

RESEARCH ARTICLE

Anti-allergic effects of sinomenine by inhibition of prostaglandin D₂ and leukotriene C₄ in mouse bone marrow-derived mast cells

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Abstract

Sinomenine is an alkaloid compound and a prominent anti-allergic agent found in the root of the climbing plant *Sinomenium acutum*. However, its effects on the bone marrow-derived mast cell (BMMC) mediated allergy and inflammation mechanism remain unknown. In this study, the biological effects of sinomenine were evaluated while focusing on its effects on the allergic mediator in PMA plus A23187-stimulated BMDCs. An investigation was also conducted to determine its effects on the production of several allergic mediators including interleukin-6 (IL-6), prostaglandin D₂ (PGD₂), leukotriene C₄ (LTC₄), β -Hexosaminidase (β -Hex), and cyclooxygenase-2 (COX-2) protein. The results revealed that sinomenine inhibited the PMA plus A23187-induced production of IL-6, PGD₂, LTC₄, β -Hex, and COX-2 protein. Taken together, these findings indicate that sinomenine has the potential for use in the treatment of allergy.

Keywords: Sinomenine, bone marrow-derived mast cell, allergy, prostaglandin D₂, leukotriene C₄

Introduction

Sinomenine (C₁₉H₂₃NO₄) is an alkaloid found in the root of the climbing plant *Sinomenium acutum*, which is native to eastern Asia. *Sinomenium acutum* is traditionally used in herbal medicine in these countries as a treatment for rheumatism and arthritis.⁽¹⁾ Recent studies have demonstrated that sinomenine has health benefits such as anti-rheumatic effects.⁽²⁾ However, the mechanism by which these health benefits occur is not fully understood.

Mast cells are one of the most important cells involved in disease related to allergic responses. When activated, mast cells release a number of biologically active molecules through various processes. Histamine, serotonin,

and serine proteases are released through exocytosis, whereas lipid mediators such as eicosanoids are released through activation of the cyclooxygenase-2 (COX-2) and 5-lipoxygenase pathways and various cytokines are synthesized *de novo*.^(3–5)

Interleukin-6 (IL-6) is an interleukin that acts as both a pro-inflammatory and an anti-inflammatory cytokine. IL-6 is secreted by T cells and macrophages to stimulate immune response to trauma, especially burns or other tissue damage leading to inflammation.^(6,7) Further, IL-6 modulates a variety of physiological events that occur in the nervous system, endocrine system, and bone metabolism.^(7,8)

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COX is a bifunctional enzyme that first catalyzes the addition of two molecules of oxygen to arachidonic acid to form the hydroperoxide prostaglandin G₂ (PGG₂), and then reduces the hydroperoxide to the alcohol, PGH₂, via peroxidase activity.⁽⁹⁾ COX has two isoforms, COX-1 and COX-2. COX-1 is constitutively expressed in most cells and is responsible for the production of prostaglandins that maintain homeostasis; however, COX-2 is upregulated in inflammatory cells in response to an inflammatory stimulus and is responsible for the production of prostaglandins at the site of inflammation.^(10–13) Therefore, there is an increasing interest in the usefulness of COX-2 inhibitors.

In the present study, sinomenine was investigated to determine if it suppresses PMA plus A23187-induced IL-6, PGD₂, and leukotriene C₄ (LTC₄) production and COX-2 protein expression in bone marrow-derived mast cells (BMMCs). The results show that sinomenine has anti-allergic activity.

Materials and methods

Animals

Male Balb/cJ mice (20–30 g) were purchased from DAMUL (Korea). All animals were kept in a temperature controlled room under a 12 h light 12 h dark cycle. The animals were provided with free access to commercial solid food (SCF Co., Ltd. Korea) and water *ad libitum*. Mice were acclimatized for at least 1 week prior to beginning the experiments.

Drugs and chemicals

Sinomenine was purchased from Wako (Osaka, Japan). RPMI 1640, penicillin, streptomycin, and FBS (fetal bovine serum) were obtained from Hyclone (Hyclone Labs Logan, UT). Bovine serum albumin (BSA), PMA, and A23187 were purchased from Sigma (St. Louis, MO). COX-2 and β -actin monoclonal antibodies and peroxidase conjugated secondary antibody were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

Preparation and activation of BMMC

BMMCs from male Balb/cJ mice were cultured for up to 10 weeks in 50% enriched medium (RPMI 1640 containing 2 mM L-glutamine, 0.1 mM nonessential amino acids, antibiotics, and 10% FBS) and 50% WEHI-3 cell-conditioned medium as a source of IL-3. After 3 weeks, >98% of the cells were BMMCs.

MTS assay for cell viability

The cell viability was examined by a 3-(4,5-dimethylthiazol-2-yl) -5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. Briefly, BMMCs were seeded at a density of 5×10^5 cells/mL in 96-well plates (Nunc, Denmark). Each batch of cells included a non-treated group as control. Sinomenine (10 or 20 μ M) was then added to each well, after which the plates were incubated for 24 h at 37°C under 5% CO₂.

Next, MTS solutions (5 mg/mL) were added to each well and the optical density was read at 490 nm. The cytotoxicity was then calculated by subtracting the ratio of the mean absorbance value for treated cells over the mean absorbance value for untreated cells from one.

ELISA

Cells were seeded at a density of 1×10^6 cells/mL in 24 well tissue culture plates and then pre-treated with various concentrations of sinomenine (10 or 20 μ M) for 30 min prior to PMA (50 nM) plus A23187 (1 μ M) stimulation. Enzyme-linked immunosorbent assay (ELISA) plates (Falcon, Becton Dickinson Labware) were then coated overnight at 4°C with anti-mouse IL-6 antibody diluted in coating buffer (0.1 M carbonate, pH 9.5) and then washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween-20. Non-specific protein binding sites were then blocked by incubating the plates with assay diluent (PBS containing 10% FBS, pH 7.0) for at least 1 h. Immediately following the incubation, each sample and IL-6 standard was added to the wells, and the plates were then incubated for 2 h. Next, working detector (biotinylated anti-mouse IL-6 monoclonal antibody and streptavidin-HRP reagent) was added to each well and the plates were incubated for an additional 1 h. Substrate solution (tetramethylbenzidine) was then added to the wells, after which the plates were incubated in the dark for 30 min. Next, the reaction was stopped using stop solution (2NH₃PO₄) and the absorbance at 450 nm was measured. All standards and samples were assayed in duplicate.

Determination of PGD₂ levels

To measure the inhibitory activity of sinomenine on COX-2, cells were suspended in enriched medium at 1×10^6 cells/mL and preincubated with aspirin (10 g/mL) for 2 h to irreversibly inactivate any pre-existing COX-1. After washing, the BMMCs were activated with PMA (50 nM) plus A23187 (1 μ M) at 37°C for 2 h in the presence or absence of sinomenine dissolved in PBS. All reactions were quenched by centrifugation at 890 g at 4°C for 5 min. The supernatant and cell pellet were frozen immediately in liquid N₂ and stored at –80°C until needed for further analysis. Under these conditions, the COX-2-dependent phase of PGD₂ generation reached 1.3 ng/10⁶ cells. The data are reported as the arithmetic mean of triplicate determinations.

Determination of LTC₄ levels

BMMCs suspended in enriched medium at 1×10^6 cells/mL were pre-treated with sinomenine for 30 min at 37°C and then stimulated with PMA (50 nM) plus A23187 (1 μ M). After 15 min of stimulation, the supernatants were isolated for further analysis by enzyme immunoassay (EIA). LTC₄ was determined using an EIA kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions. Under the conditions employed, LTC₄ reached 3.3 ng/10⁶ cells. All data are the arithmetic means of triplicate determinations.

Western blot analysis

After activation with PMA (50 nM) plus A23187 (1 μ M), BMMCs were washed once with 10 mM phosphate buffer (pH 7.4) containing 150 mM NaCl (PBS) and then lysed in PBS containing 0.1% sodium dodecyl sulfate (SDS) and 10 mM β -mercaptoethanol at 1×10^7 cells/mL. The lysate (1×10^5 cells equivalent) was then applied to 10% SDS-polyacrylamide gels. After running the gel, the protein bands were blotted onto nitrocellulose membranes (Schleicher and Schull, Dassel, Germany) using a semi-dry blotter (MilliBlot-SDE system, Millipore, Bedford, MA) according to the manufacturer's instructions. The membranes were then washed once with 10 mM Tris-buffered saline (TBS, pH 7.2) containing 0.1% Tween-20 (TBS-T), after which they were blocked for 1 h in TBS-T containing 3% skim milk. After washing the membranes with TBS-T, an antibody directed against COX-2 was added at a dilution of 1: 5000 in TBS-T. After incubation for 2 h followed by washing three times, the membranes were treated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Santa Cruz, CA) (diluted 1: 7000) in TBS-T. Finally, the protein bands were visualized using an enhanced chemiluminescence (ECL) system (Amersham Corp., Newark, NJ).

Assay of β -Hex release

β -Hexosaminidase (β -Hex), a marker of mast cell degranulation, was quantified by spectrophotometric analysis of *p*-nitrophenyl-2-acetamido-2-deoxy- β -D-glucopyranoside hydrolysis (Sigma). Briefly, after harvesting the supernatant, cells were lysed in the same volume of medium by three freeze/thaw cycles. Ten milliliters of the BMMC lysate or supernatant samples were then mixed with 50 μ L of β -Hex substrate solution (1.3 mg/mL *p*-nitrophenyl-2-acetamido-2-deoxy- β -D-glucopyranoside in 100 mM sodium citrate, pH 4.5) in the wells of a 96-well plate, after which the samples were incubated at 37°C for 90 min. The reaction was stopped by the addition of 140 μ L of 0.2 M glycine-NaOH (pH 10.7) and the absorbance was then measured at 410 nm using a microplate reader. The percentage of β -Hex released into the supernatant was calculated by the following formula: $(S/(S+P)) \times 100$, where S and P are the β -Hex contents of the supernatant and the cell pellet, respectively.

Statistical analysis

The data from the experiments are presented as the mean $\pm$ SEM. The level of statistical significance was determined by analysis of variance followed by Dunnett's *t*-test for multiple comparisons. *P* values < 0.05 were considered to be significant.

Results

The effects of sinomenine on PMA plus A23187-induced IL-6 secretion

To evaluate the effects of sinomenine on the production of IL-6, we pre-treated cells with sinomenine (10

or 20 μ M) before stimulation with PMA (50 nM) plus A23187 (1 μ M) for 6 h, after which the samples were analyzed by ELISA. As shown in Figure 1, the level of IL-6 was considerably higher after stimulation with PMA plus A23187 in BMMCs. Pre-treatment of cells with sinomenine (10 or 20 μ M) inhibited these increases in a concentration-dependent and statistically significant manner. The cytotoxic effects of sinomenine were evaluated in BMMCs by MTS assay. Treatment with sinomenine at concentrations of up to 20 μ M did not affect the viability of BMMCs (Figure 2).

The effects of sinomenine on PMA plus A23187-induced PGD₂ production

After stimulation with PMA plus A23187, cell-cultured media were harvested to measure the level of PGD₂ production. PGD₂ production was clearly detected 2 h after stimulation with PMA plus A23187. PGD₂ production also peaked in PMA (50 nM) plus A23187 (1 μ M) treated samples. Next, an investigation of the inhibitory effect of sinomenine on the PMA plus A23187-induced PGD₂ production in BMMCs was performed. Sinomenine

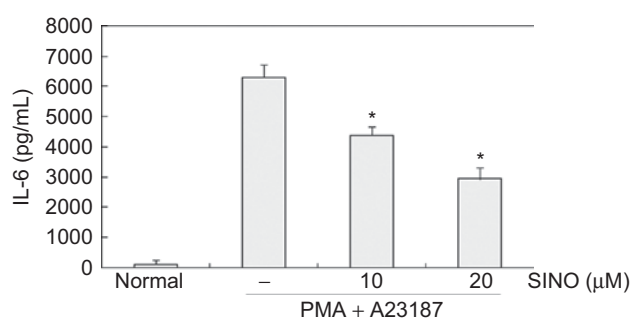


Figure 1. Effects of sinomenine on the secretion of IL-6 in PMA plus A23187-stimulated BMMCs. The cells (1×10^6 cells/mL) were pre-treated with sinomenine (10 or 20 μ M) 30 min prior to stimulation with PMA (50 nM) plus A23187 (1 μ M). Six hours after PMA plus A23187 stimulation, the IL-6 level of the supernatants was measured by ELISA. Statistical significance: **P* < 0.05, when compared to the PMA plus A23187 treated group. Significant differences between treated groups were determined using the Dunnett's *t*-test. Values shown are the mean $\pm$ SE of duplicate determinations from three separate experiments.

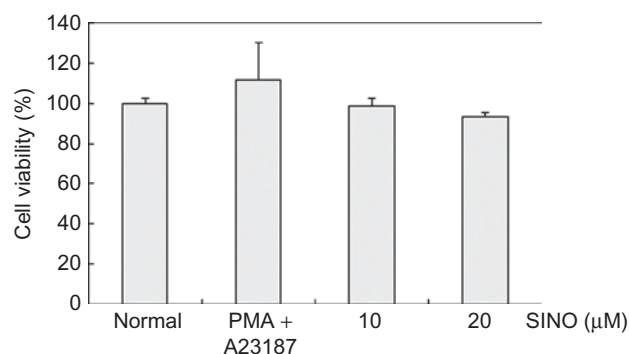


Figure 2. The cell viability of BMMCs following incubation with different doses (10 or 20 μ M) of sinomenine for 24 h as determined by an MTS assay.

significantly inhibited PMA plus A23187-induced PGD₂ production in a dose-dependent manner (Figure 3). These results show that sinomenine could inhibit PMA plus A23187-induced PGD₂ production in BMMCs.

The effects of sinomenine on PMA plus A23187-induced LTC₄ production

After stimulation with PMA plus A23187, cell-cultured media were harvested to measure the level of LTC₄ production. LTC₄ production was clearly detected 15 min after stimulation with PMA plus A23187. LTC₄ production also peaked in PMA (50 nM) plus A23187 (1 µM) treated samples. Next, an investigation of the inhibitory effects of sinomenine on the PMA plus A23187-induced LTC₄ production by BMMCs was conducted. Sinomenine sig-

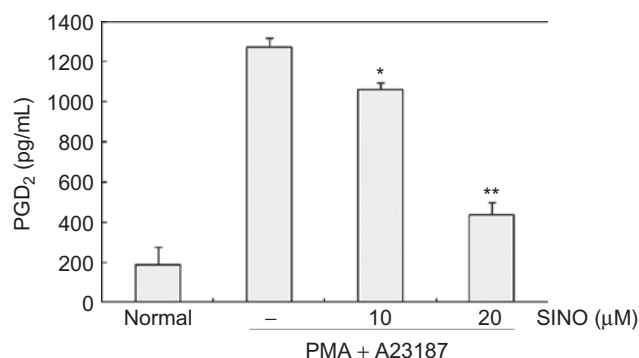


Figure 3. Effects of sinomenine on the production of PGD₂ in PMA plus A23187-stimulated BMMCs. The cells (1×10^6 cells/mL) were pre-treated with sinomenine (10 or 20 µM) 30 min prior to stimulation with PMA (50 nM) plus A23187 (1 µM). Two hours after PMA plus A23187 stimulation, the PGD₂ levels of the supernatants were measured by ELISA. Statistical significance: * $P < 0.05$ and ** $P < 0.005$, as compared to the PMA plus A23187 treated group. Significant differences between treated groups were determined using the Dunnett's *t*-test. Values shown are the mean $\pm$ SE of duplicate determinations from three separate experiments.

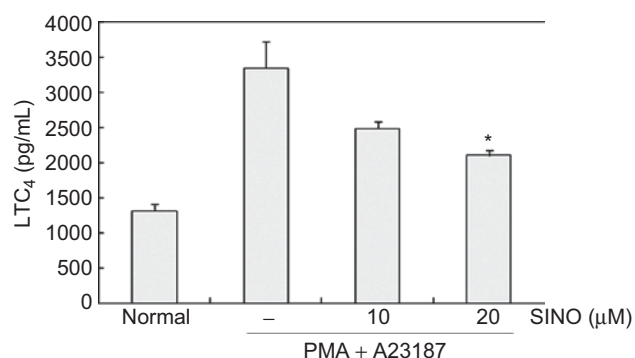


Figure 4. Effects of sinomenine on the production of LTC₄ in PMA plus A23187-stimulated BMMCs. The cells (1×10^6 cells/mL) were pre-treated with sinomenine (10 or 20 µM) 30 min prior to stimulation with PMA (50 nM) plus A23187 (1 µM). At 15 min after PMA plus A23187 stimulation, the LTC₄ levels of the supernatants were measured by ELISA. Statistical significance: * $P < 0.05$, as compared to the PMA plus A23187 treated group. Significant differences between treated groups were determined using the Dunnett's *t*-test. Values shown are the mean $\pm$ SE of duplicate determinations from three separate experiments.

nificantly inhibited the PMA plus A23187-induced LTC₄ production in a dose-dependent manner (Figure 4). These results show that sinomenine could inhibit PMA plus A23187-induced LTC₄ production in BMMCs.

The effects of sinomenine on PMA plus A23187-induced COX-2 protein expression

Western blot analysis was performed to determine if the inhibitory effects of sinomenine on PGs were related to modulation of the COX-2 expression. In unstimulated BMMCs, COX-2 protein was undetectable. However, COX-2 protein was strongly expressed in response to PMA plus A23187, and sinomenine significantly inhibited COX-2 expression (Figure 5).

The inhibitory effects of sinomenine on PMA plus A23187-induced degranulation

When mast cells are activated by various stimuli, the release of histamine occurs in a fashion similar to that of β -Hex, which is a degranulation marker. An investigation of the effects of sinomenine on the PMA plus A23187-induced β -Hex release using ELISA was performed. Pre-treatment of BMMCs with sinomenine at concentrations of 10 or 20 µM reduced β -Hex production (Figure 6).

Discussion

Allergy is a disorder of the immune system that is also often referred to as atopy. Allergic reactions occur in response to normally harmless environmental substances known as allergens, and these reactions are acquired, predictable, and rapid. Allergy occurs in one of four forms of hypersensitivity and is known as type I hypersensitivity. Allergy is characterized by excessive activation of mast cells and basophils by a type of antibody known as IgE, which results in an extreme inflammatory response.⁽¹⁴⁾

Mast cells play an important role in the induction and expression of allergic responses and have long been implicated in the pathology and mortality of anaphylaxis and other allergic disorders by virtue of their ability to be activated through Fc ϵ RI-bound antigen-specific IgE and

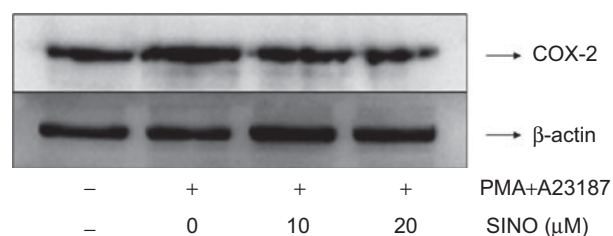


Figure 5. Effects of sinomenine on PMA plus A23187-induced COX-2 protein expressions in BMMCs. BMMCs were pre-treated with the indicated concentrations of sinomenine for 30 min prior to being incubated with PMA (50 nM) plus A23187 (1 µM) for 6 h. Equal amounts of protein (20 µg) were then separated by SDS-polyacrylamide gel electrophoresis and immunoblotted with COX-2 antibodies. Equal loading of protein was verified by β -actin.

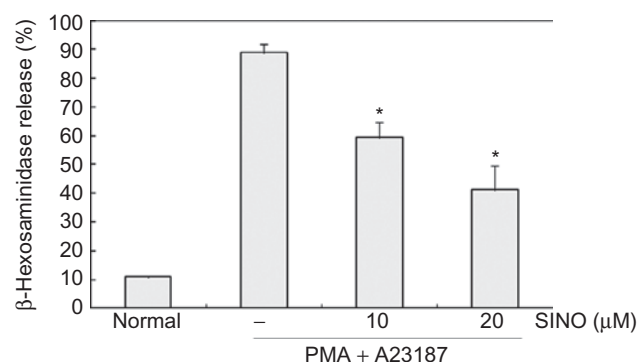


Figure 6. Effect of sinomenine on the release of β -hexosaminidase in PMA plus A23187-stimulated BMMCs. BMMCs (1×10^6 cells/mL) were pre-treated with sinomenine (10 or 20 μ M) 30 min prior to stimulation with PMA (50 nM) plus A23187 (1 μ M). At 90 min after PMA plus A23187 stimulation, the β -Hex released into the supernatant cell lysate was measured by ELISA. The procedure is described in detail in the Materials and methods section. All data are the arithmetic mean of triplicate determinations. Statistical significance: * $P < 0.05$, as compared to the PMA plus A23187 treated group. Significant differences between treated groups were determined using the Dunnett's t -test. Values shown are the mean $\pm$ SE of duplicate determinations from three separate experiments.

their concentration at surfaces that interface with the external environment.⁽³⁾ Mast cells may also be activated by various cytokines through each cytokine receptor. Activation through any of these receptors leads to the release of a number of biologically active molecules, including histamine, serotonin, proteoglycans, and neutral proteases. Among these molecules, histamine is one of the most important chemical mediators in the pathologic allergic reaction.⁽¹⁵⁾

The inhibitory effects of sinomenine on PGD_2 production were examined to determine if they occur via a direct effect of the COX-2 protein or are mediated by some other mechanism. As shown in Figure 5, the COX-2 protein was not detected in unstimulated BMMC, whereas treatment with a combination of PMA plus A23187 strongly induced the formation of detectable COX-2 protein and COX-2 protein expression was inhibited in a dose-dependent manner by sinomenine.

Arachidonic acid can also be converted to leukotrienes by the action of 5-LOX in BMMCs. The inhibition of 5-LOX is believed to be the ideal treatment for allergic diseases and asthma.⁽¹⁶⁾ Therefore, the inhibitory activity of sinomenine on the generation of LTC_4 in BMMCs was examined. As shown in Figure 4, the BMMCs stimulated with PMA plus A23187 for 15 min produced approximately 3.3 ng/mL LTC_4 , and preincubation of the BMMC with sinomenine resulted in the dose-dependent suppression of this LTC_4 biosynthesis.

The results of these investigations indicate that sinomenine can exhibit inhibitory effects on PMA plus A23187-induced IL-6, PGD_2 , and LTC_4 produc-

tion and COX-2 protein expression in BMMCs. Based on these findings, sinomenine could represent a new drug treatment for various allergic diseases. Therefore, further studies are necessary to examine its clinical application.

Declaration of interest

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