDM), penicillin and streptomycin were obtained from Hyclone (Logan, UT, USA). Bovine serum albumin, PMA and calcium ionophore A23187 were purchased from Sigma (St. Louis, MO, USA). Antibodies to TNF-α, IL-6 and IL-8, biotinylated antibodies, and recombinant human TNF-α, IL-6 and IL-8 were obtained from BD PharMingen (San Diego, CA, USA). ERK, phosphorylated ERK, p38, phosphorylated p38, COX-2,  β-actin and NF-κB p65 monoclonal antibodies and peroxidase-conjugated secondary antibody were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). An RNA extraction kit was purchased from iNtRON Biotech (Daejeon, Republic of Korea). TNF-α, IL-6, IL-8 and COX-2 oligonucleotide primers were purchased from Pioneer Corp. (Daejeon, Republic of Korea).

Cell Culture

The HMC-1 cells were grown in IMDM supplemented with 100 units/mL of penicillin, 100 µg/mL of streptomycin, and 10% fetal bovine serum (FBS) at 37°C in 5% CO₂ with 95% humidity. HMC-1 cells were treated with sinomenine (10 µM and 20 µM) for 1 hour. The cells were then stimulated with 50 nM of PMA plus 1 µM of A23187 and incubated at 37°C for the indicated time periods.

MMT Assay for Cell Viability

The cell viability was examined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Thiazolyl Blue Tetrazolium Bromide) assay. Briefly, HMC-1 cells were seeded at a density of 5 · 10⁵ cells/mL in 96-well plates (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, USA). Each batch of cells included a nontreated group as a control. Various concentrations of sinomenine were then added to each well, after which the plates were incubated for 48 h at 37°C under 5% CO₂. Next, MTT solutions (5 mg/mL) were added to each well, and the samples were cultured for an additional 4 h. The supernatant was then discarded and 100 µL of dimethyl sulfoxide (DMSO) were added to each well. Next, the optical density was read at 590 nm. The cytotoxicity was calculated by subtracting the ratio of the mean absorbance value for treated cells over the mean absorbance value for untreated cells from one.

Enzyme-Linked Immunosorbent Assay (ELISA)

The HMC-1 cells were pretreated with various concentrations of sinomenine (10 µM and 20 µM) for 1 hours before PMA plus A23187 stimulation. An enzyme-linked immunosorbent assay (ELISA) was used to assay the culture supernatants for TNF-α, IL-6, and IL-8 levels. To measure the cytokines, we used a modified ELISA method. First, we conducted a sandwich ELISA for TNF-α, IL-6, and IL-8 in duplicate in 96-well ELISA plates (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, USA). Then the supernatant was decanted into a new microcentrifuge tube, and quantitation of cytokines conducted by ELISA. ELISA plates were coated overnight at 4°C with antihuman TNF-α, IL-6, and IL-8 monoclonal antibodies antibody diluted in coating buffer (0.1 M carbonate, pH 9.5) and then washed four times with phosphate buffered saline (PBS) containing 0.05% Tween 20. The
nonspecific protein binding sites were blocked with assay diluent (PBS containing 10% FBS, pH 7.0) for at least 1 h. After washing the plates again, the test sample or recombinant TNF-α, IL-6, and IL-8 standards was added. After incubation for 2 h, a working detector (biotinylated anti-human TNF-α, IL-6, and IL-8 monoclonal antibodies and streptavidin-horseradish peroxidase reagent) was added and the mixture incubated for 1 h. Accordingly, substrate solution (tetramethylbenzidine) was added to the wells and incubated for 30 min in the dark before the reaction was stopped by the addition of 1 M H₃PO₄. The absorbance was read at 450 nm using ELISA reader (infinite M200, TECAN, Männedorf, Switzerland). All subsequent steps were conducted at room temperature, and all standards and samples were assayed in duplicate.

**RNA Extraction and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)**

Total cellular RNA was isolated using an easy-BLUE™ RNA extraction kit (iNtRON Biotech, Gyeonggi-do, Korea) according to the manufacturer’s instructions. The total RNA (2 μg) was then converted to cDNA by treating it with 200 units of reverse transcriptase and 500 ng of oligo (dT) primer in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol, and 1 mM deoxynucleotide triphosphates at 42°C for 1 h. The reaction was stopped by heating the samples at 70°C for 15 min, after which the cDNA mixture (3 μL) was used for enzymatic amplification. PCR was conducted in a reaction mixture that contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 0.2 mM deoxynucleotide triphosphates, 2.5 units of Taq DNA polymerase, and 0.1 μM each of TNF-α, IL-6, IL-8, COX-2, and β-actin primers. PCR of TNF-α and β-actin was conducted by subjecting the reaction mixtures to initial denaturation at 92°C for 5 min, followed by 30 cycles of 92°C for 1 min, 50°C for 1 min, and 72°C for 2 min, with a final extension at 72°C for 5 min17. PCR of IL-6 and IL-8 samples was conducted by heating the reaction mixtures to 95°C for 2 min, after which they were subjected to 35 cycles of 95°C for 45 s, 60°C for 45 s and 72°C for 90 s, with a final extension at 72°C for 10 min18. PCR of COX-2 samples was conducted by heating the reaction mixtures to 94°C for 5 min, after which they were subjected to 30 cycles of 94°C for 1 min, 55°C for 30 s and 72°C for 1 min, with a final extension at 72°C for 7 min19. The PCR products were then electrophoresed on 1% agarose gel and stained with ethidium bromide. The primer sequences are listed in Table I.

**Preparation of Cytoplasmic and Nuclear Extracts**

Nuclear and cytoplasmic extracts were prepared as described elsewhere20. Briefly, after activating the cells for the time periods indicated, 5 · 10⁶ cells/mL were washed with ice-cold PBS and centrifuged at 15,000 × g for 1 min. The cells were resuspended in 40 μL of a cold hypotonic buffer [10 mM Hepes/KOH, 2 mM MgCl₂, 0.1 mM EDTA, 10 mM KCl, 1 mM DTT, and 0.5 mM phenylmethylsulfonylfluoride (PMSF), pH 7.9]. We then allowed the cells to swell on ice for 15 min, followed by gentle lysis with 2.5 μL of 10% Nonide P (NP)-40 and centrifugation at 15,000 × g for 3 min at 4°C. The supernatant was collected and used as the cytoplasmic extract. The nuclear pellets were gently resuspended in 40 μL of cold saline buffer [50 mM HEPES/KOH, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, and 0.5 mM PMSF, pH 7.9]

**Table I. Primers used for RT-PCR.**

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer sequence</th>
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| TNF-α       | F: 5’-CGAGTGACAAGCTGTAGCC-3’  
R: 5’-CTACCGACCAAGGCCTCAAC-3’ |
| IL-6        | F: 5’-ATGAACTCTTTTCTCCACAAGGC-3’  
R: 5’-GAAGAGCGCCCTCTACGCTATG-3’ |
| IL-8        | F: 5’-ATGACTTCCAGCTGCGCCGCT-3’  
R: 5’-TTCACGCCCCTCTCCAAAACCTC-3’ |
| COX-2       | F: 5’-TTCAAAATGAGATTGGGAAAATGGTC-3’  
R: 5’-AGATCATCTCTGCGTACGTTC-3’ |
| β-actin     | F: 5’-CTGGCACCACGCAATGGAAG-3’  
R: 5’-ACCGACTGTGCACCTCTC-3’ |

F: forward; R: reverse.
Anti-inflammatory effect of sinomenine

and left on ice for 20 min. After centrifugation (15,000 \times g for 15 min at 4°C), the aliquots of supernatant containing nuclear proteins were frozen in liquid nitrogen and stored at −70°C until analysis. The bicinchoninic acid protein assay (Sigma Chemical Co., St Louis, MO, USA) was used for protein quantitation.

**Western Blot Analysis**

Sinomenine-pretreated HMC-1 cells (5 \cdot 10^6 cells/mL) were stimulated with PMA (50 nM) plus A23187 (1 µM). The cells were then rinsed twice with ice-cold PBS. The samples were centrifuged, after which the cell pellets were lysed in 200 µL of lysis buffer (iNtRON Biotech) for 20 min on ice. The lysates were centrifuged at 15,000 g for 5 min, after which the supernatants were immediately aliquoted and stored at −20°C until use. The protein concentrations were determined using the bicinchoninic acid protein assay (Sigma), after which 20 µg of protein from each sample was separated by 10% sodium dodecyl sulphate-polyacrylamide gels (SDS-PAGE) and then transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was then blocked with 3% BSA (bovine serum albumin) in Tween tablets/Tris buffer (TBS/T) buffer (150 mM NaCl, 20 mM Tris- HCl, 0.05% Tween 20, pH 7.4). After blocking, the membrane was incubated with primary antibodies for 18 h. The membrane was then washed with TBS/T and incubated with antimouse or antirabbit IgG horseradish peroxidase conjugated secondary antibodies. The immunoreactivity was then detected using enhanced chemiluminescence (ECL; Amersham, Milan, Italy).

**Statistical Analysis**

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Dunnett’s t-test for multiple comparisons, and the Student’s t-test for single comparisons. The data from the experiments are presented as means ± SEM. The numbers of independent experiments assessed are given in the figure legends.

**Results**

**Effects of Sinomenine on Pro-Inflammatory Cytokine Production**

Initially, we examined the cytotoxicity of sinomenine on HMC-1 cells using the MTT assay. The half maximal inhibitory concentration (IC_{50}) value of sinomenine was 52.73 µM and sinomenine did not show any cytotoxic effects up to 20 µM (data not shown). To evaluate the effect of sinomenine on the production of pro-inflammatory cytokines, we pretreated cells with sinomenine (10 and 20 µM) before stimulation with PMA (50 nM) plus A23187 (1 µM) for 8 h, and analysis using ELISA. As shown in Figure 1, the levels of TNF-α, IL-6, and IL-8 were considerably increased in HMC-1 cells after stimulation with PMA plus A23187. Pretreatment of cells with sinomenine (10 or 20 µM) inhibited these increases in a concentration-dependent and statistically significant manner.

**Effects of Sinomenine on Pro-Inflammatory Cytokine Gene Expression**

The pro-inflammatory cytokine gene expression was then analysed using RT-PCR. Enhanced TNF-α, IL-6, and IL-8 mRNA expression induced by PMA plus A23187 was inhibited by pretreatment of the cells with sinomenine (Figure 2). In particular, pretreatment with sinomenine at a concentration of 20 µM inhibited the PMA plus A23187-induced gene expression of TNF-α, IL-6 and IL-8.

**Effects of Sinomenine on COX-2 Protein and COX-2 mRNA expression**

In recent studies, the important roles of COX-2 in mast cell-mediated inflammation have been demonstrated. Thus, to determine the effects of sinomenine on COX-2 protein and COX-2 mRNA expression induced by PMA plus A23187, Western blot and RT-PCR analyses were conducted. In unstimulated HMC-1 cells, COX-2 protein and mRNA were undetectable. However, COX-2 protein and mRNA were strongly expressed in cells that were treated with PMA plus A23187, while sinomenine significantly inhibited the PMA plus A23187-induced COX-2 expression in a dose-dependent manner. RT-PCR analysis showed that the COX-2 mRNA expressions were consistent with their protein levels (Figure 3).

**Effects of Sinomenine on Activation of MAPKs**

Inhibition of the ERK1/2 and p38 MAPK pathways was recently found to attenuate pro-inflammatory cytokine secretion[34]. To examine the effect of sinomenine on PMA plus A23187-induced MAPK phosphorylation in HMC-1 cells, cells were incubated with sinomenine 30 min prior to stimulation with PMA plus A23187 for 30 min. PMA plus A23187-induced phosphorylation
of ERK1/2 and p38 MAPK was then determined by Western blot analysis. Sinomenine pretreatment significantly inhibited PMA plus A23187-induced phosphorylation of ERK1/2 and p38 MAPK in a dose-dependent manner without affecting the total protein levels of these kinases (Figure 4). These results indicate that the inhibitory effect of sinomenine on PMA plus A23187-induced MAPK phosphorylation may result in a blockage of the cytokine production and COX-2 expression in HMC-1 cells.

**Effect of Sinomenine on Phosphorylation of IkBα and Activation of NF-κB**

Stimulation of cells with inducers such as PMA plus A23187 leads to rapid phosphorylation, ubiquitination, and degradation of IkBα. This results in the release and translocation of NF-κB to the
nucleus, where it activates the expression of target genes. NF-κB activation is important for the transactivation function. Therefore, we examined the effects of sinomenine on PMA plus A23187-induced NF-κB activation in HMC-1 cells. To accomplish this, sinomenine-pretreated HMC-1 cells were stimulated with PMA plus A23187 for 1 h. Cell lysates were then prepared to analyse the phosphorylation of IκBα by Western blot analysis using an antiphospho-IκBα antibody. Phosphorylation of IκBα protein following PMA plus A23187 treatment was inhibited by sinomenine in a dose-dependent manner (Figure 5). The activation of NF-κB is necessary for the induction of the COX-2 genes. Since p65 is the major component of NF-κB activated by PMA plus A23187 in HMC-1 cells, we investigated the protein levels of p65 in the nuclear extracts by Western blot analysis. To accomplish this, HMC-1 cells were incubated with PMA plus A23187 in the presence or absence of sinomenine for 1 h. The results revealed that the PMA plus A23187-induced nuclear translocation of p65 was inhibited in a dose-dependent manner (Figure 5).

**Discussion**

Many recent studies on plant-derived anti-inflammatory compounds have investigated the potential inhibitory effects of natural products using *in vivo* and *in vitro* systems. *Sinomenium acutum* is an active component in oriental medicine and has been used since ancient times to cure various diseases such as stroke, Bell’s palsy, and edema. Sinomenine is the key bio-active compound found in *Sinomenium acutum*.

COX-2 is only expressed in response to inflammatory signals such as cytokines and stimuli. In addition, cytokines stimulate the expression of COX-2. In this study, we demonstrated that sinomenine inhibited the PMA plus A23187-induced production of COX-2 protein and mRNA in a dose-dependent manner, as shown by Western blot and RT-PCR analysis, respectively.
These results imply that sinomenine exerted its effects by inhibiting COX-2 transcription.

Furthermore, we investigated the inhibitory effect of sinomenine against PMA plus A23187-induced pro-inflammatory cytokine production in HMC-1 cells by examining its ability to inhibit the MAPK pathway and phosphorylate IkBα, which subsequently leads to the inhibition of the NF-kB signaling pathway. It has been reported that stimulating HMC-1 cells with PMA plus A23187 induces cytokine secretion via phosphorylation of MAPKs1,24,25. In this study, we demonstrated that pre-treating samples with sinomenine resulted in inhibition of PMA plus A23187-induced NF-κB phosphorylation and NF-κB activation. These findings suggest that sinomenine specifically blocks the MAPK signaling pathways.

NF-κB is a protein complex that acts as a transcription factor. NF-κB is found in many animal cell types and is involved in cellular responses to stimuli such as stress, cytokines, free radicals, ultraviolet irradiation, oxidized LDL, and bacterial or viral antigens26-30. In addition, it is well known that NF-κB plays an important role in the regulation of cell survival and the expression of pro-inflammatory cytokines31-35. NF-κB is essentially composed of p50 and p65 proteins36. In an inactivated cell, NF-κB is present in the cytosol and is linked to the IκBα inhibitory protein. The phosphorylation and degradation of the IκBα protein is followed by nuclear translocation and activation of NF-κB37. In the present study, we demonstrated that pre-treating HMC-1 cells with sinomenine resulted in inhibition of PMA plus A23187-induced IκBα phosphorylation and NF-κB activation. These results suggest that sinomenine suppresses activation of the NF-κB signaling pathway, which results in the regulation of inflammatory cytokines expression.

Conclusions

Sinomenine, an alkaloid, exerts inhibitory effects on various inflammatory mediators expression by PMA plus A23187 stimulation in HMC-1 cells. These effects occurred in response to the inhibition of ERK1/2 and p38 MAPKs phosphorylation, as well as through the phosphorylation of IκBα and subsequent regulation of the NF-κB pathway. Based on these results, sinomenine could represent a new drug treatment for various inflammatory diseases. Therefore, further studies should be conducted to examine its potential in clinical applications.

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