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Anti-inflammatory effect of *Equisetum hyemale* via suppression of nuclear factor-κB activation in human mast cells

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SUMMARY

Equisetum hyemale Linne. (EH) (Equisetaceae) has been used for the treatment of eye and skin disease, chronic eczema, pneumoconiosis and asthma in Korea and China. Human leukemic mast cells are widely distributed in the connective tissues of mammals and other vertebrates. Phorbol 12-myristrate 13-acetate (PMA) and calcium ionophore A23187 stimulated Human leukaemic mast cell line-1 (HMC-1) can produce a variety of inflammatory mediators and several pro-inflammatory and chemotactic cytokines such as TNF-α, IL-6 and IL-8. Since TNF-α, IL-6 and IL-8 are major factors during the inflammatory process, we studied the effects of EH on TNF-α, IL-6 and IL-8 release in HMC-1 stimulated with PMA and A23187. The result of this study indicate that EH inhibits TNF-α, IL-6 and IL-8 in activated HMC-1 cells via IκB/Nuclear factor-κB pathway. Therefore, EH might contribute significantly to the prevention or treatment of mast-cell mediated inflammatory diseases and EH has potential use in the therapy of chronic allergic inflammation.

Key words: *Equisetum hyemale*; TNF-α; IL-6; IL-8; NF-κB

INTRODUCTION

A whole part of *Equisetum hyemale* Linne. (EH) has been used for the treatment of eye and skin disease, a sore throat, an anal prolapse, an abscess, chronic eczema, pneumoconiosis and asthma. EH has canes of joint and lives in swampy land (Lee *et al*., 1995).

Human mast cells broadly distributed in mammalian tissues are fascinating, multifunctional, tissue-dwelling cells and one of the major effector cells in inflammatory reactions (Galli *et al*., 1994; Krishnaswamy *et al*., 2006). The binding of antigens to receptor-bound IgE and the subsequent cross-linking of the high-affinity IgE receptor (FcεRI) is the classical trigger of mast cell activation. Activated mast cells exert their biologic effects by releasing preformed and novo-synthesized mediators such as histamine, proteases, leukotrienes, prostaglandins and cytokines. Chemokines and pro-inflammatory cytokines, including TNF-α, IL-6 and IL-8 promote the development of inflammatory reaction (Beaven and Metzger, 1993; Galli, 1993; Church and Levi-Schaffer, 1997; Metcalfe *et al*.,

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1997; Kim and Lee, 1999). TNF-α induces tissue damage and is considered a major initiator of inflammation (Kim and Lee, 1999). IL-6 is a pro-inflammatory cytokine that is a potent mediator of inflammatory processes (Ershler and Keller, 2000). IL-8 is a potent neutrophil chemoattractant factor (Annelie *et al*., 1993).

NF-κB is a imperative transcription factor. NF-κB dimeric complexes composed of Rel A (p65), NFêB1 (p50) or NFκB2 (p52) subunits are normally associated with inhibitory IκB protein (α, β, γ) in the cytoplasm in an inactive state (Grimm and Baeuerle, 1993; Baeuerie and Henkel, 1994). When the cells are stimulated by extracellular stimuli, specific kinases lead to the degradation and phosphorylation of IκB and activation of NF-κB (Baeuerie and Henkel, 1994; Baumgartner *et al*., 1994). The activated NF-κB translocates to the nucleus, binds the DNA, activates inflammatory genes and finally transcribes the mediator such as TNF-α, IL-6 and IL-8 (Collart *et al*., 1990; Rao, 1994; Baumgartner *et al*., 1994; Thanos and Maniatis, 1995; Azzolina *et al*., 2003).

Although EH has been widely used for the treatment of inflammation in Korea and China, the effects of EH on inflammatory molecules in mast cell have not been studied sufficiently. To investigate the anti-inflammatory effect of EH, this report examined the effects of EH on TNF-α, IL-6 and IL-8 release, and IκB/NF-κB activation in HMC-1 in response to PMA and the A23187.

MATERIALS AND METHODS

Reagents

Iscove's Modified Dulbecco's Medium (IMDM), penicillin and streptomycin were purchased from Gibco BRL (Grand Island, NY, USA). PMA, calcium ionophore A23187, diphenyltetrazolium bromide (MTT), avidin-peroxidase and 2,2-azio-bis (ABTS) were purchased from the Sigma Chemical Co. (St. Louis, MO, USA). Recombinant TNF-α and biotinylated anti-human TNF-α were purchased from R&D system Inc. (Minneapolis, MN, USA). Recombinant IL-6 and IL-8, biotinylated anti-human IL-6 and IL-8 were purchased from Pharmingen (San Diego, CA, USA). NF-κB antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Preparation of EH

EH was purchased from Wansanyakupsa (Jeonju, South Korea) in March 2005. A voucher specimen (WME039) has been deposited at the Department of Oriental Pharmacy, College of Pharmacy, Woosuk University. An extract was obtained twice from the dried sample (200 g) with 3,000 ml of 85% MeOH under ultrasonification fo 2 h. It was evaporated and lyophilized to yield (1.9 g) an MeOH extract of EH (Yield : 4.185%), which was then stored at -20 °C until use.

Cell culture and stimulation of mast cells

HMC-1 was cultured in IMDM (Gibco, Co.) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml of penicillin, and 0.1 mg/ml of streptomycin and maintained in a humidified incubator with 5% CO_2. HMC-1 was passaged 2-3 times per week with 1:2 split ratio. For ELISA, HMC-1 was stimulated with PMA (50 nM) and A23187 (1 μM) for 24 h after the treatment of EH for 30 min. For western blotting, HMC-1 was also stimulated with PMA (50 nM) and A23187 (1 μM) for 1 h after the treatment of EH for 30 min.

Cell viability assay (MTT assay)

The effects of cytotoxicity in HMC-1 treated with final concentrations of 0.1, 0.5 and 1 mg/ml of EH extract was evaluated using a colorimetric MTT-assay, which measured the ability of viable cells to reduce the colorless tetrazolium salt MTT to the colored formazan product. The HMC-1 cells were treated with final concentrations of 0.1, 0.5 and 1 mg/ml of EH extract for 24 h at 37 °C. Following this incubation period, the 50 μl of MTT solution (5 mg/ml) was added each well. After 3 h of

incubation, the MTT formazan products were solubilized with DMSO. Absorbance of the colored solution was measured at 570 nm.

Enzyme-linked immunosorbent assay (ELISA)

HMC-1 (3×10^5 cells/well) and was cultured in the absence or in the presence of 0.1, 0.5 and 1 mg/ml of EH and stimulated with PMA (50 nM) and A23187 (1 μM) for 24 h. We then used ELISA method to assay the culture supernatants for the TNF-α, IL-6 and IL-8 protein levels. To measure the cytokines, we used a modified ELISA method (Moon *et al.*, 2007). Color development was measured at 405 nm using an automated microplate ELISA reader.

Cytoplasmic and nuclear protein extraction

HMC-1 was plated at 5×10^6 cells in 60-mm dishes. After the cells were treated with final concentrations of 0.1, 0.5 and 1 mg/ml of EH extract for 30 min, stimulated with PMA (50 nM) and A23187 (1 μM) for 1 h. The cells were scraped, washed twice with PBS, and resuspended in hypotonic buffer A (10 nM HEPES/KOH, 2 mM $MgCl_2$, 0.1 mM EDTA, 10 mM KCl, 1 mM dithiothreitol and 0.5 mM phenylmethylsulfonyl fluoride, pH 7.9) and incubated on ice for 30 min. After NP-40 were added and the cells were incubated on ice for 10 min, the supernatant from the centrifugation (2500 rpm, 20 min, 4 °C) of this mixture was collected as cytoplasmic protein. For the removal of residual cytoplasmic protein, the nuclear pellet was washed in hypertonic buffer (50 nM HEPES/KOH, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1 mM dithiothreitol and 0.5 mM phenylmethylsulfonyl fluoride, pH 7.9). The supernatant from the centrifugation (12,000 rpm, 5 min, 4 °C) of this mixture was collected as nuclear protein.

Western blotting assay

Cellular protein were extracted by a detergent lysis procedure. The 60-mm dishes of HMC-1 cells (5×10^6 cells) were scraped. Samples were heated at 95 for 5 min, and the cell extracts were separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes (Hybond-C extra, Amersham, GB) by the semi-dry procedure. Blots were blocked overnight with 5% milk powder and probed with the NF-κB and phosphate IκB antibodies (Santa cruz, CA, USA) for 2 h. The mouse anti-rabbit IgG antibody and anti-mouse IgG antibody was incubated for 90 min at room temperature, respectively, then staining visualized by the enhanced chemiluminescence technique (Pierce, USA), and exposure to X-ray film (Fuji, Tokyo, Japan)

Statistics

The data are shown as mean ± S.E.M. Statistical significance was determined by student's *t*-test, and $P < 0.05$ was considered significantly.

RESULTS

Effect of EH on cytotoxicity in HMC-1 cells

In order to examine cytotoxic effect of EH, MTT assay was performed in HMC-1. The HMC-1 was pre-treated with final concentrations of 0.1, 0.5 and 1 mg/ml of EH for 24 h. After incubating for 24 h, cell viability was measured by the method described above. As shown in Fig. 1, the concentrations of 0.1,

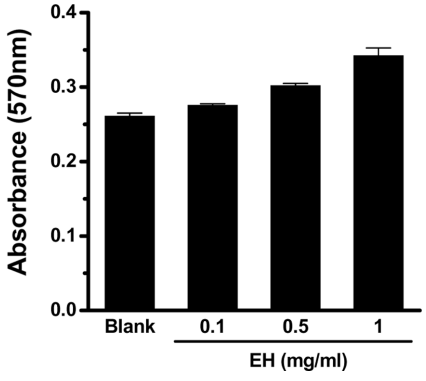

Fig. 1. Effect of EH on the cell viability on HMC-1 cells. Cell viability was evaluated by MTT assay 24 h after EH treatment as indicated concentrations in HMC-1 cells. Each data represents the mean ± S.E.M. of three independent experiments.

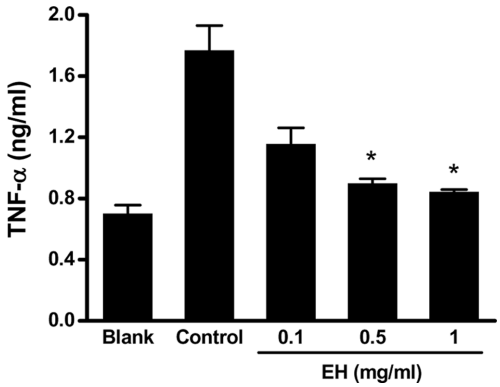

Fig. 2. Effect of EH on TNF-α expression in PMA and A23187 stimulated HMC-1 cells. Cells were pretreated with EH for 30 min prior to PMA (50 nM) and A23187 (1 μM) stimulation for 24 h. TNF-α concentrations were measured from cell supernatants using ELISA method. Values are expressed as mean ± S.E.M. of three independent experiments.

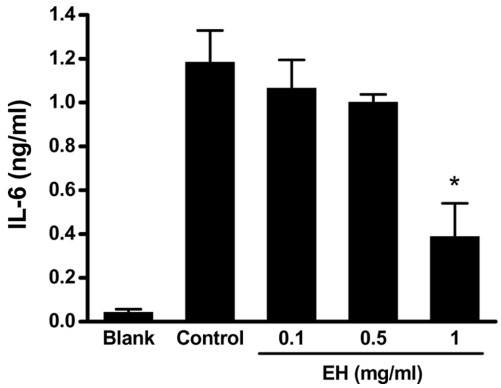

Fig. 3. Effect of EH on IL-6 expression in PMA and A23187 stimulated HMC-1 cells. Cells were pretreated with EH for 30 min prior to PMA (50 nM) and A23187 (1 μM) stimulation for 24 h. IL-6 concentrations were measured from cell supernatants using ELISA method. Values are expressed as mean ± S.E.M. of three independent experiments.

0.5 and 1 mg/ml of EH had little effect on the cell viability.

Effect of EH on TNF-α release

As the most important pro-inflammatory cytokines in mast cell mediated inflammation, TNF-α, IL-6 and IL-8 were assayed essentially and generally. To investigate the effect of EH on TNF-α release from HMC-1, culture supernatant treated with final concentrations of 0.1, 0.5 and 1 mg/ml of EH were assayed for TNF-α cytokine level by ELISA methods. Fig. 2 showed that EH inhibited the release of TNF-α in PMA and A23187 stimulated HMC-1 in a dose-dependant manner significantly.

Effect of EH on IL-6 release

To investigate the effect of EH on IL-6 release from HMC-1, culture supernatant treated with final concentrations of 0.1, 0.5 and 1 mg/ml of EH were assayed for IL-6 cytokine level by ELISA methods. Fig. 3 showed that EH inhibited the release of IL-6 in PMA and A23187 stimulated HMC-1 in a dose-dependant manner, especially in 1 mg/ml of EH.

Effect of EH on IL-8 release

To investigate the effect of EH on IL-8 release from

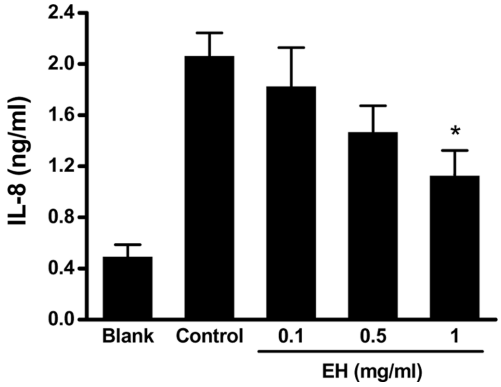

Fig. 4. Effect of EH on IL-8 expression in PMA and A23187 stimulated HMC-1 cells. Cells were pretreated with EH for 30 min prior to PMA (50 nM) and A23187 (1 μM) stimulation for 24 h. IL-8 concentrations were measured from cell supernatants using ELISA method. Values are expressed as mean ± S.E.M. of three independent experiments.

HMC-1, culture supernatant treated with final concentrations of 0.1, 0.5 and 1 mg/ml of EH were assayed for IL-8 cytokine level by ELISA methods. Fig. 4 showed that EH inhibited the release of IL-8 in PMA and A23187 stimulated HMC-1 cells in a dose-dependant manner.

Effect of EH on the IêB degradation

The release of TNF-α, IL-6 and IL-8 was regulated

PMA+A23187	−	+	+	+	+
EH (mg/ml)	−	−	**0.1**	**0.5**	**1**

Fig. 5. Effect of EH on the expression of phosphate IκBα in PMA and A23187 stimulated HMC-1 cells. Cells were pretreated with EH for 30 min prior to PMA (50 nM) and A23187 (1 μM) stimulation for 1 h. Expression of phosphate IκBα is assayed by western blot analysis.

PMA+A23187	−	+	+	+	+
EH (mg/ml)	−	−	**0.1**	**0.5**	**1**

Fig. 6. Effect of EH on the expression of p65 NF-κB in PMA and A23187 stimulated HMC-1 cells. Cells were pretreated with EH for 30 min prior to PMA (50 nM) and A23187 (1 μM) stimulation for 1 h. Expression of nucleus NF-κB is assayed by western blot analysis.

by the IκB/NF-κB pathway. To determine the effect of EH on the expression of inhibitor phosphate IκB, we performed a western blot analysis for phosphate IκB in HMC-1 stimulated by PMA and A23187. In PMA plus A23187-stimulated cells, the expression level of phosphate IκBα increased in the cytoplasm. However, Fig. 5 showed that the expression level of phosphate IκBα decreased by treatment of EH in a dose-dependant manner.

Effect of EH on the NF-κB activation

To determine the effect of EH on the expression of NF-κB, this report performed a western blot analysis for p65 protein in HMC-1 stimulated by PMA and A23187. In PMA plus A23187-stimulated cells, the expression level of NF-κB increased in the nucleus. However, Fig. 6 showed that the expression level of NF-κB decreased by treatment of EH remarkably. The result of treatment in 0.5 mg/ml of EH is no supposed blot, and further study is necessary to obtain the clarified outcome.

DISCUSSION

Mast cells are fascinating, multifunctional, tissue-dwelling cells that have been traditionally associated with the allergic response. However, recent studies suggest these cells may be capable of regulating inflammation (Krishnaswamy *et al*., 2006). Mast cells activated by PMA and A23187 are useful *in vitro* model system for studying of multifunctional effects of the immune and inflammatory reaction (Hosoda *et al*., 2002; Kim *et al*., 2003). Mast cells contain preformed and potent allergic inflammatory mediators such as histamine, heparin, proteinases, leukotriense and multifuctional cytokines (Jeong *et al*., 2006).

The cytokines especially TNF-α, IL-6 and newly synthesized IL-8 are key indicators of inflammatory symptom for the reason of critical biological role in inflammation and matrix degradation (Tetlow and Woolley, 1995; Galli *et al*., 2005). TNF-α is released as a prestored but also newly synthesized cytokine upon cell activation in mast cells (Hide *et al*., 1997). TNF-α promotes inflammation, leukocyte infiltration, granuloma formation and tissue fibrosis and is thought to be an initiator of cytokine related inflammation states by inducing other inflammatory cytokines, including IL-1β, IL-6, IL-8 and GM-CSF (Baumgartner *et al*., 1994; Arend and Dayer, 1995). IL-6 is a pleiotropic inflammatory cytokine produced by T cells, monocytes and macrophages and its local accumulation is associated with a local allergic reaction (Snick, 1990). IL-8 acts on surrounding cells such as neutrophils, T-lympocytes and eosinophils and mediates the migration of neutrophils to local inflammation as a potent activator and chemoattractant of neutrophils (Karakurum *et al*., 1994; Sengupta *et al*., 2004). Histamine inducing by cross-linking of the FcεRI is an important mediator in immediate-type allergic inflammatory reaction (anaphylactic allergic reaction) (Kemp and Lockey, 2002). In our study, EH had little effect on compound 48/80-induced systemic anaphylactic allergic reaction (data not shown). Stimulation of mast cells with

compound 48/80 initiates the activation of signal-transduction pathway, which leads to histamine release (Mousli *et al*., 1990). We speculated that EH had no effect on compound 48/80-induced histamine release from mast cells. Previous studies reported that aucubin inhibits TNF-α and IL-6 expression and scopoletin inhibits TNF-α, IL-6 and IL-8 expression through blockade of NF-κB activation, though it does not affect the release of histamine in mast cells (Jeong *et al*., 2002; Moon *et al*., 2006). Sulfasalazine, aucubin and scopoletin, which are potent and specific inhibitor of NF-κB activation without preventing AP-1 binding activity (Schoonbroodt *et al*., 1997; Wahl *et al*., 1998; Jeong *et al*., 2002). We also may speculate that EH can be a specific NF-κB inhibitor. Further study is necessary to demonstrate the accurate role of EH in the activation of mast cells.

The release of various inflammatory cytokines is regulated by transcription factors, NF-κB, AP-1, nuclear factor activated T cell, NF-IL6 and hypoxia inducible factor-1 (Rao, 1994; Galien *et al*., 1996; Fiorini *et al*., 2000; Jeong *et al*., 2003). NF-κB is consisted of predominantly two proteins, p50 and p65, also called RelA and cRel, respectively (Baeuerle and Baltimore, 1996). IκB kinase family contained IKKα (IKK-1) and IKKβ (IKK-2) is encoded on separate chromosomes and appears to regulate IκB phosphorylation in immortalized cell line and tumor cells. The IκB phosphorylation triggers proteolytic degradation. NF-κB released from IκB is translocated into the nucleus to bind the κB binding sites in the promoter regions of target inflammatory genes subsequently (DiDonato *et al*., 1997; Regnier *et al*., 1997; May and Ghosh, 1997; Hu and Wang, 1998). Present study is suggested that EH inhibits the translocation of NF-κB from the cytoplasm to the nucleus and IκBα degradation and phosphorylation. Further study is necessary to demonstrate the accurate role of EH in the activation of mast cells via p38 pathway and ERK1/2 pathway and identify active constituents from EH in inflammatory reaction.

In certain reports, EH was demonstrated that its effect on experimatal hyperlipemia in rats (Xu *et al*., 1993). Another paper showed that EH had inhibitory effect of cyclooxygenase (COX-2) and nitric oxide synthase (iNOS) in RAW 264.7 macrophage cells (Hong *et al*., 2002). In this research, we have been demonstrated that EH inhibits the TNF-α, IL-6 and IL-8 expression in PMA and A23187 stimulated HMC-1 and EH inhibits the translocation of NF-κB to the nucleus and IκBα degradation and phosphorylation to attenuate secretion of pro-inflammatory cytokines consequently.

In conclusion, the results obtained in this study present that EH might contributes significantly to the prevention or treatment of mast-cell mediated inflammatory diseases and EH has potential use in the therapy of chronic allergic inflammation.

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