Parabromophenacyl Bromide Inhibits Subepithelial Fibrosis by Reducing TGF- β_1 in a Chronic Mouse Model of Allergic Asthma

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Key Words

Parabromophenacyl bromide · Asthma · Subepithelial fibrosis · TGF- β_1 · Airway remodeling · Phospholipase A2

Abstract

Background: Our previous study showed that parabromophenacyl bromide (PBPB) inhibits the features of allergic airway inflammation and airway hyperresponsiveness (AHR). However, its effect on airway remodeling, e.g. subepithelial fibrosis in a chronic allergic asthma model, was not investigated. We examined this issue in this study. **Methods:** PBPB was administered to mice with an induced chronic asthmatic condition. AHR was estimated at the end of the experiment, followed by euthanasia. Lung sections were stained with hematoxylin and eosin, periodic acid-Schiff and Masson's trichrome to determine airway inflammation, goblet cell metaplasia and subepithelial fibrosis, respectively. Transforming growth factor- β_1 (TGF- β_1) was estimated in lung homogenates. To determine the effect of PBPB on smooth-muscle hyperplasia, immunohistochemistry against α -smooth-muscle actin was performed on the lung sections. **Results:** Chronic ovalbumin challenges in a mouse model of allergic asthma caused significant subepithelial fibrosis and elevated TGF- β_1 , along with significant AHR. PBPB attenuated subepithelial fibrosis with a reduction of lung TGF- β_1 , airway inflammation and AHR without affecting goblet cell

metaplasia. It also attenuated smooth-muscle hyperplasia with a reduction in the expression of α -smooth-muscle actin in the lungs. **Conclusion:** Our findings indicate that PBPB attenuates some crucial features of airway remodeling such as subepithelial fibrosis and smooth-muscle hyperplasia. These data suggest that PBPB could therefore be a therapeutic drug for chronic asthma.

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Introduction

The incidence of chronic inflammatory disorders such as respiratory and allergic diseases is increasing worldwide, in spite of considerable understanding of their pathogenesis and improved therapeutic strategies. The currently available standard therapies for these diseases such as steroids, β_2 -adrenergic agonists, anticholinergics, histamine H1-antagonists and leukotriene modifiers render symptomatic relief but with various severe side effects [1–3]. This necessitates the discovery of some new potent drug candidates.

Asthma is a dynamic syndrome with airway inflammation and airway remodeling [4]. Earlier, it was believed that airway inflammation itself leads to airway remodeling, but now it is believed that airway remodeling could be parallel or appears even before the inflamma-

tion or symptoms [5]. In addition, there is evidence to suggest a poor correlation between airway inflammation and airway remodeling [6]. A better antiasthmatic drug should have both anti-inflammatory and antiremodeling activities. In addition, in the chronic asthmatic condition, airway hyperresponsiveness (AHR) is well-correlated with airway remodeling [7], suggesting that AHR in the chronic asthmatic condition may be predominantly determined by airway remodeling. Among the various features of airway remodeling, subepithelial fibrosis is one of the crucial components. This is initiated by epithelial injury as injured epithelia start secreting transforming growth factor- β_1 (TGF- β_1), one of the key mediators of airway remodeling. In addition to epithelia, TGF- β_1 is secreted by fibroblasts in the chronic asthmatic condition whereas in the acute stages of asthma, the eosinophils are the major source of TGF- β_1 [8]. This further indicates not only the crucial importance of positive feedback for the development of airway remodeling but also the possible role of structural cells, such as airway epithelia and fibroblasts, to initiate the airway remodeling.

Thus, in this context, we initially identified a potent antiasthmatic agent, parabromophenacyl bromide (PBPB), in an acute mouse model of asthma [9]. PBPB is a phospholipase A2 (PLA2) inhibitor, and has been found to inhibit AHR and inflammation [9]. Most remarkably, it worked potentially in both preventive (i.e. inhibited development) as well as reversal (i.e. attenuated the developed) capacities on asthmatic features in our models [9]. However, the effect of PBPB on airway remodeling has not been studied so far. Since PBPB inhibits PLA2 and PLA2 inhibition is known for its antifibrotic effects [10, 11], we hypothesized that PBPB may also have an antiremodeling effect. In this study, we developed a chronic experimental mouse model that showed airway remodeling. We examined our hypothesis and compared the effects of PBPB with a steroid, dexamethasone (Dexa), a potent drug for asthma.

Materials and Methods

Animal Species and Acclimatization

All the mentioned experimental protocols in this study were approved by the Institutional Animals Ethics Committee, CSIR Institute of Genomics and Integrative Biology, Delhi, India. BALB/c male mice, 5–8 weeks old, weighing 15–21 g, were obtained from the Council of Scientific and Industrial Research – Central Drug Research Institute, Lucknow, India, and were acclimatized for at least 1 week under standard laboratory conditions ($25 \pm 2^\circ\text{C}$, 55% humidity) before starting the experiments.

Development of Chronic Asthma and Drug Treatments

To determine the effect of PBPB on asthmatic airway remodeling, we developed a chronic model of asthma as shown in figure 1. After acclimatization, the mice were divided into 4 groups with 6 mice in each, i.e. group 1: sham + VEH [alum-sensitized, PBS-challenged and vehicle, i.e. treated with 50% ethanol], group 2: OVA + VEH [OVA (chicken egg ovalbumin, grade V)-sensitized, OVA-challenged and VEH-treated], group 3: OVA + PBPB (OVA-sensitized, OVA-challenged and treated with 1 mg/kg PBPB twice a day, a dose based on our earlier study [9]) and group 4: OVA + Dexa (OVA-sensitized, OVA-challenged and treated with 0.75 mg/kg Dexa once a day). Each mouse was sensitized with 50 μg OVA adsorbed in 4 mg aluminium hydroxide or in 4 mg aluminium hydroxide only. These sensitizations were performed by intraperitoneal injection on days 0, 7 and 14. One week after the third sensitization, these mice were exposed to 1% OVA in normal saline or normal saline alone with a nebulizer (OMRON CX3 model, Japan) in an acrylic chamber. These challenges were performed 3 times a week (30 min a day) until day 109. From day 95 to day 109, all mice were administered VEH (10 μl), PBPB or Dexa.

In our previous study, we demonstrated that another PLA2 inhibitor, mepacrine, was able to reverse established airway remodeling [12]; mepacrine was administered for the last 2 weeks of the 4-week OVA challenge model. Various reports have demonstrated the reversal of airway remodeling with a 14-day treatment protocol [13, 14]. The major difference between our approach in this study and these published approaches was the continuation of the OVA challenge; in the previous reports, the challenge was stopped during treatment. We adopted this approach because of the substantial decrease of asthma features including collagen deposition and goblet cell metaplasia observed after the discontinuation of the OVA challenge [15].

Estimation of AHR and Lung Histopathology

On day 110, AHR was estimated in all mice as described earlier [16], followed by euthanasia to collect bronchoalveolar lavage fluid, blood and lung samples [17, 18]. Blood was centrifuged at 1,000 g for 30 min to collect sera. After measuring lung function, the mice were euthanized and the lungs were fixed with 10% formalin, processed to obtain tissue blocks [16]. Lung tissue sections were sliced at 4–5 μm and then stained with hematoxylin and eosin (HE), periodic acid-Schiff (PAS) and Masson's trichrome (MT) for the assessment of inflammatory changes, goblet cell metaplasia and subepithelial fibrosis, respectively. Inflammation score, airway mucin content and subepithelial collagen content were determined by experimentally blinded experts as we described previously [16, 19]. Briefly, to estimate the inflammation scores, HE-stained lung sections were given random numbers and analyzed by a blinded researcher, i.e. 0: no detectable inflammation, 1: occasional cuffing of immune cells, 2: 1–3 layered immune cells, 3: 3–5 layered immune cells and 4: >5 layered immune cells around the vessel (perivascular) or bronchi (peribronchial). The total score was determined as the sum of the perivascular and peribronchial scores. To estimate the subepithelial fibrosis and goblet cell metaplasia semiquantitatively, MT- and PAS-stained lung sections were photographed under a microscope with a camera (Nikon Eclipse Ti). These images were analyzed with freely available ImageJ software (<http://imagej.nih.gov/ij/>). Briefly, the images were opened via the ImageJ platform, and the subepithelial (MT) or

Fig. 1. Experimental protocol (see Materials and Methods): after acclimatization to the animal house environment, the mice were randomly divided into 4 groups and sensitized with either OVA that was adsorbed in alum or with alum only on days 0, 7 and 14. The mice were then challenged with either 1% OVA or PBS from

day 21 to day 109 (30 min a day, 3× a week). From day 95 to day 109, the mice were administered PBPB, Dexa or VEH. On day 110, the mice were assessed for AHR and then euthanized to collect the sera and lung and bronchoalveolar fluids.

epithelial (PAS) regions selected and then separated into red, green and blue channels. Color thresholds were then manually set in red (MT) or green (PAS) channelled images by a blinded expert, in such a way that the blue collagen content (MT) or magenta mucin content (PAS) matched to the color threshold by comparing the original MT- or PAS-stained images simultaneously [20]. These images were converted into binary images and the area fraction of collagen or mucin density was calculated using ‘summarize’ and ‘analyze particle’ options. This was a semiquantitative analysis, so this percentage was converted into arbitrary units (a.u.) [16, 19]. To determine the apoptotic changes, TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labelling) assay was performed in the lung sections as described earlier [16].

Enzyme-Linked Immunosorbent Assay

The lungs were homogenized in radioimmunoprecipitation assay buffer and protein estimation was performed using a bicinchoninic acid assay [16]. The levels of TGF- β_1 were estimated in the lung homogenates by ELISA using the commercially available kit (BD Pharmingen).

Statistical Analysis

All data were presented as means $\pm$ SEM. Significant differences between ≥ 2 groups were estimated using the Student t test or 1-way ANOVA. A p value ≤ 0.05 was considered significant.

Results

PBPB Treatment Attenuated AHR

Earlier, we showed the potential effects of PBPB on asthmatic features in an acute mouse model of asthma. PBPB inhibited PLA2 activity and various features of allergic airway inflammation such as methacholine-induced airway constriction, Th2 cytokines, serum IgE levels and airway eosinophilia [9]. As it is well known that asthma is a chronic condition and that most patients continue to visit the clinic after the development of asthma, the identification of drug molecules to reduce airway re-

Fig. 2. PBPB treatment attenuated AHR. AHR was estimated using invasive airway mechanics in anesthetized mice with increasing doses of methacholine. The results are expressed in the form of percentage baseline airway resistance, considering PBS aerosol values as 100%. The results are expressed as means $\pm$ SEM (n = 5–6). * p < 0.05 vs. sham + VEH; # p < 0.05 vs. OVA + VEH.

modeling would be a good strategy. On the other hand, the PLA2 signaling pathway may activate airway remodeling via the arginase/TGF- β_1 pathway [10, 11]. So we hypothesized that PBPB, a known inhibitor of PLA2, may inhibit the development of airway remodeling by affecting this pathway. To determine this, we developed a chronic model of asthma (110 days; fig. 1) and we measured airway responsiveness. OVA control mice showed increased AHR in response to higher concentrations of methacholine when compared to the sham controls; however, PBPB treatment significantly attenuated AHR in a way that was comparable to Dexa treatment (fig. 2). This indicated the ability of PBPB to reduce AHR in the chronic model of asthma.

Fig. 3. PBPB treatment attenuated airway inflammation. **a** After the measurement of AHR, the lungs were harvested, fixed in formalin and embedded in paraffin. Tissues were sectioned and stained with HE. Arrows indicate inflammatory cells. Br = Bronchi. **Inset** Higher magnification of the bronchovascular regions with the inflammatory cells. The arrowhead shows the eosinophil. **b** The inflammation score was determined as mentioned in Materials and Methods and is expressed in grades. * $p < 0.05$ vs. OVA + VEH; ** $p < 0.01$ vs. OVA + VEH.

PBPB Treatment Reduced Airway Inflammation

We found that PBPB treatment reduced AHR in the chronic model of asthma. We wanted to then determine the effect of PBPB on airway inflammation by performing HE staining of the lung sections of the mice. The OVA + VEH mice showed features of airway inflammation with the recruitment of various inflammatory cells, including eosinophils, around the bronchial and vascular regions, and this recruitment was reduced in asthmatic mice treated with PBPB (OVA + PBPB) in a similar way to the Dexa treatment (fig. 3a). The inflammation score analysis confirmed these findings (fig. 3b). PBPB also reduced the levels of Th2 cytokines such as IL-4 and IL-5 (data not shown).

PBPB Treatment Inhibited Subepithelial Fibrosis

As we found a reduction in AHR and airway inflammation with PBPB treatment in the chronic model of

asthma, we wanted to determine the effects of PBPB on epithelial injury. Our TUNEL assay on the lung sections of the mice revealed that PBPB treatment reduced the apoptotic changes in bronchial epithelia compared to VEH-treated OVA mice (data not shown). It is well known that most airway remodeling changes are induced by epithelial injury, and PBPB treatment reduced the epithelial injury, so we wanted to determine the effect of PBPB on subepithelial fibrosis, a crucial component of airway remodeling. We performed this by MT staining of the lung sections of the mice. As shown in figure 4a, OVA + VEH mice exhibited drastic subepithelial fibrosis, and this feature was reduced in the asthmatic mice treated with PBPB (OVA + PBPB). However, with Dexa treatment, the reduction was modest. The morphometry analysis confirmed these findings (fig. 4b).

Fig. 4. PBPB treatment attenuated subepithelial fibrosis. **a** Lung sections were stained with MT. Arrows indicate the fibrotic areas. Br = Bronchi. **b** Morphometry was performed as mentioned in Materials and Methods to calculate the subepithelial collagen content and is expressed in a.u. * $p < 0.05$ vs. sham + VEH; # $p < 0.05$ vs. OVA + VEH.

Fig. 5. PBPB treatment reduced the TGF- β_1 level. The lungs were homogenized with PBS and the resultant homogenates were used for the measurement of TGF- β_1 . n.s. = Nonsignificant compared to OVA + VEH. * $p < 0.05$ vs. sham + VEH; # $p < 0.05$ vs. OVA + VEH.

PBPB Treatment Reduced TGF- β_1 Levels

Since it is known that subepithelial fibrosis is attributable to elevated TGF- β_1 , we determined the levels of TGF- β_1 in the lung homogenates of mice. OVA + VEH mice had elevated TGF- β_1 , which was significantly reduced by the treatment with PBPB (fig. 5). Dexamethasone treatment did not significantly reduce the level of TGF- β_1 .

PBPB Treatment Did Not Affect Goblet Cell Metaplasia

As we found a reduction in AHR, airway inflammation and subepithelial fibrosis with PBPB treatment in the chronic model of asthma, we wanted to determine the effects of PBPB on goblet cell metaplasia. PAS staining was performed in the lung sections of the mice. OVA + VEH

Fig. 6. PBPB treatment did not attenuate goblet cell metaplasia. **a** Lung sections were stained with PAS. Arrows indicate the goblet cell metaplasia. Br = Bronchi. **b** Morphometry was performed as described in Materials and Methods to calculate the epithelial mucin content, and is expressed in a.u. n.s. = Nonsignificant compared to OVA + VEH. * $p < 0.05$ vs. sham + VEH.

mice showed increased goblet cell metaplasia. However, this was not reduced in the asthmatic mice treated with PBPB (OVA + PBPB) but the Dexa treatment reduced it drastically (fig. 6a). The morphometry analysis that estimated the epithelial mucin content confirmed these findings (fig. 6b).

PBPB Treatment Reduced the Expression of α -Smooth-Muscle Actin, a Marker of Airway Smooth Muscle

As we found a reduction in TGF- β levels in the lungs of mice with PBPB treatment in the chronic model of

asthma, and TGF- β is known to be a potent inducer of airway smooth-muscle hyperplasia, we wanted to determine the effects of PBPB on this. We performed immunohistochemistry against α -smooth-muscle actin, a specific marker of smooth muscle in the lung sections of the mice. OVA + VEH mice showed increased expression of α -smooth-muscle actin (fig. 7). This expression was markedly reduced in the asthmatic mice treated with either PBPB (OVA + PBPB) or Dexa (OVA + PBPB).

Fig. 7. PBPB treatment reduced the expression of α -smooth-muscle actin. Lung sections were immunostained with α -smooth-muscle actin. Brown areas and arrows indicate positive expression. Br = Bronchi; IHC = immunohistochemistry. Color refers to the online version only.

Discussion

The role of PLA2 in inflammation and its related diseases is relatively well-studied [21–23]. PLA2 promotes inflammation through various mechanisms such as the production of lysophospholipids, the release of arachidonic acid, which is the source of numerous proinflammatory mediators, and the release of proinflammatory cytokines [21]. As all these mechanisms are crucial in most inflammatory diseases, PLA2 is considered as a therapeutic target for such diseases and also in cancer [22, 23]. The effect of PLA2 on fibrosis has not been studied in detail, even though it has been shown that PLA2 is essential in hepatic fibrosis and fibrotic plaque formation in atherosclerosis [24, 25]. These indicate a possible link between PLA2 and fibrosis.

Initially, PBPB was used mostly in *in vitro* studies as a dissecting tool to examine the involvement of PLA2, but here we have shown, for the first time, its antiasthmatic activity in an acute mouse model of asthma with features like AHR and inflammation, even though we did not examine its effects on airway remodeling [9]. We found that PBPB treatment reduces some key features of airway remodeling such as subepithelial fibrosis and smooth-muscle hyperplasia (fig. 4, 7). These effects could be due to

multiple factors including the level of TGF- β_1 . In this study, we found a reduction of TGF- β_1 level in the PBPB-treated mice. This finding is well-supported by our earlier studies, in which we found that PBPB reduced the levels of PLA2 [9]. We also previously demonstrated the possible effect of PLA2 on airway remodeling; PLA2 can induce arginase via TGF- β_1 and the resultant arginase can lead to the production of polyamines that increase airway remodeling features like subepithelial fibrosis and the proliferation of smooth-muscle cells [10].

It is well-known that epithelial injury is an initiating event of airway remodeling because the injured airway epithelia secrete numerous growth factors including TGF- β_1 . We wanted to see whether such a pathway is involved. Interestingly, PBPB treatment reduced the apoptotic airway epithelia when compared to VEH-treated mice (data not shown). This indicated that PBPB is an epithelial protecting agent and may thus reduce the levels of TGF- β_1 . Our data are in agreement with this (fig. 5). The epithelial protective mechanism is crucial, with recent studies indeed challenging the existing inflammation-dominant theory of asthma pathogenesis [26, 27]. Evidently, most of the available genome-wide scan studies have indicated the possibility of the governing role of the airway epithelia in determining asthmatic airway in-

flammation [28]. In this context, we also found no alteration of goblet cell metaplasia with PBPB treatment (fig. 6), although the subepithelial fibrosis did diminish. This observation is critical because the survival of the epithelia is required for the conversion of airway epithelia into goblet cells [29]. This further indicates that PBPB could possibly increase the cell survival of bronchial epithelia by reducing apoptosis.

Airway remodeling in asthma, a complex event, is characterized by several features such as subepithelial fibrosis, airway inflammation, smooth-muscle hyperplasia, mucous-gland hypertrophy, goblet cell metaplasia and angiogenesis [30, 31]. However, the relations between these various phenotypes are not straightforward. For example, airway inflammation was not always necessarily correlated with AHR [32]. In addition, anti-inflammatory regimens fail to inhibit the process of airway remodeling [32]. However, airway remodeling seems to be well-correlated with AHR in the chronic asthmatic condition [7, 33]. Due to such complexity, it is very difficult to find a drug compound that can attenuate all the features. Even corticosteroids, potent anti-inflammatory drugs, cannot reduce subepithelial fibrosis, a key feature of airway remodeling; they do reduce most of the other features of airway remodeling such as inflammation and smooth-muscle hyperplasia [33, 34]. Similar to these oth-

er reports, we did not find that Dexa had any significant effects on subepithelial fibrosis or the level of TGF- β_1 , although it did reduce airway inflammation, goblet cell metaplasia and smooth-muscle hyperplasia. This could be due to the simultaneous occurrence of airway inflammation and airway remodeling changes, as various reports demonstrated that airway remodeling may appear even before the symptoms of asthma [35–37].

In conclusion, our data demonstrate that PBPB, a synthetic molecule, can reduce some crucial features of airway remodeling and could thus be a good antiasthmatic drug candidate for treating patients with chronic asthma.

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Disclosure Statement

The authors have declared that no conflict of interest exists.

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