Inhibitory Effect of Mao-Bushi-Saishin-to on Prostaglandin E$_2$ Synthesis in C6 Rat Glioma Cells

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The Kampo medicine Mao-Bushi-Saishin-to (Ma-Huang-Fu-Zi-Xi-Xin-Tang in Chinese: MBS) is containing three herbal constituents of Mao (Ephedra herb), Bushi (Aconitum tuber) and Saishin (Asiasarum root), in a ratio of 4 : 1 : 3, and has long been prescribed the treatment of various inflammatory disease.$^1$ MBS has been reported to be effective for the treatment of various symptoms of cold, especially nasal congestion and for seasonal allergic rhinitis.$^2,3$ Recently, Ikeda et al. demonstrated that the inhibitory effect on passive cutaneous anaphylaxis of MBS.$^3$ Therefore, in this report, we have studied the effect of MBS and its constituents on prostaglandin E$_2$ (PGE$_2$) production in C6 cells. $^a$

**Effect of Mao-Bushi-Saishin-to (Ma-Huang-Fu-Zi-Xi-Xin-Tang: MBS) on prostaglandin E$_2$ (PGE$_2$) production**

was investigated using C6 rat glioma cells. Mao or Saishin inhibited histamine-induced PGE$_2$ production while MBS slightly decreased and Bushi increased it. MBS and Mao inhibited and Bushi enhanced A23187-induced PGE$_2$ production while Saishin had no effect. Concomitantly, Mao inhibited, but Bushi facilitated, histamine- and A23187-induced phosphorylation of extracellular signal-regulated kinase (ERK)1/2. Treatment of MBS, Mao and also Saishin increased cAMP content. From these results, MBS inhibit PGE$_2$ production in C6 cells, mainly due to Mao but also due to Saishin at least in part, and the counteraction of Bushi. The former effect is mediated by formation of cAMP and resulting inhibition of ERK1/2-phosphorylation.

**Key words** Mao-Bushi-Saishin-to; Ephedrae Herba; C6 rat glioma cell; prostaglandin E$_2$

The Kampo medicine Mao-Bushi-Saishin-to contains three herbal constituents of Mao (Ephedra herb), Bushi (Aconitum tuber) and Saishin (Asiasarum root), in a ratio of 4 : 1 : 3, and has long been prescribed the treatment of various inflammatory disease. Mao has been reported to be effective for the treatment of various symptoms of cold, especially nasal congestion and for seasonal allergic rhinitis. Recently, Ikeda et al. described the anti-allergic mechanism of MBS treated in long-period, whereas acute effect of MBS is also reported. In the previous report, we have demonstrated that Mao increased the cAMP content in RBL-2H3 cell and this effect account for its inhibitory effect on histamine-release and thus, the inhibitory effect on passive cutaneous anaphylaxis of MBS.

In the brain, prostaglandin E$_2$ (PGE$_2$) levels are very low or undetectable in normal conditions, but can rise during inflammatory processes, multiple sclerosis, and AIDS-associated dementia. High levels of PGE$_2$ can affect the activities of several cell types, including neurons, glial, and endothelial cells, and can regulate microglia/macrophage and lymphocyte functions during inflammatory and immune processes. Therefore, the interplay between PGE$_2$ and other local factors, including pro- and anti-inflammatory cytokines, is likely to influence the outcome of inflammatory and immune responses in the central nervous system (CNS). Glial cells are assumed to be an important source of PGs in the CNS, which are similar to macrophages in that they may be activated by bacterial endotoxin and/or cytokines to produce PGE$_2$ in the CNS.

Prostanoids, arachidonic acid metabolites produced from a variety of inflammatory cells upon stimulation, are thought to be involved in the pathogenesis of diseases. Prostanoid synthesis is regulated by two successive metabolic steps, the release of arachidonic acid from membrane phospholipids by phospholipase A$_2$, and its conversion to prostanoids by cyclooxygenase. Cytosolic PLA$_2$ (cPLA$_2$) is a ubiquitously distributed 85-kDa enzyme, the activation of which is tightly regulated by postreceptor transmembrane signaling. cPLA$_2$ is activated by extracellular signal-regulated kinase (ERK)1/2 when cytosolic Ca$^{2+}$ concentrations are in the sub-micromolar or micromolar range.

It has been shown that glial cells express neurotransmitter receptors, including H$_1$ histaminergic receptor, which are coupled to intracellular Ca$^{2+}$ mobilization, resulting activation of cPLA$_2$. For the present studies we used rat C6 glioma cells because they are derived from astrocytes and express many astrocytic properties in culture.

Cyclic AMP can inhibit the ERK pathway in C6 glioma cells. Furthermore, we have previously demonstrated that baikaline, a flavonoid contained in Scutellaria root, inhibited prostaglandin E$_2$ synthesis and the phosphorylation of ERK1/2 in C6 rat glioma cells. Therefore, in this report, we have studied the effect of MBS and its constituents on prostaglandin E$_2$ synthesis in C6.

**MATERIALS AND METHODS**

**Cell Culture** C6 rat glioma cells were grown in F-10 medium containing 15% horse serum and 2.5% fetal bovine serum in a 37°C humidified incubator in an atmosphere of 5% CO$_2$ in air, as described previously.

**Assay of Prostaglandin E$_2$** C6 cells were seeded into 12-well plates at the density of 1.0×10$^5$ cells per well. The experiment was performed 2 d after cell seeding. The cells were washed twice with Eagle’s minimum essential medium (EMEM) buffered with 20 mM HEPES, pH 7.4 (EMEM-HEPES) and were preincubated with or without MBS or its constituents for 10 min. The cells were stimulated with histamine or A23187 and incubated for additional 10 min. The medium was acidified to pH 4.0 by addition of 1 N HCl, and prostaglandin E$_2$ was extracted twice with ethyl acetate. After ethyl acetate was evaporated under a stream of N$_2$ gas, the sample was dissolved in 10 mM Tris–HCl (pH 7.6). Prostaglandin E$_2$ was determined by radioimmunoassay, as described previously.

**Immunoblotting** C6 cells were seeded into 6-well plates at a density of 1.0×10$^5$ cells per well. Two days after seeding, the cells were washed twice with EMEM-HEPES and preincubated with or without MBS or its constituents for...
10 min at 37°C. After the cells were incubated with histamine or A23187 for an additional 2 min, the medium was aspirated. The cells were solubilized and separated by SDS polyacrylamide gel electrophoresis with 11% acrylamide gels.25) Electrically transferred proteins onto Immobilon polyvinylidene difluoride membranes (Millipore, Bedford, U.S.A.) were then visualized using alkaline phosphatase-conjugated goat anti-rabbit IgG (Cell signaling technology, Beverly, U.S.A.) described previously.26

**Cyclic AMP Content** The cyclic AMP 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 additional 5 min, PIPES buffer were discarded and multi-well plate were dried under 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 cyclic AMP was assayed in a competitive radioimmunoassay (Yamasa Co., Choshi, Japan).

**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)/Aconitum (Bushi)/Asiasarum root (Saishin)=4/1/3. The respective extracts from Mao; Bushi; 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 lyophilised. 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, Japan. All other chemicals were purchased from Wako (Osaka, Japan)

**Statistics** Results of the experiments are expressed as means±S.E.M. Significance was tested with Dunnett’s multiple comparison test. p values smaller than 0.05 were considered significant.

**RESULTS**

As shown in Fig. 1A, 100 μM histamine increased prostaglandin E2 synthesis 17.5±2.8 fold of resting state. Pretreatment of Mao (100 μg/ml) prevented this increment. 100 μg/ml Saishin also inhibited. Pretreatment of MBS tended to decrease and pretreatment of Bushi tended to increase it but not significant.

When one applied 10 μM A23187, a Ca2+ ionophore, to elevate intracellular Ca2+ concentration directly, PGE2 production is increased 38.8±4.0 fold of resting level. 100 μg/ml MBS or Mao attenuated this increase. Although Saishin had no effect, Bushi enhanced significantly (Fig. 1B).

Histamine (100 μM) and A23187 (10 μM) increased phosphorylation level of ERK1/2, as shown in Fig. 2. Mao (100 μg/ml) strongly inhibited. On the other hand, 100 μg/ml Bushi increased phosphorylation level.

We have examined the effect of 10 min incubation of C6 cells with MBS or its constituents on cAMP content (Fig. 3). MBS, Mao and also Saishin significantly increased cAMP level in C6 glioma cells.

![Fig. 1. Effects of Mao-Bushi-Saishin-to and Its Constituents on PGE2 Production in C6 Rat Glioma Cells](image1)

**Fig. 1. Effects of Mao-Bushi-Saishin-to and Its Constituents on PGE2 Production in C6 Rat Glioma Cells**

C6 cells were incubated with 100 μg/ml of Mao-Bushi-Saishin-to (MBS), Mao, Bushi or Saishin 10 min prior to application of 100 μM histamine (A) or 10 μM A23187 (B). Ten minutes after stimulation, the culture medium was collected and PGE2 concentration was determined. Each column was normalized with that without stimulation. * p<0.05, ** p<0.01.

![Fig. 2. Effects of Mao-Bushi-Saishin-to and Its Constituents on Phosphorylation Level of ERK1/2](image2)

**Fig. 2. Effects of Mao-Bushi-Saishin-to and Its Constituents on Phosphorylation Level of ERK1/2**

C6 cells were incubated with 100 μg/ml of Mao-Bushi-Saishin-to (MBS), Mao, Bushi or Saishin 10 min prior to application of 100 μM histamine (A) or 10 μM A23187 (B). Two minutes after stimulation, the cell lysate was performed to immunoblotting using anti-phospho-ERK1/2 antibody and visualized with chemical luminescence.

![Fig. 3. Effects of Mao-Bushi-Saishin-to and Its Constituents on cAMP Contents in C6 Cells](image3)

**Fig. 3. Effects of Mao-Bushi-Saishin-to and Its Constituents on cAMP Contents in C6 Cells**

C6 cells were treated with 100 μg/ml of MBS, Mao, Bushi or Saishin for 5 min in the presence of 1 mM IBMX and the accumulation were terminated with 5% TCA. Amount of cAMP was determined by competitive radioimmunoassay.
**DISCUSSION**

We have examined the effect of MBS and its constituents on PGE\(_2\) production in C6 rat glioma cells. In the previous report, we have shown that MBS significantly increased cAMP level at 500 to 1000 \(\mu\)g/ml in RBL-2H3 rat basophilic leukemia cells. Interestingly, 100 \(\mu\)g/ml MBS is sufficient to increase cAMP level in C6 cells which show in this study. This result indicates that C6 cells are more sensitive to MBS for cAMP production. Since 375 \(\mu\)g/ml \textit{Saishin} did not significantly increase cAMP level in RBL-2H3 cells whereas 100 \(\mu\)g/ml is sufficient to increase in C6 cells, \textit{Saishin} may contribute to the effect of MBS. \textit{i.e.}, \textit{Mao} is mainly attribute for inhibitory effect of MBS in RBL-2H3 cells, while \textit{Mao} and also \textit{Saishin} are account for the effect in C6 cells.

\textit{Saishin} inhibited histamine-induced, but not A23187-induced PGE\(_2\) production. Since increase of PGE\(_2\) stimulated C6 cells.

**REFERENCES**


