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For further information about this article or if you need reprints, please contact:

Hideaki Higashino,
Department of Pharmacology,
Kinki University School of Medicine, 377-2, Ohno-Higashi,
Osaka-Sayama, Osaka 589-8511,
Japan

Effect of Theophylline and Cyclic AMP Analogue 8-Br-cAMP on Dermatophagoides Farinae induced IL-5 mRNA of Peripheral Blood Mononuclear Cells

[1]T.P. Markova, [2]Y. Aragane, [3]A. Niwa, [3]M. Tabuchi,
[3]M. Orita, [3]K. Ooshima and [3]H. Higashino

Phosphodiesterases (PDE) catalyze the breakdown of cAMP and thus, may have potent immunomodulatory activity. Theophyllin is a non-selective PDE inhibitor and has long been used to treat atopic diseases such as asthma, based on its well-known effects of the bronchodilative and anti-inflammatory effects, although the immune modulatory activities are not completely clear yet. Because it is believed that Th2-immnuity plays a pathogenic role in asthma, we were interested to study the mechanisms of how it modulates mite-allergen-induced activation of Th2-like immunity. To address this issue, peripheral blood mononuclear cells (PBMC) were prepared from human healthy volunteers, stimulated with mite-allergen dermatophagoides farinae (Der-f), RNA prepared and semi-quantitative RT-PCR was performed using primers specific for IL-5, a representative Th2 cytokine. Consequently, it was found that theophylline at a concentration of 10^{-6} to 10^{-5} M significantly downregulated Der-f-induced expression of IL-5 mRNA. Intriguingly, theophylline (10^{-5} M) inhibited the expression of IL-5 mRNA of PBMCs from patients allergic to house dust mite. To further explore the mechanisms involved, a PKA type I inhibitor Rp-8-Br-cAMPS was added, resulting in restoration of theophylline-downregulated expression of Der-f-induced IL-5 mRNA, indicating the involvement of PKA in this event. Together, the present study demonstrates that theophylline prevents allergen-induced PKA activation and thus may, at least in part, inhibit Th2-like immune responses.

Key words: Theophylline, IL-5, mite allergen, PKA, atopy

[1]Department of Pharmacology, University of Medicine, Sofia 1431, Bulgaria
[2]Tougenkai Clinic, Osaka 550-0012, Japan
[3]Department of Pharmacology, Kinki University School of Medicine, Osaka 589-8511, Japan

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INTRODUCTION

Human homeostasis stands on fragile balance between complicated biological events. This is best-exemplified with allergies in which augmented effector immunity and/or ameliorated regulatory one plays a pathogenic role (Magnan and Vervloet, 2005). Atopic disorders significantly impair humans' quality of life and are frequently long-lasting, sometimes life-threatening (Lemanske and Busse, 2010). Asthma is a representative atopic disorder, in particular Th2 immunity is consisted of a particular subset of T cells (Levine and Wenzel, 2010). Activation of Th2 cells, also like other mature T cells, is restricted on a specific antigen that is presented on MHC class II bearing antigen presenting cells, but subsequently they, unlike other type of T cells, are capable of production of immune cytokines, IL-4 and IL-5 (Mosmann and Sad, 1996). In particular, IL-5 may control survival, adhesion and mediator release of eosinophils whose integration of lesional tissues is known to be one of the causative for atopic disorders (Finkelman *et al*., 2010). Hence, inhibition of IL-5 production could be a therapeutic target of the treatment of atopic disorders.

Phosphodiesterase (PDE) isoenzymes are differentially regulated and expressed and can be distinguished on the basis of their activity kinetics, substrate selectivity, subcellular localization, tissue distribution and inhibitor sensitivity (Reneerkens *et al*., 2009). Among these isoenzymes the AMP-specific PDE4 has been shown to be the predominant form of PDE expressed in immune and inflammatory cells (Torphy, 1998; Beavo *et al*., 1994). It has been reported that the production of several cytokines, including IL-4, IL-5, IL-13, IFN-γ, granulocyte-macrophage colony-stimulating factor is modulated by PDE inhibitors (Essayan *et al*., 1994). A large body of evidence exists which suggests that selective PDE4 inhibitors have anti-inflammatory effects and are considered as promising drugs for the treatment of allergic and inflammatory diseases (Teixeira *et al*., 1997; Torphy, 1998; Houslay and Milligan, 1997; Spina *et al*., 1998; Giembycz, 2005; Spina, 2003; MacKenzie, 2004).

Although, many effects of PDE inhibitors on mononuclear cells are already known, the mechanism underlying these effects is not completely clear yet. Currently, much research is being undertaken to develop novel PDE4 inhibitors for the treatment of allergic and inflammatory diseases including asthma (Royce and Tang, 2009) and broader knowledge about their mechanism of action may encourage us for further research and would provide new therapeutic approach for the treatment of such conditions.

Here, we wanted to elucidate the effect of a non-specific PDE inhibitor theophylline, one of the most frequently used drugs to control asthma worldwide (Urso *et al*., 2008), on IL-5 expression of human PBMCs pulsed with mite antigen dermatophagoides farinae (Der-f), at transcriptional level and how it effects on. This may shed more light on the mechanism of action of PDE inhibitors.

MATERIALS AND METHODS

Reagents: The following reagents were obtained from manufactures described as follows: Ficoll-Paque™ from Amersham Pharmacia Biotech AB, Uppsala, Sweden. 8-bromoadenosine cAMP (8-Br-cAMP), theophylline (Wako Pure Chemical Industries, Japan), Reverse transcription system was from Promega, Madison, WI, USA. Taq PCR core kit 1000 units and RNeasy mini kit were obtained from Qiagen, Tokyo, Japan. β-actin, IL-5 and primers were obtained from TaKaRa Bio Inc, Shiga, Japan. Der-f antigen from DANI, Japan.

Preparation of freshly isolated peripheral blood mononuclear cells: Human Peripheral Blood Mononuclear Cells (PBMCs) were obtained from heparinized venous blood of healthy volunteers and from patients allergic to house dust mite antigen through a density gradient centrifugation method (Ficoll-Paque®). The PBMCs were collected from the interface and washed twice with PBS.

PBMCs stimulation: The PBMCs were cultured in RPMI1640 medium supplemented with 1% L-glutamine and 1% antibiotics/antimycotics, at a density of 5×10^6 cells mL^{-1} for all experiments. Cells were pre-incubated for 30 min PKA type I inhibitor Rp-8-Br-cAMPS (1 mM). Theophylline (10^{-5} M) was added to the culture 30 min before stimulation with 1 μg mL^{-1} Der-f antigen. Cells were harvested at 18 h after stimulation with Der-f antigen.

RT-PCR: Cells were first harvested and RNA samples prepared by use of RNeasy kit (Qiagen). All RNA samples were subjected to reverse-transcription of cDNA using a reverse transcriptase system (Promega) according to the manufacturer's instructions. cDNA products were amplified with the designated primer pairs using Taq PCR Core kit (Qiagen). To perform following PCR amplification in a semi-quantitative way, serially diluted samples were amplified with β-actin primers with different cycle numbers and PCR conditions yielding exponential increase in PCR signals were used for further study.

cDNA samples then were amplified using specific primers for IL-5. The following sequences were chosen for primers:

- **β-actin (sense):** 5'-CACCTTCTACAATgAg CtgC-3'
- **β-actin (antisense):** 3'-TTCATgAggTAgTCCgTCAg -3'
- **Human IL-5 (sense):** 5'- gCTTCTgCATTTgAgTTTg CTAgCT-3'
- **HumanIL-5 (antisense):** 5'-TggCCgTCAATgTAT TTCTTTATTAA-g-3'

RESULTS AND DISCUSSION

Effect of theophylline on the Der-f-induced expression of IL-5 mRNA of human PBMCs from healthy voluntears: To determine the effect of a nonspecific phosphodiesterase inhibitor theophylline on the Der-f-induced expression of IL-5 mRNA, human PBMCs were preincubated with theophylline (10^{-6} M, 10^{-5} M) for 30 min before the addition of 1 μg mL^{-1} Der-f antigen. Cells were cultured for 18h and harvested for mRNA expression analysis. RNA was extracted thereafter, reverse-transcribed and semi-quantitative RT-PCR performed using primers specific for IL-5. In preliminary experiments, we first examined optimal concentration and time duration of Der-f stimulation to induce PBMCs activation (the induction of IL-5 mRNA was used as a readout system). To do so, PBMCs were stimulated with graded concentrations (0.1, 1, 2 and 3 μg mL^{-1}) of Der-f and harvested at various time points (from 6 to 24 h). Consequently, it was found that the stimulation with 1 μg mL^{-1} Der-f for 18 h gave rise to optimal signals of IL-5 message (data not shown). The results shown in Fig. 1 show that theophylline (10^{-6} M, 10^{-5} M) had a strong downregulating effect on Der-f-induced mRNA expression of IL-5.

Study of the effect of theophylline on IL-5 mRNA of PBMCs from patients allergic to house dust and stimulated with Der-f antigen: For studying the effect of theophylline on the expression of IL-5 mRNA from PBMCs of patients allergic to mites, cells (IgE radioimmunoassay positive for mite allergens) were isolated from allergic patients' blood and treated with Der-f and this a non-specific phosphodiesterase inhibitor. Eighteen hour later, PBMCs were harvested for RNA analysis. Our results showed that theophylline (10^{-5} M) inhibited the expression of IL-5 mRNA of PBMCs from patients allergic to mites which were stimulated

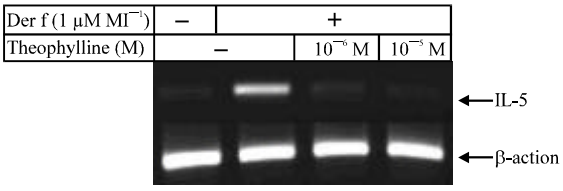

Fig. 1: Semiquantitative RT-PCR. The effect of theophylline on Der-f- induced IL-5 mRNA expression of PBMCs of healthy voluntears. PBMCs (5×10^6 cells mL^{-1}) were cultured in the presence of Der-f antigen (1 μg mL^{-1}) for 18 h with or without theophylline (10^{-6} M, 10^{-5} M). After total cell RNA extraction, RT-PCR (40 cycles) yielded a product for IL-5 (upper pannel) and product for β-actin (lower pannel) (30 cycles). RT-PCR products were separated by 2% agarose gel electrophoresis in the presence of ethidium bromide. Data from four independent experiments are shown

with 1 μg mL^{-1} Der-f *in vitro* (Fig. 2a). A quantitative representation of these data is shown in Fig. 2b. Elevations of IL-5 mRNA in response to stimulation with Der-f was statistically significant (*$p<0.05$). Pretreatment of PBMCs with theophylline (10^{-5} M) resulted in a marked inhibition of the IL-5 mRNA expression (*$p<0.05$) (Fig. 2b).

Effect of blockade of PKA activity on Der-f- upregulated IL-5 mRNA of PBMCs from healthy donors: To further elucidate mechanisms by which theophylline regulates the cytokine expression, we tested its effect on cAMP/PKA signaling pathway. In the preliminary experiments, it was observed that the cAMP analogue 8-Br-cAMP (1 mM) markedly downregulated the expression of IL-5 mRNA in human PBMCs stimulated by Der-f (data not shown). It is known that the effects of cAMP are mostly mediated through the binding to intracellular PKA kinase. To explore whether cAMP inhibits Der-f-upregulated IL-5 mRNA via activation of the PKA pathway in human PBMCs, we employed a PKA type I inhibitor Rp-8-Br-cAMPS. Cells were preincubated for 30 min with 1 mM Rp-8-Br-cAMPS and stimulated with 1 μg mL^{-1} Der-f antigen. Intriguingly, a PKA inhibitor Rp-8-Br-cAMPS (1 mM) was able to restore theophylline-downregulated expression of Der-f-upregulated IL-5 mRNA (Fig. 3a, b). This indicates the involvement of PKA in the theophylline-mediated suppressive event observed.

It is well known that cAMP controls a variety of important biological processes in immune cells

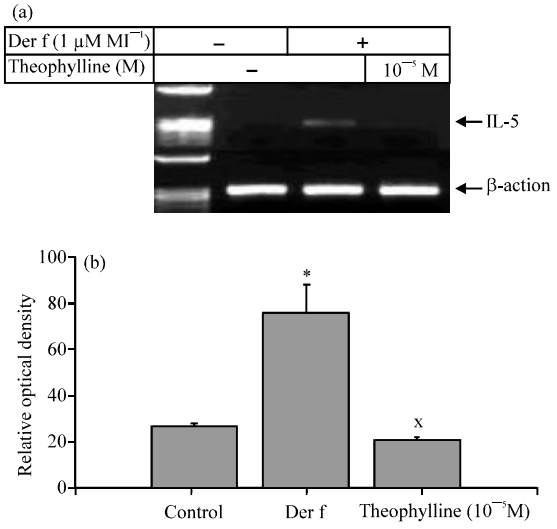

Fig. 2: (a, b) The effect of theophylline on IL-5 mRNA of PBMCs from patients allergic to house dust and stimulated with Der-f antigen. Human PBMCs from allergic patients (5×10^6 cells mL^{-1}) were treated with theophylline (10^{-5} M) (Fig. 2a) and stimulated with Der-f antigen (1 μg mL^{-1}) for 18 h. After total cell RNA extraction, RT-PCR (40 cycles) yielded a product for IL-5 (upper pannel) and product for β-actin (lower pannel) (30 cycles). RT-PCR products were separated by 2% agarose gel electrophoresis in the presence of ethidium bromide. Data from three independent experiments are shown. (b) Densitometry data are depicted for IL-5 cytokine for all subjects. Data were normalized to Der-f-treated cells; nonstimulated drug-free controls and with background values subtracted *$p<0.05$, vs. nonstimulated group; $^x p<0.05$, vs. Der-f-treated controls. The data presented are one of representative of four independent experiments

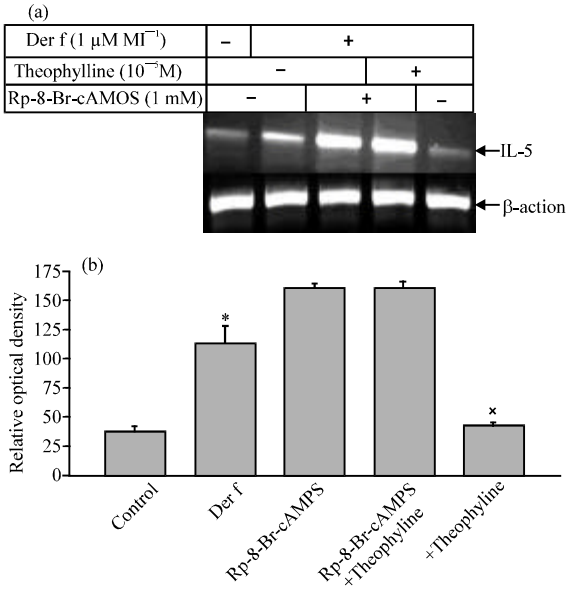

Fig. 3: (a, b) The effect of pharmacological blockade of PKA activity on Der-f-mediated expression of IL-5 mRNA. Human PBMCs (5×10^6 cells mL^{-1}) were cultured in the presence of Der-f antigen (1 μg mL^{-1}) for 18h with or without PKA type I inhibitor RP-8-Br-cAMPS (1 mM) alone or in combination with theophyllin (10^{-5} M) (Fig. 3a). Data from three independent experiments are shown. (b) Densitometry data are depicted for IL-5 cytokine for all subjects. Data were normalized to Der-f-treated cells; nonstimulated drug-free controls and with background values subtracted *$p<0.05$ vs. nonstimulated group; $^x p<0.05$ vs. Der-f-treated controls. The data presented are one of representative of four independent experiments

(Kammer, 1988) and is considered as a negative regulator of T cell activation (Anastassiou *et al*., 1992; Torgersen *et al*., 2002). However, several studies have shown conflicting results regarding the effect of cAMP-elevating agents on Th1 and Th2 cytokine production. The discrepancy could be caused by the use of different experimental systems and initial stimuli. Most published observations on IL-5 gene expression so far have been performed in T cell lines and T cell clones which may at least in part be transformed and thus are different in intracellular signaling events when compared with freshly isolated peripheral lymphocytes in our experimental setting.

It has been reported that cAMP-elevating agents increase rather than block IL-5 production in T cells stimulated by mitogens or via the TCR-CD3 complex (Betz and Fox, 1991; Snijdewint *et al*., 1993; Lee *et al*., 1993; Siegel *et al*., 1995; Schmidt *et al*., 1995). It was observed that PDE-related pharmacological agents capable of the increase in intracellular cAMP inhibited Th1 cytokine production (IL-2 and IFN-γ), but had no effect on Th2 cytokine (IL-4, IL-5 and IL-10) production (Munoz *et al*., 1990; Katamura *et al*., 1995). Klein-Hessling *et al*. (2003) reported that PKA can activate IL-5 gene expression in murine EL-4 thymoma cells. Altogether, these previous observations were generated by use of somewhat more artificial systems than the use of freshly isolated human PBMCs. First,

stimulation of T cells by mitogens may activate limited sets of signaling events. Second, stimulation of T cells with antibodies (anti-CD3 and/or anti-CD28) leads to T cell proliferation irrespective of their antigen-specificity. In the present study, PBMCs were only used, since these preparations certainly include mite-antigen specific T cells and peripheral antigen presenting cells such as monocytes and macrophages, which enables us to investigate the immune events rather closed to those in human tissues. The use of semi-quantitative RT-PCR systems also enables us to perform this study, since the stimulation index with Der-f was much less than those more artificial systems.

Recent reports have demonstrated the ability of cAMP-elevating agents to suppress cytokine secretion although the molecular mechanism underlying the inhibitory effects of cAMP remains not fully elucidated. To test the hypothesis that the mechanism of action of PDE inhibitors is via cAMP-dependent pathway, we used a cell-permeable cAMP analogue 8-Br-cAMP and studied its influence on Der-f-induced IL-5 mRNA expression. Present data showed that 8-Br-cAMP inhibits the expression of IL-5 mRNA and this suggests that the underlying mechanism of the effect of PDE inhibitors is mediated through the elevation of intracellular cAMP levels (Markova *et al*., 2007). In this context, it was reported previously that the cAMP signaling pathway represents a potentially important inhibitory influence on T cell activity (Torgersen *et al*., 2002). Most of the intracellular effects of cAMP are mediated through PKA (Kammer, 1988) which is the main intracellular target for cAMP (Skalhegg and Tasken, 1997). There are two different isozymes of PKA, termed PKA type I and PKA type II. T cells express both PKA type I and PKA type II and the inhibitory effect of cAMP on T cell proliferation is mediated through PKA I (Skalhegg *et al*., 1992). The PKA type I is thought to play an important role in cAMP-mediated effect in T cells because it colocalizes with T-Cell Receptor (TCR) during T cell activation. Skalhegg *et al*. (1994) and Braun *et al*. (1998) reported that both type I and type II PKA play key roles in mediating the inhibitory effects of cAMP on downstream antigen-driven signaling cascades. To further study the involvement of the cAMP/PKA pathway in the theophylline activities observed, we examined the effects of PKA type I inhibitor Rp-8-Br-cAMPS in this system. Treatment with a PKA type I inhibitor Rp-8-Br-cAMPS reversed the inhibitory effect of theophylline indicating that its downregulating effect on the expression of IL-5 mRNA is mainly due to activation of cAMP/PKA pathway. This observation is consistent with those by Staples *et al*. (2001) showing that cAMP-dependent inhibition of IL-5 production from human PBMCs stimulated with anti-CD3 and anti-CD28 is PKA-independent. Moreover, Staples *et al*. (2001) demonstrated that ERK and p38 MAP kinase pathways are not involved in the cAMP-mediated inhibition of IL-5. Heijink *et al*. (2002) reported that the inhibition of IL-5 by cAMP is not dependent on PKA activation and also reported the inhibitory effect of PGE2 on AP-1 and NF-κB binding activity. The observed downregulation of IL-5 mRNA may suggest a cross-talk between several downstream signaling cascades. The cAMP/PKA signaling pathway has been reported to affect T cell activation in a variety of ways, particularly through effects on the MAP kinase pathway (Ramstad *et al*., 2000). There is now enough body of evidence showing that there is a cross-talk between cAMP/PKA pathway and ERK1/2 signaling pathway (Houslay end Kolch, 2000; Stork and Schmitt, 2002).

Further elucidation of cAMP/PKA activity in this signaling pathway and possible targets of cAMP are under on-going investigation. Further work will be necessary to fully define the effect of PDE inhibitors on the expression of IL-5 and to delineate the mechanism responsible for this inhibitory effect.

In conclusion, this study allowed us to identify a novel mechanism involving cAMP/PKA in the inhibitory effect of theophylline on Der-f-induced expression of IL-5 mRNA. This study contributes to a better understanding of PDE inhibitors mechanism of action and to a better use of these potent anti-inflammatory agents.

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