Full Paper

***Ephedrae herba* in *Mao-Bushi-Saishin-To* Inhibits IgE-Mediated Histamine Release and Increases cAMP Content in RBL-2H3 Cells**

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Abstract. Acute effect of *Mao-Bushi-Saishin-To* (*Ma-Huang-Fu-Zi-Xi-Xin-Tang* in Chinese: MBS) on histamine release was investigated. The IgE-mediated anaphylactic response in Wistar rats was significantly suppressed by MBS and *Mao*. However, *Saishin* and *Bushi* had no or little effect on the antigen-mediated anaphylactic reaction. *Mao* as well as MBS but not *Saishin* nor *Bushi* inhibited IgE-mediated histamine release from rat basophilic leukemia (RBL-2H3) cells. Consistently, MBS and *Mao* but not *Bushi* nor *Saishin* increased cAMP levels in RBL-2H3 cells. However, ephedrine, methylephedrine, and pseudoephedrine, the main components in *Mao*, did not affect histamine release. From these results, increase of cAMP levels may account for the inhibitory effect of *Mao* on histamine release. Furthermore, these inhibitory actions of MBS were mainly due to *Mao* with an ingredient(s) different from ephedrines.

Keywords: *Mao-Bushi-Saishin-To*, *Ephedrae herba*, cAMP, RBL-2H3 cell, histamine release

Introduction

Mast cells are distributed widely throughout the body and are important in the development of acute inflammation, allergic disorders such as asthma, some cancers, and in defense against helminthic parasites (1). Within seconds to minutes of activation, mast cells release from their secretory granules histamine and other mediators such as enzyme and chemotactic factors. Released histamine increases vascular permeability and recruits inflammatory leukocytes (2). The rat basophilic leukemia cell line RBL-2H3 is a cultured analogue of mucosal mast cells and is an excellent model system in which to study the mechanism of secretion (3).

The Kampo medicine *Mao-Bushi-Saishin-To* (*Ma-Huang-Fu-Zi-Xi-Xin-Tang* in Chinese: MBS) contains the three herbal constituents *Mao* (*Ephedra* herb), *Bushi* (*Aconiti* tuber), and *Saishin* (*Asiasarum* root), in a ratio of 4:1:3, and has long been prescribed for the treatment of various inflammatory disease (4). MBS has been reported to be effective for the treatment of various symptoms of cold, especially nasal congestion and for seasonal allergic rhinitis (5, 6). It has been reported

that *Mao* (7) and *Saishin* (8) each have anti-allergic action and *Bushi* (9) has anti-inflammatory action.

Recently, Ikeda et al. described the anti-allergic mechanism of MBS given in long-term treatment (10), whereas the acute effect of MBS is still not clear. Here in this report, we have studied the effect of MBS on the release of histamine and its effect in vivo and in vitro.

Materials and Methods

Homologous passive cutaneous anaphylaxis (PCA) in rats

Male Wistar rats (7-week-old) were purchased from Japan SLC (Hamamatsu). Rats were sensitized i.d. with the mouse anti-dinitrophenyl (DNP) monoclonal antibody (Seikagaku Co., Tokyo) preparation (5 µg/kg) for 24 h on their shaved backs. Subsequently they were injected with 100 µl, i.p. of the drugs. After 60 min, they were anesthetized with sodium pentobarbital (40 mg/kg, i.p.), and challenged (i.v.) with 25 mg/kg dinitrophenylated bovine serum albumin (DNP-BSA; Cosmobio, Tokyo) containing 1% Evans blue. Thirty min later, the rats were killed and the amount of extravasation was evaluated by measuring long and short diameters of the area of the dye spots.

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Histamine-induced cutaneous reaction

The rats were injected with 100 μ l, i.p. of the drugs. Sixty minutes later, 1% Evans blue was introduced i.v., and subsequently histamine (0.5 mg/kg) was injected on their shaved backs. Thirty minutes later, the rats were killed and the skin was removed to measure the area of the dye as described above.

Cell culture

RBL-2H3 cells (Japanese Cancer Research Resources Bank, Wako) were grown in Eagle's minimum essential medium (EMEM; Nissui Pharmaceutical, Tokyo) with 10% heat-inactivated fetal bovine serum (ICN Pharmaceuticals, Tokyo) at 37°C in an atmosphere of 5% CO₂ in air. For experiments, the cells were seeded at a density of 10⁵ cells/well in a 12-well culture plate and were used 3 days after seeding.

Measurement of histamine release from RBL-2H3 cells

The cells plated in culture dishes were washed with a PIPES buffer consisting of 140 mM NaCl, 5 mM KCl, 0.6 mM MgCl₂, 1.0 mM CaCl₂, 5.5 mM glucose, and 10 mM piperazine-*N,N'*-bis (2-ethanesulfonic acid) (PIPES), pH 7.4. The cells were passively sensitized with 0.5 mg/mL mouse anti-DNP IgE for 60 min. The cells were then washed with PIPES buffer, incubated with drugs for 10 min, and then stimulated with 0.1 mg/mL DNP-BSA for 30 min at 37°C.

Histamine contents in the supernatant and in the cells were determined fluorometrically according to the procedure described by Shore et al. (11). Histamine release (%) was expressed as the percentage of the amount of released histamine to the total amount of histamine (released and intracellular remaining histamine). Since MBS and its constituents are brown in color, we measured the fluorescence of the sample containing MBS or its constituents without histamine and subtracted the values as background fluorescence to avoid the interference of these colors with colorimetric measurement.

cAMP content

The cAMP content was measured with a radioimmunoassay. A phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX, 1 mM), was added 5 min before the treatment with or without drugs into PIPES buffer. After an additional 5 min, PIPES buffer were discarded and the samples in the multi-well plate were dried at 37°C for 10 min. Thereafter, the cells were treated with 5% trichloroacetic acid solution. After centrifugation, trichloroacetic acid in the supernatant was removed by washing with water-saturated ether, and the succinylated cAMP was assayed in a competi-

tive radioimmunoassay (Yamasa Co., Choshi) using a gamma counting system (Packard Cobra, Albertville, MN, USA).

Materials

Drugs and their sources were as follows: *Mao-Bushi-Saishin-To* (MBS), which was the hot-water extract from the mixture of *Ephedra* herb (*Mao*)/*Aconiti* tuber (*Bushi*)/*Asiasarum* root (*Saishin*) = 4/1/3. The respective extracts from *Mao*, *Bushi*, and *Saishin* were also used. All the Chinese plants employed in this study were imported from China and their extracts were prepared by the same method: Each was extracted with a defined volume of boiling water for 1 h and then the extract was lyophilized. The same lots of these extracts and materials were used throughout the experiments. All of these MBS and its constituents were kindly supplied from Kotaro Pharm. Co., Ltd (Osaka).

Ephedrine, methylephedrine, and pseudoephedrine were isolated as reported previously (7). All other chemicals were purchased from Wako (Osaka).

Statistics

Results of the experiments are expressed as means $\pm$ S.E.M. Significance was tested with Student's *t*-test, or Dunnett's multiple comparison test when comparisons involved more than two groups. *P* values smaller than 0.05 were considered significant.

Results

Passive cutaneous anaphylaxis in rats

In the sensitized rats with anti-DNP IgE, injection of DNP-BSA caused the leak of Evans blue into the skin, which can be the index of the intensity of homologous PCA reactions. The effects of 1000 mg/kg MBS and its herbal constituents in the same amount as in MBS (500 mg/kg *Mao*, 125 mg/kg *Bushi*, or 375 mg/kg *Saishin*) on homologous PCA in rats were compared with that of 1 mg/kg ketotifen, which is an inhibitor of chemical mediator-release as well as an antagonist of H₁ receptor. MBS significantly inhibited the PCA reaction in rats to an extent comparable to ketotifen (Fig. 1).

Among three constituents, *Mao* significantly inhibited the IgE-mediated allergic reaction to a similar level as MBS. *Bushi* was not effective in this experiment, while *Saishin* showed slight inhibitory effect (Fig. 1).

MBS and *Mao* inhibited the PCA reaction in a concentration-dependent manner with the ED₅₀ values of 326 and 224 mg/kg, respectively (Fig. 2).

Histamine release from RBL-2H3 cells

DNP-BSA (0.1 μ g/mL) induced a high level of

Fig. 1. Effects of *Mao-Bushi-Saishin-To*, its constituents, and ketotifen on homologous PCA reaction in rats. Animals were passively sensitized by injection of anti-DNP IgE (5 μ g/kg, i.d.). *Mao-Bushi-Saishin-To* (MBS, 1000 mg/kg), *Mao* (500 mg/kg), *Bushi* (125 mg/kg), *Saishin* (375 mg/kg), or ketotifen (1 mg/kg) was administered i.p. 60 min prior to injection of DNP-BSA (25 μ g/kg, 0.5 ml, i.v.) containing 1% Evans blue. Thirty minutes after antigen challenge, the area of dye was measured after sacrificing the animals. Each column represents the mean $\pm$ S.E.M. of 4–6 animals. * P <0.05, ** P <0.01; Dunnett's multiple comparison test.

Fig. 2. Concentration-dependency of inhibitory effect of *Mao-Bushi-Saishin-To* and *Mao* on homologous PCA reaction in rats. Animals were passively sensitized by i.d. injection of anti-DNP IgE (5 μ g/kg). Each concentration of MBS or *Mao* was administered i.p. 60 min before injection of DNP-BSA (25 μ g/kg, 0.5 ml, i.v.) containing 1% Evans blue. Thirty minutes after antigen challenge, the area of dye was measured after sacrificing the animals. Each point represents the mean $\pm$ S.E.M. of 4 animals. * P <0.05, ** P <0.01; Dunnett's multiple comparison test.

secretion from anti DNP IgE-primed RBL-2H3 cells. MBS and *Mao* inhibited IgE-mediated histamine release in a concentration-dependent manner. The result that

Fig. 3. Concentration-inhibition relationships of *Mao-Bushi-Saishin-To* and *Mao* on antigen-stimulated histamine release from RBL-2H3 cells. RBL-2H3 cells were incubated with 0.5 μ g/ml anti-DNP IgE antibody for 1 h prior to application of MBS or *Mao* with the indicated concentrations. After incubation of 10 min, the cells were stimulated with 0.1 μ g/ml DNP-BSA for 20 min. Each point represents the mean $\pm$ S.E.M. of 3 independent experiments.

Fig. 4. Effects of three constituents of *Mao-Bushi-Saishin-To* on antigen-stimulated histamine release from RBL-2H3. RBL-2H3 cells were incubated with 0.5 μ g/ml anti-DNP IgE antibody at 1 h prior to incubation 10 min with vehicle or 200 μ g/ml of *Mao*, *Bushi*, or *Saishin*. Subsequently, the cells were stimulated with 0.1 μ g/ml DNP-BSA for 20 min. Each column represents the mean $\pm$ S.E.M. of 3 independent experiments. * P <0.05, Dunnett's multiple comparison test.

the inhibitory effect of MBS is almost identical to *Mao* with the half concentration of MBS, may be reflect the amount of *Mao* in MBS (Fig. 3).

Among the three constituents of MBS, only *Mao* showed an inhibitory effect on anti-IgE-induced histamine release, while *Bushi* and *Saishin* did not inhibit it even at the concentrations of 200 μ g/mL (Fig. 4).

Effect of ephedrine on PCA reaction in rats and histamine release from RBL-2H3 cells

Effect of ephedrine, main component in *Mao*, on the PCA reaction in rats was examined. Ephedrine did not show any significant effect on PCA even at 20 mg/kg (Fig. 5A). Ephedrine and its derivatives, methylephedrine and pseudoephedrine, did not affect the IgE-mediated histamine release in a concentration range of 50 to 200 $\mu\text{g/ml}$ (Fig. 5B).

cAMP contents in RBL-2H3 cells

As shown in Fig. 6A, MBS increased cAMP content in RBL-2H3 cells at the concentration that inhibited the histamine release from RBL-2H3 cells (Fig. 3). *Mao* also increased cAMP content (Fig. 6B). However, neither 200 $\mu\text{g/ml}$ of *Bushi* nor *Saishin* significantly increased cAMP level in RBL-2H3 cells (Fig. 6B).

Histamine-induced cutaneous reaction in rats

Effects of MBS and its constituents on histamine-induced cutaneous reaction in rats were examined to evaluate their effect on the function of released histamine. As shown in Fig. 7A, MBS and *Mao* as well as ketotifen, inhibited histamine-induced the cutaneous reaction. *Bushi* and *Saishin* also inhibited the reaction slightly but significantly. On the contrary, ephedrine showed no effect (Fig. 7B).

Discussion

We have studied the acute anti-allergic action of MBS in in vivo and in vitro. As Shibata et al. reported previously (12), MBS inhibited PCA in dose-dependent manner. When the effects of each constituent in the same amount as in MBS were compared, *Mao* inhibited PCA to the similar level as MBS did. Therefore, it is reasonable to conclude that the main part of the MBS-induced inhibition of PCA is due to *Mao*.

To determine the site of action of MBS, we examined the effects of MBS and its constituents on histamine release from IgE-activated RBL-2H3 cells. Higher concentrations of MBS inhibited histamine-release in a concentration-dependent manner; and among the three constituents, only *Mao*, but not *Bushi* nor *Saishin*, inhibited it. Comparing the concentration and inhibitory effect of MBS and *Mao*, it is reasonable to conclude that *Mao* may account for the main part of the inhibitory effect of MBS on histamine-release since the content of *Mao* in MBS is 50%.

Consistent results were observed that MBS increased cAMP content in RBL-2H3 cells with the similar concentrations that inhibit histamine release. This effect was also observed only in *Mao* and neither *Bushi* nor

Fig. 5. Effect of ephedrine on homologous PCA reaction in rats (A) and effects of the ephedrine derivatives on antigen-stimulated histamine release from RBL-2H3 cells (B). A: Animals were passively sensitized by injection of anti-DNP IgE (5 $\mu\text{g/kg}$, i.d.). Each concentration of ephedrine was administered i.p. 60 min prior to injection of DNP-BSA (25 $\mu\text{g/kg}$, 0.5 ml, i.v.) containing 1% Evans blue. Thirty minutes after antigen challenge, the area of dye was measured after sacrificing the animals. Each column represents the mean $\pm$ S.E.M. of 4–6 animals. B: RBL-2H3 cells were incubated with 0.5 $\mu\text{g/ml}$ anti-DNP IgE antibody for 1 h and then incubated with each concentration of the ephedrine, methylephedrine, or pseudoephedrine for 10 min. Finally, the cells were stimulated with 0.1 $\mu\text{g/ml}$ DNP-BSA for 20 min. Each point represents the mean $\pm$ S.E.M. of 3 independent experiments.

Saishin increased the cAMP level. It is well established that the increase of cAMP interferes with the function of mast cells (13, 14) or RBL-2H3 cells (15, 16), so the increase of cAMP may account for the inhibitory effect of MBS on histamine release in RBL-2H3 cells.

Fig. 6. Effects of *Mao-Bushi-Saishin-To* and its constituents on cAMP contents in RBL-2H3 cells. A: RBL-2H3 cells were treated with each concentration of *Mao-Bushi-Saishin-To* (MBS) for 10 min in the presence of 1 mM IBMX, and the accumulation was terminated with 5% TCA. B: RBL-2H3 cells were treated with *Mao* (500 µg/ml), *Bushi* (125 µg/ml), or *Saishin* (375 µg/ml) for 10 min in the presence of 1 mM IBMX, and the accumulation was terminated with 5% TCA. cAMP contents of the treated cells were normalized with that of the untreated cells. * $P < 0.05$, Dunnett's multiple comparison test (A). ** $P < 0.01$, Student's *t*-test (B).

MBS and *Mao* significantly inhibited the histamine-induced cutaneous reaction in rats. However, when cAMP increased in the microvasculature, it may affect the intracellular signal transduction and prevent vaso-permeabilization (17–19). Accordingly, a compound that increases cAMP may act in a similar manner to an anti-histaminergic compound. Therefore, the result that MBS and *Mao* inhibited the histamine-induced cutaneous reaction is not unexpected. However, this result does not exclude the possibility of an anti-hista-

Fig. 7. Effects of *Mao-Bushi-Saishin-To*, its constituents, and ketotifen on histamine-induced cutaneous reaction in rats. *Mao-Bushi-Saishin-To* (MBS, 1000 mg/kg), *Mao* (500 mg/kg), *Bushi* (125 mg/kg), *Saishin* (375 mg/kg), or ketotifen (1 mg/kg) was administered i.p. 60 min prior to injection of 1% Evans blue followed by i.d. application of histamine (0.5 mg/kg) (A). Each concentration of ephedrine was administered i.p. 60 min prior to injection of 1% Evans blue followed by i.d. application of histamine (0.5 mg/kg) (B). Thirty minutes after application of histamine, the area of dye was measured after sacrificing the animals. Each column represents the mean $\pm$ S.E.M. of 4–6 animals. * $P < 0.05$, ** $P < 0.01$; Dunnett's multiple comparison test.

minergic effect of MBS. Indeed, *Bushi* and *Saishin* show a small but significant inhibition of the histamine-induced cutaneous reaction. Further experiments are necessary to evaluate this possibility.

On the other hand, ephedrine showed no effect on histamine-induced cutaneous reaction and on PCA. These results are consistent with the result that ephedrine did not inhibit histamine release from RBL-

2H3 cells. However, Shibata et al. (12) demonstrated that p.o. administration of ephedrine inhibited PCA, while in our result, i.p. application did not inhibit it. The reason for this discrepancy is not clear and further studies are required.

Since ephedrine derivatives, major components of *Mao*, had no effect on histamine release, another component distinct from ephedrine derivatives in *Mao* increase cAMP. There are some reports that *Ephedra* herb or *Ephedra* root contain alkaloids, other than ephedrines, that show hypotensive activity (20–22). Since the increase of cAMP decreases blood pressure, these alkaloids may be the candidates accounting for the inhibitory effect on histamine-release. Furthermore, some flavonoids are reported as a PDE inhibitor or inhibiting anaphylaxis or an inhibitor of chemical mediator release from mast cells (for review, see 23). At this moment, there is no information about whether *Mao* contains flavonoids identical with or similar to those reported ones; however, flavonoids may be other candidates for the effect on histamine release.

Taken together, an ingredient other than ephedrine derivatives in *Mao* are responsible for the increase of cAMP and resulting inhibition of histamine release from RBL-2H3 cells and also resulting in the inhibition of the increase of vasopermeability. This effect may account for the inhibitory effect of MBS on IgE-mediated PCA.

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References

- 1 Tamasky PR, Van Arsdel PP. Antihistamine therapy in allergic rhinitis. *J Fam Pract*. 1990;30:71–80.
- 2 Hart PH. Regulation of the inflammatory response in asthma by mast cell products. *Immunol Cell Biol*. 2001;79:149–153.
- 3 Seldin DC, Adelman S, Austen KF, et al. Homology of the rat basophilic leukemia cell and the rat mucosal mast cell. *Proc Natl Acad Sci USA*. 1985;82:3871–3875.
- 4 Ukai K, Taya M, Sakakura Y, et al. Clinical evaluation of *mao-bushi-saishin-to* extract for perennial nasal allergy. *ORL Clin*. 1990;83:155–165. (text in Japanese with English abstract).
- 5 Ito H, Baba S, Takagi I, et al. Clinical studies on the prophylactic effect of Chinese herbal medicine (*Mao-Bushi-Saishin-To*) on Japanese cedar pollinosis. *Otol Fukuoka*. 1991;52:89–106. (text in Japanese with English abstract).
- 6 Ohashi Y, Nakai Y, Furuya H, et al. Clinical effect of *Mao-Bushi-Saishin-To* in patients with obstinate nasal blockage due to perennial rhinitis. *Otol Fukuoka*. 1992;85:1845–1853. (text in Japanese with English abstract).
- 7 Hikino H, Konno C, Takata H, Tamada M. Antiinflammatory principle of *Ephedra* Herbs. *Chem Pharm Bull (Tokyo)*. 1980;28:2900–2904.
- 8 Hashimoto K, Yanagisawa T, Okui Y, Ikeya Y, Maruno M, Fujita T. Studies on anti-allergic components in the roots of *Asiasarum sieboldi*. *Planta Med*. 1994;60:124–127.
- 9 Hikino H, Konno C, Takata H, et al. Antiinflammatory principles of *Aconitum* roots. *J Pharmacobiodyn*. 1980;3:514–525.
- 10 Ikeda Y, Iizuka A, Amagaya S, Ishige A, Komatsu Y, Fujihiro E. Anti-type I allergic mechanism of *Mao-bushi-saishin-to* in mice. *Jpn J Pharmacol*. 2000;82:29–33.
- 11 Shore PA, Alan B, Cohn VH Jr. A method for the fluorometric assay of histamine in tissues. *J Pharmacol Exp Ther*. 1959;127:182–186.
- 12 Shibata H, Nabe T, Yamamura H, Kohno S. *l*-Ephedrine is a major constituent of *Mao-Bushi-Saishin-To*, one of the formulas of Chinese medicine, which shows immediate inhibition after oral administration of passive cutaneous anaphylaxis in rats. *Inflamm Res*. 2000;49:398–403.
- 13 Weston MC, Anderson N, Peachell PT. Effects of phosphodiesterase inhibitors on human lung mast cell and basophil function. *Br J Pharmacol*. 1997;121:287–295.
- 14 Weston MC, Peachell PT. Regulation of human mast cell and basophil function by cAMP. *Gen Pharmacol*. 1998;31:715–719.
- 15 Ali H, Sozzani S, Fisher I, et al. Differential regulation of formyl peptide and platelet-activating factor receptors — Role of phospholipase $C\beta_3$ phosphorylation by protein kinase A. *J Biol Chem*. 1998;273:11012–11016.
- 16 Yue C, Dodge KL, Weber G, Sanborn BM. Phosphorylation of serine 1105 by protein kinase A inhibits phospholipase $C\beta_3$ stimulation by $G\alpha_q$. *J Biol Chem*. 1998;273:18023–18027.
- 17 Ikeda K, Utoguchi N, Makimoto H, Mizuguchi H, Nakagawa S, Mayumi T. Different reactions of aortic and venular endothelial cell monolayers to histamine on macromolecular permeability: role of cAMP, cytosolic Ca^{2+} and F-actin. *Inflammation*. 1999;23:87–97.
- 18 Stevens T, Garcia JGN, Shasby DM, Bhattacharya J, Malik AB. Mechanisms regulating endothelial cell barrier function. *Am J Physiol*. 2000;279:L419–L422.
- 19 Irie K, Fujii E, Ishida H, et al. Inhibitory effects of cyclic AMP elevating agents on lipopolysaccharide (LPS)-induced microvascular permeability change in mouse skin. *Br J Pharmacol*. 2001;133:237–242.
- 20 Kasahara Y, Hikino H, Tsurufuji S, Watanabe M, Ohuchi K. Antiinflammatory actions of ephedrines in acute inflammations. *Planta Med*. 1985;51:325–331.
- 21 Hikino H, Ogata K, Konno C, Sato S. Hypotensive actions of ephedradines macrocyclic spermine alkaloids of *Ephedra* roots. *Planta Med*. 1983;48:290–293.
- 22 Hikino H, Kiso Y, Ogata M, et al. Pharmacological actions of analogues of feruloylhistamine, an imidazole alkaloid of *Ephedra* roots. *Planta Med*. 1984;50:478–480.
- 23 Middleton E Jr, Kandaswami C, Theoharides TC. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacol Rev*. 2000;52:673–751.