Treatment of Ovalbumin-Induced Experimental Allergic Bronchitis in Rats by Inhaled Inhibitor of Secretory Phospholipase A₂.

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Running Title: PLA₂ inhibition for treating allergic bronchitis.
ABSTRACT
Asthma pathophysiology involves the action of inflammatory/allergic lipid mediators that are formed subsequent to membrane phospholipid hydrolysis by phospholipase-A_2 (PLA_2). Cysteinyl-leukotrienes in particular are considered potent inducers of broncho-constriction and airway remodeling. In a recent study [Offer et al., Am J Physiol 288: L523-L529, 2005] we showed that ovalbumin (OVA)-induced broncho-constriction in rats is associated with elevation of sPLA_2 activation and cysteinyl-leukotrienes production, concomitantly with suppression of cPLA_2 and prostaglandin-E_2 level. These processes were reversed when the animals were pre-treated systemically with an extracellular cell-impermeable sPLA_2 inhibitor, which also suppressed the early allergic reaction (broncho-constriction) to challenge with OVA. Subsequently, in the present study we examined the capacity of sPLA_2-inhibitor to ameliorate inflammatory and allergic manifestations (early and late bronchoconstriction) of OVA-induced allergic bronchitis in rats, when the inhibitor was administered by inhalation, to confine it to the airways. We show that treatment by inhalation of the sPLA_2-inhibitor markedly suppresses the ovalbumin-induced early and late asthmatic reactions, as expressed by broncho-constriction, airway remodeling (histology), cysteinyl-leukotrienes level in broncho-alveolar lavage (BAL), and production of TNFα and nitric oxide by BAL macrophages. Furthermore, OVA-induced broncho-constriction in sensitized, non-pre-treated rats is also inhibited by inhalation of the sPLA_2 inhibitor either prior to or after challenge. These findings verify the pivotal role of sPLA_2 in the pathophysiology of both the immediate allergic response and the inflammatory-asthmatic process. This study introduces the control of airway sPLA_2 as a novel therapeutic approach, and presents a prototype for inhaled non-steroidal cell-impermeable PLA_2 inhibitors for asthma treatment.

Keywords: Asthma, Cysteiny-leukotrienes, Nitric-oxide, TNFα, Glycosaminoglycans.
INTRODUCTION

Asthma pathophysiology involves the action of inflammatory/allergic lipid mediators produced subsequent to membrane phospholipids hydrolysis by phospholipase-A2 (PLA2), into lyso-phospholipids and arachidonic acid (AA). AA is metabolized mainly via the lipoxygenase (LOX) and cyclooxygenase (COX) pathways, producing leukotrienes (LTs) and prostaglandins (PGs), respectively. Cysteinyl LTs (CysLTs) in particular play an important role in asthma pathophysiology [1]. Lyso-phospholipids induce white cell activation, histamine secretion from mast cells, and airway smooth muscle (ASM) proliferation [2,3].

The PLA2 family consists of intracellular and secreted enzymes [4]; the intracellular enzymes include the Ca++-independent (iPLA2), which is generally considered a housekeeping enzyme, and the cytosolic (cPLA2) enzyme, which is specific to AA-carrying phospholipids, and thus considered a major AA producer. The secretory PLA2s (sPLA2), secreted by activated leukocytes and other assaulted cells [5,6], hydrolyze cell membrane phospholipids with no preference for fatty acid. sPLA2 also act as receptor ligands to induce cytokine production (TNFα, IL-6, IL-8) [7,8], mast cell survival [9], CD44 expression by eosinophils [8], and ASM cell proliferation [7]. All-in-all, PLA2s are involved directly and indirectly in inducing inflammatory/allergic processes. This pertains especially to sPLA2s, which are thus considered "the inflammatory enzymes" [6].

Although the relative contributions of cPLA2 and sPLA2 differ between cells and tissues, it is generally accepted that both types take part and often act synergistically, in producing pro-inflammatory lipid mediators [10]. However, airways seem to be an exception, in which different eicosanoids play opposing roles: PGE2, generally considered a potent broncho-dilator and can inhibit ASM proliferation [11,12], whereas CysLTs are strong broncho-constrictors and facilitate airway remodeling [13]. It has therefore been postulated that unlike other organs, the lung is a privileged site for the beneficial actions of PGE2 [12], and that in challenged airways LTs and PGs follow different routes. Accordingly, it has been reported that in inflammatory cells LTs are produced from a sPLA2-linked AA pool, while PGs are produced from a cPLA2-linked AA pool [10,14], implying opposing roles for sPLA2 and cPLA2 in asthma.

This hypothesis was strongly supported by our recent study with experimental allergic bronchitis model in rats [15], showing that ovalbumin (OVA)-induced broncho-constriction is associated with elevation of sPLA2 and CysLT levels, and suppression of cPLA2 and PGE2 levels. Furthermore, these processes were reversed by treatment with an extracellular sPLA2 inhibitor (ExPLI), suggesting a pivotal role for sPLA2 in allergic pathophysiology. Subsequently, in the present study we tested the potential of sPLA2 inhibition in ameliorating experimental allergic bronchitis, by examining the effect of ExPLI inhalation on the early and late broncho-constriction reaction, and airway inflammation and remodeling in OVA-induced experimental allergic bronchitis rats. We show that both OVA-induced broncho-constriction and lung inflammation were markedly suppressed by inhalation of sPLA2 inhibitor.

MATERIALS AND METHODS

Induction of experimental allergic bronchitis: Brown Norway rats were sensitized with ovalbumin (OVA) as previously described [15]: Briefly, subcutaneous (SC) injection of OVA with aluminum-hydroxide and intra-peritoneal (IP) injection of Bordetella Pertussis-killed organisms on day 0. Two weeks later, the rats, placed unrestrained in a 20 litre box connected to an ultrasonic nebulizer (LS 230 System Villeneuve Sur Lot, France), were subjected to repeated bronchial allergen challenge by inhalation of OVA (1 mg/ml saline) for 5 minutes, every other day through day 45.
Treatments: The ExPLI used here was HyPE, which was synthesized in the laboratory of S. Yedgar [16], by conjugating hyaluronic acid (HA, Gideon Richter, Budapest, Hungary) to dipalmitoyl phosphatidyl-ethanolamine (PE, Sigma, St Louis, MI), to form an amide bond between the PE amino group and the HA carboxylic group, and was characterization by NMR spectroscopy [16, 17].

Two treatment modes were applied:
1. HyPE inhalation through the process of the allergic bronchitis induction, to examine the ExPLI effect on the sensitization (prevention): 4 treatment groups were employed: 1. Naïve control (-/-) - no sensitization/no treatment. 2. Positive control (OVA/OVA) - Sensitization + challenge with OVA inhalation and sub-cutaneous (SC) injection of 1 ml saline before each challenge. 3. Sensitization + challenge with OVA and HyPE inhalation before every challenge (OVA/HyPE). 4. Sensitization + challenge with OVA and treatment with SC injection of dexamethasone (300 µg) before each challenge (OVA/Dx), as a conventional reference.
2. The rats were not treated with HyPE during the sensitization process, but only before or after challenge of already-sensitized rats.

For treatment with ExPLI, 5 ml HyPE solution (1 mg/ml saline) were sprayed into the 20 L cage, thus diluting the HyPE to 0.25 µg/ml aerosol. The rats inhaled the aerosol for 5 min. The rats’ respiratory rate was 120 breath/min, with an average tidal volume of 1 ml, thus reaching ventilation of 120 ml/minute. If all inhaled HyPE was absorbed in 5 min (600 ml), the maximal HyPE absorbed would be 150 µg (3 nmole) in each treatment. Each group (10 rats in each) were treated and challenged every other day from day 14 until day 45.

Assessment of pulmonary function: Unrestrained conscious rats were placed in a whole-body plethysmograph (Buxco Electronics, Troy, New York) to measure flow-derived pulmonary function (Penh), as previously described in details [15,18]. Broncho-constriction was assessed on day 20, by measuring Penh prior to and 5 min and 8 h after OVA challenge, corresponding to early and late asthmatic reaction (EAR and LAR, respectively).

Histology and Biochemistry: On day 45 the rats were anaesthetized with IP injection of sodium-pentobarbital, sacrificed by bleeding through the abdominal aorta, tracheotomized and cannulated through the trachea. Broncho-alveolar lavage (BAL) was collected by repeated washing of the lungs with 5 ml saline to a total of 50 ml. The lungs were removed, inflated and fixed with formaldehyde (4%) under pressure of 20 cm H₂O, and embedded in paraffin.

Histology: The fixed lungs were sliced longitudinally and embedded in paraffin. Histological sections, 3 µm thick, were cut and stained with 1. hematoxylin and eosin for assessments of interstitial and peri-bronchial inflammation and of airway smooth muscle (ASM) thickening; 2. Tri-chrome for assessment of sub-epithelial fibrosis (basal membrane thickness); 3. Periodic acid Schiff (PAS) for epithelial cell mucus metaplasia. Histological morphometry was performed using a computer program "ImageJ" (NIH Bethesda USA) on 3 randomly selected slides from each lung. Quantification of peribronchial cellular infiltrate in airway tissue was achieved through counting the numbers of inflammatory cells in the 50-µm region beneath the epithelium of the airway (in hematoxylin and eosin stained sections), and expressed as number of cells per millimeter of airway basal lamina length, which was measured by tracing the basal lamina in calibrated digital images. ASM and the basal membrane thickening was determined as previously described [18].
Briefly, the area of ASM cells or the basal membrane thickness were normalized to the square of the basement membrane length ($L_{bm}^2$, in $\mu m^2$) to correct for differences in airway size. Mucus metaplasia assessment was determined by the percent PAS-positively cells from the total airway epithelial cells. Only large (>2,000 $\mu m L_{bm}$) and medium size airways (1,000-2,000 $\mu m L_{bm}$) were selected as it was shown that the most significant pathological changes occur in these airways and less in the small airways [19]. Airways with a ratio of maximum-to-minimum internal diameter $\geq 2$ were considered to be cut tangentially and were not measured. The internal diameters were measured from the basement membrane on one side to the basement membrane on the opposite side of the airway.

**Cysteinyl Leukotrienes** ($LTC_4$, $LTD_4$ and $LTE_4$) were determined in BAL enzyme-immunoassay using a commercial kit (Amersham, UK) [15].

**NO and TNFα production by macrophages:** BAL macrophages were isolated and cultivated in DMEM + 10% serum for 2h, after which non-adherent cells were removed. The adherent cells were re-cultivated in DMEM +10% serum ($10^6$cells/well) for 48 hours. The culture medium was then collected for determination of nitric oxide (NO), using the Gries method for measuring the corresponding nitrite, and TNFα, using RIA kit (Amersham, UK).

**Statistical analysis** was performed using statistical software (GB-STAT, Dynamic Microsystems, Silver Spring MD). Analysis of variance (ANOVA) was used to assess significant differences between treatment groups. Tukey test was used to compare between each one of the treatment groups. A value of $p < 0.05$ was considered significant.

**RESULTS:**

**Effect of HyPE inhalation on respiratory functions in rats with OVA-induced experimental allergic bronchitis:**

To examine the ExPLI effect on sensitization of rats by OVA, the rats inhaled HyPE before every challenge as described in Methods. In parallel, another group was injected SC with dexamethasone (Dx). As shown in Figs. 1A & 1B, inhalation of HyPE was effective in reduction of broncho-constriction in both the early and late reactions (EAR and LAR, respectively).

To examine the ability of inhaled HyPE to counteract OVA-induced broncho-constriction in untreated allergic reaction, rats that were sensitized by OVA, but not pre-treated with HyPE, inhaled HyPE only once, either before or after challenge with OVA. As shown in Fig. 2, HyPE clearly decreased the OVA-induced broncho-constriction when inhaled only prior to challenge (Fig. 2A), and also reduced broncho-constriction when inhaled only after challenge (Fig. 2B).

**Prevention of airway remodeling by HyPE inhalation:**

To examine the effect of HyPE inhalation on the allergic bronchial inflammation, at the conclusion of the experiment (30 days after treatment and repeated challenge), the rats were sacrificed and tracheotomized, broncho-alveolar lavage (BAL) was collected, and lung tissues were processed for histological examination.

As demonstrated in Fig 3, peribronchiolar and perivascular cell infiltrates were composed principally of eosinophils and lymphocytes. The eosinophils were disposed in a circumferential manner around the bronchioles and vessels while the lymphocytes were either similarly disposed or arranged in primary follicles (lymphoid follicles without germinal centers). This Figure also demonstrates that the impressive ExPLI therapeutic effect
was also observed in the histological indices, expressed by the reduced inflammatory cell infiltration and airway wall thickening in the HyPE-treated asthmatic rats. The respective morphometry, presented in Table 1, shows that inhalation of HyPE reduced the peribronchial cellularity (4-I), mucus metaplasia of respiratory epithelial cells (4-II), airway smooth muscle thickening (4-III), and basal membrane thickening (4-IV), essentially to the level of the naïve rats.

Effect of HyPE inhalation on biochemical markers:
As discussed above, among the lipid mediators that are involved in asthma pathophysiology, CysLT are considered key mediators of broncho-constriction, and the inhibition of their action is currently the target of several drugs (Monelukast, Zileuton) used for treatment of asthma [20]. As the PLA₂ inhibitor controls lipid mediator production, it was of particular interest to determine its effect on CysLT production in OVA-sensitized rats. To this end, CysLT levels in the BAL of the different groups were determined by ELISA. As shown in Fig 4, the CysLT production was markedly enhanced in the sensitzes rats, but was reduced to the normal range by inhalation of HyPE.

Nitric oxide production is a characteristic marker of inflammation in asthma and is a correlative of the disease severity, although it might have dual effects on mast cells [21]. Its production has been reported to be linked to PLA₂-produced lipid mediators [22], and was found to be inhibited by ExPLIs in LPS-stimulated cells [23] and animals [24]. In line with these findings, in the present study we determined the ExPLI effect on NO production by cultured macrophages collected from the BAL of the different groups subsequent to their in-vivo treatment, without further treatment of the cultured cells. Fig. 5 shows that NO production was considerably enhanced in the BAL macrophages of the sensitized (OVA/OVA) compared to the naïve (-/-) rats, but NO production by macrophages from asthmatic rats that had been treated by HyPE inhalation, was below the level of the naïve rats.

A prominent characteristic marker of inflammatory conditions is TNFα, which is at the top of other pro-inflammatory cytokine cascade. The increase in its level correlates with inflammation severity in asthma, and its inhibition has been proposed for the treatment of severe asthma [25]. In previous studies, the ExPLIs were found effective in suppressing endotoxin-stimulated production of TNFα and other cytokines in animals and cell cultures [24, 26]. Similarly, in the present study we found that TNFα production by macrophages from the BAL of the sensitized rats was markedly augmented, but inhalation of HyPE reduced it to approximately the level of the naïve rats, as shown in Fig. 6.

DISCUSSION
Inhibition of PLA₂ activity has been considered a promising therapeutic strategy in the treatment of inflammatory/allergic diseases, as this can suppress the production of several classes of pathogenic lipid mediators (PGs, LTs, PAF, lysophospholipids). A number of PLA₂ inhibitors have been proposed for this purpose [27]. However, PLA₂ inhibitors that enter the cell might interfere with the vital PL metabolism and might impair the cell viability. In addition, as previously discussed [6], in inflammatory conditions a number of sPLAs are involved in cell lysis and lipid mediator production, and inhibiting only one or part of them has limited or no efficacy. Therefore, as has long been proposed [28], there is a clear advantage to cell-impermeable PLA₂ inhibitors that protect the cell membrane from the action of sPLA₂s, but are not internalized [6]. These requirements are provided by the ExPLIs, which are composed of a lipidic PLA₂ inhibitor conjugated with a polymeric carrier [17,29]; while the lipidic component is incorporated into the cell membrane, its internalization is prevented by the polymeric carrier. These kind of lipid conjugates have been shown to protect membranes from
diverse types of sPLA2s [29,30], and ameliorate different sPLA2-related inflammatory conditions, including endotoxin-induced sepsis in rats [24], allergic-experimental encephalomyelitis (EAE) in rats and mice [23], and TNBS-induced colitis in rats [31].

As described in the Introduction, in a preceding study we found that sensitization of rats with OVA is associated with elevation of sPLA2 and CysLT production, concomitantly with suppression of cPLA2 and PGE2 production. These processes were reversed by systemic (SC injection) treatment with the cell-impermeable PLA2 inhibitor HyPE, which also suppressed the early response (broncho-constriction) to challenge with OVA [15]. The present study shows that effective treatment of asthma can be obtained by application aimed at confining the treatment to the airway system: The inhalation of HyPE by asthmatic rats suppressed OVA-induced sensitization and airway inflammation (Fig. 1 and Table 1), concomitantly with blocking CysLT production (Fig. 4), and also reversed OVA-induced broncho-constriction in already-sensitized rats (Fig. 2). Together, the present and preceding studies provide strong evidence for the pivotal role of sPLA2 in the pathophysiology of both the immediate allergic response and the inflammatory process.

Previous studies have pointed out the involvement of PLA2 in NO production, showing that exogenous PLA2 induces iNO synthase (iNOS) expression and NO generation by alveolar macrophages, a phenomenon that contributes to lung injury [22]. In accordance with this we have found that treatment of rat glial cells with ExPLIs inhibits LPS-induced production of NO concomitantly with PGE2 production and PLA2 secretion as measured by its lipolytic activity [23]. In a study of the ExPLI effect on sepsis in rats, we have found that treatment with HyPE suppressed expression of iNOS and sPLA2-IIA mRNA in lung and kidney of rats with endotoxin-induced sepsis [24]. The present study provides further evidence for the relationship of NO production to PLA2 activity, showing that treatment with the extracellular PLA2 inhibitor suppressed the capacity of BAL macrophages to produce NO (Fig. 5).

The marked reduction in the ability of BAL macrophages to produce TNFα subsequent to in-vivo treatment of the asthmatic rats with HyPE, without further treatment of the cultured cells (Fig. 6), may be attributed to PLA2 activity. However, the dependence of TNFα production on PLA2 enzymatic activity is unclear. In a previous study from our laboratory, it was found that ExPLIs inhibited LPS-induced production of different cytokines in cultured endothelial cells [26], which would suggest a relation to PLA2 activity. However, in that study we could not document a dependence of cytokine production on PLA2 lipolytic activity. On the other hand, treatment with ExPLIs inhibited the LPS-induced activation of NFk-B, which mediates TNFα production, in cultured endothelia cells [26], as well as TNFα production in septic rats [24]. As discussed in the Introduction, sPLA2 enzymes can induce cytokine production via a receptor-mediated process, independent of their enzymatic activity [7]. It is thus possible that the ExPLIs’ effect on cytokine production is due to suppression of PLA2 action as a receptor ligand, rather than its enzymatic activity.

The lipolysis-independent effects of the ExPLIs may be attributed to their being membrane-anchored glycosaminoglycans (GAGs) or GAG-like molecules: Cell surface GAGs are known to protect the cell from exogenous damaging agents, such as endotoxins, reactive oxygen species, degradation enzymes (e.g., heparanase) and cytokines [32,33]. A number of studies have shown that sPLA2 action on the cell membrane is inhibited by cell surface GAG, such as heparin [34] and hyaluronic acid [29, 35]. In a study of the synergistic action of hydrogen peroxide (HPO) and sPLA2, we have previously observed that only after the cell surface GAGs are degraded by the HPO, is the cell membrane rendered accessible to lysis by exogenous sPLA2 [32]. Although the relationship between GAG function and the action of inflammatory mediators is not unequivocally clear, it is well-documented that GAG
stripping exposes cells and tissues to damaging agents. Accordingly, it has been proposed that enrichment of cell surface GAGs would assist in protection of the cell from infection and inflammatory/allergic assaults [36]. These requirements are fulfilled by the ExPLIs, which are composed of PLA₂-inhibiting molecules, such as N-derivatized phosphatidyl-ethanolamine, to polymeric carriers (e.g., hyaluronic acid or chondroitin sulfates). Due to their structure, these lipid-conjugates exhibit a multiple effect in cell protection: The lipid moiety, which incorporates into the cell membrane, suppresses membrane PL hydrolysis by endogenous sPLA₂ and the subsequent production of lipid mediators [23,29-31]. At the same time, the polymeric carrier mimics the cell surface GAG in protecting cells from exogenous sPLA₂s and other inflammatory agents, such as reactive oxygen [32,35]. The soundness of this concept has been demonstrated in several in-vivo studies, as cited above [23,24,31]. Of the different GAGs, hyaluronic acid (HA) is of particular relevance to the present study, since it specifically binds to CD44 on lymphocytes and eosinophils and activates them. Accordingly, HA binding to lymphocyte CD44 prevents their rolling and subsequent extravasation to inflammatory sites [37]. This makes HyPE-like lipid-conjugates particularly suitable for the treatment of asthma, and this indeed is demonstrated by the findings of the present study.

All-in-all, the results and considerations summarized above introduce a novel therapeutic approach in the treatment of allergic/inflammatory diseases, and present the lipid-conjugates, which concomitantly control PLA₂ action and mimic the cell surface GAG function, as a prototype of potential multi-functional, non-steroidal inhaled drugs for comprehensive treatment of asthma.

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Competing interests: According to the regulations of the Hebrew University (by which Saul Yedgar, the corresponding author, is employed), the rights to the results of this research belong to Yissum, the Hebrew University Research and Development Company. Yissum have licensed the technology pertaining to the PLA₂ inhibitors used in this study, to Morria Biopharmaceutical Plc. The present study or the authors were not financially supported by Morria or any other commercial entity.
Table 1

<table>
<thead>
<tr>
<th></th>
<th>Naïve</th>
<th>Asthma</th>
<th>Asthma +HyPE</th>
<th>Asthma +Dx</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Inflammatory cells infiltration</td>
<td>1.16 ± 0.11</td>
<td>11.41 ± 1.81</td>
<td>1.44 ± 0.21</td>
<td>1.41 ± 0.16</td>
</tr>
<tr>
<td>II. ASM thickening</td>
<td>3.96 ± 0.39</td>
<td>40.20 ± 4.78</td>
<td>5.03 ± 0.65</td>
<td>7.10 ± 0.96</td>
</tr>
<tr>
<td>III. BM thickening</td>
<td>0.76 ± 0.20</td>
<td>8.22 ± 1.20</td>
<td>0.70 ± 0.09</td>
<td>0.69 ± 0.08</td>
</tr>
<tr>
<td>IV. Mucus metaplasia</td>
<td>12.0 ± 1.3</td>
<td>67.0 ± 3.2</td>
<td>13.0 ± 4.1</td>
<td>11.0 ± 3.5</td>
</tr>
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Effect of HyPE on remodeling of OVA-sensitized rat airway; histological morphometry:
I – Infiltration of inflammatory cells into the space between intima and adventicia of the bronchi, expressed as number of inflammatory cells per mm of basal membrane. II – Airway smooth muscle (ASM) thickening, expressed as width of ASM per length of basal membrane (µm/mm). III – Thickening of basal membrane (BM), expressed as width (µm) per length (mm) of BM. IV – Mucus metaplasia of respiratory epithelial cells, expressed as the percent of PAS positive cells from total epithelial cells. Each datum is Mean ± SEM for 10 rats. For all parameters, statistical analysis showed p < 0.001 between Asthma (non-treated asthmatic rats) and the other groups, and no significant difference was observed between HyPE-treated and Naïve or Dx-treated rats.
LEGENDS TO FIGURES:

Figure 1: Effect of HyPE inhalation on early (upper panel) and late (lower panel) broncho-constrictive reactions induced by ovalbumin inhalation: Broncho-constriction, expressed as the percent change in Penh (16) was induced in OVA-sensitized rats by inhalation of OVA, and measured before allergen challenge, and 5 min and 8 h after allergen challenge (see Methods). Each datum is Mean ± SEM for 10 rats. Combined statistical tests yielded $p < 0.01$ between OVA/OVA and the other groups for both EAR and LAR.

Figure 2: Immediate effect of HyPE inhalation on OVA-induced (broncho-constriction) in OVA-sensitized rats. A. Prevention of broncho-constriction: OVA- sensitized rats (not treated with HyPE during sensitization) were subjected to Penh determination (baseline), then inhaled HyPE (circles) or saline (squares) and subjected to Penh determination 5 min later, then challenged with OVA inhalation, and subjected to Penh determination 5 min later. B. Relief of broncho-constriction: OVA- sensitized rats (not treated with HyPE during sensitization) were subjected to Penh determination (baseline), then inhaled OVA and subjected to Penh determination 5 min later, then inhaled HyPE (circles) or saline (squares) and subjected to Penh determination 5 min later. Each datum is Mean ± SEM for 5 rats. In A, $P < 0.05$ between HyPE and saline-treated and between OVA + HyPE. In B, $P < 0.01$ between HyPE and saline-treated rats.

Figure 3: Effect of HyPE inhalation on structural change in airways (airway remodeling) of OVA-sensitized rats: Rats were subjected to OVA inhalation every other day for 30 days. For treatment with HyPE, the rats inhaled HyPE aerosol for 5 min before every allergen inhalation. The rats were sacrificed on Day 45. A – Staining with hematoxylin eosin for detection of inflammatory cell infiltration and changes in smooth muscle cell (ASM) thickness. B – Staining of connective tissue (collagen) with Mason-Trichrom, for detection of changes in basal membrane thickness. C - Staining with Periodic Acid Schiff (PAS) for detection of mucus metaplasia of respiratory epithelial cells. 1, 2, 3 and 4 depict tissues of Naïve, OVA/OVA, HyPE-treated and Dx-treated rats, respectively.

Figure 4: Effect of HyPE inhalation on Cysteinyl leukotrienes (LTC4, LTD4 and LTE4) level in the BAL of OVA-sensitized rats (see Methods for details): Broncho-alveolar lavage (BAL) was collected upon sacrifice and CysLT levels were determined by EIA, as described in Methods. Each datum is Mean ± SEM for 10 rats. $P < 0.01$ between OVA/OVA and HyPE-treated rats. No significant difference between HyPE-treated and the Naïve or Dx-treated rats.

Figure 5: Effect of HyPE inhalation on NO production by macrophages collected from the BAL of OVA-sensitized rats: Macrophages, collected from the BAL of the different groups, were cultured without further treatment with HyPE or Dx, and NO production was determined by the corresponding nitrate level in the culture medium. Each datum is Mean ± SEM for 10 rats. NO level was reduced compared to OVA/OVA and naïve rats by both HyPE ($p < 0.001$ and $p < 0.001$ respectively) and Dx ($p < 0.001$ and $p < 0.001$, respectively).

Figure 6: Effect of HyPE inhalation on TNFα production by macrophages collected from the BAL of OVA-sensitized rats (see Methods for details): Macrophages, collected from the BAL of the different groups, were cultured without further treatment with HyPE or Dx, and TNFα secreted to the culture medium was determined as described in Methods.
Each datum is Mean ± SEM for 10 rats. \( p < 0.001 \) between OVA/OVA and HyPE-treated rats. No significant difference between HyPE-treated, Naive and Dx-treated rats.

**REFERENCES**


Figure 2

A

B

Baseline | Treatment | OVA

Baseline | OVA | Treatment
Figure 4

![Graph showing CysLT (pg/µg protein) levels in different groups: Naive, OVA/OVA, OVA/OVA + HyPE, and OVA/OVA + Dx. The graph indicates higher CysLT levels in the OVA/OVA group compared to other groups.]
Figure 6

![Graph showing TNF levels in different groups.

- **Naive**
- **OVA/OVA**
- **OVA/OVA + HyPE**
- **OVA/OVA+Dx**

The graph illustrates that the TNF level in the OVA/OVA group is significantly higher compared to the other groups.}