

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

In-Young Choi¹, Ho-Jeong Na¹, Jae-Young Um¹, Hyung-Min Kim¹, Seung-Heon Hong², Kuk-Jin Sim³, Bong-Keun Song³, Gi-Hye Nam⁴, Se-Young Choung⁴ and Hyun-Ja Jeong^{5,*}

¹Department of Pharmacology, College of Oriental Medicine, Institute of Oriental Medicine, Kyung Hee University, 1 Hoegi-Dong, Dongdaemun-Gu, Seoul, 130-701, Republic of Korea; ²College of Pharmacy, Wonkwang University, Iksan, Jeonbuk, 570-749, Republic of Korea; ³College of Oriental Medicine, Wonkwang University, Iksan, Jeonbuk, 570-749, Republic of Korea; ⁴Department of Hygienic Chemistry, College of Pharmacy, Kyung Hee University, 1 Hoegi-Dong, Dongdaemun-Gu, Seoul, 130-701, Republic of Korea; ⁵Biochip Research Center, Hoseo University, Asan, Chungnam 336-795, Republic of Korea

Received for publication January 15, 2010; accepted February 12, 2010

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- α (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 (*Typhaceae*) is robust semi-aquatic perennial plants that grow in fresh or slightly brackish water up to two meters in depth. It is found throughout the state 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/Fc ϵ RI-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

*Correspondence: Hyun-Ja Jeong, Biochip Research Center, Hoseo University; Asan, Chungnam 336-795, Republic of Korea. Tel.: +82415409681; Fax: +82415429681; E-mail: hjeong@hoseo.edu

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 (1×10^6 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 (5×10^6 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 μ g 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. Immunodetection 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 \pm 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

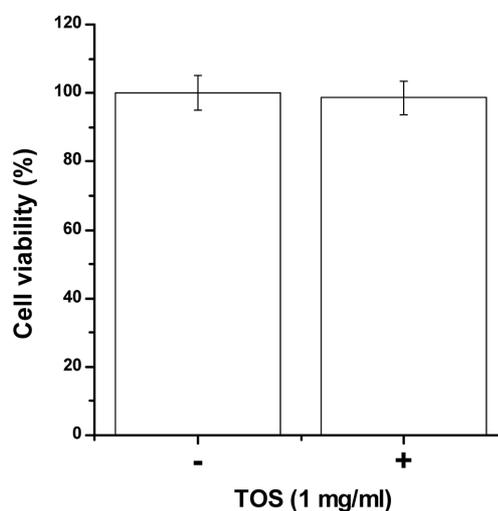


Fig. 1. Effect of TOS on cell viability in HMC-1 cells. Cell viability was evaluated by MTT assay at 8 h after TOS treatment (1 mg/ml) in HMC-1 cells. Data represent the mean \pm S.E.M. of three independent experiments.

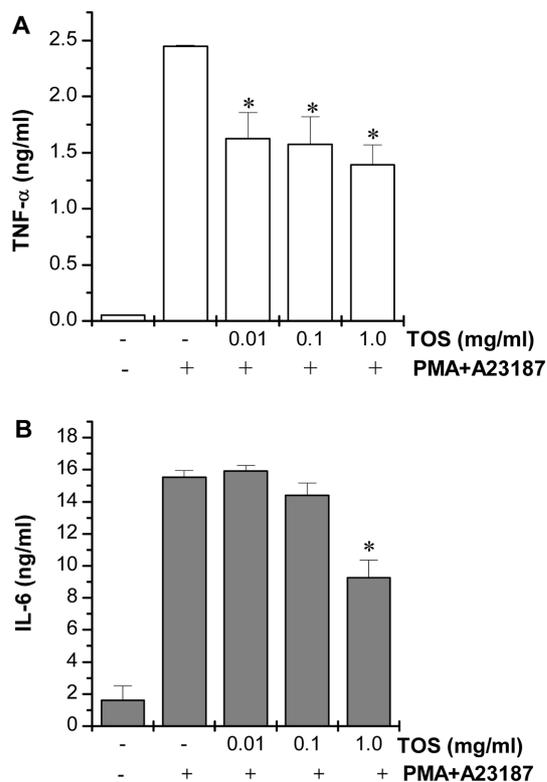


Fig. 2. Inhibition of TNF- α (A) and IL-6 (B) production by TOS on PMA plus A23187-stimulated HMC-1 cells. Cells were pretreated with TOS for 30 min and then stimulated with PMA plus A23187 for 8 h. TNF- α and IL-6 concentrations were measured from cell supernatant using ELISA method. Values are mean \pm S.E.M. of duplicate determinations from three separate experiments. * $P < 0.001$; significantly different from the stimulated group.

15.54 \pm 0.42 ng/ml in PMA plus A23187-stimulated cells ($P < 0.001$). However, the IL-6 production was significantly decreased to 9.27 \pm 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

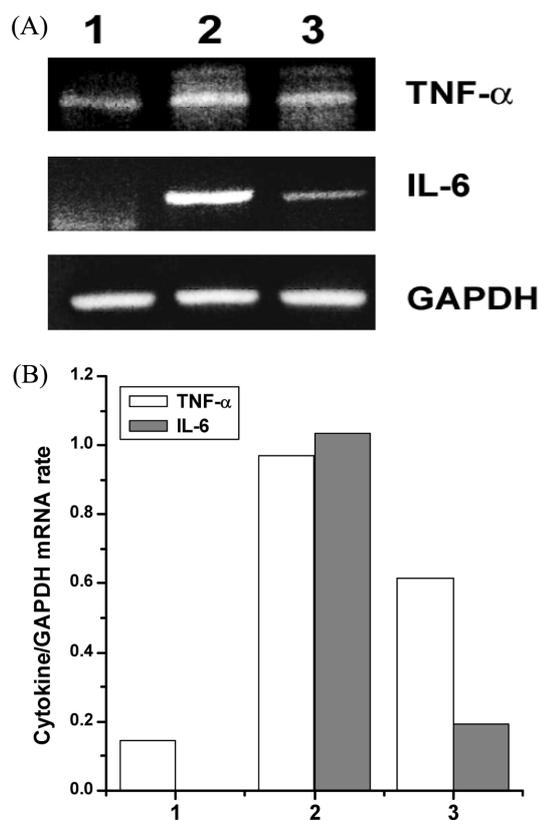


Fig. 3. Effect of TOS on TNF- α and IL-6 mRNA expression in HMC-1 cells. HMC-1 cells were treated with TOS (1 mg/ml) for 30 min and then stimulated with PMA plus A23187 for 6 h. Messenger RNA was measured using RT-PCR method (A). The mRNA levels were measured by densitometry (B). Results are representative of three independent experiments, and the band intensities correspond to the levels of cytokines/GAPDH. Lane 1, control (no stimulation); lane 2, PMA plus A23187 treatment; lane 3, PMA plus A23187 plus TOS treatment.

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

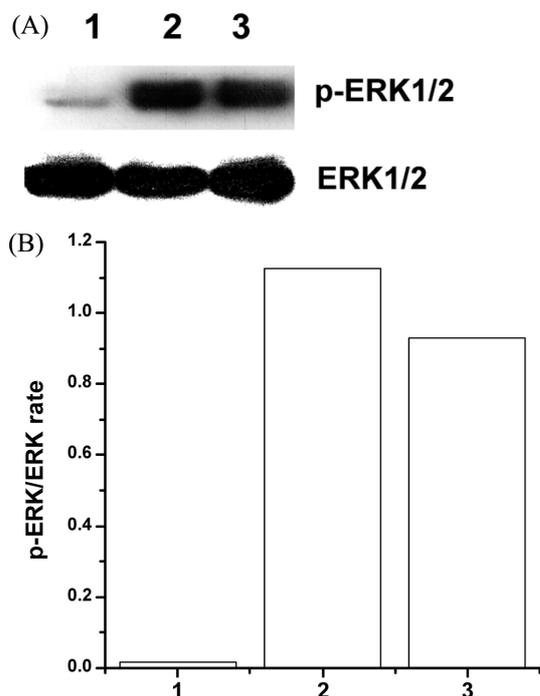


Fig. 4. Effect of TOS on p-ERK1/2 activation. HMC-1 cells were pretreated with or without TOS (1 mg/ml) for 30 min prior to PMA plus A23187 stimulation. Phosphorylated ERK1/2 was evaluated by using Western blot analysis (A). The protein levels were measured by densitometry (B). Results are representative of three independent experiments, and the band intensities correspond to the levels of p-ERK/ERK. Lane 1, control (no stimulation); lane 2, PMA plus A23187 treatment; lane 3, PMA plus A23187 plus TOS treatment.

mg/ml TOS blocked PMA plus A23187-induced ERK1/2 phosphorylation (Fig. 4).

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 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.

ACKNOWLEDGEMENTS

This research was supported by the Academic Research fund of Hoseo University in 2009 (2008-0501).

REFERENCES

- Alonzi T, Fattori E, Lazzaro D, Costa P, Probert L, Kollias G. (1998) Interleukin 6 is required for the development of collagen-induced arthritis. *J. Exp. Med.* **187**, 461-468.
- Andersson K, Sundler R. (2000) Signalling to translational activation of tumour necrosis factor- α expression in human THP-1 cells. *Cytokine* **12**, 1784-1787.
- Bradding P, Holgate ST. (1999) Immunopathology and human mast cell cytokines. *Crit. Rev. Oncol. Hematol.* **31**, 119-133.
- Camussi G, Albano E, Tetta C, Bussolino F. (1991) The molecular action of tumor necrosis factor- α . *Eur. J. Biochem.* **202**, 3-14.
- Choi IY, Moon PD, Koo HN, Myung NY, Kim SJ, Lee JH, Han SH, Moon G, Seo SY, Sung HJ, Park RK, Jeong HJ, Um JY, Kim HM, Hong SH. (2007) Observations of Forsythia koreana methanol extract on mast cell-mediated allergic reactions in experimental models. *In Vitro Cell Dev. Biol. Anim.* **43**, 215-221.
- Feldmann M, Brennan F M, Maini RN. (1996) Role of cytokines in rheumatoid arthritis. *Annu. Rev. Immunol.* **14**, 297-440.
- Hoeck J, Woisetschlager M. (2001) STAT6 mediates eotaxin-1 expression in IL-4 or TNF- α -induced fibroblasts. *J. Immunol.* **166**, 4507-4515.
- Hosoda M, Yamaya M, Suzuki T, Yamada N, Kamanaka M, Sekizawa K, Butterfield JH, Watanabe T, Nishimura H, Sasaki H. (2002) Effects of rhinovirus infection on histamine and cytokine production by cell lines from human mast cells and basophils. *J. Immunol.* **169**, 1482-1491.
- Kang OH, Jang HJ, Chae HS, Oh YC, Choi JG, Lee YS, Kim JH, Kim YC, Sohn DH, Park H, Kwon DY. (2009) Anti-inflammatory mechanisms of resveratrol in activated HMC-1 cells: pivotal roles of NF-kappaB and MAPK. *Pharmacol Res.* **59**, 330-337.
- Kim SH, Shin TY. (2009) Anti-inflammatory effect of leaves of *Eriobotrya japonica* correlating with attenuation of p38 MAPK, ERK, and NF-kappaB activation in mast cells. *Toxicol In Vitro.* **23**, 1215-1219.
- Kim SJ, Park JS, Myung NY, Moon PD, Choi IY, An HJ, Kim NH, Na HJ, Kim DH, Kim MC, An NH, Kim IK, Lee JY, Jeong HJ, Um JY, Kim HM, Hong SH. (2008) *Scrophularia buergeriana* Regulates Cytokine Production In Vitro. *Immunopharmacol. Immunotoxicol.* **22**, 1-13.
- Krishnaswamy G, Ajitawi O, Chi DS. (2006) The human mast cell: an overview. *Methods Mol. Biol.* **315**, 13-34.
- Lipsky PE. (2006) Interleukin-6 and rheumatic diseases. *Arthritis Res Ther.* **8**, Suppl 2:S4.
- Meng H, Tonnesen MG, Marchase MJ, Clark RA, Bahou WF, Gruber BL. (1995) Mast cells are potent regulator endothelial cell adhesion molecule ICAM- α and VCAM-1 expression. *J. cell Physiol.* **165**, 40-53.
- Murrant T, Bihari D. (2000) Anaphylaxis and anaphylactoid reactions. *Int. J. Clin. Pract.* **54**, 322-328.
- Oh JW, Lee HB, Park IK, Kang JO. (2002) Interleukin-6, interleukin-8, interleukin-11, and interferon-gamma levels in nasopharyngeal aspirates from wheezing children with respiratory syncytial virus or influenza A virus infection. *Pediatr. Allergy Immunol.* **13**, 350-356.
- Sato E, Nelson D, Koyama S, Hoyt J C, Robbins, A. (2001) Inflammatory cytokines modulate eotaxin release by human lung fibroblast cell line. *Exp. Lung Res.* **27**, 173-183.
- Schaeffer HJ, Weber MJ. (1999) Mitogen-activated protein kinases specific messages from ubiquitous messengers. *Mol. Cell. Biol.* **19**, 2435-2444.