Evaluation on efficacy of modern medicine ketotifen using traditional delivery science acupuncture in allergic animal models

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Pharmaceutical acupuncture (PA) is a traditional oriental therapeutic technique that combines acupuncture with herbal medicine. We investigated the effect of mast cell stabilizer, ketotifen PA to efficiently and safely regulate various allergic reactions. Ketotifen was administered through PA on the ST36 acupoint in allergic animal models. Ketotifen PA significantly decreased compound 48/80-induced mortality, histamine release, and ear swelling. Ketotifen significantly reduced the level of intracellular Ca^{2+} increased by compound 48/80 in human mast cell line, HMC-1 cells. Compared to oral administration, ketotifen PA significantly suppressed the dye effusion on passive cutaneous anaphylaxis (PCA). The level of IL-1β was significantly decreased by ketotifen PA in sera after PCA. Ketotifen also significantly inhibited IL-1β production and mRNA expression through the regulation of caspase-1 and nuclear factor-κB activation in HMC-1 cells. In conclusion, we found that ketotifen PA was more effective than oral administration and may serve as a new and useful therapy.

Key words: Ketotifen – Pharmaceutical acupuncture – Allergic reactions – Mast cells – IL-1β – Caspase-1.

Allergies are a significant health problem that negatively affects the lifestyle of modern people. Allergic diseases are caused by complex interactions between genes, environments, and behaviors. Major allergic diseases occur in the airway, gastrointestinal tract, and skin [1, 2]. Anaphylactic reactions, life-threatening allergic diseases, are associated with the mast cell. The mast cell is an immune cell that contributes to the immunoglobulin E (IgE)/FcεRI-mediated activation mechanism in allergies. In immediate hypersensitive immune reactions, mast cells release inflammatory/pro-inflammatory chemical mediators, including cytokines, chemokines, and histamine [3-5]. In the activated mast cells, intracellular Ca^{2+} acts as a second messenger and increases in intracellular Ca^{2+} are essential for mast cell degranulation. Forced increases in intracellular Ca^{2+} by agents such as compound 48/80 induce anaphylaxis [6, 7].

Activated mast cells produce histamines and other inflammatory mediators, such as eicosanoids, proteoglycans, proteases, and several proinflammatory cytokines, especially interleukin (IL)-1β [8]. The expression of inflammatory cytokines, including IL-1β, is dependent on the activation of a transcription factor, nuclear factor (NF)-κB. When NF-κB binds to a specific consensus DNA element on the promoter of target genes, it initiates the transcription of various genes. NF-κB normally resides in the cytoplasm with its endogenous inhibitor, IκB (α, β, or γ forms). However, when NF-κB is activated, it translocates to the nucleus, binds DNA, and activates genes [9]. Caspase-1 is a member of the caspase family and an IL-1β converting enzyme. It plays a crucial role in the regulation of inflammation [10, 11]. Caspase-1 transgenic mice overexpressing caspase-1 induce serious allergic reactions by increasing the levels of histamine and IgE [12].

Ketotifen is a non-competitive histamine-1 (H1) receptor antagonist and mast cell stabilizer used for treating diseases such as allergies, asthma, and gastric mucosal damage [13, 14]. In the pharmacokinetics of ketotifen oral administration with a tablet, C_{max} in serum is 2 to 4 h. Ketotifen in a liquid formula (such as syrup) has a significantly higher absorption rate compared to the tablet, but both have a half-life of about 12 h [15]. Recently, Klooker *et al*. reported that ketotifen decreases the visceral hypersensitivity from irritable bowel syndrome [16]. However, ketotifen still induces side effects such as headache, drowsiness, and vomiting.

Pharmaceutical acupuncture (PA) is a traditional oriental therapeutic technique that combines acupuncture with herbal treatment. This technique involves injecting an herbal extract into certain acupoints according to oriental medical theory [17]. Although the oral administration of ketotifen has an anti-allergic effect, the clinical use of ketotifen is limited by its side effects and patient's conditions. PA is an effective and safe intervention to treat allergies, arthritis, rheumatism, and pain (especially lower back pain) [18].

The ST36 acupoint (Zusanli), the most important and most frequently used acupoint on the stomach meridian, is considered to be the main point of regulation of gastrointestinal function, promoting gastrointestinal peristalsis and detoxification and protecting the mucosal barrier [19]. ST36 point is one of the most frequently used points in acupuncture practice, and also in acupuncture research [20]. In this study, we investigated the effect of ketotifen administered through PA on the ST36 acupoint in various allergic animal models.

I. MATERIALS AND METHODS

1. Reagents

We purchased ketotifen fumarate, phorbol 12-myristate 13-acetate (PMA), A23187 (calcimycin; $C_{29}H_{37}N_3O_6$), compound 48/80, 2-bis(2-aminophenoxy)ethane-N,N,N0,N0-tetraacetic acid acetoxymethyl ester (BAPTA-AM), anti-dinitrophenyl (DNP)-IgE, DNP-human serum albumin (HSA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2'-azino-bis (3-ethylbenzithiazoline-6-sulphonic acid) tablets substrate (ABTS), dimethyl sulfoxide (DMSO), phosphate-buffered saline (PBS), bovine serum albumin (BSA), o-phthaldialdehyde (OPA), bicinchoninic acid (BCA), avidin-peroxidase, OVA, and Evans blue from Sigma Chemical Co. (St. Louis, MO, United States); Isocove's Modified Dulbecco's Medium (IMDM), penicillin, streptomycin, and fetal bovine serum (FBS) from Gibco BRL (Grand Island, NY, United States); Anti-mouse and human IL-1β antibody (Ab), biotinylated anti-mouse and human IL-1β Ab, and recombinant mouse (rm) and human (rh) IL-1β from Pharmingen (Sandiego, CA, United States) and R&D Systems (Minneapolis, MN, United States).

2. Culture of HMC-1 cells

The human mast cell line (HMC-1) was grown in IMDM sup-

plemented with 100 unit/mL penicillin, 100 mg/mL streptomycin and 10% heat-inactivated FBS at 37 °C in 5 % CO_2 with 95 % humidity.

3. Animals

The original stock of male ICR mice (4 weeks old) was purchased from the Dae-Han Experimental Animal Center (Eumsung, Chungbuk, Republic of Korea). The animal care and experimental procedures were performed under approval from the animal care committee of Kyung Hee University [KHUASP(SE)-12-022].

4. Histamine assay

The HMC-1 cells (2×10^5) was for 40 min prior to stimulation with compound 48/80 incubated for 25 min. Histamine in sera and the cell supernatant were measured by the OPA spectrofluorometric procedure [21]. The fluorescent intensity was measured at 440 nm (excitation at 360 nm) with a spectrofluorometer.

5. Compound 48/80-induced systemic anaphylaxis

Mice were given an intraperitoneal (i.p.) injection of the mast cells degranulator, compound 48/80 (8 mg/kg). The period for observation of mortality was based on the control mice, which had died within 20 min of receiving compound 48/80. Mortality was monitored for 40 min after the induction of anaphylactic shock.

6. Compound 48/80-induced ear swelling response

Compound 48/80 was injected intradermally (100 μg/site) into the dorsal side of a mouse ear using a microsyringe with a 28-gauge hypodermic needle. Ear thickness was measured with a digimatic micrometer (Mitutoyo, Japan) under mild anesthesia. The ear swelling response corresponded to an increase of thickness above baseline control values and was determined 40 min after compound 48/80 or saline injection. Ketotifen (oral or PA, ST36 acupoint; 2 mm lateral to the anterior tubercle of the tibia and 3 mm below the knee joint) was administered 1 h before compound 48/80-injection. The values obtained appear to represent the effect of compound 48/80 rather than the effect of saline injection (physical swelling), since the ear swelling response evoked by physiologic saline almost returned to baseline thickness within 40 min

7. Intracellular calcium measurement

To measure the intracellular calcium in a time-dependent manner, the HMC-1 cells (1×10^5) were pretreated with Fura-2/AM in IMDM containing 10 % FBS for 30 min. After being washed twice with a calcium free medium containing 0.5 mM EGTA, the cell suspension was placed into a 96-well plate and pretreated with ketotifen (0.02, 0.2, and 2 μg/mL) and then stimulated with PMACI. The kinetics of the intracellular calcium was recorded every 10 s at 440 nm (excitation at 360 nm) in a spectrofluorometer.

8. Passive cutaneous anaphylaxis (PCA)

An IgE dependent PCA was generated by sensitizing the skin with an intradermal injection of anti-DNP-IgE followed 48 h later with an injection of DNP-HSA into the mice tail vein. The DNP-HSA was diluted in PBS. The mice were injected intradermally with 100 ng of anti-DNP-IgE into each of three dorsal skin sites that had been shaved 48 h earlier. The sites were outlined with a water-insoluble red marker. 48 h later, each mouse received an injection of 200 μL of the 1:1 mixture of 1 mg/mL DNP-HSA in PBS and 4 % evans blue via the tail vein. One hour before this injection, ketotifen was administered orally or injected into ST36 acupoint (PA 0.02, 0.2, and 2 mg/kg). The mice were euthanized 40 min after the intravenous challenge. The dorsal skin of each mouse was removed for measurement of the pigment area. The amount of dye was then determined colorimetrically after extraction with 0.5 ml of 1 M KOH and 4.5 mL of a mixture of acetone and phosphoric acid (with the ratio of 5:13). The absorbent intensity of the extraction was measured at 620 nm in a spectrophotometer, and the amount of dye was calculated with the Evans blue measuring line.

9. Enzyme-linked immunosorbent assay (ELISA)

HMC-1 cells (3×10^5) were treated with ketotifen (0.02, 0.2, and 2 μg/mL), for 1 h prior to stimulation with PMA plus A23187 (PMACI) incubated for 8 h. IL-1β in serum and cell supernatant were measured by ELISA. The ELISA was performed by coating 96-well plates with 1 mg/well of capture Ab. Before the subsequent steps in the assay, the coated plates were washed twice with 1 × PBS containing 0.05 % Tween 20 (PBST). All reagents and coated wells used in this assay were incubated for 2 h at room temperature. The standard curve was generated from known concentrations of cytokine, as provided by the manufacturer. After exposure to the medium, the assay plates were exposed sequentially to each of the biotin-conjugated secondary antibodies, and AP, and ABTS substrate solution containing 30 % H_2O_2. The plates were read at 405 nm. Appropriate specificity controls were included, and all samples were run in duplicate. Cytokine level in spleen and nasal mucosa was divided according to the total protein. Protein was determined using a BCA.

10. MTT assay

HMC-1 cell aliquots (3×10^5 cells/mL) were cultured in microplate wells for 8 h after treatment by ketotifen (0.02, 0.2, and 2 μg/mL) and incubated with 20 μL of an MTT solution (5 mg/mL) for additional 4 h at 37 °C under 5 % CO_2 and 95 % air. Consecutively, 250 μL of DMSO was added to extract the MTT formazan and the absorbance of each well at 540 nm was read by an automatic microplate reader.

11. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cell according to the manufacturer's specification using easy-BLUETM RNA extraction kit (iNtRON Biotech, Korea). The concentration of total RNA in the final elutes was determined by spectrophotometry. Total RNA (2.5 mg) was heated at 65 °C for 10 min and then chilled on ice. Each sample was reverse-transcribed to cDNA for 90 min at 37 °C using cDNA synthesis kit (Amersham Pharmacia Biotech, Piscataway, NJ, United States). PCR was performed with the following primers for human IL-1β (5' GGG GTA CCT TAG GAA GAC ACA AAT TG 3'; 5' CCG GAT CCA TGG CAC CTG TAC GAT CA 3'), and human GAPDH (5' CCT GCT TCA CCA CCT TCT TG 3'; 5' CAA AAG GGT CAT CAT CTC TG 3') was used to verify whether equal amounts of RNA were used for reverse transcription and PCR amplification from different experimental conditions. The annealing temperature was 50°C for human IL-1β and 60 °C for GAPDH. Products were electrophoresed on a 1.5 % agarose gel and visualized by staining with ethidium bromide.

12. Western blot analysis

The cells were prepared by a detergent lysis procedure. Samples were heated at 95 °C for 5 min, and briefly cooled on ice. Following centrifugation at 15,000 × g for 5 min, 50 mg aliquots were resolved by 10 % SDS-PAGE. Resolved proteins were electrotransferred overnight to nitrocellulose membranes in 25 mM Tris, pH 8.5, 200 mM glycerin, 20 % methanol at 25 V. Blots were blocked for at least 2 h with PBST containing 5 % nonfat dry milk and then incubated with primary antibodies for 1 h at room temperature. Blots were developed by peroxidase-conjugated secondary antibodies, and proteins were visualized by enhanced chemiluminescence procedures (Amersham Bioseciences, Piscataway, NJ, United States) according to the manufacturer's instructions.

13. Caspase-1 assay

Caspase-1 activity was measured according to the manufacturer's