

## Effects of Anti-Asthma Agents on Cytokine and Prostaglandin Production in Ovalbumin-Sensitized Splenocytes

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**Abstract** – The cytokines which is produced by allergen-specific T helper (Th) cells play a pivotal role in the pathogenesis of asthma. Asthma is caused by exaggerated T-helper 2 (Th2)-based immune responses. It is suggested that controlling such Th2-based response is necessary for asthma therapy. The current therapies for asthma focus primarily on control of symptoms and suppression of inflammation, without affecting the underlying cause. So, we examined that anti-asthmatic drugs might have play a certain role in Th2/Th1 balance. Splenocytes isolated from ovalbumin (OVA)-sensitized mice cultured with anti-asthmatic drugs. It is well known that Th2 and Th1 immune responses can balance one another, as Th2 mediators suppress Th1 responses and Th1 mediators similarly inhibit Th2 responses. But salmeterol inhibits both of Th1 and Th2 mediators, which salmeterol is a suppressor of immune responses not only a suppressor of Th2-based immune responses. Aminophylline is a weak suppressor of immune responses. But ipratropium and cromoglycate don't have any suppressor effect to Th2-driven responses. They only have suppressor effect to Th1 immune responses. Salmeterol, ipratropium, aminophylline, and cromoglycate augmented mRNA levels of CRTH2, EP2, and IP2 receptors in OVA-sensitized splenocytes. It is well known that the up-regulation of CRTH2 - PGD<sub>2</sub> receptor - results in restraint of eosinophil recruitment and that the increment of IP and EP2 - PGI<sub>2</sub> and PGE<sub>2</sub> receptor, respectively - may induce the accumulation of cAMP that decrease the effector function of T cells. Moreover salmeterol and cromoglycate increase the mRNA expression of PGD<sub>2</sub> synthase. These findings indicate that anti-asthma agents may alleviate the immunological responses that cause the asthmatic diseases.

**Keywords:** Asthma, Ovalbumin, BALB/c mice, T helper 2 cell, Cytokines, Prostaglandins

### INTRODUCTION

Asthma is a chronic inflammatory disease of the airways that is a major health problem worldwide, and the morbidity and mortality caused by asthma are on the rise. Asthma is characterized by intermittents and airway hyperresponsiveness to a wide variety of stimuli (Peters, 2003). The inflammatory characteristic of asthma includes infiltration of the airway by inflammatory cells that release various cytokines and inflammatory mediators. These mediators result in an increase in airway edema and mucus secretion, hy-

per trophy and hyperplasia of airway smooth muscle, and increased airway vascularity, all of which contribute to air-flow obstruction (Houtman and van den Worm, 2005). It is generally accepted that exaggerated T-helper 2 (Th2)-driven responses result in the development of asthma responses in genetically susceptible individuals. Thus, CD4<sup>+</sup> T cells producing Th2 cytokines play a prominent role in the lungs of asthmatic subjects, particularly because interleukin-4 (IL-4) and IL-10 enhance immunoglobulin E (IgE) production and mast cell growth and IL-5 induces eosinophil accumulation. It is suggested that controlling such Th2-based response is necessary for asthma therapy.

Lipid mediators represent a family of signaling molecules with great impact on inflammation. The main biologically active prostanoids being implicated in in-

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flammation of the respiratory tract include prostaglandin D<sub>2</sub> (PGD<sub>2</sub>), prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), prostacyclin (also termed prostaglandin I<sub>2</sub>, PGI<sub>2</sub>) (Nauta *et al.*, 2008). PGD<sub>2</sub> is released from mast cells and Th2 cells and modulates the physiology of the airways by causing bronchoconstriction and mucous production (Marom *et al.*, 1981; Black *et al.*, 1986; Nagata *et al.*, 1999). The PGD<sub>2</sub> level in bronchoalveolar lavage (BAL) fluid increases in responses to antigen provocation and is used as a marker for activation of mast cells *in vivo* (Miadonna *et al.*, 1990; Turner *et al.*, 1995). The prostanoid PGE<sub>2</sub> is regarded as a potent inflammatory mediator due to its effects on vasodilation and vascular permeability (Sturm *et al.*, 2008). Although the role of PGE<sub>2</sub> in allergic inflammation is unclear, this prostaglandin may have beneficial bronchoprotective and anti-inflammatory effects as well. PGI<sub>2</sub> plays a protective role in allergic lung inflammation (Takahashi *et al.*, 2002). PGs clearly play an important role in the development and maintenance of allergic inflammation in the lung. Also it has different function to specific PGs and their receptor interactions. Current *in vivo* animal studies suggest that PGD<sub>2</sub> increase allergic phenotype, whereas PGE<sub>2</sub> and PGI<sub>2</sub> restrain the allergen induced inflammatory response such as asthma.

The current therapies for asthma focus primarily on control of symptoms and suppression of inflammation, without affecting the underlying cause. Salmeterol,  $\beta_2$ -adrenoceptor agonist, is one of most effective bronchodilators and is increased cAMP level in bronchial cells. Aminophylline that has been extensively used in the therapy of asthma inhibit phosphodiesterase which degrading cAMP level in bronchial cells. Ipratropium, muscarinic receptor antagonist, has been used for the therapy of asthma for a long time. And cromoglycate, which was analogue of a bischromone, worked as mast cell stabilizers for a short time.

Although most patients with asthma are now able to lead a normal life through the use of current medications for alleviation of symptoms, the controlling of Th2-biased response is necessary for more effective asthma therapy since asthma is actually caused by overzealous T-helper 2 (Th2)-biased immune responses. Therefore, we examined whether anti-asthmatic drugs in market - salmeterol, ipratropium, aminophylline, and cromoglycate - have play a certain role in Th2/Th1 balance and PGs mediator synthesis.

## MATERIALS AND METHODS

### Animals

Male BALB/c mice, 6-7 weeks old, were obtained from

the Orient Bio Inc. (Gyeonggi, Korea). Animals were housed in an environmentally controlled, pathogen-free animal facility for the duration of experiments. They were given free access to tap water and OVA-free chow daily, and maintained under the following laboratory conditions of constant temperature ( $21 \pm 3^\circ\text{C}$ ), relative humidity ( $50 \pm 10\%$ ), and illumination (12 hr light/dark cycles) until the end of the experiment.

### Sensitization and challenge

For sensitization and challenge, the mice were received 50  $\mu\text{g}$  of OVA (Sigma-Aldrich, Deisenhofen, Germany) and 10 mg of aluminum potassium sulfate dodecahydrate (alum, Sigma-Aldrich, Deisenhofen, Germany) in 200  $\mu\text{l}$  of PBS intraperitoneally on day 0 and 7.

### Splenocytes isolation and culture

Seven days after sensitization, mice were sacrificed by cervical dislocation, the spleens were excised and disaggregated. Splenocytes were suspended at  $4 \times 10^6$  cells/well in RPMI 1640 (Cellgro, VA, USA) with 10% heat inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin and streptomycin (Cellgro, VA, USA). Asthma drugs, ipratropium bromide monohydrate, cromolyn sodium salt (disodium cromoglycate), salmeterol xinafoate, aminophylline (Sigma-Aldrich, Deisenhofen, Germany), were dissolved at 10 mM in methanol. Splenocytes were cultured in the 24-well plates in the presence or absence of 200  $\mu\text{g}/\text{ml}$  OVA and each asthma agents at 0.1, 1, 10 and 100  $\mu\text{M}$  for 72 hrs.

### Measurement of cytotoxicity

Splenocytes were suspended at  $2 \times 10^6$  cells/well in RPMI 1640 and cultured with anti-asthma agents in the 96-well plates for 24 hrs. After cultured, 10  $\mu\text{l}$  of MTT [3-(4,5-dimethylthiazolyl)-2, 5-diphenyltetrazolium bromide] (5 mg/ml, Sigma-Aldrich, Deisenhofen, Germany) solution were added to each well and incubated for 4 hrs. To each well, 100  $\mu\text{l}$  of a solubilization solution (0.04 N HCl in isopropanol) were added, and the optical density of the yellow reaction product was evaluated at 570 nm wavelength.

### Enzyme-linked immunosorbent assay (ELISA)

The 96-well plates were coated overnight with the primary antibody. The wells were washed three times with PBS containing 0.05% Tween20 (PBS-T) and incubated with blocking solution and then washed four times with PBS-T. Samples and diluted standards were added to the plate and incubated. After four times washing, the biotinylated secondary antibody was added. After 30 min in-

cubation, the wells were washed six times and avidin-conjugated alkaline phosphatase was added for 30 min. The substrate solution was added and the plates were left at room temperature for 5 min before adding 1 M NaOH stop buffer. The colored product was read at 405 nm.

### RT-PCR (Reverse transcription - polymerase chain reaction)

Total RNA was isolated from each sample using Trizol reagent (Invitrogen, CA). RNA was reverse-transcribed at 42°C for 1 hr. PCR was performed after quantitative normalization for each gene by a densitometry using GAPDH gene expression. The primer sequences are as follows Table I PCR products were electrophoresed and visualized by ethidium bromide staining.

**Table I.** Primer sequences for PCR

Target genes	Nucleotide sequences
IL-4 (312 bp)	5' - TCA ACC CCC AGC TAG TTG TC 3' - ATC GAA AAG CCC GAA AGA GT
IL-10 (451 bp)	5' - CTT GCA CTA CCA AAG CCA CA 3' - TTT TCA CAG GGG AGA AAT CG
IFN- $\gamma$ (388 bp)	5' - TTT GAG GTC AAC AAC CCA CA 3' - CGC AAT CAC AGT CTT GGC TA
TNF- $\alpha$ (532 bp)	5' - GTG CCA GCC GAT GGG TTG TCA C 3' - AGG CCC ACA GTC CAG GTC ACT G
CRTH2 (458 bp)	5' - GCA GAG AGG TAA GGC CTG TG 3' - TTC TGC AGA CAC CTG CAT TC
PGD <sub>2</sub> synthase (408 bp)	5' - TGT TTT GGA GGT GGA AGG AC 3' - GAA ATG GCA GGG ATA GCT TG
EP <sub>2</sub> receptor (419 bp)	5' - CTG GAT ACG GAA TTG GTG CT 3' - ACG ATA AGC AGC AGG AGC AT
IP receptor (414 bp)	5' - CCA TTT CCC ATC CCC TAT CT 3' - CGA GGG AGG AAG AGT GAG TG
GAPDH (548 bp)	5' - ACC CAG AAG ACT GTG GAT GG 3' - TGT GAG GGA GAT GCT CAG TG

### Statistics

Data in text and figures are expressed as the mean  $\pm$  SD. Two-group comparisons were evaluated with Student's *t*-tests. Differences were considered statistically significant at  $p < 0.05$ ,  $p < 0.01$ , and  $p < 0.001$ .

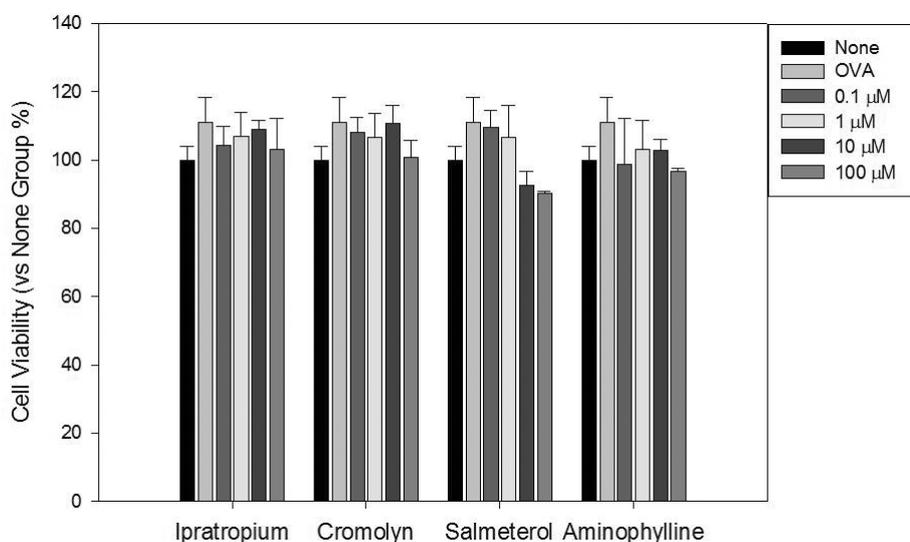
## RESULTS

### The cytotoxicity of anti-asthma drugs on splenocytes

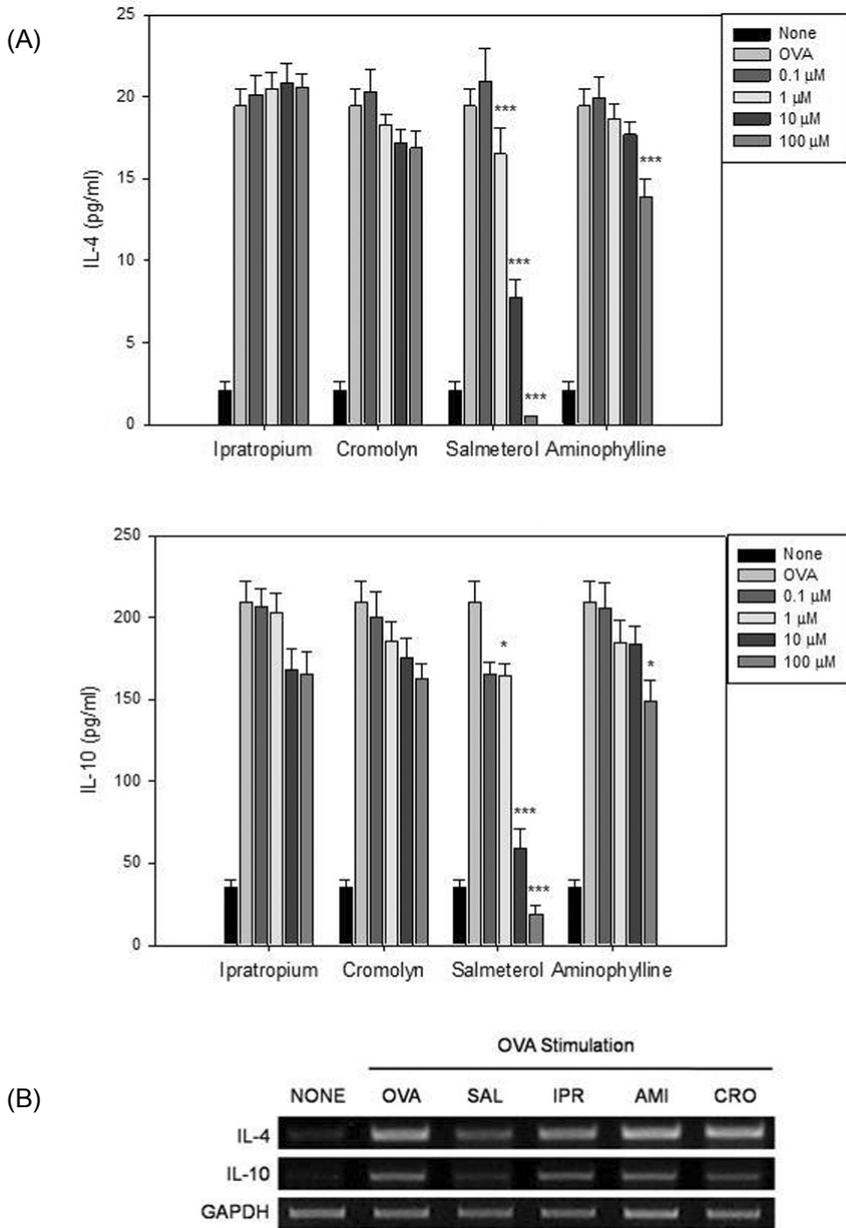
Splenocytes were suspended at  $2 \times 10^6$  cells/well in the 96-well plates and cultured with various concentrations of ipratropium, cromolyn, salmeterol and aminophylline for 24 hrs. After cultured, cytotoxicity was measured by MTT assay. The cell toxicity was less than 10 percents in maximum concentration (100  $\mu$ M) of each drug (Fig. 1).

### The production of Th2 cytokines

Previously study showed that salmeterol decreased IL-4 in human peripheral blood mononuclear cells (PBMC) and cromolyn reduced IL-4 secretion in PBMC from atopics (Mohede *et al.*, 1996; Oh *et al.*, 2002). We detected elevated levels of IL-4 and IL-10 in splenocytes sensitized by OVA and significantly reduced by salmeterol on concentration dependently. Aminophylline mildly decreased IL-4 and IL-10 secretion. Ipratropium increased in the levels of IL-4 that was unexpected and showed slight decline the levels of IL-10. Cromolyn reduced levels of IL-4 and IL-10 in a concentration-dependent manner, but their declines were weak (Fig. 2A). We measured mRNA levels of IL-4 and IL-10 which are typical Th2 cytokines and have pivotal role in asthma. They were increased in splenocytes sensitized by OVA and addition of salmeterol declined their mRNA expression. The other drugs did not show notice-



**Fig. 1.** Cytotoxicity of anti-asthma drug-sin splenocytes. BALB/c mice were intraperitoneal injected twice with 50  $\mu$ g OVA and 10 mg Alum in day 0 and 7. Mice were sacrificed at day 14 and splenocytes were isolated. Splenocytes cultured with 200  $\mu$ g/ml OVA and/or various concentration of anti-asthma drugs for 24 hrs. Cytotoxicity was determined by MTT method. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$  versus OVA group.



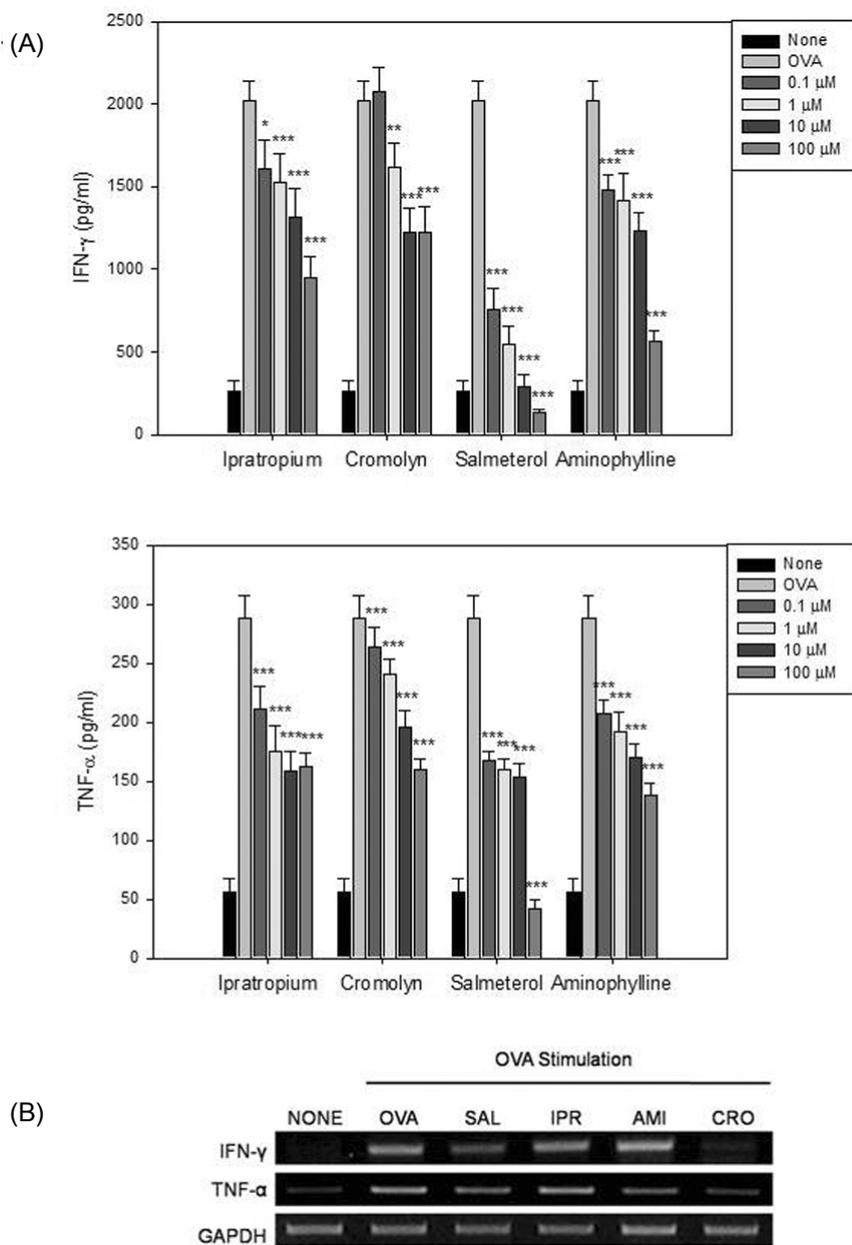
**Fig. 2.** Production and mRNA expression of Th2 cytokine in OVA-stimulated splenocytes. (A) Splenocytes cultured with 200 μg/ml OVA and/or various concentration of anti-asthma drugs for 72 hrs. IL-4 and IL-10 levels were detected in splenocyte culture supernatants by ELISA. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.0001$  versus OVA group. (B) Splenocytes cultured with OVA and each 10 μM of anti-asthma drugs for 24 hrs. mRNA levels of IL-4 and IL-10 were detected by RT-PCR. NONE: negative control, OVA: positive control, SAL: salmeterol, IPR: ipratropium, AMI: aminophylline, CRO: cromolyn.

able change in IL-4. They are merely decreased a little mRNA level of IL-10 (Fig. 2B).

**The production of Th1 cytokine**

Previous study showed that salmeterol decreased IFN-γ in human PBMC and disodium cromoglycate reduced TNF-α in human lung *ex-vivo* model (Mohede *et al.*, 1996; Matsuo *et al.*, 2000). We detected that augmented levels of IFN-γ and TNF-α in splenocytes sensitized by OVA and all drugs significantly reduced their on concentration dependently. Salmeterol decreased the cytokines less than control group which don't sensitized by OVA.

Aminophylline significantly decreased IFN-γ and TNF-α, comparatively mildly decreased level of IL-4 and IL-10. Ipratropium and cromolyn showed decline to half of IFN-γ and TNF-α levels (Fig. 3A). And we measured levels of IL-2 and IL-12p40, but anti-asthma drugs didn't play remarkable change to the cytokines (data not shown). We detected mRNA levels of IFN-γ and TNF-α which are typical Th1 cytokines and were increased in splenocytes sensitized by OVA. Salmeterol and aminophylline declined mRNA levels both of IFN-γ and TNF-α. Cromolyn strongly decreased mRNA level of TNF-α, but it didn't affect IFN-γ gene expression. Ipratropium did not play remarkable change



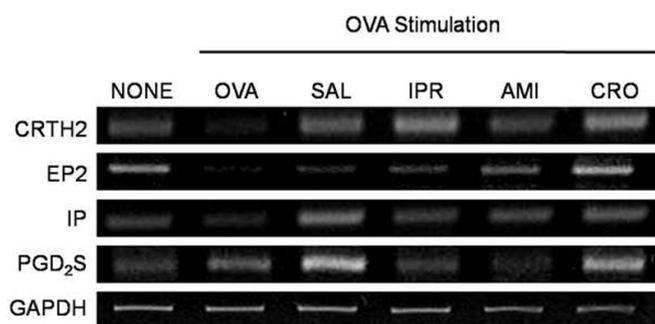
**Fig. 3.** Production and mRNA expression of Th1 cytokine in OVA-stimulated splenocytes. (A) Splenocytes cultured with 200  $\mu\text{g/ml}$  OVA and/or various concentration of anti-asthma drugs for 72 hrs. IFN- $\gamma$  and TNF- $\alpha$  levels were detected in splenocyte culture supernatants by ELISA. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.0001$  versus OVA group. (B) Splenocytes cultured with OVA and each 10  $\mu\text{M}$  of anti-asthma drugs for 24 hrs. mRNA levels of IFN- $\gamma$  and TNF- $\alpha$  were detected by RT-PCR. NONE: negative control, OVA: positive control, SAL: salmeterol, IPR: ipratropium, AMI: aminophylline, CRO: cromolyn.

to the expression levels of both cytokines (Fig. 3B).

#### mRNA expression of prostanoid synthases and receptors

We detected mRNA levels of CRTH2 which are PGD<sub>2</sub> receptor and PGD<sub>2</sub> synthase (PGD<sub>2</sub>S). CRTH2 was decreased in splenocytes sensitized by OVA. All of anti-asthma drugs augmented mRNA expression of CRTH2, especially ipratropium robustly elevated mRNA expression of CRTH2. The level of PGD<sub>2</sub>S didn't show remarkable changes by OVA stimulation. Interestingly, its expression was decreased by ipratropium and aminophylline, but increased by salmeterol and cromolyn (Fig. 4). We esti-

mated mRNA expressions of EP2, which is PGE<sub>2</sub> receptor, and PGE<sub>2</sub> synthase (PGE<sub>2</sub>S). The four agents for study induced the increment of EP2 gene expression although its expression level was decreased in OVA-stimulated splenocytes. However the expression level of PGE<sub>2</sub>S gene has no changes in whole experimental conditions (data not shown). Then, we examined the mRNA expressions of PGI<sub>2</sub> receptor (IP) that is related to signaling of anti-inflammatory activation. The expression of IP gene was decreased in splenocytes stimulated by OVA. Salmeterol remarkably increased mRNA expression of IP and the other agents mildly elevated its expression as control group.



**Fig. 4.** mRNA expression of prostaglandin-related genes. Splenocytes cultured with OVA and/or each 10  $\mu$ M of anti-asthma drugs for 24 hrs. mRNA levels of CRTH2, EP2, IP, and PGD<sub>2</sub>S were detected by RT-PCR. NONE: negative control, OVA: positive control, SAL: salmeterol, IPR: ipratropium, AMI: aminophylline, CRO: cromolyn.

## DISCUSSION

The prevalence of asthma has increased dramatically over the past decades. Allergen-specific T helper (Th) cells play a pivotal role in the pathogenesis of asthma. Asthma is caused by exaggerated T-helper 2 (Th2)-based immune responses in genetically susceptible individuals. Asthma-related Cytokines are associated with Th2 cytokines and diverse inflammatory mediators are involved in this process. Th2 cytokines, IL-3 and IL-13 increase the production of IgE, mast cell growth and PGD<sub>2</sub>. This results in the aggregation of Th2 cells, eosinophils and basophiles in the airway (Nagata *et al.*, 1999; Matsuoka *et al.*, 2000). IL-6 and IL-9 also induce the growth of master cells and cause the contraction of the blood vessels in the airway (Wills-Krap *et al.*, 1998; Umetsu and Dekruyff, 1998). PGE<sub>2</sub>, an inflammation mediator, elevates the levels of IL-6 in master cells, decreases the levels of TNF- $\alpha$ , a Th1 cytokine, and eventually increases inflammatory response (Leal-Berumen *et al.*, 1995). PGI<sub>2</sub> has been known to protect allergic lungs from the inflammation and to lower the function of effector T cells (Narumiya *et al.*, 1993). TXA<sub>2</sub> is known to be involved in the process of airway contraction and anaphylaxis. It also accelerates allergic airway anaphylaxis and the airway contraction (Nagai *et al.*, 1993). The activation of master cells activates LTB<sub>4</sub>, which induces the asthma by the increase of aggregation and the activation of neutrophils, monocytes and eosinophils (Wenzel *et al.*, 1991). Current drug therapy for asthma is highly effective and has evolved. But, the current therapies for asthma focus primarily on control of symptoms and suppression of inflammation, without affecting the underlying immunological cause.

Salmeterol dramatically decreased the production of Th1 and Th2 cytokine - IFN- $\gamma$ , TNF- $\alpha$ , IL-4, and IL-10 - and their mRNA expression. These finding suggested that salmeterol is a suppressor of whole immune responses not that a suppressor of Th2-biased immune responses. Aminophylline moderately decreased the production of IL-4 and IL-10 cytokines and significantly suppressed the secretion of IFN- $\gamma$  and TNF- $\alpha$ . These data supported that aminophylline is a weak suppressor of immune responses. Ipratropium and cromolyn do not have suppressor effect to Th2-driven responses and they only have suppressor effect to Th1 immune responses.

Splenocytes isolated from OVA-challenged BALB/c mice were cultured and re-stimulated by OVA. They exhibited a little expression of PGs receptor gene such as CRTH2, EP2, and IP. However, salmeterol, ipratropium, aminophylline, and cromolyn induced the great expression of PGs receptor mRNA. They are well known that the up-regulation of interaction between CRTH2 receptor and PGD<sub>2</sub> can results in the restraint of eosinophil recruitment and that elevated signaling through the EP2 and IP receptor may increase cAMP concentration. Interestingly, increment of cAMP levels may reduce effector function of T cells. Furthermore the cells cultured with salmeterol and cromolyn revealed the large mRNA expression of PGD<sub>2</sub> synthase (PGD<sub>2</sub>S) that produce the ligand of CRTH2 receptor, PGD<sub>2</sub>. The increment of CRTH2 receptor and PGD<sub>2</sub>S expression in salmeterol and cromolyn treatment may consequently boost their interaction that suppresses the recruitment of eosinophil.

In this article, we examined comparison mechanisms of current anti-asthma drugs in allergic asthma mouse model. We estimated their effects on Th1 and Th2 cytokines secretion and prostaglandins expression, which are essential mediators of asthma, in OVA-stimulated splenocytes. Salmeterol and aminophylline suppressed the both of Th1 and Th2 type immunities. Unexpectedly, ipratropium and cromolyn inhibited the only Th1-type response, which can reduce the differentiation and proliferation of Th2 cells, the major reason of asthmatic diseases. It is considered that the inhibition of Th1 cytokine production by ipratropium and cromolyn may aggravate the inflammation in asthma subjects. But the two agents did not induced Th2-type immune response. In addition, it is well known that Th1 and Th2 cells together affect the exacerbation of the inflammation in chronic asthmatic response, albeit only Th2 response for initiation of the disease. Therefore the reduction of Th1 cytokine secretion is as important as the suppression of Th2 immunity. Meanwhile, it is appeared that salmeterol, aminophylline, ipratropium, and cromolyn in-

creased the mRNA expression of PG receptors that can induce the increment of the intracellular cAMP concentration. The high cAMP concentration in T lymphocytes suppressed their inflammatory functions. These findings maybe support the development and application of the immunology-based antasthmatic remedy.

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