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ORIGINAL ARTICLE

Effect of Procaterol, a β_2 Selective Adrenergic Receptor Agonist, on Airway Inflammation and Hyperresponsiveness

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ABSTRACT

Background: β-agonists are frequently used as bronchodilators for asthma as not only a reliever but also a controller, and their utility has increased with the development of long-acting β_2 selective drugs. Although anti-inflammatory effects of β_2 selective-agonists have been reported *in vitro*, side effects on augmentation of airway hyperresponsiveness by chronic use of β_2 selective-agonists have been described in several reports. In this study, we investigated the effects of procaterol, a second-generation β_2-agonist, on airway inflammation *in vivo* using an antigen-specific murine model of asthma.

Methods: Mice immunized with ovalbumin (OVA) + alum and challenged with inhaled ovalbumin were orally administered procaterol during the challenge. After inhalation, the mice were tracheostomized and placed in a body box under controlled ventilation to measure airway resistance before and after acetylcholine inhalation.

Results: Administration of procaterol at a clinical dose equivalent did not augment airway hyperresponsiveness, inflammation of the airway wall, or subsequent airway wall thickening induced by OVA inhalation. BALF cell analysis revealed that the eosinophil number in the BALF was significantly reduced in procaterol-treated mice compared to untreated mice.

Conclusions: Oral administration of procaterol at a clinical dose did not augment airway responsiveness, but did reduce eosinophil inflammation.

KEY WORDS

airway hyperresponsiveness, allergic inflammation, eosinophil, murine model, β_2 adrenergic receptor agonist

INTRODUCTION

Currently, the main target of asthma therapies is chronic airway inflammation.[1-3] The steroid inhaler has become a basic long-term therapy for management of chronic airway inflammation.[4] For combination therapy, steroid inhalers have been supplemented with long-acting β_2 agonists, theophylline, or leukotriene receptor agonists.[5-7] Clinically, it has been observed that addition of β_2 selective-agonists is more effective than doubling the dose of steroid inhaler.[4,8-10]

Studies *in vitro* have demonstrated that β_2 selective-agonists possess anti-inflammatory effects. β_2 selective-agonists increase cyclic AMP levels, which in turn inhibit mast cell and eosinophil degranulation, induction of apoptosis, and cytokine production.[11-16] In contrast, human studies as well as *in vivo* studies have shown that chronic use of β_2 agonists worsen airway hyperresponsiveness.[17-19] The anti-inflamma-tory effects of salmeterol, a new long-acting β_2 agonist, have been intensively studied.[11,20-22] Salmeterol shows superior anti-inflammatory activities over salbutamol,[23] and in addition it possesses synergistic effects with steroids.[24-26] However, there are contradictory data regarding the anti-

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inflammatory effects and the synergistic effects of salmeterol.[27-29] In this study, we investigated the *in vivo* effects of a clinical dose of procaterol on airway inflammation as well as on airway hyperresponsiveness. Procaterol is β2-selective full agonist that is used as a rescue from asthmatic attack when inhaled and as a controller when taken orally. We found that a clinical oral dose of procaterol did not augment airway responsiveness. Rather, procaterol exhibited a tendency to reduce eosinophil infiltration.

METHODS

MEASUREMENT OF SERUM PROCATEROL CONCENTRATIONS

All mice were orally administered procaterol (Otsuka Pharmaceutical Co. Ltd, Tokyo, Japan) dissolved in distilled water at doses of 0.1, 1, or 10 mg/kg in a volume of 10 mL/kg. At 1 hour and 6 hours after administration, a venous blood sample was collected from the large abdominal vein of mice anesthetized with ether. The blood was transferred to a sample tube and centrifuged at 3000 rpm for 30 minutes, and the serum was frozen until analysis by liquid chromatography-tandem mass spectrometry. Each sample comprised sera obtained from 5 mice.

TREATMENT OF MICE

Specific pathogen-free male A/J mice (10–12 weeks old) with native airway hyperresponsiveness to acetylcholine (ACh)[30,31] were purchased from SLC (Shizuoka, Japan). Mice were bred in the animal facilities of Teikyo University School of Medicine under Specific Pathogen-Free (SPF) conditions. Care and use of the animals followed the guidelines of the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research.

The mice were initially immunized four times with 10 μg OVA + 2 mg alum on days 0, 28, 35, and 49. ELISA titers of OVA-specific IgE were significantly elevated after the immunizations as previously reported.[32] After immunization, the mice were divided into four groups for administration of inhaled challenge from day 49 to day 63 (inhalation of 20 mg/ml OVA for 10 minutes every other day, total 7 times): (1) 0.9 M NaCl, (2) OVA, (3) OVA + procaterol (orally) (4) OVA + dexamethasone (1 mg/kg, intraperitoneally). Procaterol in distilled water and dexamethasone was dissolved in saline and saline only was administered as a control. Procaterol and dexamethasone were administered once a day, at 1 hour before each OVA inhalation. Four to six mice were used in each group for one experiment.

ASSESSMENT OF AIRWAY RESPONSIVENESS

Twenty-four hours after the final OVA inhalation, airway responsiveness was analyzed. The mice were anesthetized with pentobarbital and were tracheostomized. The animals were connected to a Harvard ventilator with 0.25 ml tidal volume and a respiratory frequency of 120/minute, as previously reported,[33] after which they were given an injection of pancuronium bromide. Airway resistance (Raw) was measured using a whole-body plethysmograph (Buxco Electronics, Inc., Troy, NY). ACh was administered by ultra-nebulization for 3 minutes. Data were expressed as [(Raw after inhalation of ACh/Raw before inhalation) ×100 (%)].

BALF CELL ANALYSIS AND HISTOLOGICAL EXAMINATION

BALF was obtained from selected mice by intubating and washing the lungs with 1 ml of saline until the recovered fluid reached 5 ml. BALF was centrifuged at 1500 rpm for 10 minutes at 4℃. Pellets were dissolved in 1 ml PBS and the number of the cells was counted. Cytospin specimen was obtained by rotating at 640 rpm for 2 minutes. Then, the cells were stained with Diff Quik (International Reagents Corporation) and the cell differentiation counts were examined by microscope.

The lungs were fully inflated using 10 cm H_2O pressure and fixed with 20% formaldehyde for hematoxylin-eosin (HE) and elastica van Gieson (EVG) staining.

ANALYSIS OF MRNA EXPRESSION

Lungs of mice were frozen in liquid nitrogen immediately after harvest and were used for RNA extraction. Each lung tissue was moved quickly into 1 ml ISOGEN (Nippon Gene Co., Ltd., Tokyo, Japan). Lung tissue was homogenized and total RNA was extracted, using a modified acid guanidium-phenol-chloroform method. To synthesize cDNA, 5 μg of total RNA was incubated with 5 mM $MgCl_2$, 1 mM dNTP mixture, 0.25 U reverse transcriptase, 1 U RNase inhibitor, and 0.125 μM oligo (dT) (Takara Biochemicals, Tokyo, Japan). Amplification cycles were 42℃ for 15 minutes, 99℃ for 5 minutes, and then 5℃ for 5 minutes using a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA).

The mRNA levels of cytokines were quantified by real-time polymerase chain reaction (PCR) using the Light Cycler-Fast Start DNA Master SYBR Green I kit (Roche Diagnostics, Mannheim, Germany) for amplification of cDNA. The reaction was undertaken in 20 μl, containing 3 mM MgCl2, 1 μM primers, FastStart Taq DNA polymerase, dNTP mix and SYBR Green I (Light Cycler-Fast Start DNA Master SYBR GreenI kit, Roche Diagnostics). Quantification was performed with a standard curve obtained using 5 dilutions of cDNA. Results are shown as ratios of the level of mRNAs standardized to the level of β-actin mRNA. The primers used were as follows: β-actin 5'-CCTGTATGCCTCTGGTCGTA-3' 5'-CCATCTCCTG-CTCGAAGTCT-3' 260bp, IL-13 5'-GAGGAGCTGAG-CAACATCAC-3' 5'-GCAATATCCTCTGGGTCCTG-3'

Table 1 Serum Procaterol Concentrations in A/J Strain Mice

Dose of Procaterol	Serum Concentrations of Procaterol (ng/mL)	
	1 hour after administration	6 hours after administration
0.1 mg/kg	0.212	0.032
1 mg/kg	3.272	0.557
10 mg/kg	23.861	4.098

Each value is the mean of 2 samples. Each sample comprised serum obtained from 5 animals.

157bp, eotaxin 5'-TCCCCAACACACTACTGAAG-3' 5'-AGGCTCTGGGTTAGTGTCAA-3' 217bp, TGF-β1 5'-AACAACGCCATCTATGAG-3', 5'-ATTCCGTCTCCT-TGGTT-3' 294bp.

STATISTICAL ANALYSIS

Data were statistically analyzed by Student's t-test and ANOVA. Statistical significance was accepted at $p < 0.05$.

RESULTS

INFLUENCE OF PROCATEROL ON AIRWAY HYPERRESPONSIVENESS

First, we tried to set the concentration of procaterol at a clinical dose. A single clinical dose of procaterol in humans reaches 0.2 ng/mL of serum level.[34,35] We found that oral administration of 0.1 mg/kg procaterol reached the human effective serum concentration (Table 1). Next, we examined whether continuous treatment with procaterol (0.1 mg/kg) augments airway responsiveness. The dose response curve was examined using three different mice in each group as shown in Figure 1A. OVA inhalation significantly increased airway responsiveness after 2.5 mg and 5 mg ACh inhalation ($p < 0.05$). Treatment with procaterol before OVA inhalation did not augment airway responsiveness under these conditions (Figs. 1A, B). Because we examined airway hyperresponsiveness 25 hours after the final procaterol administration, the direct effect of bronchodilation by procaterol was negligible. We hypothesize that the slight decrease in airway response by procaterol is attributable to its influence on airway inflammation.

EFFECT OF PROCATEROL ON AIRWAY INFLAMMATION

Next, we examined the influence of procaterol on airway inflammation. BALF cell analysis was performed 24 hours after the final inhalation of OVA. The total number of cells in BALF was significantly increased in OVA-treated groups compared with the non-treated groups ($p < 0.01$). Macrophages were dominant in non-treated groups. In contrast, an increase in eosinophils was prominent in OVA-treated groups ($p < 0.01$) (Fig. 2). A significant decrease in eosinophil number was observed in OVA inhalation group ($p < 0.05$) (Fig. 2). We also performed histological examination with HE staining and EVG staining. Histological analysis confirmed deceased infiltration of eosinophils in the submucosal area in procaterol-treated mice (Fig. 3A). EVG staining showed that subepithelial fibrosis, which represents airway remodelling, did not worsen after procaterol treatment (Fig. 3B).

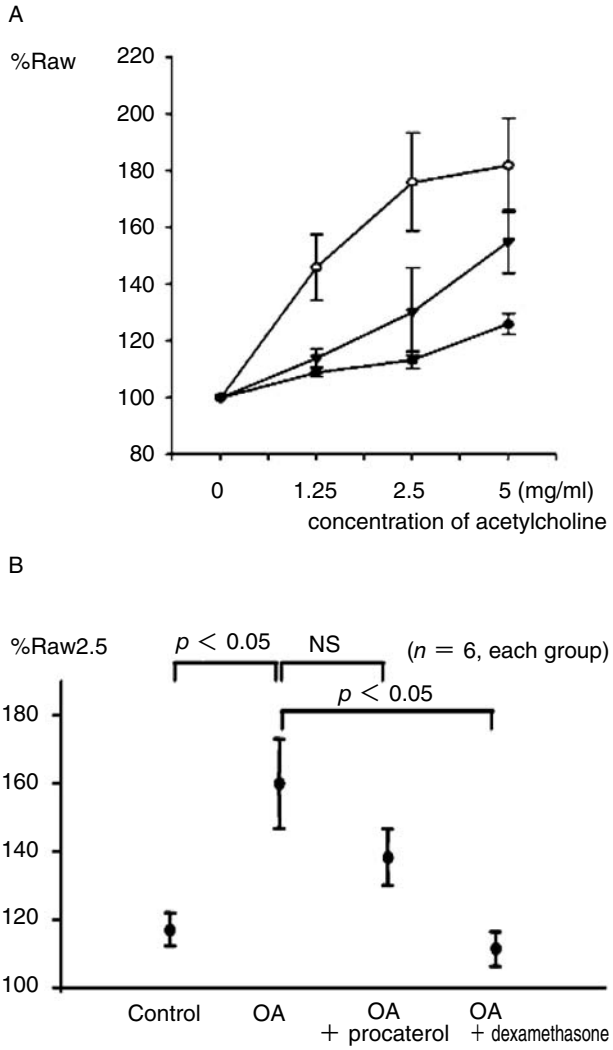

Fig. 1 Airway Responsiveness. Bronchoconstriction induced with ACh inhalation. ACh 1.25, 2.5 5 mg/ml were inhaled for 3 minutes. Raw was measured using whole body plethysmographs as described in the Methods. ACh-evoked changes in Raw are expressed as a percentage of Raw observed before ACh inhalation (100%). Data shown are the mean ± SEM. (**A**) Dose response curve. ○ OVA ▼ OVA + procaterol ● control mice (**B**) Airway responsiveness at 2.5 mg/ml Ach.

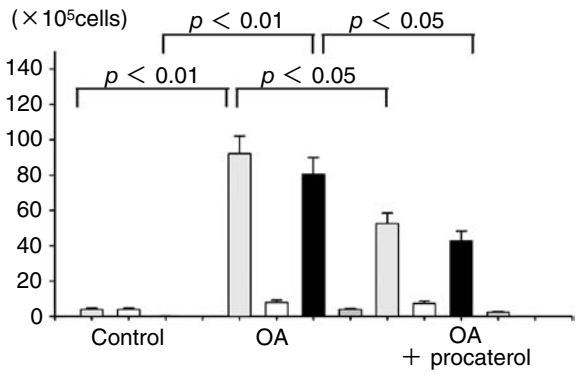

Fig. 2 BALF Cell Analysis. Lungs were subjected to lavage through intubations until 5 ml of BALF was obtained. Cells present in the BALF were pelleted, resuspended in 1 mL of saline and placed on glass slides, where they were counted and fixed by Cytospin. Slides were then stained with Diff Quik, and cell differentiation was assessed microscopically. Each bar indicates means ± SEM of seven mice. □ Total cells □ macrophage ■ eosinophils □ lymphocytes. Similar experiments were undertaken at least three times.

EFFECT OF PROCATEROL ON CYTOKINE mRNA AND PROTEIN SYNTHESIS

β_2 agonists have been reported to suppress cytokine production *in vitro*. Next, we examined the effect of procaterol on cytokine mRNA synthesis *in vivo*. Figure 4 shows that procaterol itself did not significantly reduce IL-13 and eotaxin mRNA, the products of which mediate eosinophil-associated inflammation. TGF-β, which is involved in airway remodelling, also exhibited no change after procaterol administration.

DISCUSSION

β_2 selective agonists function as bronchiodilators and are used as relievers and controllers.[34,35] Recently, inhaled steroids have been recognized as the most effective anti-inflammatory drugs and are the most common choice for controlling asthma.[36] A combined therapy comprising inhaled steroids and a long acting β_2 selective agonist is recommended for controlling asthma.[25] Synergistic effects have been reported, and in fact, the combined therapy is more effective than doubling the dose of inhaled steroids.[4,8-10] Thus new aspects of the usefulness in β_2 selective agonists are considered. However, the adverse effects of chronic

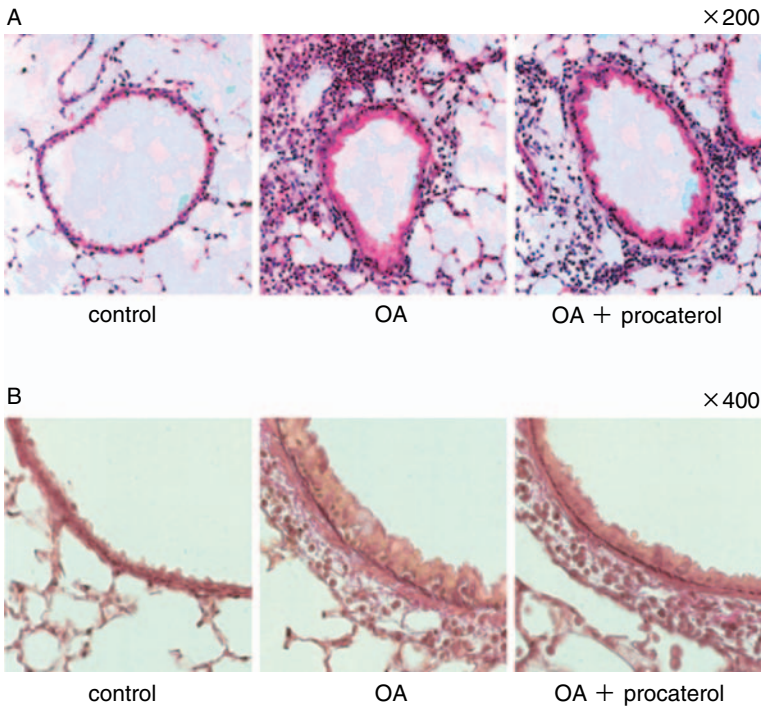

Fig. 3 Histological Analysis. Hematoxylin eosin (**A**) and Elastica van Gieson staining (**B**) were performed on paraffin-embedded sections. Lungs were extracted and fixed overnight with intra-tracheal infusion of 10% formalin maintaining the airway pressure at 10 cm H_2O lung.

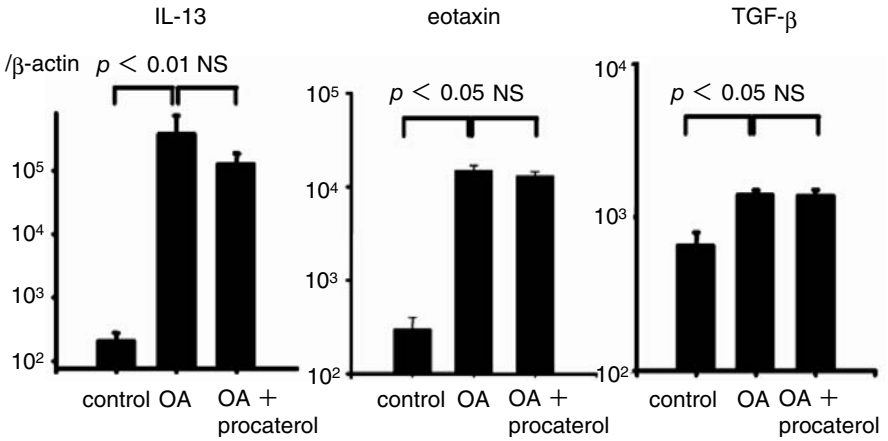

Fig. 4 Effect of Procaterol on IL-13, Eotaxin and TGF-β Gene Expression. RNA was extracted from whole lung of OVA treated mice and mRNA expression for various genes was determined by real-time PCR. Data are mean ± SEM of three different mice.

use by inhalation, including increases in airway hyperresponsiveness, have been reported.[17,37,38] The short acting β_2 selective agonist, salubutamol, has been shown to worsen the airway hyperresponsiveness in animal model and in human studies.[17-19,37-39] While the anti-inflammatory activities of salmeterol, a long acting β_2 selective agonist have been reported,[11,13,23] some reports deny the effects.[27,28] In this study, we investigated the effect of procaterol, a β_2 selective full agonist, which is commonly used as controller by oral tablet administration. We found that clinical dose of oral procaterol did not augment airway responsiveness and airway remodelling. Rather, procaterol significantly reduced eosinophil infiltration.

Mast cells, eosinophils, and smooth muscle cells at the site of asthmatic inflammation possess β_2 receptors,[40] and β_2 agonists have been reported to block mast cell and eosinophil degranulation.[13,14] β_2 agonists function by increasing the concentration of intracellular cAMP,[41] which result in inhibition of cytokine synthesis and induction of apoptosis on eosinophils *in vitro*.[42] However, a report which studied spontaneous apoptosis showed that β_2 agonists and cAMP increasing reagents decrease apoptosis of eosinophils.[43] In contrast to spontaneous apoptosis, it has been shown that cytokine mediated survival of eosinophils is inhibited by the increase of cAMP, through accelerated induction of apoptosis.[16,44] Theophylline, another cAMP increasing drug, has been shown to reduce cytokine mediated eosinophil survival, which is relevant to the *in vivo* condition.[45-47] We can hypothesize that the *in vivo* effects of a decrease in eosinophils occurred via induction of apoptosis. Another possible mechanism of a decrease in airway inflammation by procaterol is down-regulation of adhesion. Procaterol has been proven to reduce adhesion molecules in vitro studies.[16,48] It was also reported that systemic administration of tulobuterol, a β_2 selective agonist, attenuates eosinophil adhesion to endothelial cells, which results in reduction of eosinophil inflammation.[49] Systemic but not inhalational administration can modulate endothelial cells.[49]

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