Effect of Procaterol, a $\beta_2$ Selective Adrenergic Receptor Agonist, on Airway Inflammation and Hyperresponsiveness

Hiroyuki Tashimo¹, Naomi Yamashita¹,², Hirofumi Ishida¹, Hiroyuki Nagase¹, Tetsuya Adachi¹, Junichi Nakano¹, Koichi Yamamura¹, Tomoko Yano¹, Hisanao Yoshihara¹ and Ken Ohta¹

ABSTRACT

Background: $\beta$-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 $\beta_2$ selective drugs. Although anti-inflammatory effects of $\beta_2$ selective-agonists have been reported in vitro, side effects on augmentation of airway hyperresponsiveness by chronic use of $\beta_2$ selective-agonists have been described in several reports. In this study, we investigated the effects of procaterol, a second-generation $\beta_2$-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, $\beta_2$ 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 $\beta_2$ agonists, theophylline, or leukotriene receptor agonists.⁵⁻⁷ Clinically, it has been observed that addition of $\beta_2$ selective-agonists is more effective than doubling the dose of steroid inhaler.⁴⁻⁶¹⁰ Studies in vitro have demonstrated that $\beta_2$ selective-agonists possess anti-inflammatory effects. $\beta_2$ 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 $\beta_2$ agonists worsen airway hyperresponsiveness.¹⁷⁻¹⁹ The anti-inflammatory effects of salmeterol, a new long-acting $\beta_2$ 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-
inflammatory effects and the synergistic effects of salmeterol. 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) 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. 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, intraperitonally). 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, 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°C. 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 H2O 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 MgCl2, 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°C for 15 minutes, 99°C for 5 minutes, and then 5°C 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 Green I 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’-CAACATCAC-3’ 5’-CCATCTCTG-CTCGAAGTCT-3’ 5’-GCAATATCCTCTGGTGTCGTA-3’ 260bp, IL-13 5’-CACGAGCTGAGTCTGTATGCCTGTATGCCTGTA-3’ 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 (%)].
Table 1 Serum Procaterol Concentrations in A/J Strain Mice

<table>
<thead>
<tr>
<th>Dose of Procaterol</th>
<th>Serum Concentrations of Procaterol (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 hour after administration</td>
</tr>
<tr>
<td>0.1 mg/kg</td>
<td>0.212</td>
</tr>
<tr>
<td>1 mg/kg</td>
<td>3.272</td>
</tr>
<tr>
<td>10 mg/kg</td>
<td>23.861</td>
</tr>
</tbody>
</table>

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'-ATTCCGTCCTCCTTGGTT-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. 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.

Effects 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 bronchodilators and are used as relievers and controllers. Inhaled steroids have been recognized as the most effective anti-inflammatory drugs and are the most common choice for controlling asthma. A combined therapy comprising inhaled steroids and a long acting β_2_ selective agonist is recommended for controlling asthma. Synergistic effects have been reported, and in fact, the combined therapy is more effective than doubling the dose of inhaled steroids. Thus new aspects of the usefulness in β_2_ selective agonists are considered. However, the adverse effects of chronic
use by inhalation, including increases in airway hyperresponsiveness, have been reported.\textsuperscript{17,37,38} The short acting \( \beta_2 \) selective agonist, salbutamol, has been shown to worsen the airway hyperresponsiveness in animal model and in human studies.\textsuperscript{17-19,37-39} While the anti-inflammatory activities of salmeterol, a long acting \( \beta_2 \) selective agonist have been reported,\textsuperscript{11,13,23} some reports deny the effects.\textsuperscript{27,28} In this study, we investigated the effect of procaterol, a \( \beta_2 \) agonist 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 spontaneous eosinophil infiltration.

Mast cells, eosinophils, and smooth muscle cells at the site of asthmatic inflammation possess \( \beta_2 \) receptors,\textsuperscript{40} and \( \beta_2 \) agonists have been reported to block mast cell and eosinophil degranulation.\textsuperscript{13,14} \( \beta_2 \) agonists function by increasing the concentration of intracellular cAMP,\textsuperscript{41} which result in inhibition of cytokine synthesis and induction of apoptosis on eosinophils \textit{in vitro}.\textsuperscript{42} However, a report which studied spontaneous apoptosis showed that \( \beta_2 \) agonists and cAMP increasing reagents decrease apoptosis of eosinophils.\textsuperscript{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.\textsuperscript{16,44} Theophylline, another cAMP increasing drug, has been shown to reduce cytokine mediated eosinophil survival, which is relevant to the \textit{in vivo} condition.\textsuperscript{45-47} We can hypothesize that the \textit{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.\textsuperscript{16,48} It was also reported that systemic administration of tulobuterol, a \( \beta_2 \) selective agonist, attenuates eosinophil adhesion to endothelial cells, which results in reduction of eosinophil inflammation.\textsuperscript{49} Systemic but not inhalational administration can modulate endothelial cells.\textsuperscript{49}

**REFERENCES**

5. Tsuda T, Matsuse H, Machida I et al. Evaluation of theophylline or pranlukast, a cysteinyl leukotriene receptor 1 antagonist, as add-on therapy in uncontrolled asthmatic patients with a medium dose of inhaled corticosteroids. \textit{Allergy Asthma Proc.} 2005;\textbf{26}:287-291.
11. Butchers PR, Vardey CJ, Johnson M. Salmeterol. A potent and long-acting inhibitor of inflammatory mediator re-


47. Yasui K, Hu B, Nakazawa T, Agematsu K, Komiyama A.
