Bakumondo-to (Mai-Men-Dong-Tang) increases β_1-adrenergic receptor mRNA expression in rat alveolar type II cells

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Abstract

Bakumondo-to (Mai-Men-Dong-Tang) has been used for the treatment of bronchitis and pharyngitis accompanying severe cough. Although the evidence for the efficacy of Bakumondo-to is increasing, the molecular basis of the action has not been established. To determine whether Bakumondo-to has a regulatory effect on gene expression, in the present study, we examined the effect of Bakumondo-to on β_1- and β_2-adrenergic receptor mRNA levels in cultured alveolar type II cells. Bakumondo-to increased β_1-adrenergic receptor mRNA concentration-dependently, whereas it did not change that of β_2- receptor. The effect of Bakumondo-to was considerably similar to that of dibutyryl cAMP. H-89 inhibited the increase in β_1-adrenergic receptor mRNA induced by Bakumondo-to. Furthermore, Bakumondo-to increased cAMP content in alveolar type II cells. These results suggest that Bakumondo-to increases β_1-adrenergic receptor gene expression, and this effect is through the activation of cAMP-dependent signaling.

Key words alveolar type II cell, Bakumondo-to, β-adrenergic receptor, cyclic AMP, RNase protection assay.

Abbreviations Bakumondo-to (Mai-Men-Dong-Tang), 麦門冬湯; cAMP, adenosine 3', 5'-cyclic monophosphate; CTP, cytidine 5'-triphosphate; dibutyryl cAMP, dibutyryladenosine 3', 5'-cyclic monophosphate; DMEM, Dulbecco's modified Eagle's medium; PMA, phorbol myristate acetate.

Introduction

Clinical usage of traditional herbal medicines for various airway diseases has been increasing. Herbal medicines tend to have minor side effects and sometimes produce remarkable efficacy, especially to treat chronic inflammation. Bakumondo-to (Mai-Men-Dong-Tang), which is composed of *Ophiopogonis Tuber, Pinelliae Tuber, Zizyphi Fructus, Glycyrrhiza Radix, Ginseng Radix* and *Oryzae Fructus,* has been used for the treatment of bronchitis and pharyngitis accompanying severe cough.[1,2] There is increasing evidence that Bakumondo-to inhibits cough and airway hyperresposibility,[3,4] activates mucociliary transport,[5] and increases pulmonary surfactant secretion.[6] However, the molecular basis for these effects has not been established. In the light of our previous study and other studies,[4-7] herbal medicines seem to modify the responses to various hormones and cytokines. Considering these results, herbal medicine might regulate the gene expression of receptors for hormones or cytokines. However, it has not been clear whether Bakumondo-to has any regulatory effect on gene expression.

β-Adrenergic receptor regulates many aspects of lung physiological functions including pulmonary surfactant synthesis and secretion by alveolar type II

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cells,[8,9] relaxation of bronchial smooth muscle,[10,11] and activation of mucociliary transport.[12] There are three subtypes in β-adrenergic receptor, β_1, β_2 and β_3.[13] β_1- and β_2-receptors exist in various cell types in the lung, while β_3 does not.[13] Alveolar type II cells express both β_1- and β_2-receptors,[14,15] and increased expression of mRNA for the receptors is associated to increase in the response to cathecolamines.[15] In the present study, therefore, we examined the effect of Bakumondo-to on mRNA level for β_1- and β_2-adrenergic receptors in primary cultured rat alveolar type II cells.

Materials and Methods

Materials: The rats were purchased from Kyudo Farm (Fukuoka, Japan). Extract of Bakumondo-to was a gift from Tsumura Co. Ltd. (Tokyo, Japan). Bulk powder of Bakumondo-to was dissolved into DMEM in a tube by sonication for 20 min. The solution was centrifuged twice and precipitates were removed. It was then sterilized by passing through a filter unit. (Toyo Roshi Kaisha, Ltd, Tokyo Japan), and prepared into several concentrations by using DMEM. Dexamethasone, triiodothyronine, phorbol myristate acetate (PMA) and dibutyryl-adenosine 3', 5'-cyclic monophosphate (dibutyryl cAMP) were purchased from Sigma (St Louis, MO, USA). H-89 and H-85 were from Seikagaku Co. (Tokyo, Japan). [α-^{32}P] cytidine 5'-triphosphate (CTP) (specific activity: 30 TBq / mmol) was from Amersham (Amersham, UK). Fetal bovine serum was from JHR Bioscience (Lenexa, KS, USA).

Alveolar type II cells isolation and culture: Alveolar type II cells were isolated from the lungs of adult pathogen-free male Wistar rats (180-200 g), as described previously.[15] Briefly, trypsin was used to dissociate the cells from the lung tissues. The resultant cell suspension was incubated on rat IgG-coated plastic petri dishes for 30 min, to remove the non-type II cells. The isolated alveolar type II cells were suspended at 1.0×10^6 cells/ml in DMEM containing, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10 % fetal bovine serum which was charcoal-dextran treated for depletion of hormones. The cells were dispensed onto plastic culture dishes at a density of 4×10^5 cells/cm^2 and cultured in 5 % CO_2/95 % air at 37°C. In all experiments, nonadherent cells were removed from the dishes by washing with DMEM after 24 h cultivation.

Measurement of β-adrenergic receptor mRNA levels: Total cellular RNAs were extracted from cultured alveolar type II cells by the acid guanidinium thiocyanate-phenol-chloroform method. Extracted total RNAs were checked for integrity by staining with ethidium bromide following electrophoretic separation in agarose gel containing formaldehyde. Only undegraded samples were used.

β_1- and β_2-adrenergic receptor mRNA levels were determined by RNase protection method as described previously.[14] A 172-bp fragment of β_1-adrenergic receptor cDNA, corresponded to nucleotides +774 to +945 of coding region,[16] and a 183-bp fragment of β_2-adrenergic receptor cDNA, nucleotides -1 to +182[17] were subcloned into pBluescript with a standard method. A 224-nucleotide-long radiolabeled antisense RNA probe for β_1-adrenergic receptor and a 251-nucleotide-long probe for β_2-adrenergic receptor were produced and [α-P^{32}] CTP labeled from pBluescript constructs by mean of an *in vitro* transcription kit (Stratagene) according to the supplier's recommendations.

Total cellular RNA (10 μg) was hybridized with either β_1- or β_2-adrenergic receptor radiolabeled antisense RNA probes (1×10^5 cpm) in 80 % deionized formamide, 100 mM sodium citrate, 300 mM sodium acetate and 1 mM EDTA for 24 h at 60°C. After this hybridization, samples were 10-fold diluted with a buffer containing 10 mM Tris (pH 7.5), 5 mM EDTA, 300 mM NaCl, 2.5 U/ml RNase A and 100 U/ml RNase T, and were incubated at 37°C for 30 min.

After this digestion, the RNase resistant hybrids were precipitated with 7.5 % trichloroacetic acid for 10 min on ice, and collected by vacuum filtration on Whatman GF/C glass-fiber filters. The filters were washed with 7.5 % trichloroacetic acid, dried, and subjected to liquid scintillation spectrometry. The levels of β_1- and β_2-adrenergic receptor mRNA were quantified by use of a standard curve generated by plotting the amounts of sense RNA, produced by *in vitro* transcription, versus the amount of RNase resistant hybrids formed by prior hybridization of sense

RNA with radiolabeled antisense RNA probe.

cAMP assay: The isolated type II cells were cultured 24 h, after which the medium was removed and the cells were washed three times with fresh DMEM without FBS, antibiotics or Bakumondo-to. Fresh DMEM was then added, and the cells were returned to the incubator. After a 30-min preincubation in the fresh medium, drugs or solvent vehicle were added, and the incubation was continued for 5 min, after which the medium was aspirated and the cells were extracted with ice-cold 0.1 N HCl. The extract was immediately frozen, and lyophilized. The sample was reconstituted and acetylated, and the cAMP content was determined as described by the radioimmunoassay kit manufacturer.[15]

Others: Data are presented as the mean±S.E.M. Duncan's multiple-range test was used for statistical analysis. $P<0.05$ was considered to be significant.

Results

Effect of Bakumondo-to on β-adrenergic receptor mRNA expression

Rat alveolar type II cells express both β_1- and β_2-adrenergic receptor mRNAs, as reported previously.[14,15] To examine the effect of Bakumondo-to on these mRNAs expression, we cultured rat alveolar type II cells in the presence of Bakumondo-to for 24 h. Bakumondo-to (1 mg/ml) did not affect cell density, morphology, or cause cytotoxicity as assessed by trypan blue uptake. In control cells, the steady state levels of mRNA for β_1- and β_2-adrenergic receptors were 0.62±0.05 and 1.85±0.12 amol/μg total cellular RNA, respectively (n=5). The level of β_1-adrenergic receptor mRNA was increased by the incubation with Bakumondo-to in a concentration-dependent manner (Fig. 1). At 1 mg/ml, Bakumondo-to caused 2-fold increase in the mRNA. This effective concentration of Bakumondo-to is similar to the value reported for surfactant phospholipid secretion in alveolar type II cells.[6] In contrast, the level of β_2-adrenergic receptor mRNA was not changed (Fig. 1). The effect of Bakumondo-to on the steady state level of β_1-adrenergic receptor at various incubation times is shown in figure 2. Significant increase in the mRNA was not noted within 2 h, although 4 or 24 h of incubation significantly increased the mRNA.

Mechanism of Bakumondo-to-induced mRNA expression

To assess the mechanism of Bakumondo-to-in-

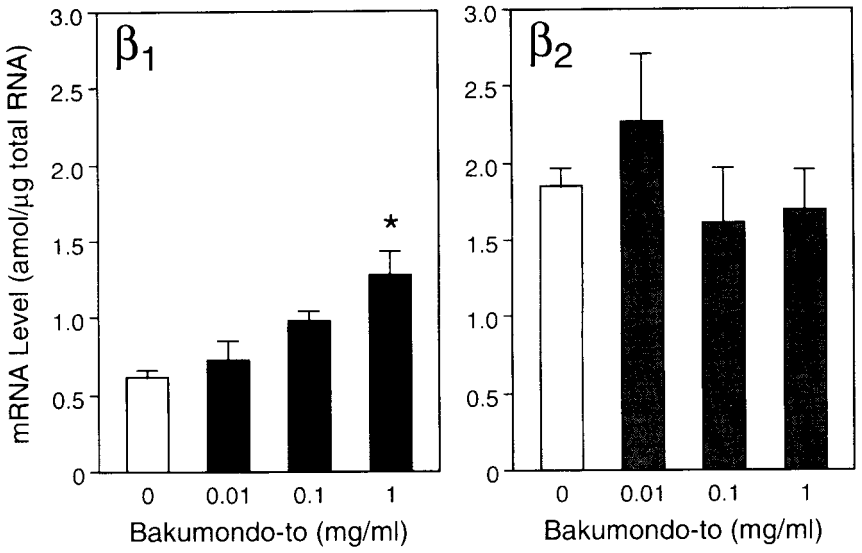

Fig. 1 Concentration-dependent effect of Bakumondo-to on mRNA levels for β_1- and β_2-adrenergic receptors in alveolar type II cells.
Cells were cultured for 24 h with indicated concentration of Bakumondo-to. Total cellular RNA was extracted from cultured cells. The level of mRNA for β_1- and β_2- adrenergic receptors were determined by the RNase protection assay. Each bar represents the mean ± S.E.M. from 4 different experiments. * indicates a significant difference from the control group at $p<0.05$.

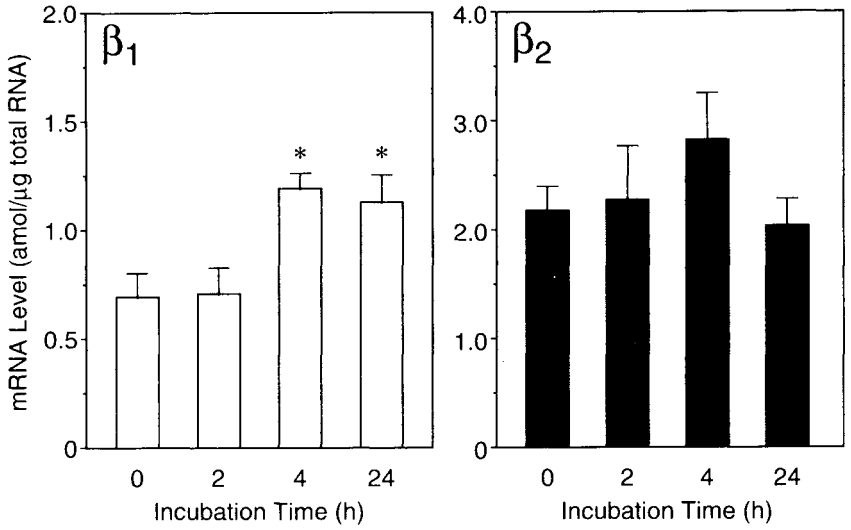

Fig. 2 Time course of the effect of Bakumondo-to on mRNA levels for β_1- and β_2-adrenergic receptors in alveolar type II cells.
Cells were cultured with 1 mg/ml Bakumondo-to for the time indicated, after which total cellular RNA was extracted and mRNA for β_1- and β_2- adrenergic receptors were determined as described in Fig. 1. Each bar represents the mean ± S.E.M. from 4 different experiments. * indicates a significant difference from the control group at $p<0.05$.

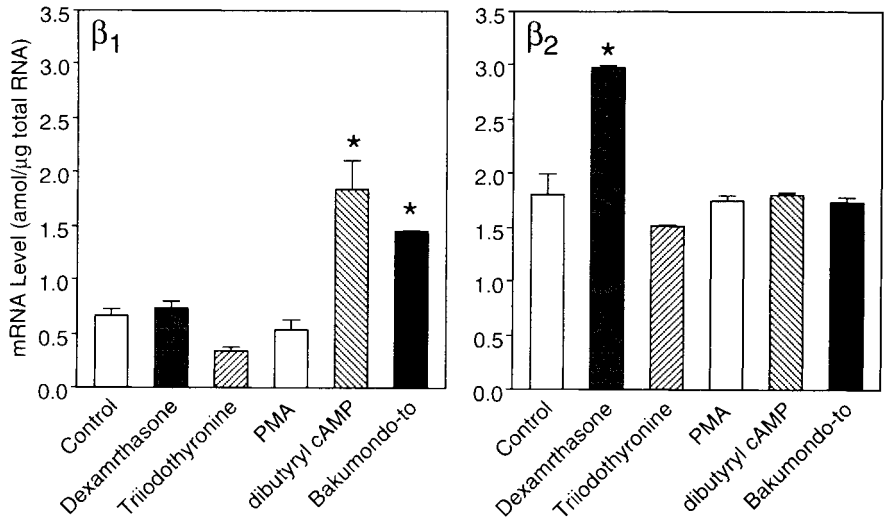

Fig. 3 Effects of dexamethasone, triiodothyronine, PMA, dibutyryl cAMP and Bakumondo-to on mRNA levels for β_1- and β_2-adrenergic receptors in alveolar type II cells.
Cells were cultured with 1 μM dexamethasone, 10 μM triiodothyronine, 10 nM PMA, 1 mM dibutyryl cAMP or 1 mg/ml Bakumondo-to for 24 h. Total cellular RNA was extracted and mRNA for β_1- and β_2- adrenergic receptors were determined as described in Fig. 1. Each bar represents the mean ± S.E.M. from 4 different experiments. * indicates a significant difference from the control group at $p<0.05$.

duced increase in mRNA level, we compared the effect of this medicine with those of dexamethasone, triiodothyronine, dibutyryl cAMP and PMA which are respective transcription regulators for β_1-adrenergic receptor mRNA. Among these drugs, dibutyryl cAMP increased β_1-adrenergic receptor mRNA (Fig. 3). On

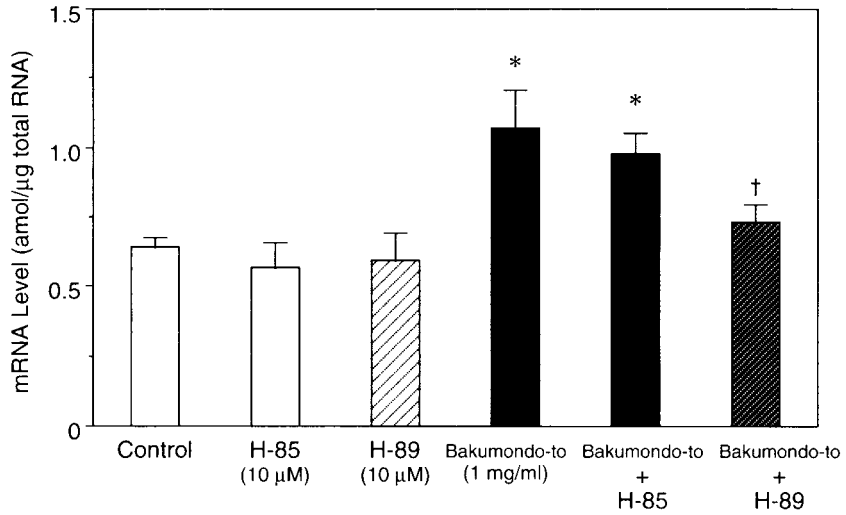

Fig. 4 Effect of H-89 on Bakumondo-to-induced increase in mRNA for β_1-adrenergic receptor in alveolar type II cells.
Cells were cultured with indicated drugs for 24 h. Total cellular RNA was extracted and mRNA for β_1-adrenergic receptor was determined as described in Fig. 1. Each bar represents the mean ± S.E.M. from 4 different experiments. * and † indicate significant differences from the control group and from Bakumondo-to alone at $p<0.05$, respectively.

the other hand, mRNA for β_2-adrenergic receptor was increased by only dexamethasone (Fig. 3). We then examined the effect of H-89, a cyclic AMP-dependent protein kinase inhibitor, on Bakumondo-to-induced increase in β_1-adrenergic receptor mRNA. Bakumondo-to-induced β_1-adrenergic receptor mRNA expression was completely abolished by the simultaneous application of 10 μM H-89, whereas H-85, control reagent for H-89, did not affect the increase in mRNA by Bakumondo-to (Fig. 4).

We finally determined the effect of Bakumondo-to on intracellular cAMP content in type II cells. As shown in Table I, Bakumondo-to significantly ($p<0.05$) increased the cAMP content, whereas the increase in cAMP was less than that by isoprenaline, a β-adrenergic agonist.

Table I Effect of Bakumondo-to on cAMP content in alveolar type II cells.

Treatment	n	cAMP content (pmol/well)
Control	4	177 ± 5
Isoprenaline (10 μM)	4	1671 ± 88[a]
Bakumondo-to (1 mg/ml)	4	511 ± 21[b]

Alveolar type II cells were isolated from rats and cultured overnight. The medium was then removed and the cells were incubated in fresh medium for 30 min. Bakumondo-to or isoprenaline were then added and the incubation continued for 5 min, after which cAMP content was measured. The data are mean ± S.E.M. from 4 experiments. [a] and [b] are significantly different from the control group at $p<0.01$ and <0.05, respectively.

Discussion

Alveolar type II cell produces and secretes pulmonary surfactant which is composed of phospholipids and apoproteins.[18] The increase in pulmonary surfactant secretion lowers the surface tension at the air-liquid interface in the lung and provides alveolar stability.[18] In addition to this vital role, pulmonary surfactant activates alveolar macrophages to prevent airway infection,[19] and stimulates mucociliary transport to accelerate airway clearance.[20,21] We previously reported that Bakumondo-to increases pulmonary surfactant secretion from cultured type II cells.[6] Therefore, type II cell is believed to be responsive to Bakumondo-to. Additionally, we reported that glucocorticoid increased β_2-adrenergic receptor mRNA in type II cells, and this increase in mRNA was accompanied with an increase in the surfactant secretion in response to β-agonist.[15] Therefore, we assumed that mRNA for β-adrenergic receptor in type II cells may be a useful marker to investigate the

effect of Bakumondo-to on gene expressions, and to analyze its regulatory mechanisms.

Our findings indicated that Bakumondo-to increased β_1-, but not β_2-adrenergic receptor mRNA expression in alveolar type II cells. Generally, the transcription rate is important to change the mRNA level. The promoter region of β_1-adrenergic receptor gene contains glucocorticoid-, thyroid-, AP-1- and cAMP-responsive sequences.[22] Therefore, we examined the effects of dexamethasone, triiodothyronine, PMA, and dibutyryl cAMP to compare with that of Bakumondo-to. Among these gene expression stimulators, only dibutyryl cAMP could increase the mRNA for β_1-adrenergic receptor in type II cells (Fig. 3). In agreement with this, H-89, a cAMP-dependent protein kinase inhibitor, significantly inhibited the increase in mRNA by Bakumondo-to (Fig. 4), and Bakumondo-to increased cAMP content in alveolar type II cells (Table I). Therefore, we assumed that Bakumondo-to increases β_1-adrenergic receptor mRNA, at least in part, by activating cAMP-dependent signaling. As described above, there is cAMP response element in β_1-adrenergic receptor gene.[22] Therefore, Bakumondo-to may increase mRNA for β_1-adrenergic receptor by increasing transcription rate. However, it was not possible to determine this, as actinomycin D and 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole, transcription inhibitors, were toxic to the cells in our culture system.

In the current study, the mRNA level for β_2-adrenergic receptor was increased by dexamethasone (Fig. 3). This result is consistent with our previous finding [15] and with other studies using human lung tissue[23] and rat heart cell line.[24] Therefore, the failure of dexamethasone to increased mRNA for β_1-adrenergic receptor in type II cells is not due to the difference of cell types. The glucocorticoid response element on β_1-adrenergic receptor gene is not likely to be functional. On the other hand, triiodothyronine has been reported to increase β_1-adrenergic receptor mRNA in rat ventricular myocytes,[25] and PMA has been reported in rat glioma cells.[26] In the current study, neither triiodothyronine nor PMA increased the mRNA for either β_1- or β_2-receptor mRNAs in type II cells (Fig. 3). We, therefore, assume that the deficiency of signaling mechanism(s), e.g., receptor and kinase, is involved in the failure of the response to these transcriptional stimulators.

In lung, β_1-adrenergic receptor mediates pulmonary surfactant secretion,[8] fluid clearance from alveolar space[27] and decreases smooth muscle tone,[28] as well as β_2-receptor. Therefore, it is possible that the increase in mRNA expression for β_1-adrenergic receptor by Bakumondo-to is related to the upregulation of these functions. Further studies are clearly needed to clarify the physiological significance of the increase in β_1-adrenergic receptor mRNA by Bakumondo-to. To our knowledge, however, the cardiovascular side effect by clinically used Bakumondo-to has not been reported, although β_1- receptor is abundantly expressed in the cardiovascular system.[29] Therefore, the increase in β_1-adrenergic receptor mRNA induced by Bakumondo-to may be through the specified mechanism for alveolar type II cells. To establish the molecular basis for the effect of Bakumondo-to, underlining mechanism(s) for cAMP increase by this medicine is probably important.

Conclusion

Bakumondo-to increases mRNA for β_1-adrenergic receptor, but not for β_2-receptor in alveolar type II cells. The activation of cAMP-dependent signaling may be important for this gene expression by Bakumondo-to. These results suggested that Bakumondo-to has a regulatory effect on gene expression.

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和文抄録

麦門冬湯は激しい咳を伴う咽頭炎や気管支炎に用いられる漢方方剤である。麦門冬湯の有効性に関する薬理学的情報は増えつつあるが，それらの作用の分子レベルでの機序については十分に解明されていない。本研究では，麦門冬湯が遺伝子発現調節作用をもつか否かについて調べるために，培養肺胞II型上皮細胞のβ_1-およびβ_2-アドレナリン受容体mRNA発現に対する作用を検討した。麦門冬湯はβ_1-受容体mRNAを濃度依存的に増加

させたが，β_2-受容体 mRNA 量には影響しなかった。麦門冬湯の作用は dibutyryl cAMP の作用とよく一致した。また，cAMP 依存性プロテインキナーゼ阻害薬である H-89 は，麦門冬湯による β_1-受容体 mRNA の増加をほぼ完全に抑制した。さらに，麦門冬湯は II 型細胞の細胞内 cAMP 量を有意に増加させた。これらの結果から，麦門冬湯は肺胞 II 型上皮細胞の β_1-アドレナリン受容体 mRNA 発現を促進し，その機序として cAMP 依存性の情報伝達系が重要であることが考えられた。

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