**Typha orientalis** inhibits inflammatory cytokine expression through suppression of ERK phosphorylation in HMC-1 cells

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SUMMARY

*Typha orientalis*’ stem (TOS) is traditionally used as an herbal medicine for difficulty in urination, galactophoritis purulenta, whooping cough, and allergic dermatitis. However, its effect in experimental models remains unknown. Here, we report the effect of TOS on the phorbol 12-myristate 13-acetate (PMA) plus calcium ionophore A23187-induced inflammatory cytokine production and extracellular signal-regulated kinase (ERK) activation in the human mast cell line, HMC-1. TOS inhibited PMA plus A23187-induced cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-6. Maximal inhibition rate of TNF-α and IL-6 production by TOS (1 mg/ml) was about 44.02%, and 45.20%, respectively ($P < 0.05$). In addition, TOS inhibited the expression of TNF-α and IL-6 mRNA under the same condition. Moreover, TOS partially blocked PMA plus A23187-induced ERK phosphorylation. These results suggested TOS could inhibit the cytokine production through blocking of ERK activity.

Key words: Human mast cell; *Typha orientalis*’ stem; Phorbol 12-myristate 13-acetate; A23187; Tumor necrosis factor-α; Interleukin-6; Extracellular signal-regulated kinase

INTRODUCTION

*Typha orientalis* Presl (*Typhaeae*) is robust semi-aquatic perennial plants that row in fresh or slightly brackish water up to two meters in depth. It is found throughout the stat in lakes, dams, irrigation channels, marshes and along the banks of rivers where the water flow is slow and dissolved nutrient levels are high. In the present study, we investigated the anti-inflammatory properties of *Typha orientalis*’ stem (TOS) in phorbol 12-myristate 13-acetate (PMA) plus calcium ionophore A23187-stimulated human mast cell line, HMC-1.

Mast cells have been associated with immediate hypersensitivity, an immune reaction resulting from the release of chemical mediators and cytokines following IgE/FcεRI-mediated activation (Murrant and Bihari, 2000; Krishnaswamy et al., 2006). Moreover, the significant contribution of mast cells has become evident in pathogenesis of diverse inflammatory
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Diseases such as allergy, parasitic diseases, atherosclerosis, malignancy, asthma, pulmonary fibrosis, and arthritis (Bradding and Holgate, 1999; Krishnaswamy et al., 2006). Activated mast cells release both preformed and newly synthesized cytokines such as tumor necrosis factor (TNF-α) and interleukins. TNF-α has been shown to induce the chemotaxis of neutrophils and T cells, and stimulate the expression of adhesion molecules (Meng et al., 1995), an important mediator of immune and inflammatory response. Also, interleukin (IL)-6 is a pro-inflammatory cytokine, a potent mediator of inflammatory processes. Originally identified as a B-cell differentiation factor, IL-6 can also activate vascular endothelial cells, upregulating expression of certain chemokines and adhesion molecules, and facilitating leukocyte recruitment directly to sites of inflammation (Lipsky 2006). The mitogen-activated protein kinase (MAPK) cascade is a major signaling pathway in many cells (Schaeffer et al., 1999). The induction of most cytokine genes requires activation of the extracellular signal-regulated kinase (ERK) 1/2 (Andersson and Sundler, 2000). Herein we present evidence that TOS inhibited PMA plus A23187-induced TNF-α and IL-6 production as well as ERK1/2 activation in mast cells.

MATERIALS AND METHODS

Reagents
Fetal bovine serum (FBS), and Iscove’s Modified Dulbecco’s Medium (IMDM) were purchased from Gibco BRL (Grand Island, NY, USA). PMA, A23187, avidin-peroxidase, 2, 2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid), 3-(4, 5-dimethylthiazol-2-yl)-diphenyl-tetrazolium bromide (MTT), and other reagents were obtained from Sigma (St. Louis, MO, USA). Anti-human TNF-α and IL-6 antibody (Ab), biotinylated anti-human TNF-α and IL-6 Ab, and recombinant human (rh) TNF-α and IL-6 were purchased from R&D Systems (Minneapolis, MN, USA). Phospho-specific ERK1/2 (p-ERK1/2) and ERK1/2 were purchased from Santa Cruz Biotech. Inc. (Santa Cruz, CA, USA). For RT-PCR, commercially available primer sets of TNF-α and IL-6 were prepared from Bioneer (Daejeon, Korea). Taq polymerase was purchased from TaKaRa Biotech (Shiga, Japan).

Preparation of TOS
TOS was prepared by decocting the dried stem of *Typha orientalis* with boiling distilled water. The duration of decoction was about 2 h. The decoction was filtered, lyophilized and kept at 4°C. The yield of extraction was about 10% (w/w). The water extract powder was dissolved in sterile saline (100 mg/ml).

Culture of HMC-1 cells
Human mast cell line, HMC-1, were grown in IMDM medium supplemented with 100 U/ml penicillin, 100 g/ml streptomycin, 10 nM monothioglycerol and 10% heat-inactivated FBS at 37°C in 5% CO₂.

MTT assay
To test the viability of cells, MTT colorimetric assay was performed as described previously (Choi et al., 2007). HMC-1 cells (1 × 10⁶ cells/ml) were incubated for 8 h after stimulation in the absence or presence of TOS (1 mg/ml). After addition of MTT solution, the cells were incubated at 37°C for 4 h. The crystallized MTT was dissolved in dimethyl sulfoxide and measured the absorbance at 540 nm.

ELISA
Secreted TNF-α and IL-6 level in supernatants from HMC-1 cells was measured by a sandwich enzyme-linked immunosorbent assay (ELISA) according to manufacturer’s protocol (R&D Systems). Absorption of the avidin-horseradish peroxidase color reaction was measured at 405 nm and compared with serial dilutions of human TNF-α and IL-6 recombinant as a standard.
RT-PCR

Total RNA was isolated from HMC-1 cells with Easy-blue (Intron, Korea) according to the manufacturer’s instruction. And 1 mg of total RNA was converted to cDNA by reverse transcriptase at 37°C for 1h using a first-strand cDNA synthesis kit (Intron, Korea). The PCR amplification consisted of 35 cycles (94°C, 45 s; 60°C, 45 s; 72°C, 1.5 min) with the commercial oligonucleotide primer sets for human TNF-α, IL-6, and GAPDH. The final PCR products were electrophoresed on 2% agarose gels.

Western blot analysis

Cell extracts were prepared by detergent lysis procedure. Cells (5 × 10⁶ cells) were harvested, washed once with phosphate buffered saline (PBS), and resuspended in lysis buffer. Samples were vortexed for lysis for a few seconds every 15 min at 4°C for 1 h and centrifuged at 12,000 rpm for 5 min 4°C. Samples were heated at 95°C for 5 min, and cooled on ice followed by centrifugation at 12,000 rpm for 5 min. 20 µg cytosolic proteins were loaded and separated on 12% SDS-polyacrylamide gels. After electrotransferring onto nitrocellulose membrane (Amersham Pharmacia Biotech UK limited, England) at 4°C, the membrane was blocked with 5% nonfat dry milk in PBS containing 0.05% Tween-20 (PBST) for 1 h. After slightly washing with PBST, membrane was probed with primary Ab for 1 h and washed three times with PBST. Horseradish peroxidase-conjugated secondary Ab was incubated. Immuno-detection of the protein was done using enhanced chemiluminescence detection kit (Amersham Pharmacia, Piscataway, NJ) and then exposed to X-ray film.

Statistical analysis

The results were expressed as mean ± S.E.M. for a number of experiments. Statistical significance was compared between each treated group and control by analysis of variance (ANOVA), with post hoc test of the means according to Tukey’s method. For all tests, P value less than 0.05 was considered significant.

RESULTS

Effect of TOS on HMC-1 viability

To test cytotoxic effect of TOS, we performed MTT assay in HMC-1 cells. Fig. 1 shows the viability of cells 8 h incubation after stimulation. TOS did not affect cell viability and had no toxicity on HMC-1 cells (Fig. 1).

Effect of TOS on cytokine production from HMC-1 cells

We examined the inhibitory effect of TOS on the PMA plus A23187-induced production of TNF-α and IL-6 from HMC-1 cells. Culture supernatant was assayed for each cytokine levels by ELISA method. TOS pretreatment in cells inhibited PMA plus A23187-induced TNF-α and IL-6 production. The concentration of TNF-α was 0.05 ± 0.01 ng/ml in non-stimulated cells, and 2.448 ± 0.011 ng/ml in PMA plus A23187-stimulated cells (P < 0.001). However, the TNF-α production was significantly decreased to 1.39 ± 0.17 ng/ml by the treatment of 1 mg/ml TOS (P < 0.001). The concentration of IL-6 was 1.62 ± 0.89 ng/ml in non-stimulated cells, and
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15.54 ± 0.42 ng/ml in PMA plus A23187-stimulated cells ($P < 0.001$). However, the IL-6 production was significantly decreased to 9.27 ± 1.09 ng/ml by the treatment of 1 mg/ml TOS ($P < 0.001$, Fig. 2).

**Effect of TOS on TNF-α and IL-6 mRNA expression in HMC-1 cells**

In order to determine whether TOS can regulate TNF-α and IL-6 mRNA expression, total RNA was isolated from HMC-1 cells and reverse-transcribed into cDNA. The cDNA was amplified by PCR with primers specific for TNF-α and IL-6. TNF-α and IL-6 mRNA expression was increased compared with the absence of PMA plus A23187. But TOS (1 mg/ml) suppressed the TNF-α and IL-6 expression (Fig. 3).

**Effect of TOS on ERK1/2 phosphorylation**

MAPKs are serin/threonine kinases that become activated by phosphorylation on threonine and tyrosine residues upon extracellular stimuli (Schaeffer et al., 1999). PMA plus A23187 rapidly induced phosphorylation of ERK1/2, with no change in total ERK1/2 levels. However, treatment with 1
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DISCUSSION

In the present study, we showed that TOS pretreatment profoundly inhibited the expression of TNF-α and IL-6 in PMA plus A23187-stimulated mast cells. Also, TOS inhibited PMA plus A23187-induced ERK1/2 phosphorylation.

HMC-1 cells activated by PMA plus A23187 are useful in vitro model system for studying of multifunctional effects of the immune and inflammatory reactions (Hosoda et al., 2002; Kim et al., 2008). We showed that TOS inhibited inflammatory cytokines such as TNF-α, and IL-6 from PMA plus A23187-stimulated HMC-1 cells. TNF-α is an essential cytokine in many pathological conditions such as allergic diseases, rheumatoid arthritis and pulmonary fibrosis (Camusi et al., 1991). TNF-α induced production and release of eosinophil chemotactic factors such as eotaxin from fibroblasts and epithelial cells (Hoeck and Woisetschlager, 2001; Sato et al., 2001). IL-6 plays a major role in triggering and sustaining the allergic inflammatory response. Elevated levels of IL-6 have been reported to occur in the bronchoalveolar lavage fluid of patients with asthma (Oh et al., 2002). In the present study, TOS remarkably inhibited the production of TNF-α and IL-6. It was associated with blocking gene expression (Fig. 3).

The induction of most cytokine genes requires activation of the ERK1/2 (Shapiro et al., 1995). There are reports that Eriobotrya japonica and resveratrol inhibit the cytokine production via activation of pERK in stimulated HMC-1 cells (Kang et al., 2009; Kim and Shin, 2009). We next tested the effect of TOS for PMA plus A23187-induced ERK1/2 activation. The present results show that TOS dramatically inhibited PMA plus A23187-induced ERK1/2 phosphorylation. The present results show that TOS dramatically inhibited PMA plus A23187-induced ERK1/2 phosphorylation in HMC-1 cells (Fig. 4).

In conclusion, TOS inhibited the expression of TNF-α and IL-6 in mast cells. In addition, TOS partially blocked PMA plus A23187-induced ERK1/2 phosphorylation. These results suggested TOS could inhibit the cytokine expression through blockade of ERK1/2 activity. Given these findings, the regulation of the ERK1/2 pathway by TOS in HMC-1 cells could form the basis of a new strategy for the treatment of mast cell-mediated inflammatory diseases.

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REFERENCES


