

Inhaled Carbenoxolone Prevents Allergic Airway Inflammation and Airway Hyperreactivity in a Mouse Model of Asthma

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Key Words

Airway hyperreactivity · Airway inflammation · Carbenoxolone · Cytokines · Eosinophils · Mucus secretion

Abstract

Background: Asthma is a chronic respiratory disease, which needs a safer medication preferably in inhalation form. In view of this, we have evaluated the effect of inhaled carbenoxolone (CBX), a herbal-derived compound, on asthma in a mouse model. **Methods:** Mice were sensitized and challenged with ovalbumin (OVA) to develop certain characteristic features of asthma such as airway hyperreactivity (AHR), airway eosinophilia, lung inflammation and mucus hypersecretion. To evaluate the effect of CBX on the above asthmatic features, CBX (2.5, 5 and 10 mg/ml, 3 ml) or vehicle (water) was given by inhalation. AHR was determined using whole-body plethysmography. Infiltration of eosinophils was estimated by microscopy. Lung inflammation and mucus hypersecretion were assessed using hematoxylin and eosin, and periodic acid-Schiff staining, respectively. Th-2 cytokines, IL-4 and IL-5 were measured in bronchoalveolar lavage (BAL) fluid and IgE in sera. To identify the possible mode of CBX action, we measured corticosterone levels in the BAL fluid and 5-lipoxygenase (5-LO) expression in the lungs. **Results:** CBX (5 mg/ml) inhalation markedly alleviated AHR ($p = 0.0032$) and reduced lung inflammation and mucus hypersecretion. Also, it prevented the increase in IL-4 ($p = 0.0192$),

IL-5 ($p = 0.0116$) and eosinophils ($p < 0.0005$) in the BAL fluid, and OVA-specific IgE levels ($p = 0.00061$) in sera. 5-LO expression was also markedly reduced. However, corticosterone levels were not affected. **Conclusions:** Inhaled CBX alleviates the asthmatic features in mice and could be a potent nebulized therapy in clinical asthma.

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Introduction

Carbenoxolone (CBX, 3 β -hydroxy-11-oxoolean-12-en-3-O-oic acid 3-hemisuccinate, fig. 1) is a hemisuccinate of glycyrrhetic acid, which is a major active constituent of licorice (*Glycyrrhiza glabra*) root. It is used in the treatment of gastric ulcers in humans [1, 2] and has been attributed numerous pharmacological activities like antiviral, anti-tumor, hepatoprotective and anti-inflammatory effects in experimental animals [3–6]. Recently, CBX has been demonstrated to block gap junction in animal models and expected to be an anti-epileptic agent for humans [7].

Asthma is a respiratory disease which is characterized by reversible airway obstruction, airway hyperresponsiveness and airway inflammation. The pathological features include infiltration of the airways by lymphocytes and eosinophils, damage and loss of bronchial epithelium, mucus hypersecretion, mast cell degranulation, hyper-

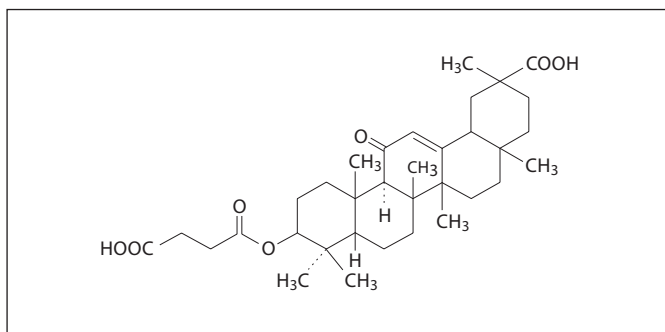


Fig. 1. Structure of carbenoxolone (3 β -hydroxy-11-oxoolean-12-en-3-O-oic acid 3-hemisuccinate).

plasia and collagen deposition in the epithelial sub-basement membrane area [8]. Despite the increased understanding of the pathogenesis of asthma and drug development strategies, its incidence is increasing rapidly all over the world [9]. The existing therapy is not adequate, and currently available drugs such as steroids, β_2 -adrenoceptor agonists and phosphodiesterase inhibitors have numerous adverse effects [10–12]. There is an intense need to identify new molecules preferably from natural origin with negligible or no adverse effects for treating this epidemic disease. In view of this, several herbal compounds such as curcumin, luteolin, glycyrrhizin, verproside and cannabinoids have been identified as anti-asthmatic agents in animal models of asthma [13–17], and efforts are still continued to search other potent herbal molecules. Based on its anti-inflammatory activity [5, 6], we speculated that CBX may also possess anti-asthmatic activity.

In the present study, we have evaluated its effect on certain asthmatic features in mice, such as allergen-induced airway hyperreactivity (AHR), pulmonary inflammation, mucus hypersecretion, and increased Th-2 cytokines and IgE. To understand its mechanism of action, we have also examined its effect on steroid (corticosterone) levels and 5-lipoxygenase (5-LO) expression.

Materials and Methods

Animals

Experimental protocols were approved by the Institutional Animals Ethics Committee. BALB/c male mice (8–10 weeks old, weighing 18–20 g) were obtained from the Vallabhbhai Patel Chest Institute, Delhi, India, and acclimatized for 1 week under laboratory conditions ($25 \pm 2^\circ\text{C}$, 55% humidity) before starting the experiment.

Development of Experimental Asthma

The asthmatic features, AHR and airway inflammation, were induced by ovalbumin (OVA) sensitization and OVA challenge as shown in the protocol (fig. 2). Mice were sensitized with 0.2 ml i.p. saline suspension containing 50 μg OVA (Sigma, USA) and 4 mg alum (aluminum hydroxide) on days 0, 7 and 14. From days 19 to 26, mice were subjected to daily OVA aerosol (2%) inhalation (fig. 2) in a Perspex box chamber ($20 \times 20 \times 10 \text{ cm}^3$) for 30 min. The aerosol was generated by a nebulizer (Omron CX3, USA) with an airflow rate of 10 l/min. Developed AHR and airway inflammation were examined as described below. Control (sham-sensitized) mice received intraperitoneal injections of 0.2 ml saline containing 4 mg alum and were challenged with saline alone. They were also checked for airway reactivity and airway inflammation.

Treatment of Mice with CBX

Mice were randomly divided into five groups (6 mice in each), and each group was named according to the treatment. For example, SHAM/SAL/VEH means that the mice were sham-sensitized, saline-challenged and inhaled vehicle; and OVA/OVA/CBX 5 mg means that the mice were OVA-sensitized, OVA-challenged and inhaled CBX 5 mg/ml. Mice in group 1 were sham-sensitized, saline-challenged and inhaled vehicle (3 ml water); mice in group 2 were OVA-sensitized and OVA-challenged and treated with vehicle alone; mice in groups 3–5 were sensitized and challenged with OVA and given CBX (carbenoxolone disodium salt, Sigma; 2.5, 5 and 10 mg/ml, respectively, 3 ml, inhalation) twice daily from days 18 to 26 (fig. 2). Mice were housed in a Perspex box ($20 \times 10 \times 10 \text{ cm}^3$) and, using an Omron nebulizer (CX3 model, USA), 3 ml of CBX solution were aerosolized into the box until it was finished (8–10 min).

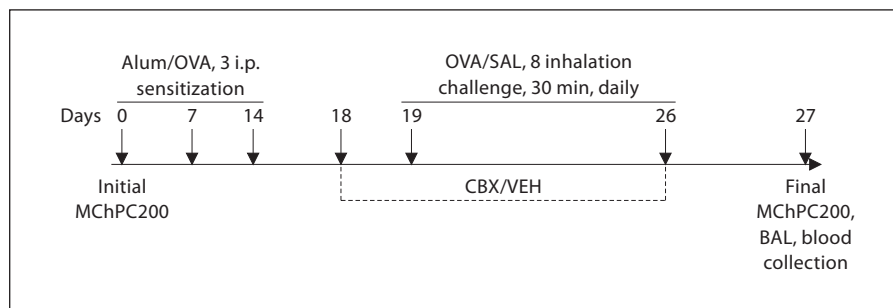
Measurement of Airway Reactivity to Methacholine (MCh)

Airway function of the mice was measured as enhanced pause (Penh) by whole body plethysmography (Buxco Electronics, USA), as described by Hamelmann et al. [18]. Airway reactivity to MCh (Sigma-Aldrich, USA) was determined before sensitization (on day 0) and 24 h after the last OVA aerosol challenge (on day 27). Each individual mouse was housed in the whole body plethysmograph and acclimatized, and 100 μl of PBS (as vehicle) aerosol was given to obtain the basal Penh level. Subsequently, aerosol of progressively doubled concentrations of MCh (3.1, 6.25, 12.5, 25, 50 and 100 mg/ml PBS, 100 μl) was each given for 60 s until the Penh level had increased 2-fold or more of the basal level. The aerosol was generated by an ultrasonic nebulizer (Aeronob Pro, Buxco). An MCh dose-Penh percentage curve was prepared for each mouse, and the dose which produced a 200% increase in its basal Penh level (MChPC200) was derived by interpolation of the curve and was used as an index of airway reactivity.

Collection of Bronchoalveolar Lavage (BAL) Fluid and Blood Samples

After measurement of MCh-induced airway reactivity, mice were sacrificed on day 27 using an overdose of thiopentone sodium (100 mg/kg, i.p.). Blood samples were collected via heart puncture; sera were separated by centrifugation at 3,000 rpm for 10 min and kept at -70°C until analyzed for IgE. The trachea was cannulated and BAL fluid was collected by repeated lung washing with 0.5 ml of PBS four times from each mouse. About 1.5 ml of BAL fluid was recovered per mouse and centrifuged (400 g, 4°C ,

Fig. 2. Experimental protocol: mice were sensitized by intraperitoneal injection of OVA on days 0, 7 and 14 and challenged with OVA aerosol for 8 consecutive days (30 min daily) from days 19 to 26. To evaluate the effect of CBX on asthmatic parameters, mice were given CBX by inhalation from days 18 to 26. On day 27, each mouse was challenged with increasing concentrations of MCh to determine MCh-induced airway reactivity.



10 min), and the supernatant was kept at -70°C until analyzed for cytokine and corticosterone levels.

Cell Counts and Lung Histology

BAL cells were washed three times with PBS and the pellet was resuspended in 200 μl of cold PBS. A small portion of this suspension was taken for total cell counts using Neubauer's chamber. For differential counts, cytopsin preparations were made and stained with Leishman's stain. The cells were identified and counted by standard morphology; 200 cells were counted and the percentage of eosinophils was calculated. For lung histology, the excised lung portion was fixed in 10% buffered formalin. The fixed, paraffin-embedded tissues were cut into 4- μm sections and stained with hematoxylin and eosin (HE), and periodic acid-Schiff (PAS) to assess inflammation and mucus secretion, respectively. Histopathological analysis was done in 4 mice from each group.

Immunohistochemistry

Immunohistochemical localization of 5-LO was performed using the antigen retrieval method. The paraffin-embedded lung tissues were cut into 4- μm sections. After deparaffinization, endogenous peroxidase was quenched with 2 ml of H_2O_2 in 48 ml of PBS for 30 min. Antigen retrieval was done by heating the sections dipped in citrate buffer (pH 6.0). Non-specific adsorption was minimized by incubating the section in 150 μl of rabbit sera in 10 ml of PBS. The sections were incubated overnight with anti-5-LO antibody at a dilution of 1:100 (Santa-Cruz, USA). Sections were washed with PBS and incubated with secondary antibody conjugated with HRP enzyme. Specific labeling was detected by adding diaminobenzidine, which forms a brown-colored product. The sections were counterstained with hematoxylin and mounted with DPX. Photomicrographs of the stained sections were taken using a Samsung digital camera (model: SDC-313PD) coupled to a bright-field microscope (Nikon YS100, Nikon, Japan).

Measurement of Cytokines, IgE and Corticosterone

The cytokines IL-4 and IL-5 were measured in BAL fluid supernatant and OVA-specific IgE levels in sera were measured by enzyme-linked immunosorbent assay (ELISA), as described previously [14, 19]. Corticosterone in the BAL fluid was assessed using an ELISA kit obtained from Neogen (USA).

Statistics

All data were presented as means ($\pm$ SEM). Student's *t* test was applied to detect significant differences. A *p* value <0.05 was considered significant.

Results

CBX Inhibits AHR to MCh

To investigate the effect of inhaled CBX on MCh-induced AHR, mice were treated with CBX aerosol (2.5, 5, and 10 mg/ml, 3ml) or vehicle as described in the protocol (fig. 2), and MChPC200 values were determined 24 h after the last OVA challenge (i.e. on day 27). As shown in figure 3, there was a significant decrease in the MChPC200 values in OVA/OVA/VEH mice (11.3 ± 1.2 mg/ml PBS) compared with baseline (34.6 ± 3.4 mg/ml PBS, $p = 0.0011$), while there was no change between the initial and final MChPC200 values of the SHAM/SAL/VEH group. This demonstrates that OVA-sensitized and OVA-challenged mice had developed AHR to MCh while normal control (sham-sensitized and saline-challenged mice) had no AHR. Treatment of mice with CBX (OVA/OVA/CBX 2.5, 5 and 10 mg/ml, 3ml) increased MChPC200 in a dose-dependent manner, showing attenuation of AHR to MCh. At a dose of 5 mg/ml CBX, MChPC200 values increased up to 96.6% ($p = 0.0032$) of the basal level (fig. 3). An apparent increase induced by the 10 mg/ml dose over the 5 mg/ml dose was seen; however, no significant change ($p = 0.19$) was noted. Therefore, the 5 mg/ml dose of CBX seems to be the optimally working dose to attenuate AHR to the normal level in our mouse model.

CBX Prevents the Increase in IL-4 and IL-5 in the BAL Fluid

To examine the effect of CBX on IL-4 and IL-5 levels in the airways, we measured them in the BAL fluid by ELISA and compared the values between different groups. As shown in figure 4, IL-4 levels were found to be markedly increased in the BAL fluid of OVA/OVA/VEH (445.8 ± 10.9 pg/ml) compared with SHAM/SAL/VEH (64.0 ± 8.1 , $p = 0.00018$; fig. 4). The inhalation of CBX prevented the increase in IL-4 in a dose-dependent manner (OVA/OVA/CBX 2.5 mg/ml, 11%, $p = 0.048$;

Fig. 3. CBX inhibits MCh-induced AHR. Experiments were performed as described in figure 2, and MChPC200 values were determined on day 27 to examine the effect of CBX on airway reactivity. Means $\pm$ SEM; n = 6 in each group. White bars = Initial MChPC200 values (before starting sensitization); grey bars = MChPC200 values on day 27 after vehicle or CBX inhalation treatment. ^ap = 0.0011 vs. initial MChPC200; ^bp = 0.0129, ^cp = 0.0032, ^dp = 0.0025, vs. OVA/OVA/VEH.

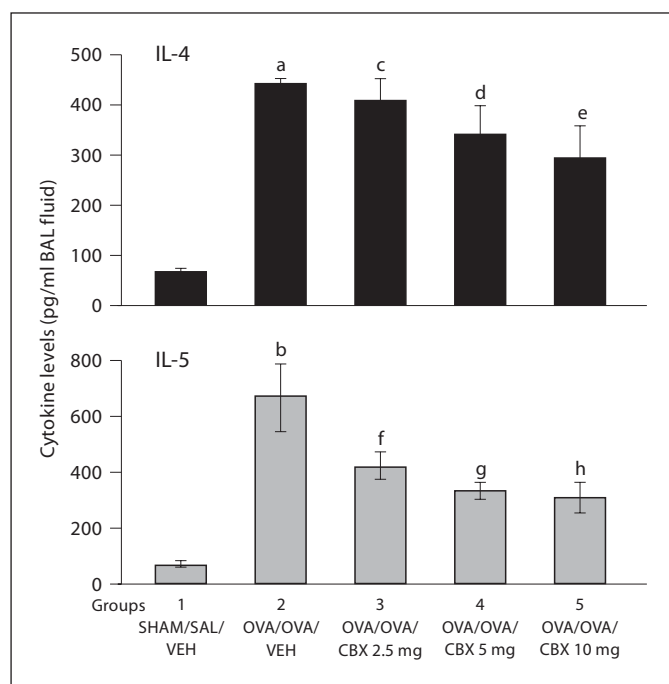
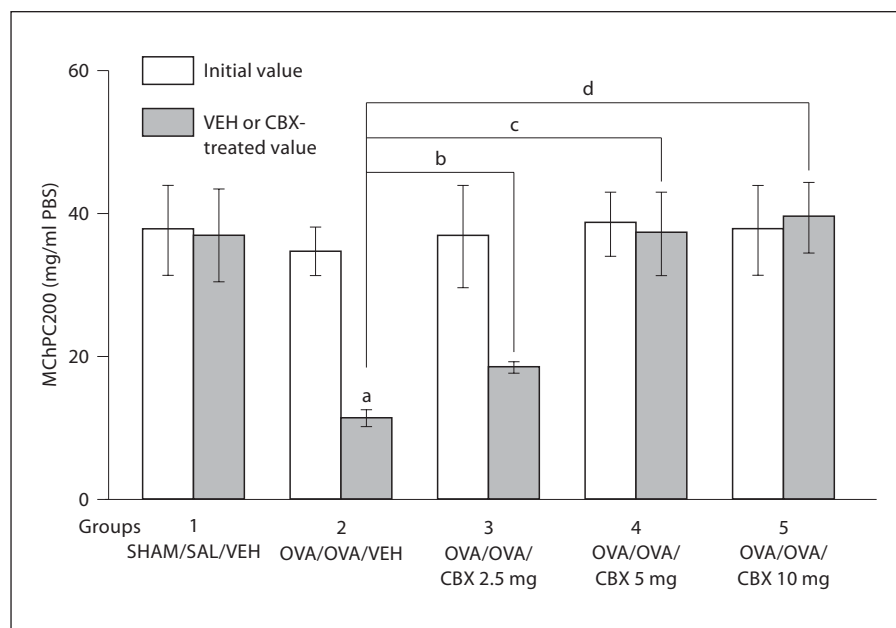


Fig. 4. Effect of CBX treatment on IL-4 and IL-5 levels in the BAL fluid. Means $\pm$ SEM; n = 6 in each group. ^ap = 0.00018, ^bp = 0.00026, vs. SHAM/SAL/VEH; ^cp = 0.048, ^dp = 0.0192, ^ep = 0.013, ^fp = 0.037, ^gp = 0.0116, ^hp = 0.0104, vs. OVA/OVA/VEH.

5 mg/ml, 23%, p = 0.0192; 10 mg/ml, 34%, p = 0.013 compared with OVA/OVA/VEH).

IL-5 levels were also highly increased in OVA/OVA/VEH (671.78 $\pm$ 122.0 pg/ml) compared with SHAM/SAL/VEH (68.2 $\pm$ 15.7, p = 0.00026). CBX treatment also induced a dose-dependent decrease in IL-4 and IL-5 levels. Notably, inhibition was more pronounced for IL-5 than IL-4 levels (IL-5: OVA/OVA/CBX 2.5 mg/ml, 37.2%, p = 0.037; 5 mg/ml, 50.3%, p = 0.0116; 10 mg/ml, 54.3%, p = 0.0104 vs. OVA/OVA/VEH; fig. 4). The optimum inhibition of both cytokines was noted at the dose of 5 mg/ml, with no additional significant benefit of the 10 mg/ml dose.

CBX Prevents the Increase in OVA-Specific Serum IgE Levels

To test the effect of CBX in OVA-specific IgE, serum samples were collected from mice treated with vehicle or different concentrations of CBX and the levels of OVA-specific serum IgE were measured. OVA-specific IgE levels were markedly increased in OVA/OVA/VEH mice compared to SHAM/SAL/VEH mice (0.64 $\pm$ 0.02 vs. 0.07 $\pm$ 0.021 OD_{450 nm}, p = 0.00021; fig. 5). CBX treatment decreased IgE levels dose dependently (OVA/OVA/CBX 2.5 mg/ml, 10.7%, p = 0.0353; 5 mg/ml, 35.1%, p = 0.00061, and 10 mg/ml, 41.2%, p = 0.0005 vs. OVA/OVA/VEH mice). However, compared with 5 mg/ml, 10 mg/ml did not show any significant change.

Table 1. Effect of CBX on total cell and eosinophil numbers, and the percentage of eosinophils in the BAL fluid

	SHAM/SAL/VEH	OVA/OVA/VEH	OVA/OVA/CBX 2.5	OVA/OVA/CBX 5	OVA/OVA/CBX 10
Total cells, n/ml $\times 10^5$	0.74 $\pm$ 0.15	16.47 $\pm$ 0.98 ^a	9.83 $\pm$ 0.37 ^b	3.81 $\pm$ 0.38 ^c	2.83 $\pm$ 0.37 ^c
Eosinophils, n/ml $\times 10^5$	0.012 $\pm$ 0.005	6.215 $\pm$ 1.073 ^a	2.284 $\pm$ 0.657 ^b	0.549 $\pm$ 0.102 ^c	0.374 $\pm$ 0.081 ^c
Eosinophils, %	1.5 $\pm$ 0.43	37.8 $\pm$ 5.87 ^a	23.0 $\pm$ 6.1 ^b	14.5 $\pm$ 2.39 ^c	13.5 $\pm$ 2.36 ^c

^a $p < 0.0001$ vs. SHAM/SAL/VEH; ^b $p < 0.05$ and ^c $p < 0.0005$, vs. OVA/OVA/VEH.

CBX Prevents Airway Eosinophils in the BAL Fluid and Lung Inflammation

Total cell and eosinophil counts were dramatically increased in OVA/OVA/VEH mice compared with SHAM/SAL/VEH mice (table 1). CBX treatment showed a significant, dose-dependent reduction in both total cell numbers and eosinophil counts.

The effect of CBX was also investigated in the lung tissue to examine the inflammation status and was found very effective. The extent of the inflammation and the cellular infiltration in the mice lungs were assessed by HE staining of the paraffin-embedded sections. Representative micrographs are shown in figure 6. The lungs of SHAM/SAL/VEH mice showed normal lung parenchyma with no sign of inflammation (fig. 6a). However, the lungs of OVA/OVA/VEH mice showed dense perivascular and peribronchial distribution of inflammatory cells (fig. 6b). CBX treatment reduced the inflammation significantly. Whereas the 2.5 mg/ml dose inhibited inflammation only mildly (fig. 6c), OVA/OVA/CBX 5 and OVA/OVA/CBX 10 mice showed a marked reduction in both peribronchial and perivascular inflammation, and the lung architecture (fig. 6d, e) was very similar to that of normal mice (fig. 6a).

CBX Reduces Mucus Secretion in Airways

To determine the effect of CBX on mucus hypersecretion, the paraffin-embedded lung sections were used for PAS staining. As shown in figure 7, the OVA/OVA/VEH group showed marked mucus hypersecretion in the epithelial wall lining of bronchi, whereas the SHAM/SAL/VEH group showed no mucus secretion. Thus, CBX treatment inhibited mucus secretion in a dose-dependent manner.

CBX Inhibits 5-LO Expression in the Lungs

To examine the effect of CBX on 5-LO in the lung tissue, immunohistochemistry was done as described. As shown in figure 8, SHAM/SAL/VEH mice showed little

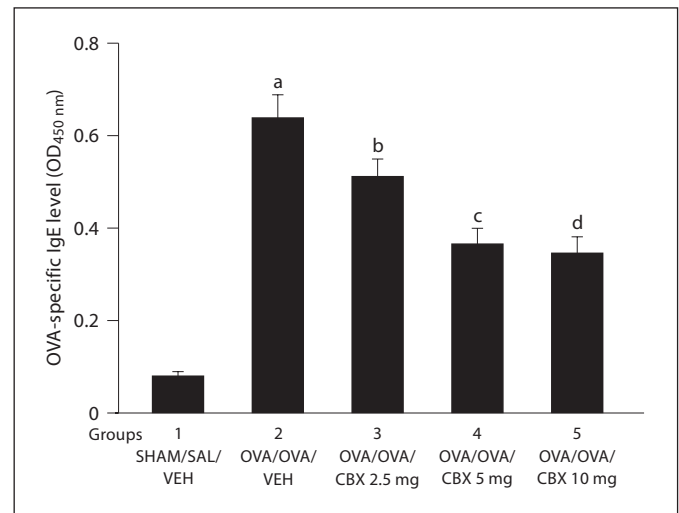


Fig. 5. CBX prevents the increase in OVA-specific serum IgE levels measured by ELISA. Means $\pm$ SEM; $n = 6$ in each group. ^a $p = 0.00021$ vs. SHAM/SAL/VEH; ^b $p = 0.035$, ^c $p = 0.00061$, ^d $p = 0.0005$, vs. VA/OVA/VEH.

or negligible 5-LO expression, whereas OVA/OVA/VEH showed a significant increase. Interestingly, CBX treatment inhibited 5-LO in a dose-dependent manner.

Effect of CBX on Corticosterone Levels

To test whether CBX, a known inhibitor of 11β -hydroxysteroid dehydrogenase, could affect its substrate cortisol/corticosterone, corticosterone levels were measured in the BAL fluid. As shown in table 2, OVA/OVA/VEH mice showed a nonsignificant increase (5.465 ± 0.945 ng/ml) in the corticosterone level compared with SHAM/SAL/VEH mice (4.635 ± 0.655 ng/ml). Most importantly, CBX treatment showed no effect on the corticosterone level compared with SHAM/SAL/VEH controls (table 2).

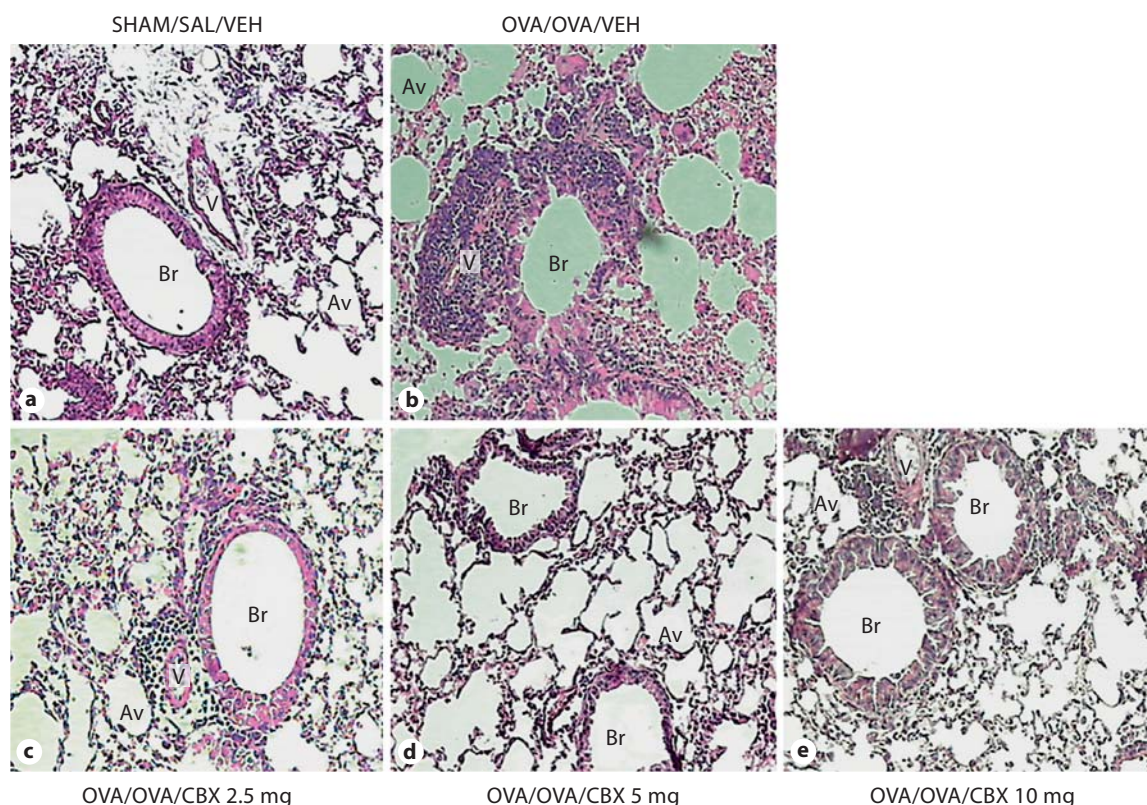


Fig. 6. Lung histopathology. **a** Photomicrograph shows the lung parenchyma of normal (SHAM/SAL/VEH) mice with bronchi (Br), vessel (V) and alveoli (Av) without inflammation and inflammatory cells. **b** OVA/OVA/VEH mice show dense inflammatory cell infiltration both in peribronchial and perivascular areas. Histograms of OVA/OVA 5 (**d**) and 10 mg mice (**e**) with no significant inflammation in peribronchial and perivascular areas resemble the normal (**a**) histogram. A representative section of three independent experiments is shown. **a–e** $\times 250$.

Discussion

As asthma is a chronic airway inflammatory disease and needs a long-term medication, respirable (inhalation) drug delivery is preferable due to its rapid onset of action and minimum side effects compared to the same drug delivered by oral, intraperitoneal or intravenous routes [20, 21]. In the present study, we delivered CBX by inhalation to mice and demonstrated for the first time that inhalation treatment of this compound markedly inhibited the asthmatic features, airway inflammation and AHR, and also modulated the immunological parameters (IL-4, IL-5 and IgE) involved in the pathogenesis of asthma.

The allergen challenge in the airways of sensitized animals produces an increase in Th-2 cytokines, espe-

cially IL-4, IL-13 and IL-5, which are important candidates for the induction of inflammation. IL-4 and IL-13 promote allergen-specific IgE formation by B lymphocytes [22, 23]. IgE binds to the high affinity receptor (Fc ϵ RI) on pulmonary mast cells, which upon cross-linkage by allergen causes mast cell degranulation and release of numerous inflammatory mediators such as histamine, leukotrienes and cytokines. These potent substances cause early asthmatic reactions and are also involved in the late asthmatic phase such as airway inflammation and AHR. IL-4 also upregulates endothelial vascular cell adhesion molecule-1 on the endothelium, which may interact with very late antigen-4 on eosinophils and promotes their eosinophil recruitment into the airways [24]. IL-5 is one of the key cytokines

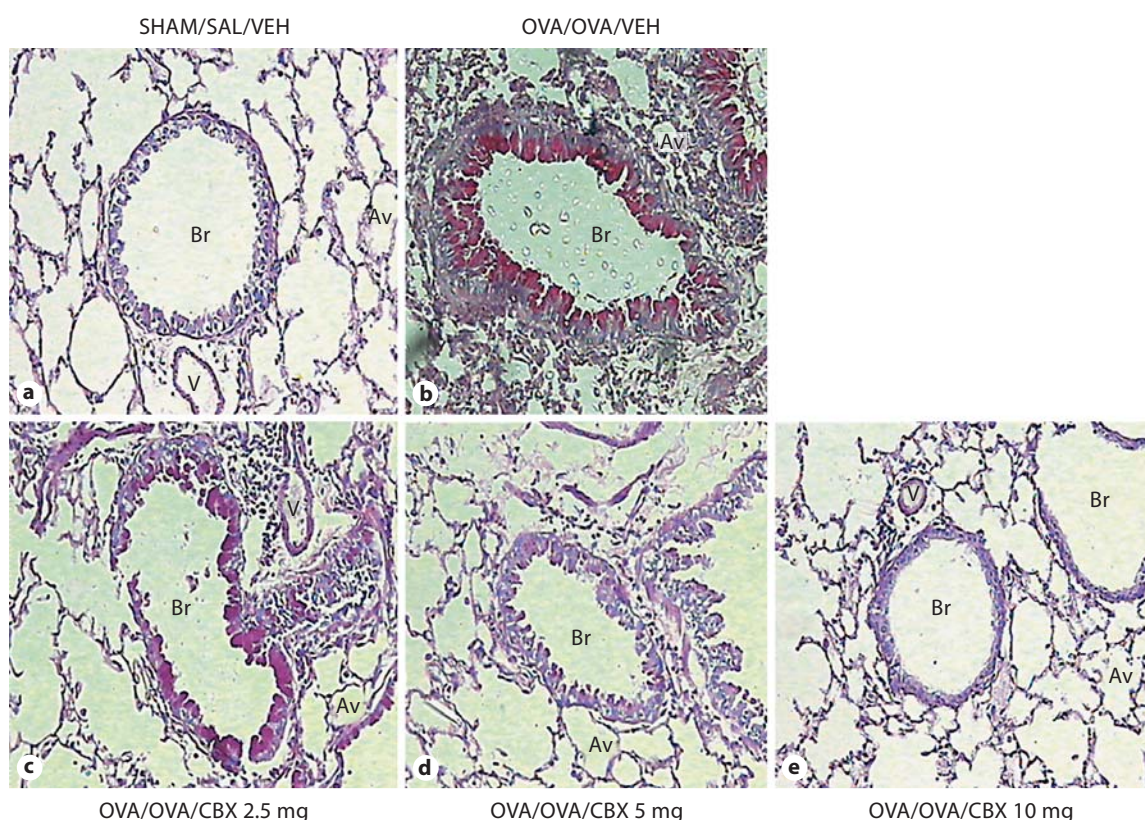


Fig. 7. CBX reduces mucus secretion in the airways. PAS staining of lung sections of OVA/OVA/VEH shows more staining (online version: pink staining) in the bronchi (Br), indicating hypersecretion of mucus compared with SHAM/SAL/VEH. CBX treatment markedly reduces mucus secretion. Av = Alveoli; V = vessel.

Table 2. Effect of CBX on corticosterone levels in the BAL fluid

	SHAM/SAL/VEH	OVA/OVA/VEH	OVA/OVA/CBX 2.5	OVA/OVA/CBX 5	OVA/OVA/CBX 10
Corticosterone, ng/ml	4.65 ± 0.662	5.458 ± 0.947	5.425 ± 0.912	5.692 ± 0.756	4.333 ± 0.500

responsible for maturation, growth and recruitment of eosinophils, and it also promotes the chronic presence of eosinophils in the airways by reducing their apoptosis [25]. The treatment of CBX markedly attenuated the levels of IL-4, IL-5 and IgE. The CBX-induced reduction in IgE could be attributed to the decreased IL-4 level, which could decrease the release of mast cell mediators. The marked reduction in eosinophil counts by CBX may be attributed to the reduced level of IL-5 in the lung fluid.

The attenuation of AHR in mice could be attributed to all these properties of CBX.

CBX is a known inhibitor of 11 β -hydroxysteroid dehydrogenase, an enzyme that catalyzes the conversion of cortisol (in humans)/corticosterone (in rodents) to its respective inactive metabolites cortisone/11-dehydrocorticosterone [26, 27] and is ascribed a role in the inhibition of inflammation (i.e. via an increase in cortisol/corticosterone levels) [28]. In the present study, however, there

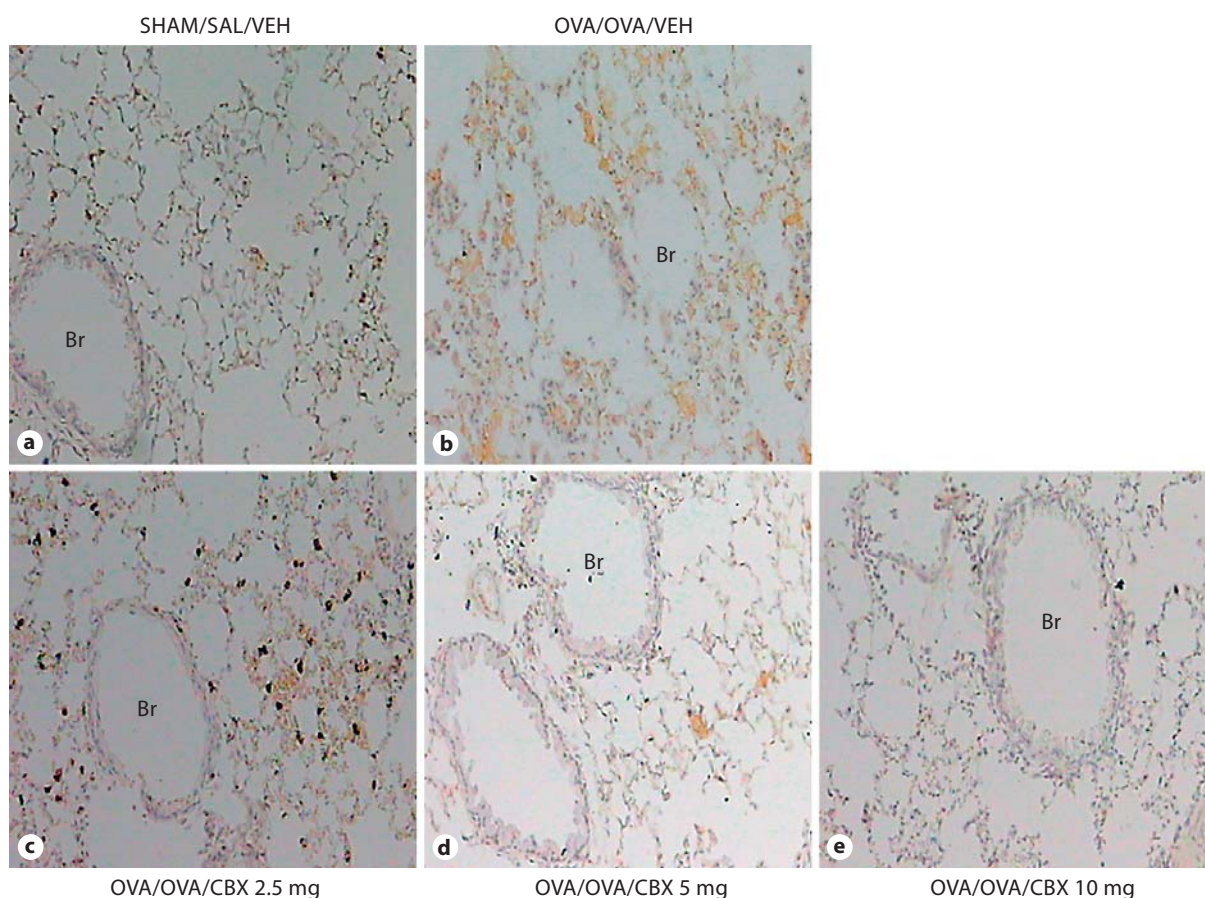


Fig. 8. Immunohistochemical localization of 5-LO in the lung tissue. (Online version: brown color indicates the sites of 5-LO expression.) The SHAM/SAL/VEH group showed very low expression while the OVA/OVA/VEH group showed high 5-LO expression in the lung tissue. CBX treatment markedly inhibited 5-LO expression. Br = Bronchi.

was no effect of any CBX dose on corticosterone levels. Our results are in agreement with an earlier clinical study showing that the lower concentration of its related compound did not affect the corticosteroid level [29]. Therefore, another pathway may be involved in the pathogenesis of asthma at this dose range of CBX, e.g. 5-LO and phosphodiesterase enzymes, established therapeutic targets for developing anti-asthmatic drugs [30, 31]. Interestingly, we observed that CBX markedly inhibited 5-LO expression, indicating an important role in anti-asthmatic activity. Therefore, future studies investigating other pathways may be of interest.

In conclusion, the inhalation of CBX markedly alleviates asthmatic features such as airway inflammation,

mucus hypersecretion and AHR, and inhibits 5-LO expression, Th-2 cytokines (IL-4 and IL-5) and allergen-specific IgE in a mouse model. CBX could, therefore, be a potent molecule for developing an effective nebulized drug in the treatment or management of clinical asthma.

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